

Evaluation of Ion Mobility and Targeted QToF acquisition modes for the quantitative analysis of peptides

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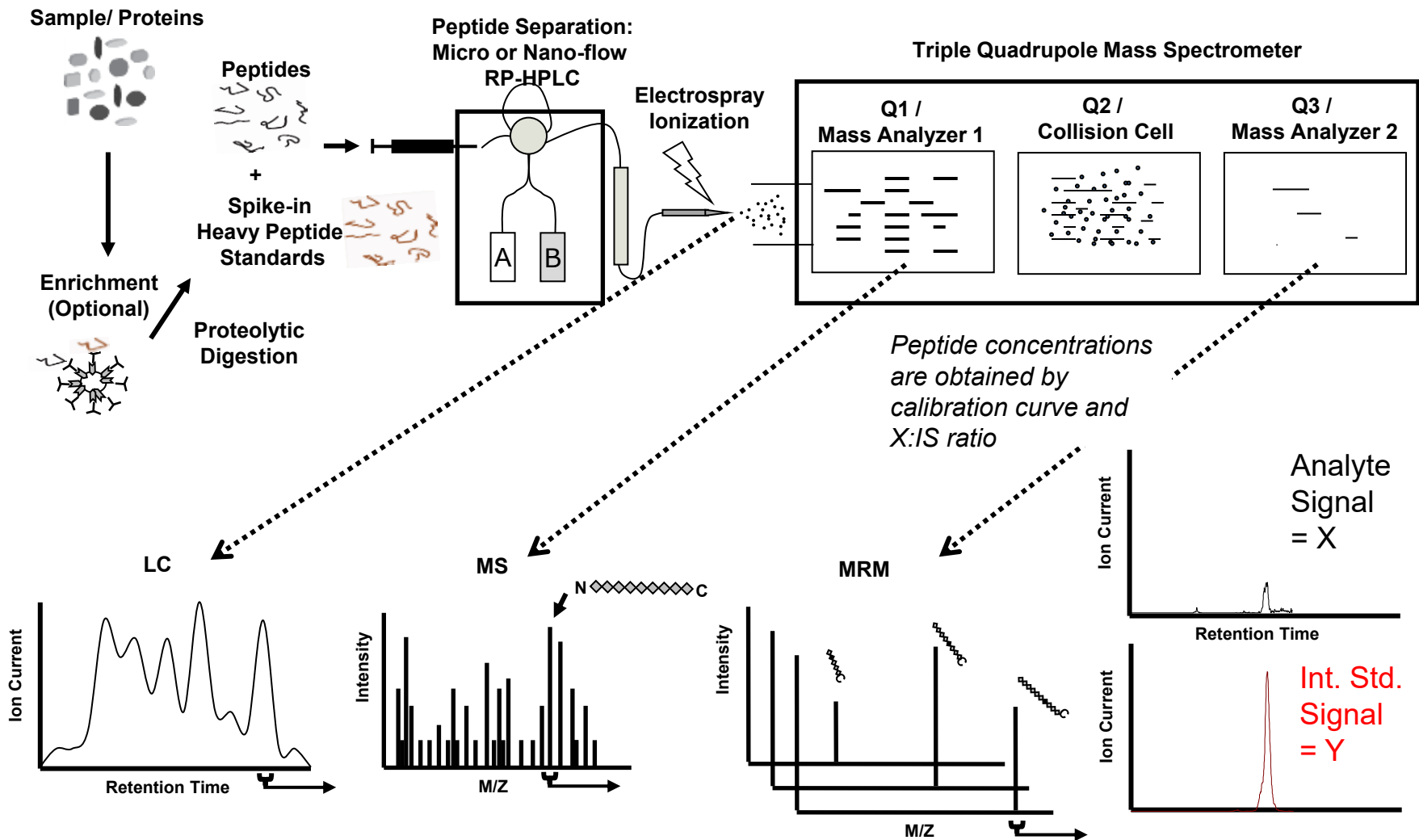
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**ASMS – San Antonio
2016**

LC-MS Based Protein Quantitation

- Pharma industry is moving to adopt LC-MS based quantitation in parallel with immunoassay techniques
- Regulatory requests to have “more specific” assays to enable better understanding of new therapeutics
- Current trends moving towards more complex molecules (ADC's, bispecifics, etc.) and more complex treatments (co-dosing of mAb's and small molecules, or mixtures)
- Development of advanced capabilities/skills in both discovery and development stages are needed to support future Biologics pipelines

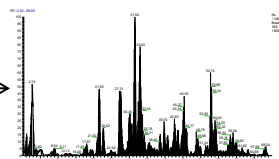
Protein Quantitation by MS: Multiple Reaction Monitoring (MRM)



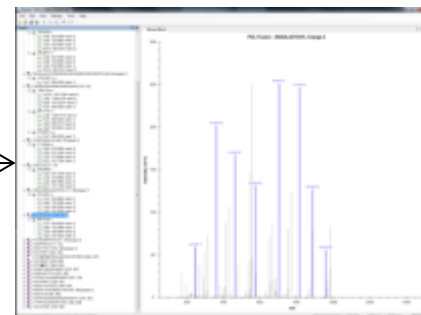
Merck Current Protein Assay Development



Nanoacquity Orbi Velos



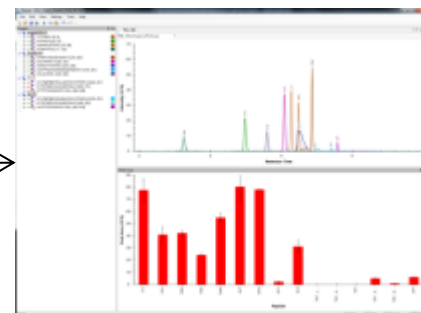
~ 1 day



TQS w/ ionKey

→ Peptide selection → CE Opt → Matrix Check →
≤10 min Methods

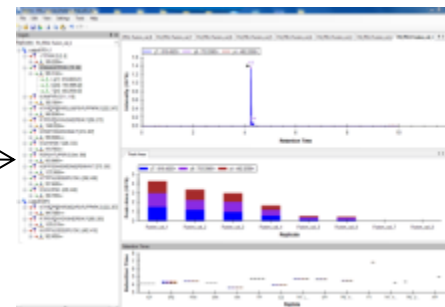
~ 1 day



AssayMAP

→ Std Curve → Immuno Capture → Digestion →

~ 2 days



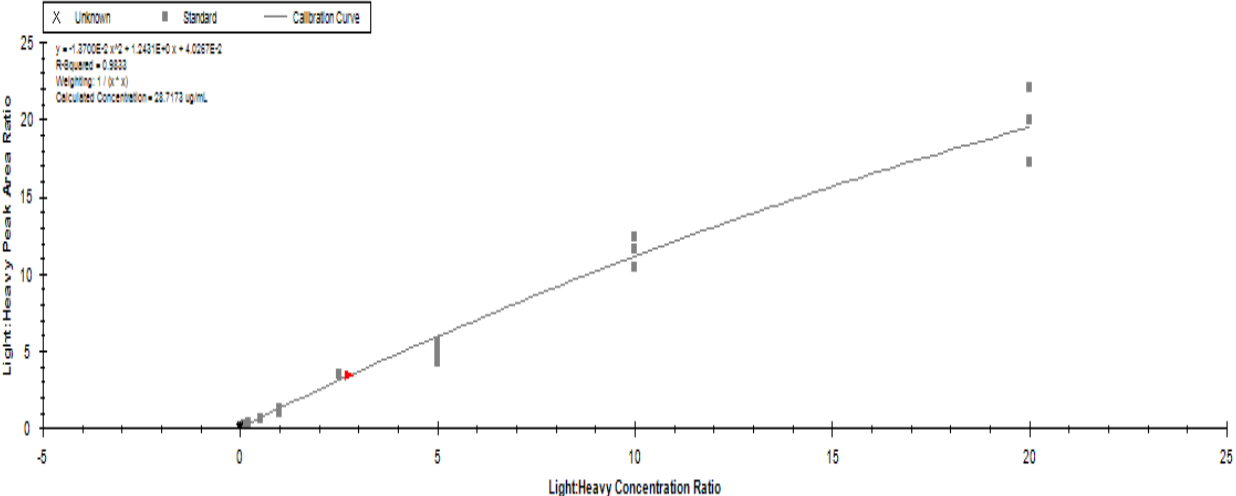
Current Sensitivity for mAb PK study by QQQ-MRM (and Microflow)

- Sample preparation:
 - Immunoprecipitation
 - Beads: Protein G Magnetic Dynabeads (100uL beads)
 - Sample: 20uL spiked SiluLite plasma + 25ug/mL SilumAb + TBS
 - React 1hr RT, Wash 3x TBS, Elute 1hr 100uL 0.1%TFA
 - Reduce/Alkylate/Digest
 - DTT reduction, IAM alkylation, 4hr trypsin digestion

