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Don‘t miss a thing on your peptide mapping journey

How to get full coverage peptide maps using high resolution accurate mass spectrometry

Kai Scheffler, PhD
BioPharma Support Expert ,LSMS Europe

Outline

- What is the exact goal of the analysis?
  - sequence elucidation/verification
  - sequence variant analysis, amino acid substitution
  - analysis of modifications: glycosylation, deamidation, sulfation, oxidation
  - disulfide bridge analysis
  - distinguishing Leu/Ile, Asp/isoAsp
- Mass spectrometry portfolio: which instrument is applicable?
- Method setup: what is the most suitable method?
- MS methods:
  - what are the critical parameters?
  - how do they influence the success of the analysis?

MS Portfolio for BioPharma Characterization

Thermo Scientific™ Ion Trap LC-MS systems

Full MS with dd MS^2 (CID, HCD, ETD)

- MSn

Thermo Scientific™ Exactive™ Plus Orbitrap™ Mass Spectrometer

Full MS only

- (HCD fragmentation, no precursor selection)

Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap™ mass spectrometers

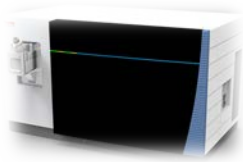
Full MS only

- Full MS with dd MS^2 (HCD)

Thermo Scientific™ LTQ Orbitrap XL™ Hybrid Ion Trap-Orbitrap™ Mass Spectrometer

Full MS only (OT detection)

- Full MS with dd MS^2 (CID, HCD, ETD)
- MSn



Thermo Scientific™ Orbitrap™ Fusion™ Tribrid™ Mass Spectrometers

Full MS only (OT detection)

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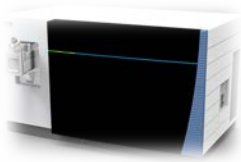
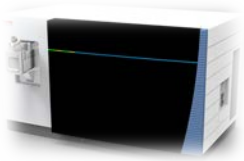


Thermo Scientific™ Orbitrap™ Fusion™ Tribrid™ Mass Spectrometers

- Full MS only (OT detection)
- Full MS with dd MS^2 (CID, HCD, ETD)
- MSn

Analysis type	Ion Trap LC-MS	Exactive™ Plus Orbitrap™	Q Exactive™ hybrid quadrupole-Orbitrap™	LTQ Orbitrap XL™ Hybrid Ion Trap-Orbitrap™	Orbitrap™ Fusion™ Tribrid™
Peptde mapping/sequence confirmation	✓ low res, low mass accuracy	☑ Full MS only	✓	✓	✓
Sequence variant analysis	✓	☑ Confirmation of expected ones only	✓	✓	✓
Analysis of PTMs and other modifications	✓	☑ Confirmation of expected ones only	✓	✓	✓
Disulfide bond analysis	✓	☑ Confirmation of expected ones only	✓	✓	✓

MS Portfolio for BioPharma Characterization



Analysis type	Thermo Scientific™ Ion Trap LC-MS systems	Thermo Scientific™ Exactive™ Plus Orbitrap™ Mass Spectrometer	Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap™ mass spectrometers	Thermo Scientific™ LTQ Orbitrap XL™ Hybrid Ion Trap-Orbitrap™ Mass Spectrometer	Thermo Scientific™ Orbitrap™ Fusion™ Tribrid™ Mass Spectrometers
	• Full MS with dd MS^2 (CID, HCD, ETD) • MSn	• Full MS only • (HCD fragmentation, no precursor selection)	• Full MS only • Full MS with dd MS^2 (HCD)	• Full MS only (OT detection) • Full MS with dd MS^2 (CID, HCD, ETD) • MSn	• Full MS only (OT detection) • Full MS with dd MS^2 (CID, HCD, ETD) • MSn
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Analysis of PTMs and other modifications	✓	☑ Confirmation of expected ones only	✓	✓	✓
Disulfide bond analysis	✓ ETD enabled systems only	☑ Confirmation of expected ones only	✓	✓ ETD enabled systems only	✓

Method Setup

Before you setup, refine and start the MS method, you need to ensure you have an optimized LC-MS setup:

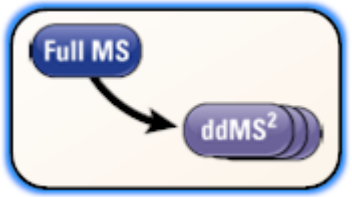
- Calibration
- Source settings:
  - ❑ probe position in the source housing (probe depth, x/y position)
  - ❑ source voltage
  - ❑ gas flows (sheath/aux gas) optimized for LC flow rate
  - ❑ S-lens

Peptide Mapping Method Setup

Method 1:

Full MS scans only

Method 2:



Full MS scans with data dependent MS^2 scans

Peptide Mapping Method Setup

Method 1:

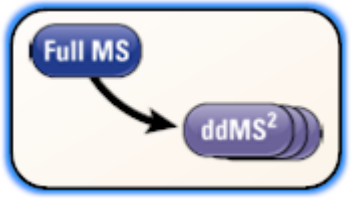
Full MS scans only

- Sequence **confirmation** based on intact accurate precursor masses
- **Confirmation** of known and expected modifications
- **Confirmation** of disulfide bridges

Critical parameters:

- **Mass range**
- AGC target setting
- Fill time

Method 2:



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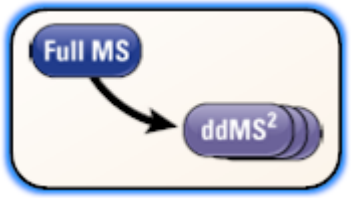


Full MS scans with data dependent MS^2 scans

- Sequence confirmation based on intact accurate precursor masses **and MS^2 spectra**
- Confirmation of known and expected modifications and **identification of unknowns**
- Confirmation of disulfide bridges and **identification of unknowns**

Critical parameters:

- **Mass range**
- **Charge state range**
- AGC target setting MS + MS^2
- **Maximum injection time MS/MS^2**
- Fixed first mass (MS^2)
- Intensity threshold
- **TopN**
- Collision energy (NCE)

Peptide Mapping Method Setup

Method 1:

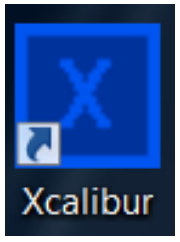
Full MS scans only

- Sequence **confirmation** based on intact accurate precursor masses
- **Confirmation** of known and expected modifications
- **Confirmation** of disulfide bridges

Critical parameters:

- **Mass range**
- AGC target setting
- Fill time

For Exactive MS series the **MS method setup and data acquisition** can be done via Thermo Scientific™Xcalibur™ Software and/or Thermo Scientific™ Chromeleon™ Chromatography Data System Software



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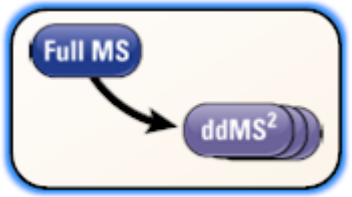

