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LSMS

The importance of *sample preparati*on for the analysis of proteins and peptides by liquid chromatography with mass spectrometric detection

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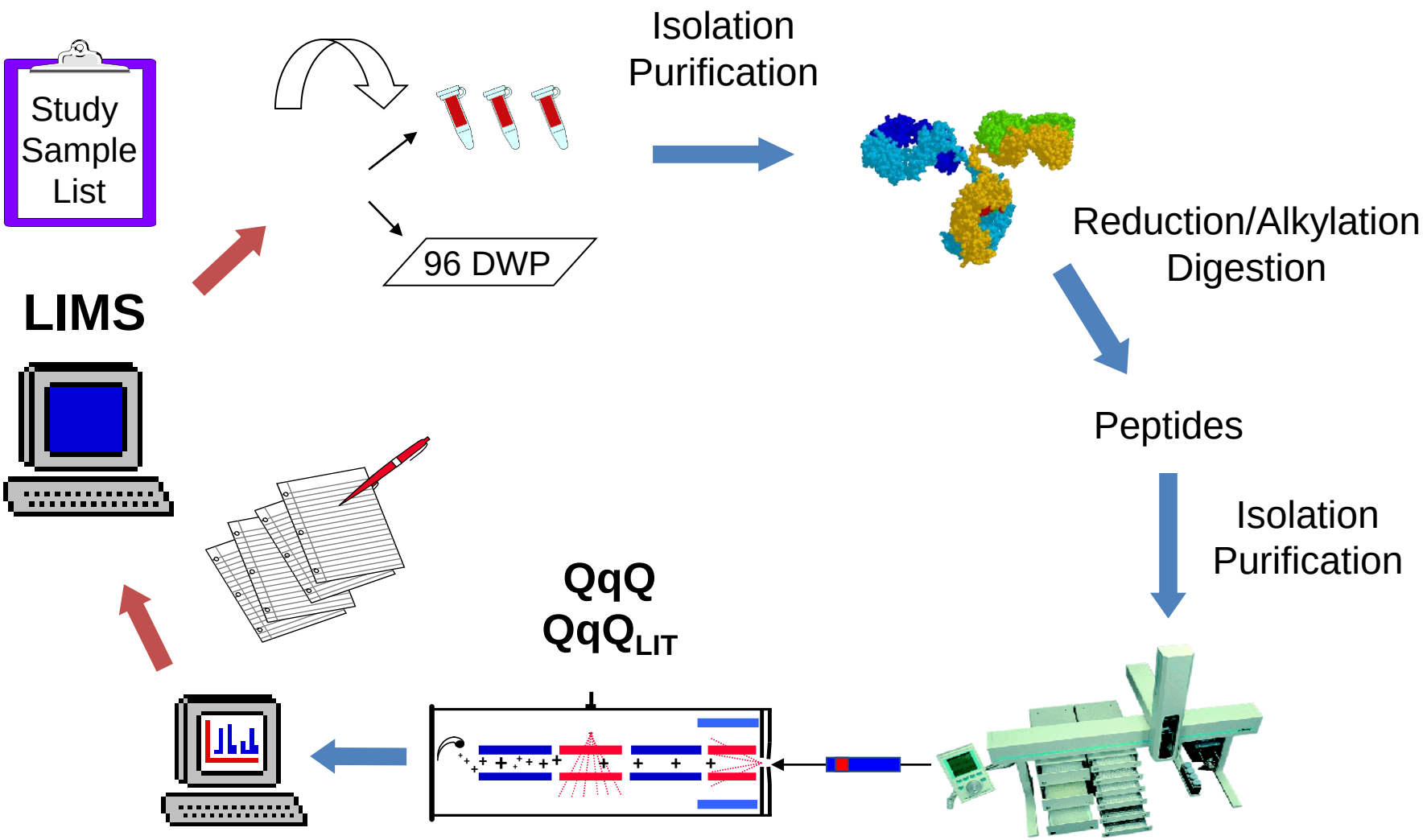
Challenges in the analysis of peptides and proteins

- Selectivity : thousands of proteins and peptides are present in plasma
- Sensitivity : large dynamic range of concentrations
- Matrix effects : co-eluting analytes can affect the ionization process and therefore the limit of quantification
- Sample throughput : Proteomics deals with moderate samples sets while bioanalysis deals with large number of samples

Sample Preparation Strategies for Peptide/Protein Analysis in Plasma

Peptides 300-1000 Da	Peptides 1000-5000 Da	Proteins 5-30 kDa	Proteins > 30 kDa
Protein Precipitation Liquid-Liquid Extraction Solid-Phase Extraction Affinity Enrichment	Abundant Protein Depletion Protein Precipitation Solid-Phase Extraction Affinity Enrichment	Partial Protein Precipitation Abundant Protein Depletion Protein Digestion Affinity Enrichment	Abundant Protein Depletion Affinity Enrichment (protein) Protein Digestion LLE SLE Affinity Enrichment (peptide)

Quantitative Peptide/Protein Analysis Workflow



Sample Preparation Steps for Protein Analysis by LC-SRM/MS

1) Removal of abundant proteins
2) Reduction/alkylation
3) **Overnight trypsin digestion**
4) Removal of un-digested proteins

Mostly
24 hours

Do we need to optimize the process?

Tryptic Digestion of Whole Plasma

Plasma Volume	Albumins	Globulins
50 µl	2.1 mg	1.23 mg
1000 µl	42 mg	24.5 mg

To digest 50 µl of plasma about 70 µg of trypsin (1/50) are needed per sample, for 96 samples -> 6.7 mg of trypsin

Promega Trypsin Gold, Mass Spectrometry Grade 100µg US$100.13

6700 US$ for 96 samples

Can We Accelerate Trypsin Digestion ?

Why 18 hours at 37°C ?

Microwave digestion

Thermomixer digestion

30 minutes at 37, 60°C ?

Quantitation of mAb in Human Plasma

Recombinant monoclonal antibody (mAb), MW = 145'000 Da

Heavy Chain sequence

```
1   QVQLVESGGGVVQPGRSLRLSCAASGFTFSVYGMNWVRQAPGKGLEWVAIIWYDGDNQYY  60
61  ADSVKGRFTISRDNSKNTLYLQMNGLRAEDTAVYYCARDLRTGPFDYWGQGTLVTVSSAS  120
121 TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGL  180
181 YSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPS  240
241 VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST  300
301 YRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMT  360
361 KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQ  420
421 GNVFSCSVMHEALHNHYTQKSLSLSPGK                                  448
```

Number of amino acids: 448 MW = 49'253.6 Da pI = 8.49

Light Chain sequence

```
1   EIVLTQSPDFQSVTPKEKVTITCRASQSIGSSLHWYQQKPDQSPKLLIKYASQSFSGVPS  60
61  RFSGSGSGTDFTLTINSLEAEDAAAYYCHQSSSLPFTFGPGTKVDIKRTVAAPSVFIFPP  120
121 SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT  180
181 LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC                            214
```

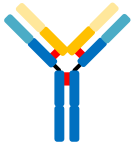
Number of amino acids: 214 MW = 23'357.9 Da pI = 6.49

Internal Standard and Protein Analysis

Protein

No IS

Protein
+
Labeled Protein (IS)
Threonine labeled with $^{13}C/^{15}N$

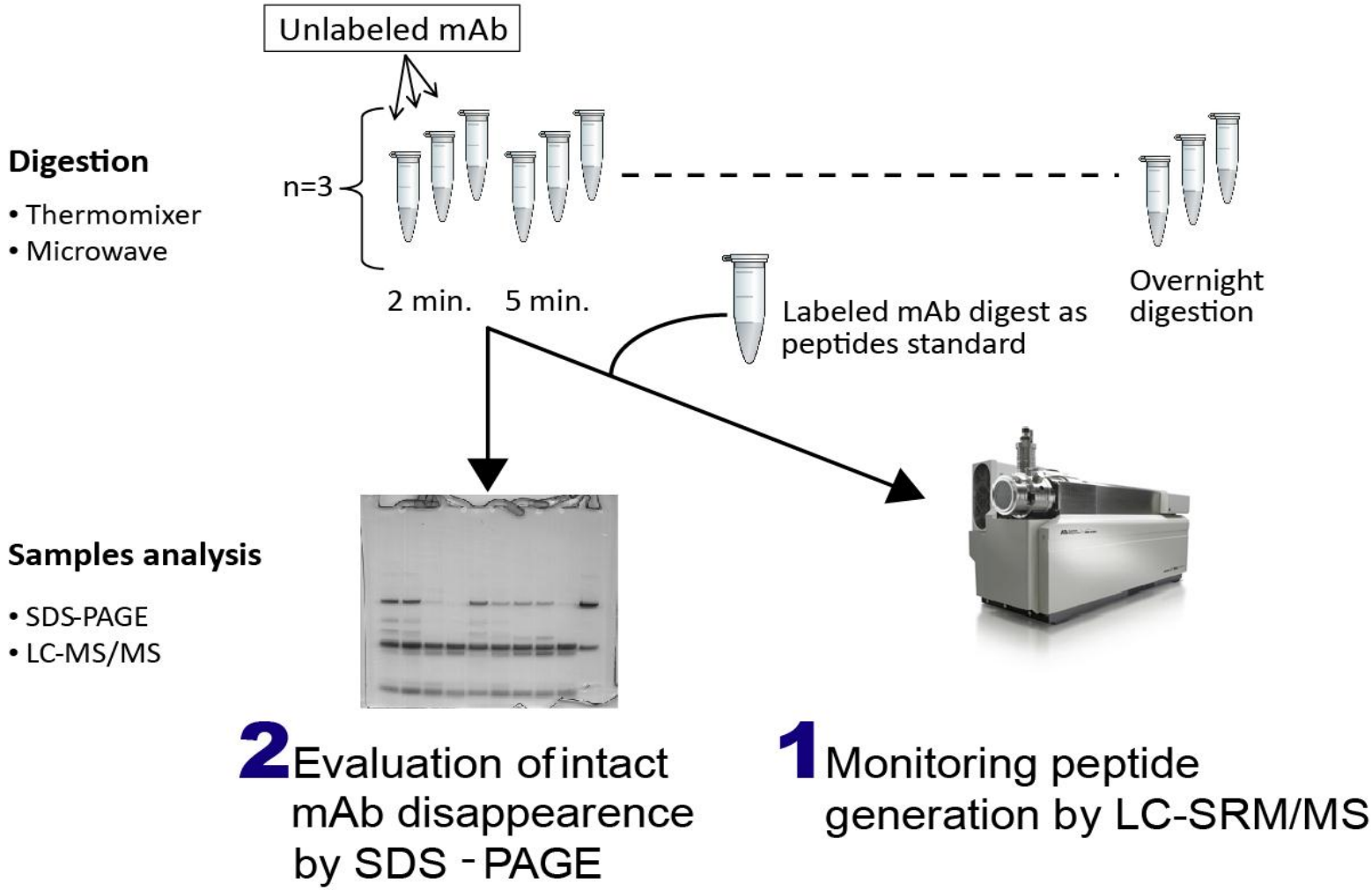


Protein
+
Labeled peptide (IS)

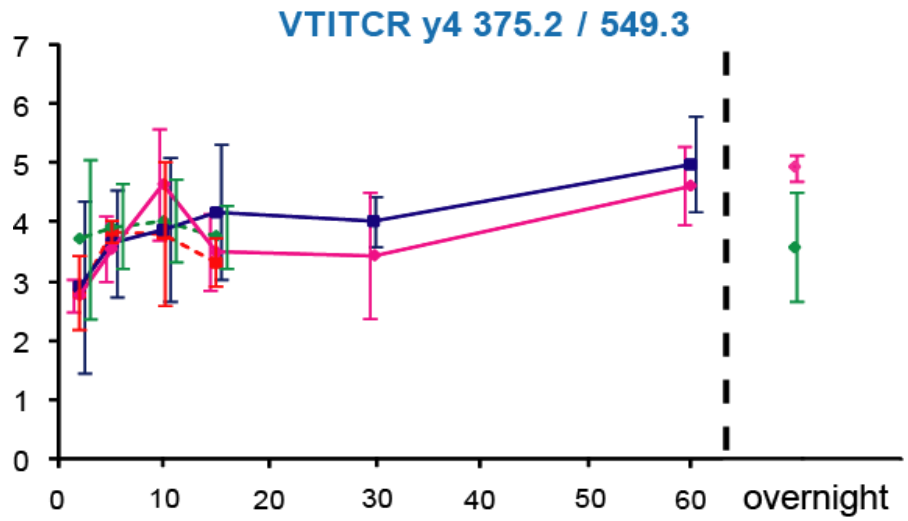
Digestion

Peptides → Signature Peptide + Labeled signature peptide → SRM LC-MS/MS quantitation