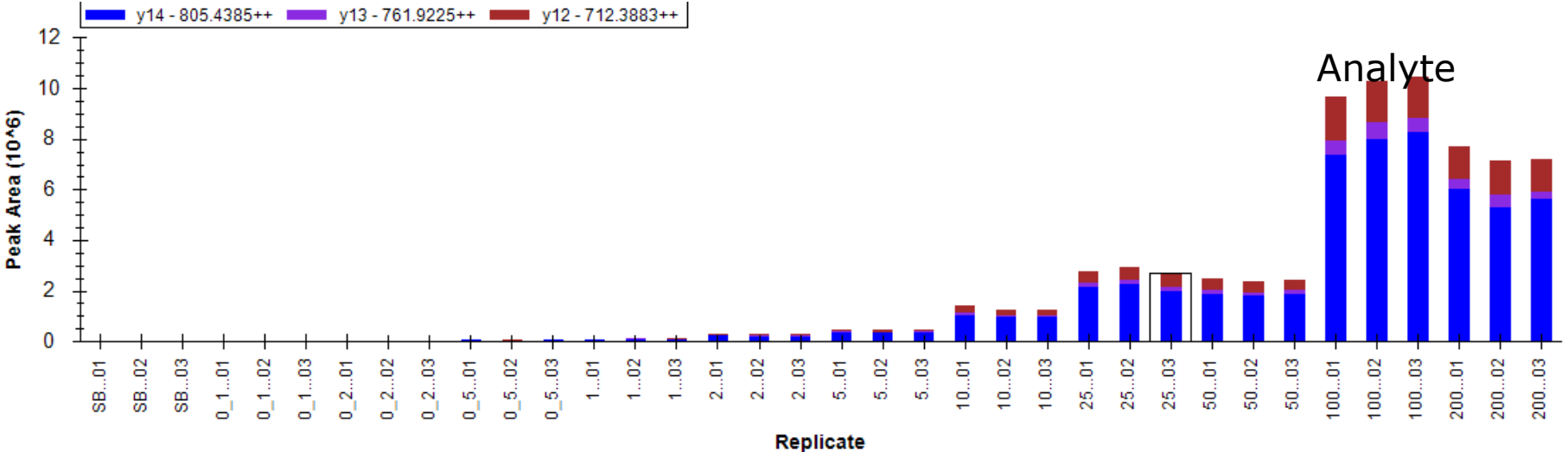
- LC/MS
 - ***M-class Acquity with IonKey on Xevo TQS***
 - A: 0.1% FA in H₂O; B: 0.1% FA in ACN
 - 3uL/min flow rate, 12 min run time, 10-55% B in 7 min
 - 1uL injection
 - Typically **0.2-0.5ug/mL LLOQ**

QQQ Peptide Intensity for Standard Curve

TQS-IonKey Platform



Std Conc (ug/mL)	Calc Conc (ug/mL)	Accuracy
0.2	0.2	-2%
0.5	0.5	0%
1	1.1	9%
2	2.1	7%
5	4.6	-7%
10	8.8	-12%
25	28.9	16%
50	40.8	-18%
100	103.4	3%
200	206.6	3%



Conditions

■ LC Conditions – Acquity I-Class

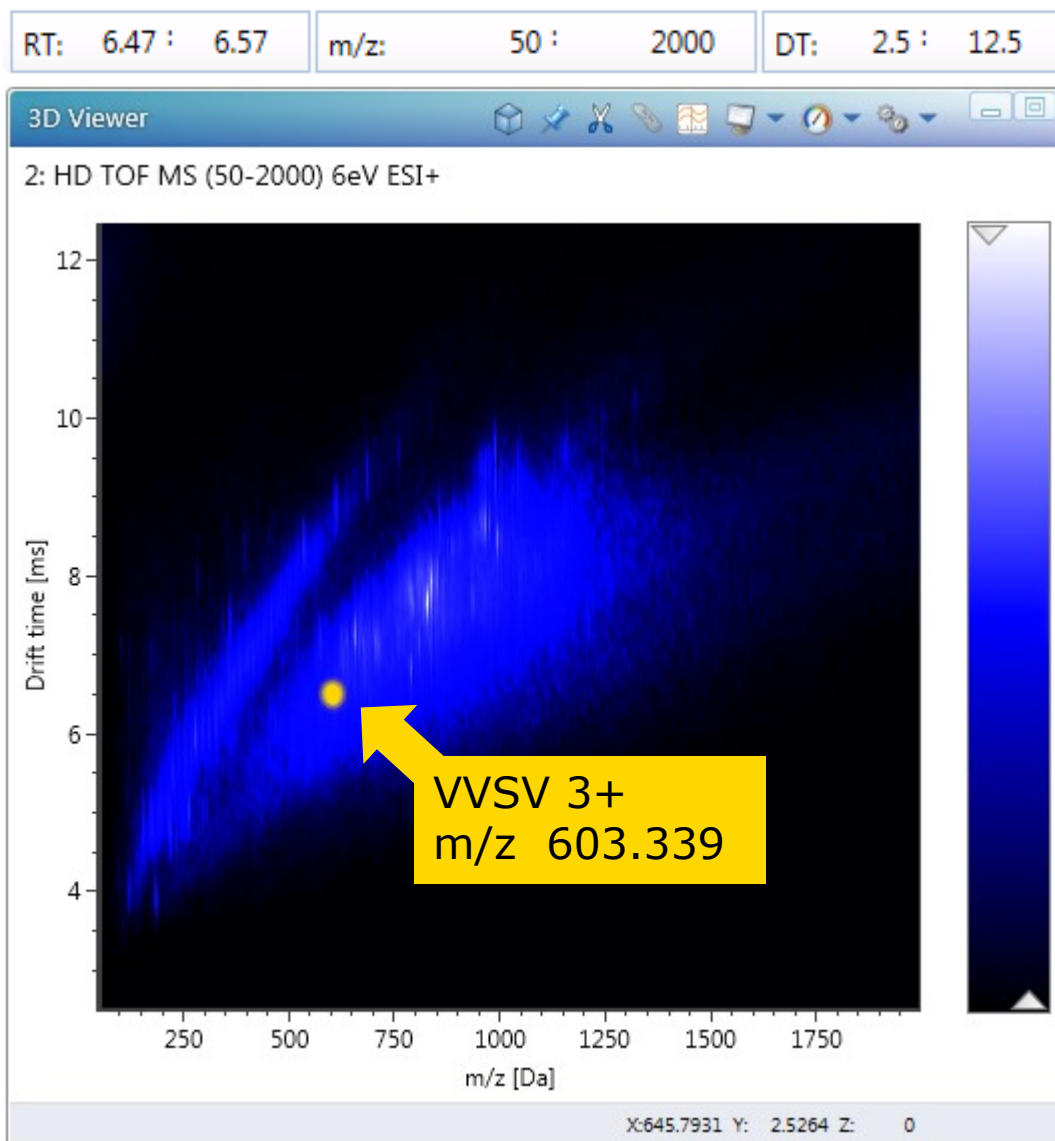
- Mobile Phase A: Water + 0.1% formic acid, B: ACN + 0.1% formic acid
- 2% to 40% B over 9 minutes, 400uL/min
- ACQUITY UPLC BEH C18 Column, 1.7 μm , 2.1 mm X 100 mm
- Column Temp 40C
- Injection Volume 1uL

■ MS – Vion IMS QTof

- Acquisition Modes
 - HDMS – Fullscan w IMS (50-2000 m/z, sensitivity mode)
 - HSMRM – TofMRM w IMS (SIM Mode and MRM Mode)
- Source
 - Capillary: 0.7 kV
 - Desolvation Temp: 400C
 - Desolvation Gas: 800L/h



