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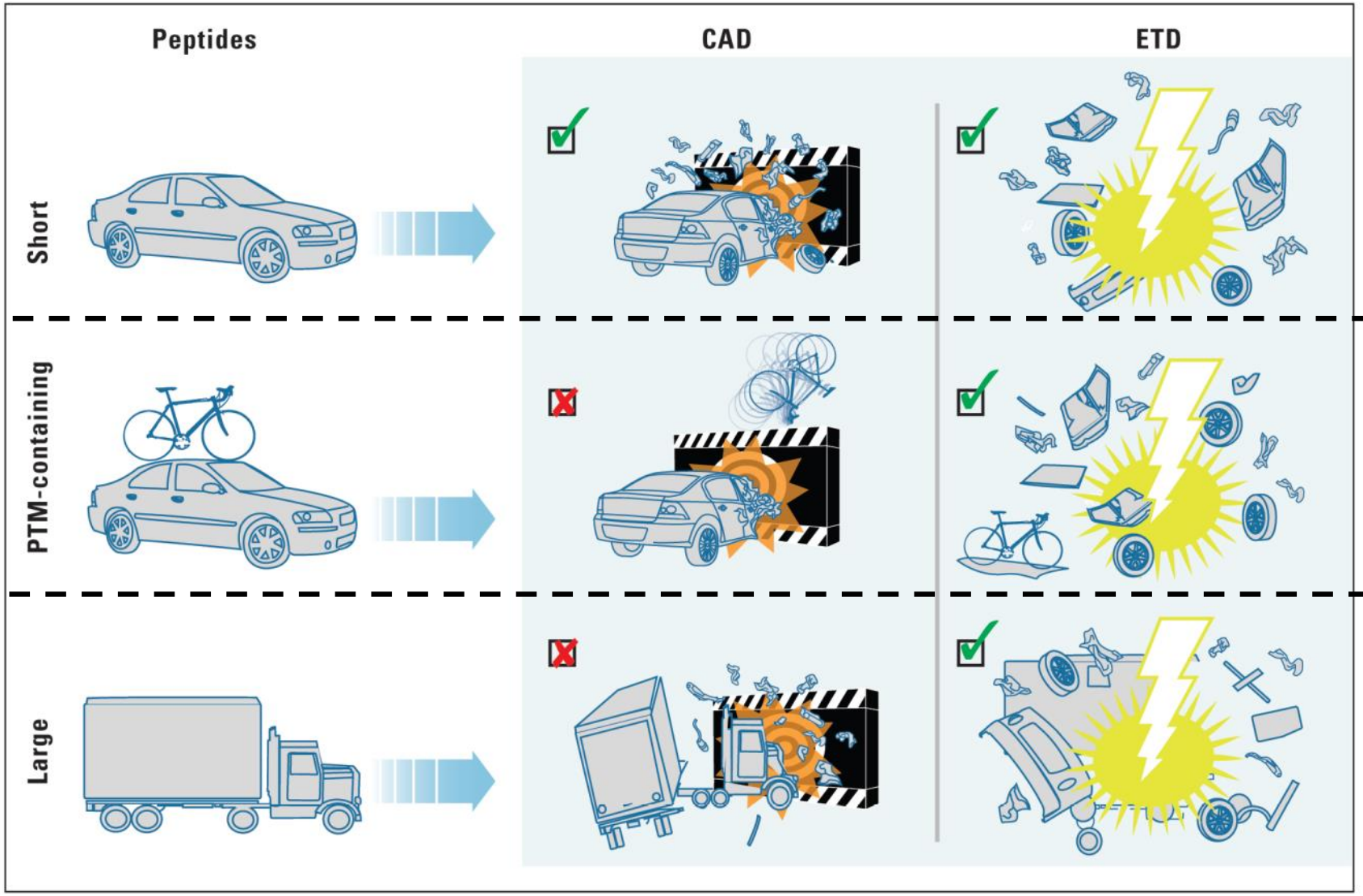
Advanced Fragmentation Techniques for BioPharma Characterization

Global BioPharma Summit

Different modes of fragmentation to answer different questions or for different assays



Peptide Mapping

Site occupancy of PTM for mAb or site of conjugation for ADC

Top-Middle down

(Figure from Coon et al., Analytical Chemistry, 2009)

MS Tools for Major Biopharma Characterization Workflows

Thermo Scientific™ Q Exactive™ MS Family

For all the routine characterization workflows.

Mode of fragmentation: HCD

Thermo Scientific™ Orbitrap Fusion™ Tribrid™ MS Family

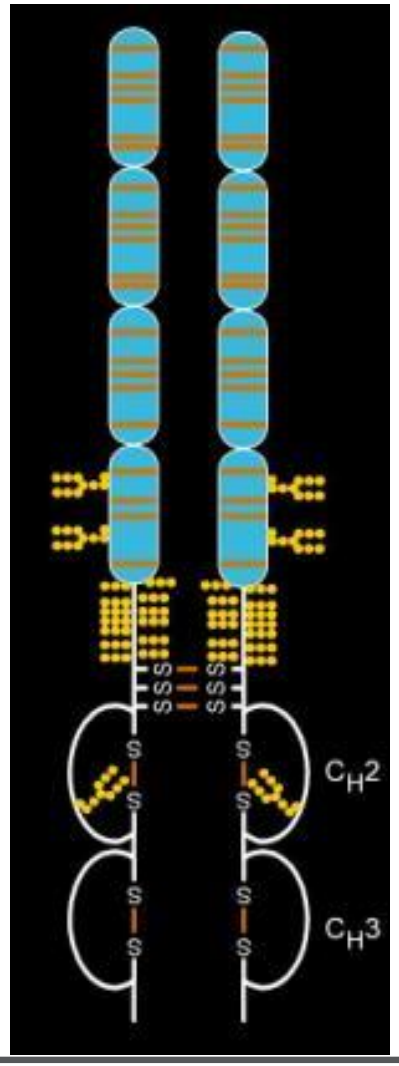
From routine to the most challenging tasks.

Mode of fragmentation: HCD
CID
ETD
EThcD
ETciD
(UVPD - not commercial)

Etanercept: O-glycosylation

O-glycosylation of Etanercept

Etanercept
(trade name Enbrel)



LPAQVAF**T**PYAPEPGSTC_{18}RLREYYDQTAQM$C_{31}C_{32}$SKC_{35}SPGQHAKVFC_{45}TKTSDTVC_{53}DS
C_{56}EDSTYTQLWNWVPEC_{71}LSC_{74}GSRC_{78}SSDQVETQAC_{88}TREQNRIC_{96}TC_{98}RPGWYC_{104}ALS
KQEGC_{112}RLC_{115}APLRKC_{121}RPGFGVARPGTETSDVVC_{139}KPC_{142}APGTFS**N**TTSSTDIC_{157}R
PHQIC_{163}NVVAIPG**N**ASMDAVC_{178}TSTSPTR**S**MAPGAVHLPQPV**ST**RSQH**T**QP**T**PEP**ST**AP**ST**
SFLLPMGP**S**PPAEGSTGDEPKS**C**$_{240}$DKTH**T**C_{246}PP**C**$_{249}$PAPELLGGPSVFLFPPKPKDTLMIS
RTPEVTC_{281}VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY**N**STYRVVSVLTVLHQDWLN
GKEYKC_{341}KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTC_{387}LVKGFYP
SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC_{445}SVMHEALHNH
YTQKSLSLSPGK

1) 3 N-glycan sites
2) 13 reported O-glycosylation sites

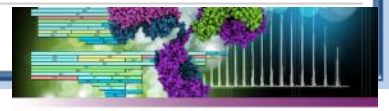
Sialic acids from the O-glycan were removed prior trypsin digestion

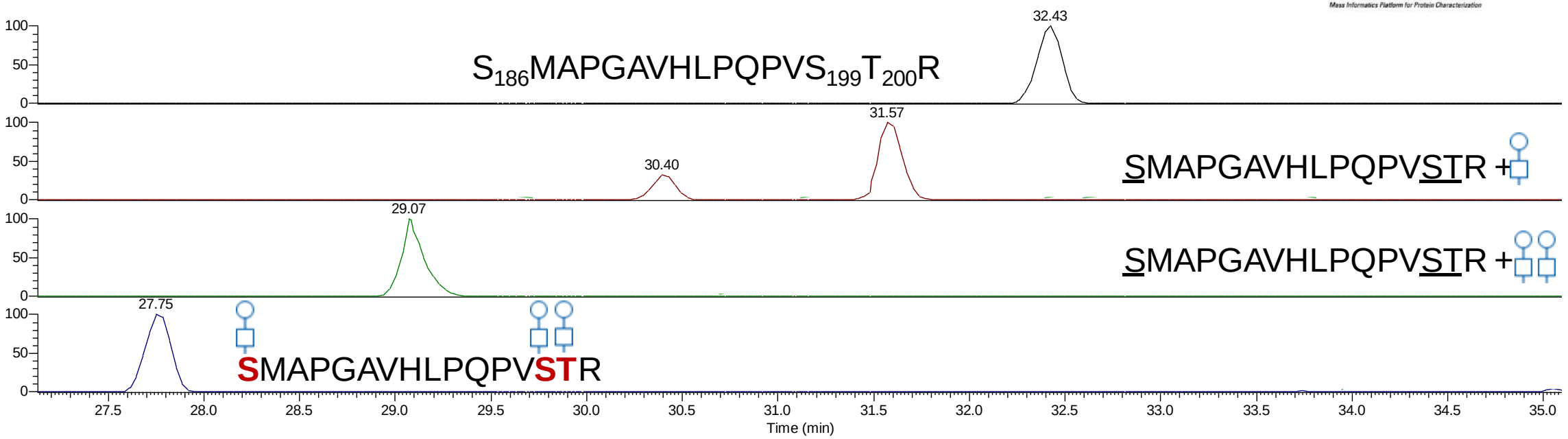
Peptide mapping was performed on the tryptic digest

Results for peptide SMAPGAVHLPQPVSTR after processing HCD data

HCD

	No.	Peptide Sequence	Modification	Delta (ppm)	RT (min)	M/Z	Charge State	Mono Mass Exp.	Mono Mass Theo.	MS Area
1	1339	SMAPGAVHLPQPVSTR	None	-2.15	32.41	824.434	2	1646.8527	1646.8562	14,750.08
2	855	SMAPGAVHLPQPVSTR	O_core	-2.09	30.41	1007.501	2	2011.9843	2011.9885	185,478.92
3	1076	SMAPGAVHLPQPVSTR	O_core	-2.33	31.59	1007.500	2	2011.9838	2011.9885	756,140.69
4	599	SMAPGAVHLPQPVSTR	O_core ,O_core	-2.90	29.08	1190.066	2	2377.1138	2377.1207	33,328,382.00
5	378	SMAPGAVHLPQPVSTR	O_core ,O_core ,O_core	-2.29	27.75	1372.633	2	2742.2466	2742.2529	201,459.91





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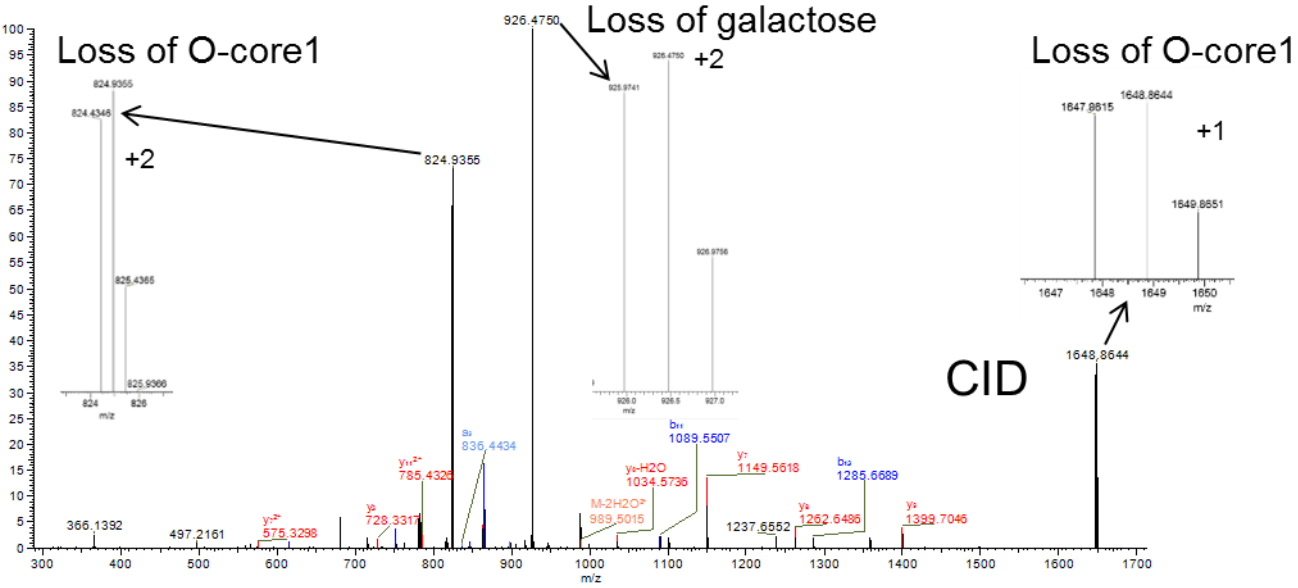
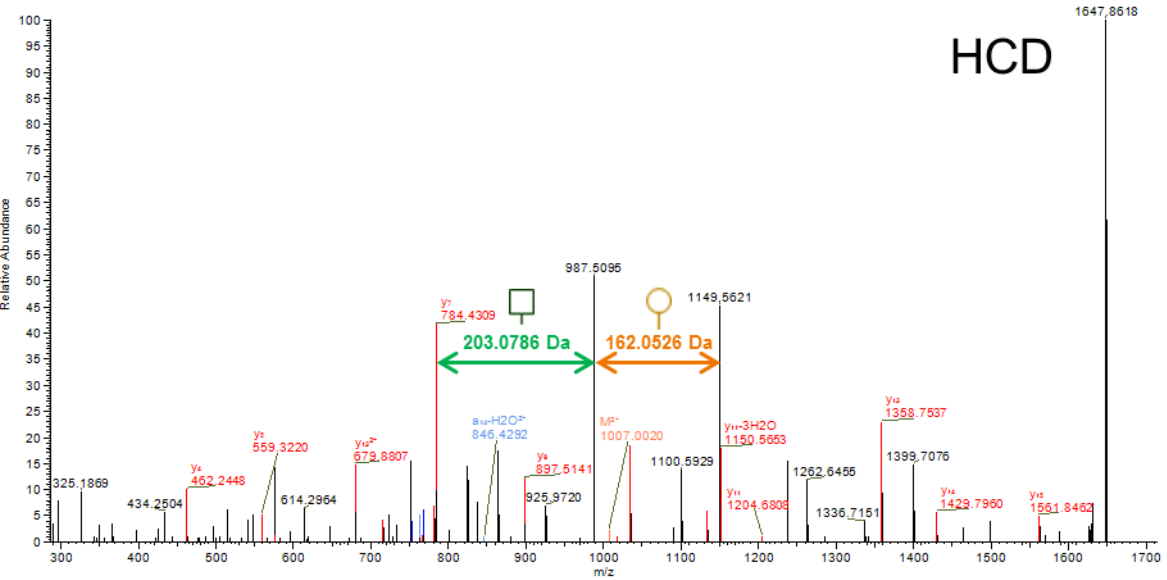
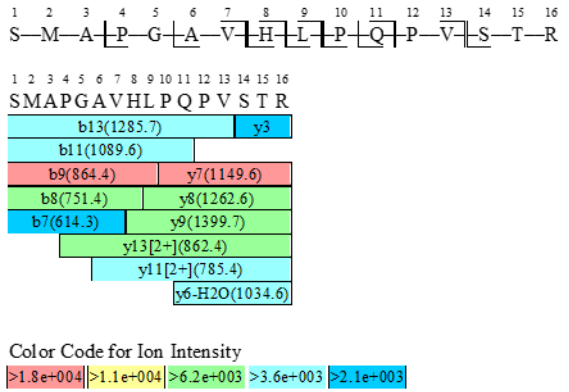
CID and HCD spectra of peptide SMAPGAVHLPQPVSTR + 1 O-core1 (365.1322 Da)





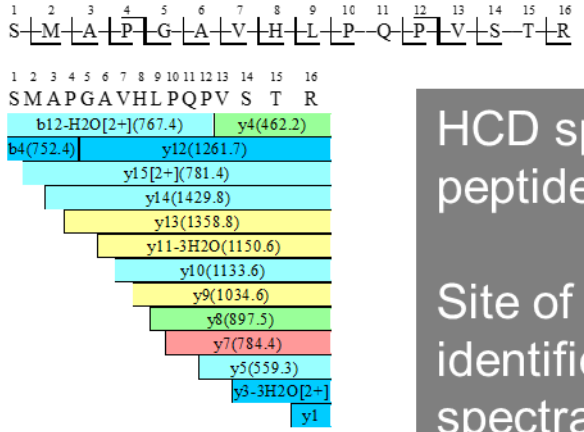
Average Structural Resolution = 1.8 residues



Color Code for Ion Intensity

>1.8e+004	>1.1e+004	>6.2e+003	>3.6e+003	>2.1e+003

Average Structural Resolution = 1.1 residues



Color Code for Ion Intensity

>1.6e+004	>9.0e+003	>5.1e+003	>2.9e+003	>1.6e+003

HCD spectrum provides good peptide backbone fragmentation.

Site of glycosylation cannot be identified with the HCD or CID spectra.

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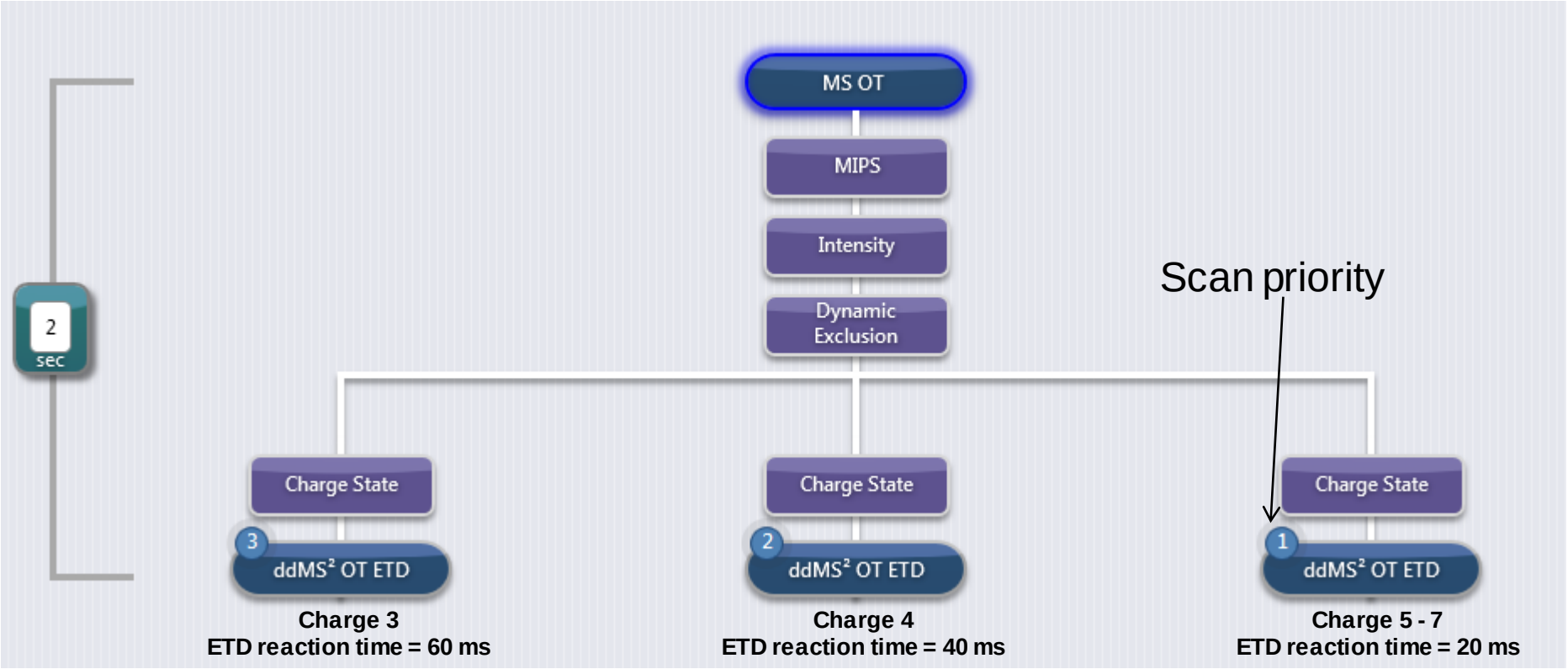
Data acquisition method on a Fusion Lumos: ETD methods

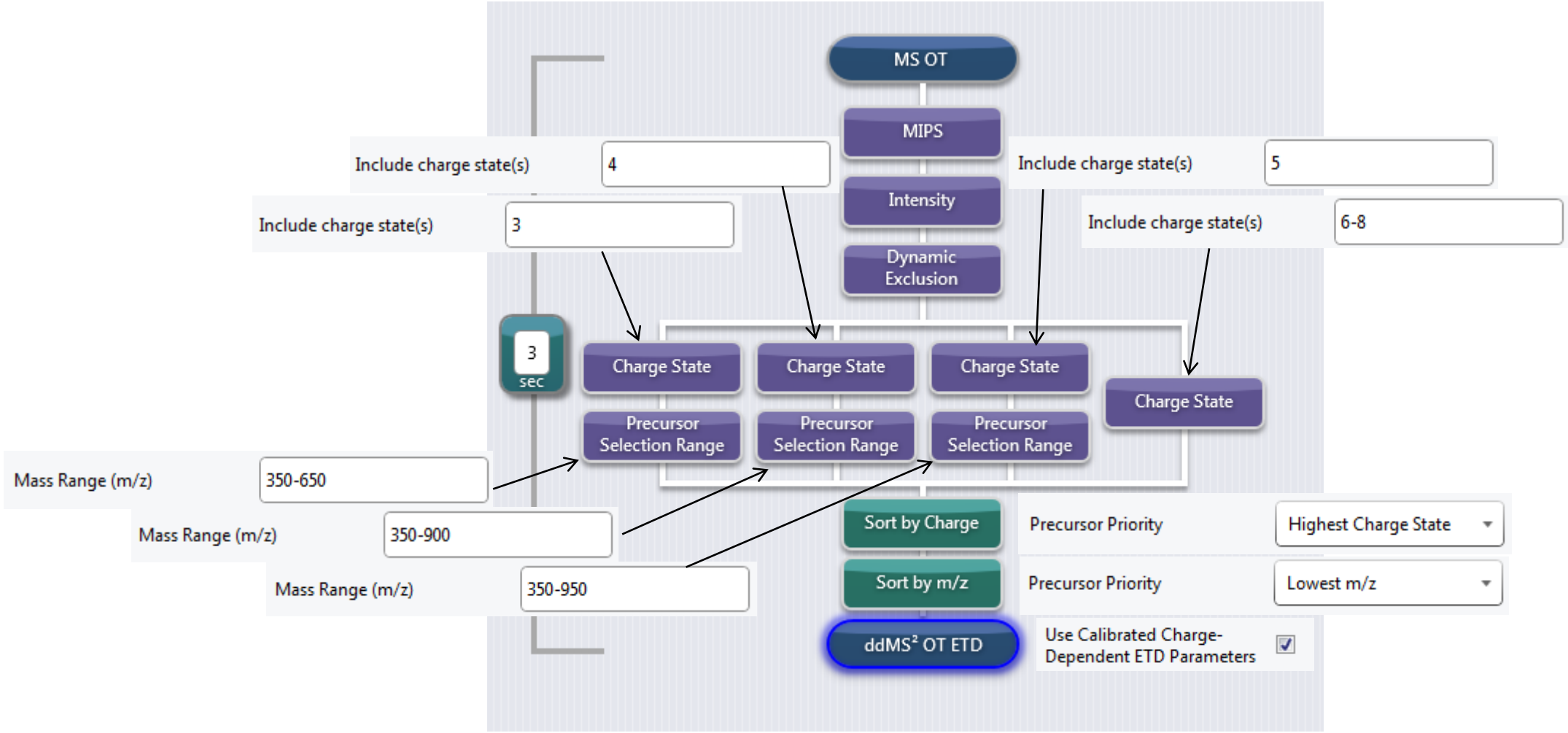
Flexibility and ease of use to design acquisition method



DDA method used to collect data to identify the sites of O-glycosylation.