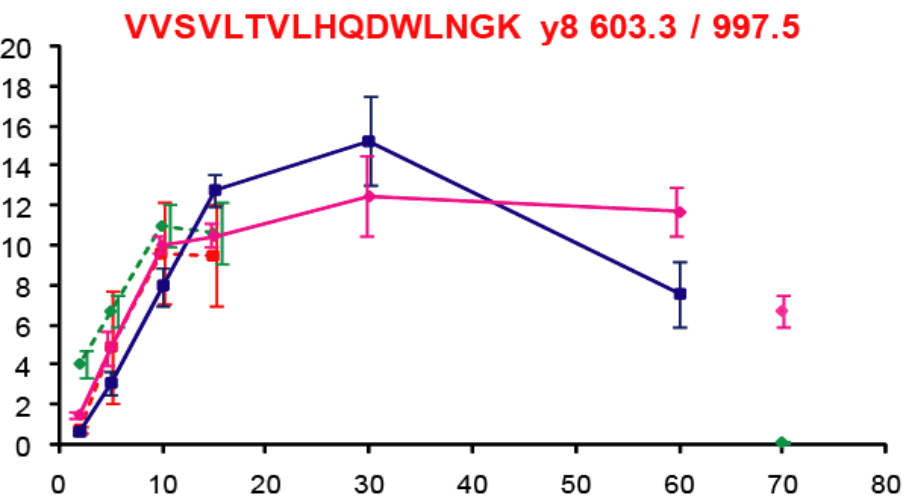
Evaluation of Accelerated Digestion

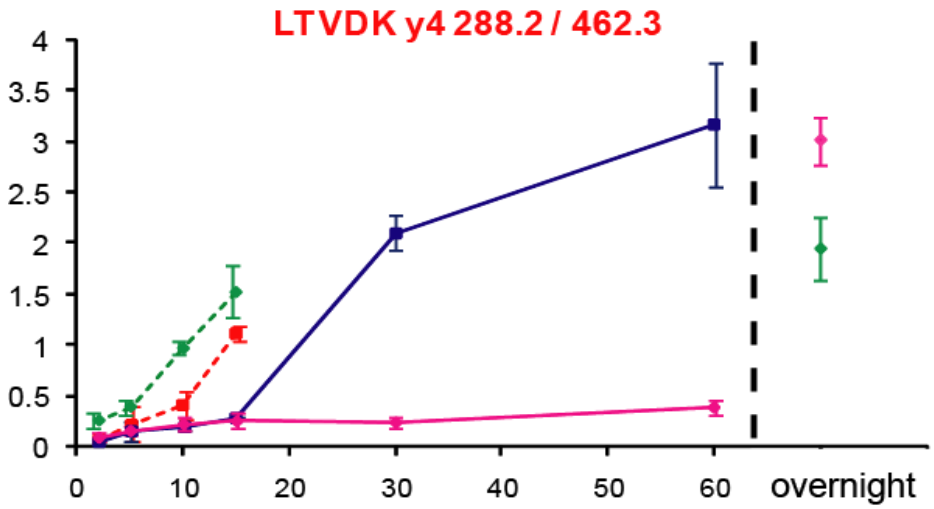


Peptides Kinetic Formation

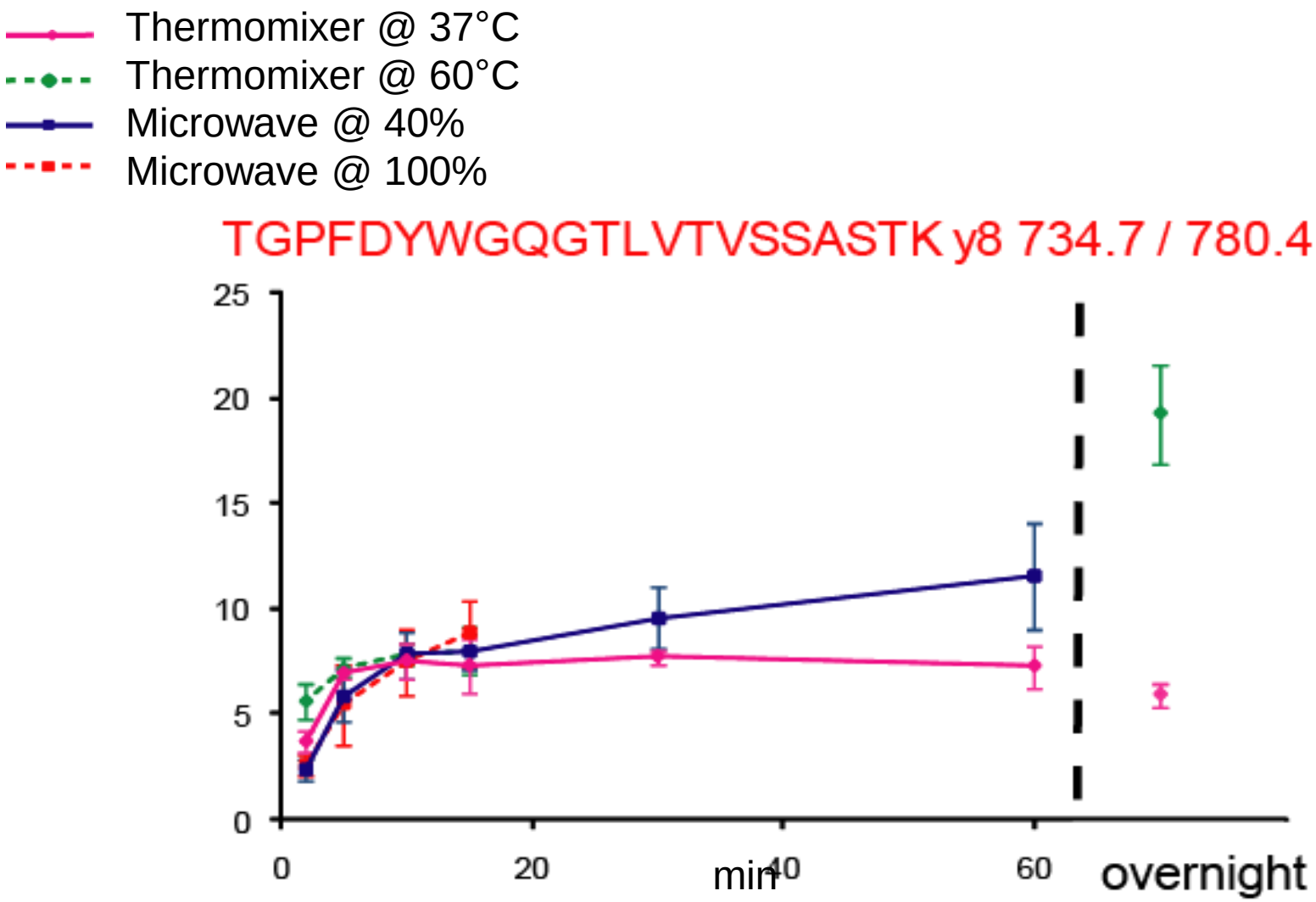


Thermomixer @ 37°C
Thermomixer @ 60°C
Microwave @ 40%
Microwave @ 100%

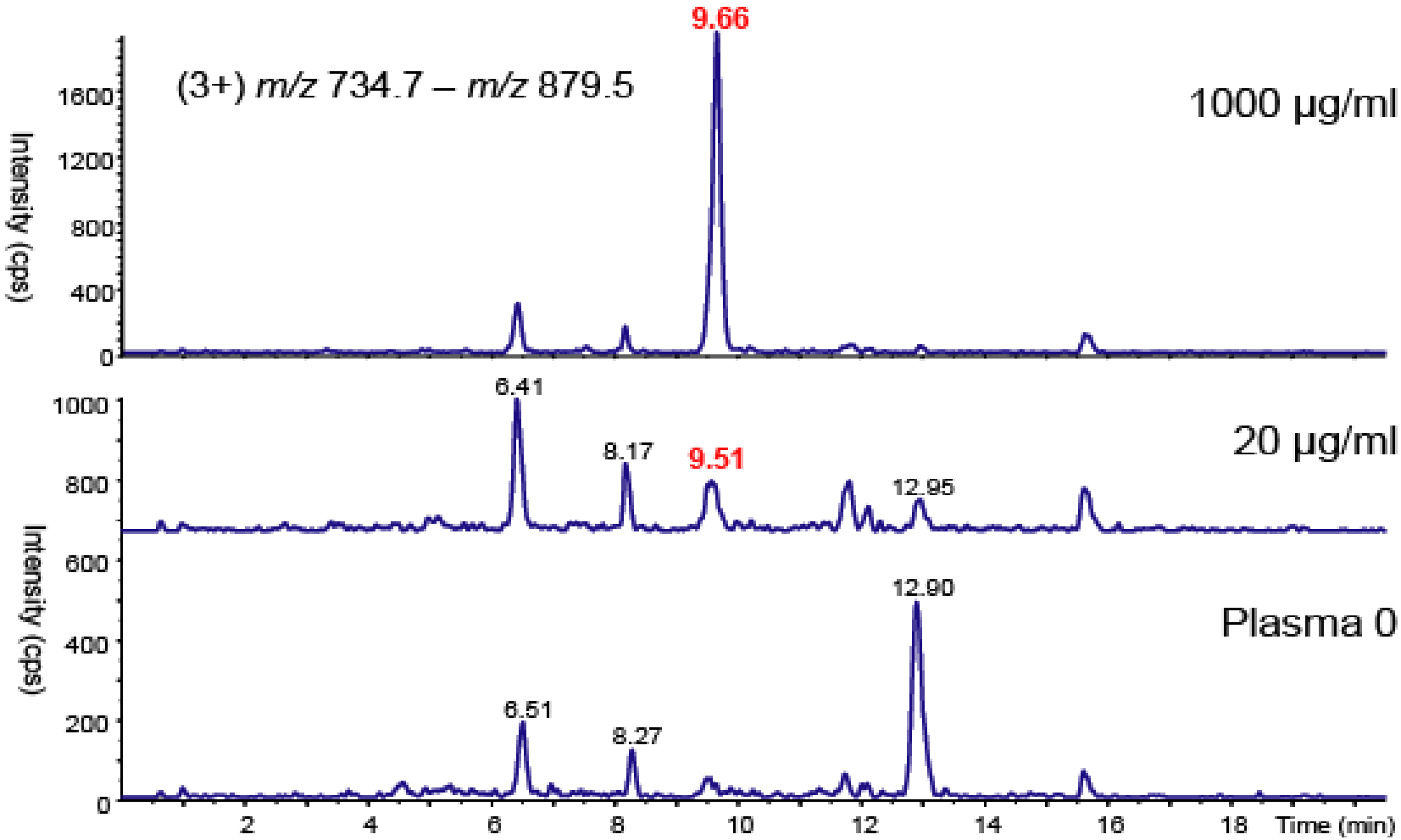




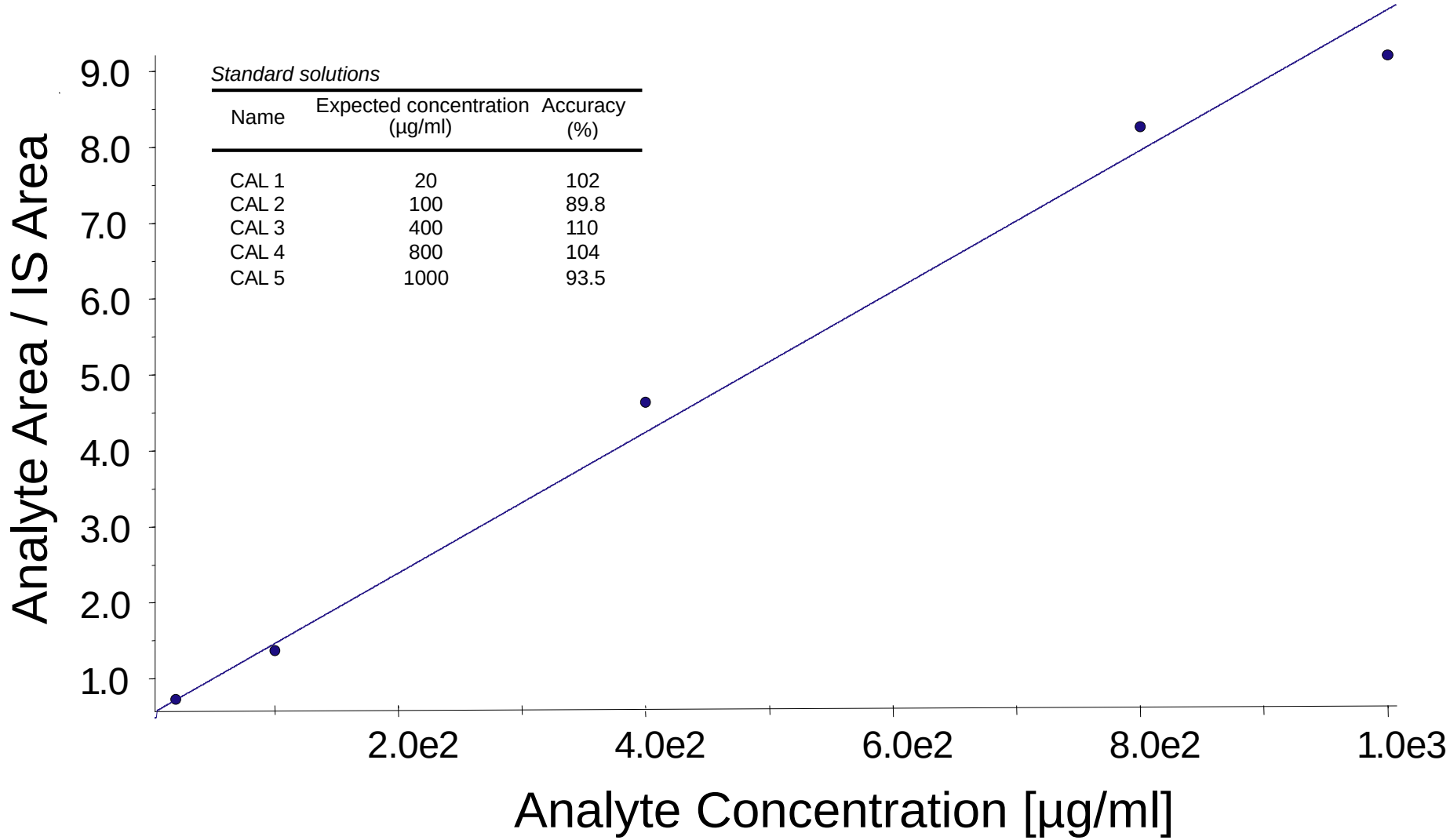
Signature Peptide for mAb's Quantitation in Human Plasma



Accelerated Digestion in Human Plasma



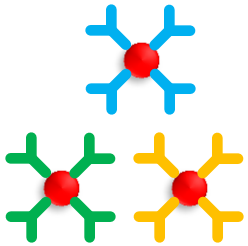
Calibration Curve in Human Plasma



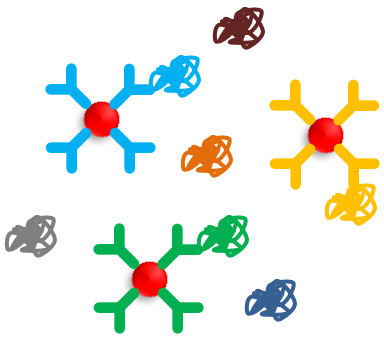
Standard solutions

Name	Expected concentration (µg/ml)	Accuracy (%)
CAL 1	20	102
CAL 2	100	89.8
CAL 3	400	110
CAL 4	800	104
CAL 5	1000	93.5