Travelling Wave IMS



Advantages

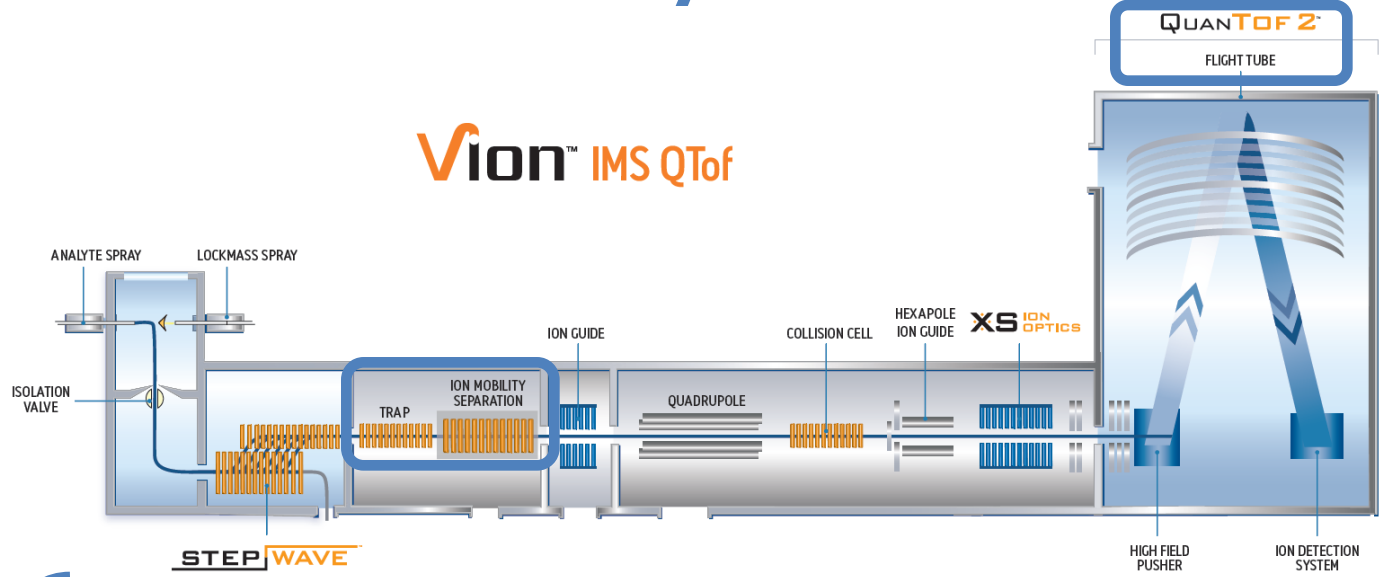
- *Fullscan or Targeted IMS Modes*
- *CCS Value is reproducible measure that can be used to identify or filter ions like m/z*
- *Charge state and size dependent*

Can we:

- Use the property to select ions for quantitation
- Get good linearity?
- Get good sensitivity?

IMS-QToF

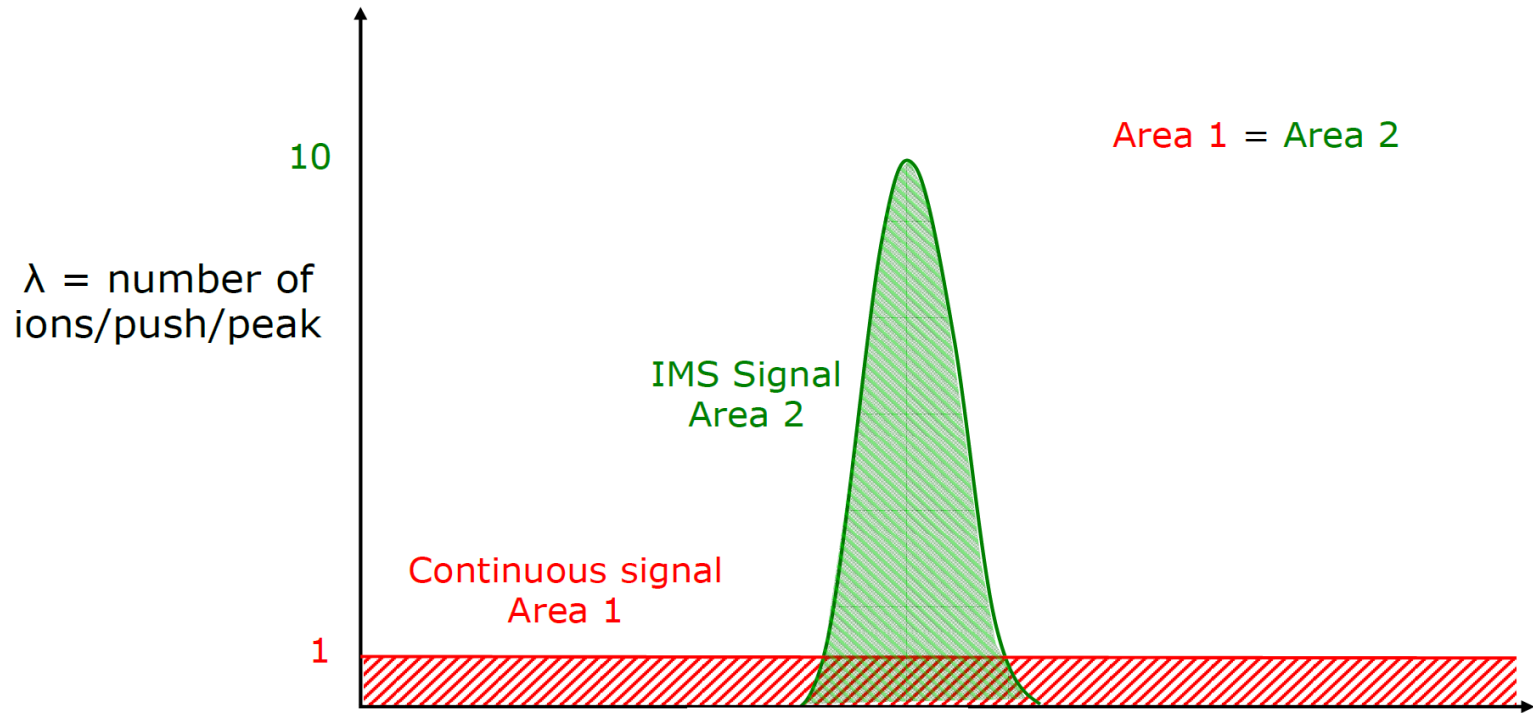
Mechanisms of Selectivity



Non CE tuned	Measure CCS (IMS) of molecule	Highly specific XICs with many points across the peak	IMS - Fullscan
	Using Ion Mobility to clean up Ions prior to entering Quadrupole	+ Target Enhancement	IMS - ToF MRM (SIM Mode)
CE Tuning required	Optional Fragment selected ions	+ Target Enhancement	IMS - ToF MRM

The Challenge of Ion Mobility

> Linear Dynamic Range



Non-IMS vs IMS mode of operation

Signals are more 'bunched' up

- This increases the number of ions hitting the detector per push per mass peak
- Quan ToF 2 specifically designed to address this limitation and make IMS routine