Data acquisition method on a Fusion Lumos: ETD methods

Flexibility and ease of use to design acquisition method



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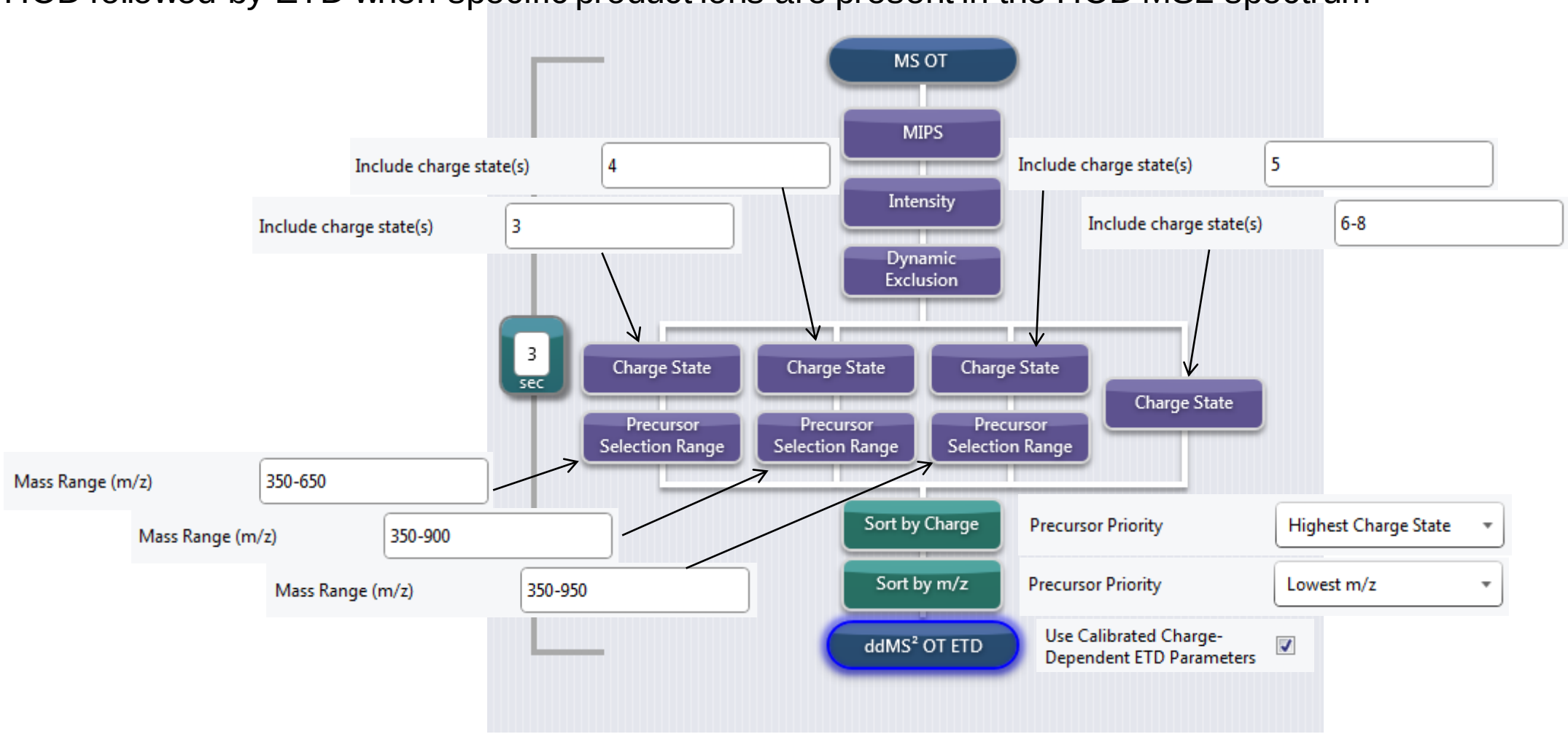
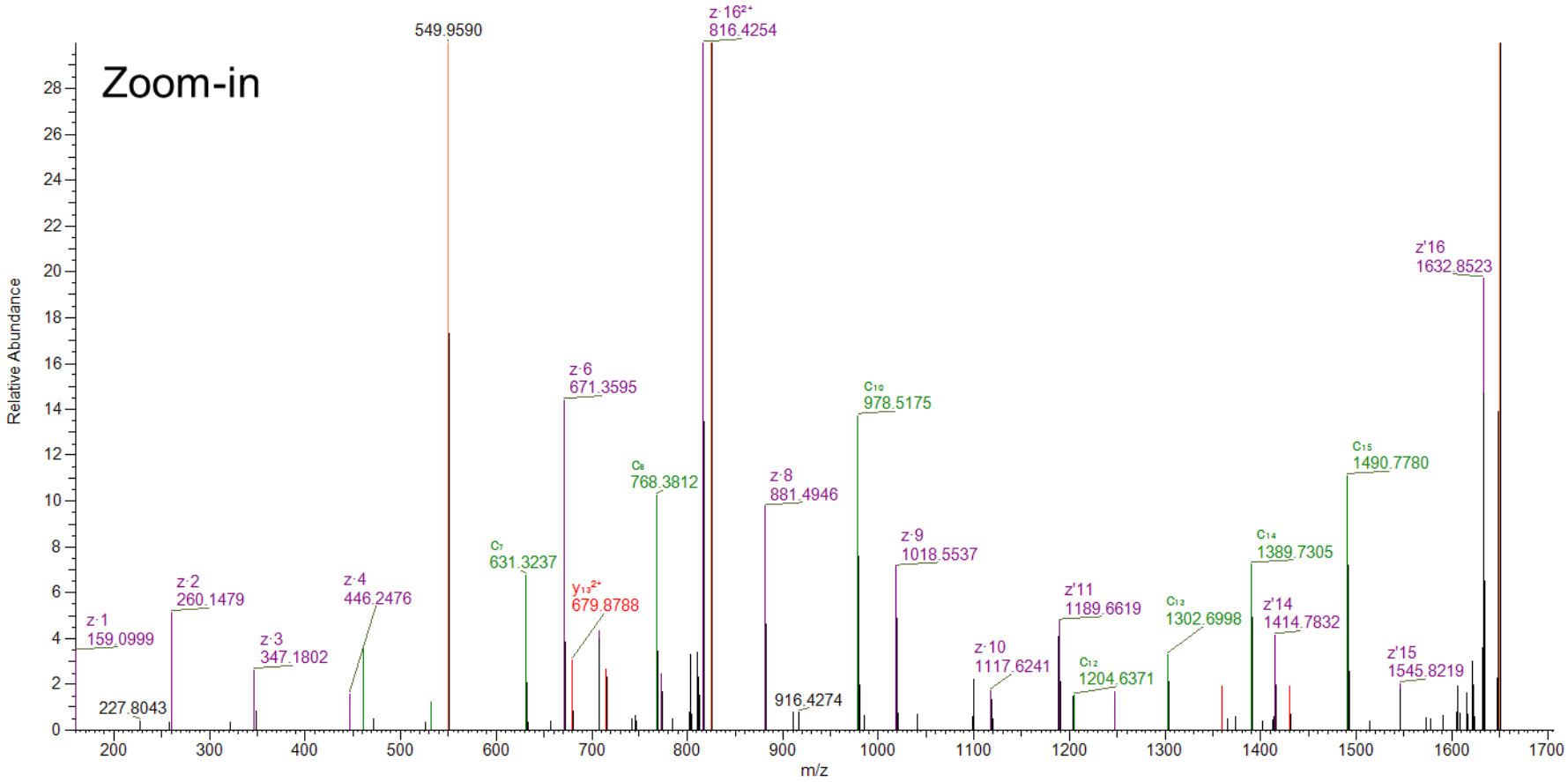
Data acquisition method on a Fusion Lumos: ETD methods

Other useful methods:

* HCD followed by ETD for every precursors.
* HCD followed by ETD when specific product ions are present in the HCD MS2 spectrum

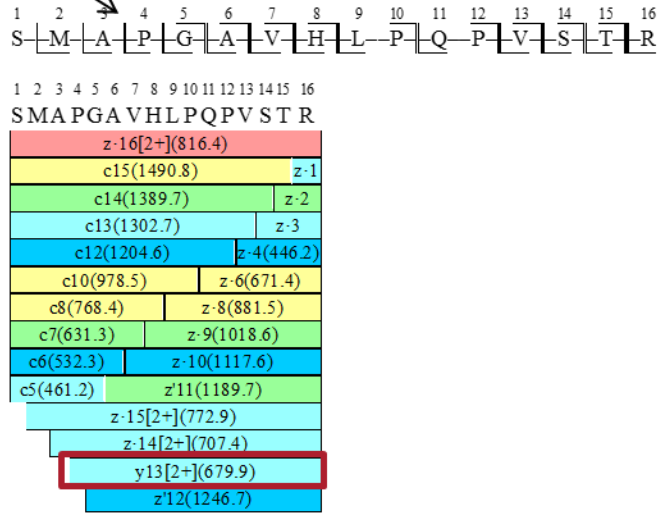


ETD spectrum of SMAPGAVHLPQPVSTR (No O-core1 tag)



y_{13}^{2+}

Average Structural Resolution = 1.1 residues



Color Code for Ion Intensity

>1.8e+004	>9.1e+003	>4.7e+003	>2.4e+003	>1.3e+003

Excellent peptide coverage

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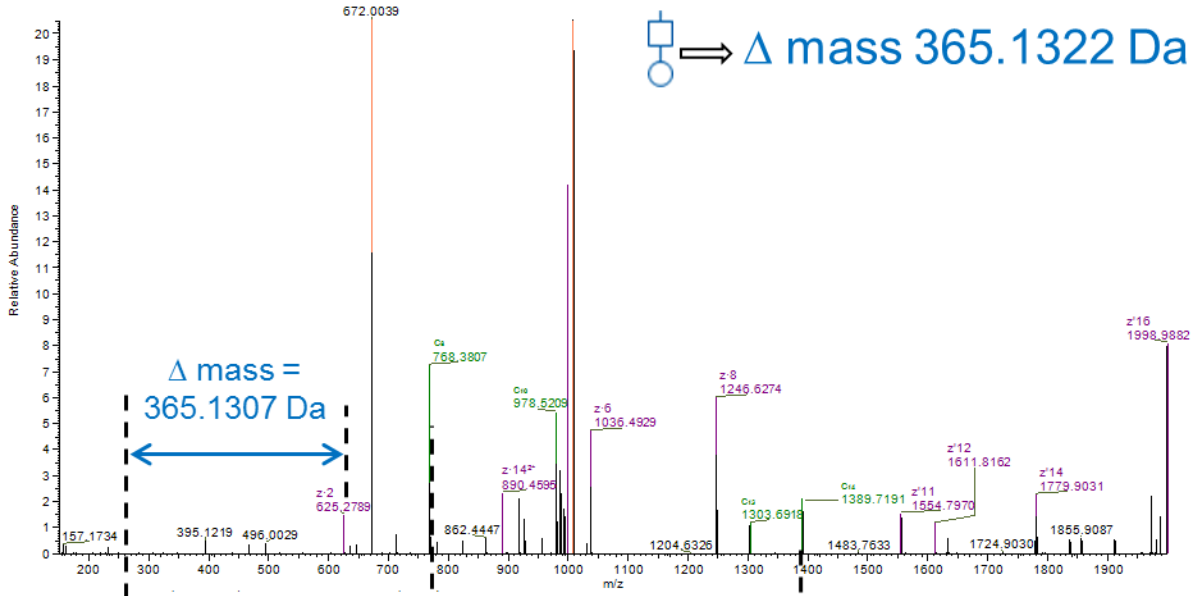
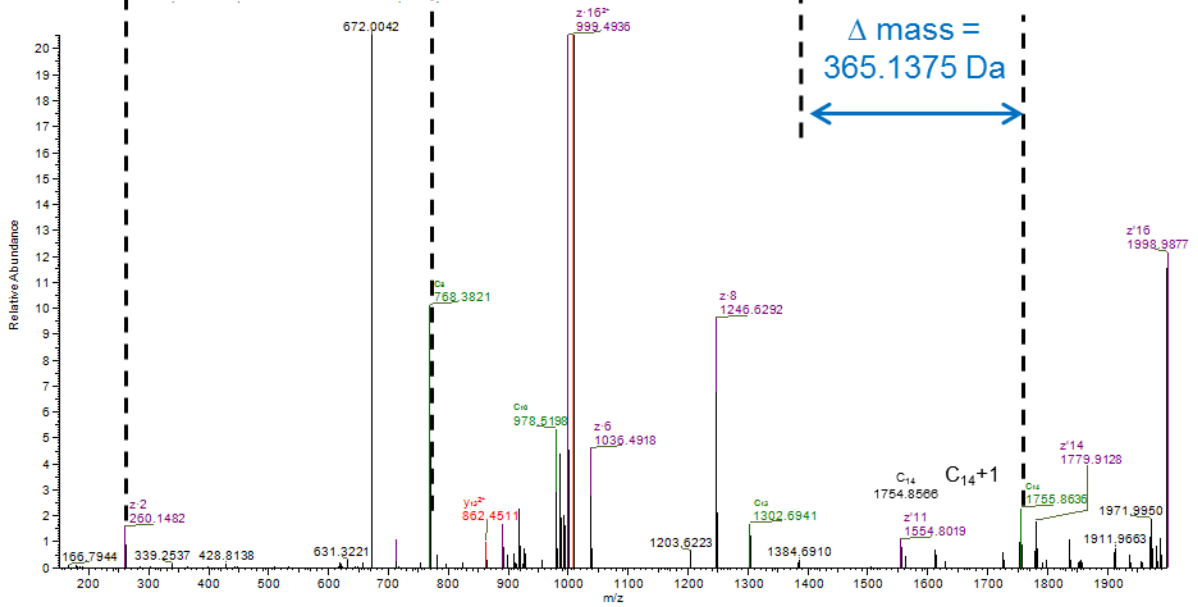
ETD MS/MS of O-glycan structural isomers: SMAPGAVHLPQPVSTR + 1 core1 tag.

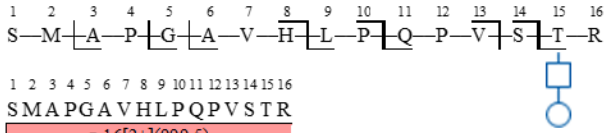
RT = 30.40 min



RT = 31.77 min



Average Structural Resolution = 2.0 residues



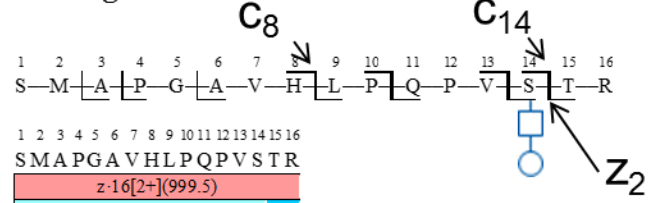
z·16[2+](999.5)
c14(1389.7) z·2
c13(1303.7)
c10(978.5) z·6(1036.5)
c8(768.4) z·8(1246.6)
z·14[2+](890.5)
z'12(1611.8)
z'11(1554.8)

Color Code for Ion Intensity

>2.0e+005 | >1.2e+005 | >7.5e+004 | >4.6e+004 | >2.8e+004

For both O-glycopeptides with 1 O-core1 tag, the sites of glycosylation were identified with the ETD spectra.

Average Structural Resolution = 2.0 residues



z·16[2+](999.5)
c14(1755.9) z·2
c13(1302.7) z·3
c10(978.5) z·6(1036.5)
c8(768.4) z·8(1246.6)
z'14(1779.9)
y13[2+](862.5)
z'11(1554.8)

Color Code for Ion Intensity

>4.5e+005 | >2.4e+005 | >1.3e+005 | >7.0e+004 | >3.8e+004

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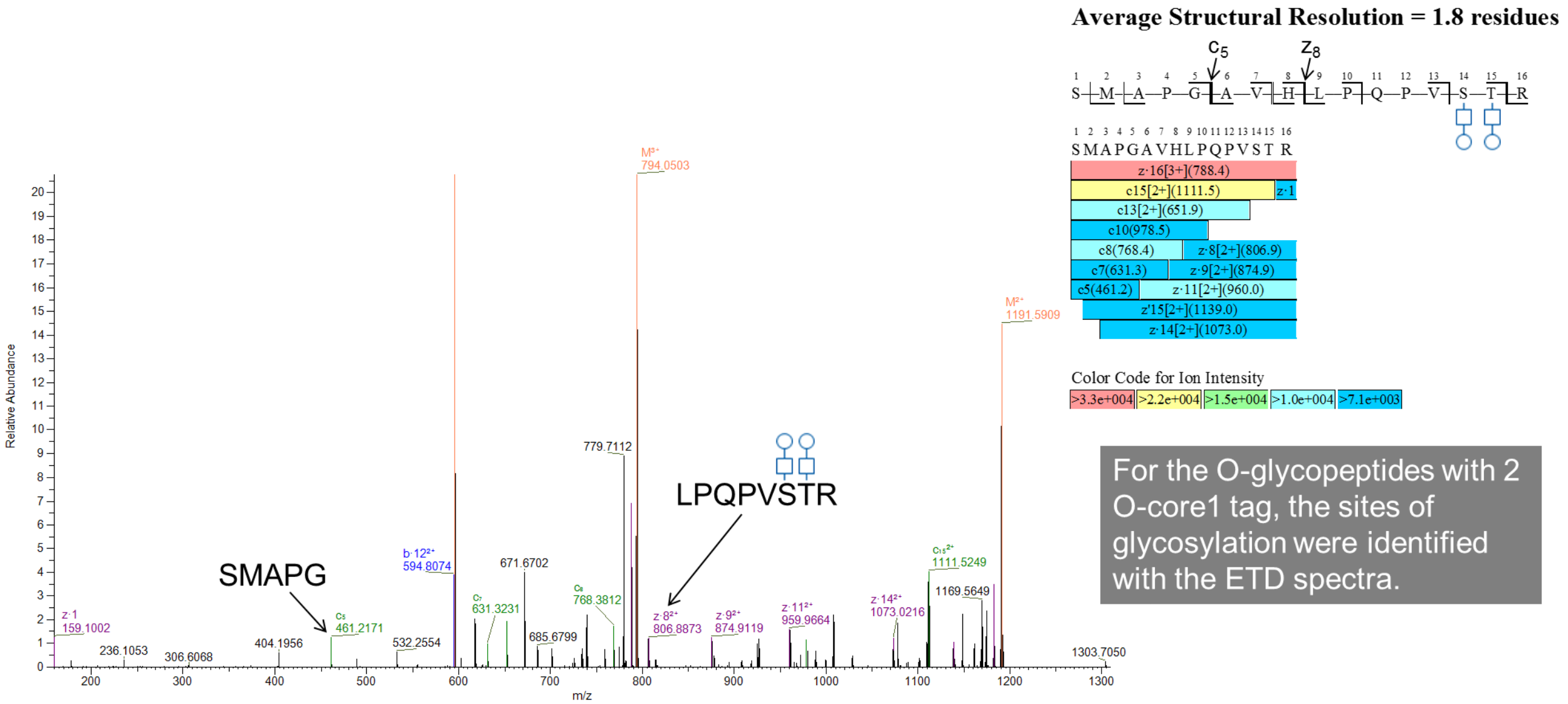
ETD spectrum of SMAPGAVHLPQPVSTR + 2 core1 tag.



For the O-glycopeptides with 2 O-core1 tag, the sites of glycosylation were identified with the ETD spectra.