Immunocapture Techniques coupled to MS

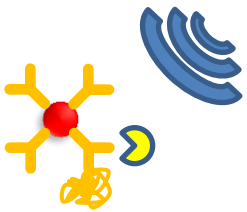


Immobilization of antibodies on **magnetic beads**

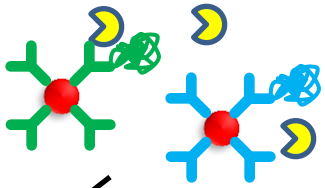


Capture of targeted proteins (in plasma samples)

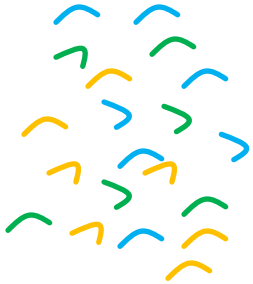
Tryptic digestion directly on beads



Microwave assisted digestion

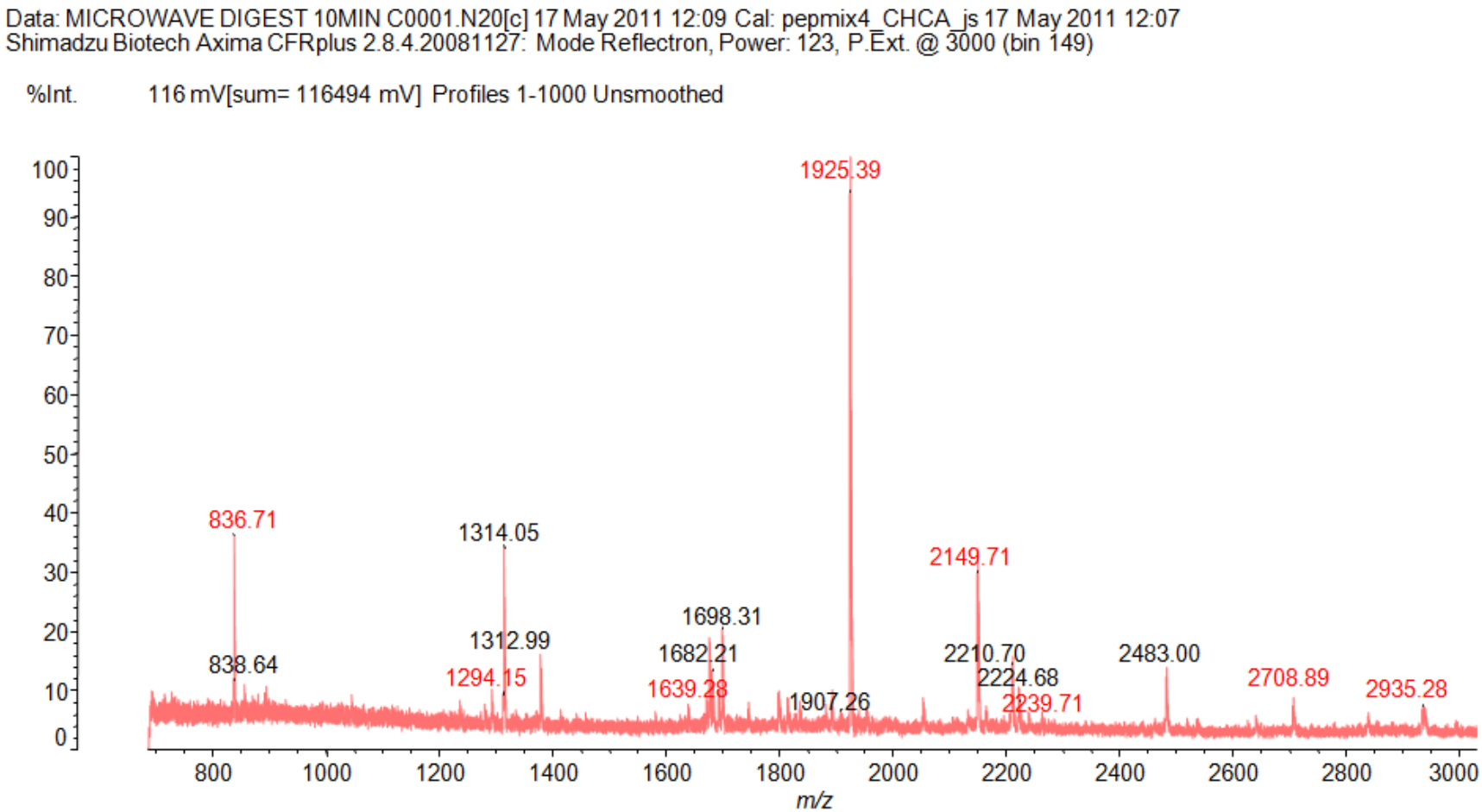


Removal of the magnetic beads



LC-SRM/MS analysis

Goat IgG Captured by Magnetic Beads Coated with Protein G



- 10 min digestion assisted by microwave

- 44% coverage for the heavy chain

How Selective is Liquid Chromatography with Selected Reaction Monitoring Quantitation?

Protein: **Osteopontin** (P10451-1 | OSTP_HUMAN)

```
1   MRIAVICFCL LGITCAIPVK QADSGSSEEK QLYNKYPDAV ATWLNPDPSQ 50
51  KQNLLAPQNA VSSEETNDFK QETLPSKSNE SHDHMDDMDD EDDDDHVDSQ 100
101 DSIDSNDSDD VDDTDDSHQS DESHHSDESD ELVTDFPTDL PATEVFTPVV 150
151 PTVDTYDGRG DSVVYGLRSK SKKFRRPDIQ YPDATDEDIT SHMESEELNG 200
201 AYKAIPVAQD LNAPSDWDSR GKDSYETSQL DDQSAETHSH KQSRLYKRKA 250
251 NDESNEHSDV IDSQELSKVS REFHSHEFHS HEDMLVVDPK SKEEDKHLKF 300
301 RISHELDSAS SEVN                                       314
```

MW (average) = 33’713.53 Da pI = 4.35 (mature protein)

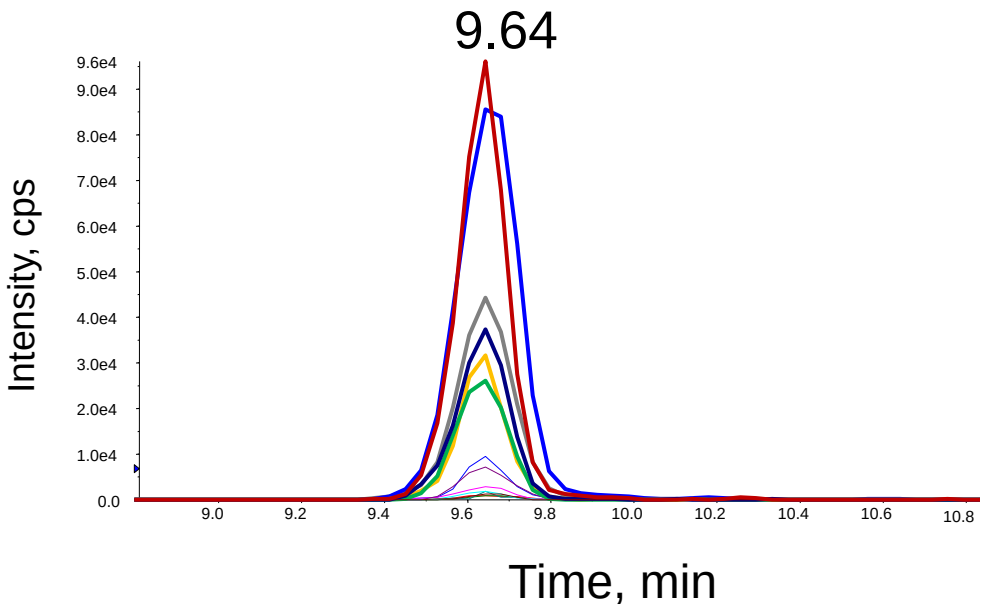
4 isoforms are described in UniProtKB (www.uniprot.org/uniprot/P10451#section_alternative)

Heavily phosphorylated and glycosylated protein

SRM Selectivity in Human Plasma
(Osteopontin phosphopeptide)

EFH**S***HEFH**S***HEDMLVVDPK Precursor *m/z* 480.8 z=5^{+}

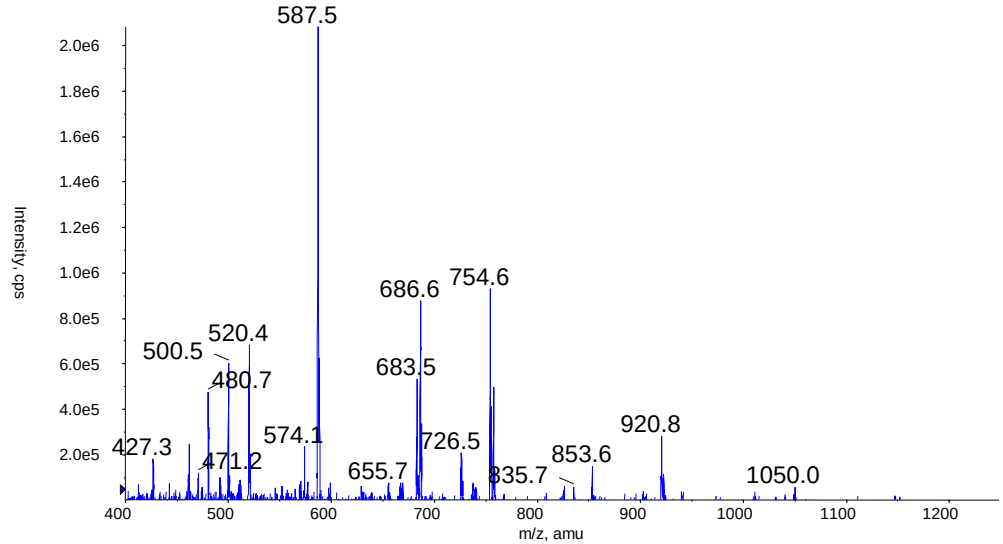
Product m/z	ion	charge
501.2	b4	1
588.3	y14	3
377.8	b8	3
922.3	b14	4
461.7	b14	3
511.2	b16	4
401.2	y7	2
757.7	y18	3
251.1	b4	2
633.9	y15	3
277.1	b2	1
414.2	b3	1
577.5	b13	3
497.5	y16	4

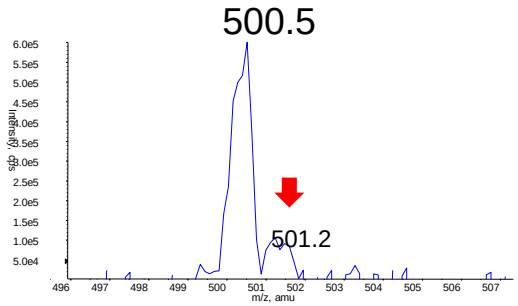


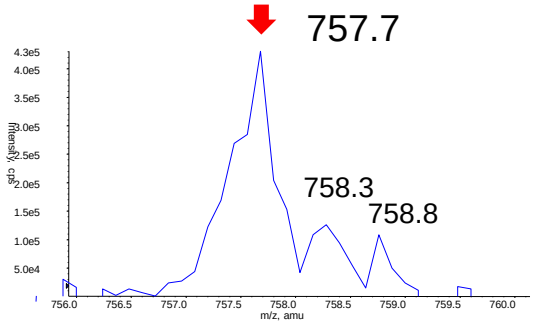
14 transitions at the same retention

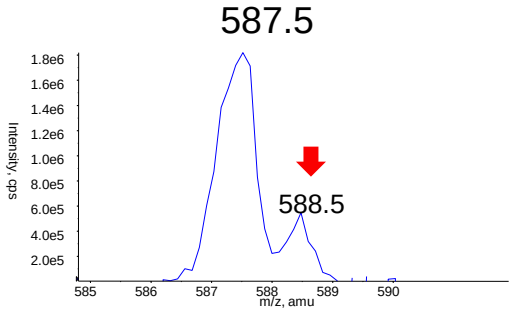
Product Ion MS/MS Spectrum of *m/z* 480.8

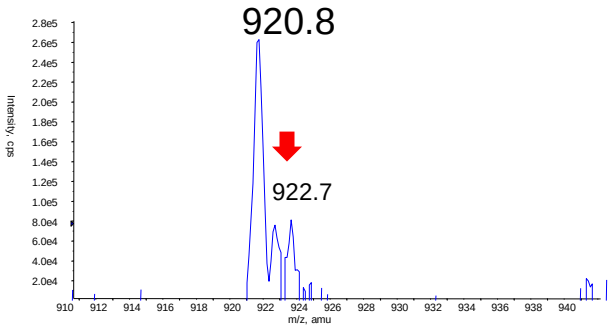
Product m/z	ion	charge
501.2	b4	1
588.3	y14	3
377.8	b8	3
922.3	b14	4
461.7	b14	3
511.2	b16	4
401.2	y7	2
757.7	y18	3
251.1	b4	2
633.9	y15	3
277.1	b2	1
414.2	b3	1
577.5	b13	3
497.5	y16	4

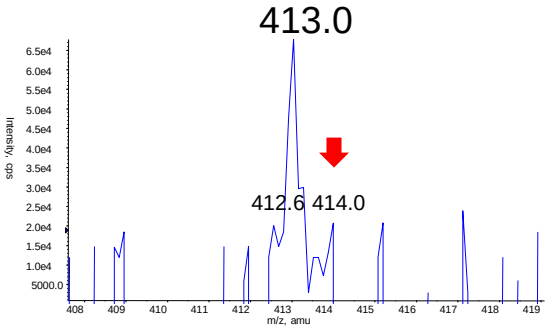










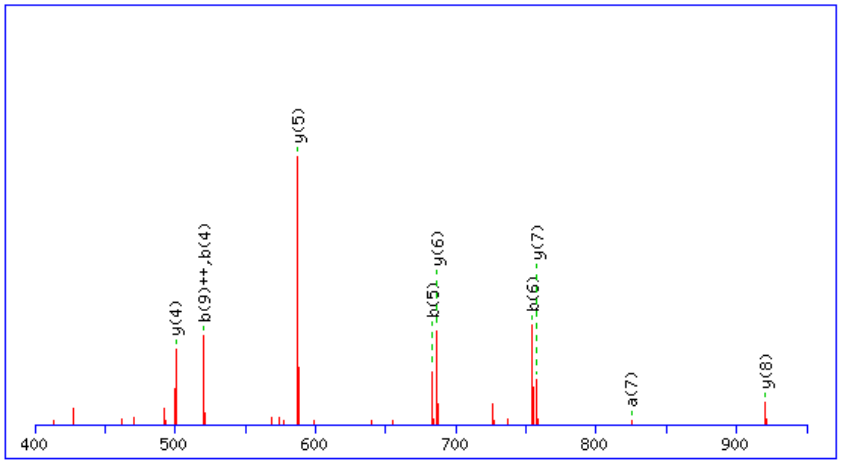


Database Search (UniProtKB_SP)