HDMS – IMS Enabled Fullscan

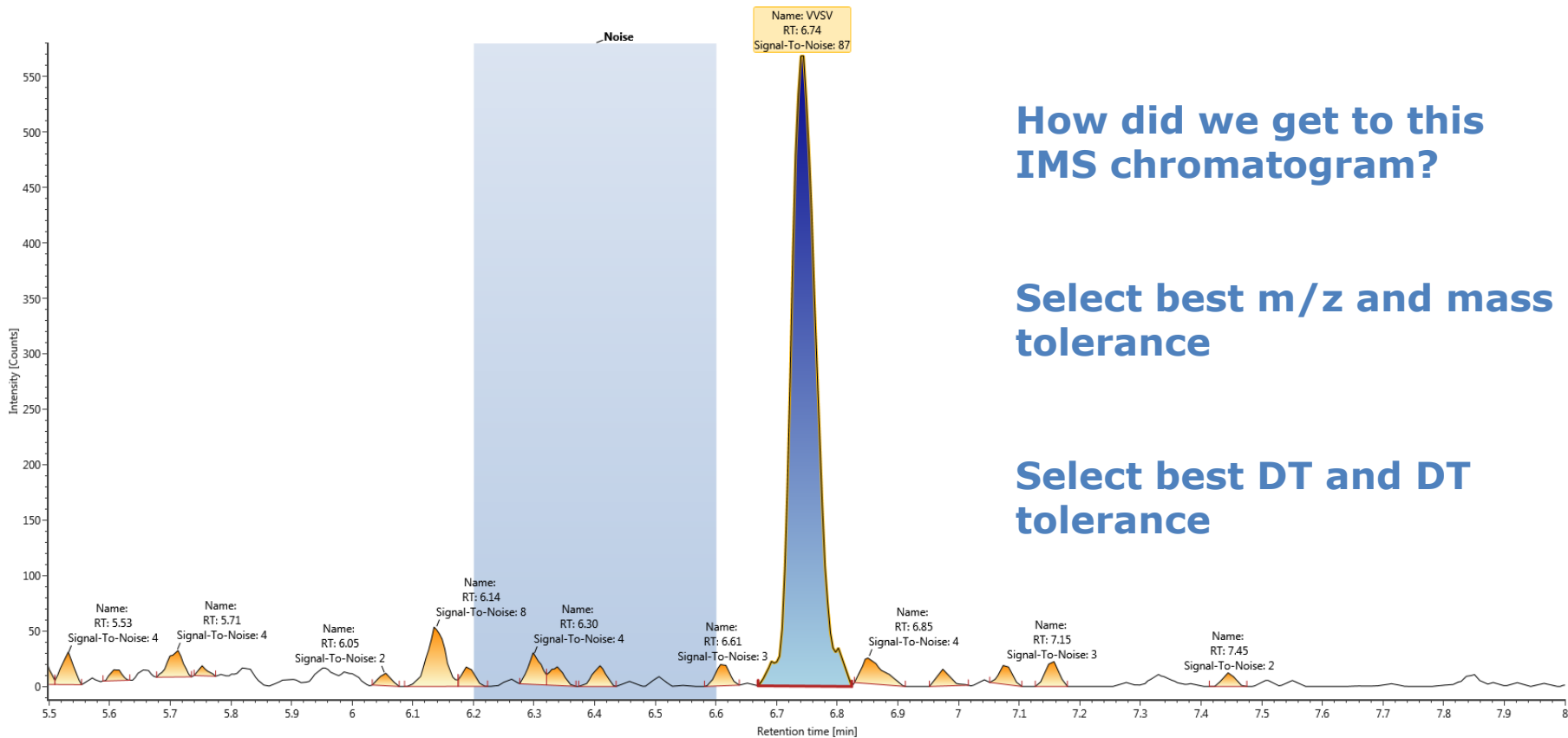
MS XIC

+IMS



Item name: D1

Channel name: 2: +603.3393 (25.0 PPM) : DT=6.30 to 6.78 ms : HD TOF MS (50-2000) 6eV ESI+ : Integrated : Smoothed



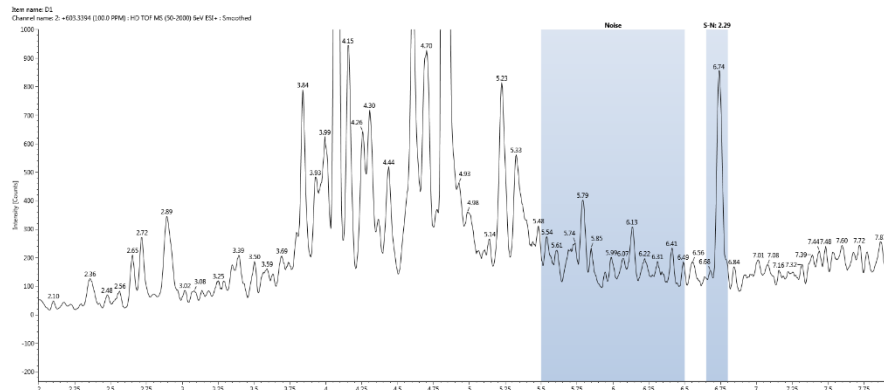
How did we get to this
IMS chromatogram?

Select best m/z and mass
tolerance

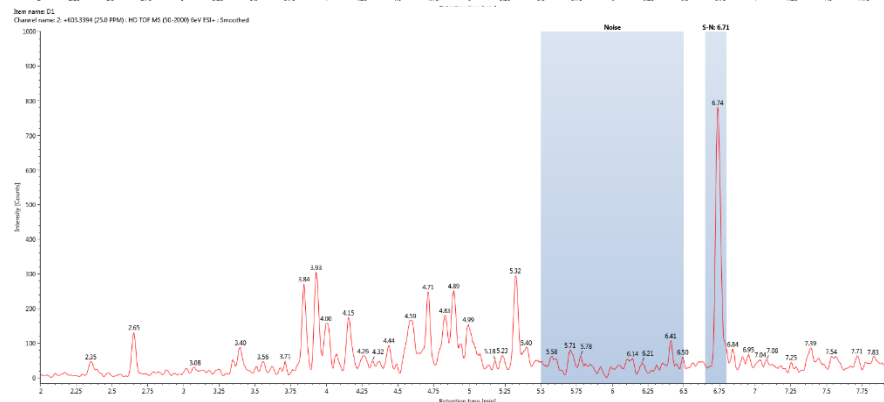
Select best DT and DT
tolerance

Improving Selectivity for HRMS Combining MS and IMS

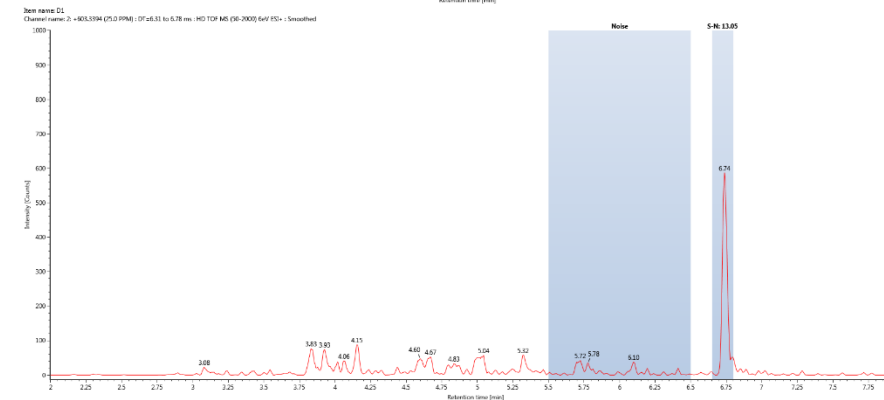
VSV Peptide, 1 ug/mL,
1uL injection



**100 ppm
S/N 2.3**



**25 ppm
S/N 6.7**



**25 mDa + IMS
S/N 13.0**

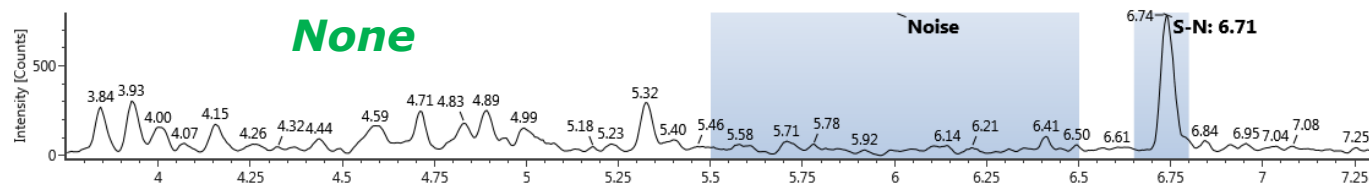
HRMS

+IMS

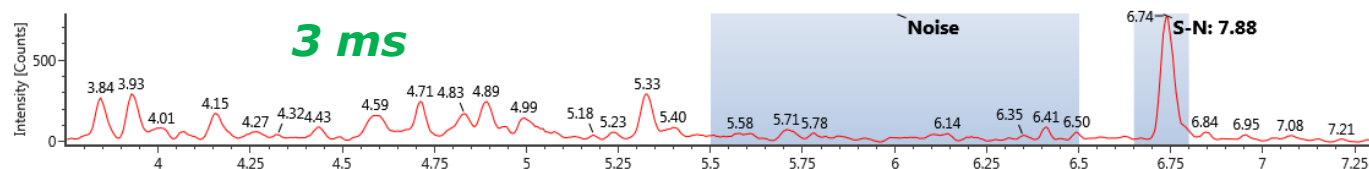


IMS – Tunable Parameter for better S/N

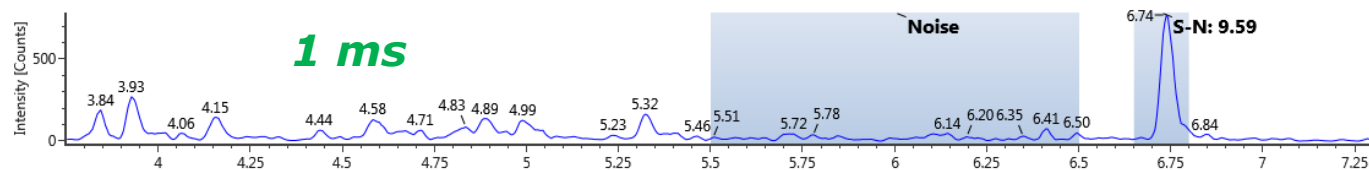
Item name: D1
Channel name: 2: +603.3394 (25.0 PPM) : HD TOF MS (50-2000) 6eV ESI+ : Smoothed



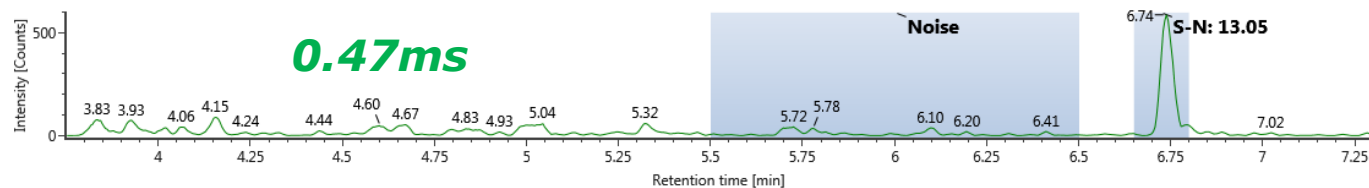
Item name: D1
Channel name: 2: +603.3394 (25.0 PPM) : DT=5.05 to 8.05 ms : HD TOF MS (50-2000) 6eV ESI+ : Smoothed



