Discovery of Bioactive Proteins Derived from Scorpion Venom using Two Dimensional Mass Spectrometry

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Pharmacological values of Scorpion Venom

- 1400 species worldwide[1]
- Mesobuthus Martensii, commonly known as Manchurian Scorpion
- Used in traditional Chinese medicines for:
  - Cardiovascular problems[2]
  - Drug dependence[3]
  - Chronic pain[4]
  - Diabetes[5]
  - Tumours[6]

- Previous research mainly focused on purification of bioactive venom peptides, and sequences are searched against the database.[3-7]

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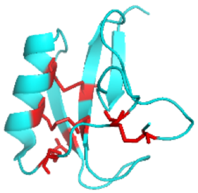
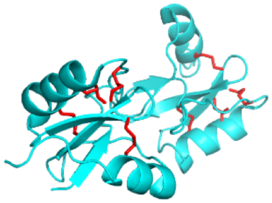
1) Sridhara, S.; Chakravarthy, A. K.; Kalarani, V.; Reddy, D. C., Diversity and Ecology of Scorpions: Evolutionary Success Through Venom, Springer Singapore: 2016; pp 57-80
2) Wang, R.; Moreau, P.; Deschamps, A.; de Champlain, J.; Sauvé, R.; Foucart, S.; Bai, L.; Lu, X.-R., Toxicon 1994, 32 (2), 191-200.
3) Liu, Y.-F.; Ma, R.-L.; Wang, S.-L.; Duan, Z.-Y.; Zhang, J.-H.; Wu, L.-J.; Wu, C.-F., Protein Expression and Purification 2003, 27 (2), 253-258.
4) Goudet, C.; Chi, C.-W.; Tytgat, J., Toxicon 2002, 40 (9), 1239-1258.
5) Ortiz, E.; Gurrola, G. B.; Schwartz, E. F.; Possani, L. D., Toxicon 2015, 93, 125-135.
6) D'Suze, G.; Rosales, A.; Salazar, V.; Sevcik, C., Toxicon 2010, 56 (8), 1497-1505. Xu, X.; Duan, Z.; Di, Z.; He, Y.; Li, J.; Li, Z.; Xie, C.; Zeng, X.; Cao, Z.; Wu, Y.; Liang, S.; Li, W., Journal of Proteomics 2014, 106, 162-180.
7) 3Guan, R.-J.; Xiang, Y.; He, X.-L.; Wang, C.-G.; Wang, M.; Zhang, Y.; Sundberg, E. J.; Wang, D.-C., Journal of Molecular Biology 2004, 341 (5), 1189-1204.

Proteins from Scorpion Venom

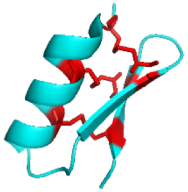
- Complex mixture of bioactive proteins
- 113 (proteins) **reviewed**, 103 (genome sequence) **unreviewed** entries from UniProt
- Heavily modified (such as amidation of C-terminus, hydroxylation of proline, and bromination of tryptophan)[1] and crosslinked with multiple disulphide bonds
- Only partial genome sequencing is available
- De novo sequencing is essential for many of these proteins.



alpha-like neurotoxin BmK M1[2]



insect-specific toxin BmK IT-AP[3]



Toxin BmBKTx1[4]

Heavily disulphide bonded biomolecules from scorpion venoms

~ 1 disulphide bond / 1kda

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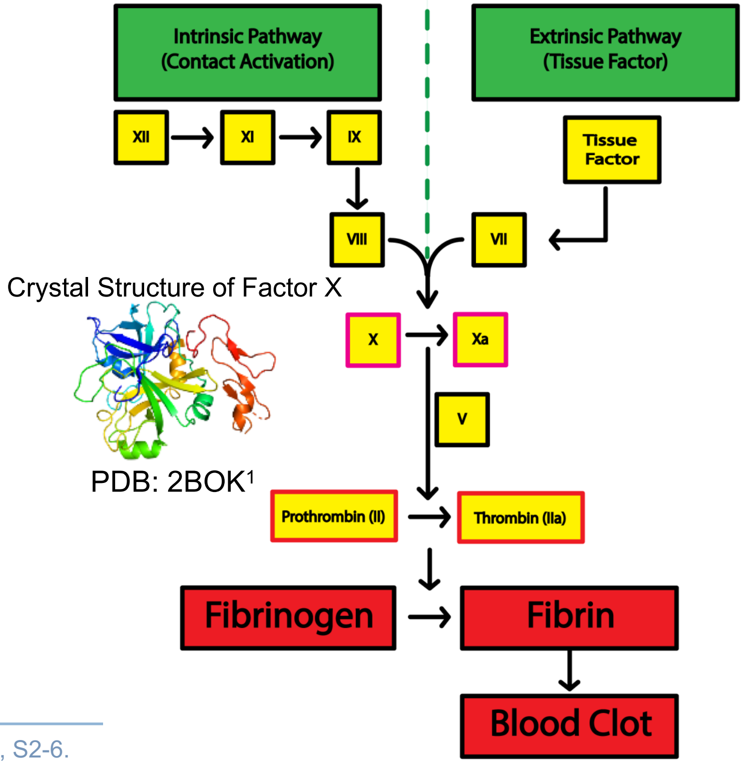
1) Lewis, R. J.; Garcia, M. L., Nat Rev Drug Discov 2003, 2 (10), 790-802.
2) Guan, R.-J.; Xiang, Y.; He, X.-L.; Wang, C.-G.; Wang, M.; Zhang, Y.; Sundberg, E. J.; Wang, D.-C., Journal of Molecular Biology 2004, 341 (5), 1189-1204.
3) Mandal, K.; Pentelute, B. L.; Tereshko, V.; Kossiakoff, A. A.; Kent, S. B. H., Journal of the American Chemical Society 2009, 131 (4), 1362-1363.
4) Li, C., Guan, R.-J., Xiang, Y., Zhang, Y. & Wang, D.-C. (2005). Acta Cryst. D61, 14-21

Search for new anticoagulants

- Thrombotic diseases
  - Lead to high blood pressure, heart failure and stroke
  - Current anticoagulants have many disadvantages such as increased gastrointestinal bleeds, bleeding under skin, vomiting blood and bleeding in the brain
- Previous researches shown that scorpion venoms posses anticoagulation properties[2,3]
- Inhibition of Factor Xa (active form of Factor X)
  - Catalyses the conversion of prothrombin to thrombin[4]
  - One molecule of Factor Xa leads to the formation of approx. 1000 molecules of thrombin[5]
  - Thrombin converts the soluble fibrinogen to insoluble fibrin strands



Crystal Structure of Factor X

PDB: 2BOK[1]

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1) Nutescu, E. A.; Shapiro, N. L.; Chevalier, A.; Amin, A. N., Cleve Clin J Med 2005, 72 Suppl 1, S2-6.
2) Brazón, J., D'Suze, G., D'Errico, M.L., Arocha-Piñango, C.L., Guerrero, B., 2009, Arch.Toxicol. 83, 669–678.
3) Song, Y.M., Tang, X.X., Chen, X.G., Gao, B.B., Gao, E., Bai, L., Lv, X.R., 2005, Toxicon 46, 230–235
4) Hoffman M, Monroe DM. Coagulation 2006: a modern view of hemostasis. Hematol Oncol Clin North Am 2007;21:1–11.
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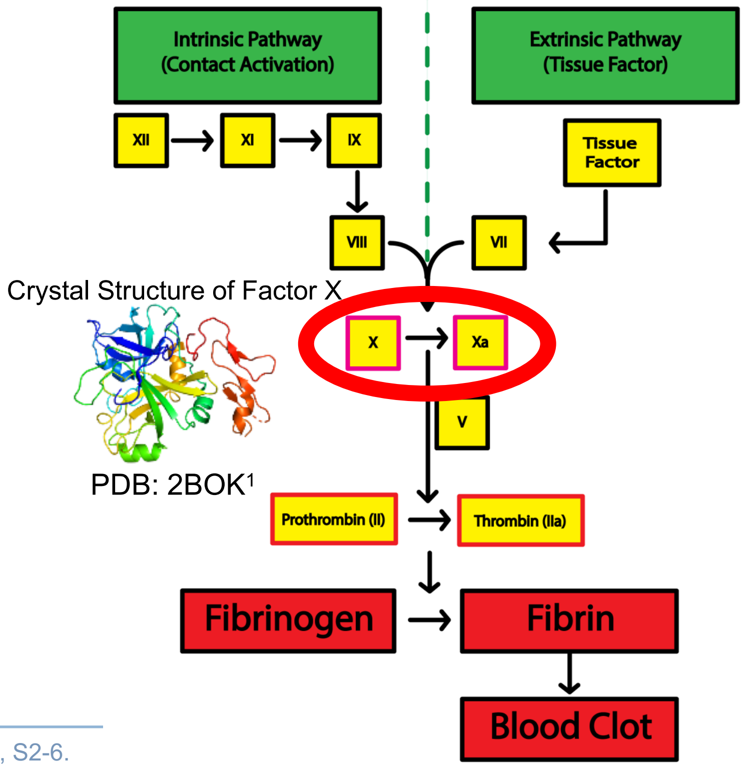

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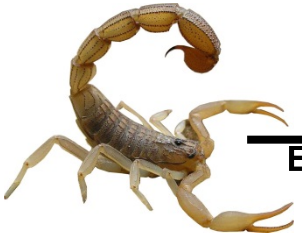
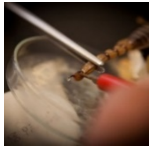
Standard Proteomics Methods

- Better understand the scorpion venom





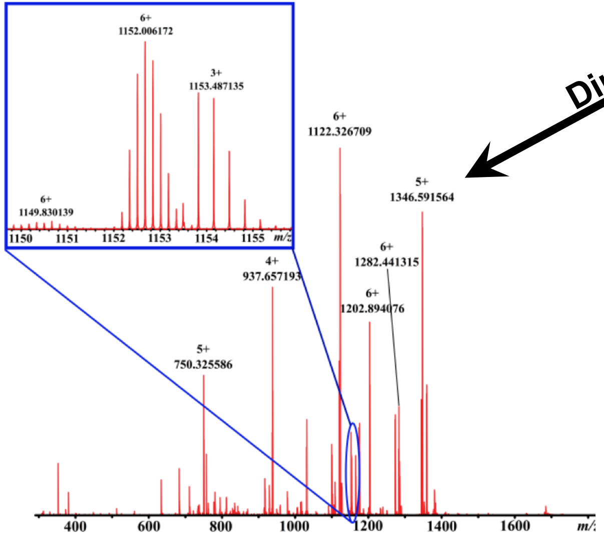
Venom Milking
Electrical Stimulation
(8-12 V, 128 Hz)

Venom
(freeze-dried)

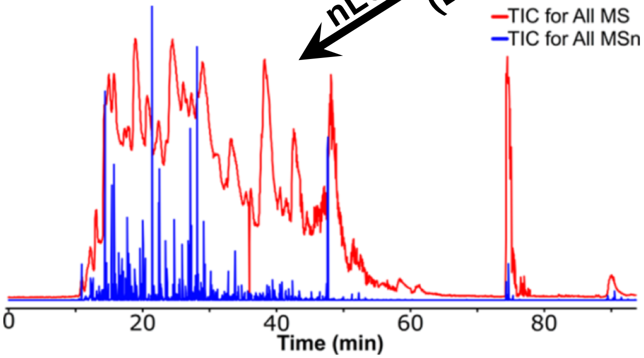
Reduction, Alkylation
Trypsin Digestion

Direct Infusion
(Top-down)

nLC-MS/MS (CAD)
(Bottom-up)



12T Bruker Solarix



Direct Infusion: MS scan (top-down)

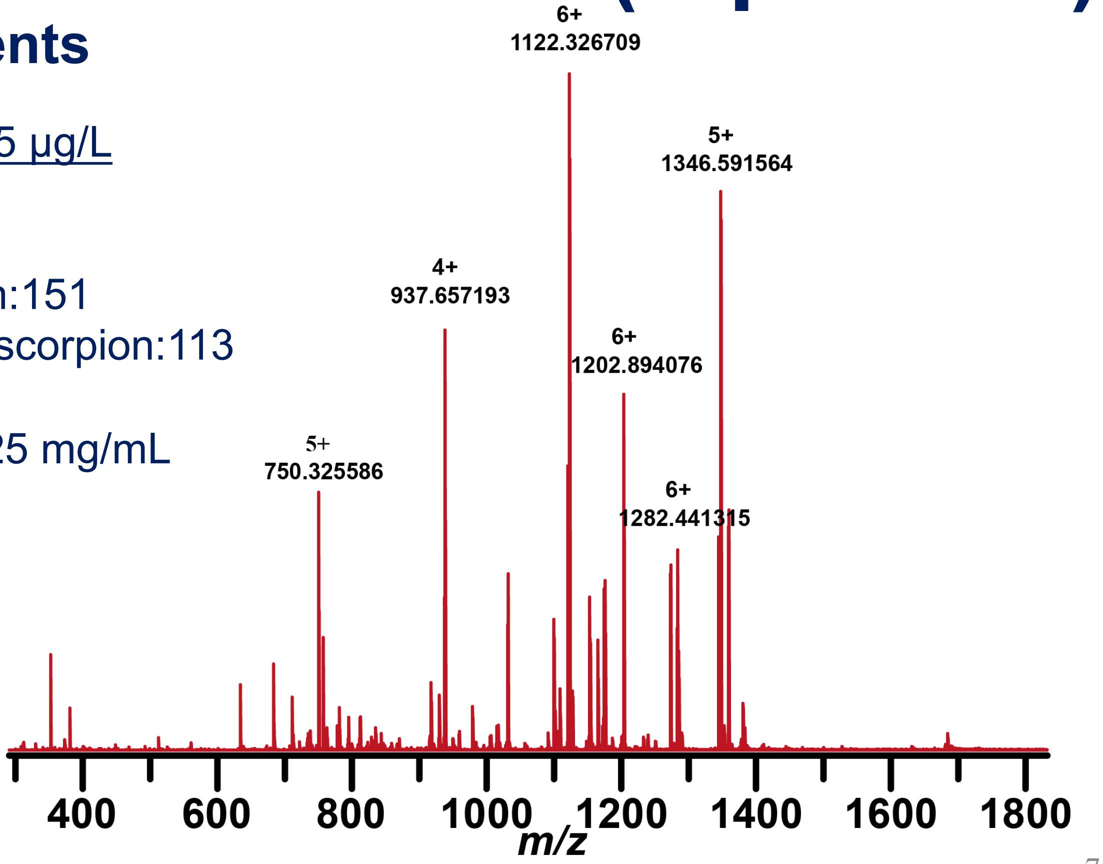
- Selected Assignments

In vitro inhibition (IC50):15.75 µg/L

Counted No. Proteins

- Observed in crude venom:151
- UniProt Database of this scorpion:113

Concentration of sample: 0.25 mg/mL



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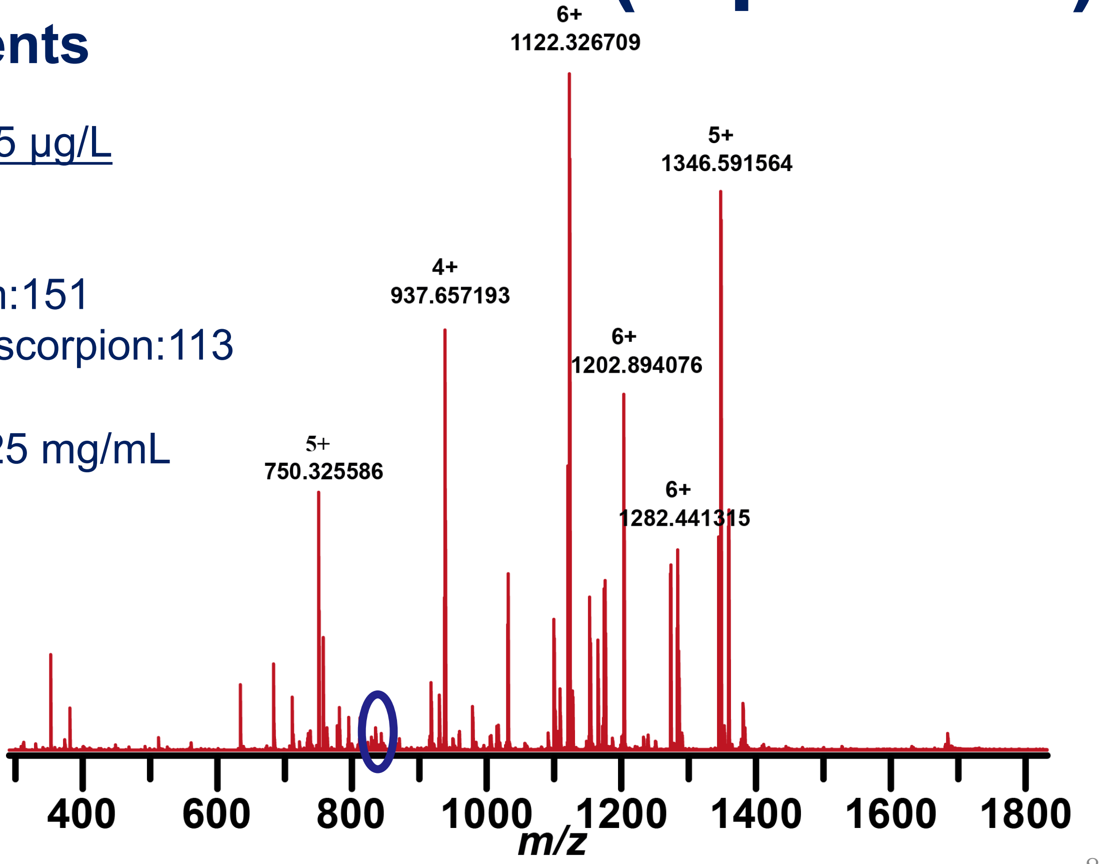

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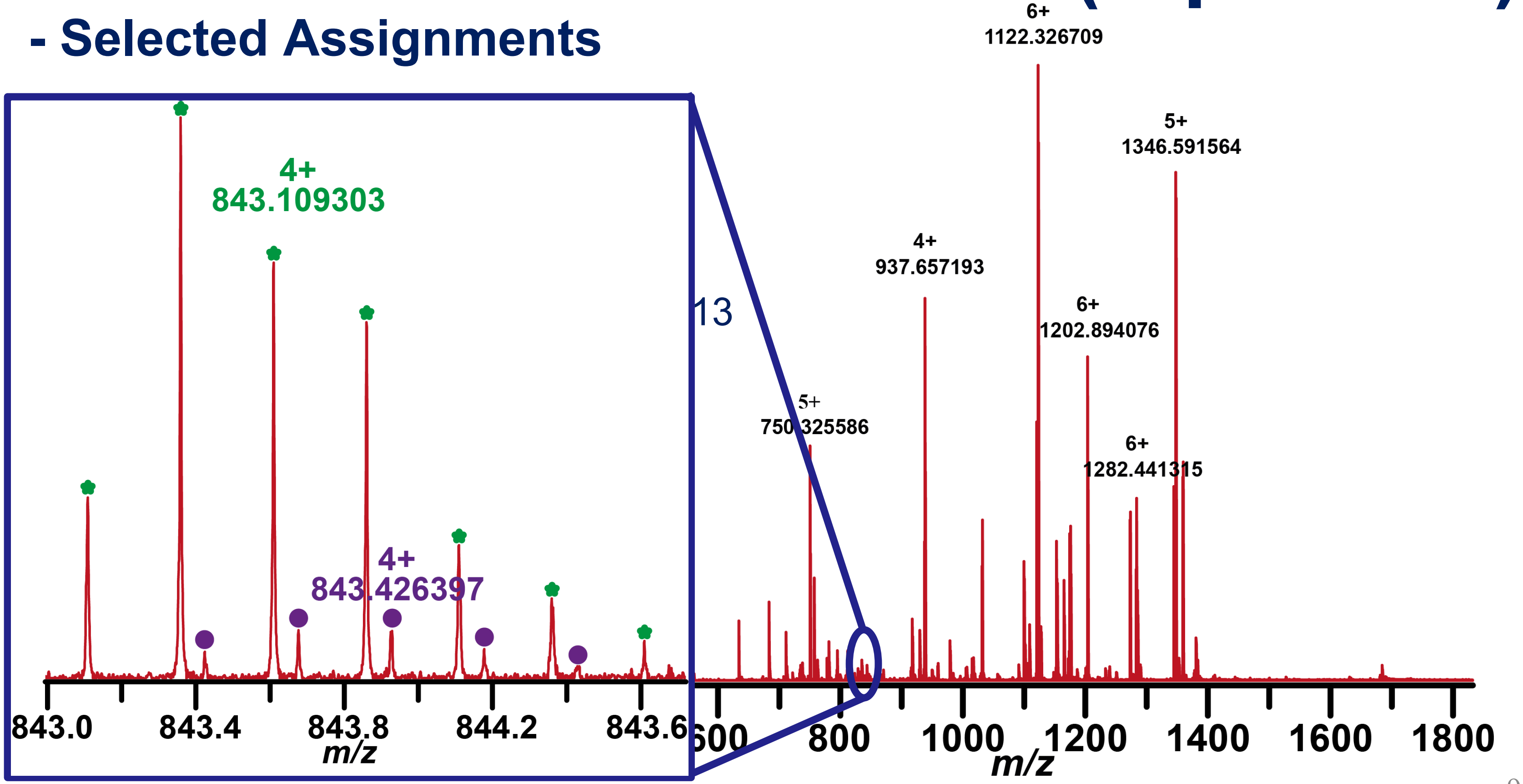
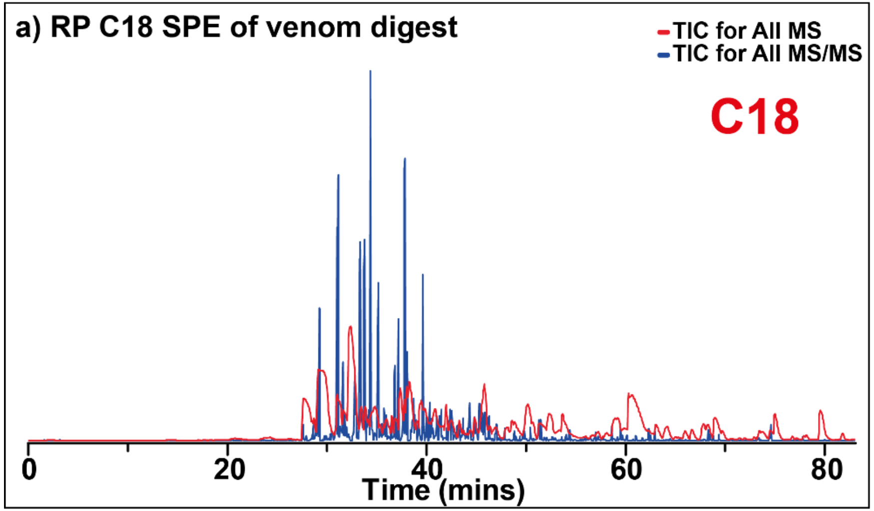
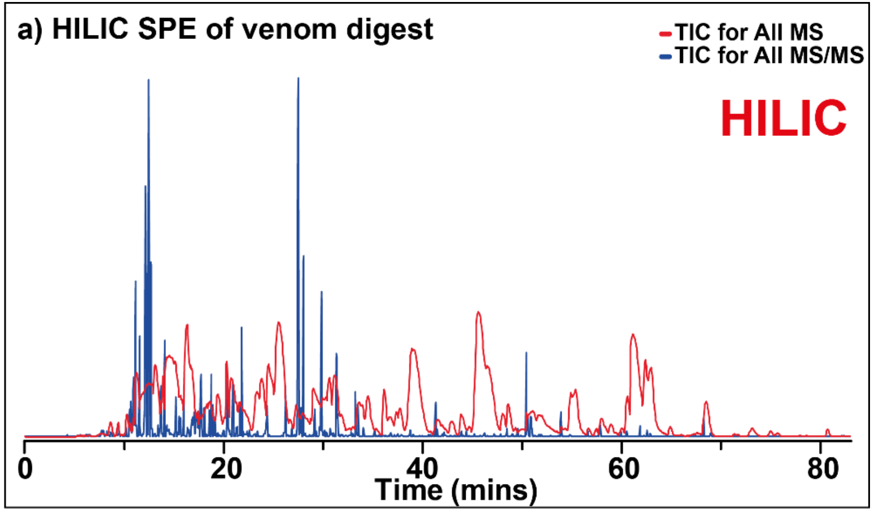