MS/MS Fragmentation of **RHPEYAVSVLLR**
Found in **ALBU_BOVIN**, Serum albumin precursor (Allergen Bos d 6) (BSA) - Bos taurus (Bovine)

Match to Query 1: 1439.408172 from(480.810000,3+)
Data file C:\Documents and Settings\LESUR\Desktop\Mascot_temp\mas99.tmp

Click mouse within plot area to zoom in by factor of two about that point
Or, Plot from 400 to 950 Da Full range



#	a	a++	a*	a*++	b	b++	b*	b*++	Seq.	y	y++	y*	y*++	#
1	129.11	65.06	112.09	56.55	157.11	79.06	140.08	70.54	R					12
2	266.17	133.59	249.15	125.08	294.17	147.59	277.14	139.07	H	1283.71	642.36	1266.68	633.85	11
3	363.23	182.12	346.20	173.60	391.22	196.11	374.19	187.60	P	1146.65	573.83	1129.63	565.32	10
4	492.27	246.64	475.24	238.12	**520.26**	260.63	503.24	252.12	E	1049.60	525.30	1032.57	516.79	9
5	655.33	328.17	638.30	319.66	**683.33**	342.17	666.30	333.65	Y	**920.56**	460.78	903.53	452.27	8
6	726.37	363.69	709.34	355.17	**754.36**	377.69	737.34	369.17	A	**757.49**	379.25	740.47	370.74	7
7	**825.44**	413.22	808.41	404.71	853.43	427.22	836.40	418.71	V	**686.46**	343.73	669.43	335.22	6
8	912.47	456.74	895.44	448.22	940.46	470.74	923.44	462.22	S	**587.39**	294.20	570.36	285.68	5
9	1011.54	506.27	994.51	497.76	1039.53	**520.27**	1022.51	511.76	V	**500.36**	250.68	483.33	242.17	4

Found "Albumin" peptide
RHPEYAVSVLLR *m/z* 480.6 z=3$^+$

Target peptide
EFHSHEFHSHEDMLVVDPK phosphorylated *m/z* 480.8 z=5$^+$

What Can We Do To Improve Selectivity To Avoid False Positive Results?

Sample preparation
Chromatography (UHPLC, multidimensional, …)

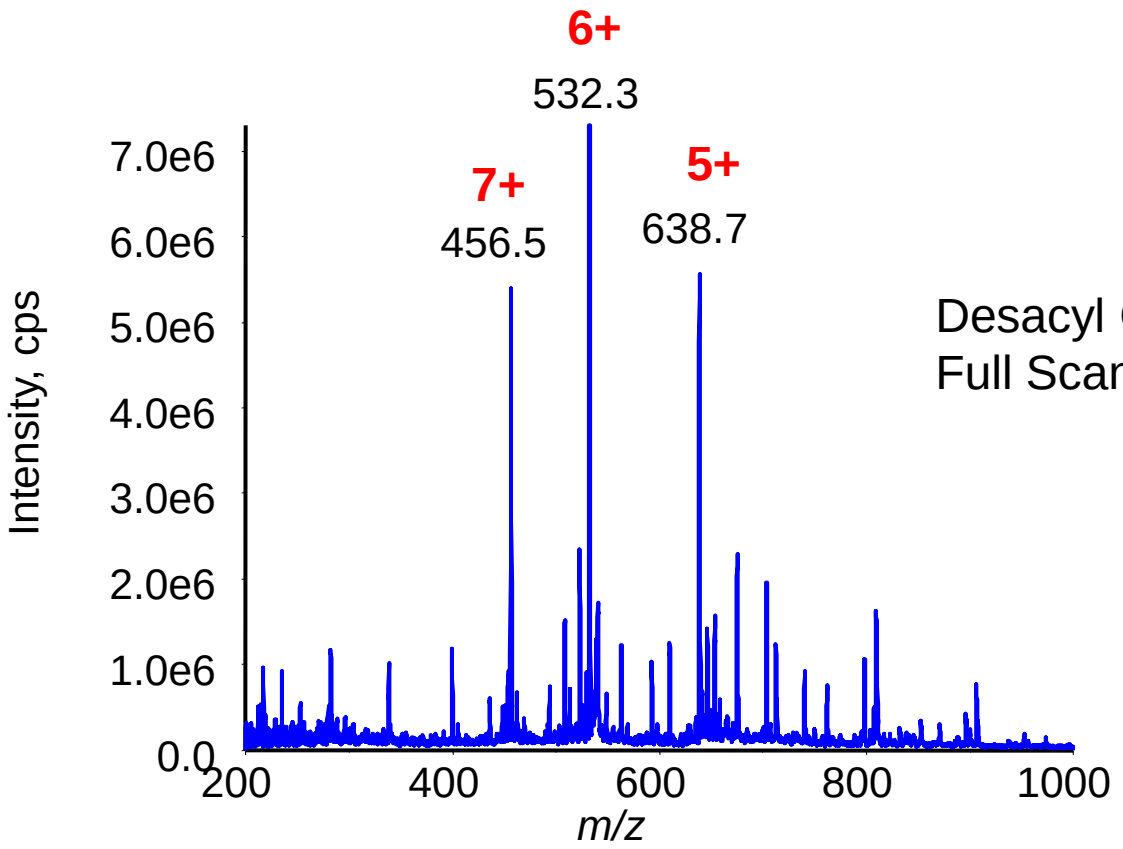
1) Multiple MS (SRM^3)
2) High Resolution: but How Much ?
3) Ion Mobility Spectrometry (DMS)
……