Item name: D1
Channel name: 2: +603.3394 (25.0 PPM) : DT=6.05 to 7.05 ms : HD TOF MS (50-2000) 6eV ESI+ : Smoothed



Item name: D1
Channel name: 2: +603.3394 (25.0 PPM) : DT=6.31 to 6.78 ms : HD TOF MS (50-2000) 6eV ESI+ : Smoothed



S/N

6.7

7.9

9.6

13.1

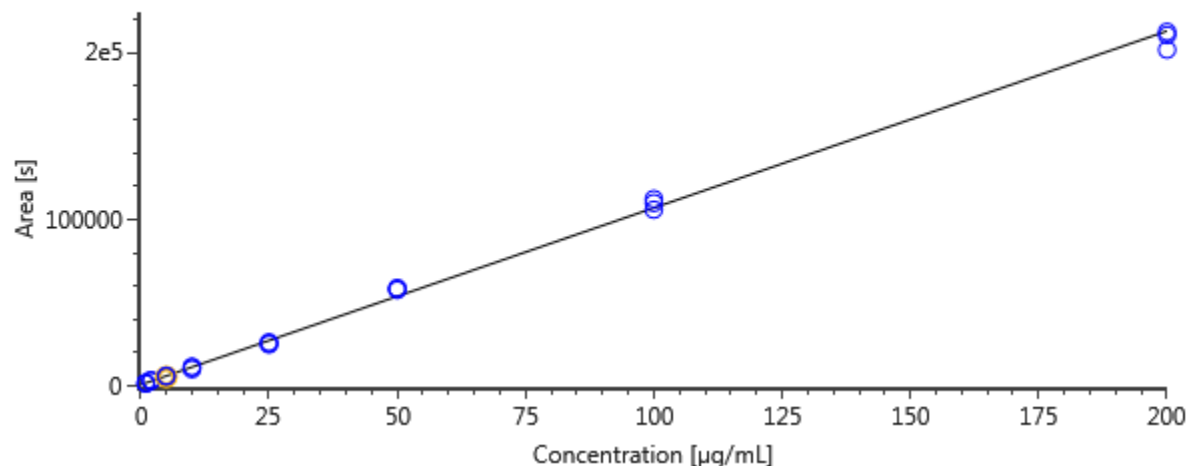
Increasing
Selectivity

Replicates/Linearity 1-200 ug/mL – Vion HDMS

Item name	% Deviation (%)	X Value	Y Value
D1	-20.57	1.000	1632.908
D1	-16.91	1.000	1671.759
D1	5.96	1.000	1914.646
E1	20.92	2.000	3357.457
E1	12.31	2.000	3174.657
E1	20.51	2.000	3348.796
F1	-11.80	5.000	5472.572
F1	2.16	5.000	6213.834
F1	-7.14	5.000	5719.860
G1	-1.23	10.000	11277.501
G1	1.59	10.000	11577.586
G1	-12.04	10.000	10130.056
H1	-6.10	25.000	25717.569
H1	-8.84	25.000	24990.996
H1	-4.09	25.000	26250.437
A2	7.17	50.000	57693.834
A2	8.83	50.000	58573.023
A2	9.15	50.000	58744.312
B2	2.77	100.000	109918.833
B2	-1.03	100.000	105889.896
B2	5.07	100.000	112359.986
C2	-0.23	200.000	212677.722
C2	-1.18	200.000	210674.958
C2	-5.28	200.000	201955.681

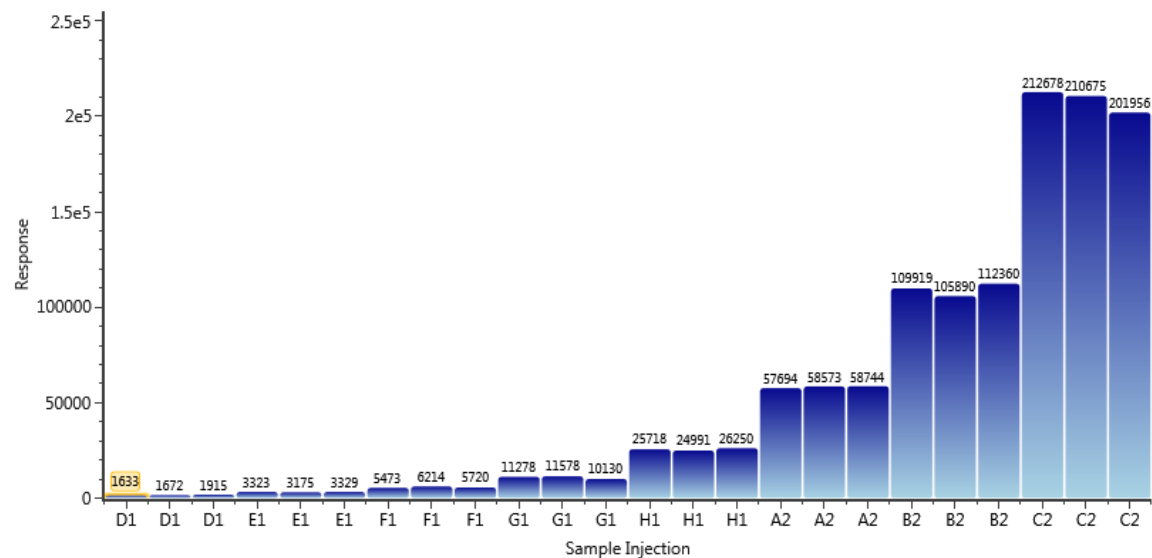
Calibration component: VVSV
Equation: $Y = 1.06e3X + 784$
Weighting: 1/X

% RSD (%): 14.797
R²: 0.997380



Component: VVSV

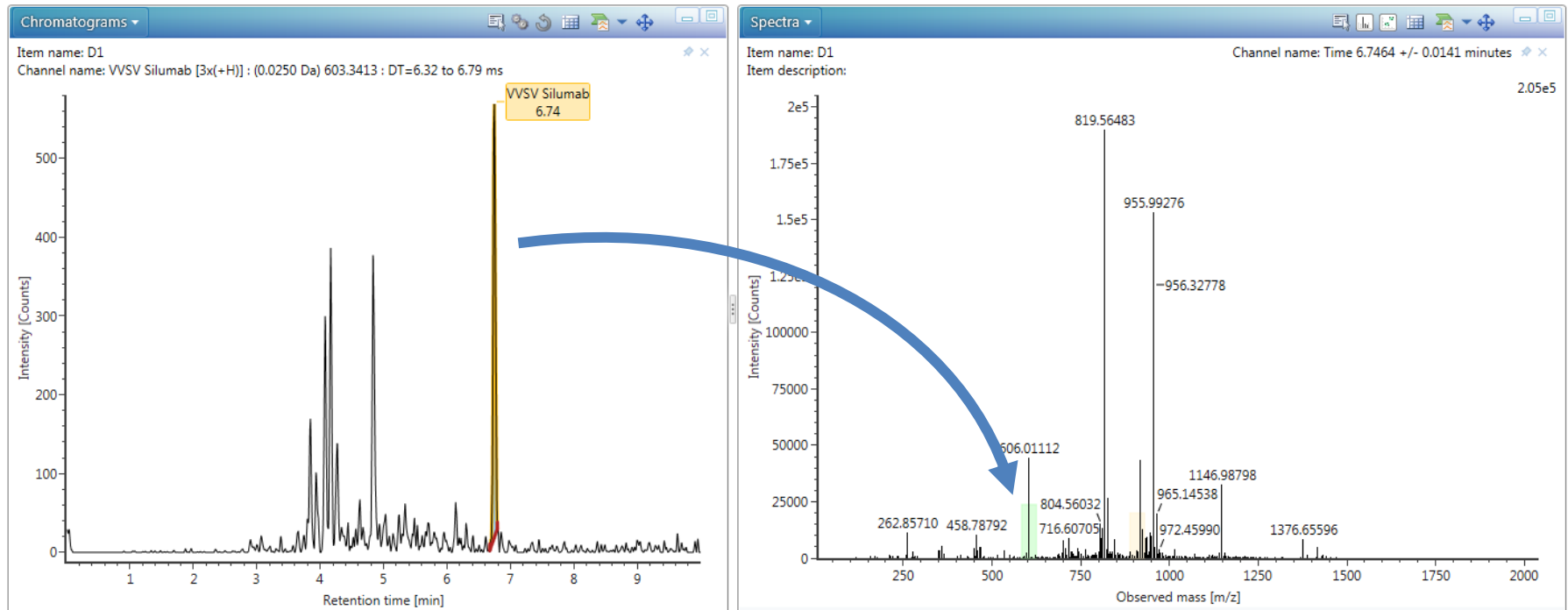
Summarized by: Response



What does the (spectral) data actually look like?