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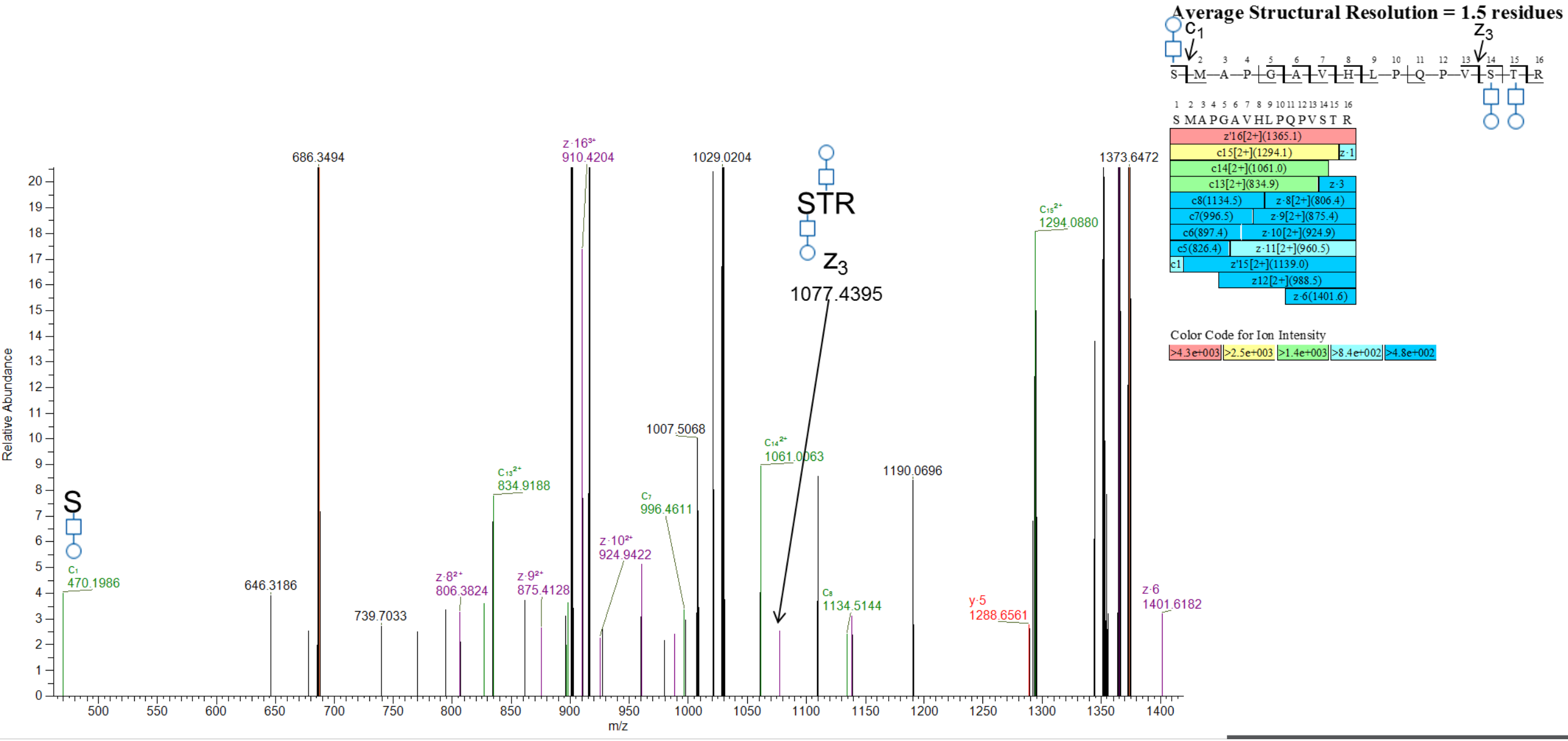
ETD spectrum of SMAPGAVHLPQPVSTR + 3 core1 tag.



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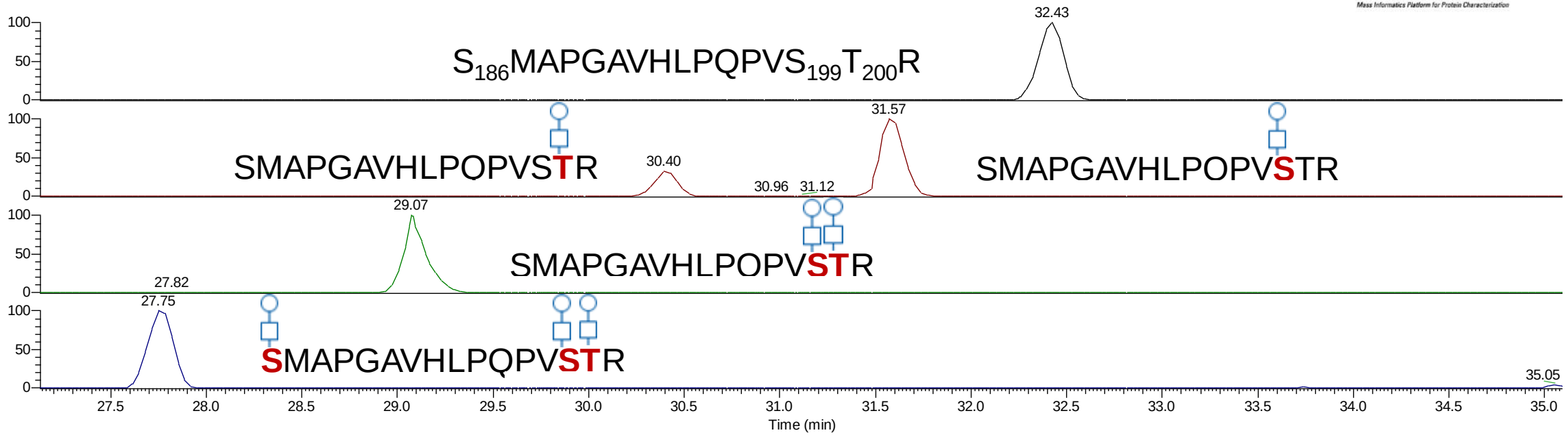
Results for peptide SMAPGAVHLPQPVSTR after processing ETD data

ETD

	Peptide Sequence	Modification	Site	Delta (ppm)	Confidence Score	RT (min)	M/Z	Charge State	Mono Mass Exp.	Mono Mass Theo.
	Aa sma	Aa	Aa	=	=	=	=	=	=	=
1	SMAPGAVHLPQPVSTR	None		-1.19	100.0%	32.35	549.959	3	1646.8542	1646.8562
2	SMAPGAVHLPQPVSTR	O_core	~T200	-0.94	100.0%	30.35	672.003	3	2011.9866	2011.9885
3	SMAPGAVHLPQPVSTR	O_core	~S199	-1.06	100.0%	31.54	672.003	3	2011.9863	2011.9885
4	SMAPGAVHLPQPVSTR	O_core ,O_core	S199,T200	-1.97	100.0%	29.07	595.537	4	2377.1160	2377.1207
5	SMAPGAVHLPQPVSTR	O_core ,O_core ,O_core	S186,S199,T200	-2.11	100.0%	27.71	686.569	4	2742.2471	2742.2529

All of the O-glycosylation sites of peptide SMAPGAVHLPQPVSTR were successfully identified



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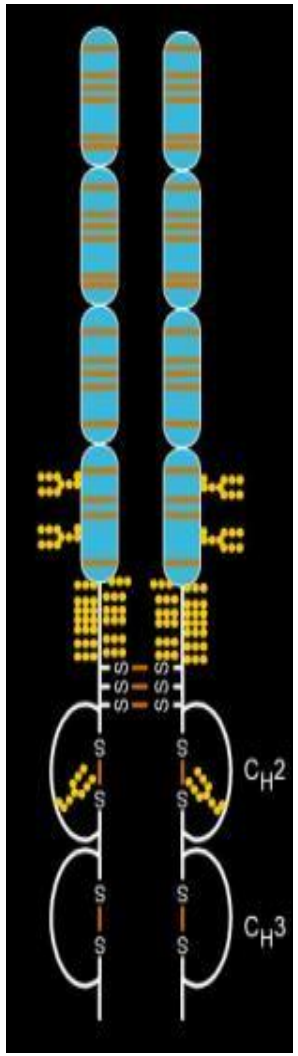
Etanercept: leveraging ETD and MSn for disulfide bond mapping

Disulfide bond mapping: Etanercept

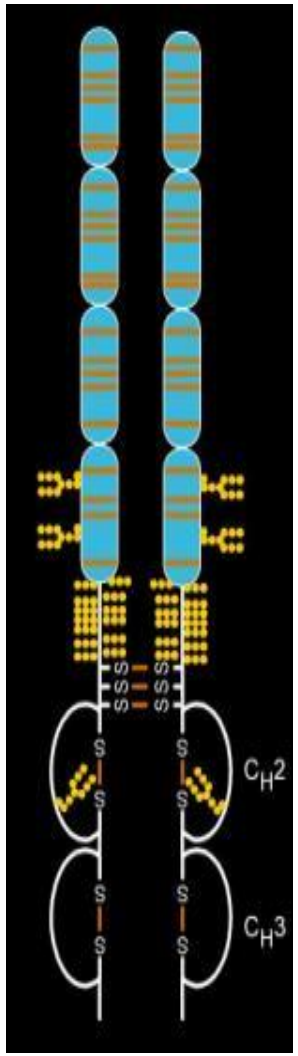
Etanercept
(trade name Enbrel)



LPAQVAF**T**PYAPEPGSTC_{18}RLREYYDQTAQM$C_{31}$$C_{32}SKC_{35}$SPGQHAK
VFC_{45}TKTSDTVC_{53}DSC_{56}EDSTYTQLWNWVPEC_{71}LSC_{74}GSRC_{78}SSD
QVETQAC_{88}TREQNRIC_{96}TC_{98}RPGWYC_{104}ALSKQEGC_{112}RLC_{115}APL
RKC_{121}RPGFGVARPGTETSDVVC_{139}KPC_{142}APGTFS**N**TTSSTDIC_{157}
RPHQIC_{163}NVVAIPG**N**ASMDAVC_{178}TSTSPTR**S**MAPGAVHLPQPV**ST**
RSQH**T**QP**T**PEP**ST**AP**STS**FLLPMGP**S**PPAEGSTGDEPKSC_{240}DKTH**T**
C_{246}PPC_{249}PAPELLGGPSVFLFPPKPKDTLMISRTPEVTC_{281}VVVDV
SHEDPEVKFNWYVDGVEVHNAKTKPREEQY**N**STYRVVSVLTVLHQDW
LNGKEYKC_{341}KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEM
TKNQVSLTC_{387}LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS
FFLYSKLTVDKSRWQQGNVFSC_{445}SVMHEALHNHYTQKSLSLSPGK

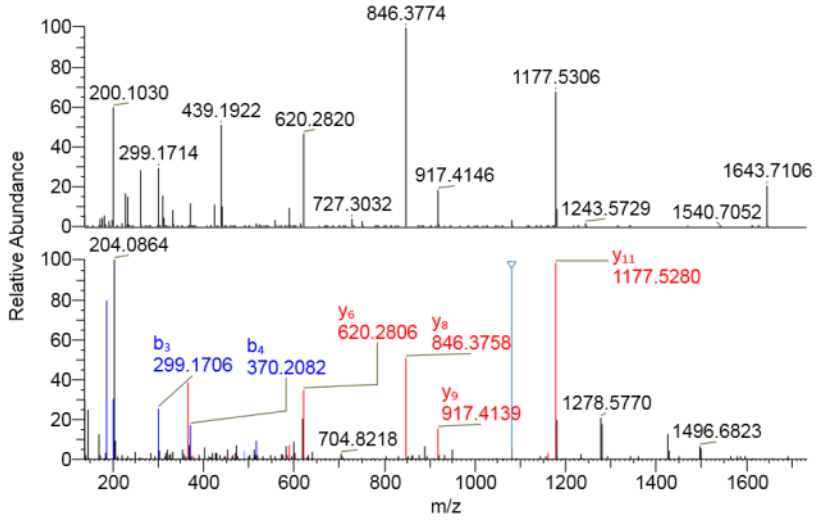
Disulfide bond mapping: Etanercept

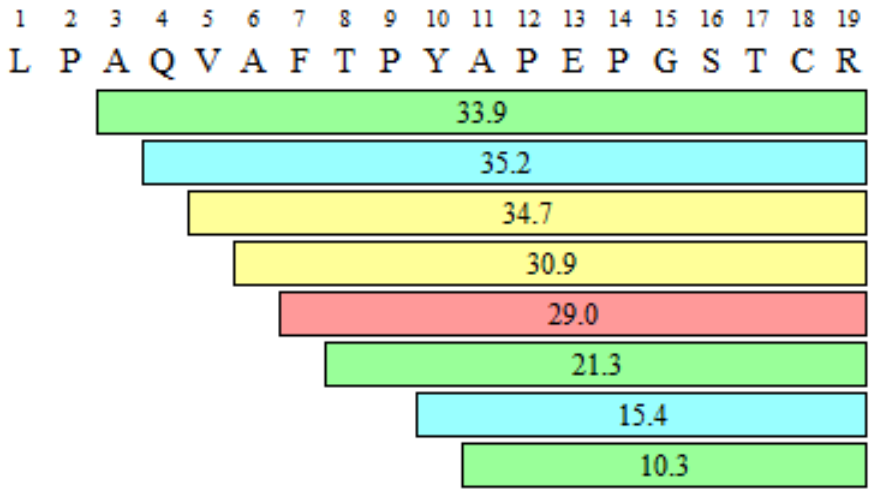
Etanercept
(trade name Enbrel)



LPAQVAFTPYAPEPGSTC$_{18}$RLREYYDQTAQMC$_{31}$C$_{32}$SKC$_{35}$SPGQHAK

	Peptide Sequence	Modification	Site	Delta (ppm)	Confidence Score	ID Type	RT (min)	M/Z	Charge State	Mono Mass Exp.	Avg Mass Exp.	Mono Mass Theo.	MS Area
1	AQVAFTPYAPEPGSTCR	nonspecific,O_Core1	T8	1.03	100.0%	MS2	33.86	1080.996	2	2158.9751	2160.12	2158.9729	135,861.53





The N-terminal side of this sample is not as expected. The first 2 amino acids are missing.

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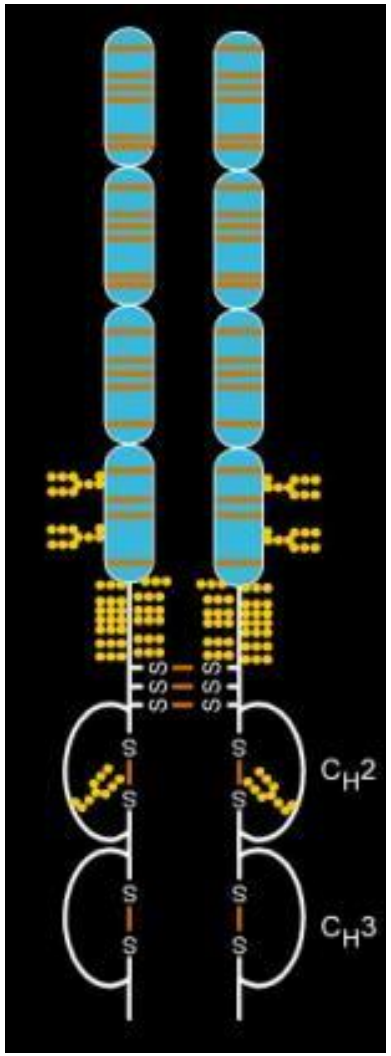
Disulfide Bond Mapping: Etanercept

Etanercept
(trade name Enbrel)

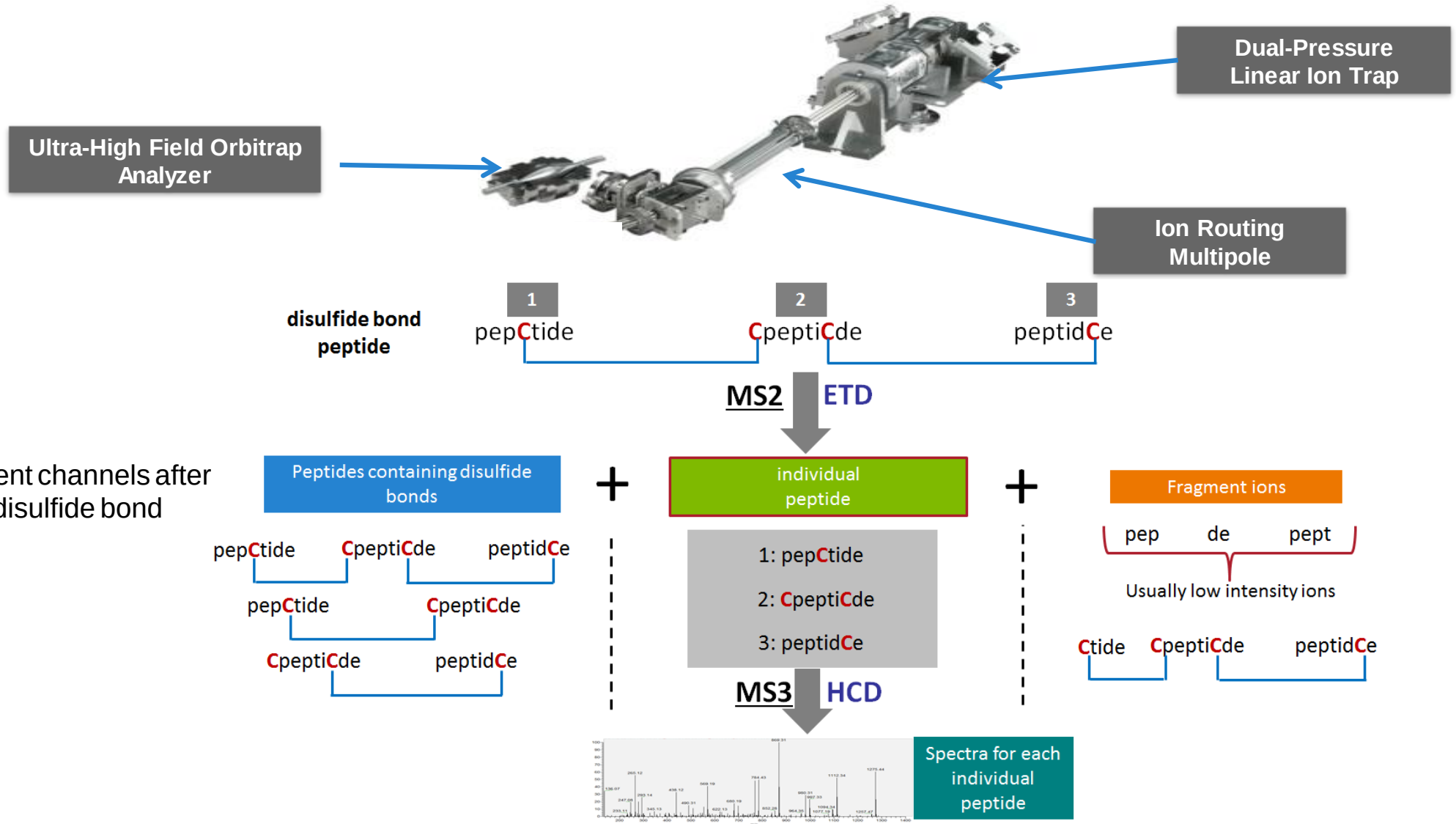


LPAQVAF**T**PYAPEPGSTC_{18}RLREYYDQTAQM$C_{31}C_{32}$SKC_{35}SPGQHAKVFC_{45}TKTSDTVC_{53}DS
C_{56}EDSTYTQLWNWVPEC_{71}LSC_{74}GSRC_{78}SSDQVETQAC_{88}TREQNRIC_{96}TC_{98}RPGWYC_{104}ALS
KQEGC_{112}RLC_{115}APLRKC_{121}RPGFGVARPGTETSDVVC_{139}KPC_{142}APGTFS**N**TTSSTDIC_{157}R
PHQIC_{163}NVVAIPG**N**ASMDAVC_{178}TSTSPTR**S**MAPGAVHLPQPV**ST**RSQH**T**QP**T**PEP**ST**AP**ST**
SFLLPMGP**S**PPAEGSTGDEPKS**C**$_{240}$DKTH**T****C**$_{246}$PP**C**$_{249}$PAPELLGGPSVFLFPPKPKDTLMIS
RTPEVTC_{281}VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY**N**STYRVVSVLTVLHQDWLN
GKEYKC_{341}KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTC_{387}LVKGFYP
SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC_{445}SVMHEALHNH
YTQKSLSLSPGK

The N-terminal side peptide contains one cysteine.
The HCD search result of the non-reduced sample suggests that the N-terminal peptide is linked to two other peptides through disulfide bonds.