Ghrelin in Rat Plasma

GSS[1]FLSPEHQKAQQRKESKKPPAKLQPR

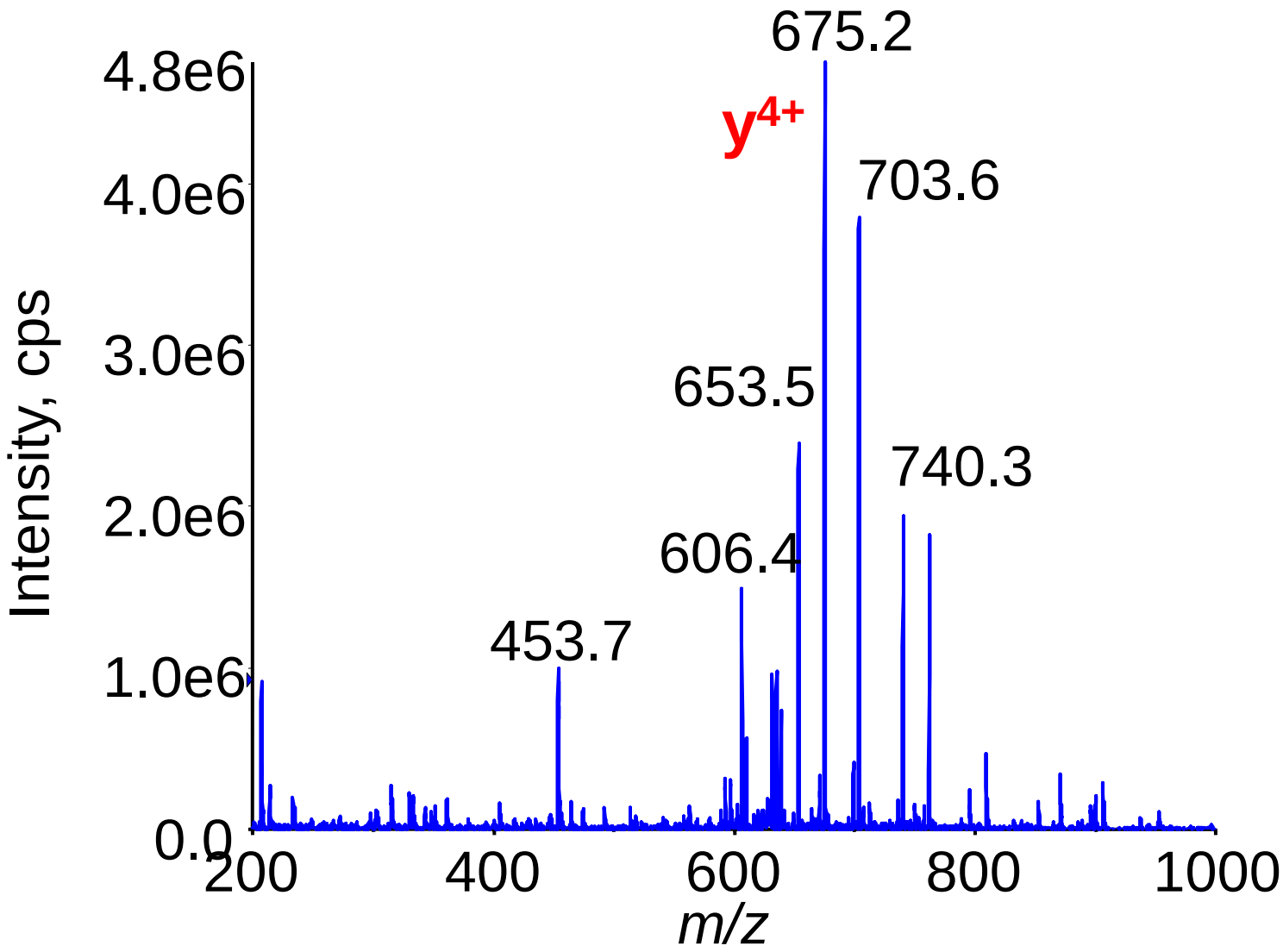
S[1]) with octanoyl 3314.6 u

S) without 3188.6 u

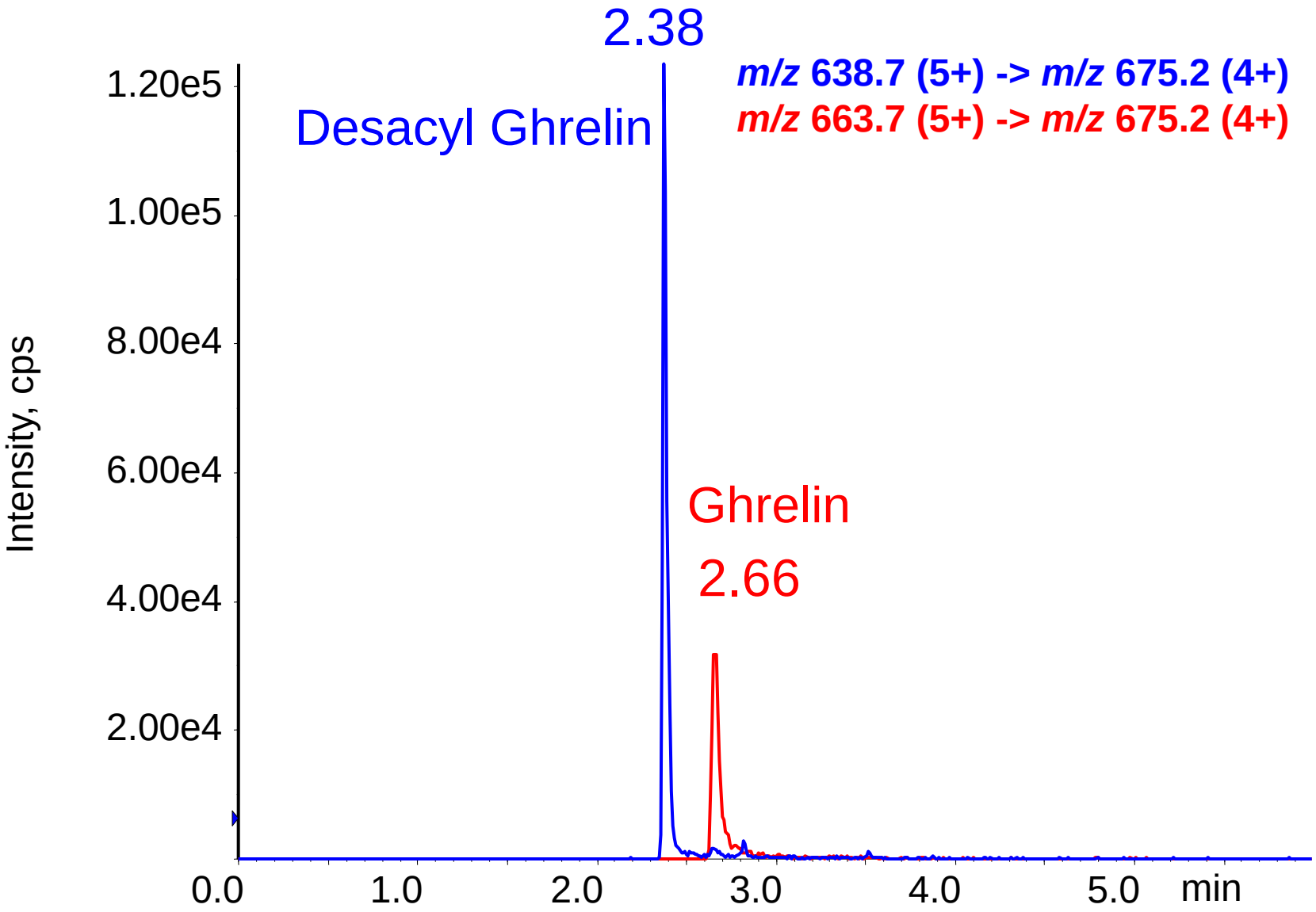


Desacyl Ghrelin
Full Scan MS Spectrum

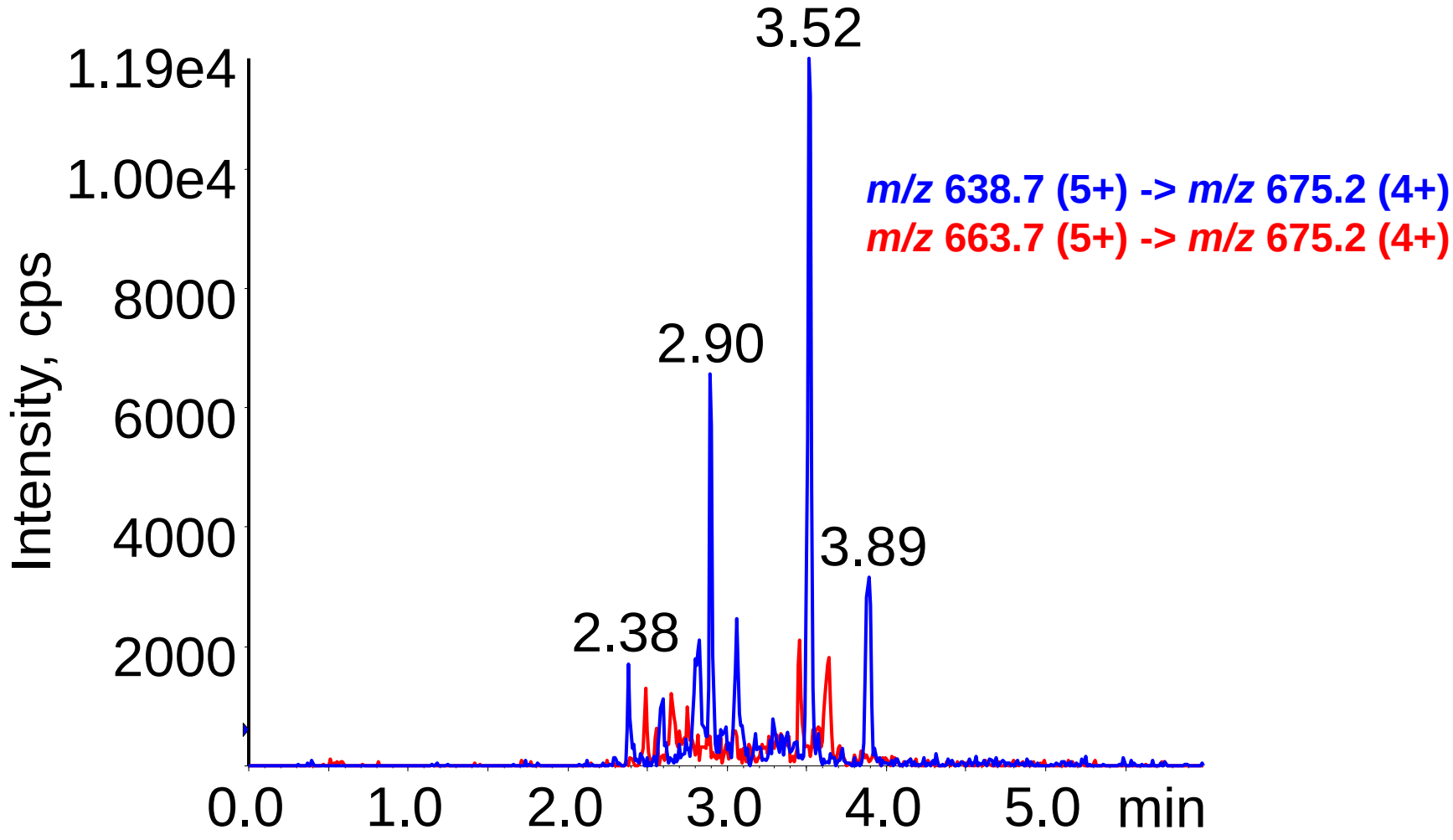
Product Ion Spectrum from 5^+ Precursor



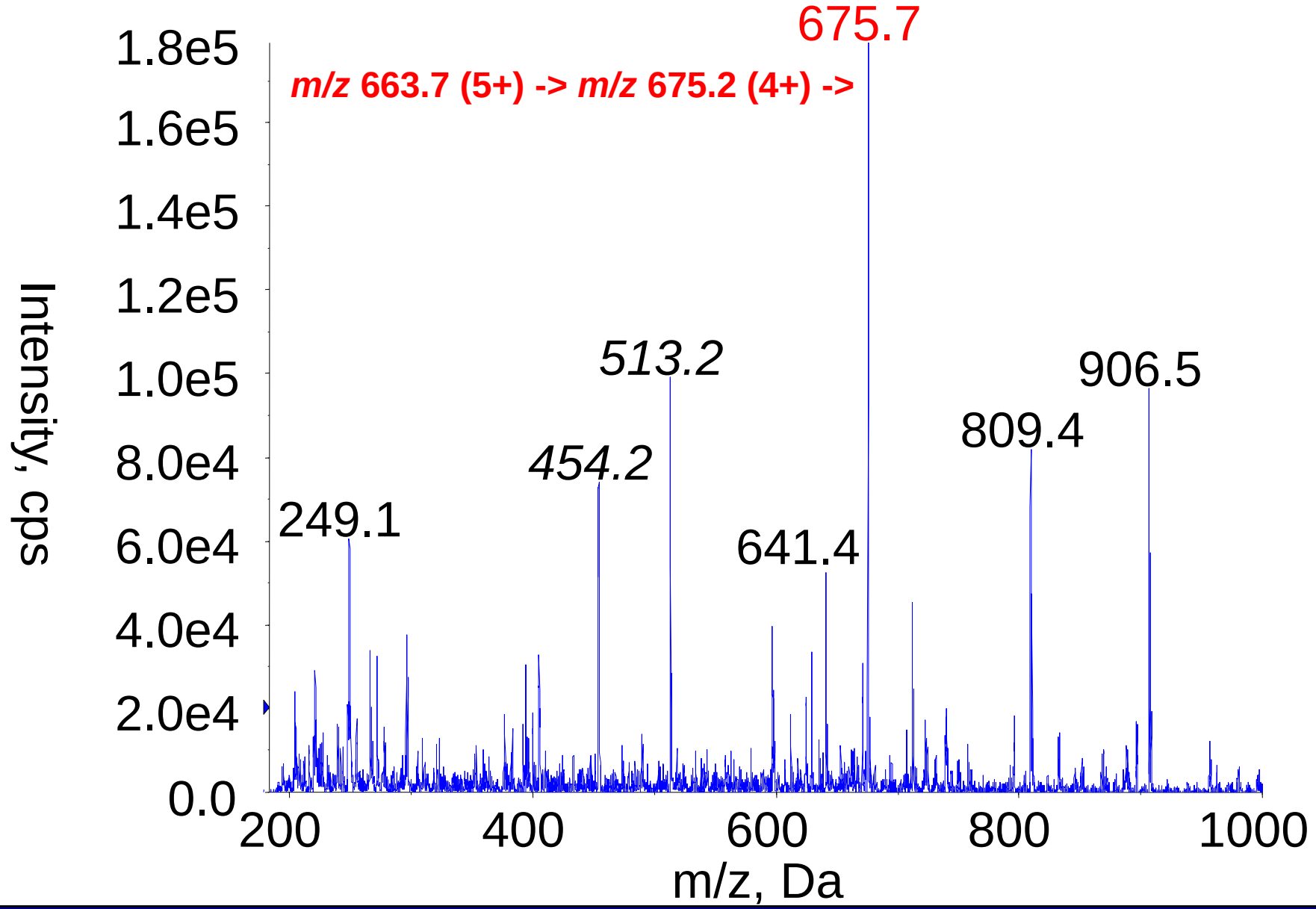
LC-SRM/MS Chromatogram (standard)



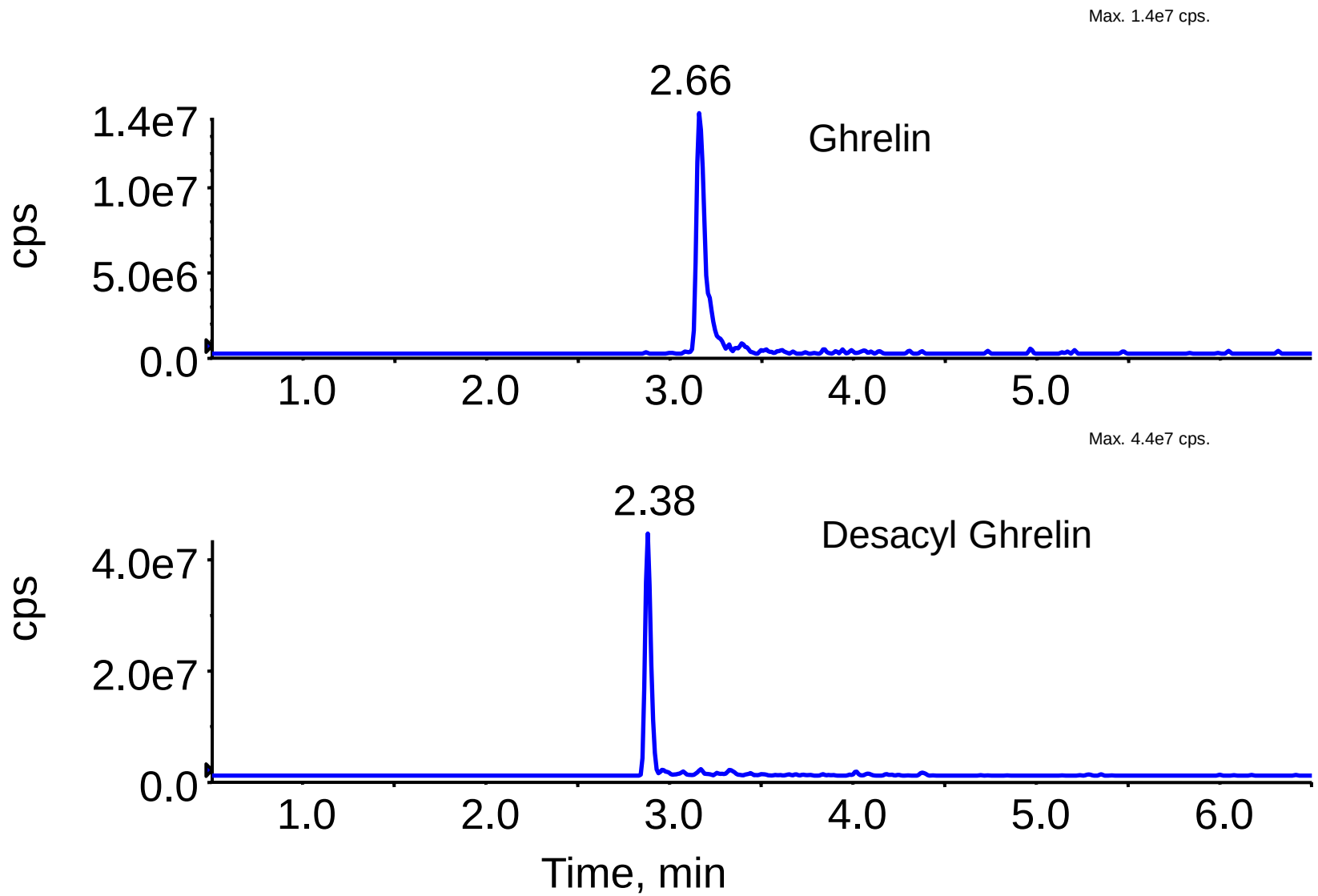
LC-SRM/MS Analysis Rat Plasma after Protein Precipitation with $HClO_4$



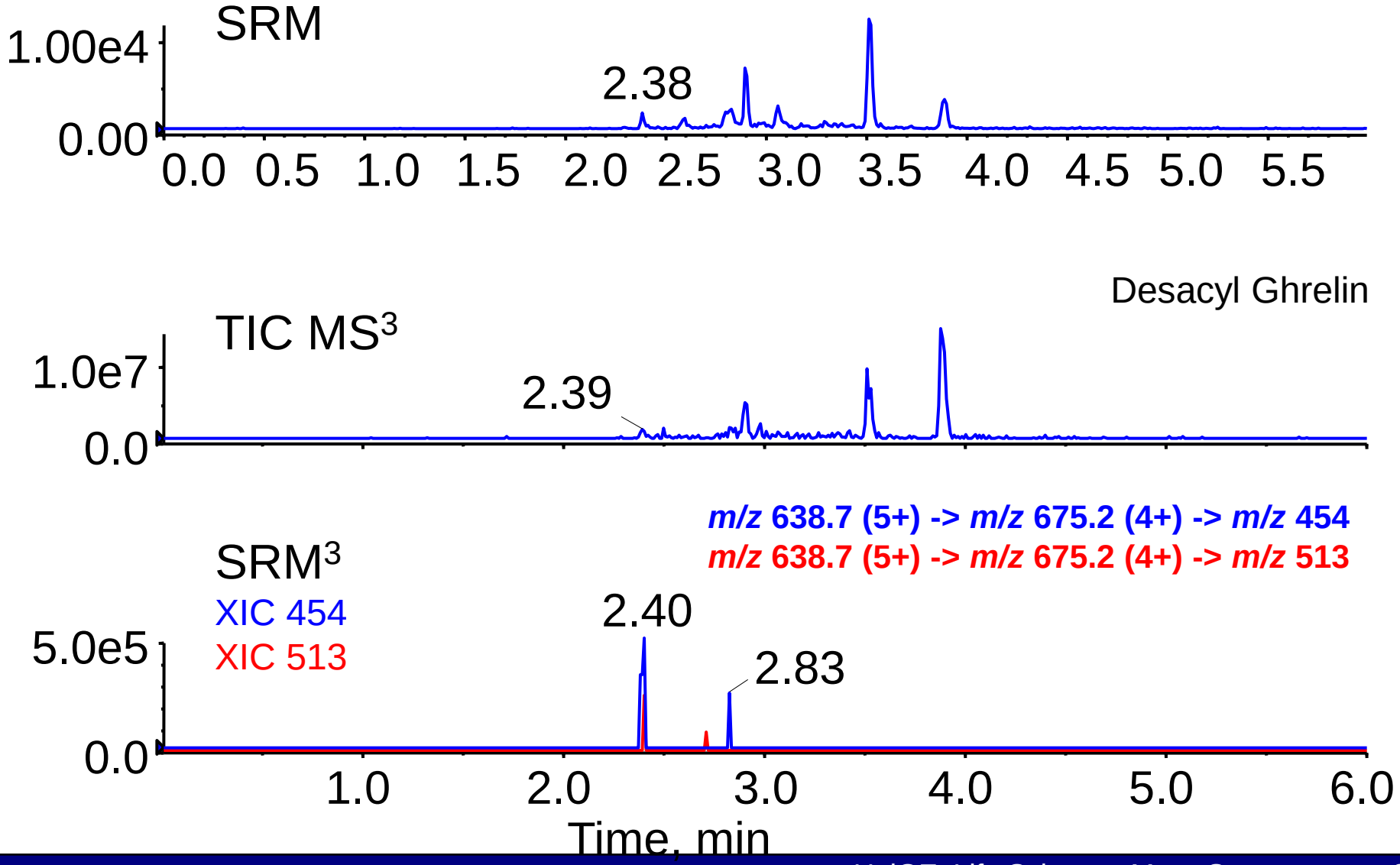
1. MS^3 Spectrum of Desacyl Ghrelin (standard)