Great for characterization, method development

Great for quantitation (can pull out many other peptides from same data)



BUT what if we want to maximize sensitivity and simplicity, in a more locked down method which would be suitable for a routine assay ...

Targeted Modes

- Options beyond IMS Fullscan

Select IMS Window (Narrow or Wide)



Select Precursor (Tof SIM)



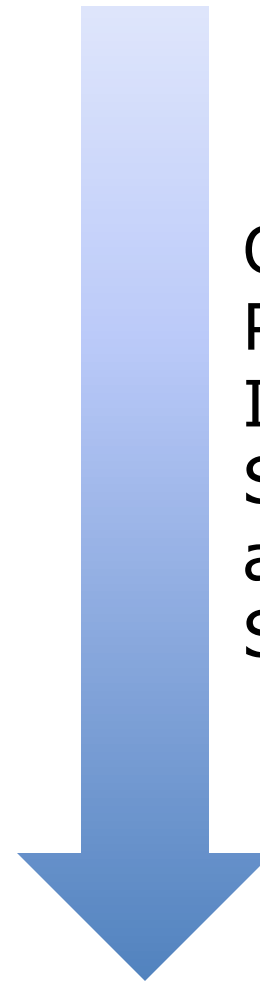
Select Quad Window (Narrow or Wide)



Select Fragment Ion, Tune CE (Tof MRM)



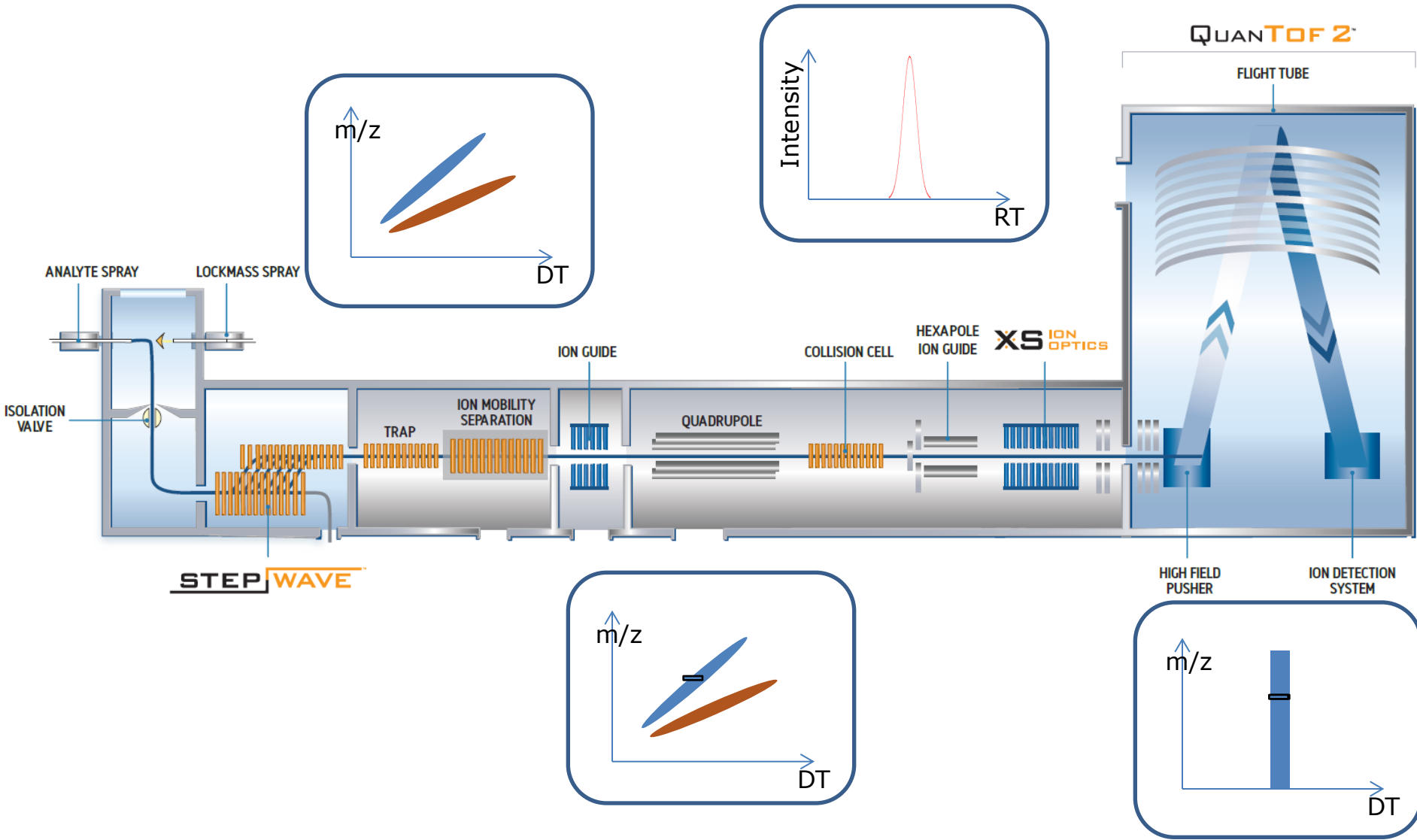
Select ppm/mDa XIC window (maximize S/N)



Options to
Potentially
Increase
Selectivity
and
Sensitivity

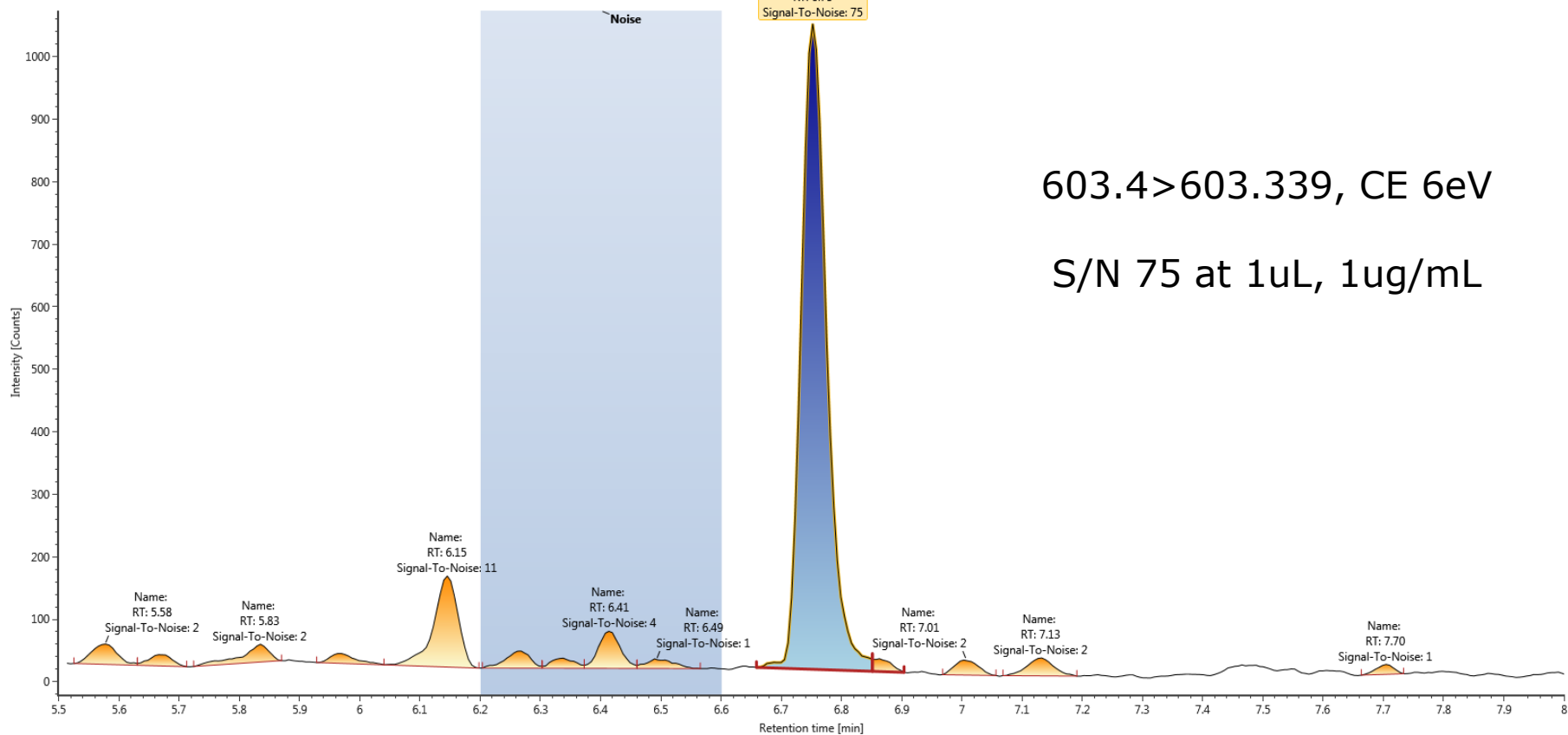
High Selectivity - HS MRM

Ion Mobility Enabled ToF MRM



HS MRM (SIM Mode)