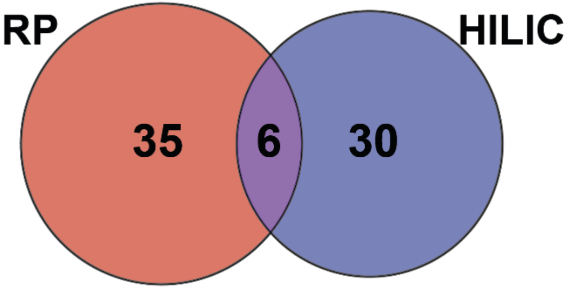
nLC-FTICR-MS/MS (CAD)





NanoLC-MS/MS: Total runtime: 90 minutes
Column: RP C18 (15 cm)

No. Proteins:
Observed vs Mascot search: 151 vs 42 (71 peptides)



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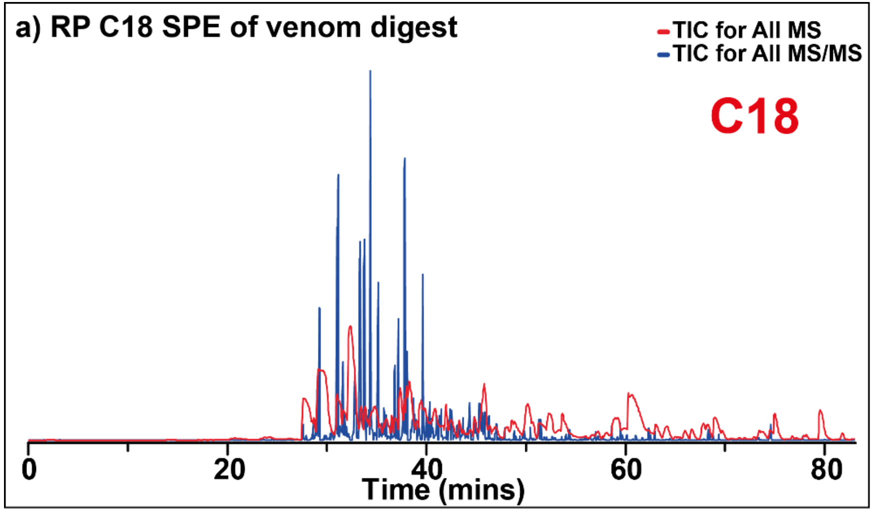

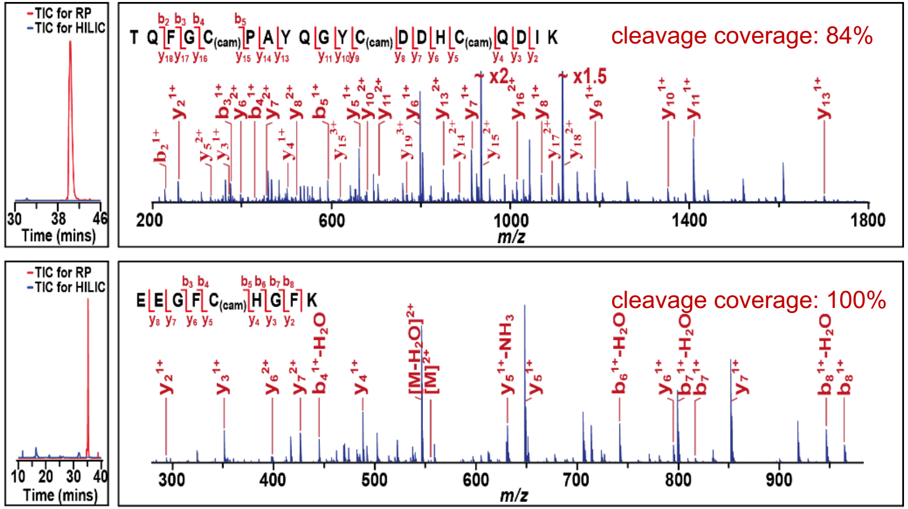

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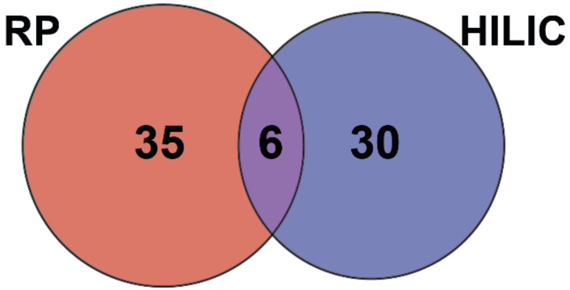

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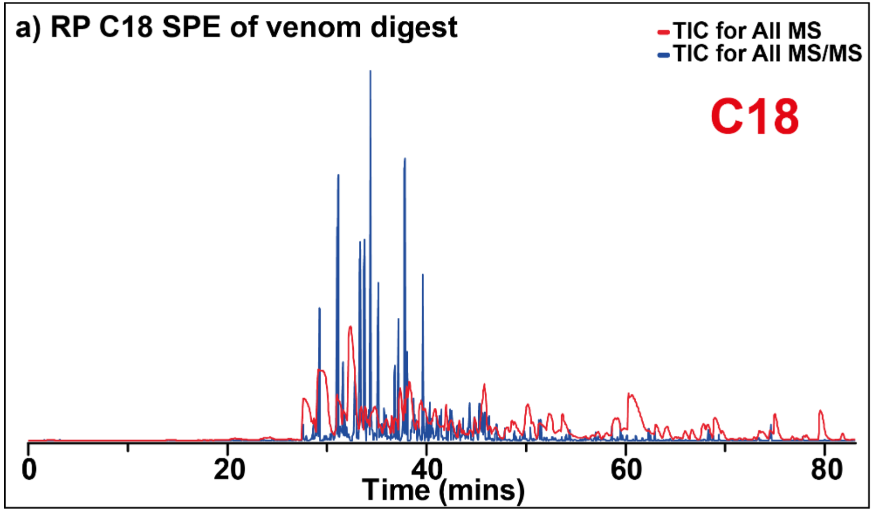

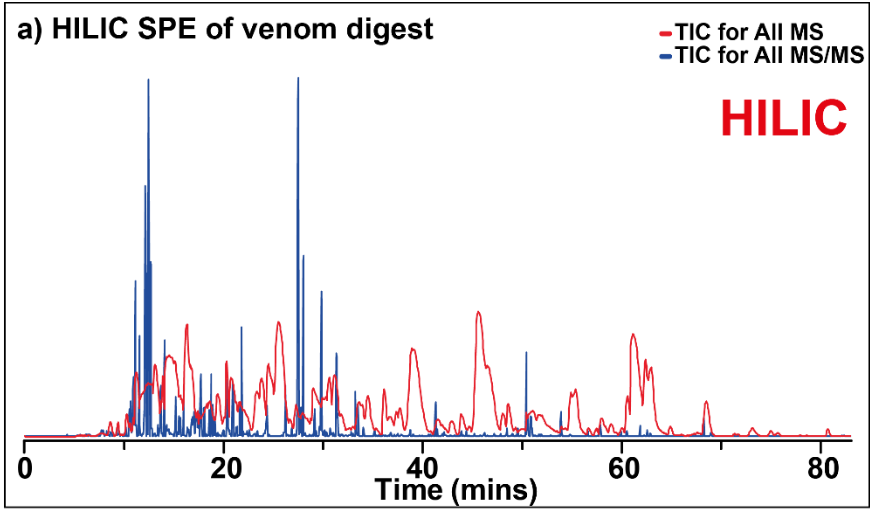

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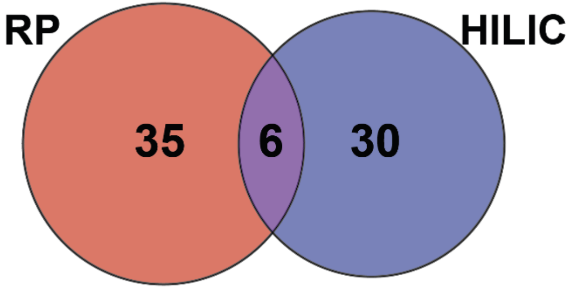

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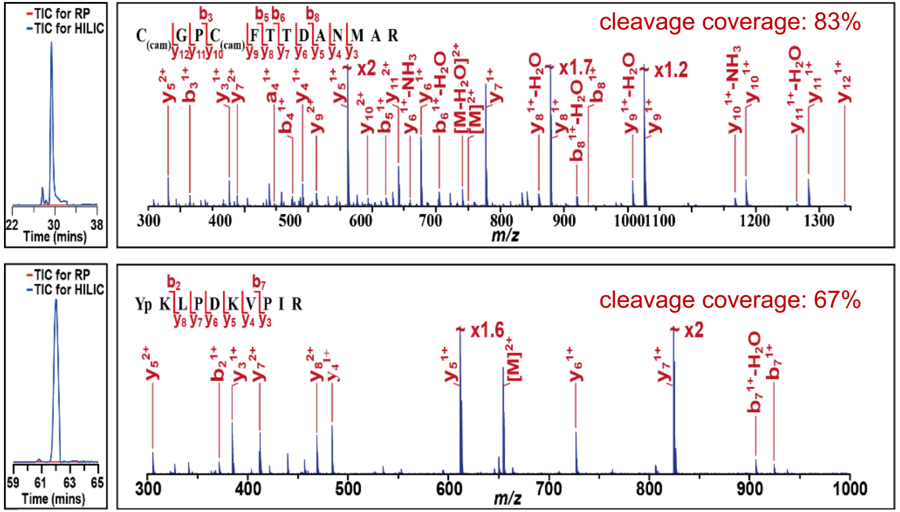

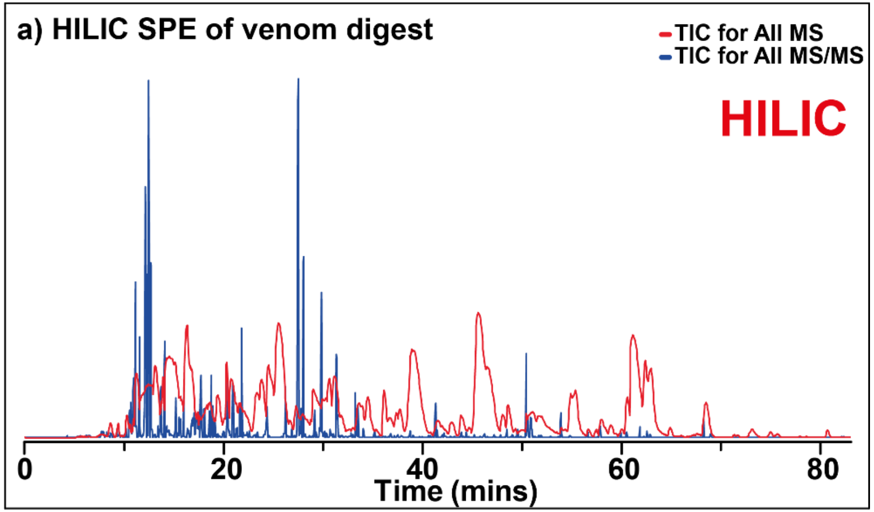

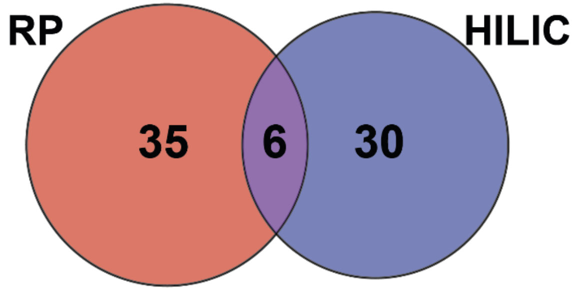

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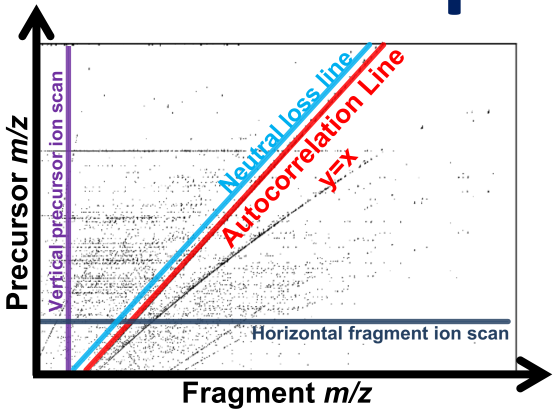
Two Dimensional Mass Spectrometry

- Provides complementary information to nLC-MS/MS
- New method for proteomics
- Suitable for venom mixtures
- 2D contour plot from 3D plot
- Data-independent
- Suitable for complex samples
- Simple experimental set-up
- Potential to avoid the need for separation
- All precursors and their fragments ions can be visualised on one spectrum



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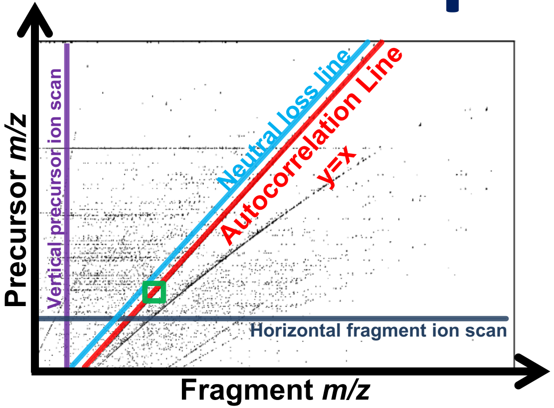

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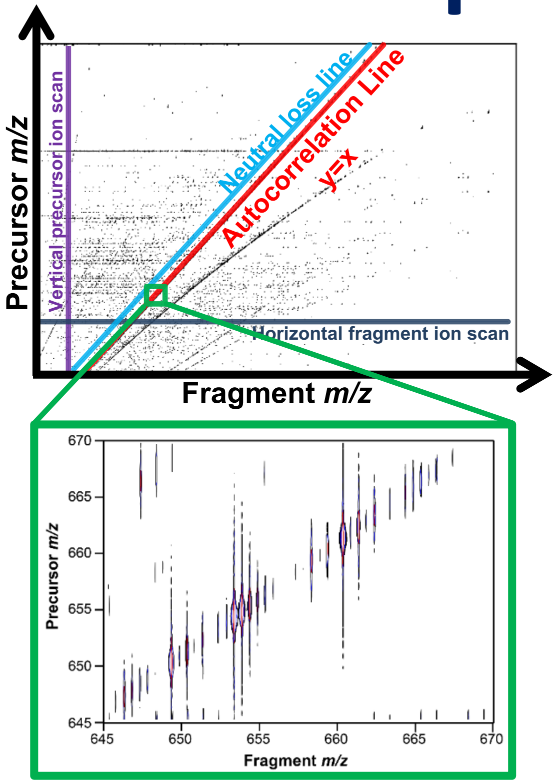

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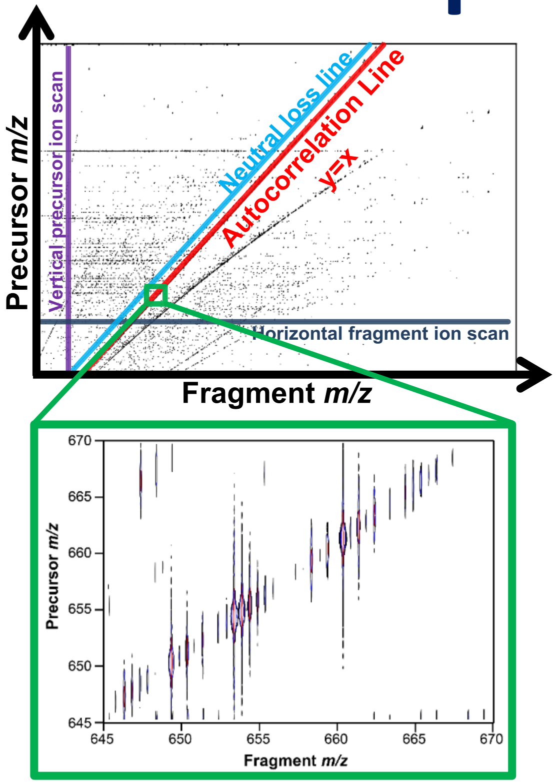
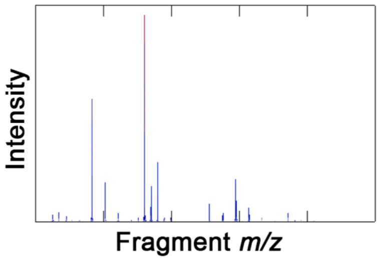


2DMS spectrum rotate around x-z axis



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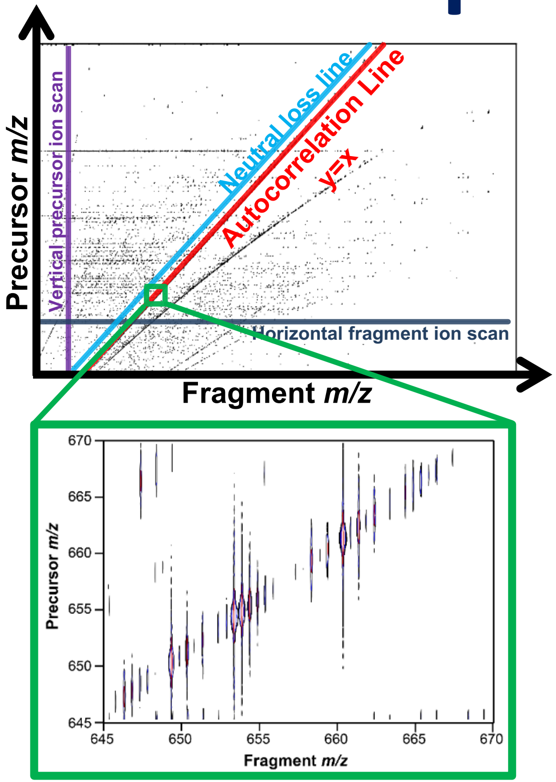
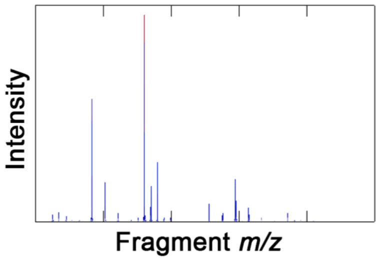
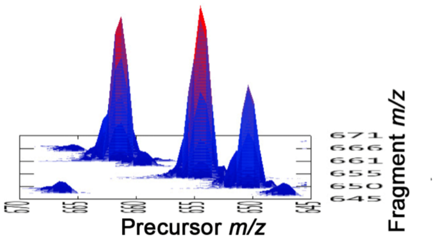


2DMS spectrum rotate around x-z axis



2DMS spectrum rotate around x-y axis



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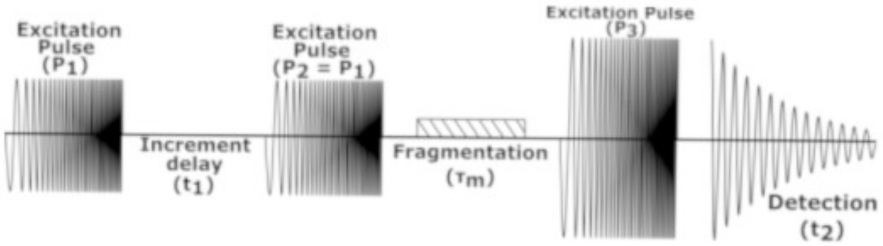

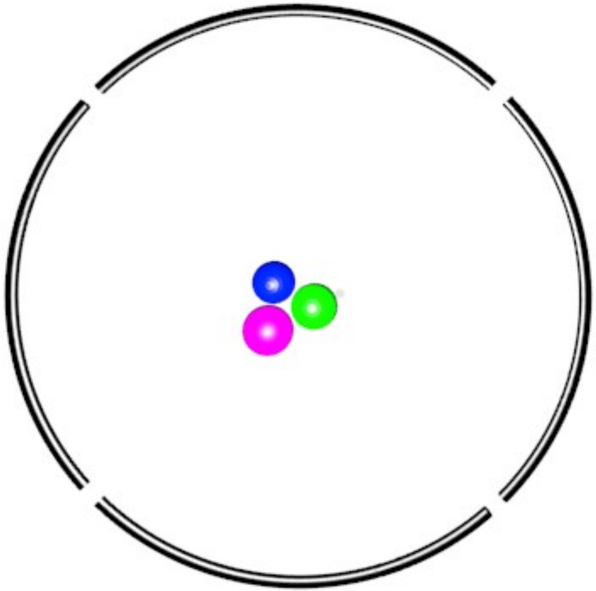

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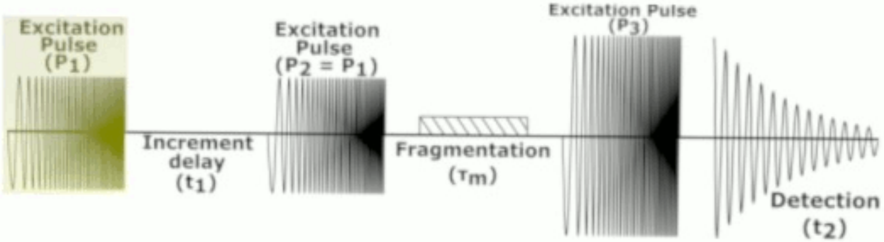
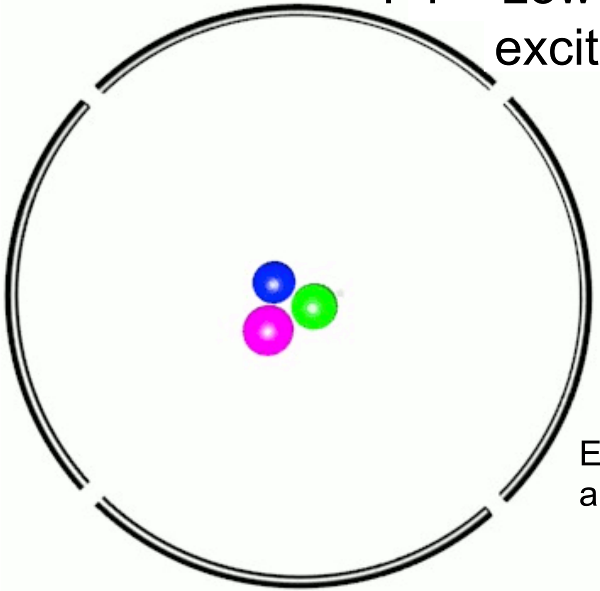


P1 – Low energy pulse excites ions



Excites ions into a higher orbit

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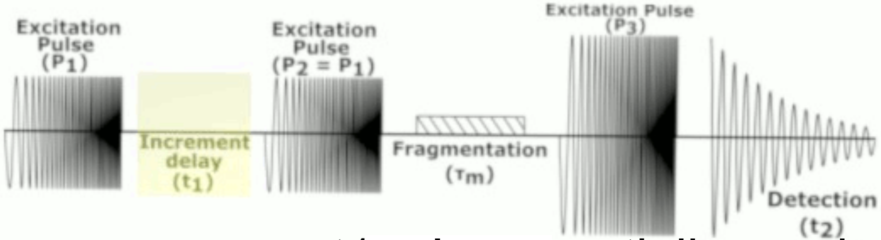
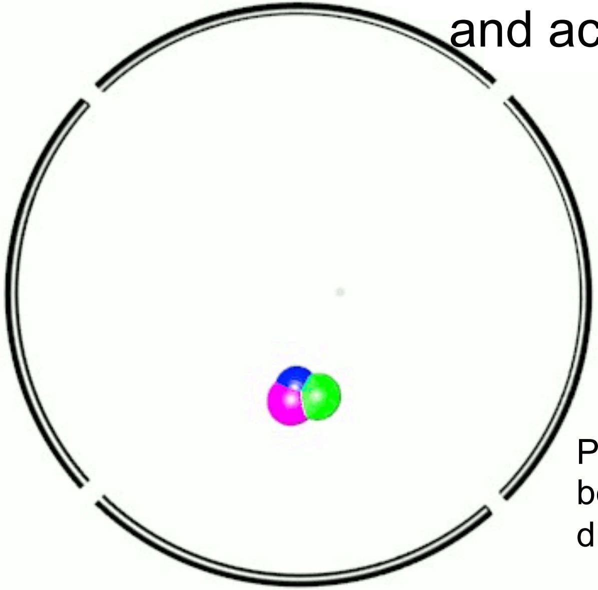


t1 – Ions spatially resolve and acquire phase



Phase of ions will be different with different delays

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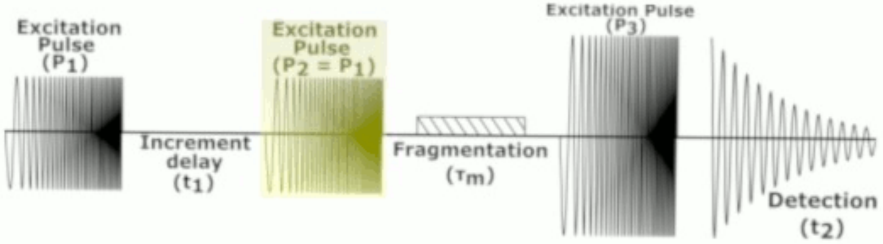