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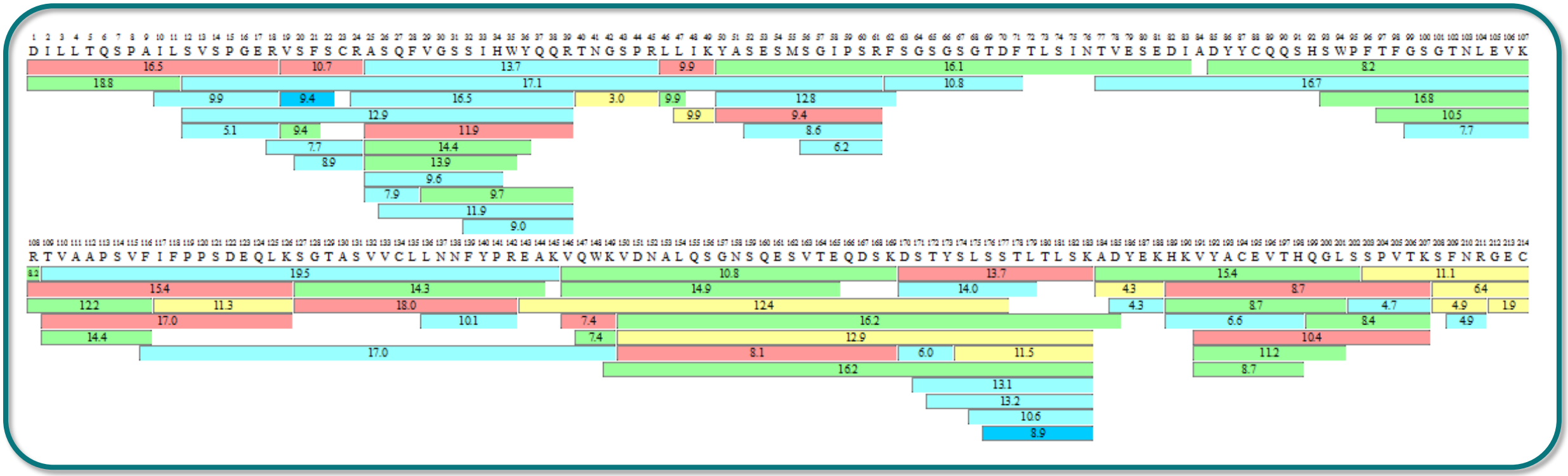
- Collision energy (NCE)

Critical Parameter 1: Mass Range

- Parameter relates to the mass range for monitoring intact precursor ions (m/z)
- Suggested to be set **to 200-2000 m/z** for peptide mapping
- Suggested LC-MS setup is running **without a trap column** and starting the MS method **without a delay time**



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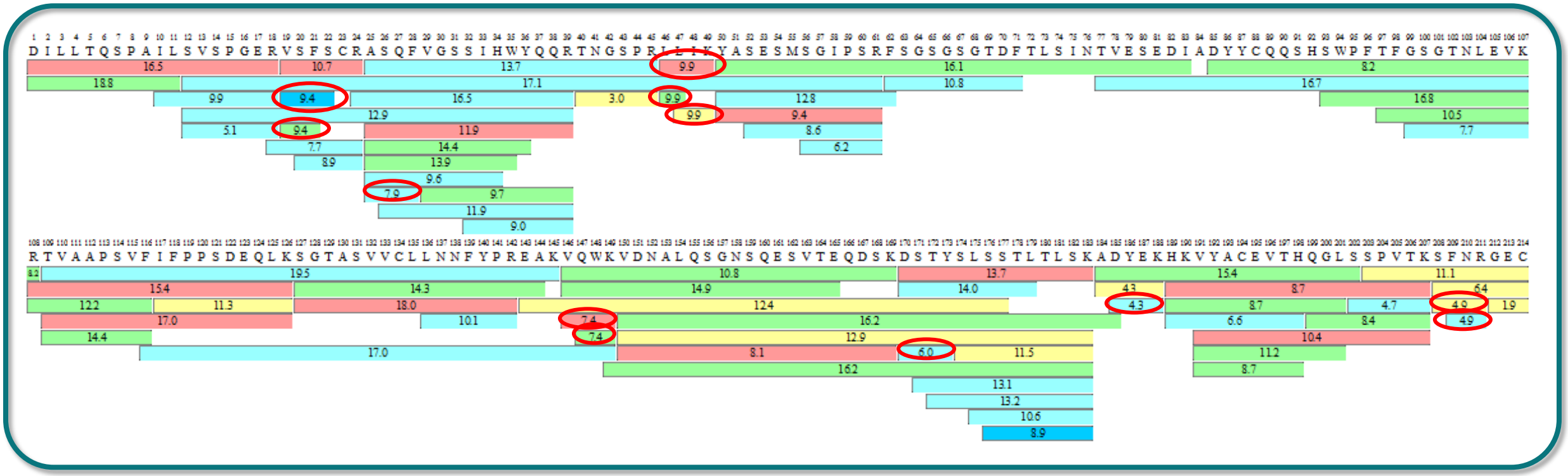