Leveraging ETD and MSn for disulfide bond mapping

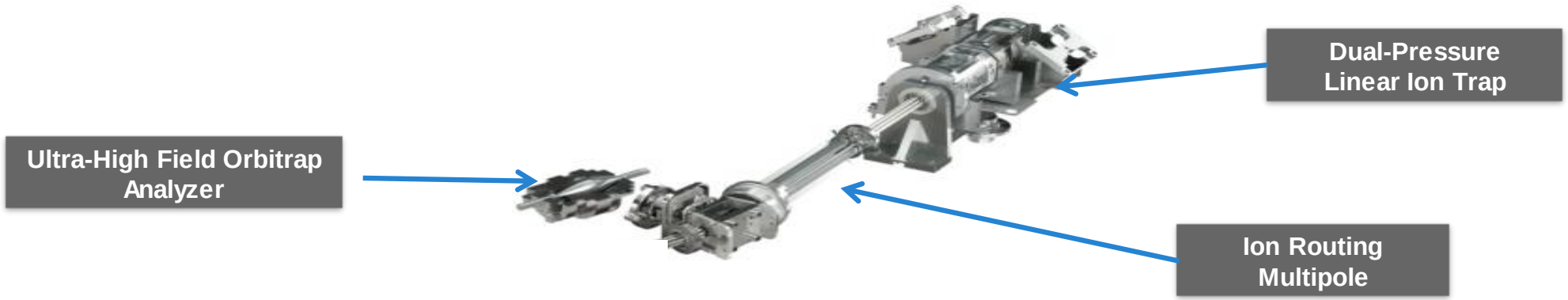
Workflow of the MS2 ETD – MS3 HCD for disulfide bond peptide identification



3 fragment channels after ETD of disulfide bond peptide

Leveraging ETD and MSn for disulfide bond mapping

Workflow of the MS2 ETD – MS3 HCD for disulfide bond peptide identification



disulfide bond peptide

1 pepCtide

2 CpeptiCde

3 peptidCe

MS2 ETD

3 fragment channels after ETD of disulfide bond peptide

Peptides containing disulfide bonds

pepCtide CpeptiCde peptidCe

pepCtide CpeptiCde

CpeptiCde peptidCe

\+

individual peptide

1: pepCtide

2: CpeptiCde

3: peptidCe

MS3 HCD

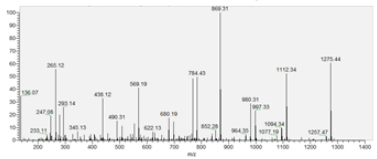
\+

Fragment ions

pep de pept

Usually low intensity ions

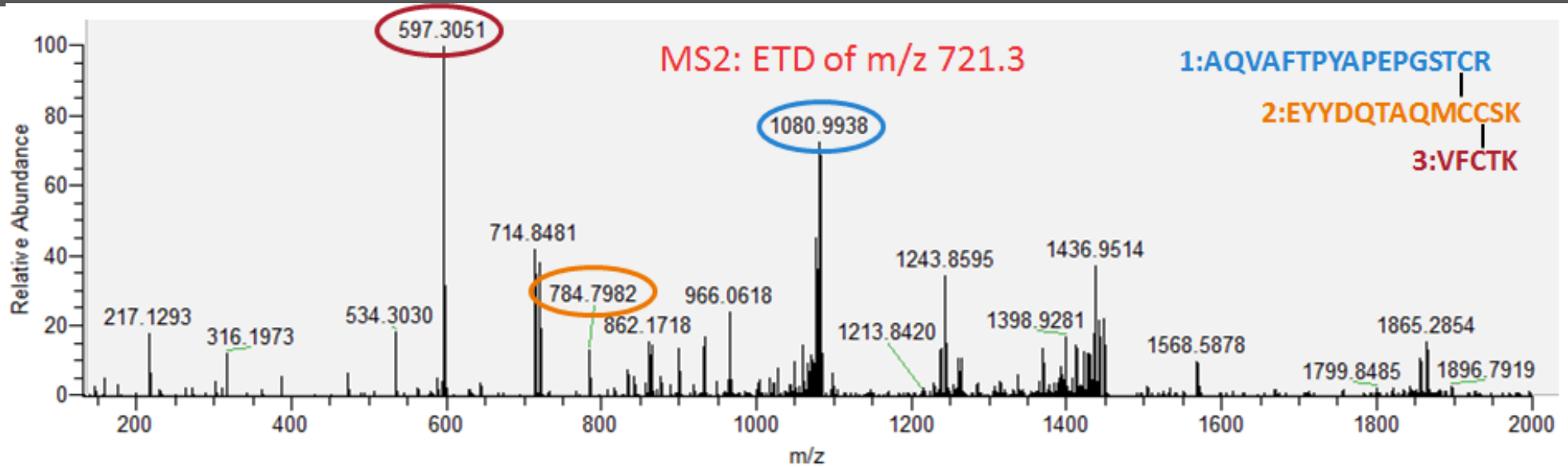
Ctide CpeptiCde peptidCe



Spectra for each individual peptide

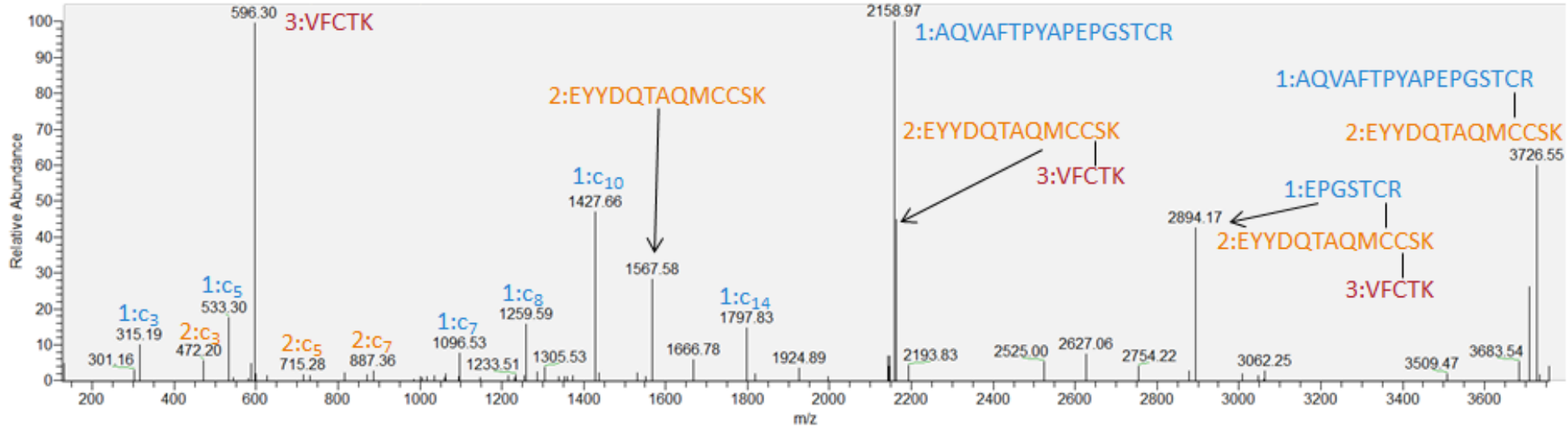
Peptide: AQVAFTPYAPEPGSTC$_{18}$R-EYYDQTAQMC$_{31}$C$_{32}$SK-VFC$_{45}$TK

MS2 ETD Spectrum



Deconvolution with Xtract

deconvoluted spectrum



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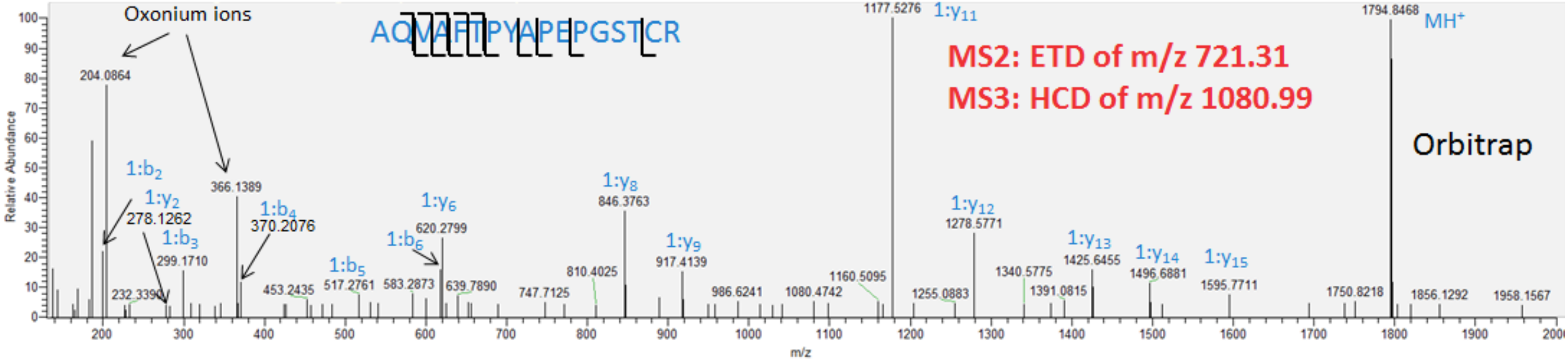
Peptide: AQVAFTPYAPEPGSTC$_{18}$R-EYYDQTAQMC$_{31}$C$_{32}$SK-VFC$_{45}$TK

MS3 HCD spectrum of AQVAFTPYAPEPGSTC$_{18}$R.



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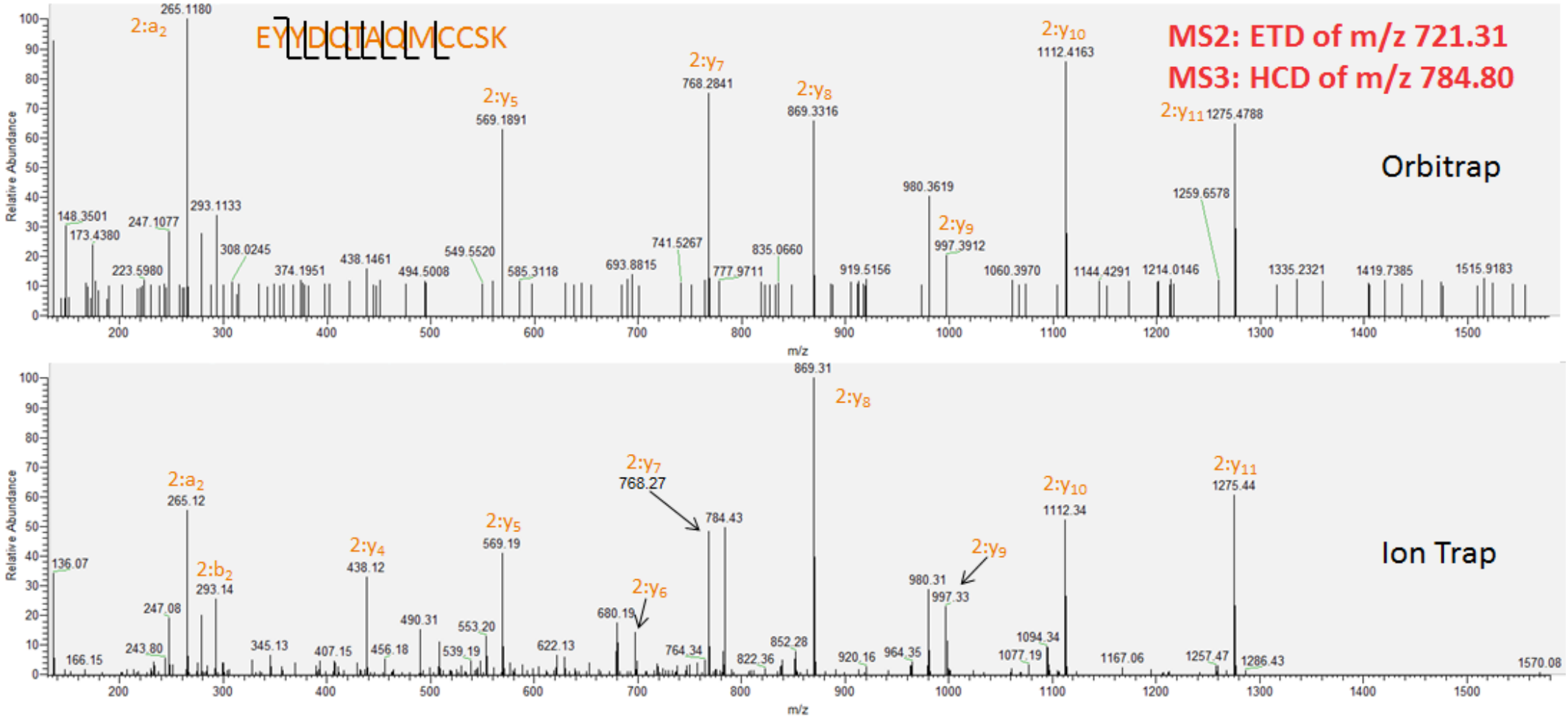
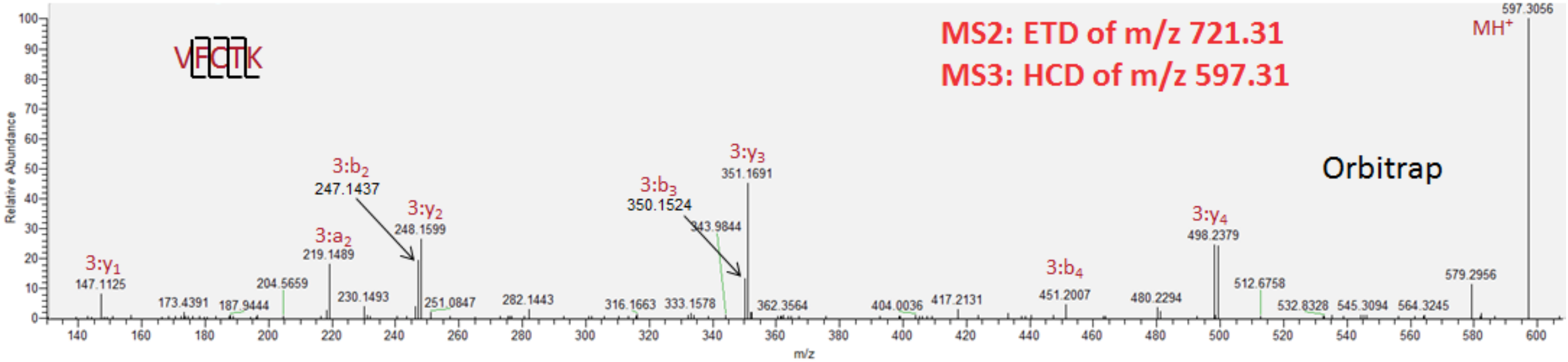
MS3 HCD spectrum of EYYDQTAQMC$_{31}$C$_{32}$SK using a) the Orbitrap or b) the ion trap mass analyzers.



ThermoFisher SCIENTIFIC

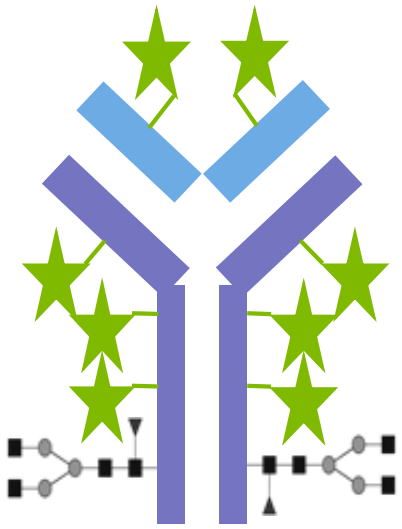
MS3 HCD spectrum of VFC$_{45}$TK.



Antibody Drug Conjugates

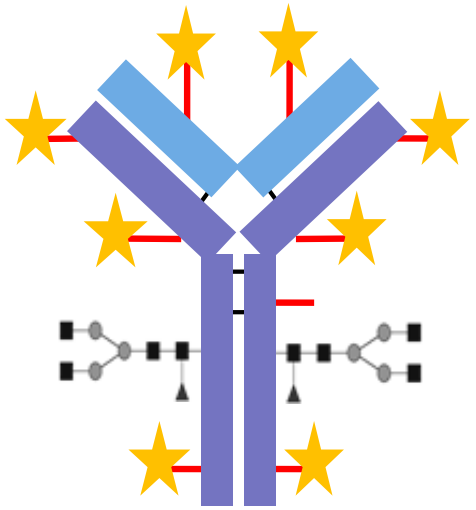
ADCs are heterogeneous mixture

Brentuximab vedotin
Adcetris®



Cysteine-linked

Trastuzumab Emtansine
Kadcyla®



Lysine-linked

FDA-approved ADCs

Light Chain: 12 Lysines
Heavy Chain: 32 Lysines

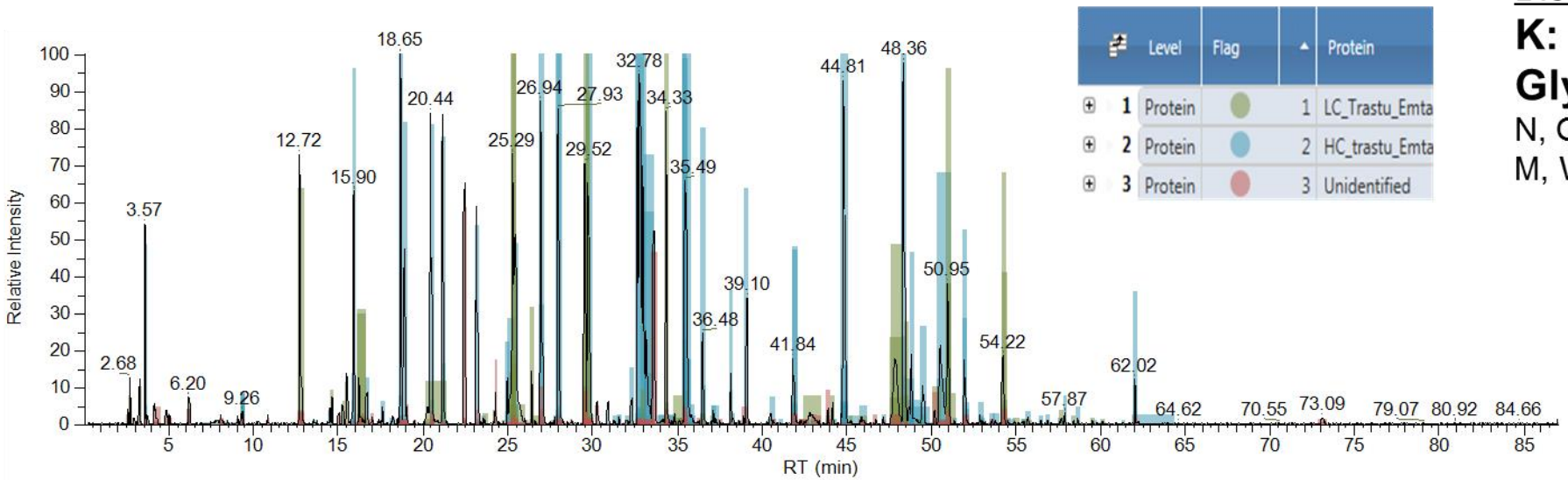
Peptide mapping

Goal: Identifying conjugated peptides and sites of conjugation

Trastuzumab emtansine peptide mapping by SMART Digest™

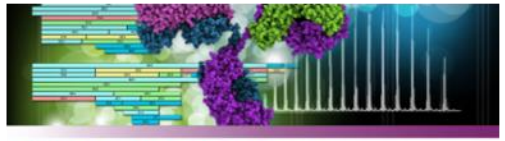
Trastuzumab emtansine

SMART Digest (75 min) - reduction
(non optimized protocol)



Level	Flag		Protein
1 Protein		1	LC_Trastu_Emta
2 Protein		2	HC_trastu_Emta
3 Protein		3	Unidentified

Proteins	Number of MS Peaks	MS Peak Area	Sequence Coverage	Abundance (mol)
1:LC_Trastu_Emta	1029	25.2%	100.0%	38.62%
2:HC_trastu_Emta	2479	62.8%	100.0%	61.38%
Unidentified	25270	12.0%		



BioPharma Finder Variable PTMs

K: MCC-DM1

Glycan: CHO

N, Q: Deamidation

M, W: Oxidation

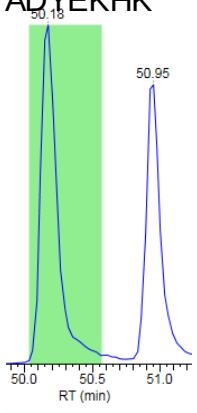
ThermoFisher SCIENTIFIC

Trastuzumab emtansine: conjugated peptides

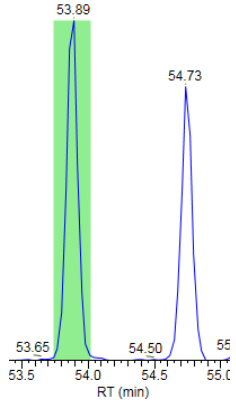
Subset of identified conjugated peptides

	No.	Peptide Sequence	# K	Modification	Site	Delta (ppm)	ID Type	RT (min)	M/Z	Charge State	Mono Mass Exp.	Mono Mass Theo.	MS Area	Protein
1	21049	ADYEKHK	2	DM1	K188	0.60	MS2	50.16	616.272	3	1845.7949	1845.7938	20,170,934.00	LC_Trastu_Emta
2	21419	ADYEKHK		DM1	K188	-0.13	MS2	50.94	616.272	3	1845.7936	1845.7938	14,392,560.00	LC_Trastu_Emta
3	22848	AKGQPR	1	DM1	K343	-0.09	MS2	53.88	806.878	2	1611.7408	1611.7410	1,016,677.25	HC_trastu_Emta
4	23430	AKGQPR		DM1	K343	-0.17	MS2	54.75	806.878	2	1611.7407	1611.7410	790,552.56	HC_trastu_Emta
5	26843	ALPAPIEKTISK	2	DM1	K337	0.64	MS2	64.62	742.381	3	2223.1206	2223.1192	462,441.38	HC_trastu_Emta
6	26950	ALPAPIEKTISK		DM1	K337	0.42	MS2	65.11	742.381	3	2223.1201	2223.1192	353,500.62	HC_trastu_Emta
7	25889	ASQDVNTAVAWYQQKPGK	2	DM1	K39	0.40	MS2	60.66	983.794	3	2946.3564	2946.3553	99,419.80	LC_Trastu_Emta
8	25971	ASQDVNTAVAWYQQKPGK		DM1	K39	0.56	MS2	61.18	983.794	3	2946.3569	2946.3553	78,092.43	LC_Trastu_Emta
9	23503	ASQDVNTAVAWYQQKPGKAPK	3	DM1	~K42	0.58	MS2	54.93	1082.521	3	3242.5420	3242.5401	3,348,808.00	LC_Trastu_Emta
10	23773	ASQDVNTAVAWYQQKPGKAPK		DM1	~K42	0.20	MS2	55.50	1082.521	3	3242.5408	3242.5401	2,925,363.50	LC_Trastu_Emta
11	27147	DSTYSLSSTLTLSKADYEK	2	DM1	K183	0.50	MS2	66.91	1023.135	3	3064.3821	3064.3806	72,910.94	LC_Trastu_Emta
12	27210	DSTYSLSSTLTLSKADYEK		DM1	K183	0.10	MS2	67.35	1023.136	3	3064.3809	3064.3806	42,332.44	LC_Trastu_Emta
13	22964	TKPR	1	DM1	K293	-1.18	MS2	54.16	729.342	2	1456.6698	1456.6715	1,648,967.12	HC_trastu_Emta
14	23556	TKPR		DM1	K293	-0.01	MS2	55.02	729.343	2	1456.6715	1456.6715	1,267,997.38	HC_trastu_Emta