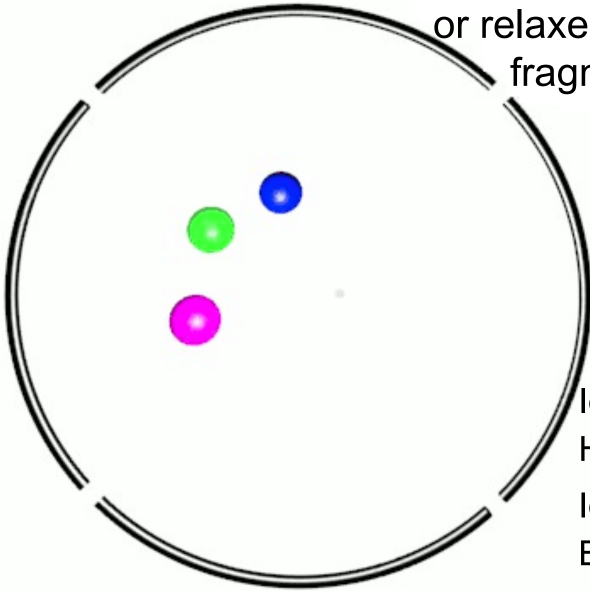

P2 – Further excites or relaxes ions into/ out of fragmentation region

Ions in phase:
Higher orbit

Ion out of phase:
Back to centre

Two Dimensional Mass Spectrometry

- New method for proteomics
- Suitable for venom mixtures
- Provides complimentary information to nLC -MS/MS
- 2D contour plot from 3D plot
- Data-independent
- Suitable for complex samples
- Simple experimental set-up
- Without the need for separation
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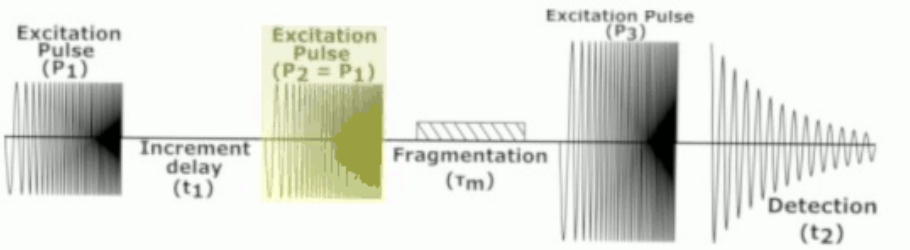
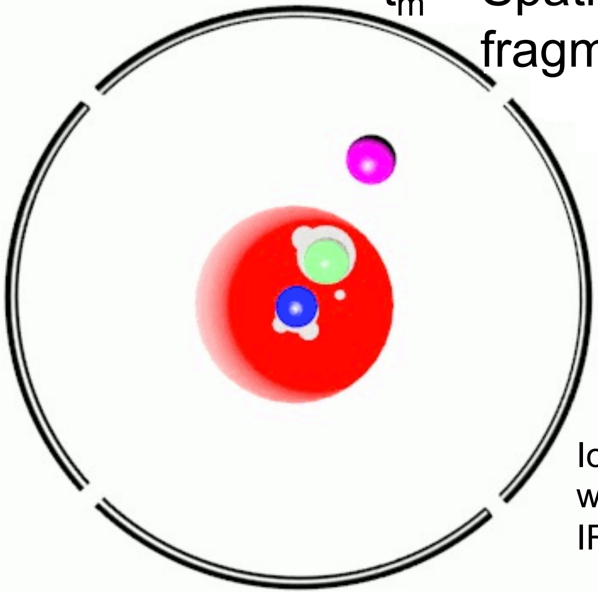


t_m – Spatially resolved fragmentation



Ions in the centre will fragmented by IRMPD

Two Dimensional Mass Spectrometry

- New method for proteomics
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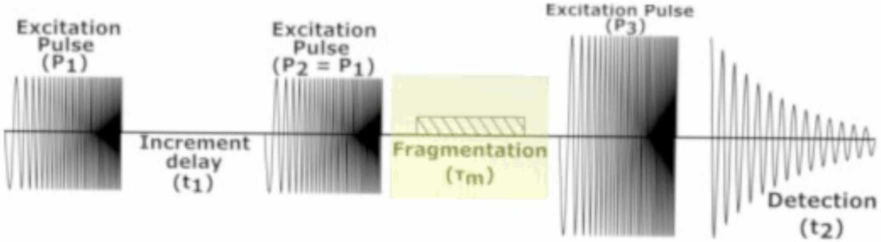
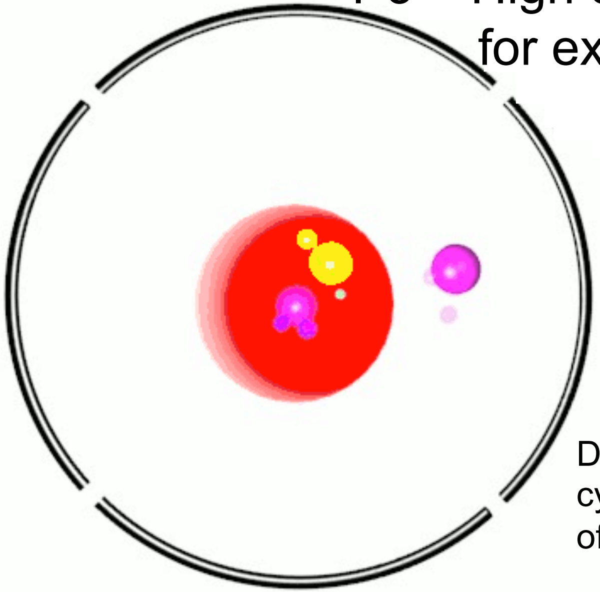


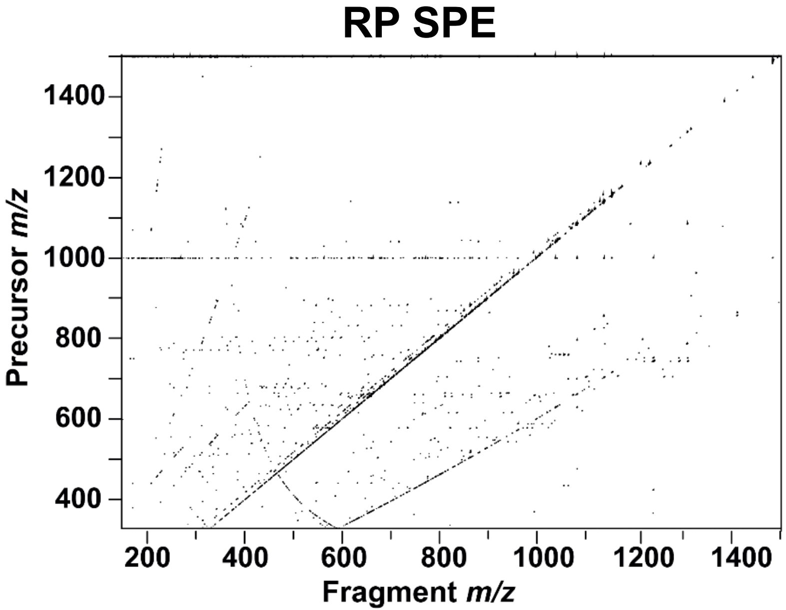
P3 – High energy pulse for excite/ detect



Detection of the cyclotron frequency of ions

2DMS of Venom Tryptic Digests (IRMPD)

- Potential application of 2D proteomics in venoms

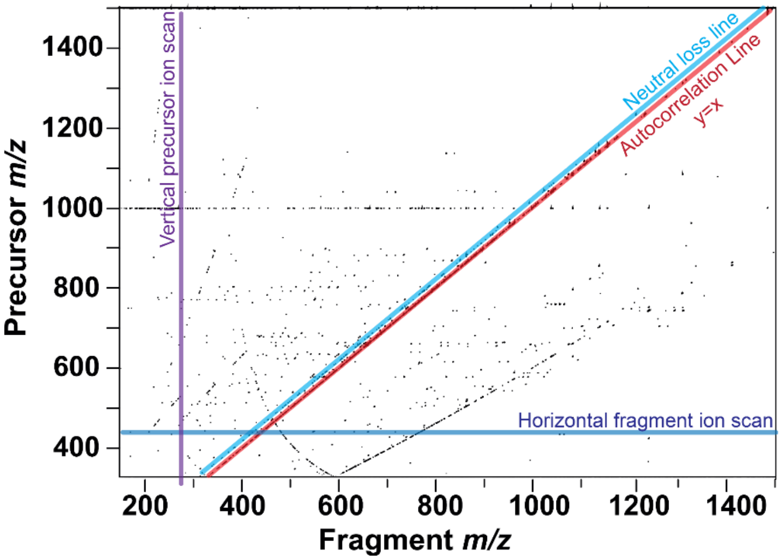


- ➢ Direct infusion
- ➢ All fragments are shown in 1 spectrum
- ➢ 1M x 8192
- ➢ Acquisition time: 3 hours
- ➢ File size: 93 GB
- ➢ Processing time: 4 hours on cluster computers

2DMS of Venom Tryptic Digests (IRMPD)

- Potential application of 2D proteomics in venoms

RP SPE



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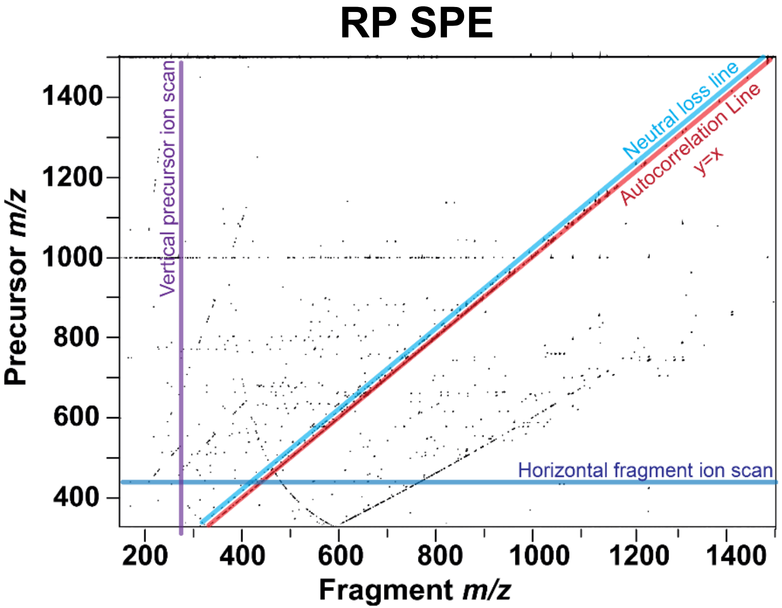

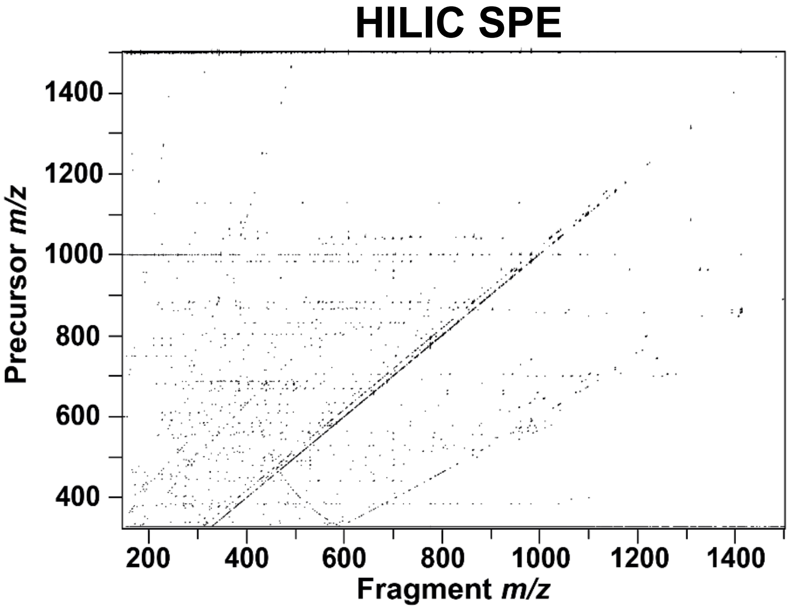

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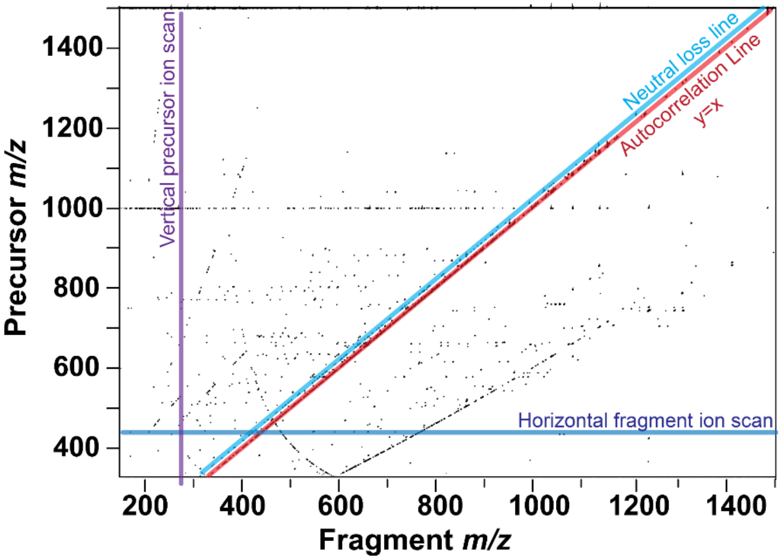
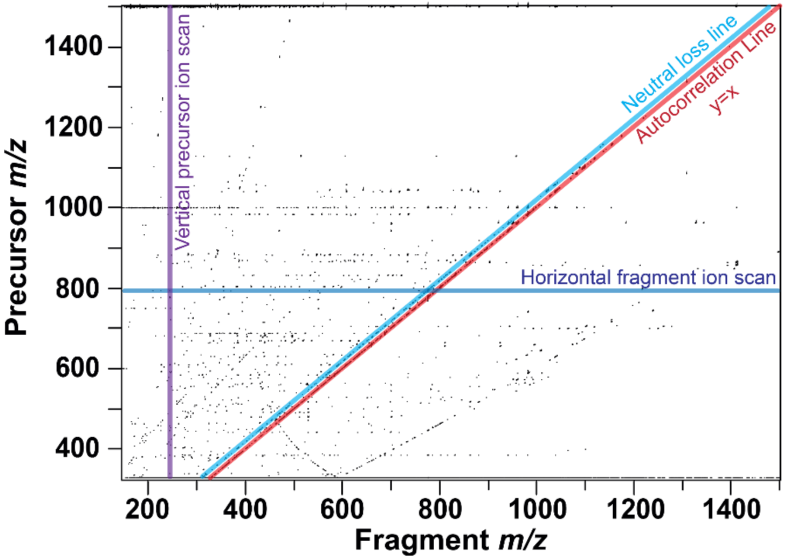
- Potential application of 2D proteomics in venoms

RP SPE

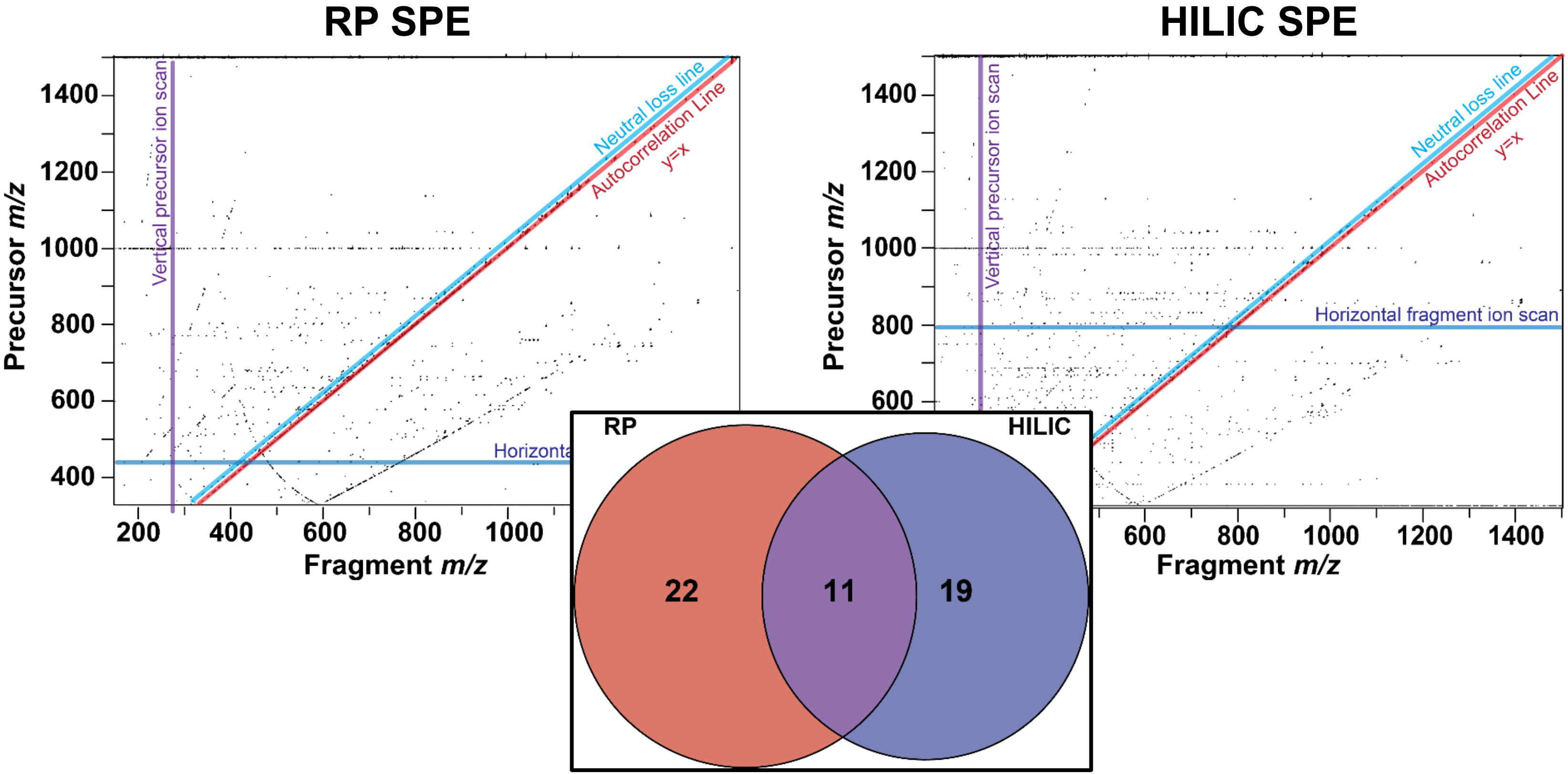


HILIC SPE



2DMS of Venom Tryptic Digests (IRMPD)

- Potential application of 2D proteomics in venoms


RP SPE

HILIC SPE

2DMS of Venom Tryptic Digests (IRMPD)

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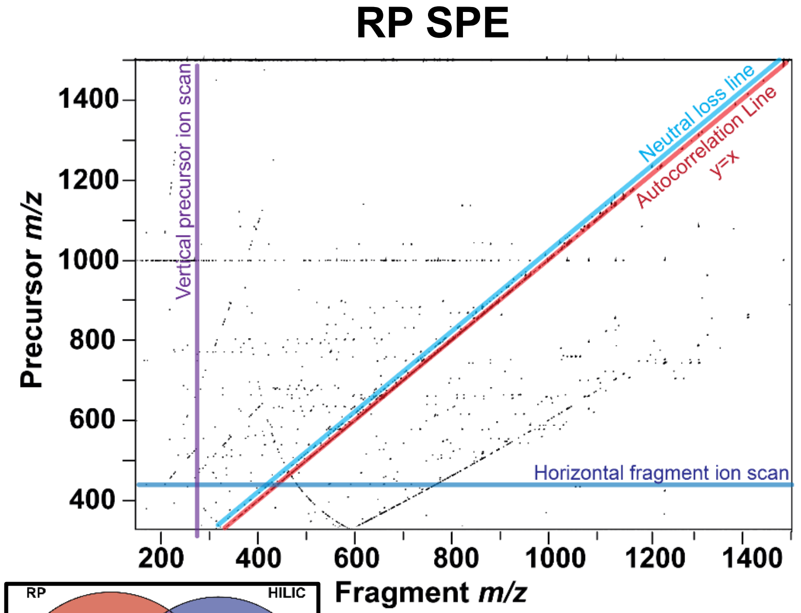

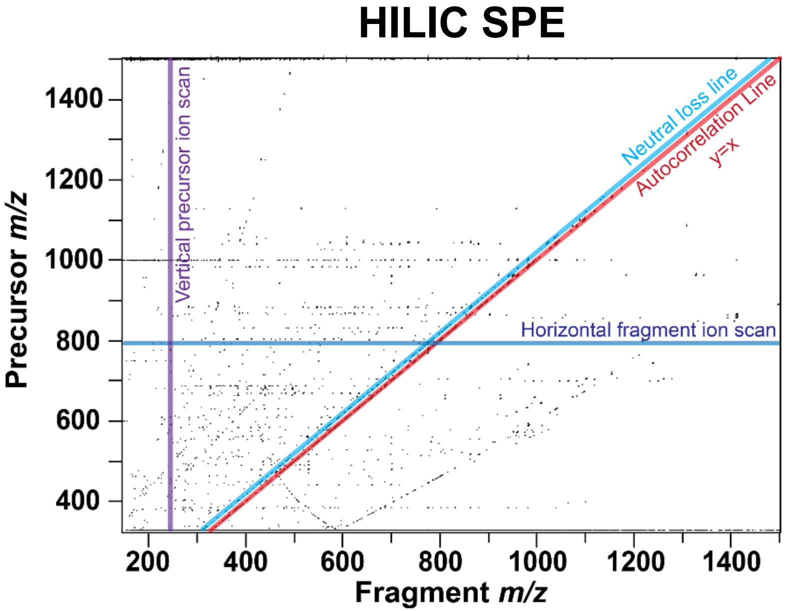

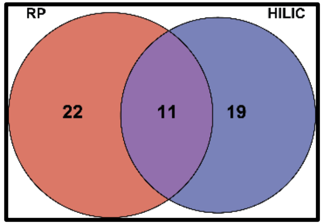

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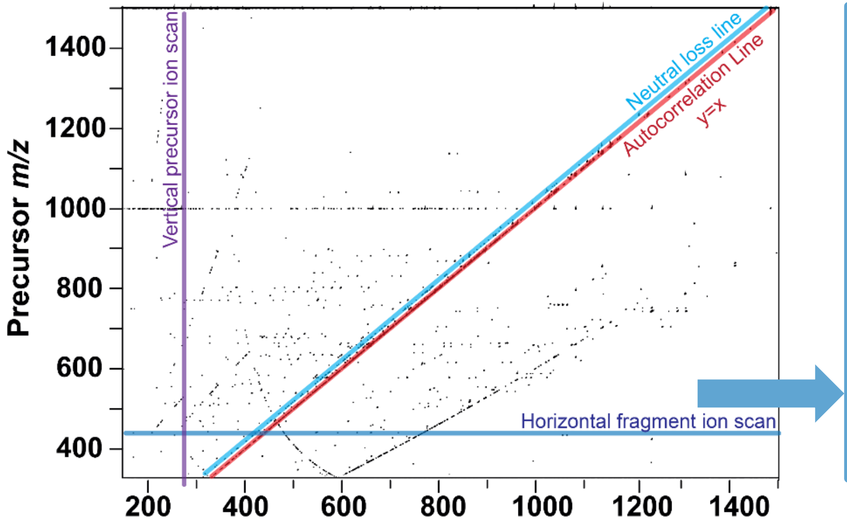