Item name: D1
Channel name: 5: +603.3390 (0.0200 Da) : HS TOF MRM M/Z=603.339, DT=6.45ms > 603.339 6eV ESI+ : Integrated : Smoothed



603.4 > 603.339, CE 6eV

S/N 75 at 1uL, 1ug/mL

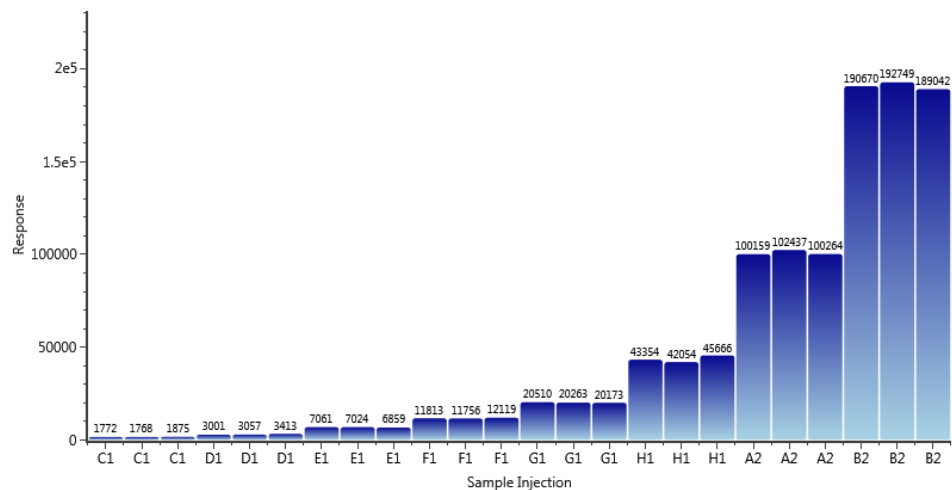
HS MRM (SIM Mode)

0.5
μg/mL

Item name	% Deviation (%)	X Value	Y Value
D1	2.93	1.000	3001.381
D1	5.82	1.000	3056.549
D1	24.49	1.000	3412.800
F1	12.95	5.000	11813.232
F1	12.35	5.000	11755.713
F1	16.15	5.000	12118.537
G1	2.06	10.000	20510.159
G1	0.76	10.000	20262.585
G1	0.29	10.000	20173.283
H1	-11.28	25.000	43353.919
H1	-14.01	25.000	42053.804
H1	-6.44	25.000	45665.855
A2	3.90	50.000	100159.230
A2	6.29	50.000	102437.446
A2	4.01	50.000	100263.594
B2	-0.61	100.000	190669.567
B2	0.48	100.000	192748.813
B2	-1.46	100.000	189041.793

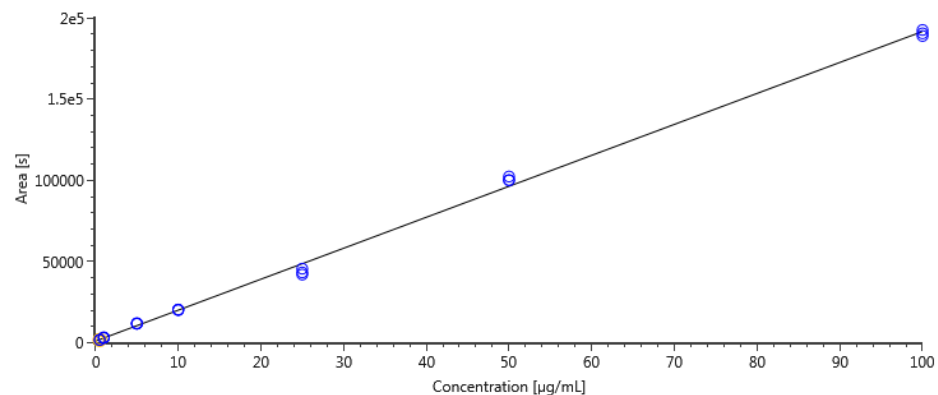
Component: VVSV Silumab

Summarized by: Response



Calibration component: VVSV Silumab
Equation: $Y = 1.91e3 \cdot X + 1.04e3$
Weighting: 1/X

% RSD (%): 16.525
R²: 0.996778

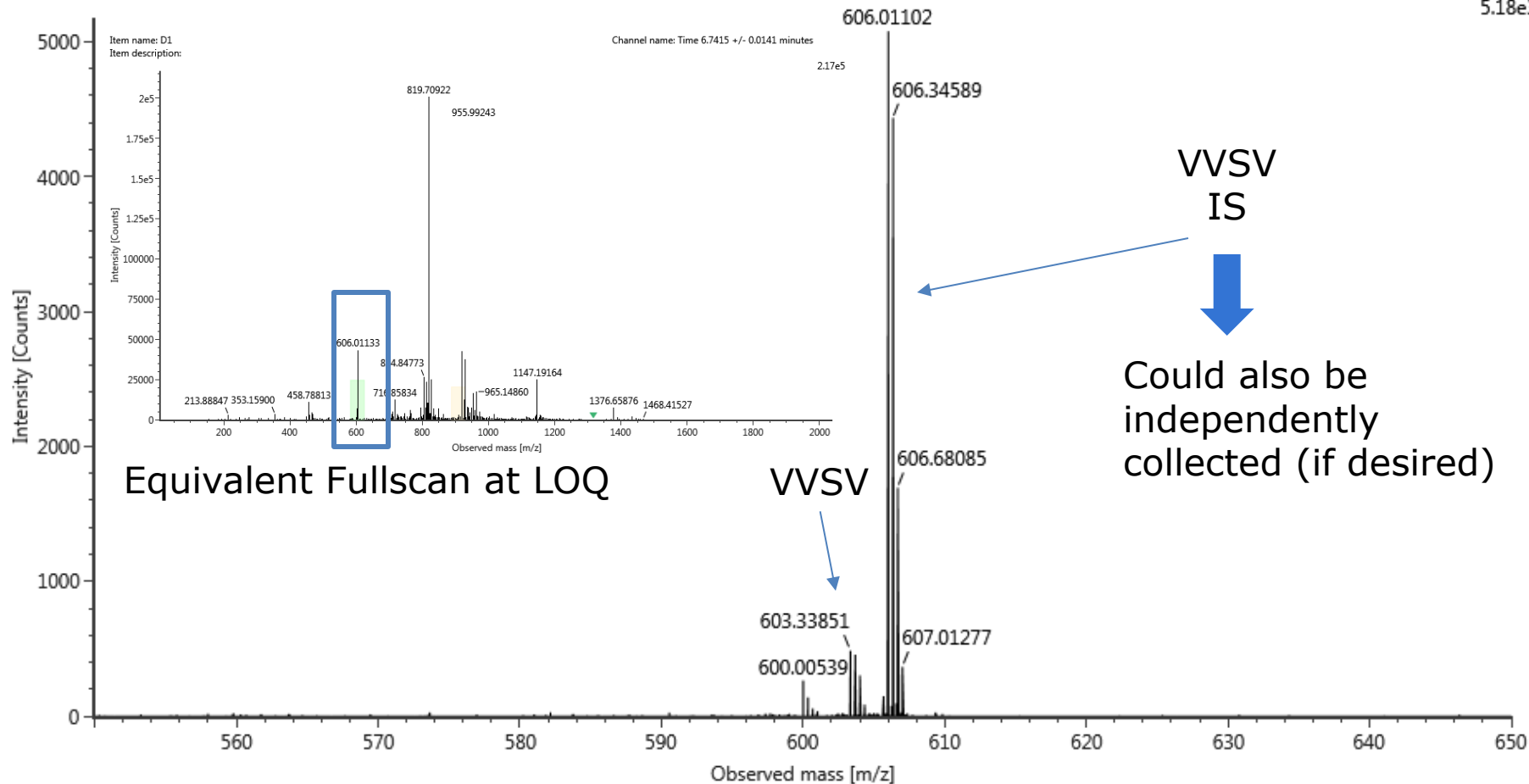


HS MRM (SIM Mode) Spectral Data/Complexity

Item name: D1
Item description:

Channel name: 5: RT=6.7522 mins : DT=6.45 ms : HS TOF MRM M/Z=603.339, DT=6.45ms > 603.339 6eV ESI+