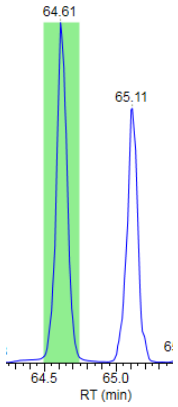
ADYEKHK



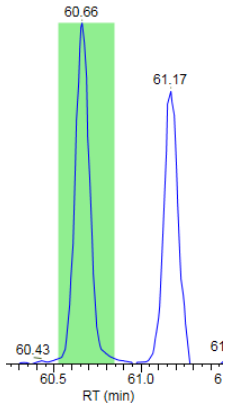
AKGQPR



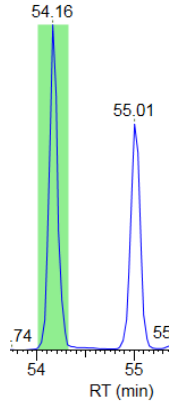
ALPAPIEKTISK



ASQDVNTAVAWYQQKPGK



TKPR

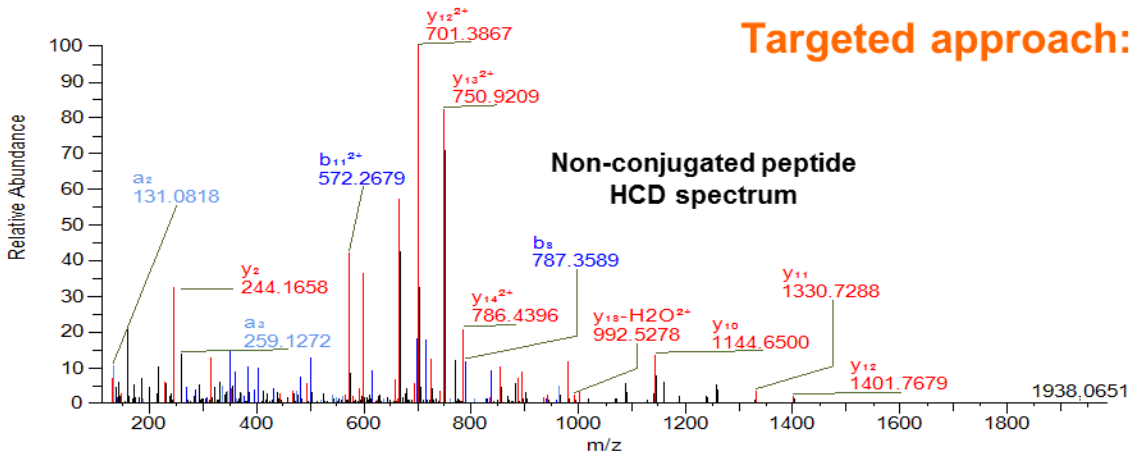


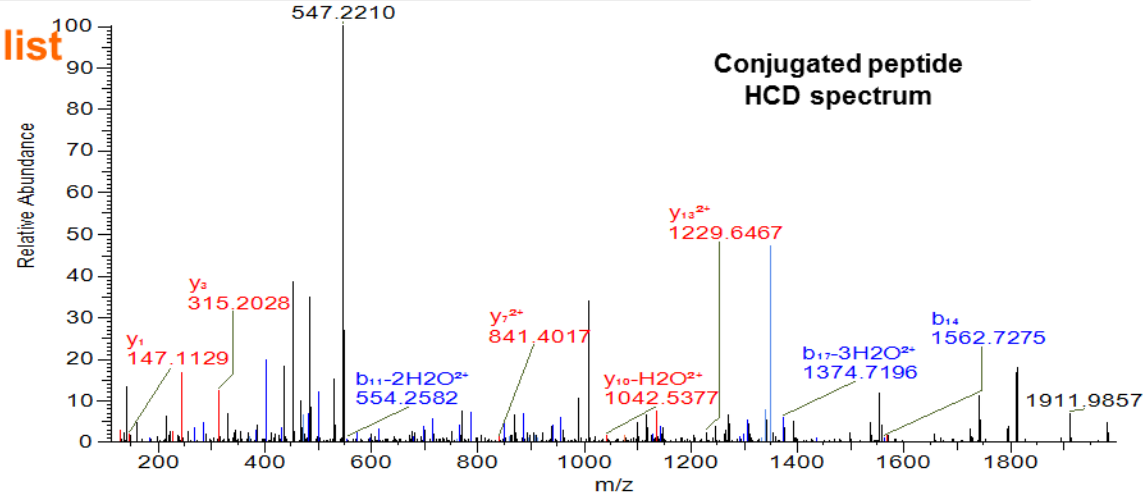
ThermoFisher SCIENTIFIC

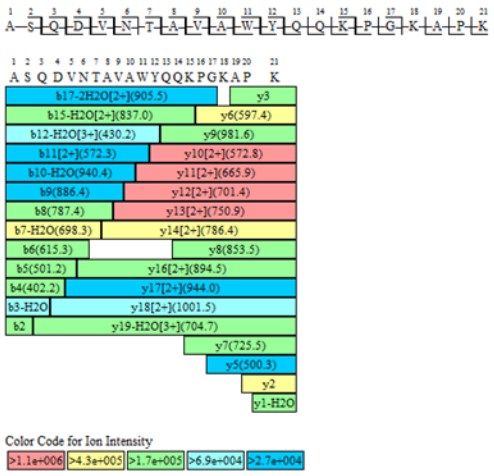
Identification of peptide ASQDVNTAVAWYQQKPGKAPK

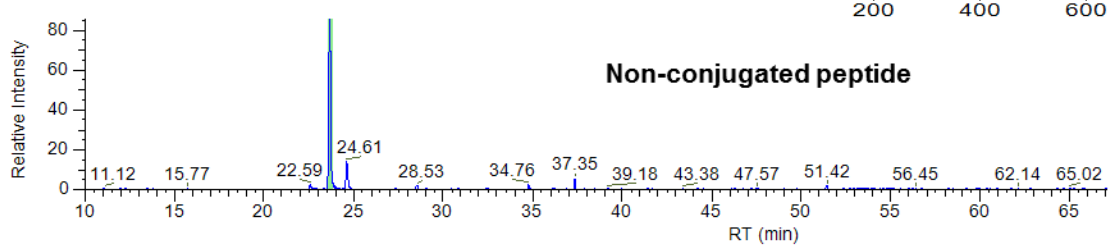
	Identification	Peptide Sequence	Modification	Site	Delta (ppm)	ID Type	RT (min)	M/Z	Charge State	Mono Mass Exp.	Mono Mass Theo.	MS Area
1	1:A25-K45 = 2286.17568m	ASQDVNTAVAWYQQKPGKAPK	None		-0.70	MS2	23.65	763.400	3	2286.1741	2286.1757	822,589.50
2	1:A25-K45 = 2286.17568m(~K42+DM1)	ASQDVNTAVAWYQQKPGKAPK	DM1	~K42	0.58	MS2	54.93	1082.521	3	3242.5420	3242.5401	3,348,808.00
3	1:A25-K45 = 2286.17568m(~K42+DM1)	ASQDVNTAVAWYQQKPGKAPK	DM1	~K42	0.20	MS2	55.50	1082.521	3	3242.5408	3242.5401	2,925,363.50

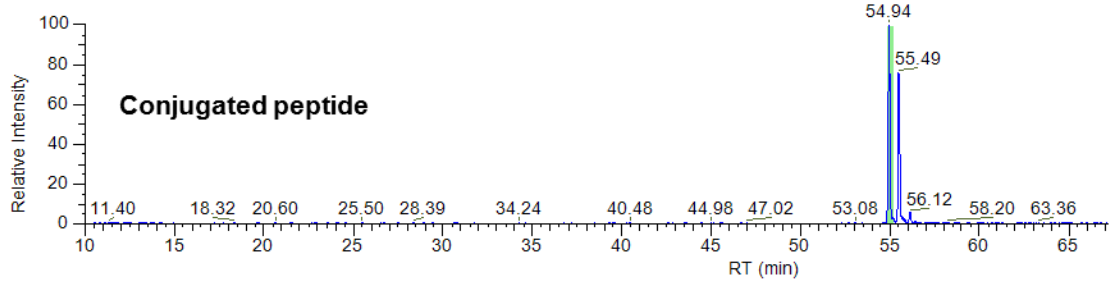
Targeted approach: inclusion list

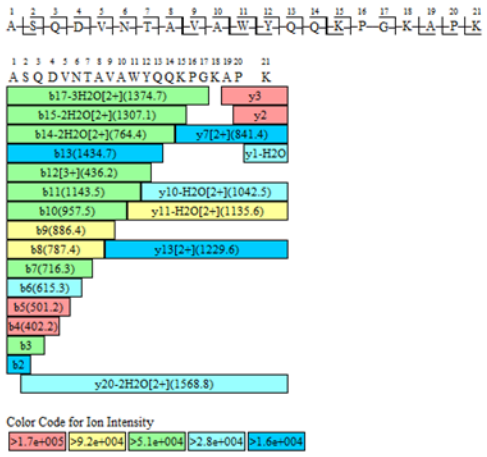










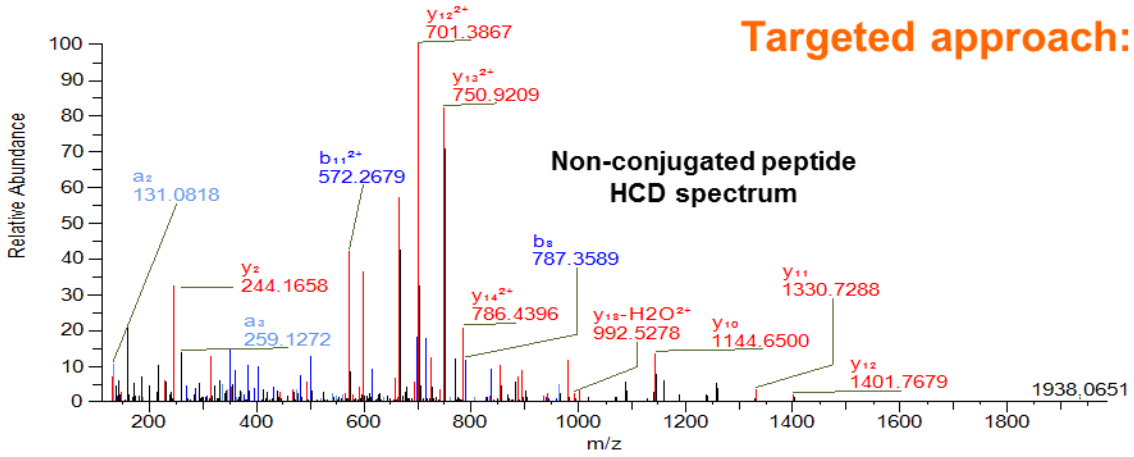


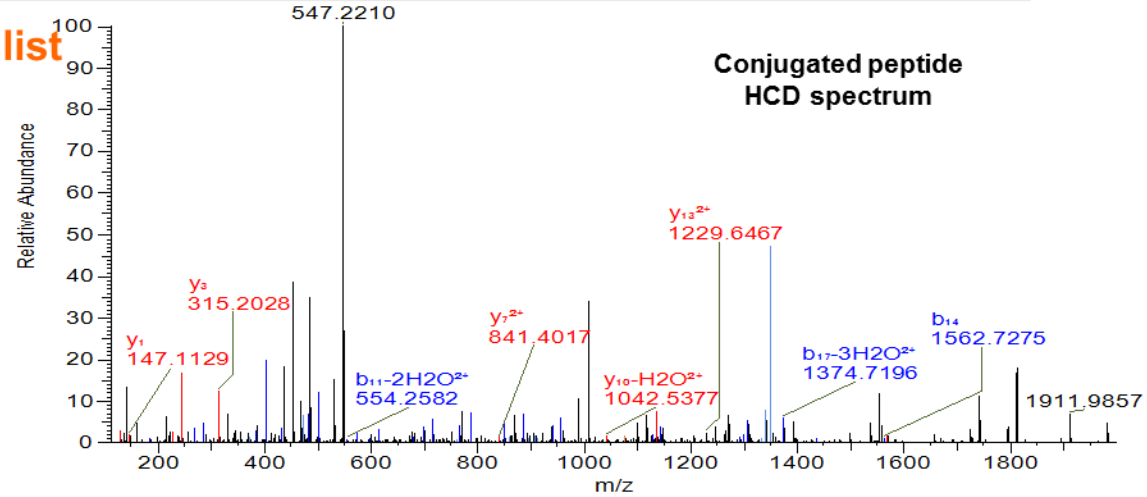
ThermoFisher SCIENTIFIC

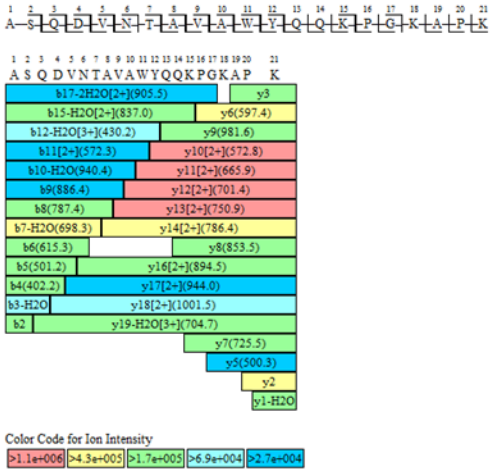
Identification of peptide ASQDVNTAVAWYQQKPGKAPK

	Identification	Peptide Sequence	Modification	Site	Delta (ppm)	ID Type	RT (min)	M/Z	Charge State	Mono Mass Exp.	Mono Mass Theo.	MS Area
1	1:A25-K45 = 2286.17568m	ASQDVNTAVAWYQQKPGKAPK	None		-0.70	MS2	23.65	763.400	3	2286.1741	2286.1757	822,589.50
2	1:A25-K45 = 2286.17568m(~K42+DM1)	ASQDVNTAVAWYQQKPGKAPK	DM1	~K42	0.58	MS2	54.93	1082.521	3	3242.5420	3242.5401	3,348,808.00
3	1:A25-K45 = 2286.17568m(~K42+DM1)	ASQDVNTAVAWYQQKPGKAPK	DM1	~K42	0.20	MS2	55.50	1082.521	3	3242.5408	3242.5401	2,925,363.50

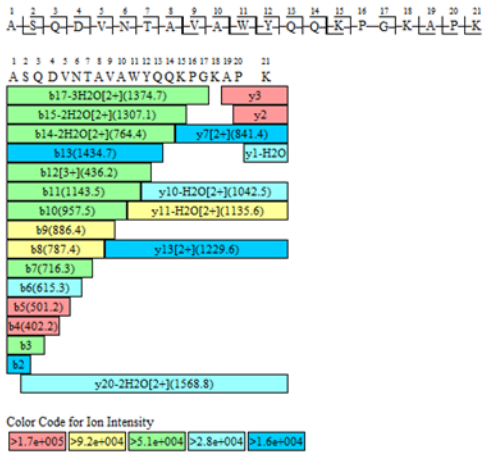
Targeted approach: inclusion list





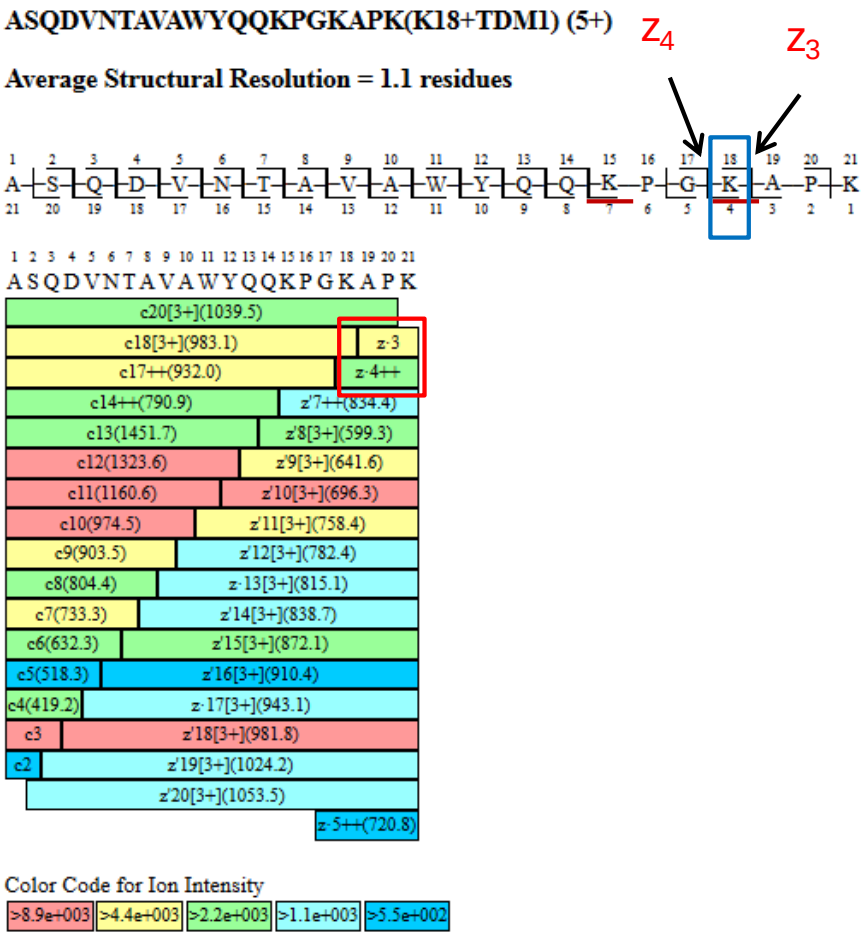


The HCD spectra of the non-conjugated peptide contains numerous intense y fragments ions. On the opposite, most of y ions for the conjugated peptide are at low intensity.
The HCD spectra of the conjugated peptide contains a characteristic neutral loss ion at m/z 547.2.



ThermoFisher SCIENTIFIC

Trastuzumab emtansine site occupation: ETD spectrum

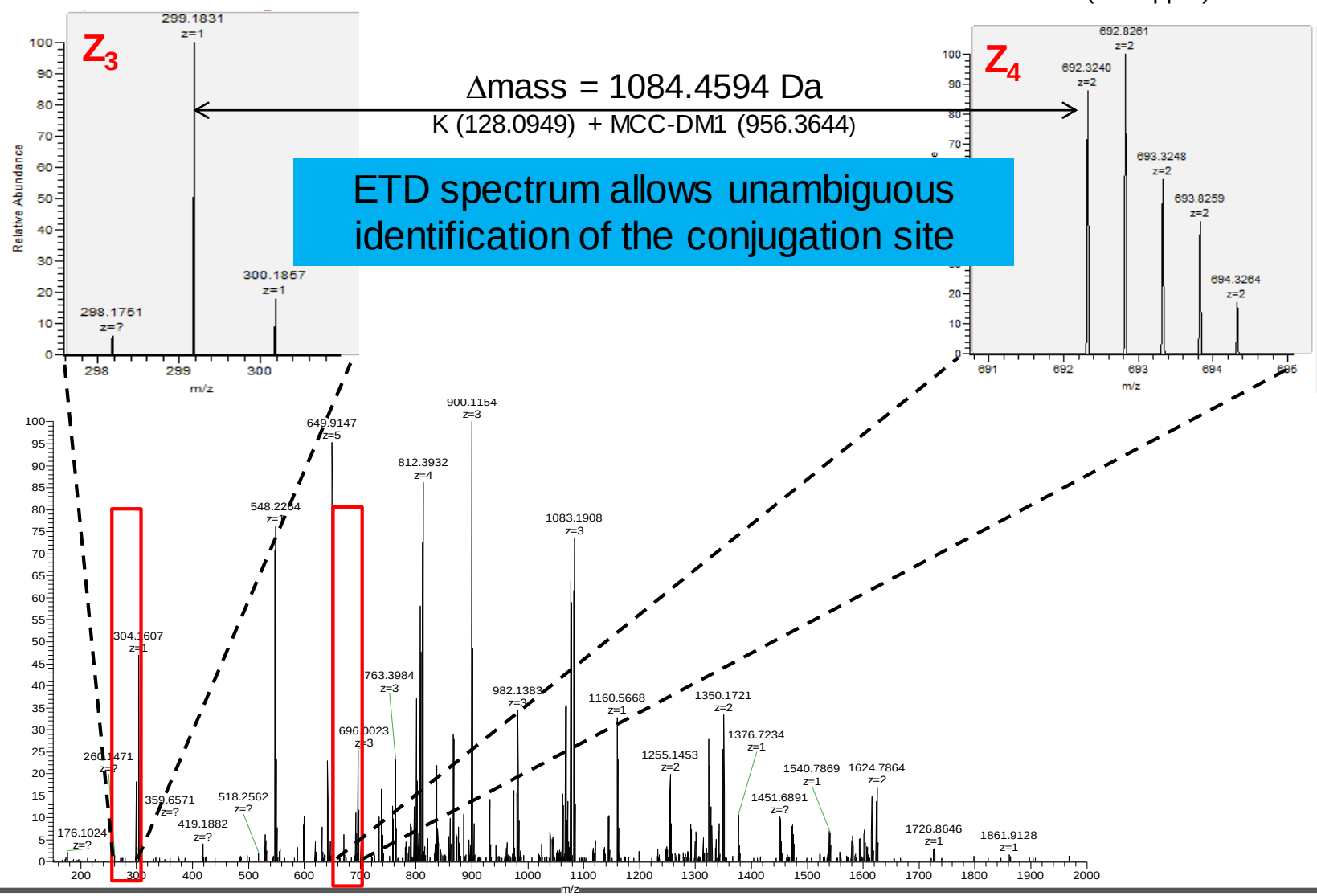


ASQDVNTAVAWYQQ**K$_{39}$**PG**K$_{42}$**AP**K**

45.99 min; m/z= 649.916 (+5); ETD

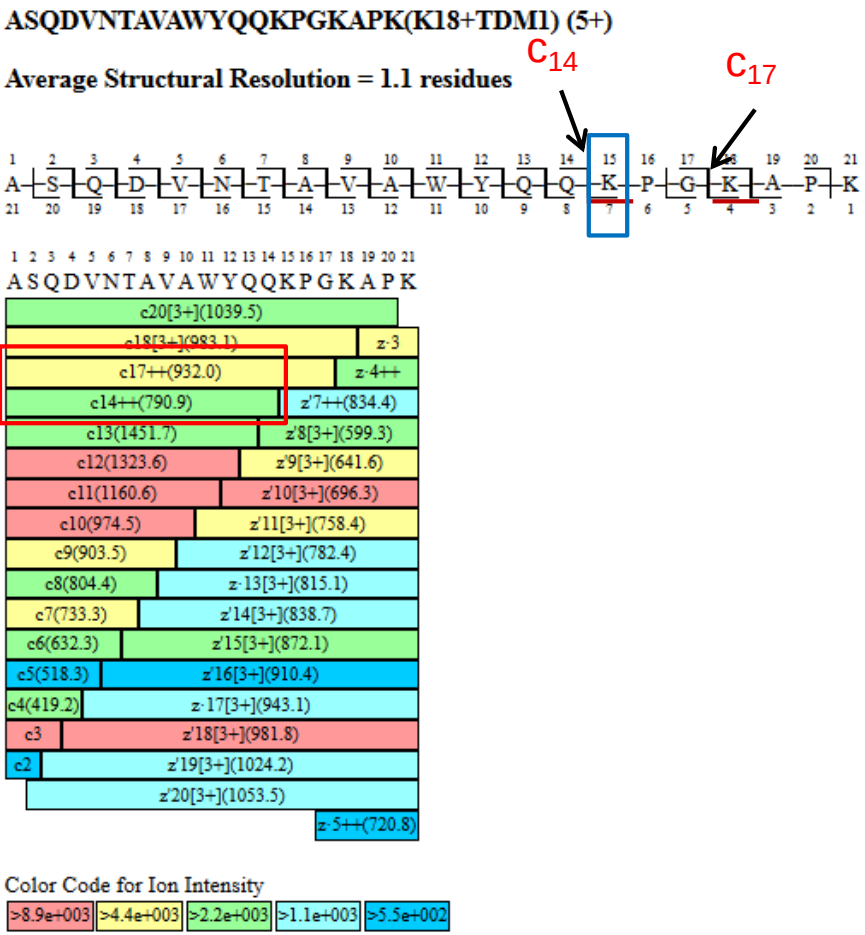
299.1840 (+1)
(-3.0 ppm)