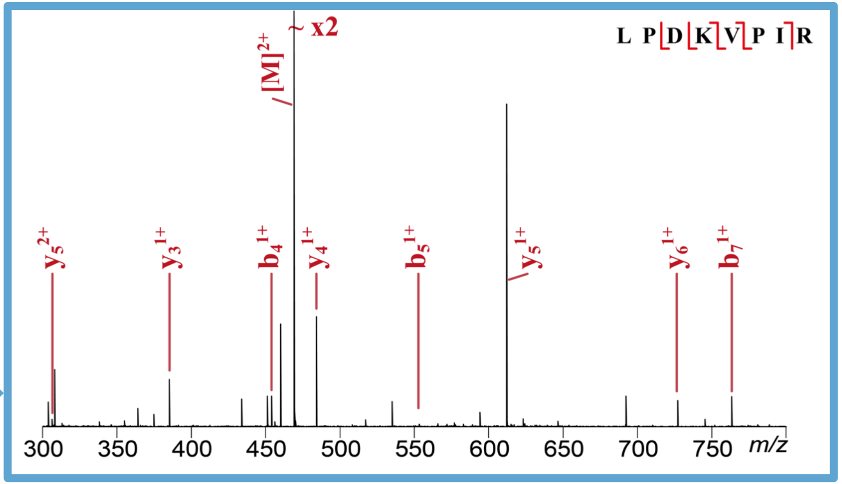

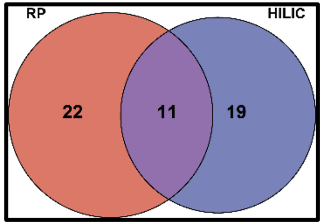

2DMS of Venom Tryptic Digests (IRMPD)

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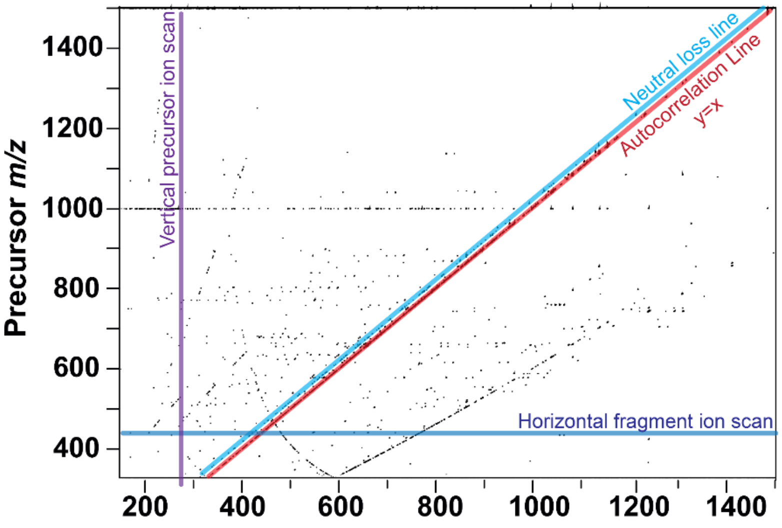

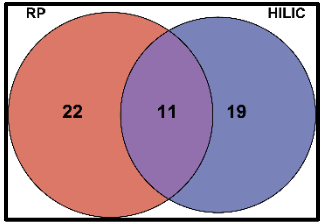

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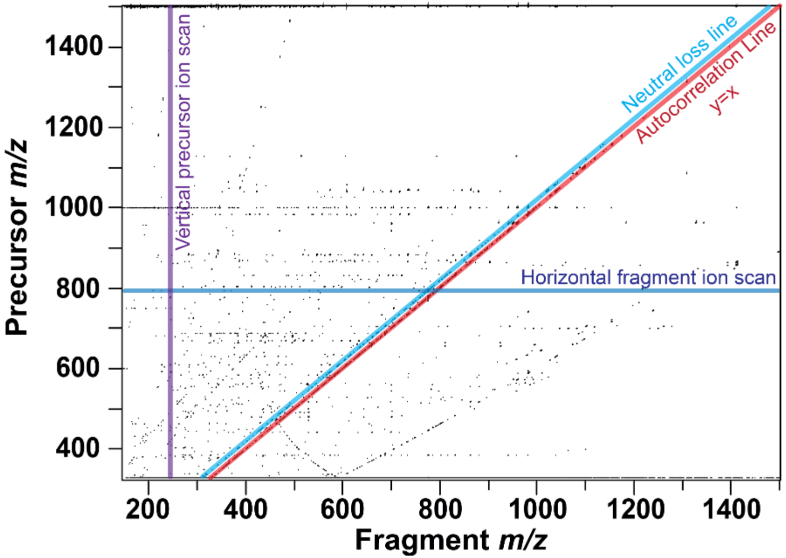

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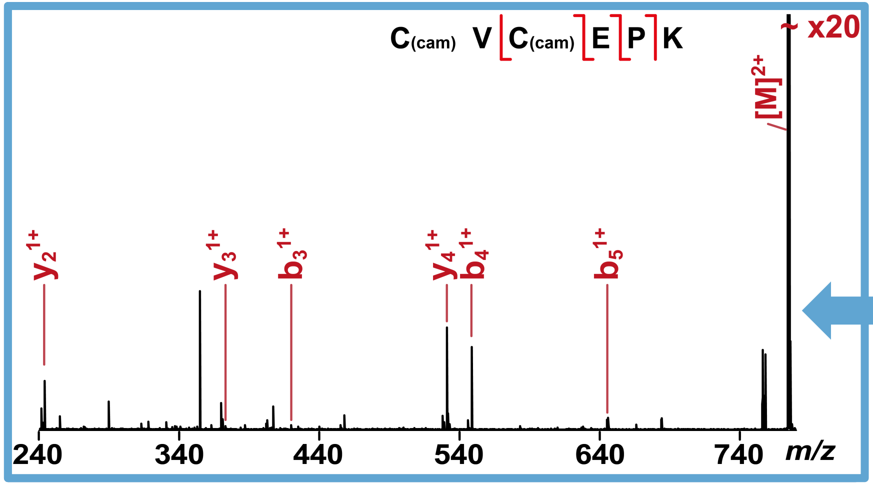

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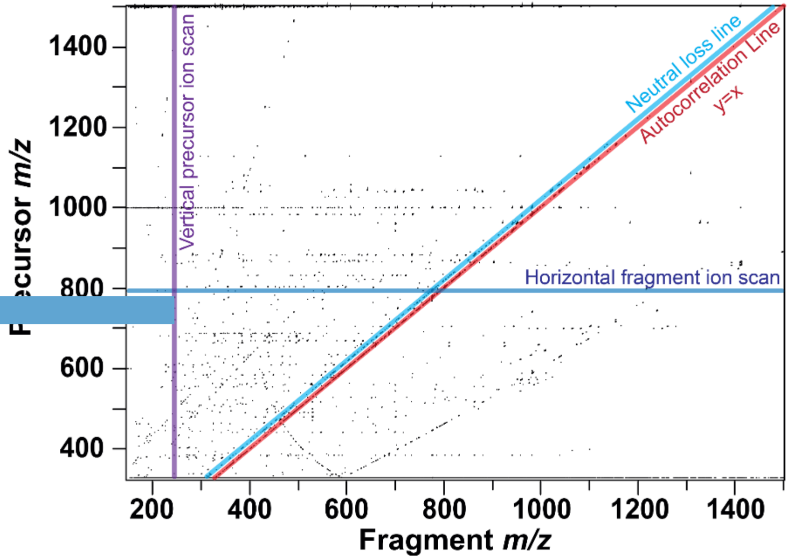
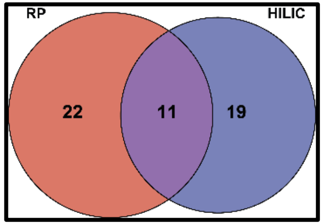
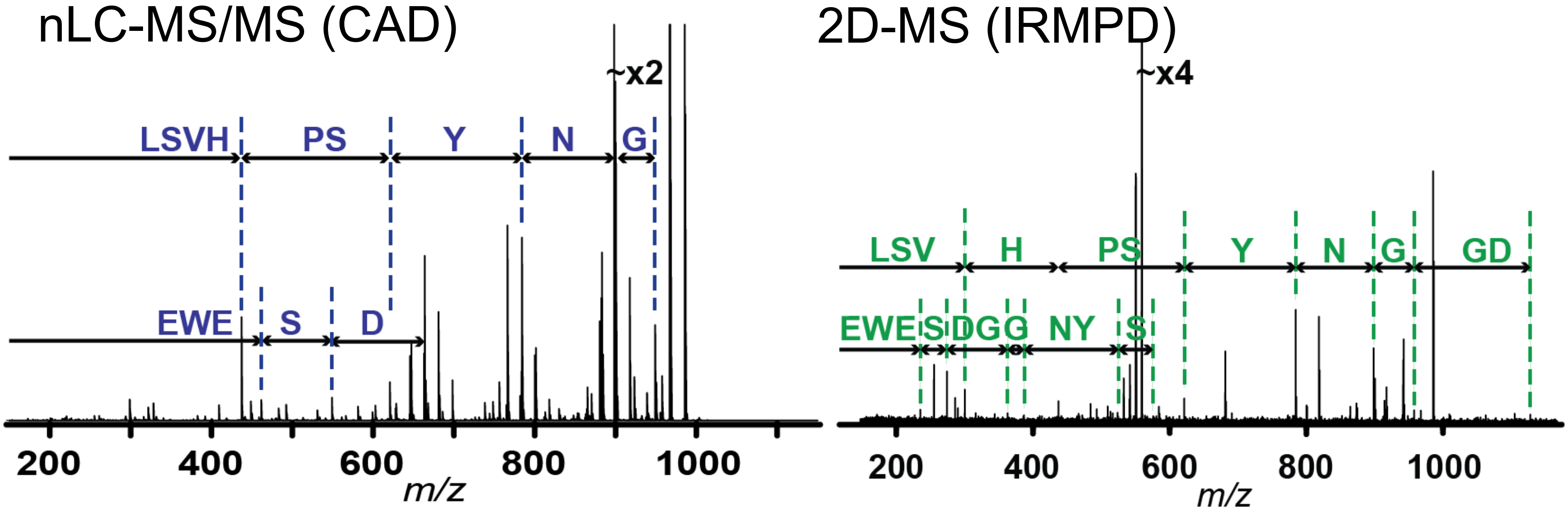




De Novo of Venom Peptide: nLC vs 2D

- Advantages of using both 2D and nLC

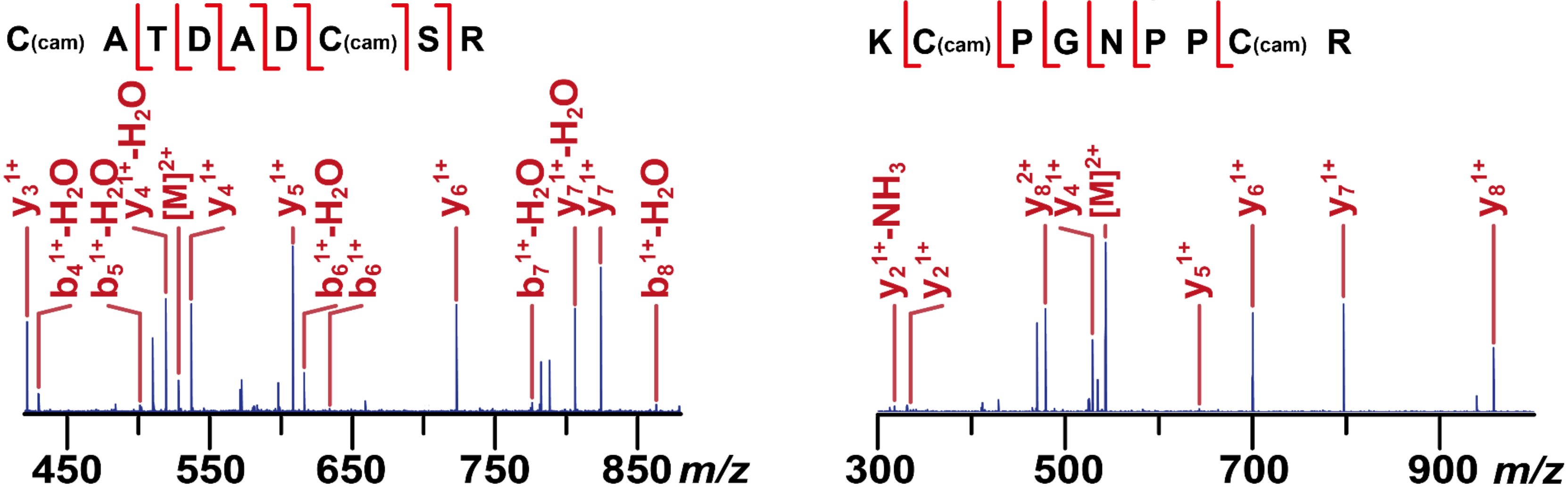


*amidated

Both techniques provide complementary information for de novo sequencing

Bottom-up Limitations

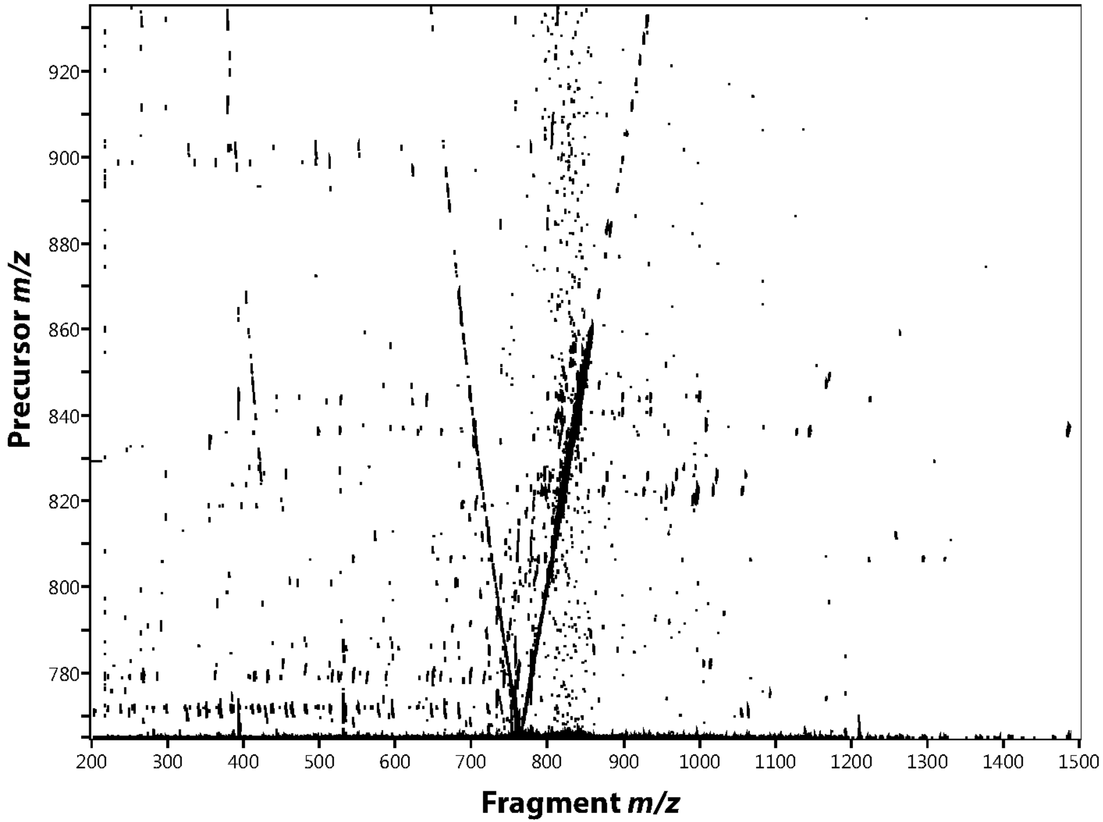
Difficult to tell which peptide come from which undigested proteins



<u>Possible Proteins</u>

I3Y3N9: T P F AIR CATDADCSR KCPGNPPCR NG F CACT
G9BNN9: T P F EVR CATDADCSR KCPGNPPCR NG L CACT
G9BNP2: T R F AIR CATDADCSR KCPGNPPCR NG F CACT
Q9BJX2 :T P F EVR CATDADCSR KCPGNPPCR NG F CACT
Q95NK7: T P F AIK CATDADCSR KCPGNPPCR NG F CACT

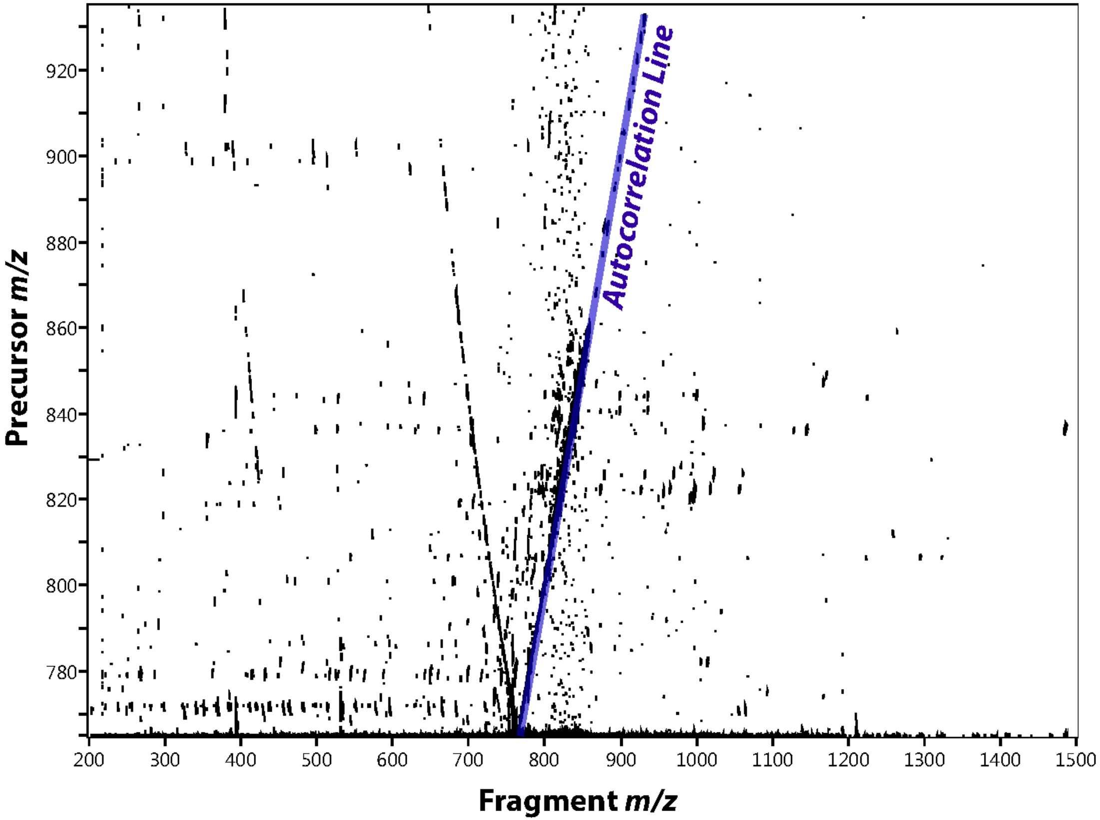
2DMS of Crude Venom Proteins (Top-down)



Advantages：

- Direct infusion
- Mass range isolation is only needed to obtain better dynamic range
- Acquire all fragments in 1 spectrum
- Acquisition: 3 hours
- 0.25 mg/mL sample
- 1M x 8192

2DMS of Crude Venom Proteins



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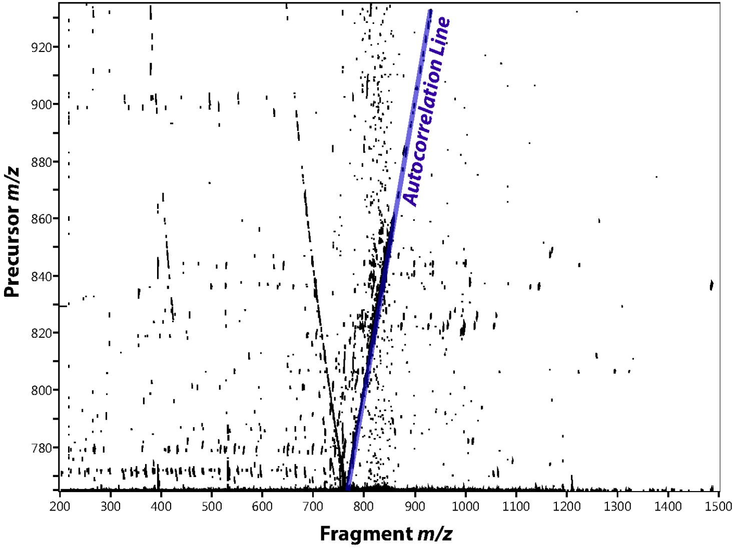
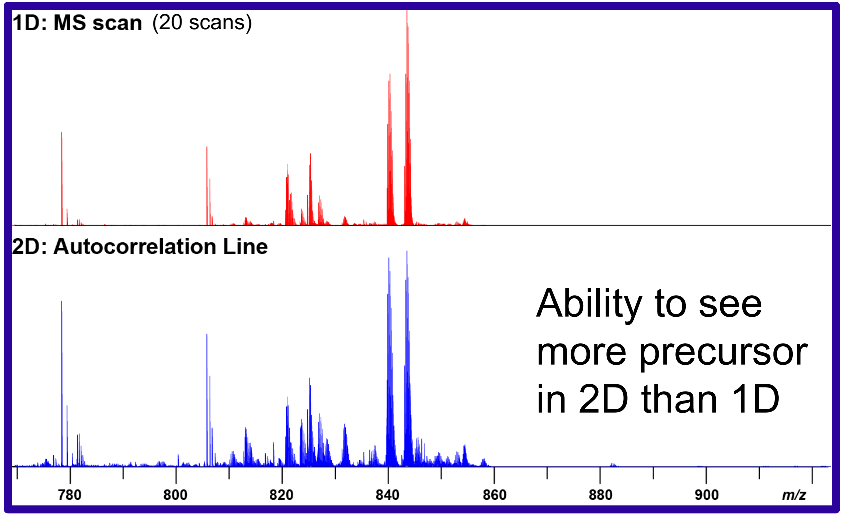
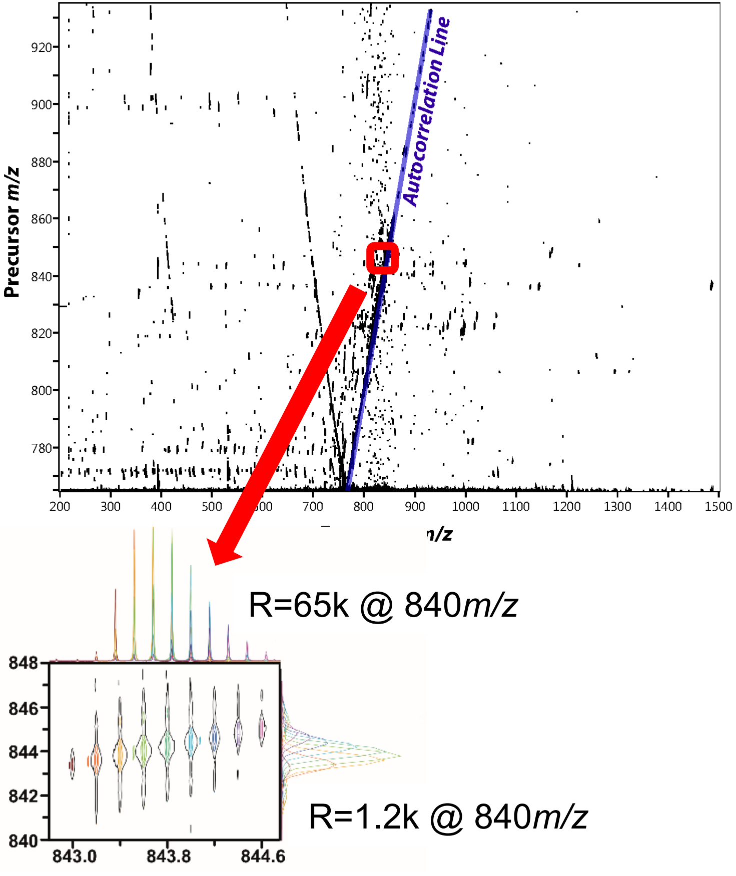
2DMS of Crude Venom Proteins