5.18e3

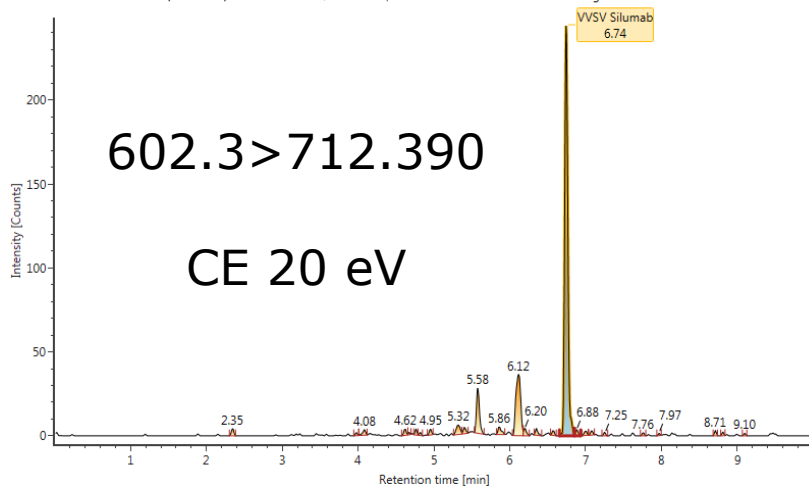


HS MRM

- Tuned Fragment Ion

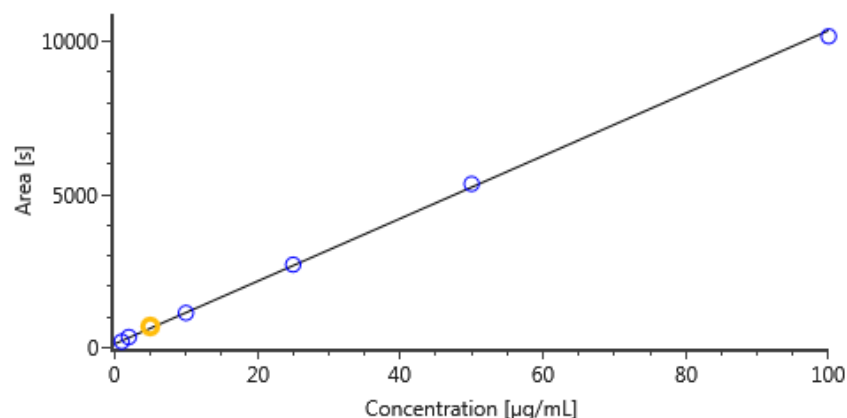
Item name	Level	Ignore	% Deviation (%)	X Value	Y Value	Calculated X value
D1	Level 4	<input type="checkbox"/>	-23.49	1.000	206.824	0.765
E1	Level 5	<input type="checkbox"/>	11.10	2.000	355.977	2.222
F1	Level 6	<input type="checkbox"/>	11.78	5.000	700.734	5.589
G1	Level 7	<input type="checkbox"/>	-0.95	10.000	1142.631	9.905
H1	Level 8	<input type="checkbox"/>	1.30	25.000	2721.282	25.324
A2	Level 9	<input type="checkbox"/>	2.14	50.000	5357.025	51.068
B2	Level 10	<input type="checkbox"/>	-1.87	100.000	10175.190	98.127

Channel name: 2: +712.3900 (0.0250 Da) : HS TOF MRM M/Z=603.339, DT=6.45ms > 712.39 20eV ESI+ : Integrated : Smoothed

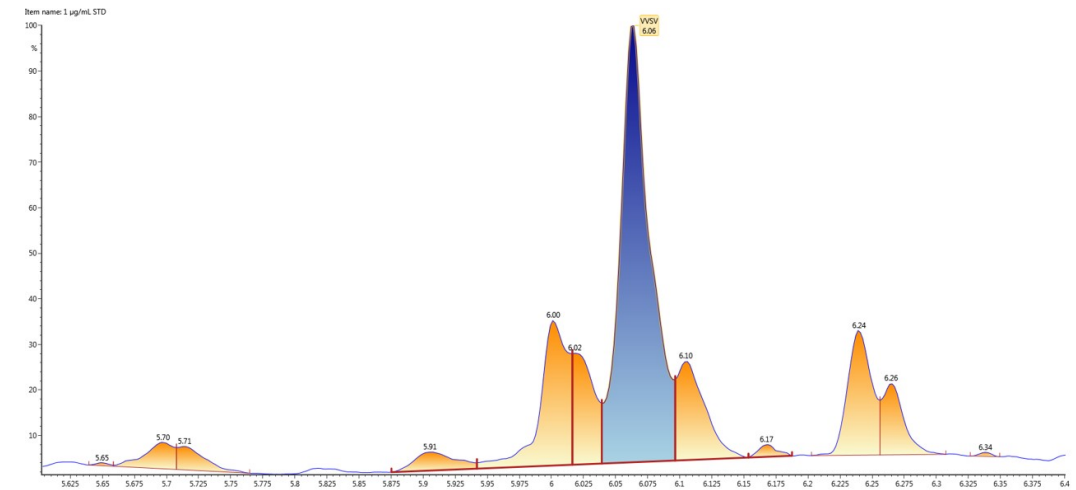


Calibration component: VVSV Silumab
Equation: $Y = 102 * X + 128$
Weighting: 1/X

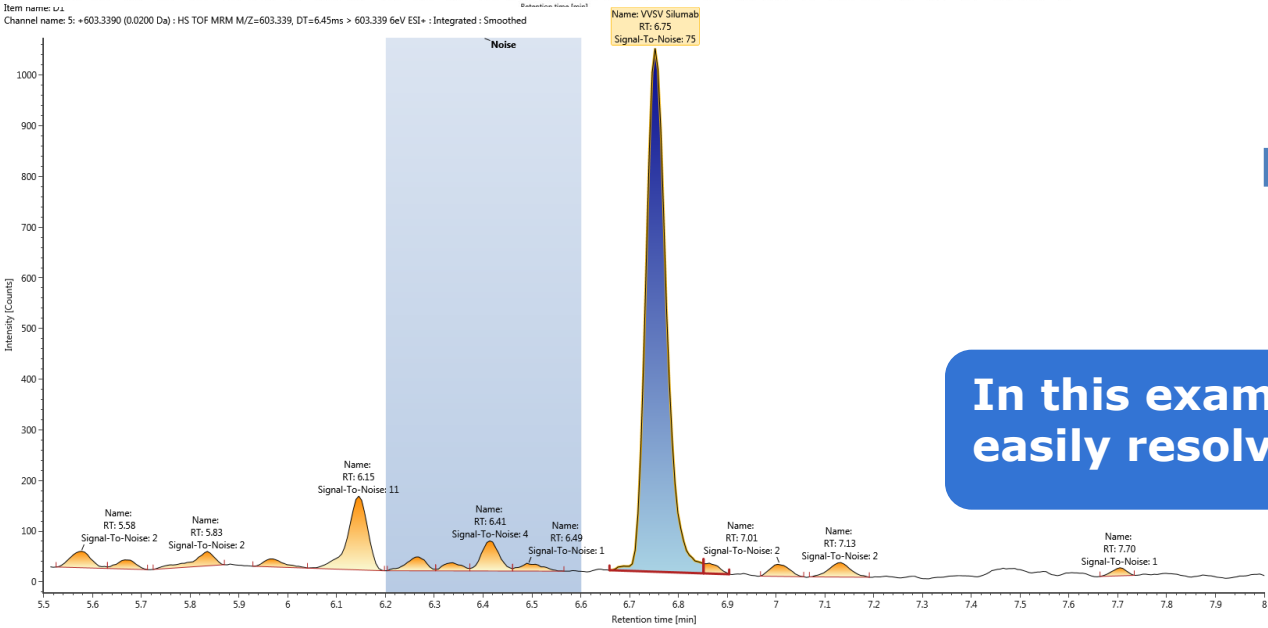
% RSD (%): 7.974
 R^2 : 0.998729



VVSV Peptide Comparison Tof MRM vs HS MRM (SIM Modes)



Tof MRM – 3uL



HS MRM – 1uL

In this example, Ion Mobility easily resolves what m/z can not

Conclusions

- IMS and HRMS data highly reproducible and accurate
- Method development through quantitative assay possible on a single platform
- IMS for Quantitation
 - Dynamic range limitations from previous generations not limiting for assay
 - IMS is a tunable property of the molecule, enables selectivity beyond mass/quad based properties.

Future Work

- Continue optimizing MRM modes, ToF MRM, HSMMRM (SIM and MRM modes)
- Define best practices, when to use IMS, which defaults work best
- Evaluate effects of Analytical Scale vs Microflow for ToF Platforms

Acknowledgements

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