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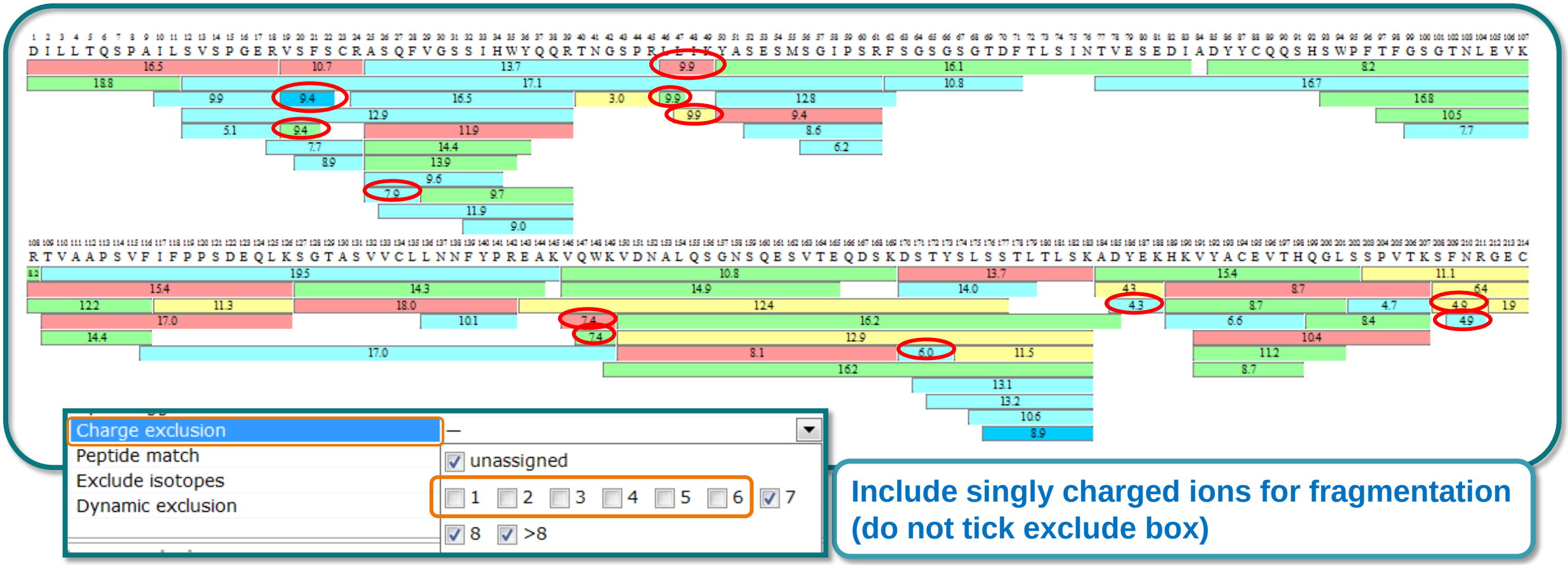

Include singly charged ions for fragmentation (do not tick exclude box)

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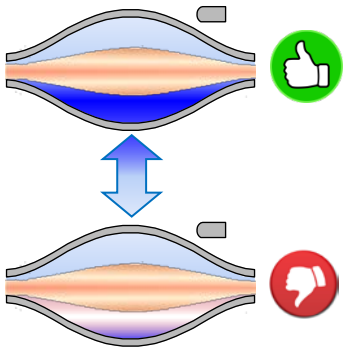
Critical Parameters 2 & 3: AGC Target Setting MS + MS^2 / Maximum Inject Time

AGC target values depend on the particular instrument used:

AGC target	Exactive	Q Exactive	Hybrid OT	OT Fusion/Lumos
Full MS	1-3e6	3e6	3e6	2e5/4e5
MS^2 OT	---	1e5	1e5	5e4/1e5
MS^2 IT	---	---	1e4	1e4/1e4