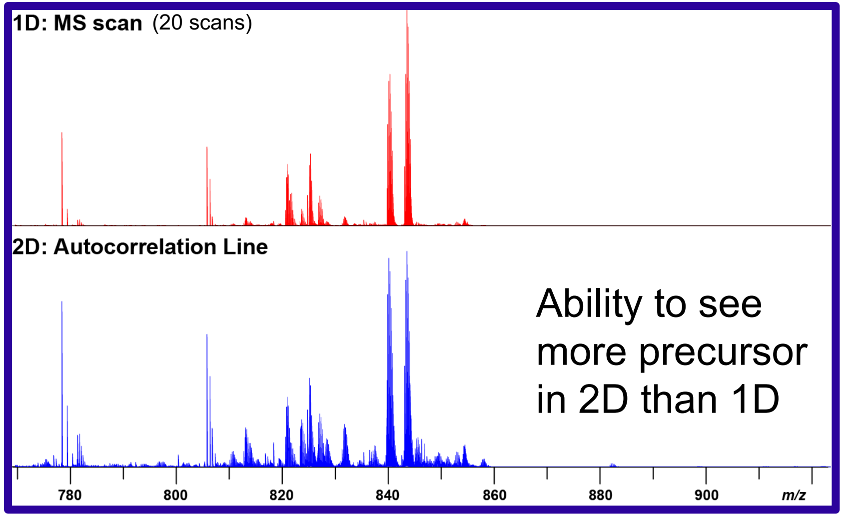
Ability to see more precursor in 2D than 1D

2DMS of Crude Venom Proteins



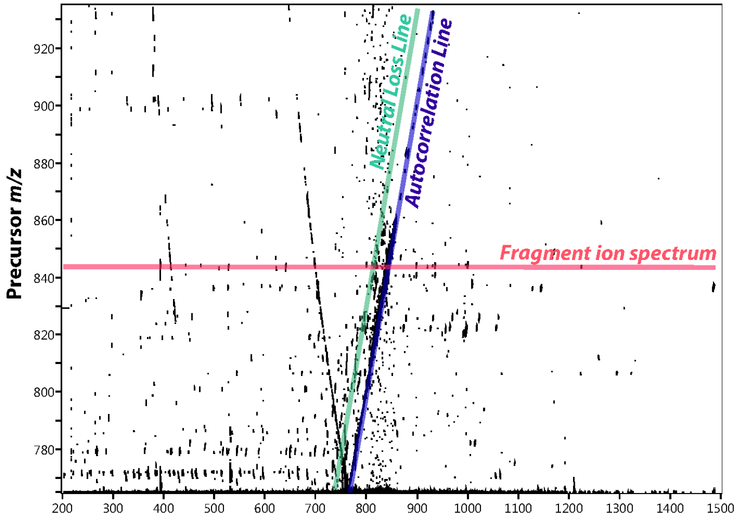
R=65k @ 840*m/z*

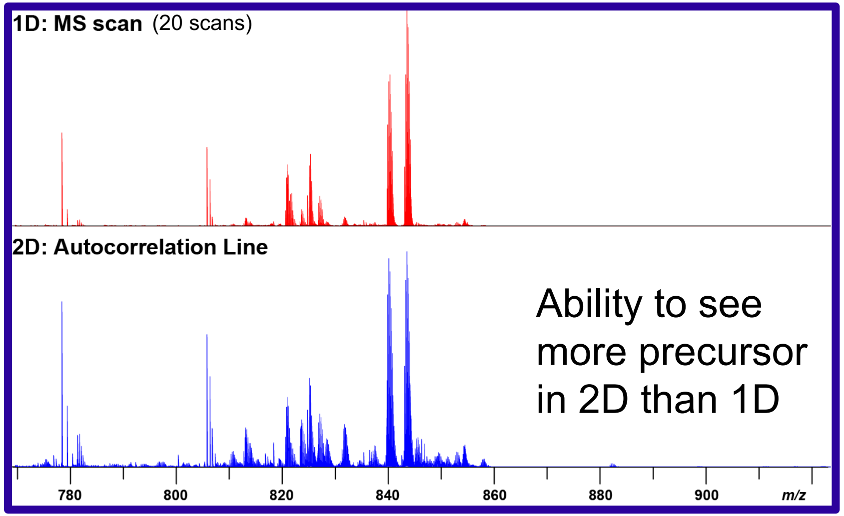
R=1.2k @ 840*m/z*



Ability to see more precursor in 2D than 1D

2DMS of Crude Venom Proteins





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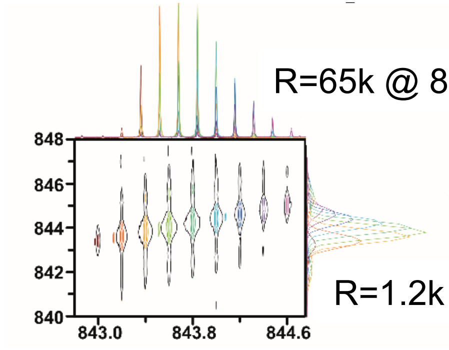

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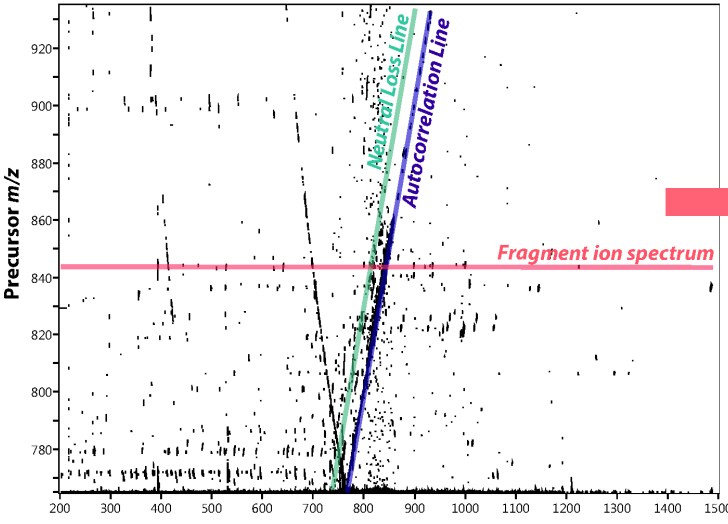
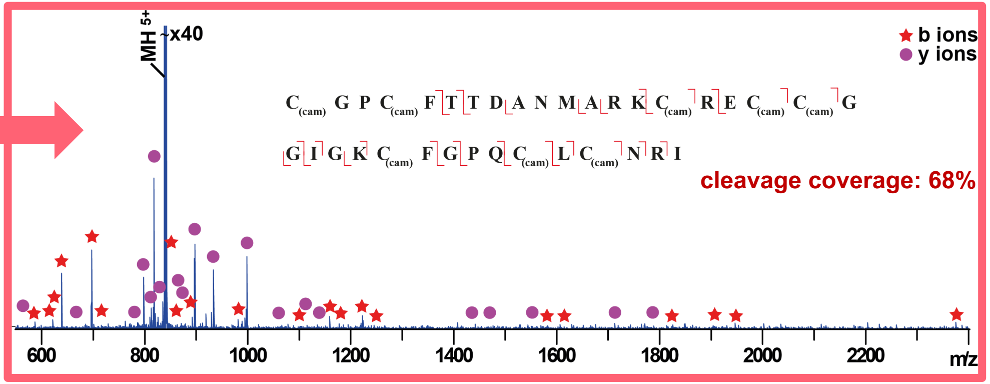
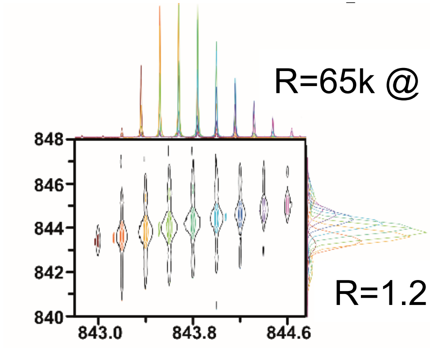
2DMS of Crude Venom Proteins



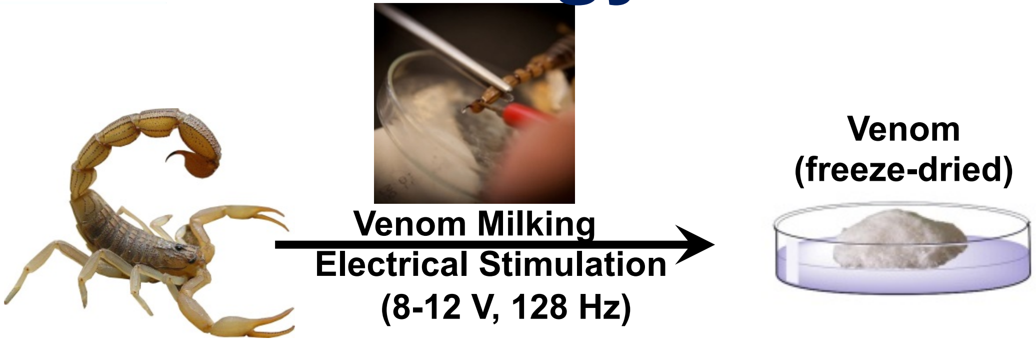




R=65k @ 840*m/z*

R=1.2k @ 840*m/z*

Methodology of obtaining active species



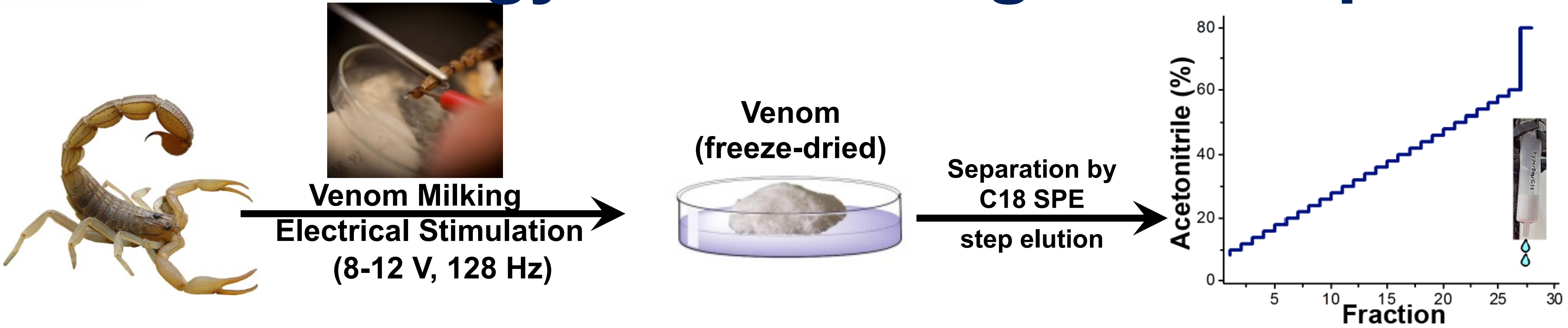
Venom Milking

Electrical Stimulation

(8-12 V, 128 Hz)

Venom
(freeze-dried)

Methodology of obtaining active species



Venom Milking
Electrical Stimulation
(8-12 V, 128 Hz)

Venom
(freeze-dried)

Separation by
C18 SPE
step elution

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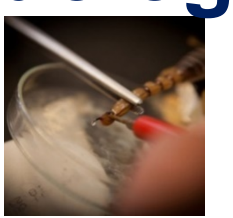

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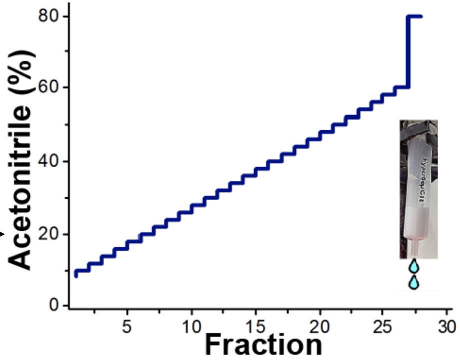
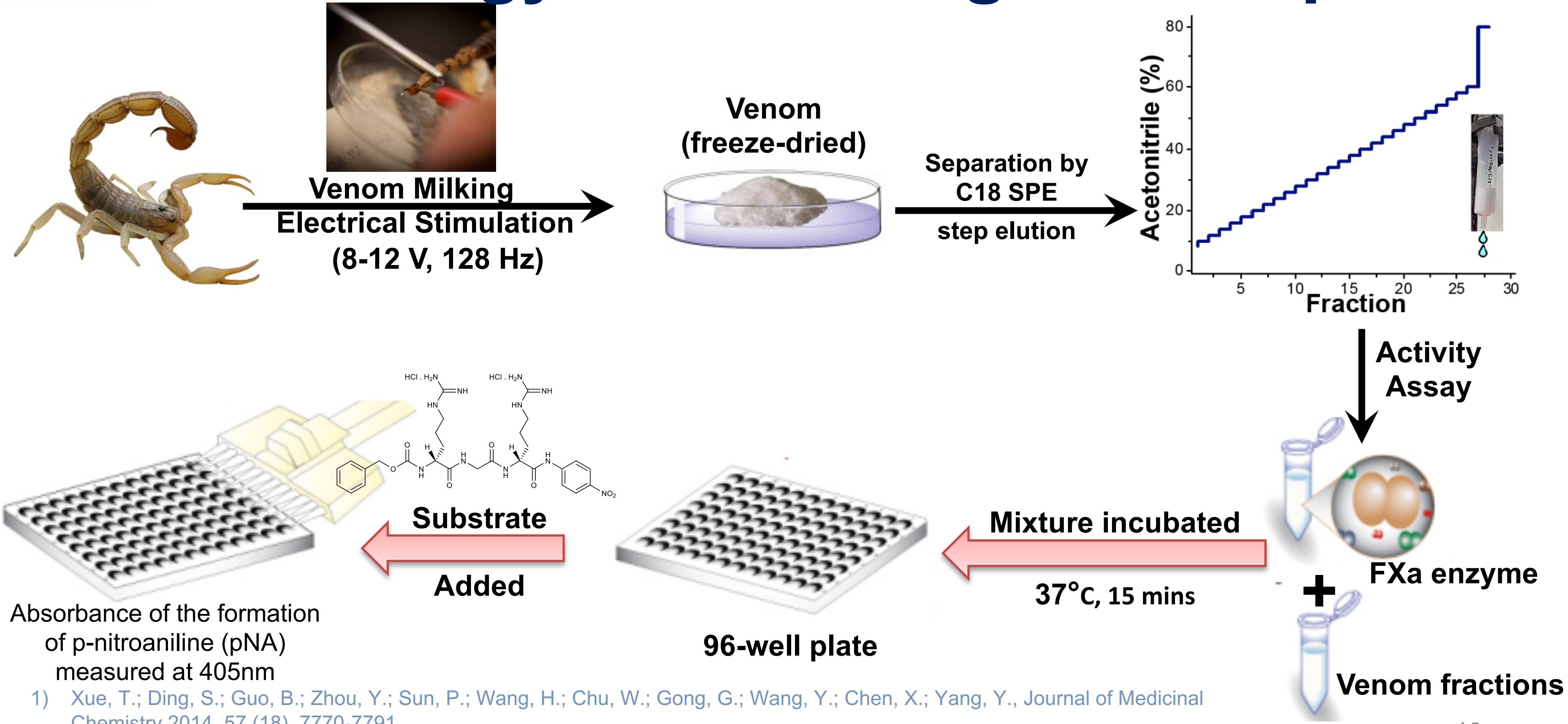


Activity
Assay

Methodology of obtaining active species



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Electrical Stimulation
(8-12 V, 128 Hz)

Venom
(freeze-dried)

Separation by
C18 SPE
step elution

Activity Assay

FXa enzyme

+

Venom fractions

Mixture incubated

37°C, 15 mins

96-well plate

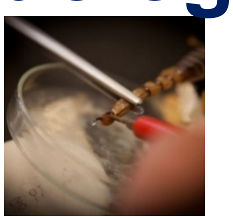
Substrate

Added

Absorbance of the formation of p-nitroaniline (pNA) measured at 405nm

1) Xue, T.; Ding, S.; Guo, B.; Zhou, Y.; Sun, P.; Wang, H.; Chu, W.; Gong, G.; Wang, Y.; Chen, X.; Yang, Y., Journal of Medicinal Chemistry 2014, 57 (18), 7770-7791.

Methodology of obtaining active species



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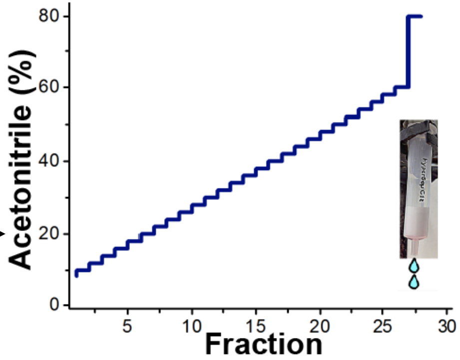
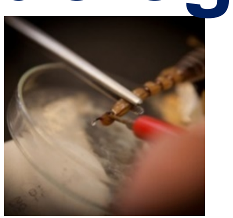


Activity
Assay

Methodology of obtaining active species



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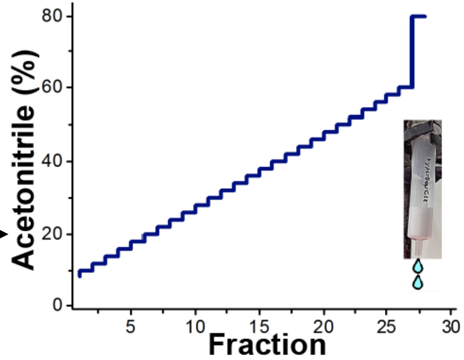

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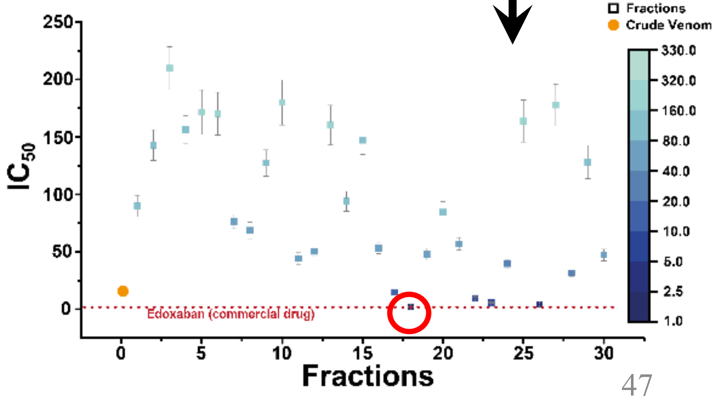
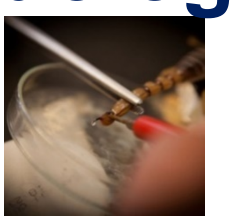


Methodology of obtaining active species



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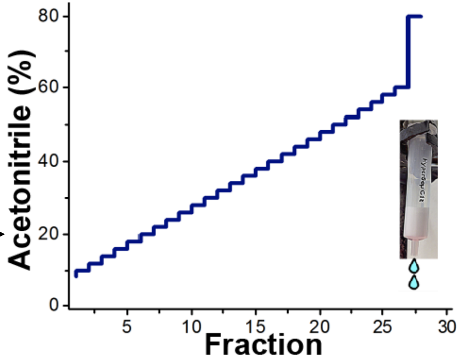
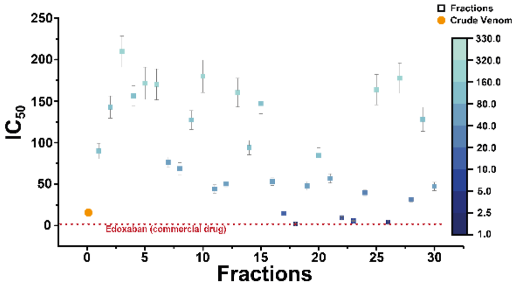
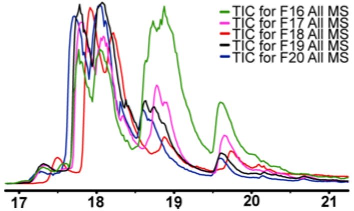


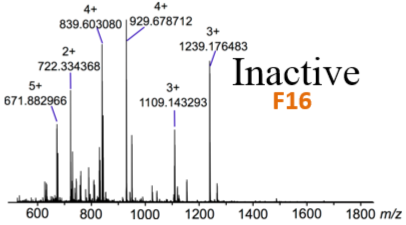
Activity
Assay

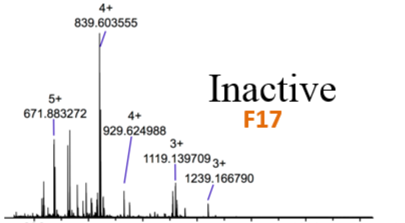


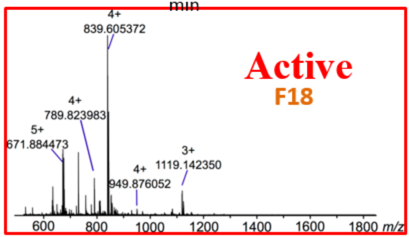
Effective gradient: 10 minutes

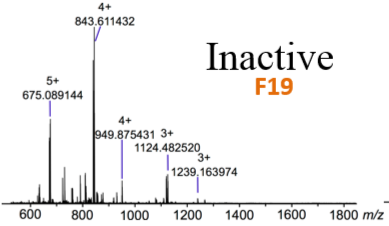
Column: RP C18

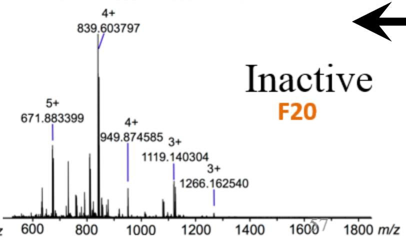












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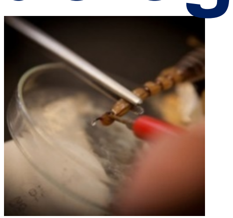
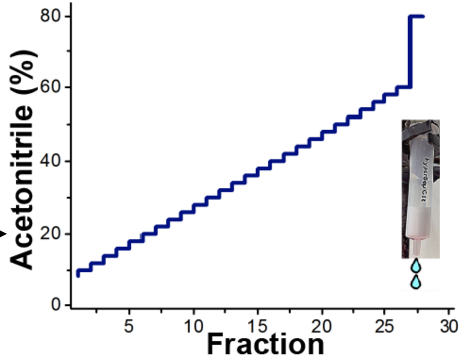


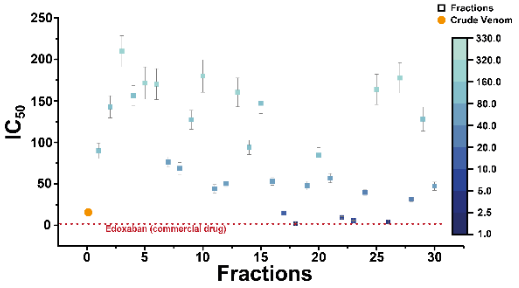
Venom Milking
Electrical Stimulation
(8-12 V, 128 Hz)

Venom
(freeze-dried)

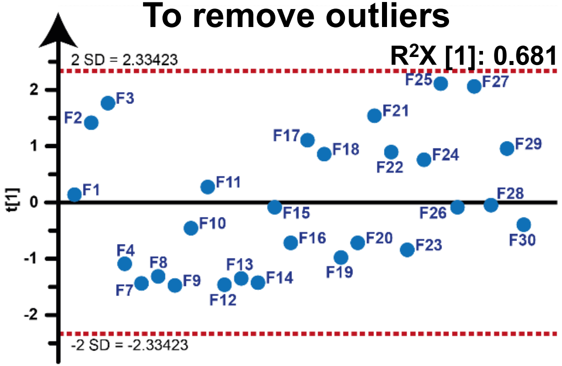
Separation by
C18 SPE
step elution



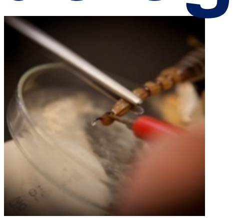
Activity
Assay



PCA:
To remove outliers



Methodology of obtaining active species



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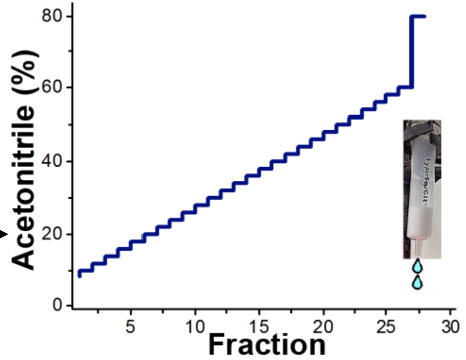
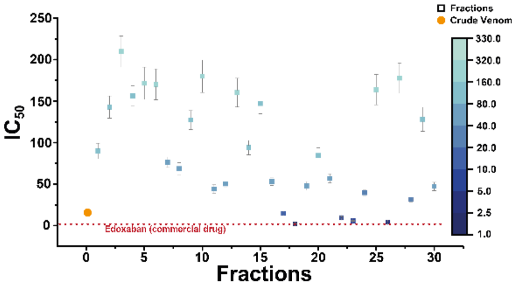