692.3253 (+2)
(-1.9 ppm)



ThermoFisher SCIENTIFIC

Trastuzumab emtansine site occupation

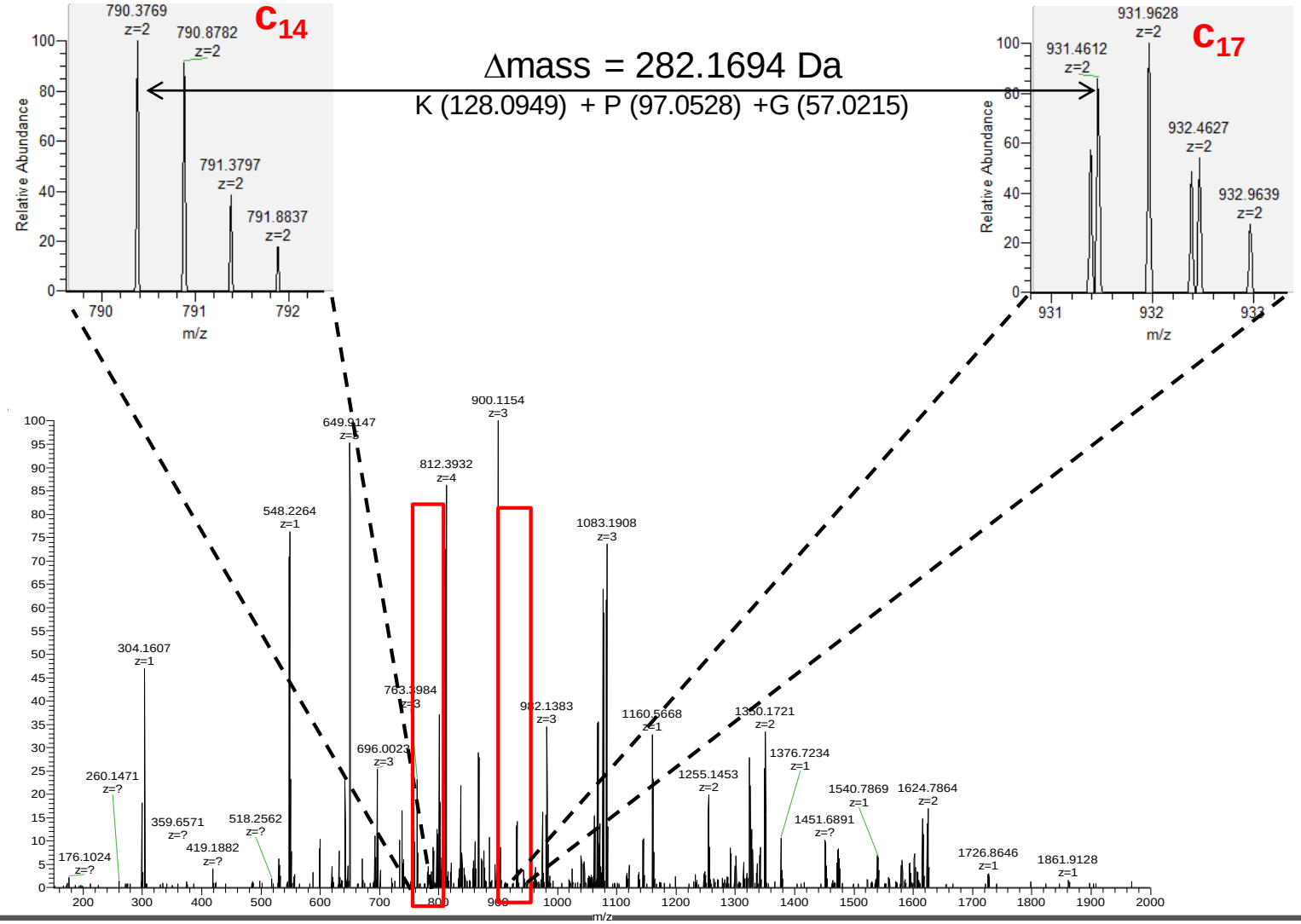


ASQDVNTAVAWYQQ**K**PG**K**AP**K**

45.99 min; m/z= 649.916 (+5); ETD

790.3785 (+2)
(-3.3 ppm)

931.4632 (+2)
(-2.1 ppm)



ThermoFisher SCIENTIFIC

Trastuzumab emtansine: conjugated peptides

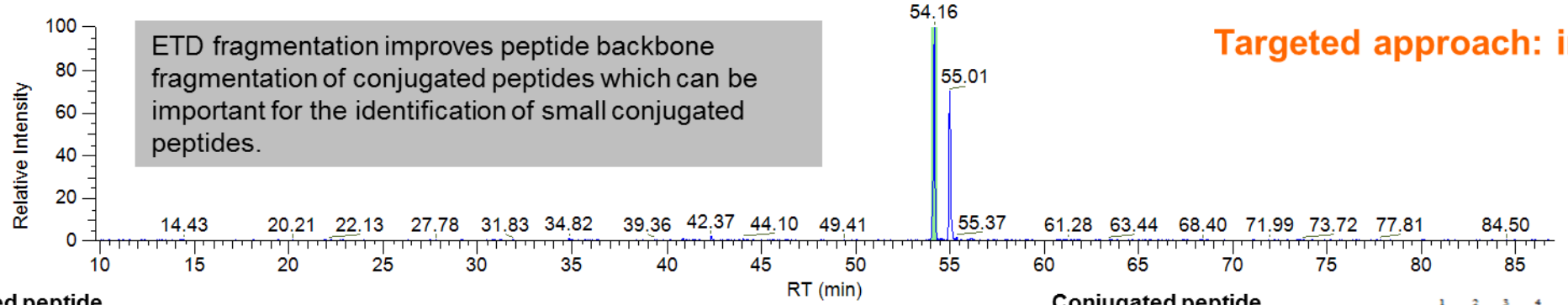
Subset of identified conjugated peptides

	No.	Peptide Sequence	# K	Modification	Site	Delta (ppm)	ID Type	RT (min)	M/Z	Charge State	Mono Mass Exp.	Mono Mass Theo.	MS Area	Protein
1	21049	ADYEKHK	2	DM1	K188	0.60	MS2	50.16	616.272	3	1845.7949	1845.7938	20,170,934.00	LC_Trastu_Emta
2	21419	ADYEKHK		DM1	K188	-0.13	MS2	50.94	616.272	3	1845.7936	1845.7938	14,392,560.00	LC_Trastu_Emta
3	22848	AKGQPR	1	DM1	K343	-0.09	MS2	53.88	806.878	2	1611.7408	1611.7410	1,016,677.25	HC_trastu_Emta
4	23430	AKGQPR		DM1	K343	-0.17	MS2	54.75	806.878	2	1611.7407	1611.7410	790,552.56	HC_trastu_Emta
5	26843	ALPAPIEKTISK	2	DM1	K337	0.64	MS2	64.62	742.381	3	2223.1206	2223.1192	462,441.38	HC_trastu_Emta
6	26950	ALPAPIEKTISK		DM1	K337	0.42	MS2	65.11	742.381	3	2223.1201	2223.1192	353,500.62	HC_trastu_Emta
7	25889	ASQDVNTAVAWYQQKPGK	2	DM1	K39	0.40	MS2	60.66	983.794	3	2946.3564	2946.3553	99,419.80	LC_Trastu_Emta
8	25971	ASQDVNTAVAWYQQKPGK		DM1	K39	0.56	MS2	61.18	983.794	3	2946.3569	2946.3553	78,092.43	LC_Trastu_Emta
9	23503	ASQDVNTAVAWYQQKPGKAPK	3	DM1	~K42	0.58	MS2	54.93	1082.521	3	3242.5420	3242.5401	3,348,808.00	LC_Trastu_Emta
10	23773	ASQDVNTAVAWYQQKPGKAPK		DM1	~K42	0.20	MS2	55.50	1082.521	3	3242.5408	3242.5401	2,925,363.50	LC_Trastu_Emta
11	27147	DSTYSLSSTLTLSKADYEK	2	DM1	K183	0.50	MS2	66.91	1023.135	3	3064.3821	3064.3806	72,910.94	LC_Trastu_Emta
12	27210	DSTYSLSSTLTLSKADYEK		DM1	K183	0.10	MS2	67.35	1023.136	3	3064.3809	3064.3806	42,332.44	LC_Trastu_Emta
13	22964	TKPR	1	DM1	K293	-1.18	MS2	54.16	729.342	2	1456.6698	1456.6715	1,648,967.12	HC_trastu_Emta
14	23556	TKPR		DM1	K293	-0.01	MS2	55.02	729.343	2	1456.6715	1456.6715	1,267,997.38	HC_trastu_Emta

Identification of peptide TKPR

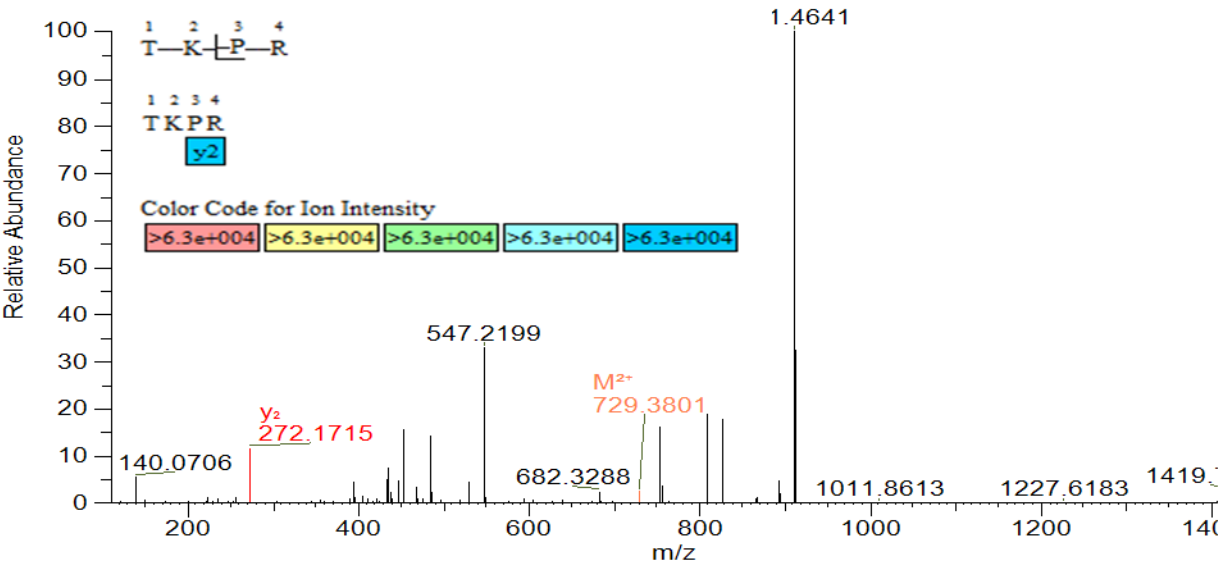
		Identification	Peptide Sequence	Modification	Site	Delta (ppm)	ID Type	RT (min)	M/Z	Charge State	Mono Mass Exp.	Mono Mass Theo.	MS Area
1		2:T292-R295 = 500.30708m(K293+DM1)	TKPR	DM1	K293	-1.18	MS2	54.16	729.342	2	1456.6698	1456.6715	1,648,967.12
2		2:T292-R295 = 500.30708m(K293+DM1)	TKPR	DM1	K293	-0.01	MS2	55.02	729.343	2	1456.6715	1456.6715	1,267,997.38

ETD fragmentation improves peptide backbone fragmentation of conjugated peptides which can be important for the identification of small conjugated peptides.

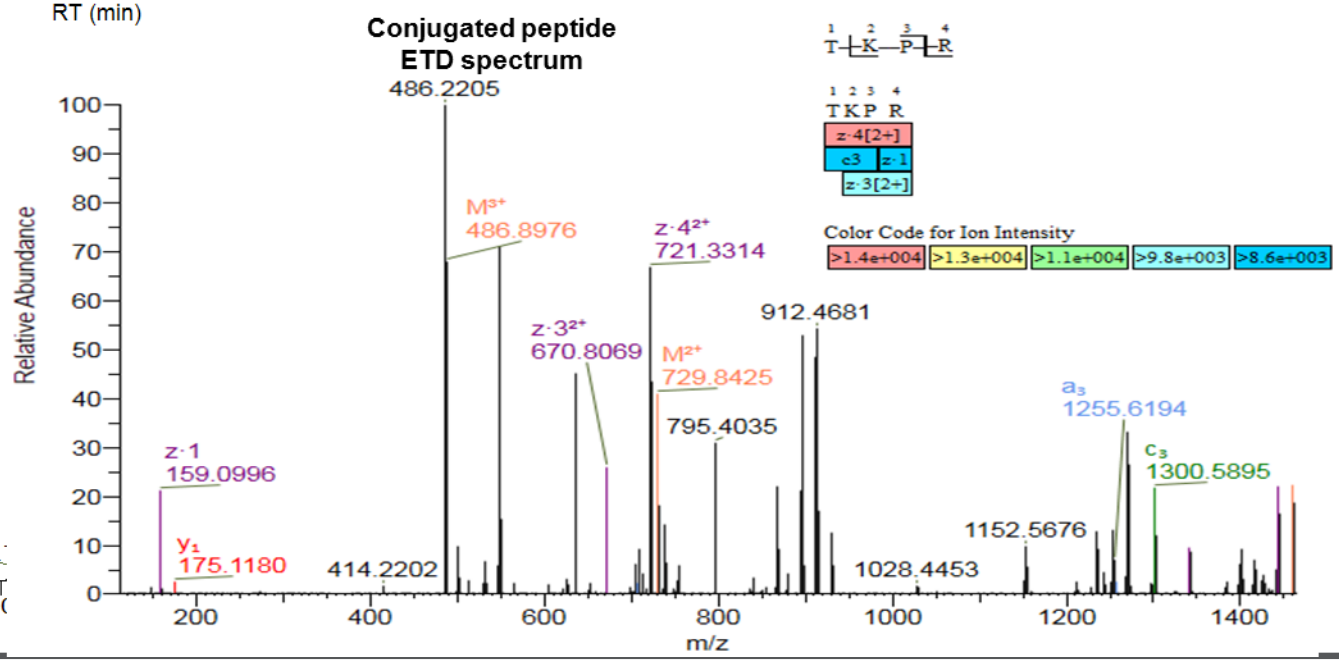


Targeted approach: inclusion list

Conjugated peptide HCD spectrum



Conjugated peptide ETD spectrum



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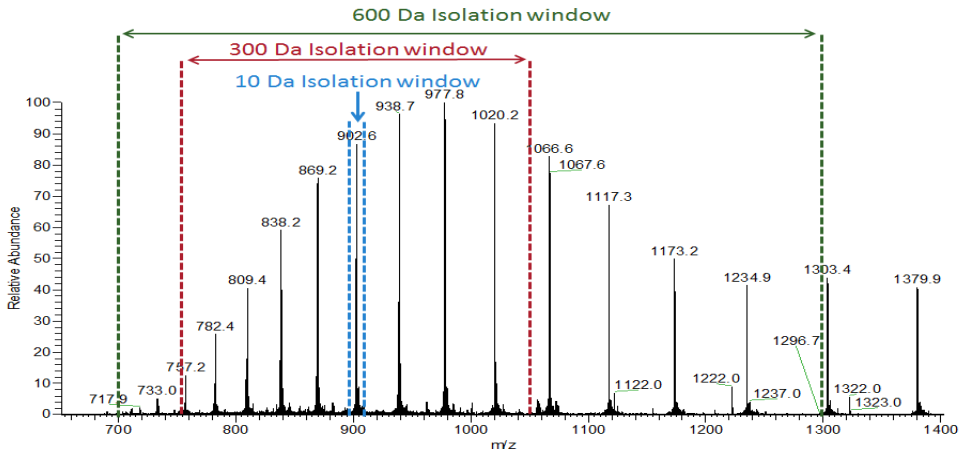
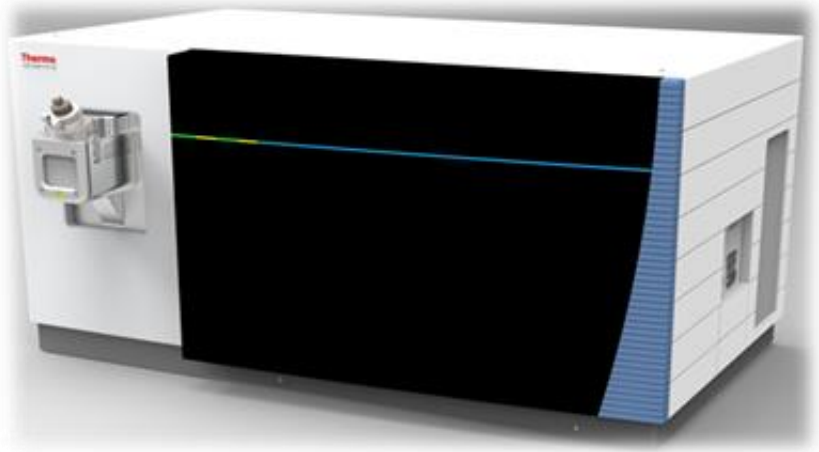
Middle-down: ETD

Top-Down Sequencing

Main parameters that can be controlled for ETD fragmentation on an Orbitrap™ Fusion™ mass spectrometer.

- Isolation window



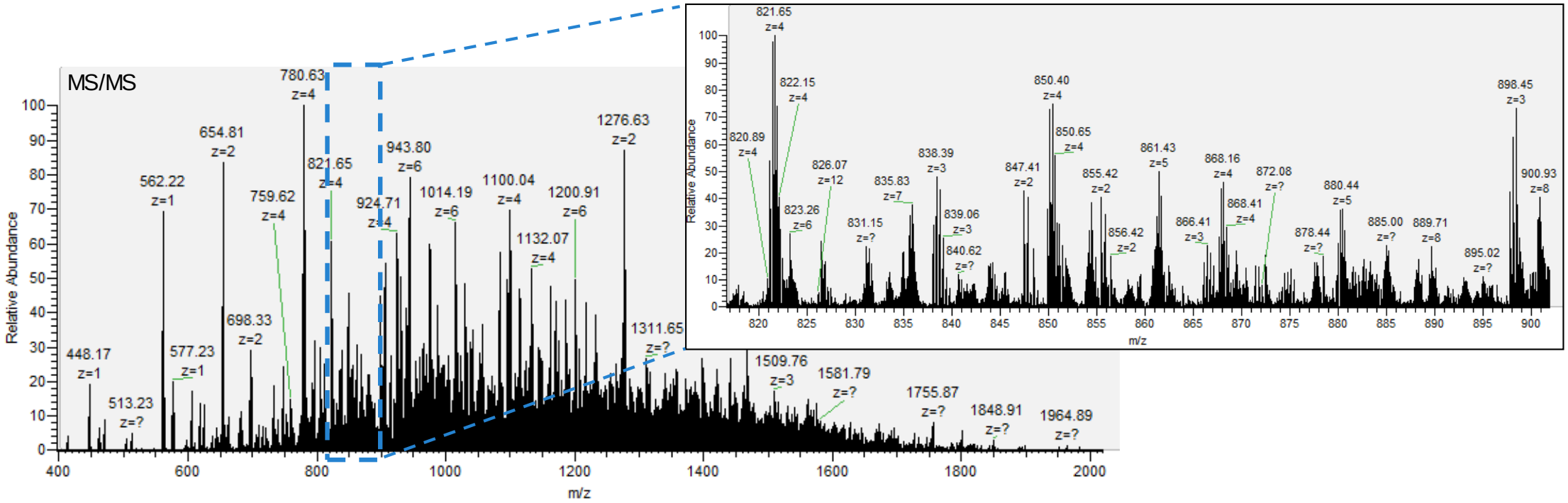


- AGC target for precursor ions and reagent
- Reaction time
- Supplemental energy

Top-Down sequencing: Trastuzumab

Light Chain of Trastuzumab

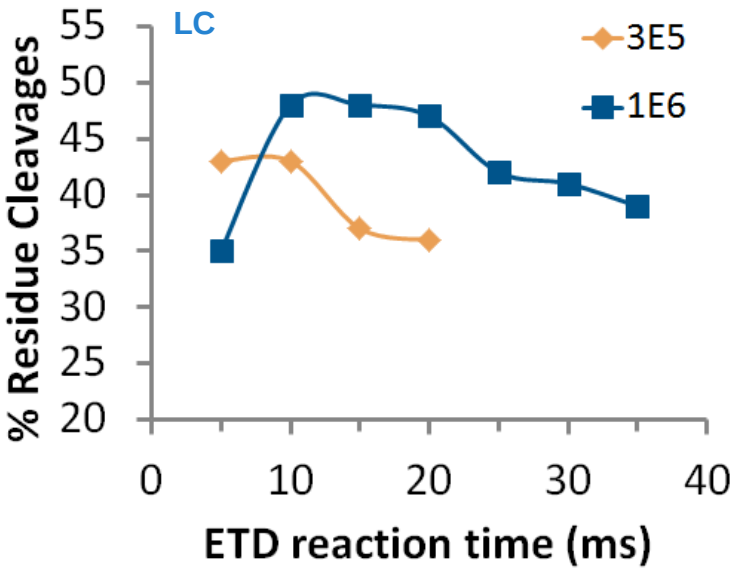
ETD settings: 300Da isolation window, 3E5 precursor, 7E5 reagent, 10 ms reaction time