LC-MS^3 traces (standard)

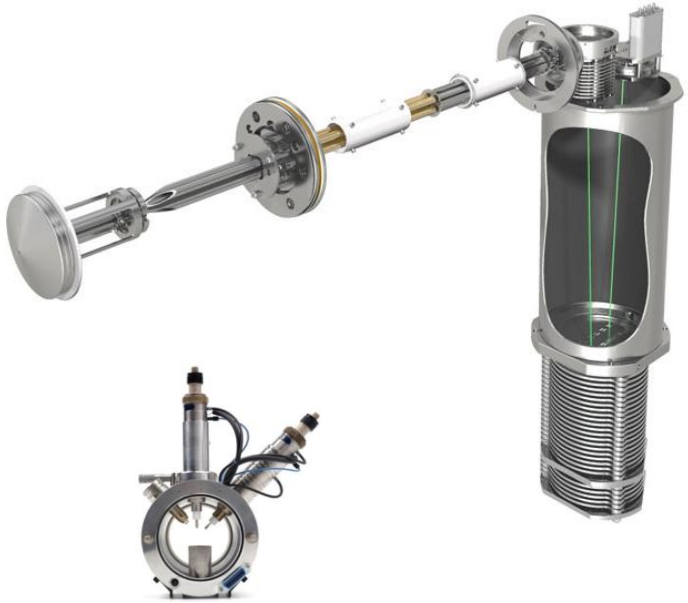


LC-MS analysis Rat Plasma after Protein Precipitation with $HClO_4$

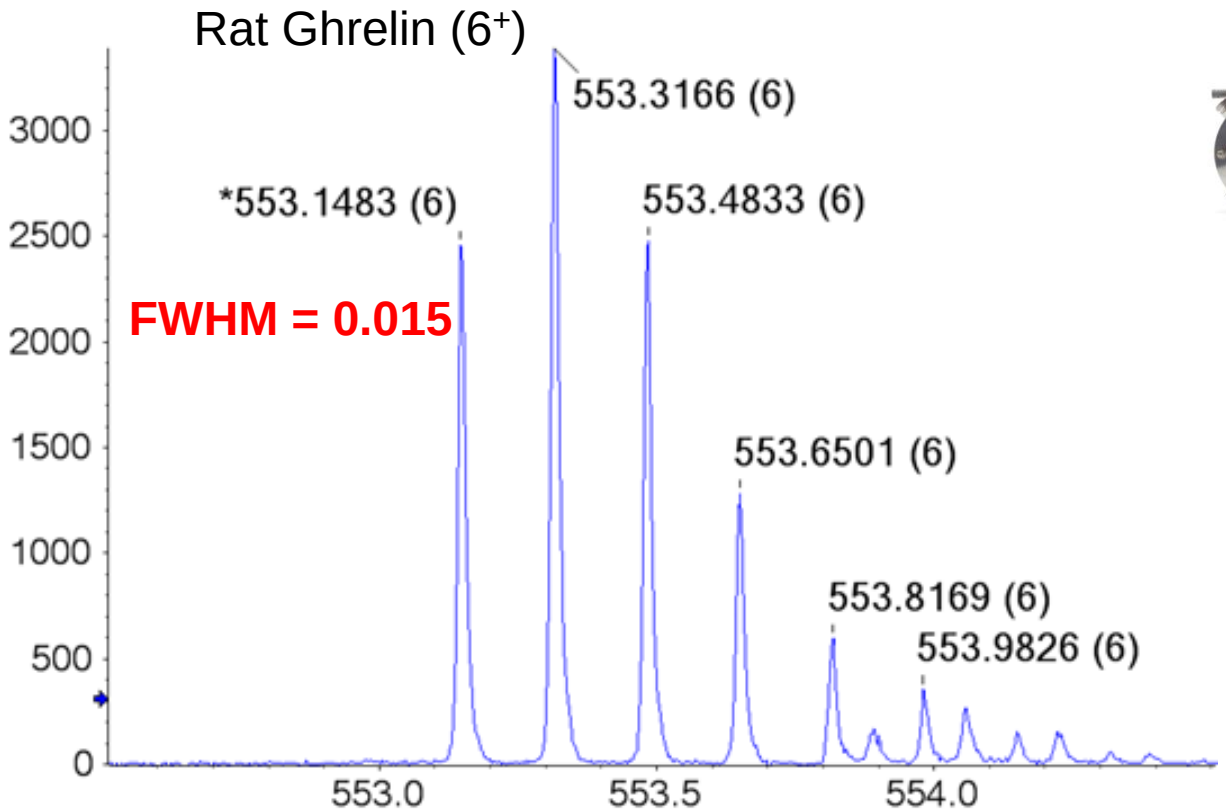


2. High Resolution and Accurate Mass Measurements

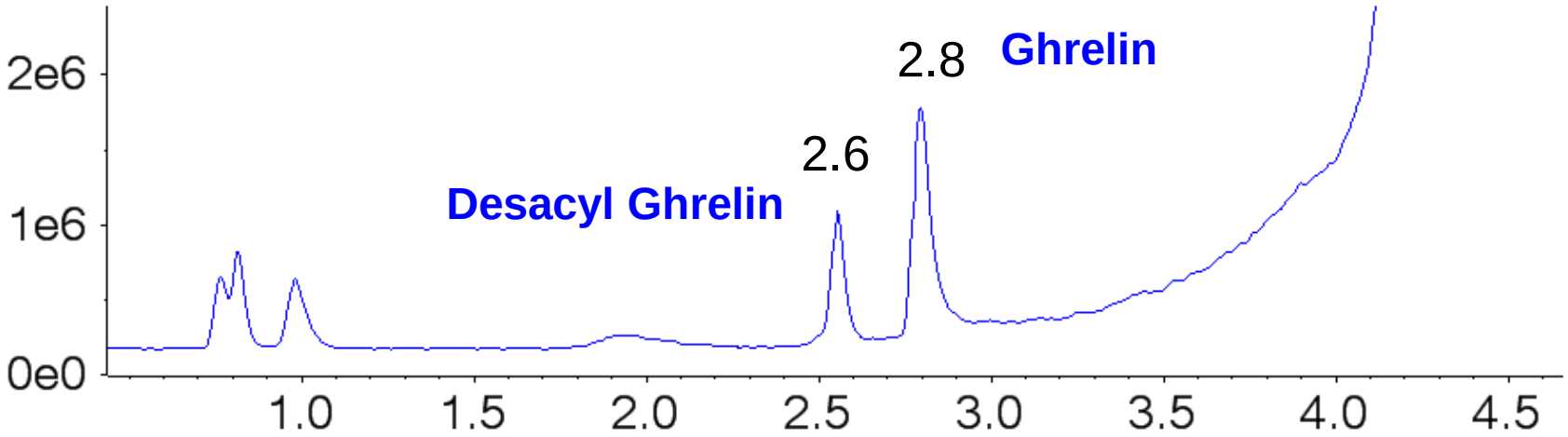
TOF MS and MS/MS ≤ 100 msec
Resolution ≥ 30’000
Mass Accuracy ≤ 2 ppm