Activity
Assay



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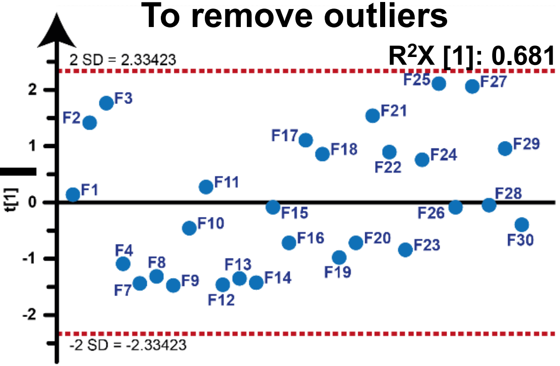
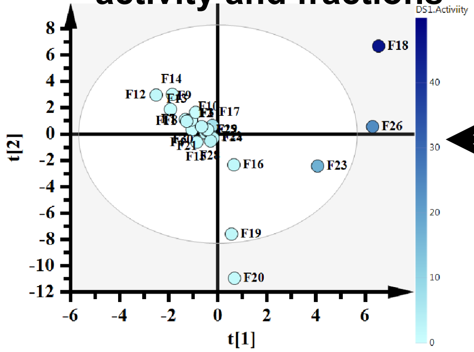
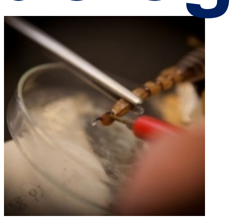


PLS:
To find correlation between activity and fractions



Methodology of obtaining active species



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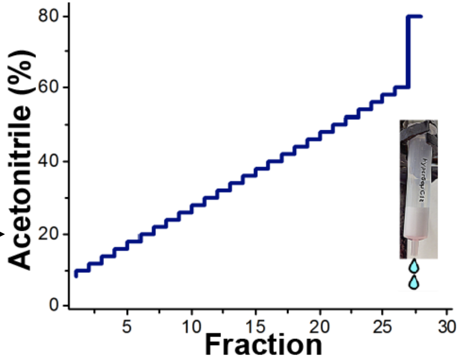
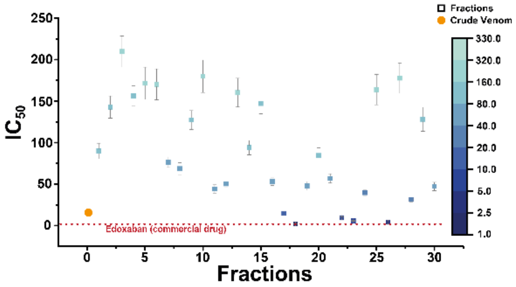


Activity
Assay



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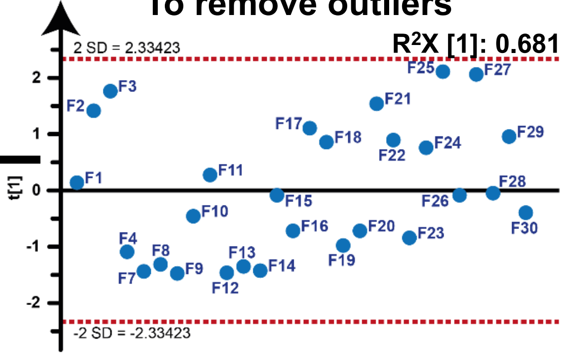

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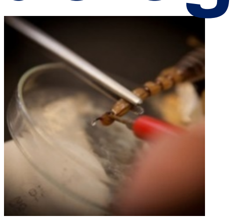

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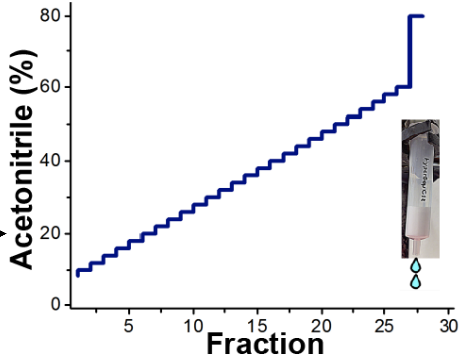
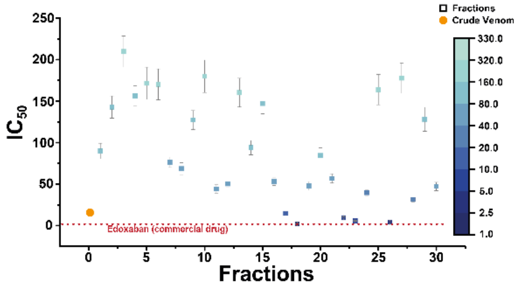


Activity
Assay



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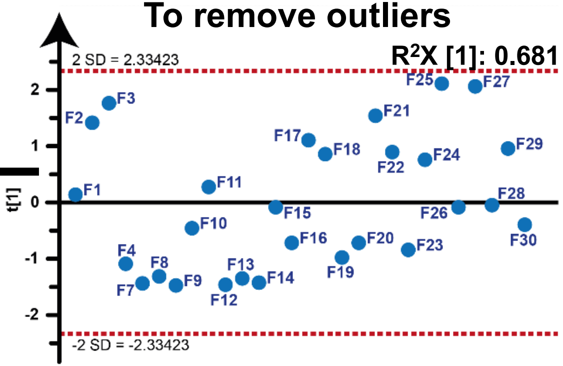

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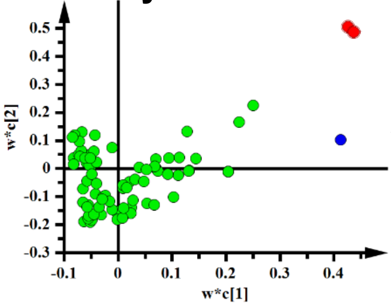

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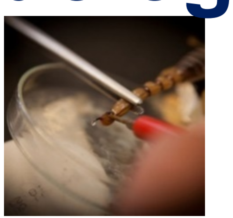

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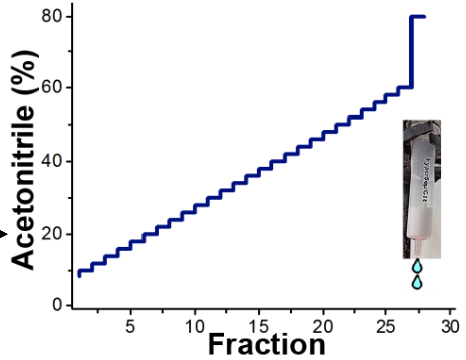
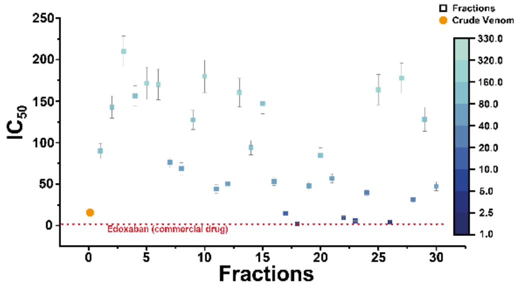


Activity
Assay



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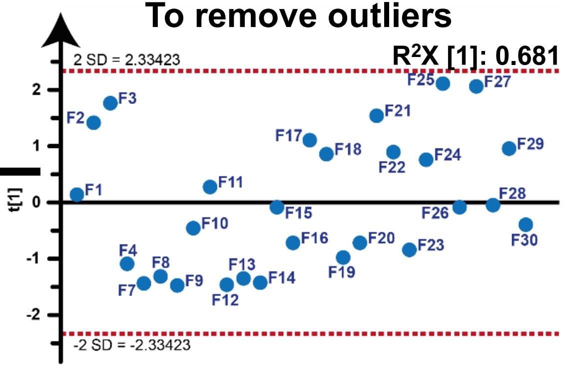

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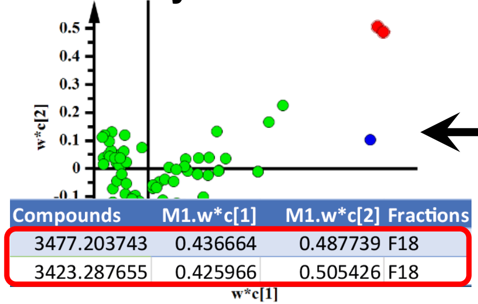
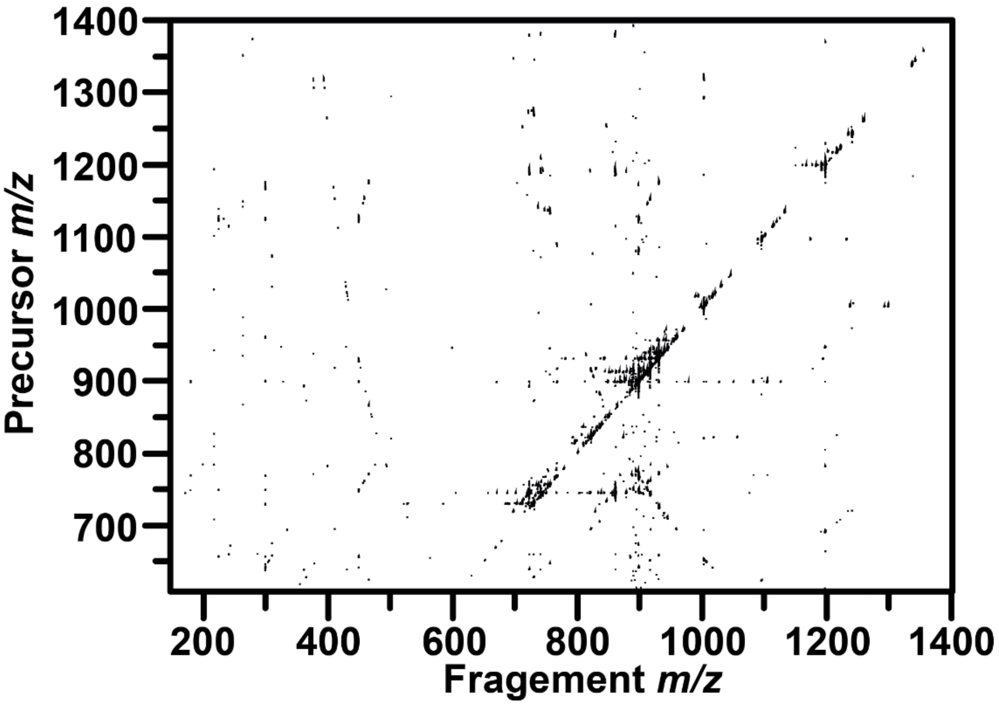


Compounds	M1.w*c[1]	M1.w*c[2]	Fractions
3477.203743	0.436664	0.487739	F18
3423.287655	0.425966	0.505426	F18

2DMS of potential active species (IRMPD)

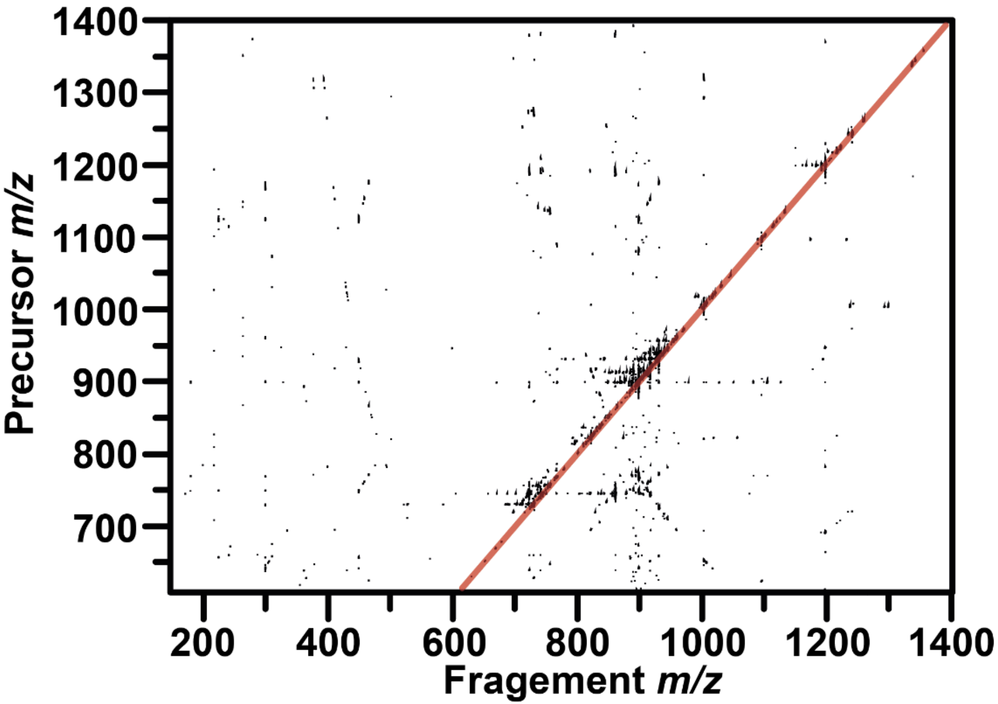
- Application of 2D with unknown sequence



- 2DMS of HPLC fraction
- 1M x 4096
- Acquisition time: 1.5 hours
- Processing time: 2.5 hours

2DMS of potential active species (IRMPD)

- Application of 2D with unknown sequence



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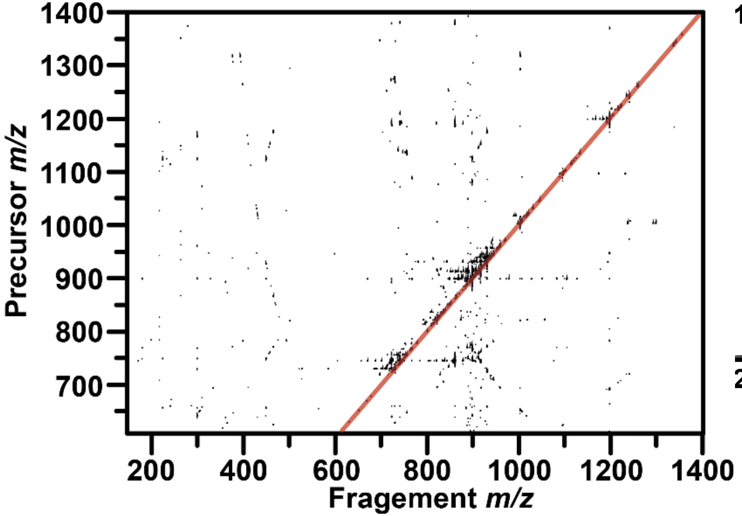
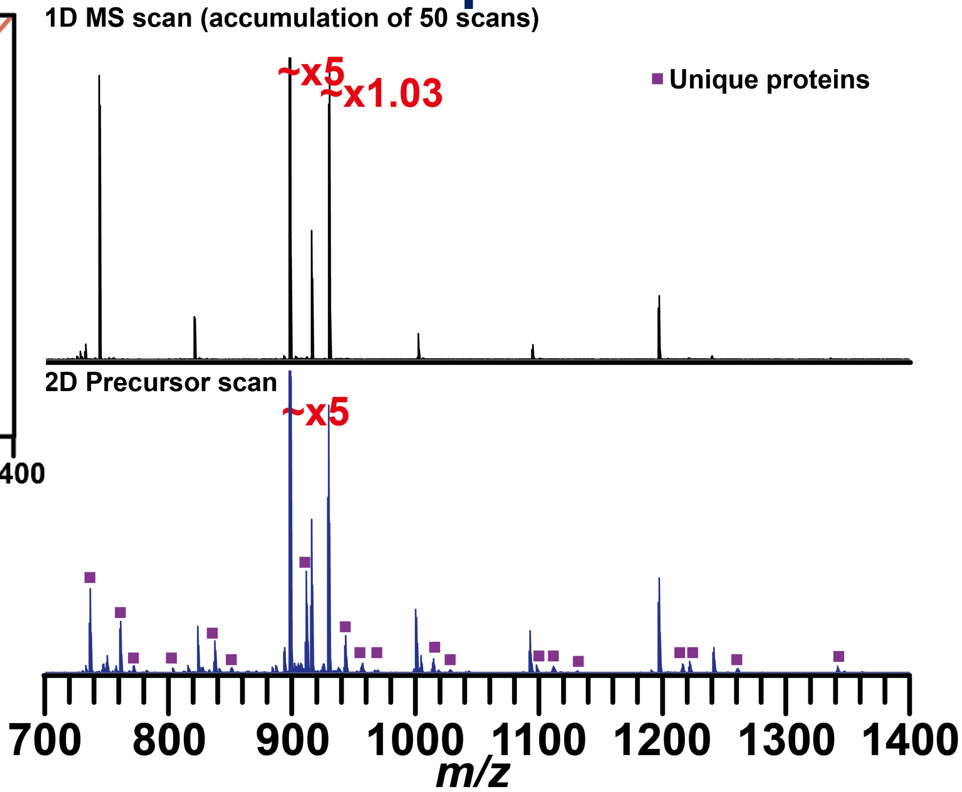
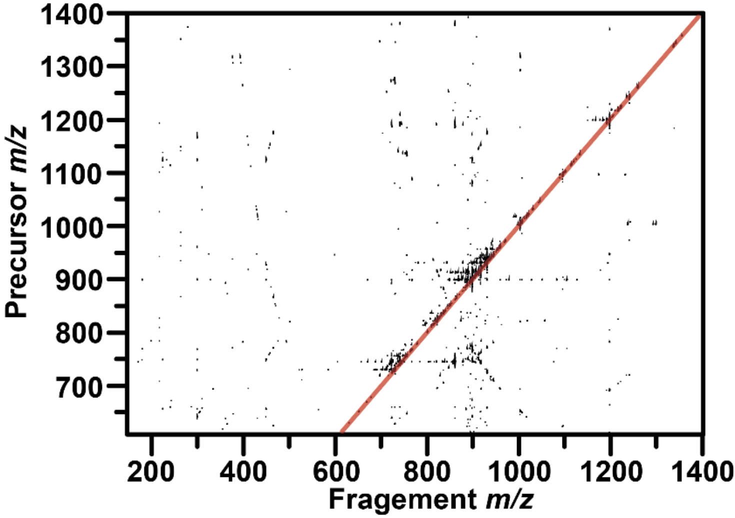


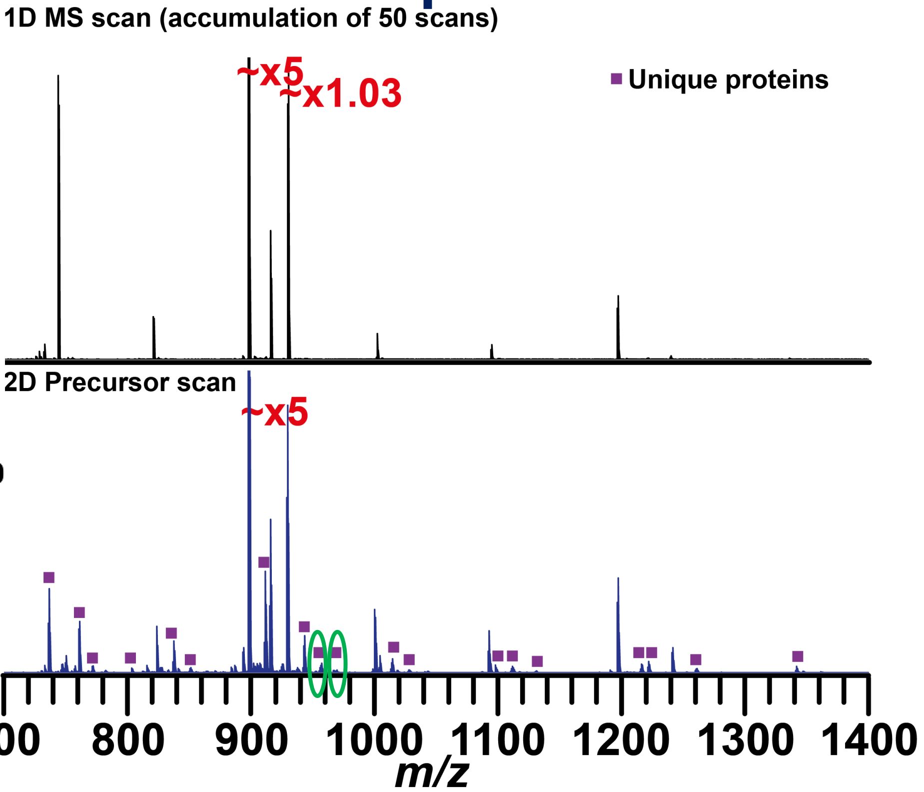

1D MS scan (accumulation of 50 scans)

2D Precursor scan

2DMS of potential active species (IRMPD)

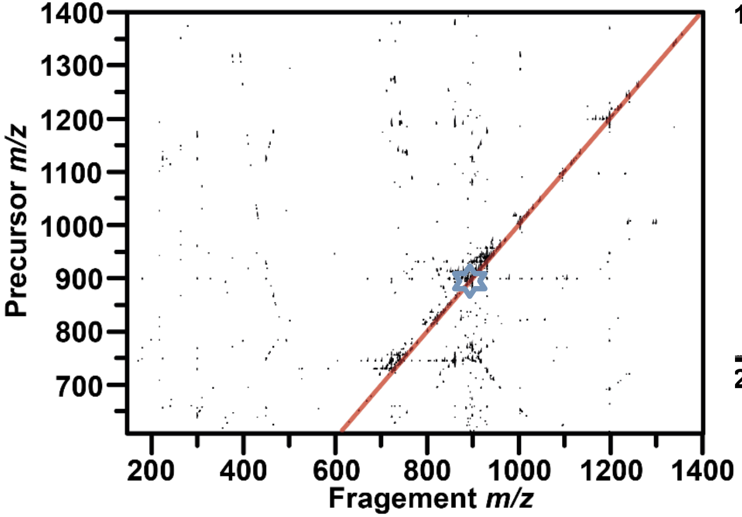
- Application of 2D with unknown sequence

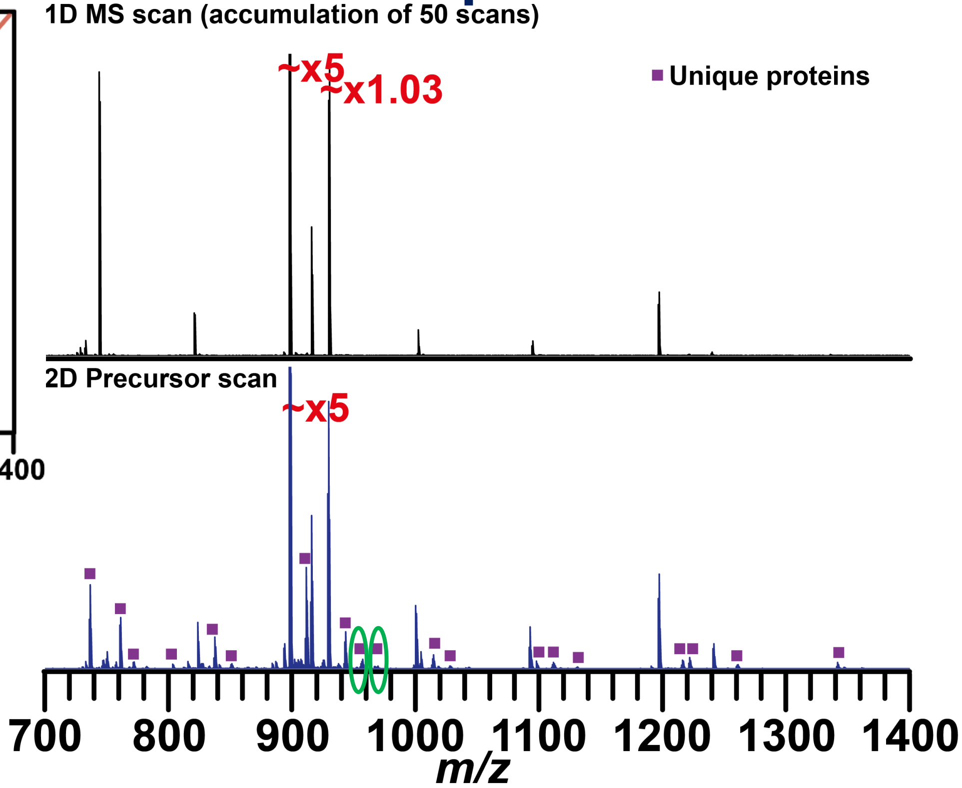




2DMS of potential active species (IRMPD)