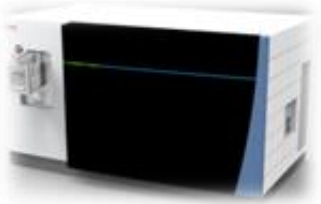


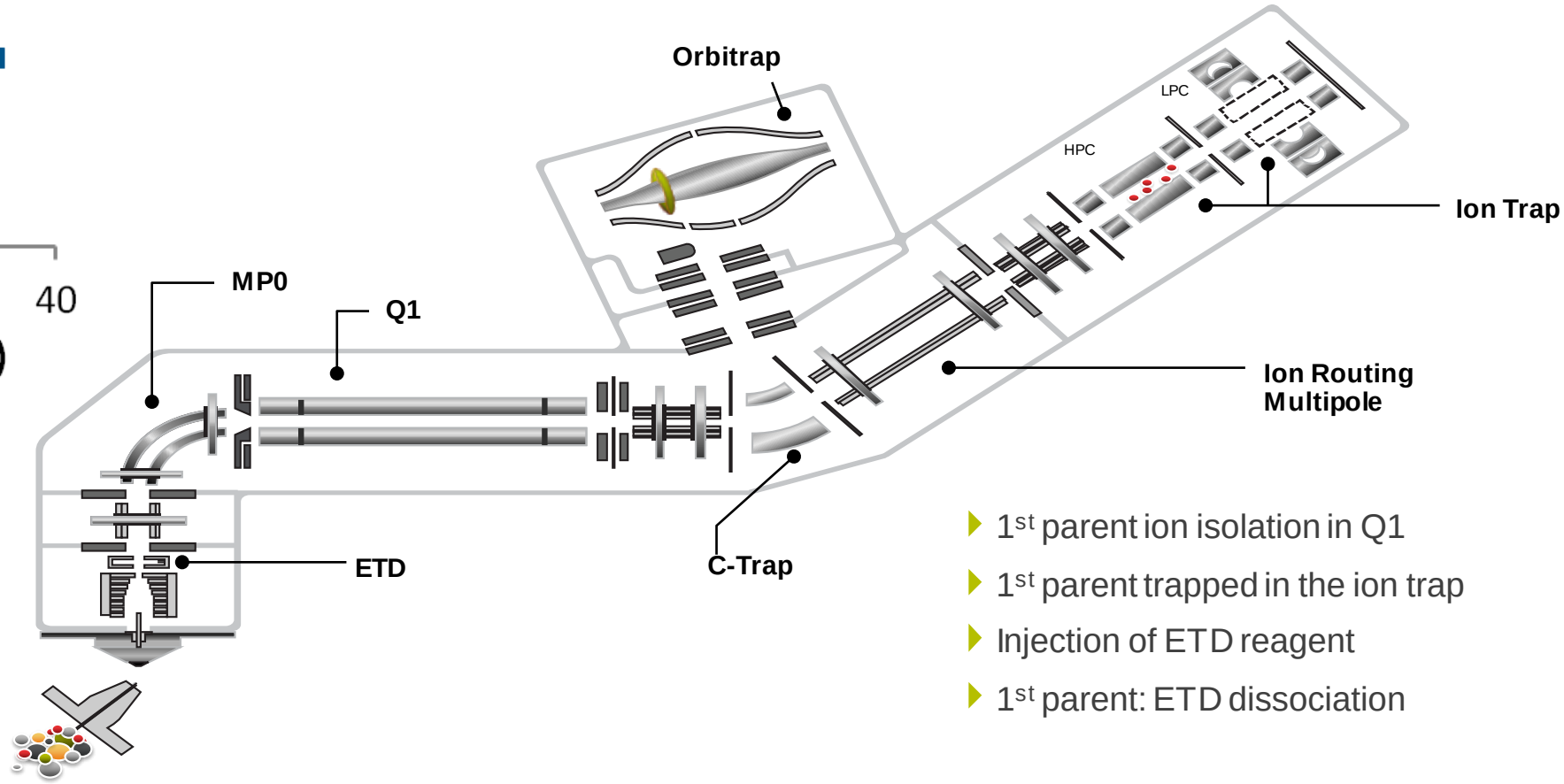
Due to the complexity of the spectra in top-down analysis, high resolution is required

Effect of parent ion AGC and ETD reaction time on sequence coverage (Trastuzumab)

% residue cleavages for AGC target value of 3E5 or 1E6 at different ETD reaction times







- 1st parent ion isolation in Q1
- 1st parent trapped in the ion trap
- Injection of ETD reagent
- 1st parent: ETD dissociation

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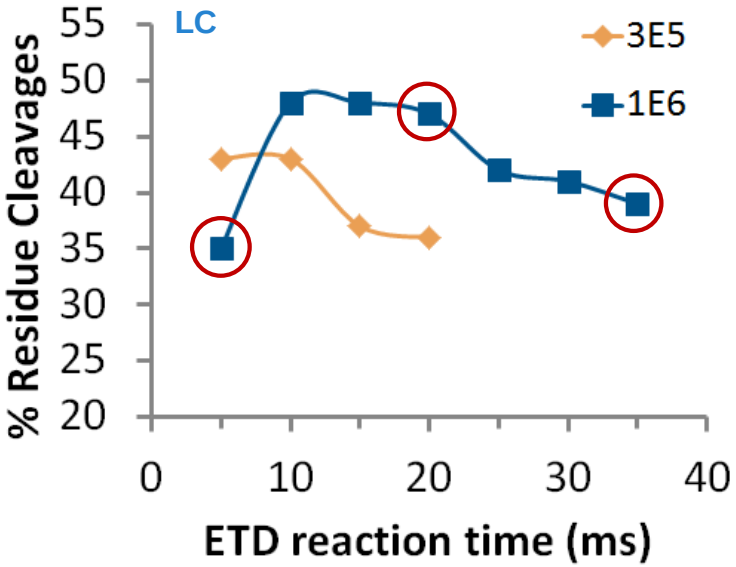
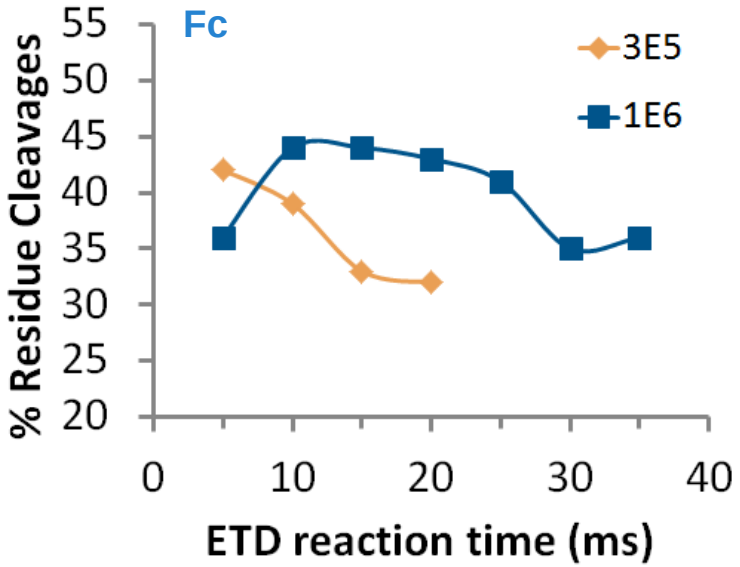
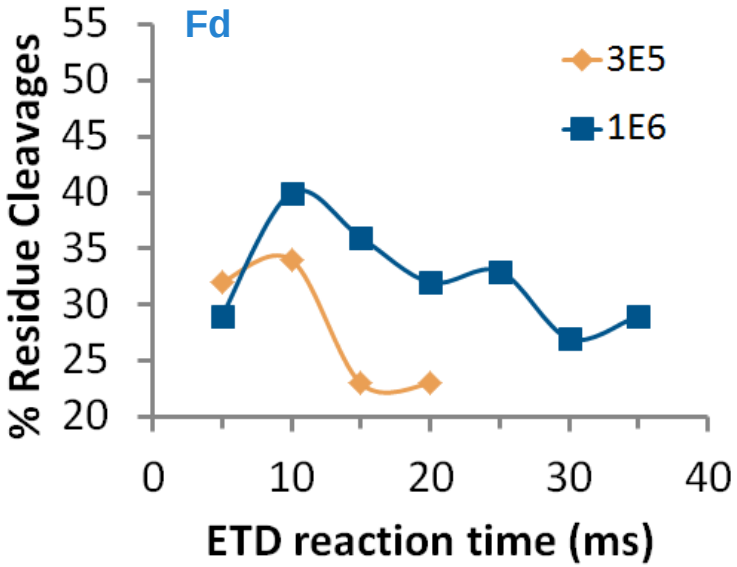
Effect of parent ion AGC and ETD reaction time on sequence coverage (Trastuzumab)

% residue cleavages for AGC target value of 3E5 or 1E6 at different ETD reaction times

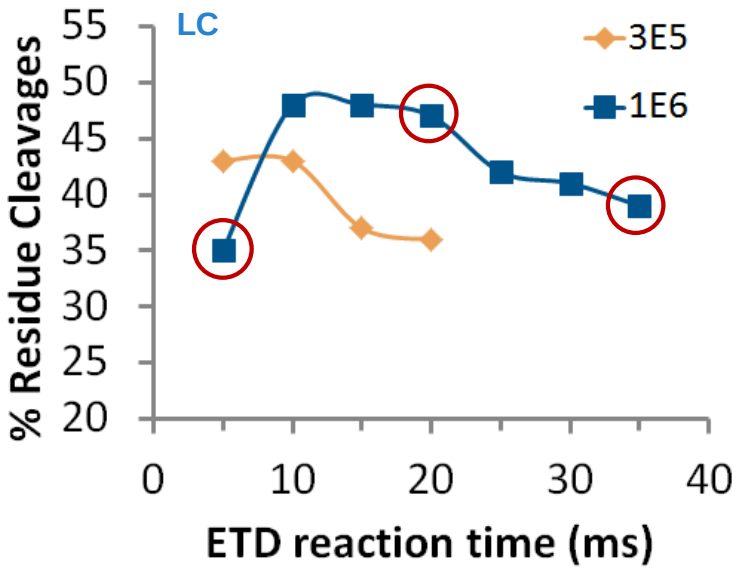


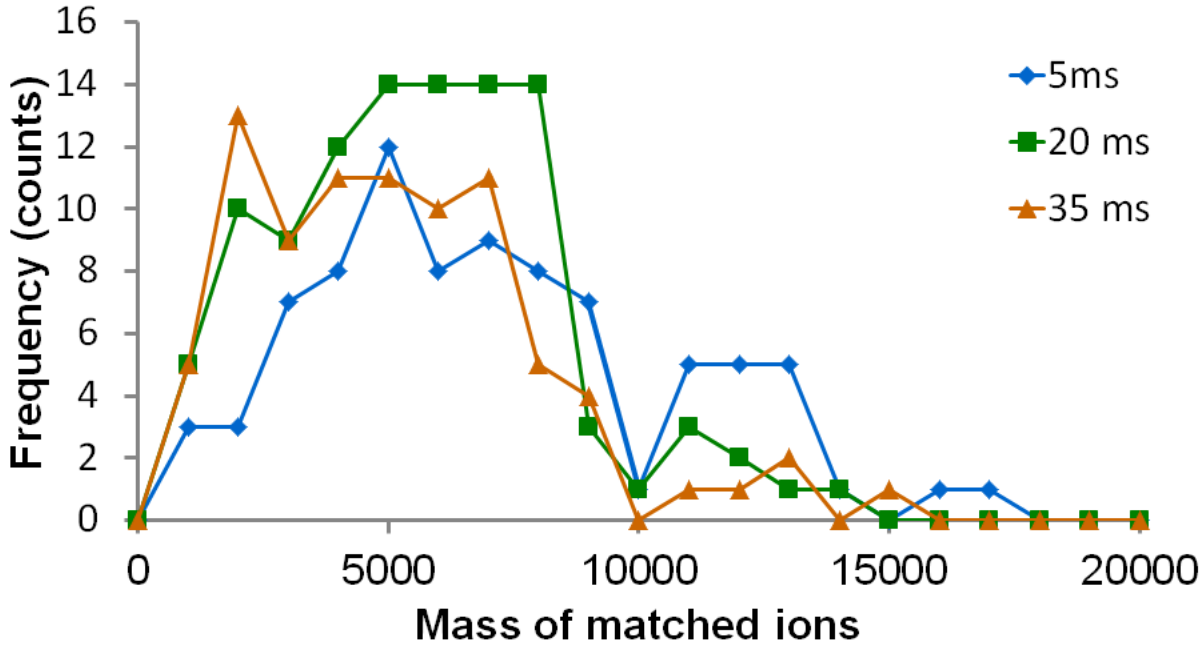




Effect of parent ion AGC and ETD reaction time on sequence coverage (Trastuzumab)

% residue cleavages for AGC target value of 3E5 or 1E6 at different ETD reaction times





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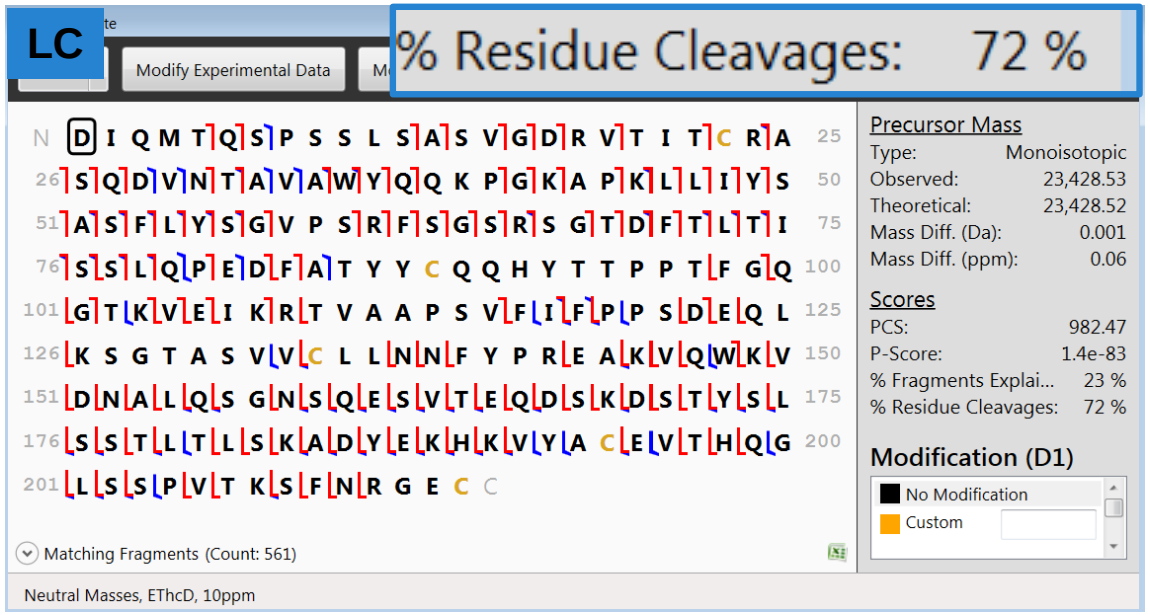
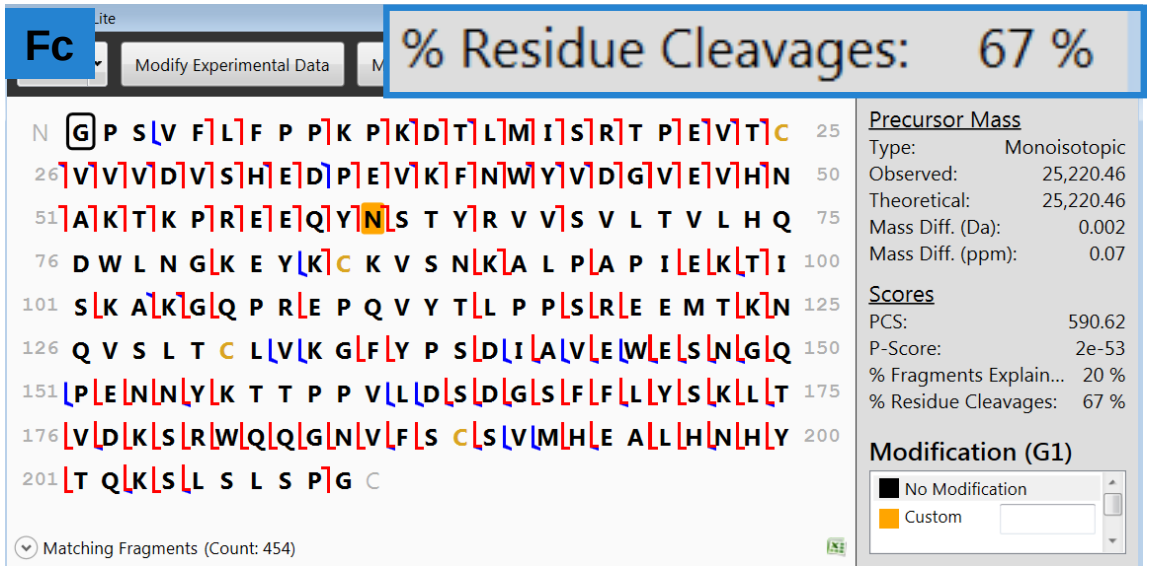
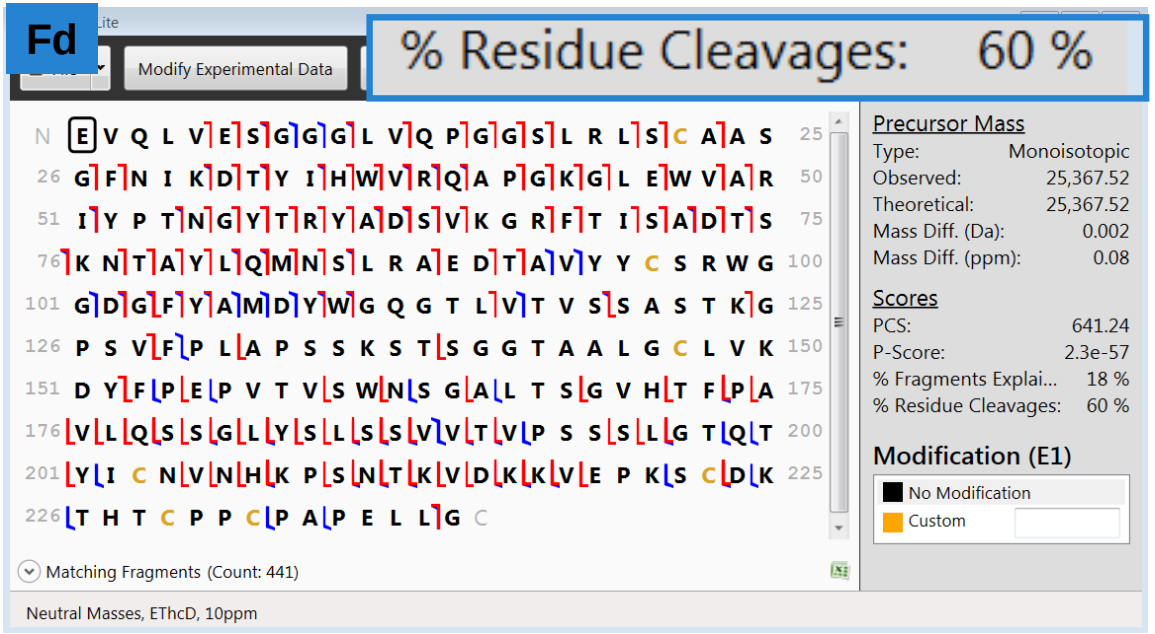
Middle Down: Orbitrap Tribrid Fusion Lumos







High sequence coverage for the light chain, Fc and Fd were obtained from the combined ETD and EThcD experiments.