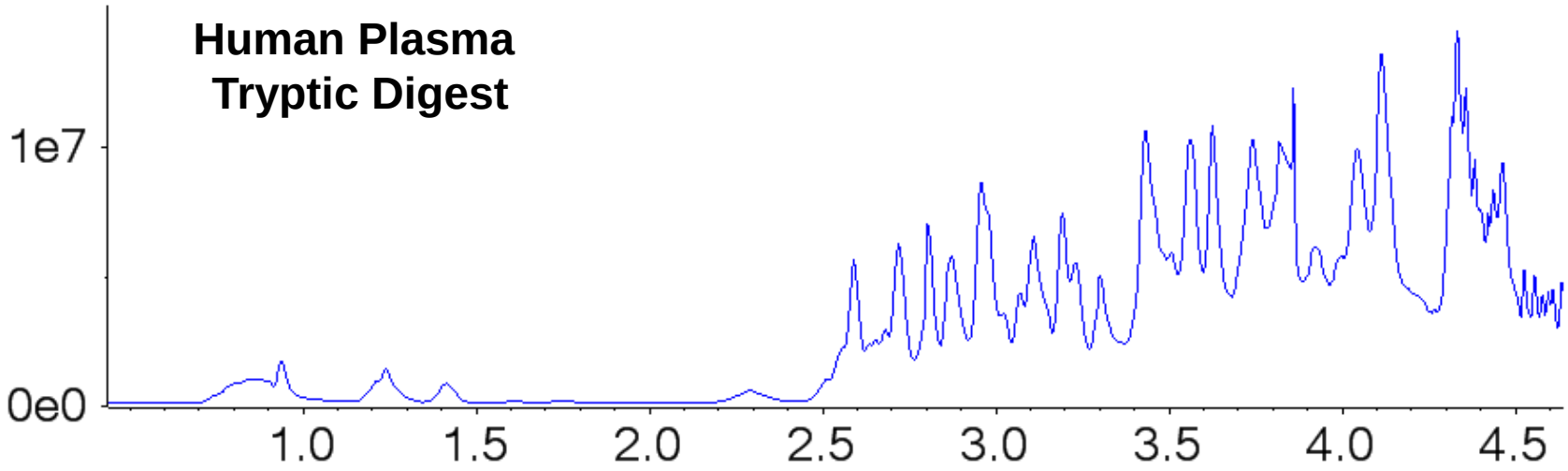


TripleTOF 5600
AB Sciex

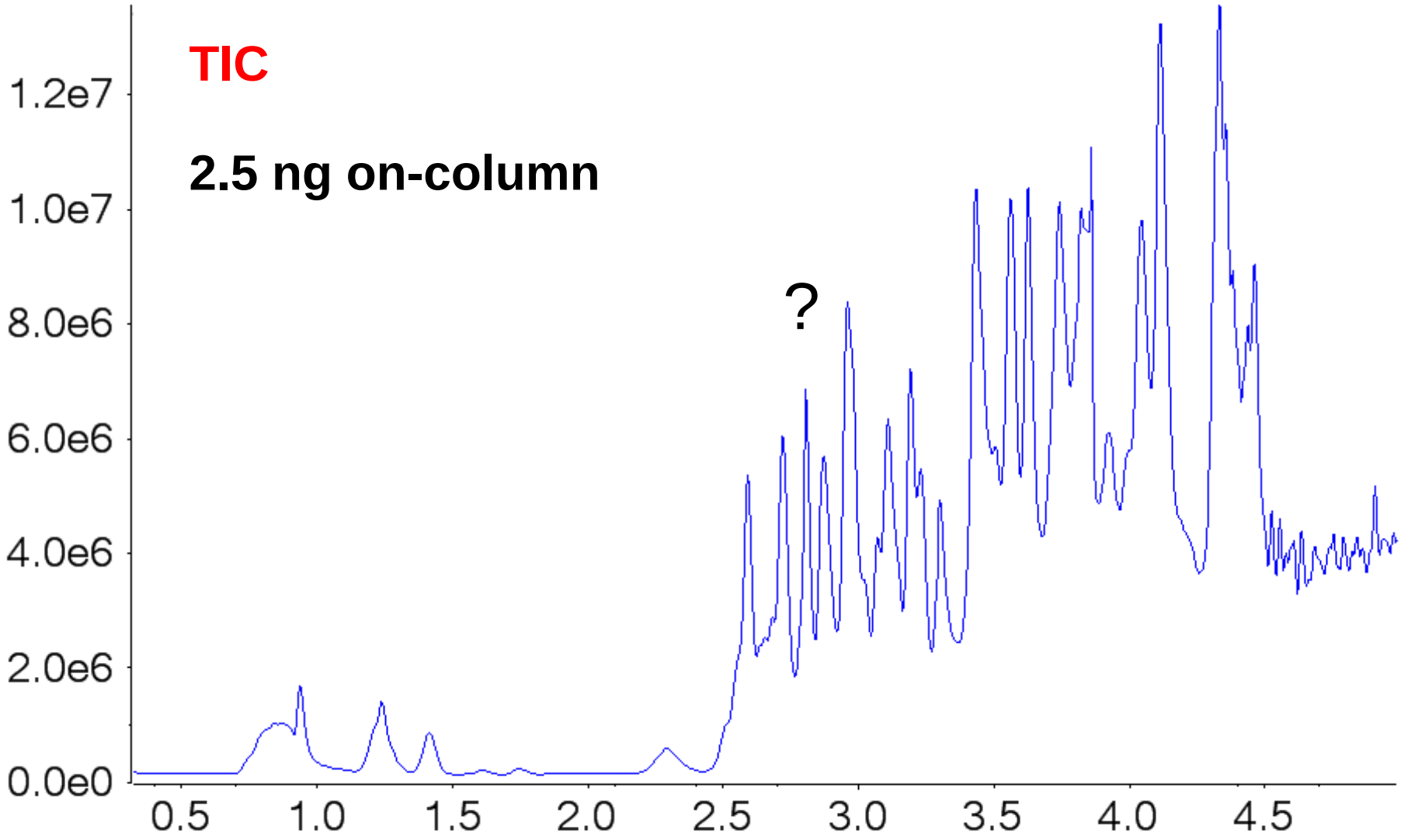


LC-TOF/MS

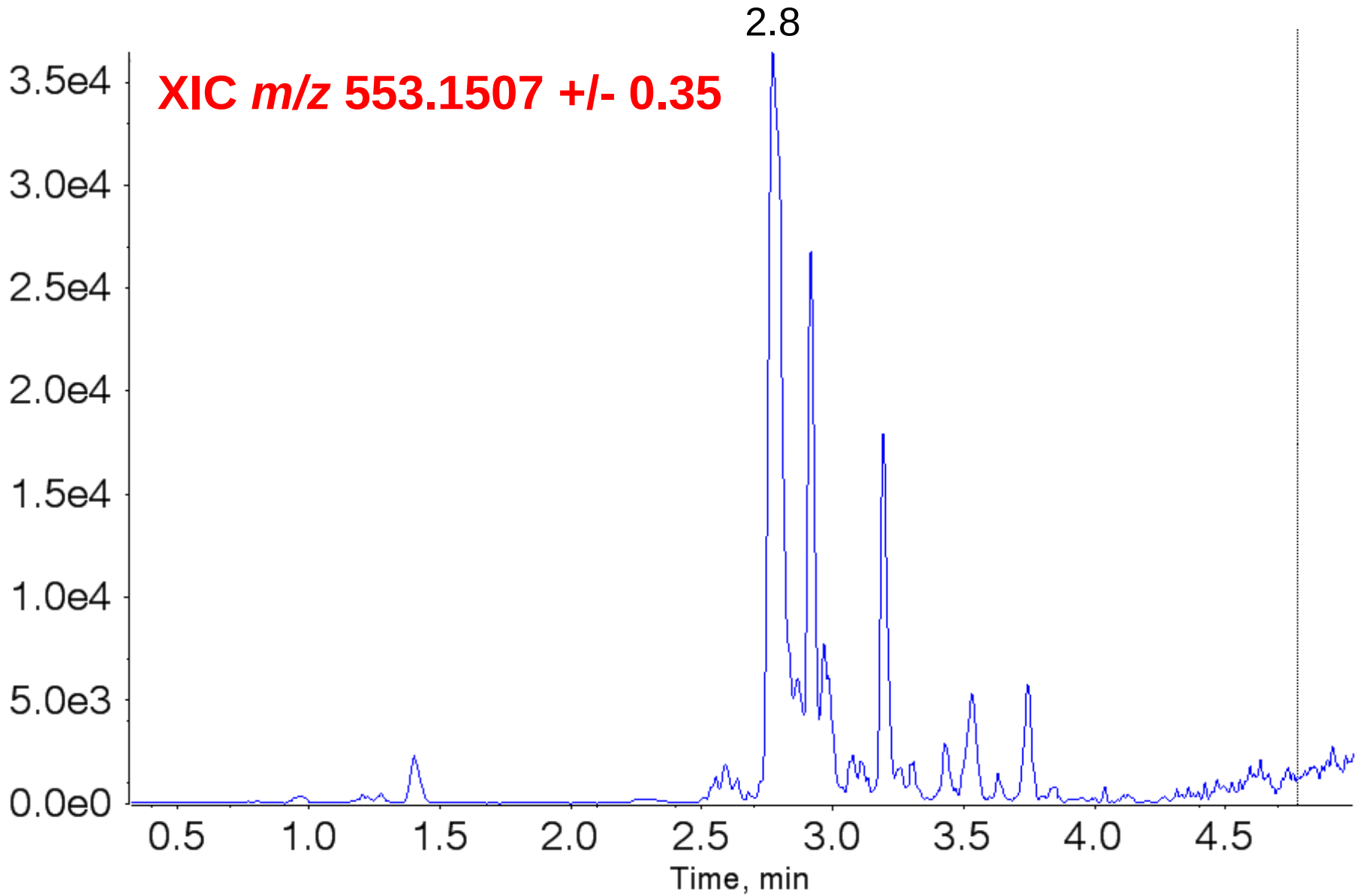

2.8
Ghrelin
2.6
Desacyl Ghrelin
2e6
1e6
0e0
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5


Human Plasma
Tryptic Digest
1e7
0e0
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5

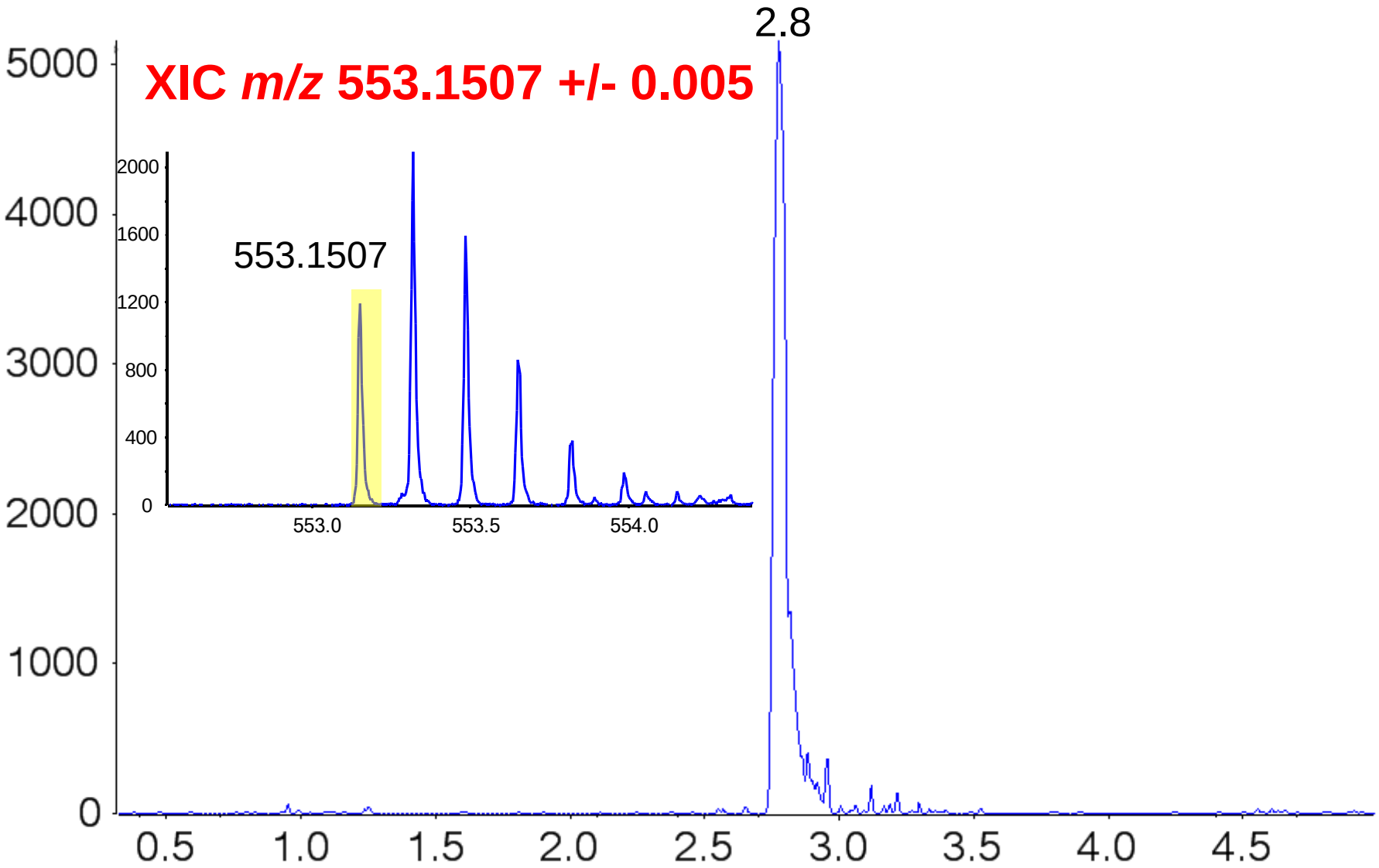
Human Plasma Digest Spiked with Rat Ghrelin



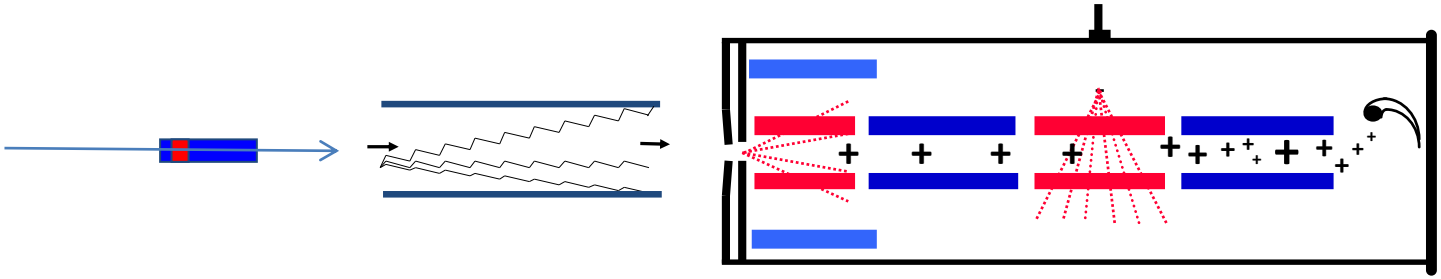
Human Plasma Digest Spiked with Rat Ghrelin



Human Plasma Digest Spiked with Rat Ghrelin



3. Differential Ion Mobility Spectrometry – Triple Quadrupole Linear Ion Trap (DMS-QqQ$_{LIT}$)

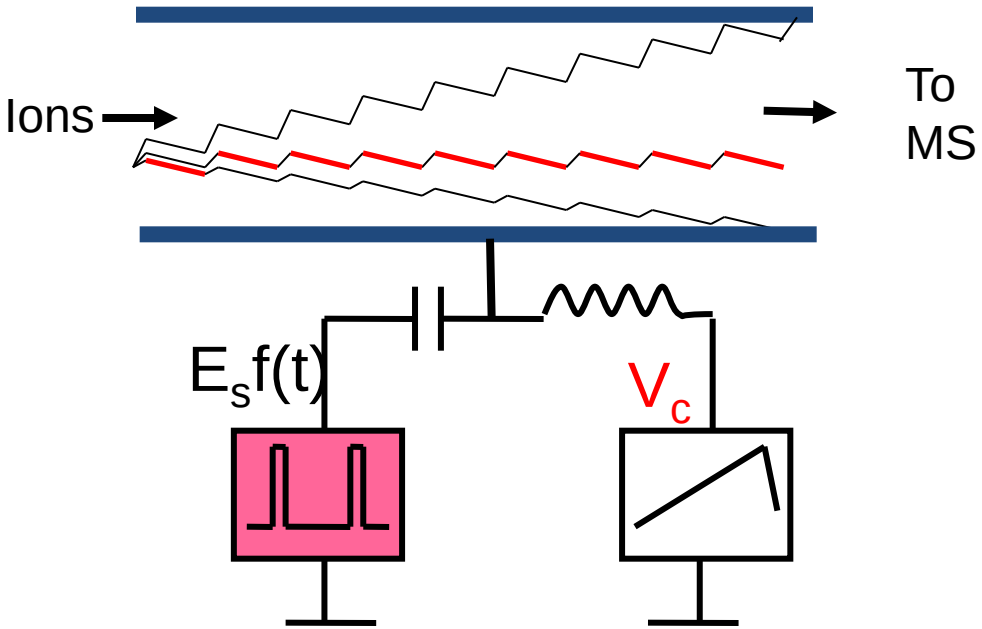


1st Dimension (HPLC) *2nd Dimension (DMS)* *3rd Dimension (MS or MS/MS)*

GS**S**[1]FLSPEHQKAQQRKESKKPPAKLQPR

Rat Ghrelin and Desacyl Ghrelin

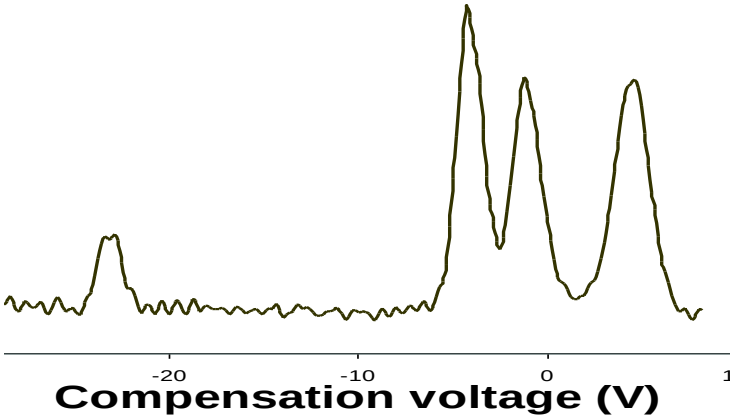
Differential Ion Mobility Spectrometry (DMS)