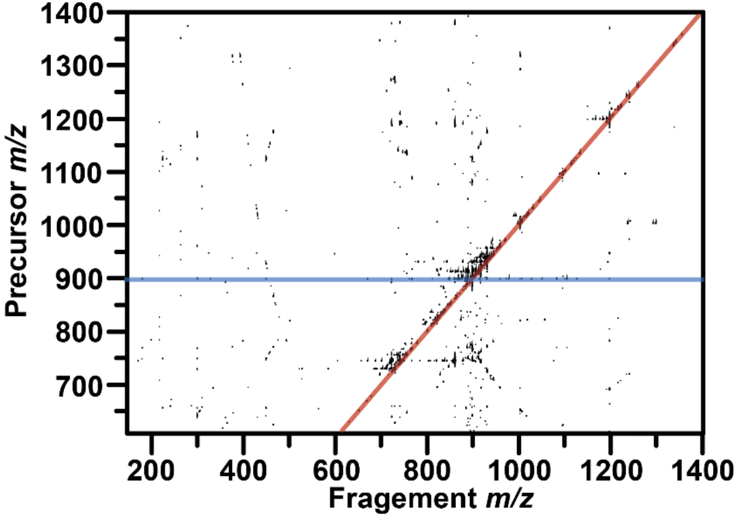
- Application of 2D with unknown sequence



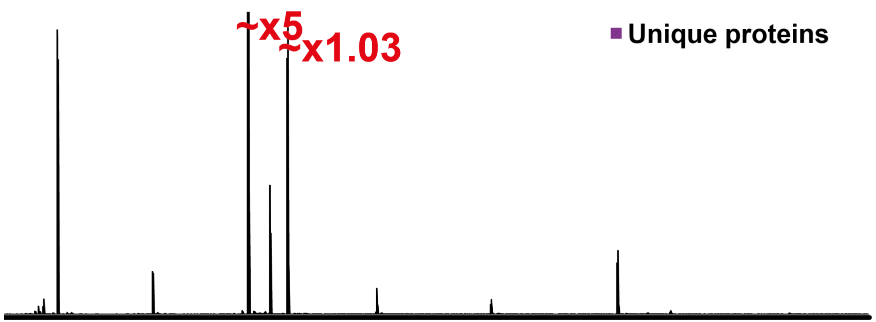


2DMS of potential active species (IRMPD)

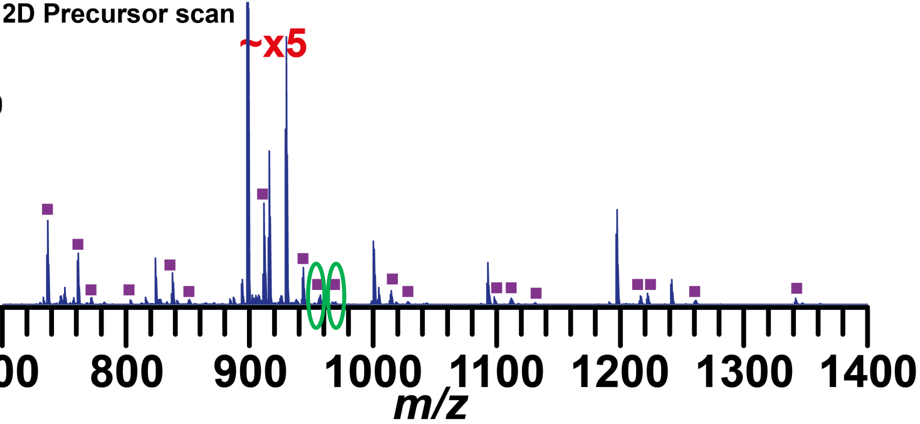
- Application of 2D with unknown sequence



1D MS scan (accumulation of 50 scans)

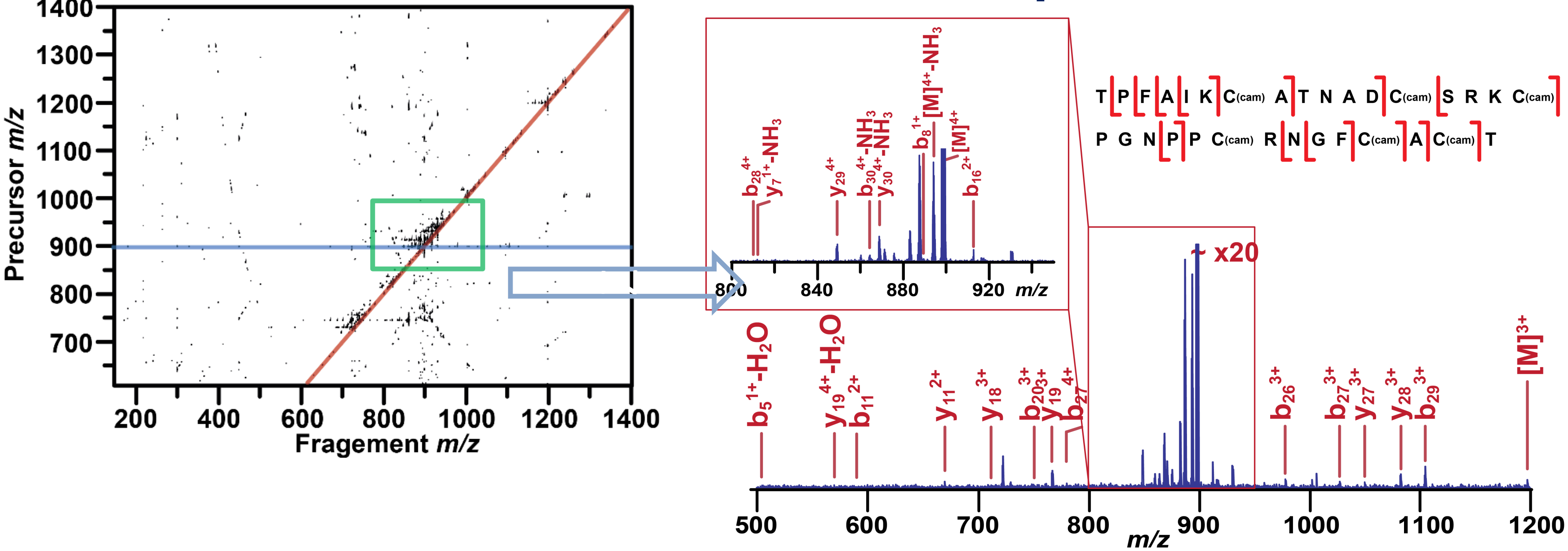


2D Precursor scan



2DMS of potential active species (IRMPD)

- Application of 2D with unknown sequence



2DMS of potential active species (IRMPD)

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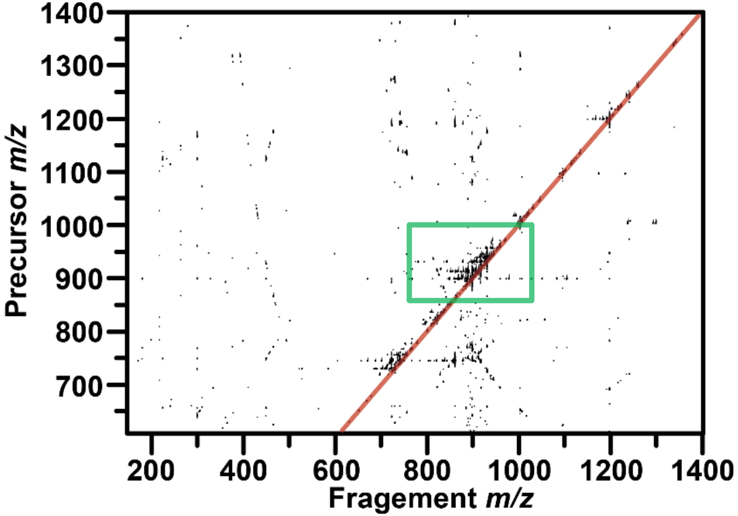
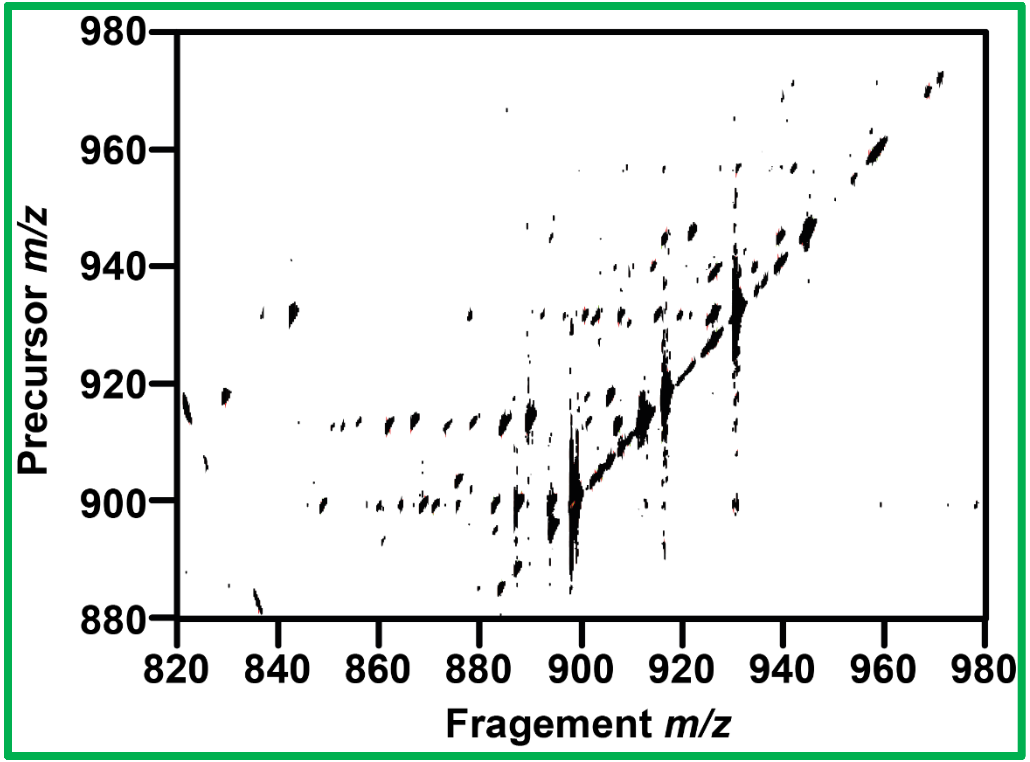
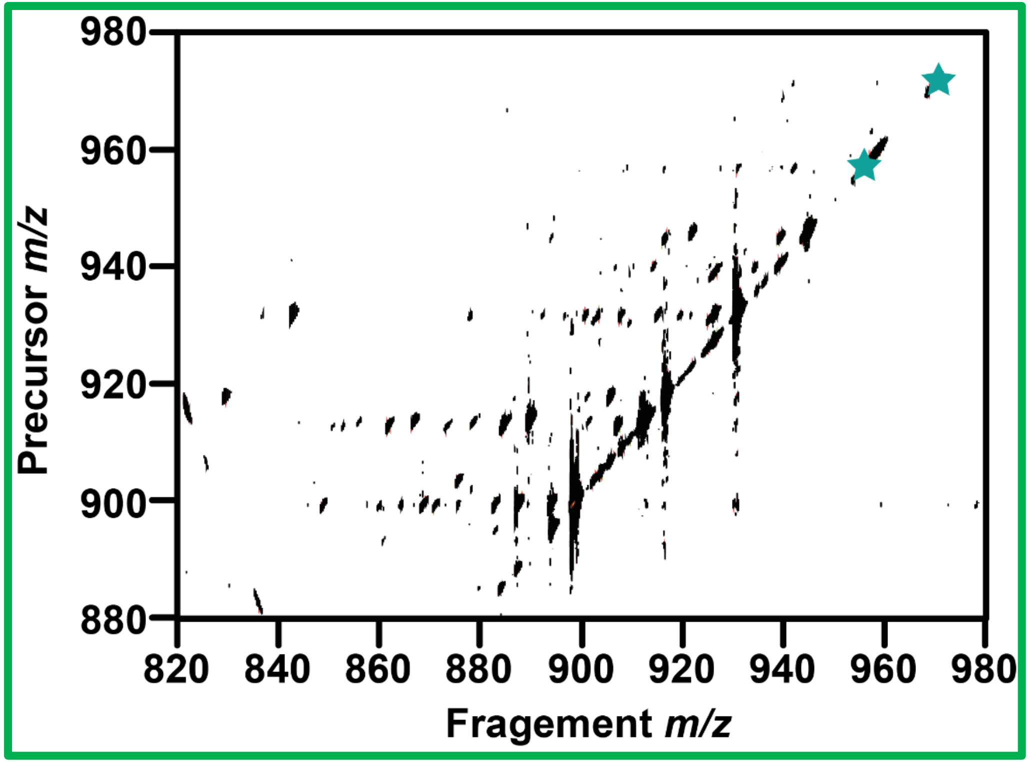




2DMS of potential active species (IRMPD)

- Application of 2D with unknown sequence

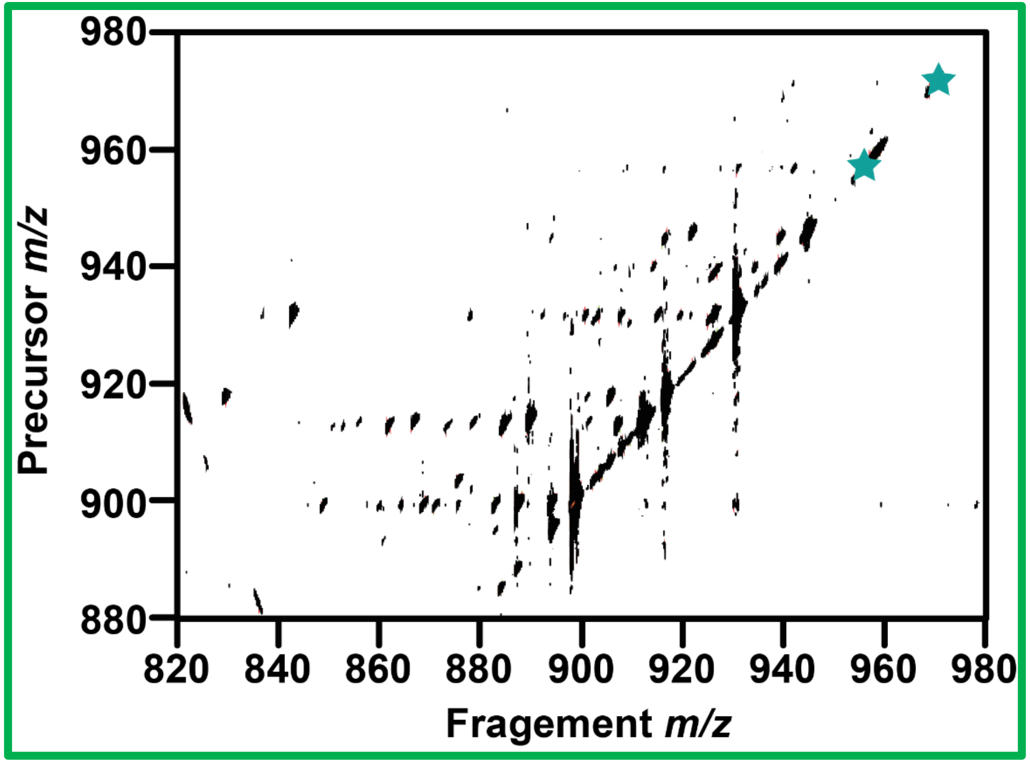
- Target species(★)



2DMS of potential active species (IRMPD)

- Application of 2D with unknown sequence

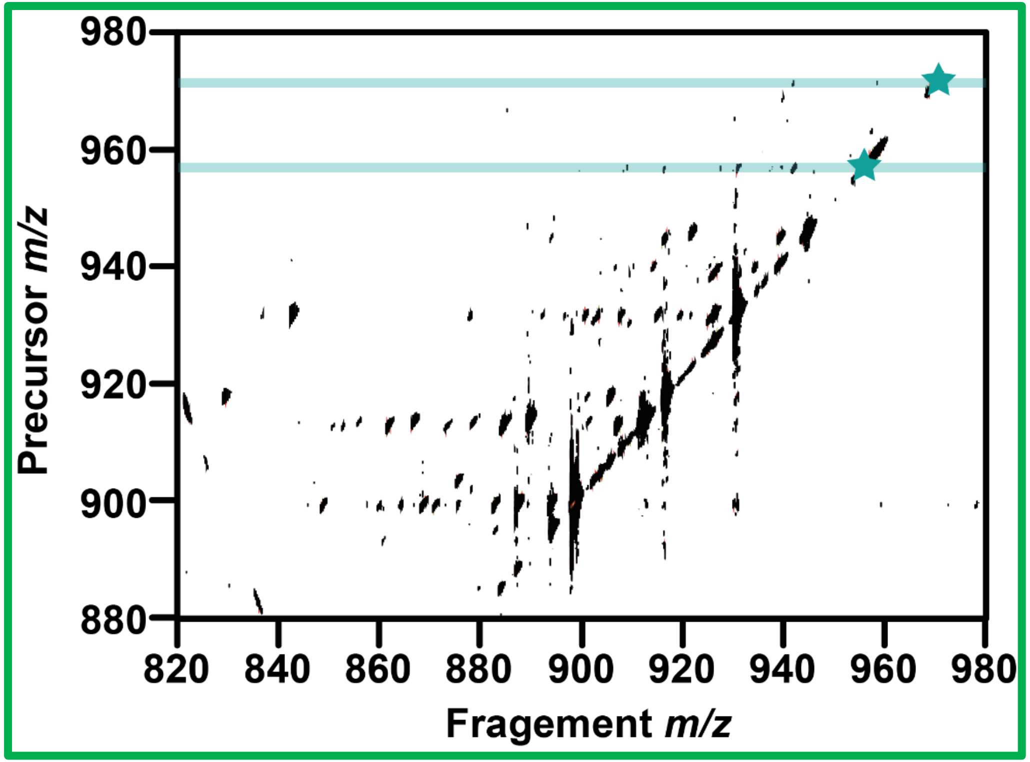
- Target species(★)
- 971*m/z* and 955 *m/z*



2DMS of potential active species (IRMPD)

- Application of 2D with unknown sequence

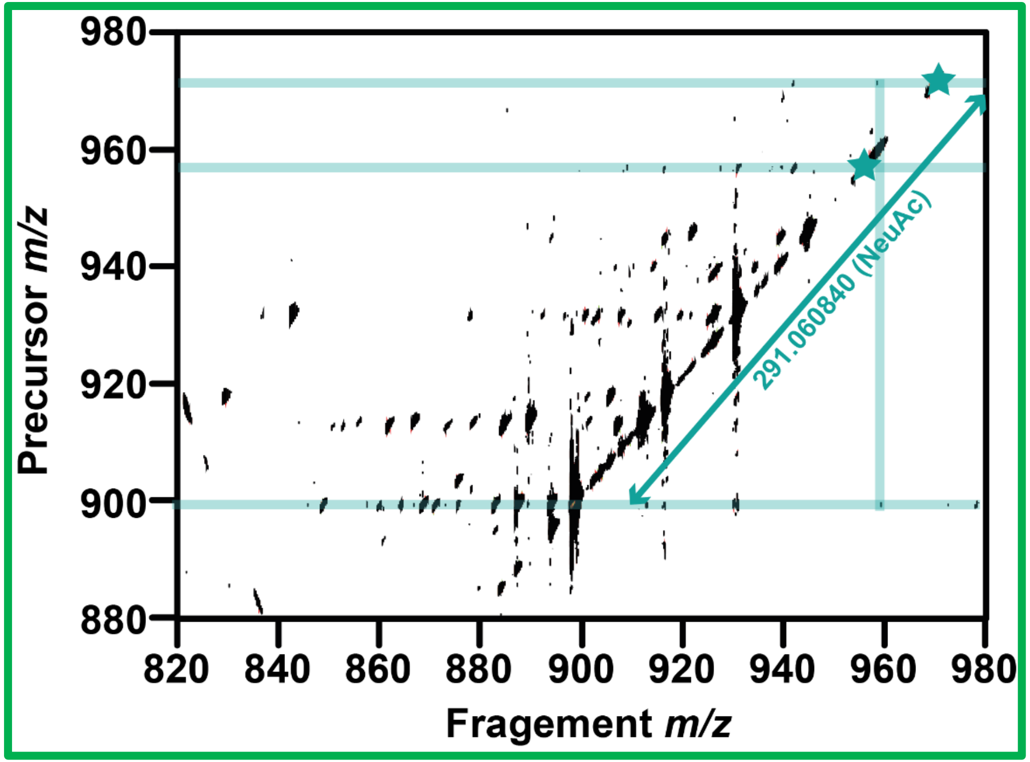
- Target species(★)
- 971*m/z* and 955 *m/z*



2DMS of potential active species (IRMPD)

- Application of 2D with unknown sequence

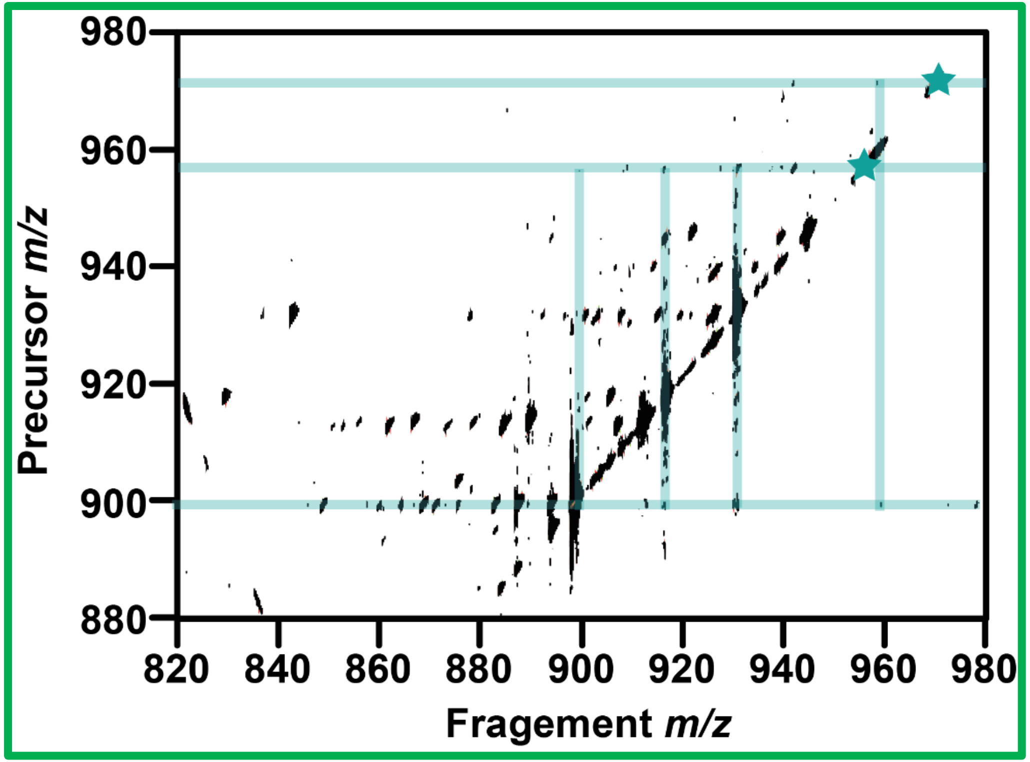
- Target species( ) ★
- 971 *m/z* and 955 *m/z*
- 971 *m/z* vs 897 *m/z*
  - Δmass: 291.060840
  - NeuAc



2DMS of potential active species (IRMPD)

- Application of 2D with unknown sequence

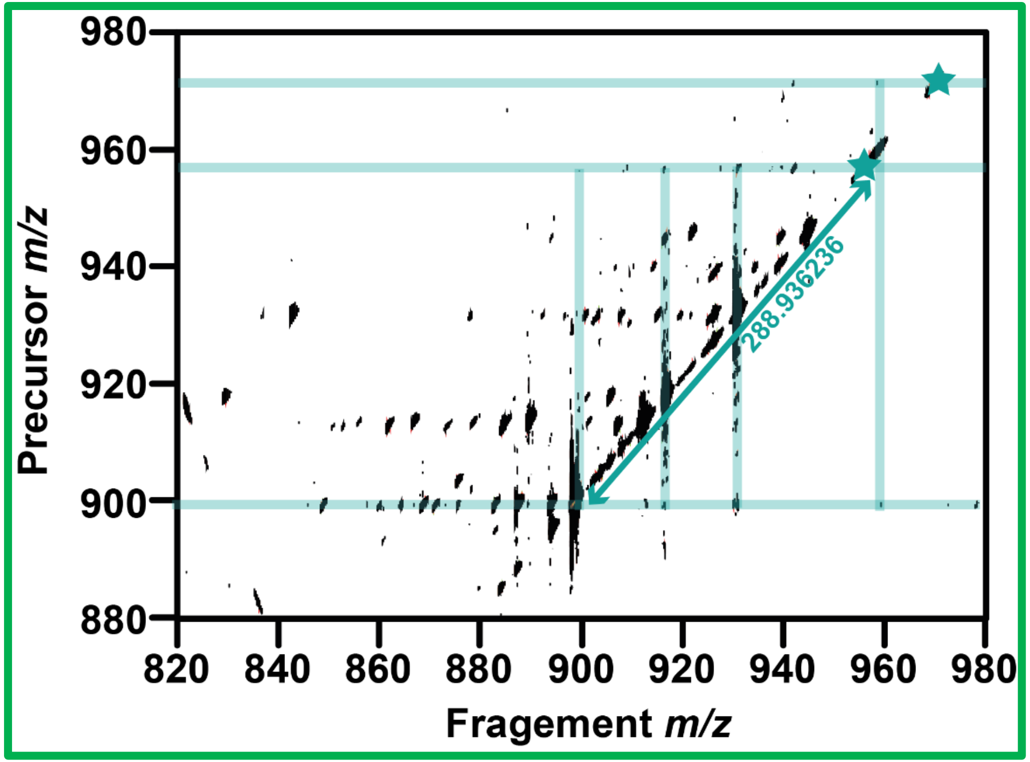
- Target species( ) ★
- 971 *m/z* and 955 *m/z*
- 971 *m/z* vs 897 *m/z*
  - Δmass: 291.060840
  - NeuAc
- 955 *m/z* has very similar fragmentation pattern to 897 *m/z*