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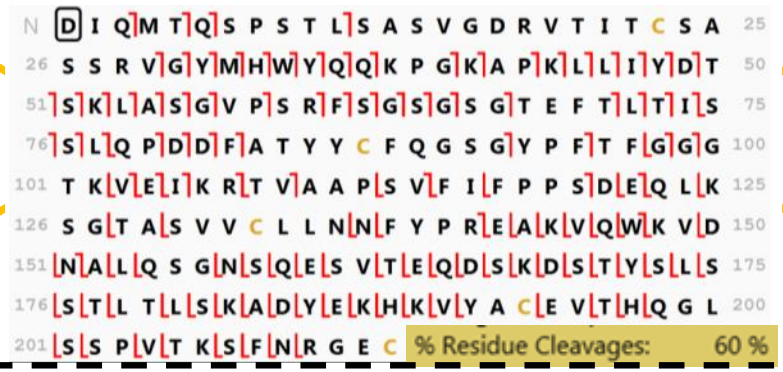
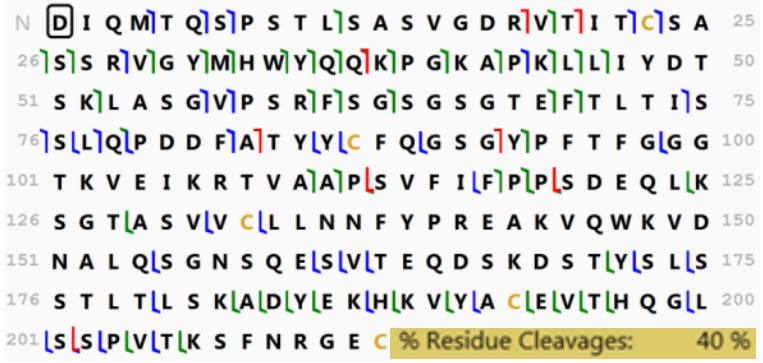
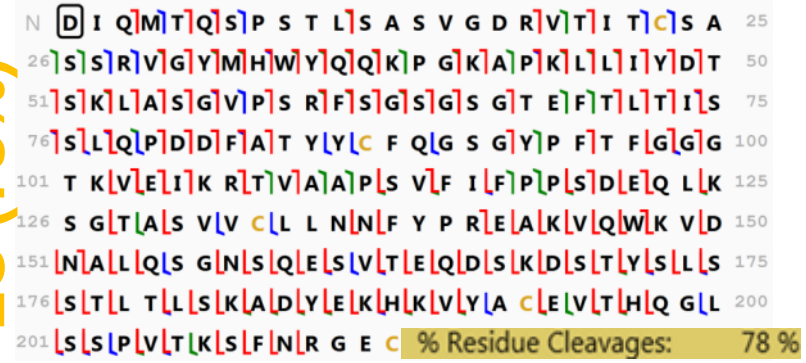
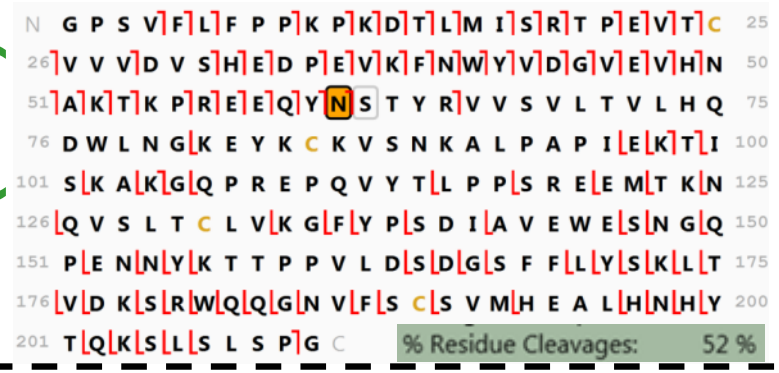
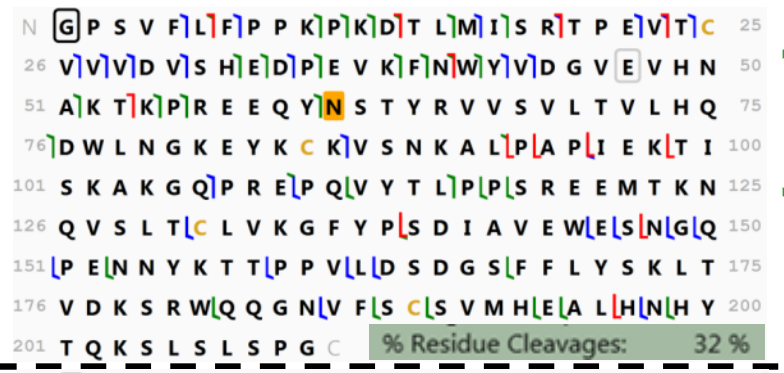
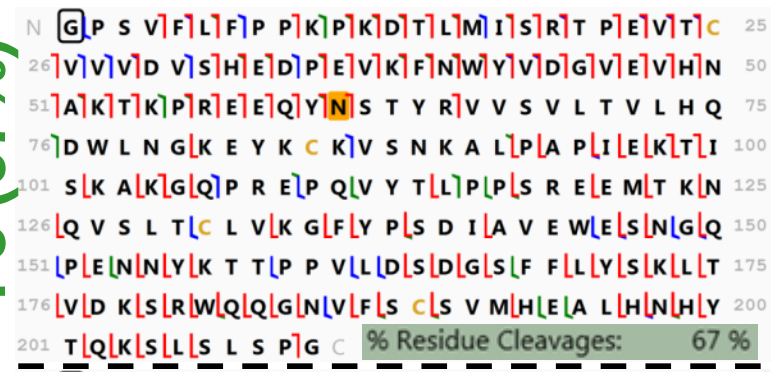
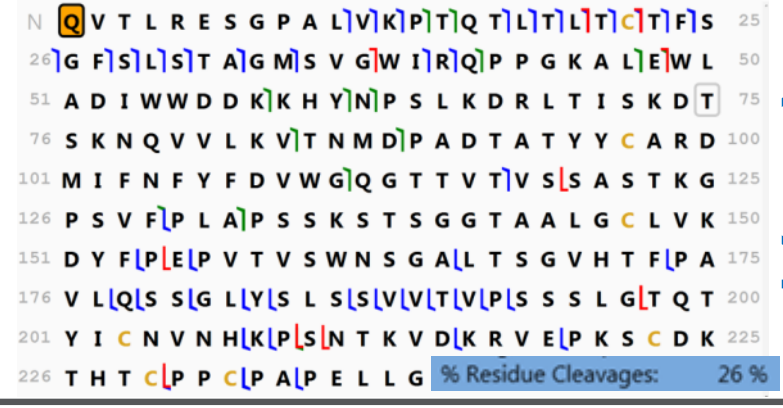
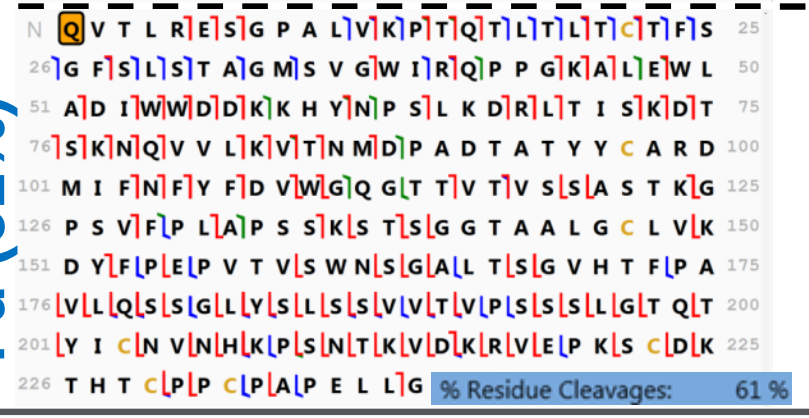
NIST mAb: middle down experiment using ETD and UVPD

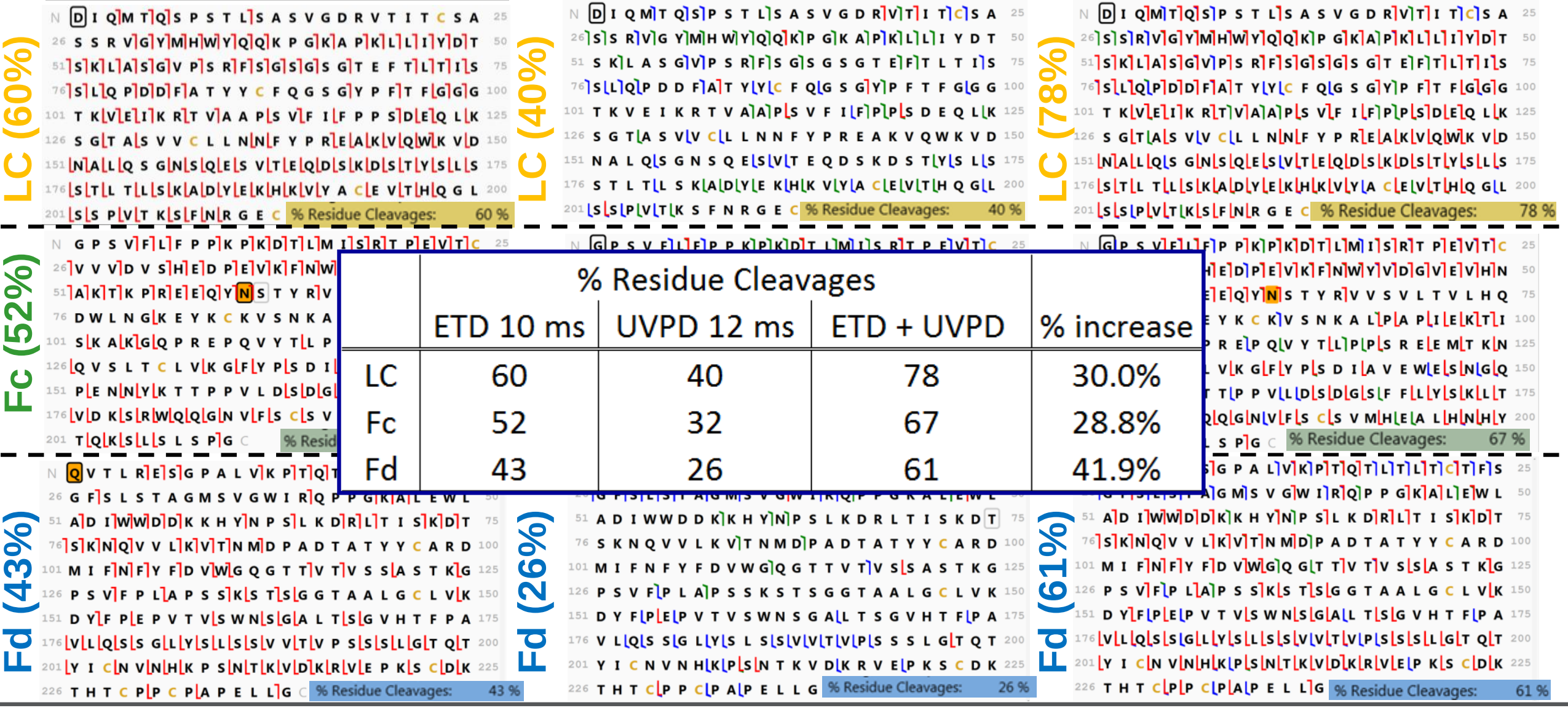
	One acquisition with ETD 10 ms	One acquisition with UVPD 12 ms	ETD 10 ms + UVPD 12 ms
LC (60%)			
Fc (52%)			
Fd (43%)			

NIST mAb: middle down experiment using ETD and UVPD

One acquisition with ETD 10 ms

One acquisition with UVPD 12 ms

ETD 10 ms + UVPD 12 ms



	% Residue Cleavages			
	ETD 10 ms	UVPD 12 ms	ETD + UVPD	% increase
LC	60	40	78	30.0%
Fc	52	32	67	28.8%
Fd	43	26	61	41.9%

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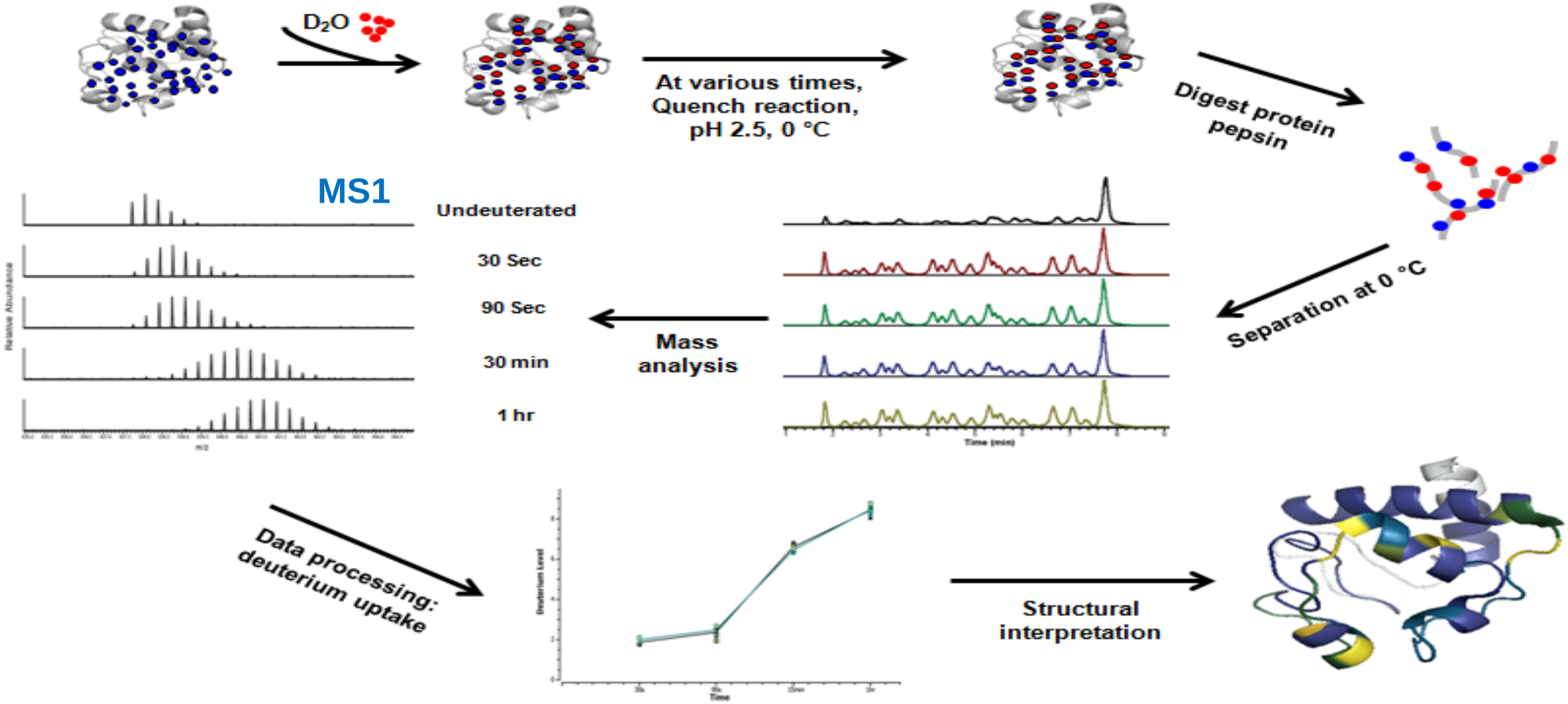
Hydrogen Deuterium Exchange

HDX-MS workflow



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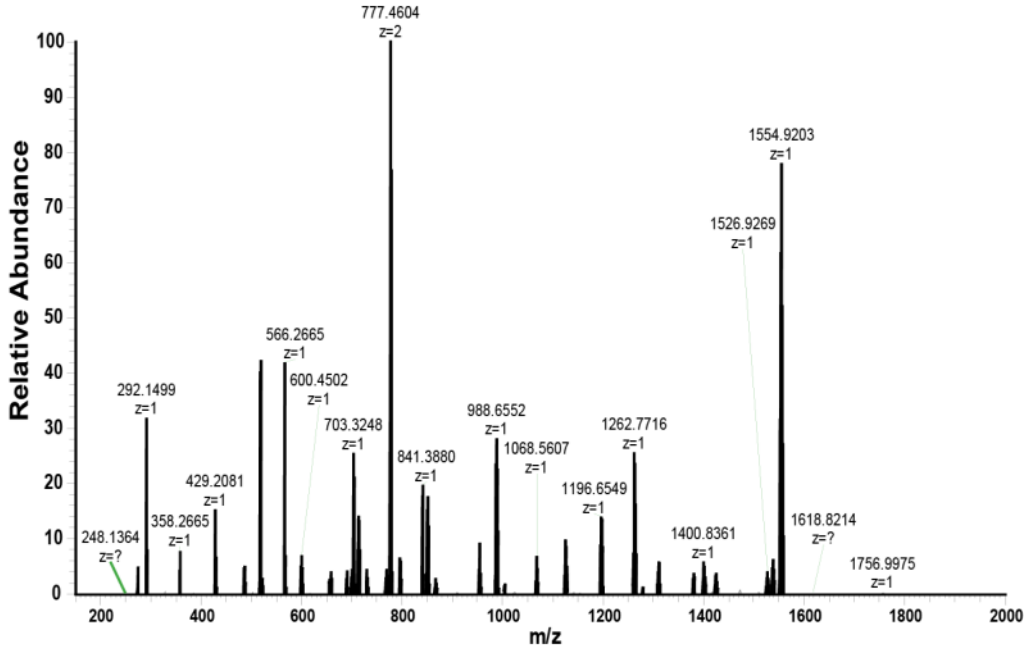
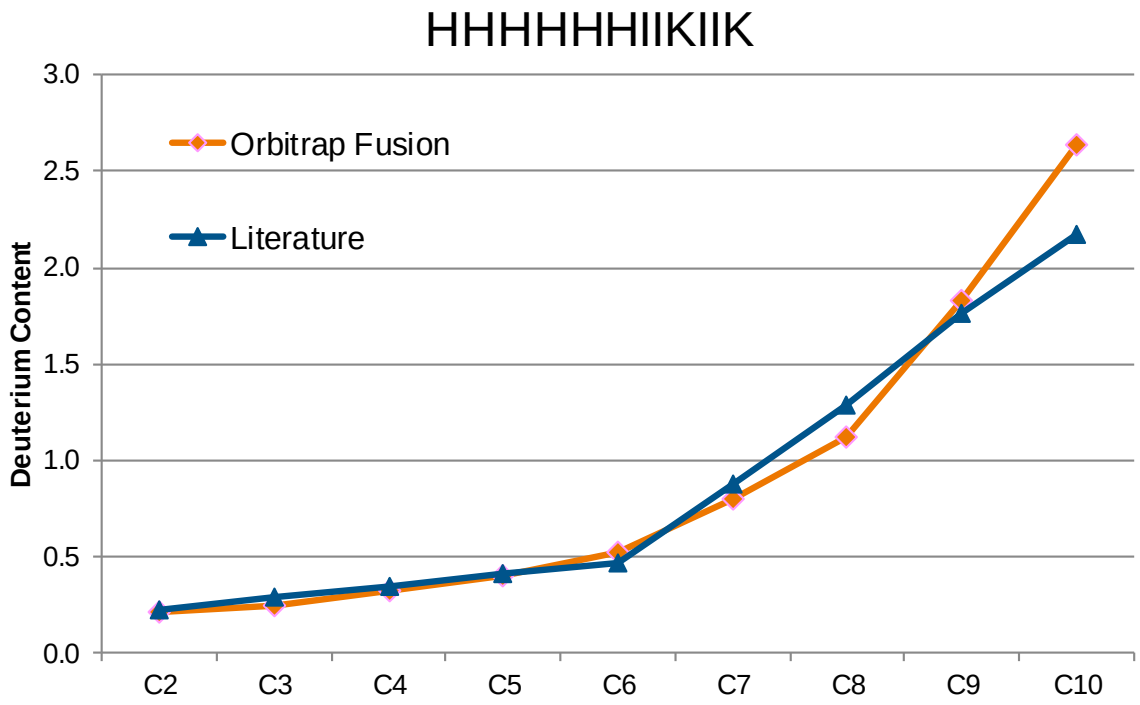
Moving from MS only to MS/MS HDX experiment using ETD

Under normal operation condition, minimal scrambling is observed on the ETD Spectrum





(Martin Zehl, Kasper D. Rand, Ole N. Jensen, and Thomas J. D. Jørgensen J. AM. CHEM. SOC. 2008, 130, 17453–17459 9 17453)

•Minimal deuterium scrambling ETD measurement with different instruments and source conditions
(No deuterium is retained on Histidine due to fast exchange with solvent)

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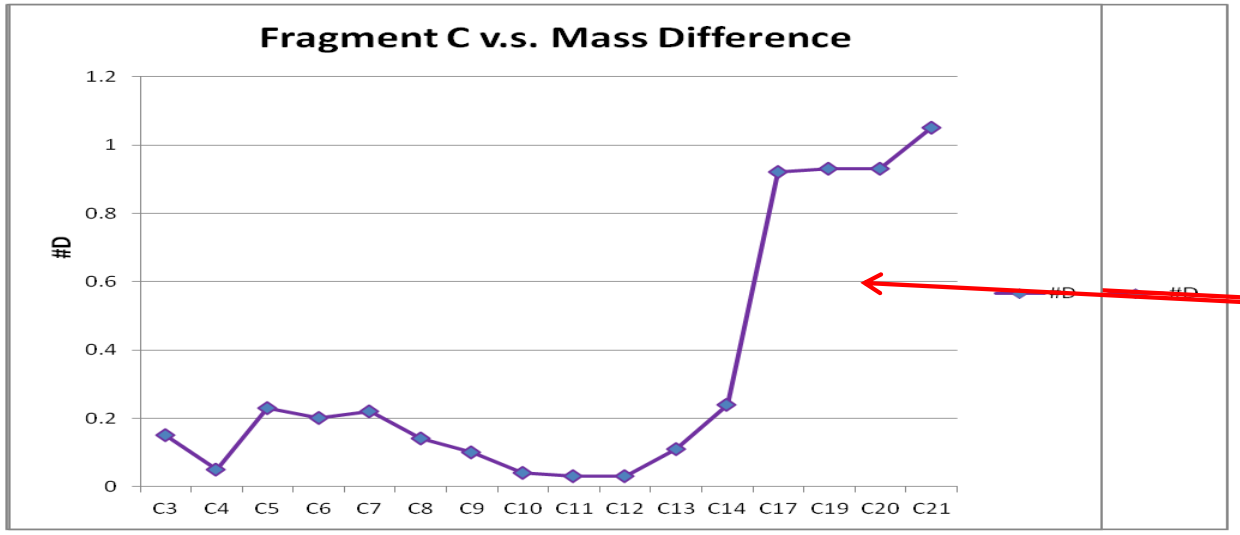
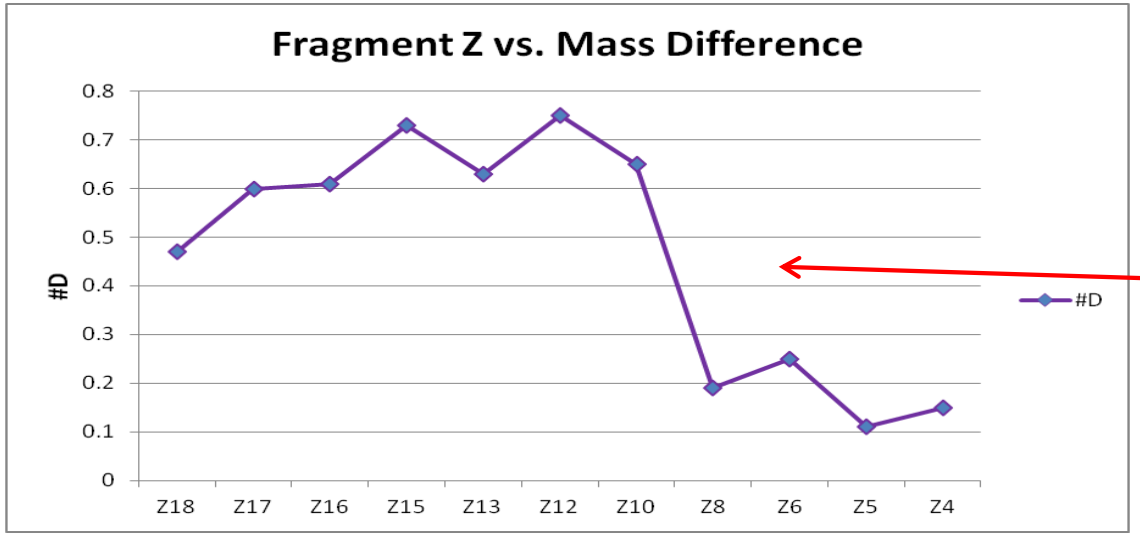
Moving from MS only to MS/MS HDX experiment using ETD



Pinpoint the Protein Ligand Binding Site with ETD

Deuterium labeled protein and protein+ligand biding sample's C and Z fragments mass differences vs. sequence position. From C3 to C14 mass differences were from 0.03 to 0.2. Start from C17, the Δ mass increased to around 1 and stay at the same level for the rest of the C fragments. C14 was identified as significant change area.



Significant change was observed around Z_8-Z_{10} is consistent with the C fragments plot. Combine the two plot results, the potential binding site was predicted.

Summary

- ETD is a must have type of fragmentation for in-depth characterization of biotherapeutic proteins.
- High sequence coverage for middle-down experiments allows quick characterization assays.
- Orbitrap Fusion Lumos offers multiple types of fragmentation and ease of use to tackle the most challenging task.

Thank you !

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