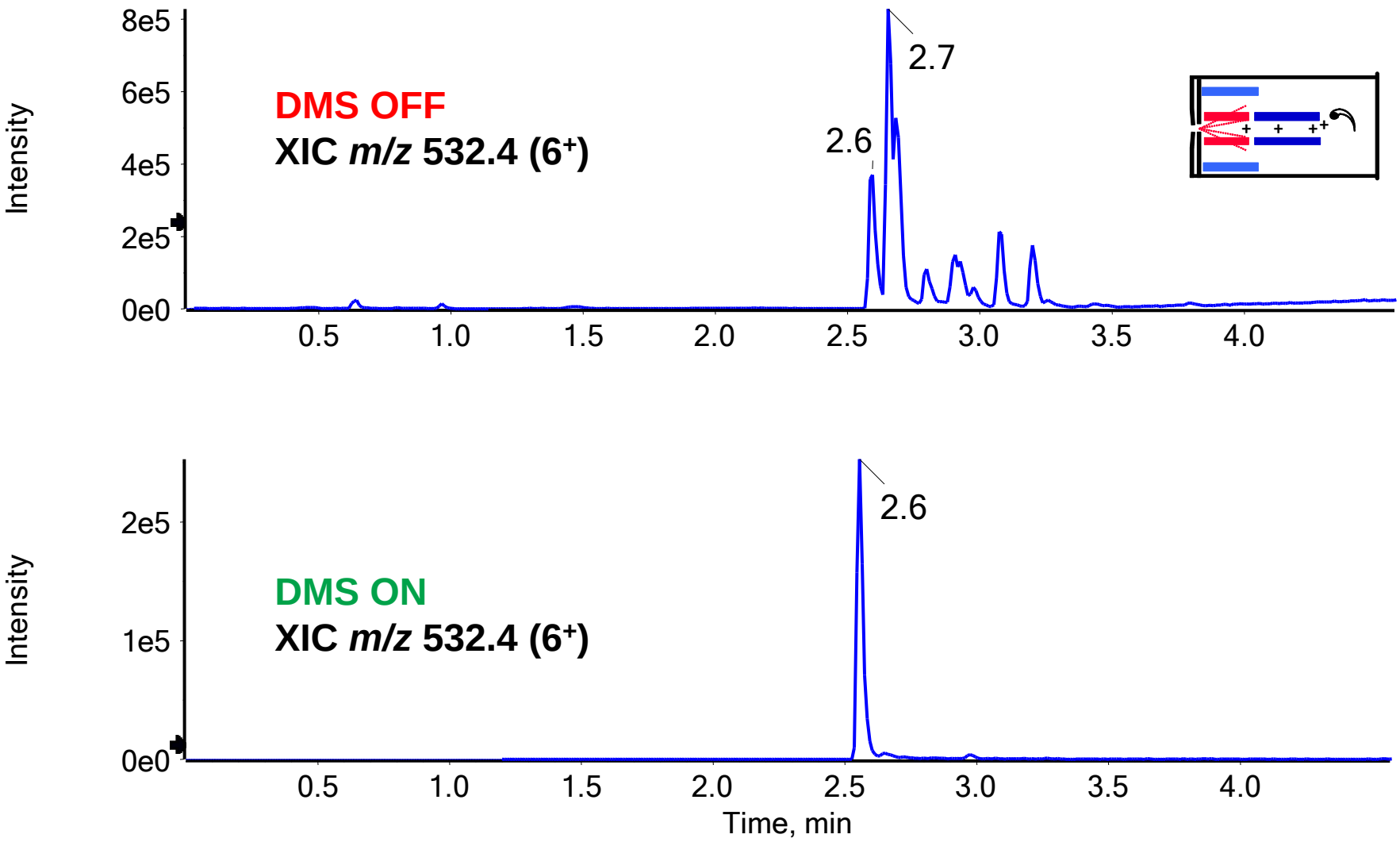
$K(E)=K_o(1+\alpha(E))$

a) **When V_c is constant and is tuned for certain ions species, DMS is operated as ions filter**

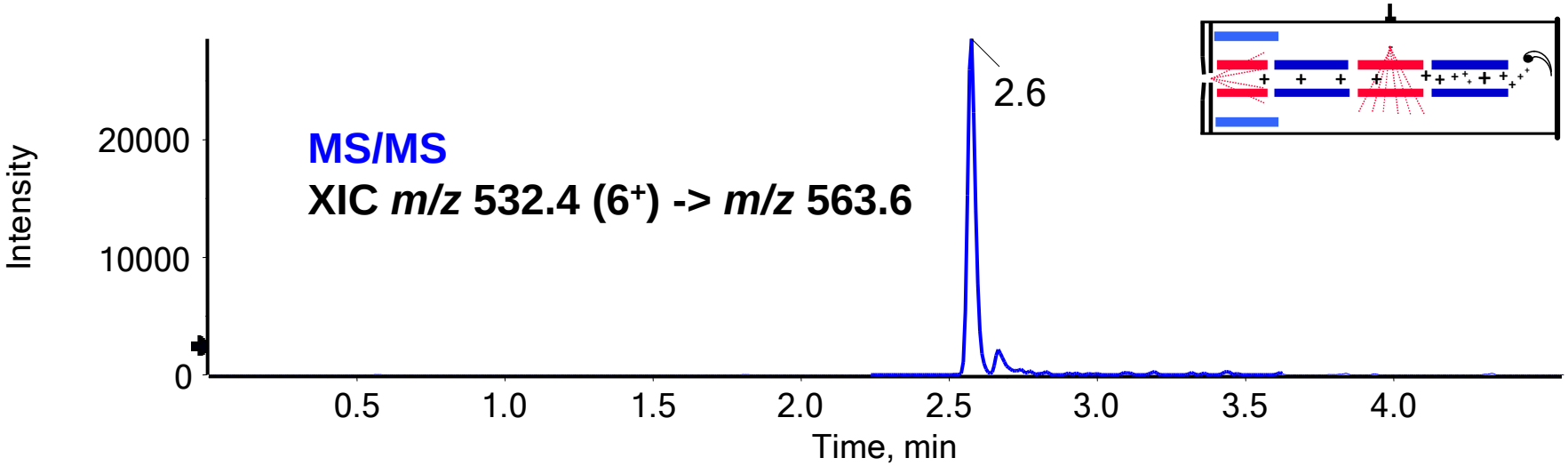
b) **When V_c is a function of time, DMS provides spectral information**

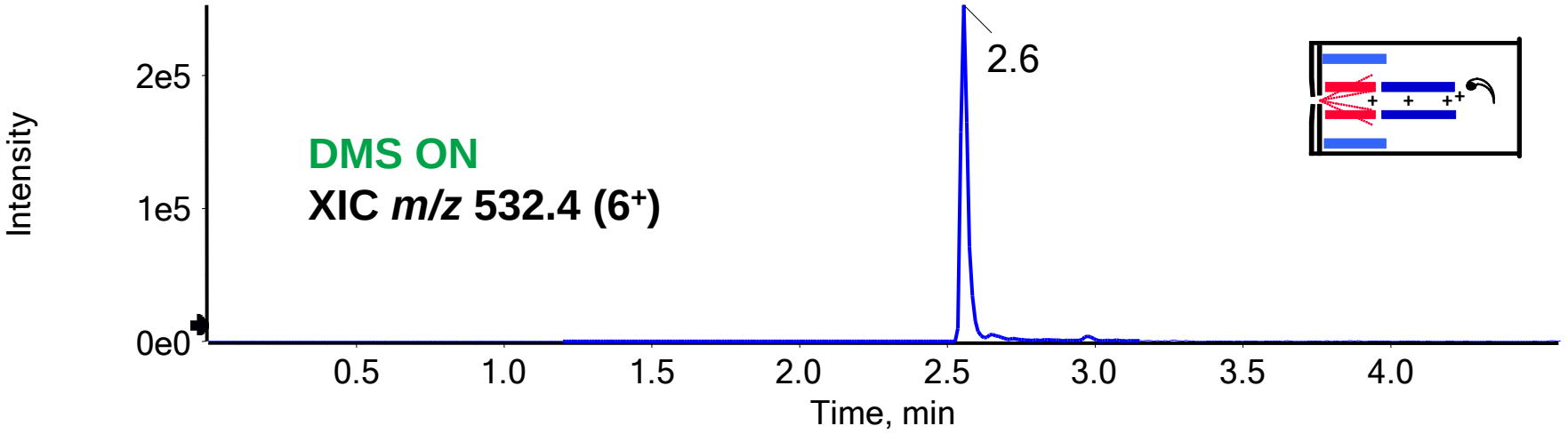


LC-DMS-Single Ion Monitoring/MS

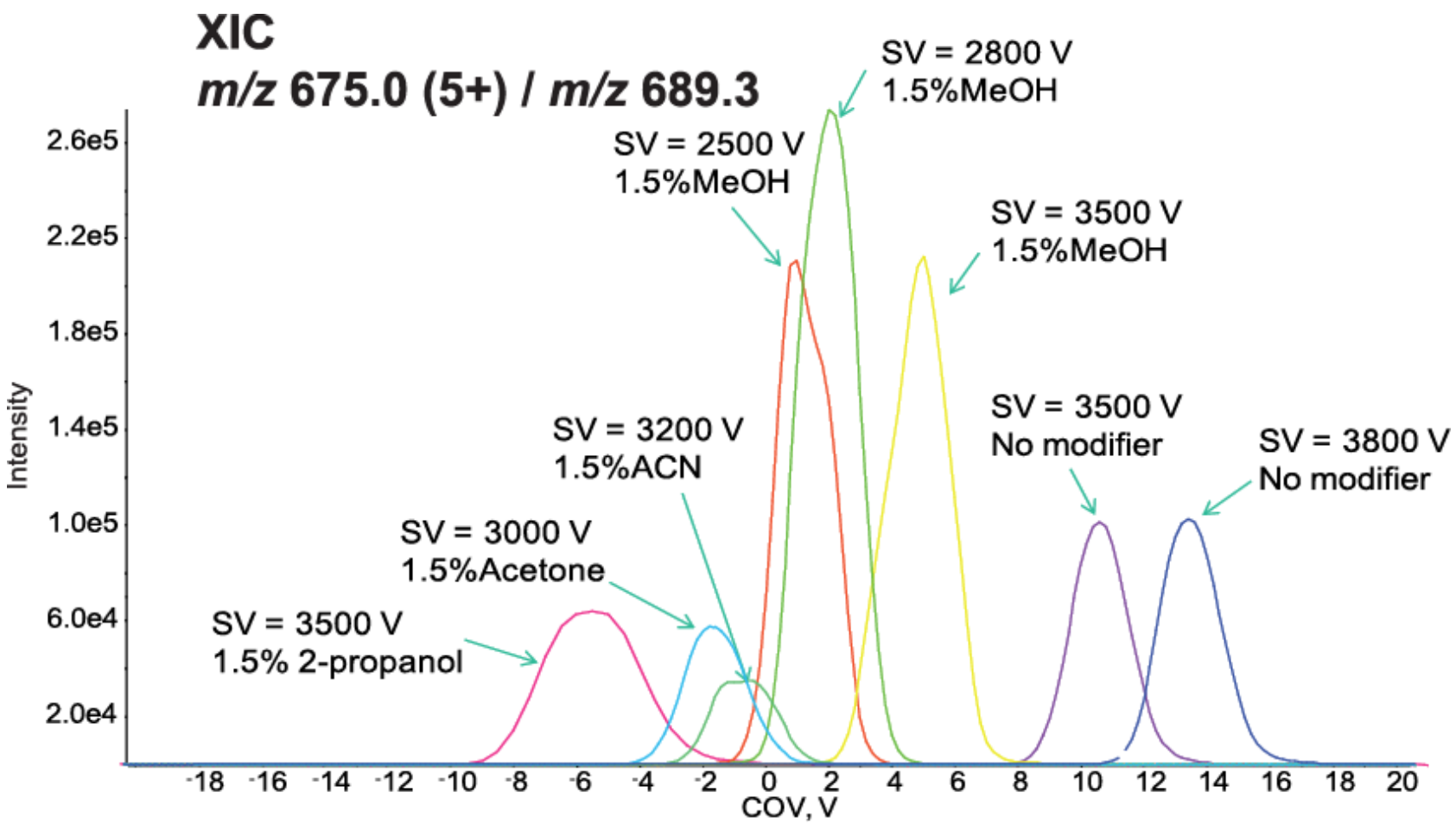


LC-SRM/MS *vs.* LC-DMS/SIM

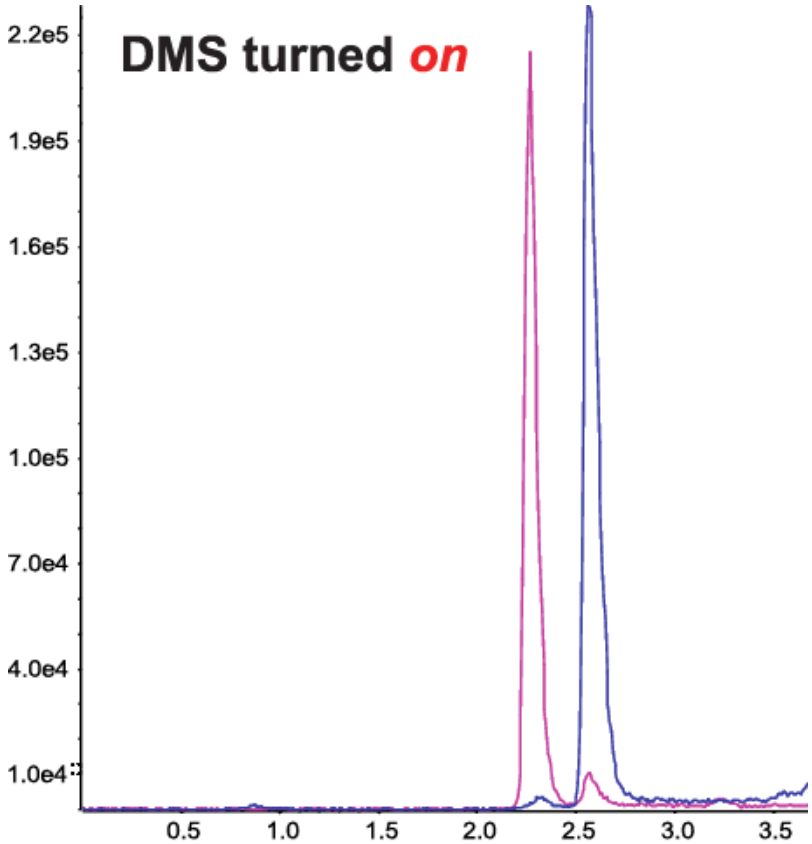




Differential Ion Mobility Spectrometry (DMS) With Organic Modifiers (Additional Selectivity)



LC-DMS SIM/SIM Analysis of Rat Ghrelin Spiked in Human Plasma



Ghrelin			
nominal concn (ng/ml)	measured concn (ng/ml)	RSD (%)	accuracy (%)
5	4.96	7.5	99
10	9.74	7.1	97
50	46.84	7.0	94
250	264.70	9.8	106
500	494.57	7.5	99
1000	996.07	3.9	100
Desacyl-ghrelin			
10	10.30	12.3	103
50	47.81	12.7	96
250	281.87	8.7	113
500	514.57	6.0	103
1000	927.97	6.4	93

Modifier: methanol 1.5 % in N_2, SV 2800 Volts

Conclusions

- For proteins and peptides LC-SRM/MS analysis, the sample preparation strategies strongly depend on the size of the protein/peptide.

- Protein digestion can be accelerated by increasing the temperature. The advantage of microwave versus Thermomixer is still not clear.

- Contrary to small molecules, the Selected Reaction Monitoring mode is not as selective for multiply charged peptides.

- Selectivity can be improved from the MS side by applying high resolution approaches or using differential ion mobility spectrometry (DMS). *Waiting for the combination of both……!*

- Organic modifiers in DMS positively affects the resolution power and the selectivity but can also affects the ionization process.

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