2DMS of potential active species (IRMPD)

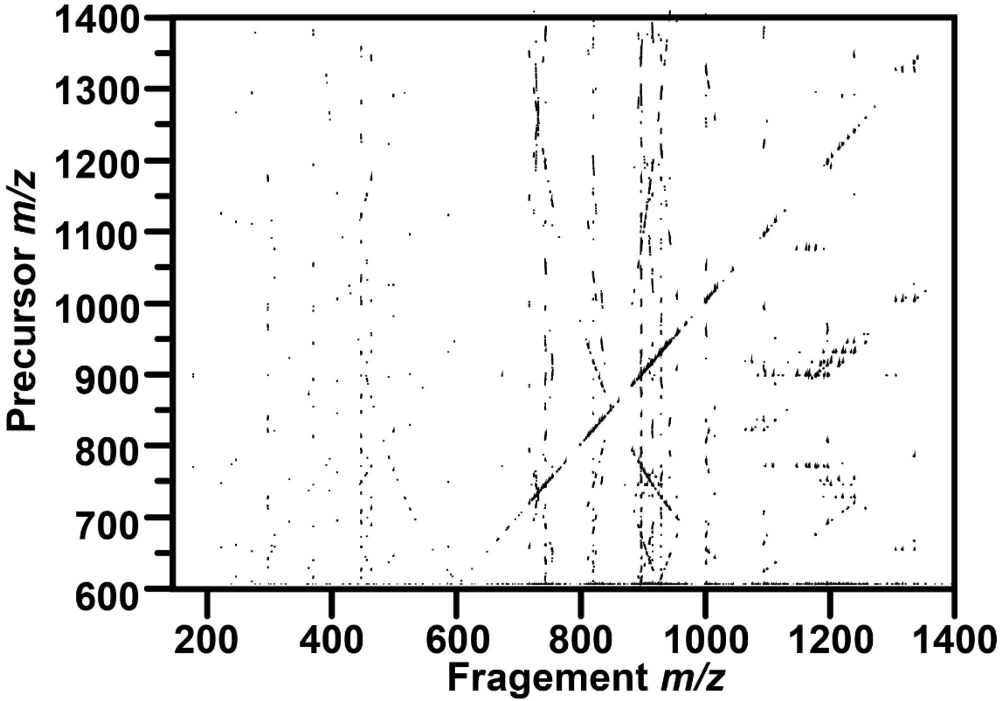
- Application of 2D with unknown sequence

- Target species(★)
- 971 *m/z* and 955 *m/z*
- 971 *m/z* vs 897 *m/z*
  - Δmass: 291.060840
  - NeuAc
- 955 *m/z* has very similar fragmentation pattern to 897 *m/z*
  - *Maybe related species*
- 955 *m/z* vs 897 *m/z*
  - Δmass: 288.936236
  - No potential modification mass
  - Future works



2DMS of potential active species (ECD)

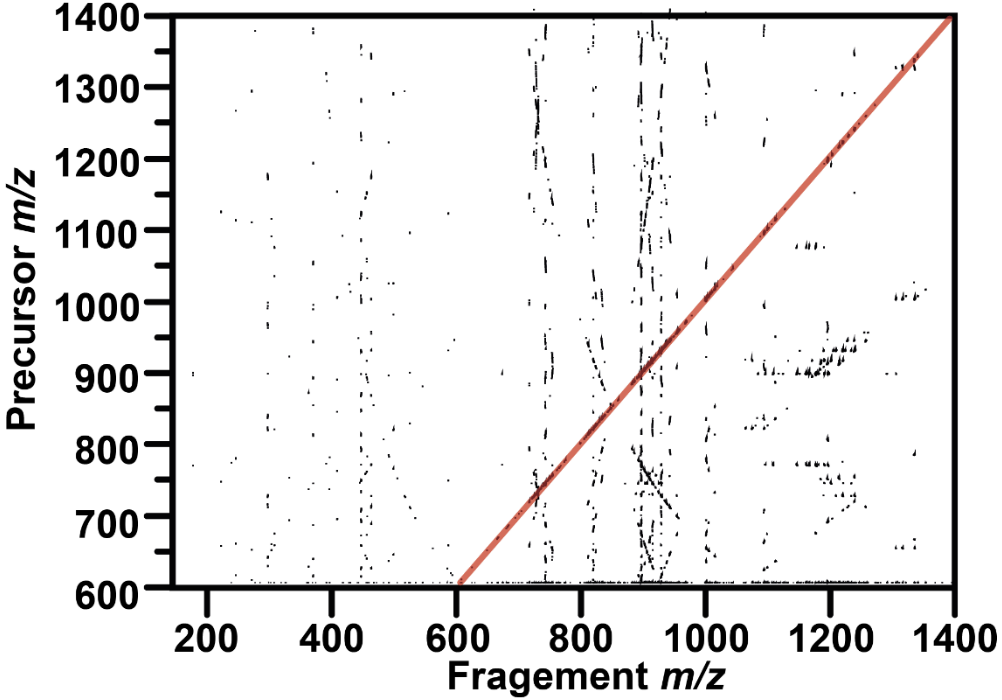
- Application of 2D with unknown sequence



- 2DMS of HPLC fraction
- 1M x 4096
- Acquisition time: 1.5 hours
- Processing time: 2.5 hours

2DMS of potential active species (ECD)

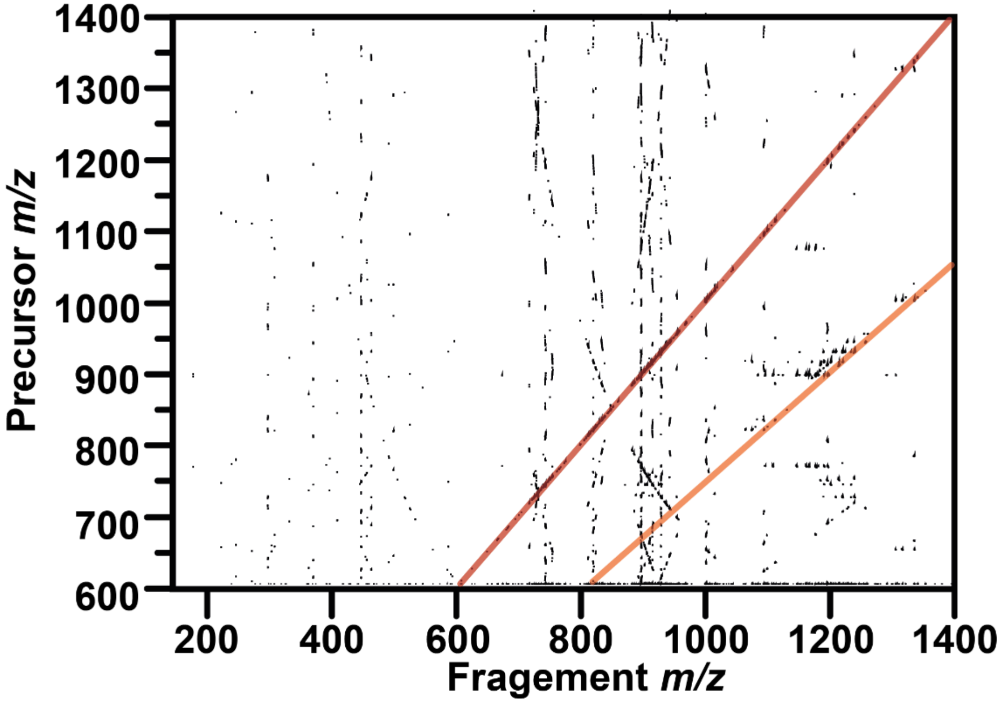
- Application of 2D with unknown sequence



- Autocorrelation Line

2DMS of potential active species (ECD)

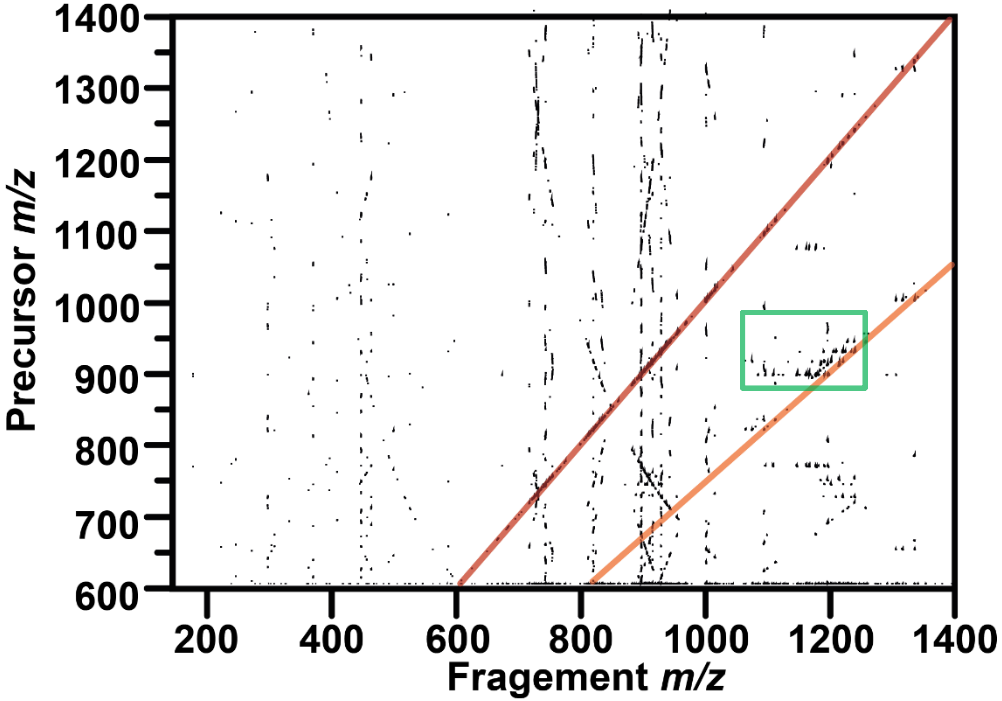
- Application of 2D with unknown sequence



- Autocorrelation Line
- Charge reduced species

2DMS of potential active species (ECD)

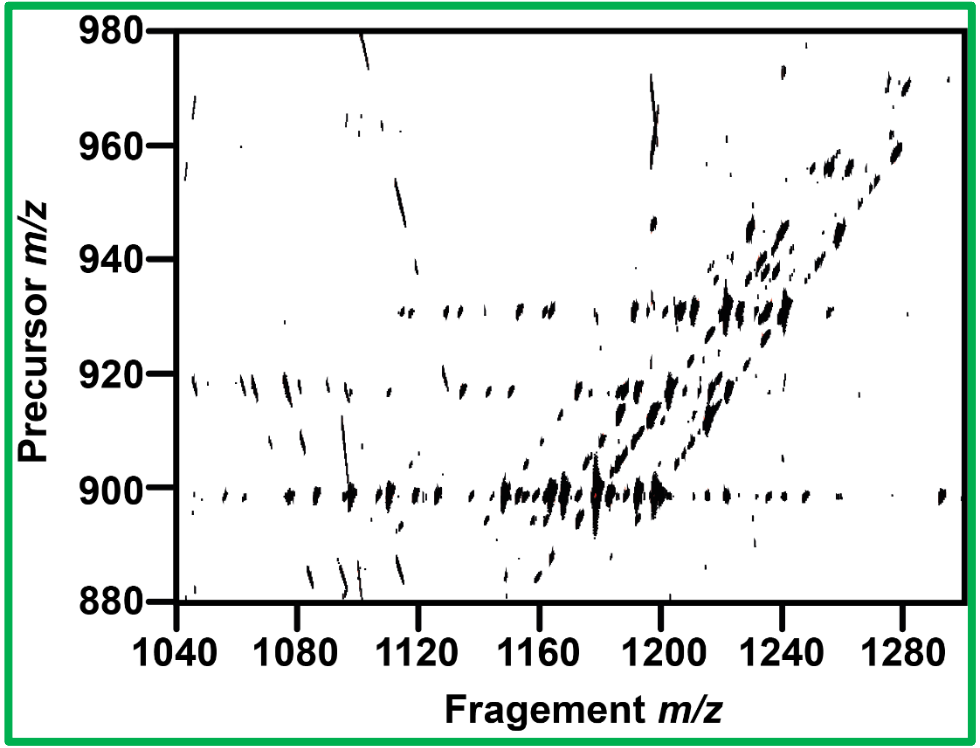
- Application of 2D with unknown sequence



- Autocorrelation Line
- Charge reduced species

2DMS of potential active species (ECD)

- Application of 2D with unknown sequence



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2DMS of potential active species (ECD)

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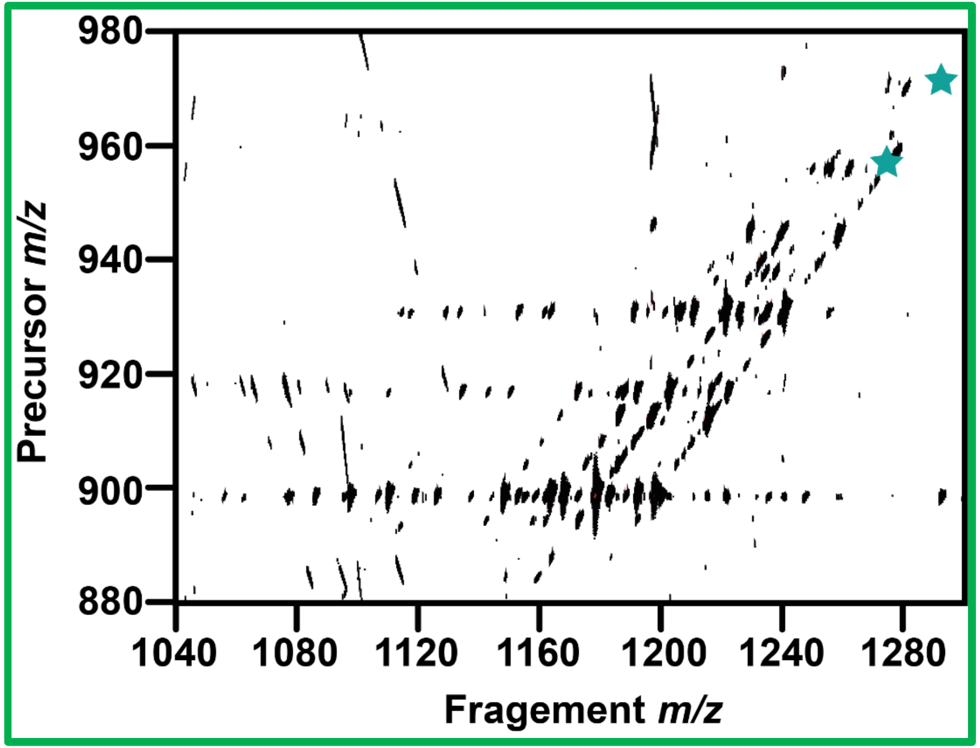

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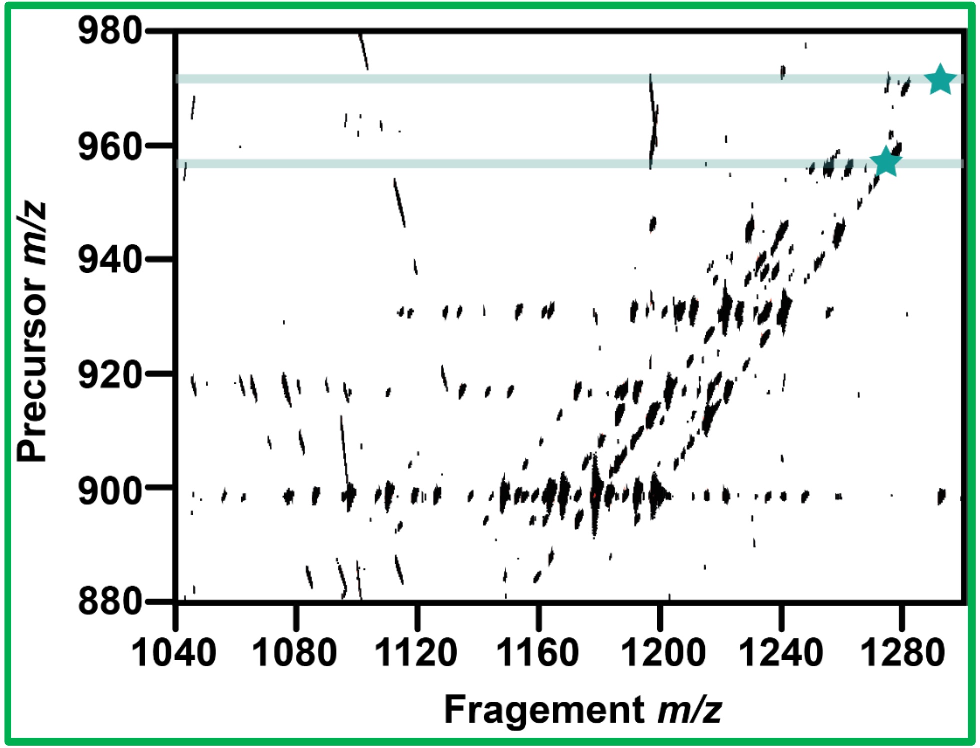

- Autocorrelation Line
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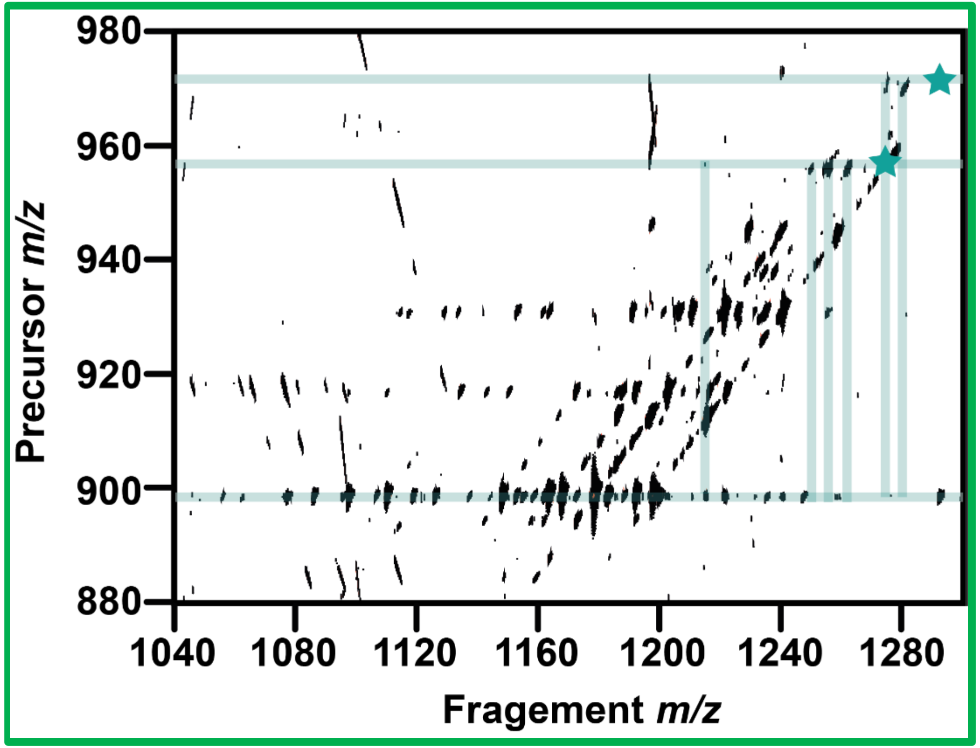
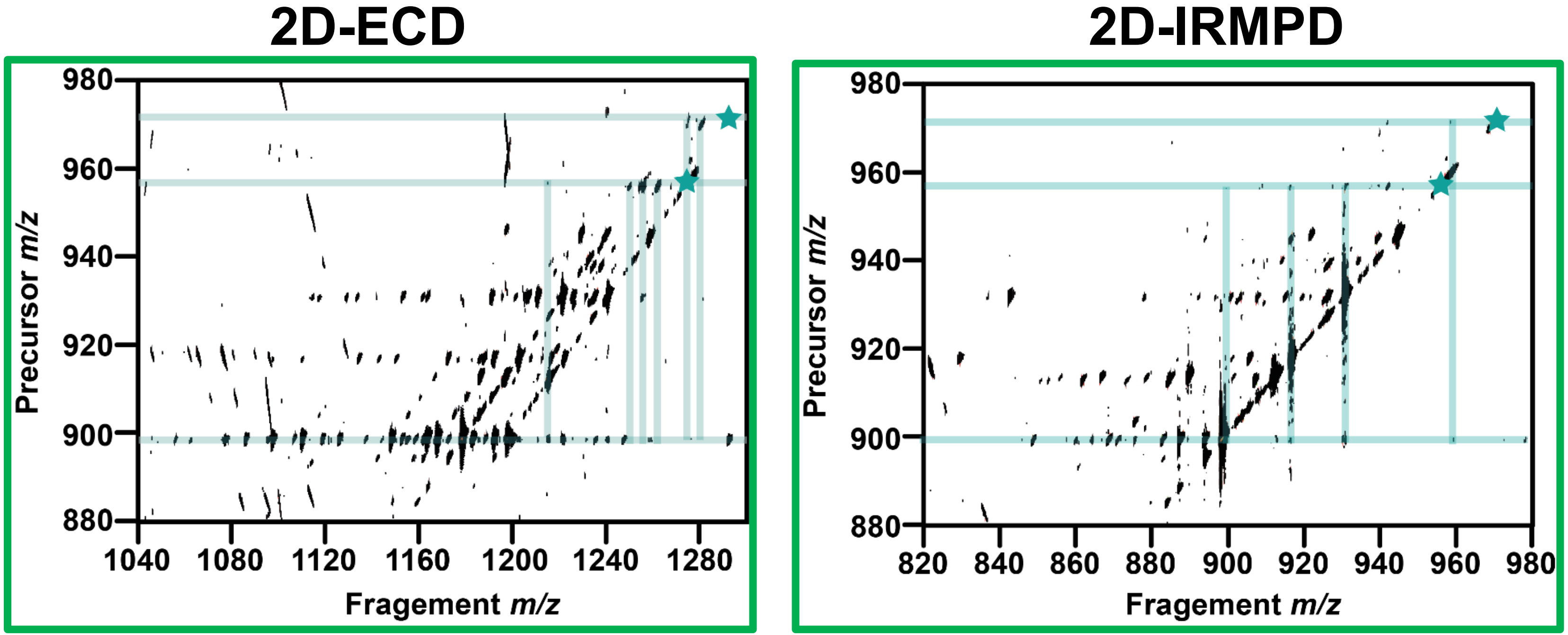


- Autocorrelation Line
- Charge reduced species
- Fragemntation pattern is very different

2DMS of potential active species (ECD)

- Application of 2D with unknown sequence



Fragmentation pattern suggest that this is a labile modification

Conclusions

- Standard methods
  - Difficult to find new potential pharmaceutical targets
- 2D-FTICR-MS can provide additional information to nLC which is essential to de novo sequencing of unknown proteins and peptides
- Bioactivity assays coupled with PLS helps to narrow down potential pharmaceutical targets
- Potential, novel FXa inhibitor protein sequenced
- 2D allows for easy identification of labile modifications
- Future works
  - Locate the modification site of the NeuAc
  - Find our the modification of the protein with the m/z of 955

Acknowledgement

University of Warwick:
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Dr. Mark Barrow
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