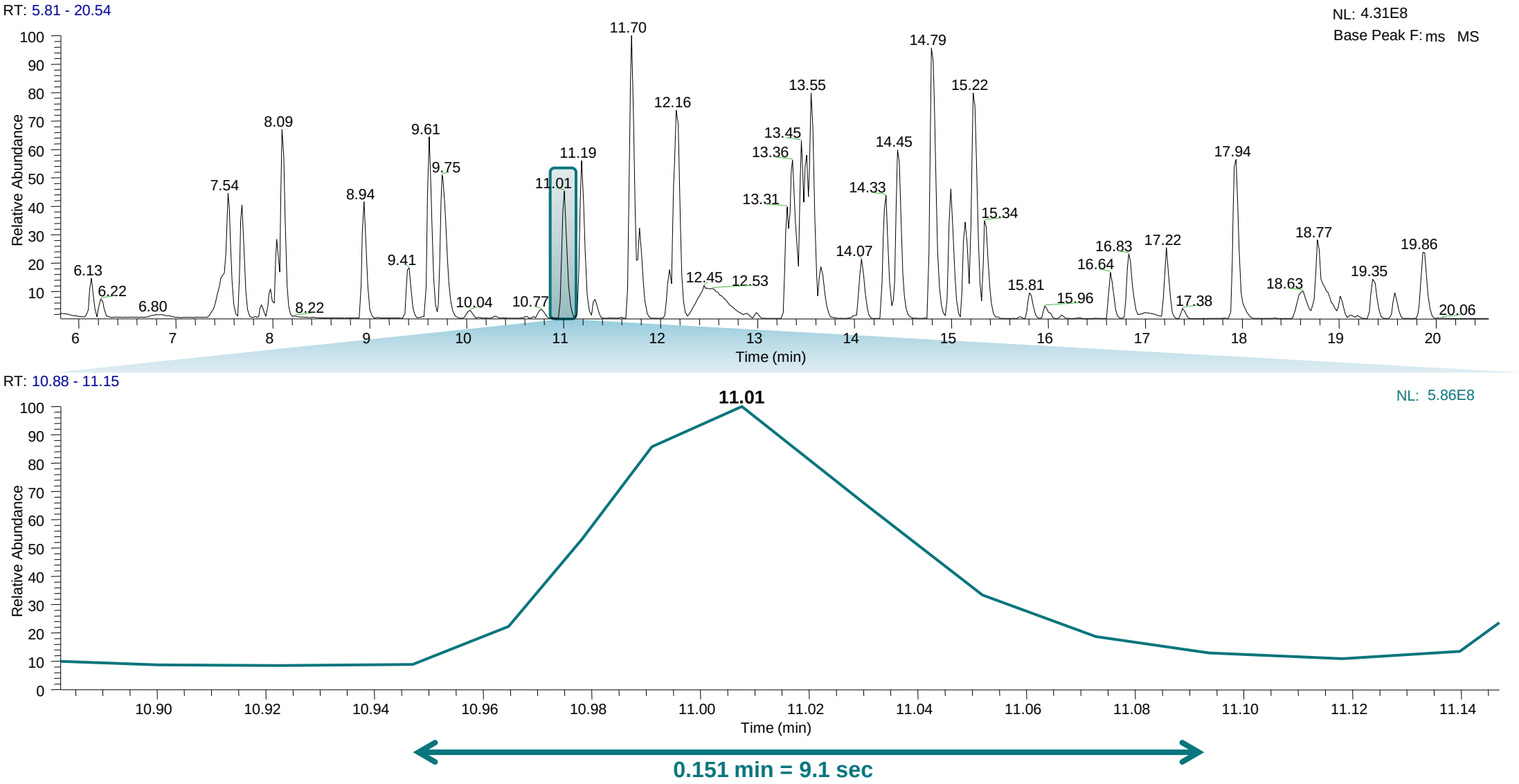


Optimal max. inject times need to be in accordance with:

- TopN set in the method
- Gradient length
- Sample amount injected

Max. Inject time	Exactive	Q Exactive	Hybrid OT	OT Fusion/Lumos
Full MS	50-100 ms	50-100 ms	50-100 ms	50-100 ms
MS^2 OT	---	150-200 ms	150-200 ms	150-200 ms
MS^2 IT	---	---	100-200 ms	100-200 ms

Critical Parameter 4: TopN with N=?



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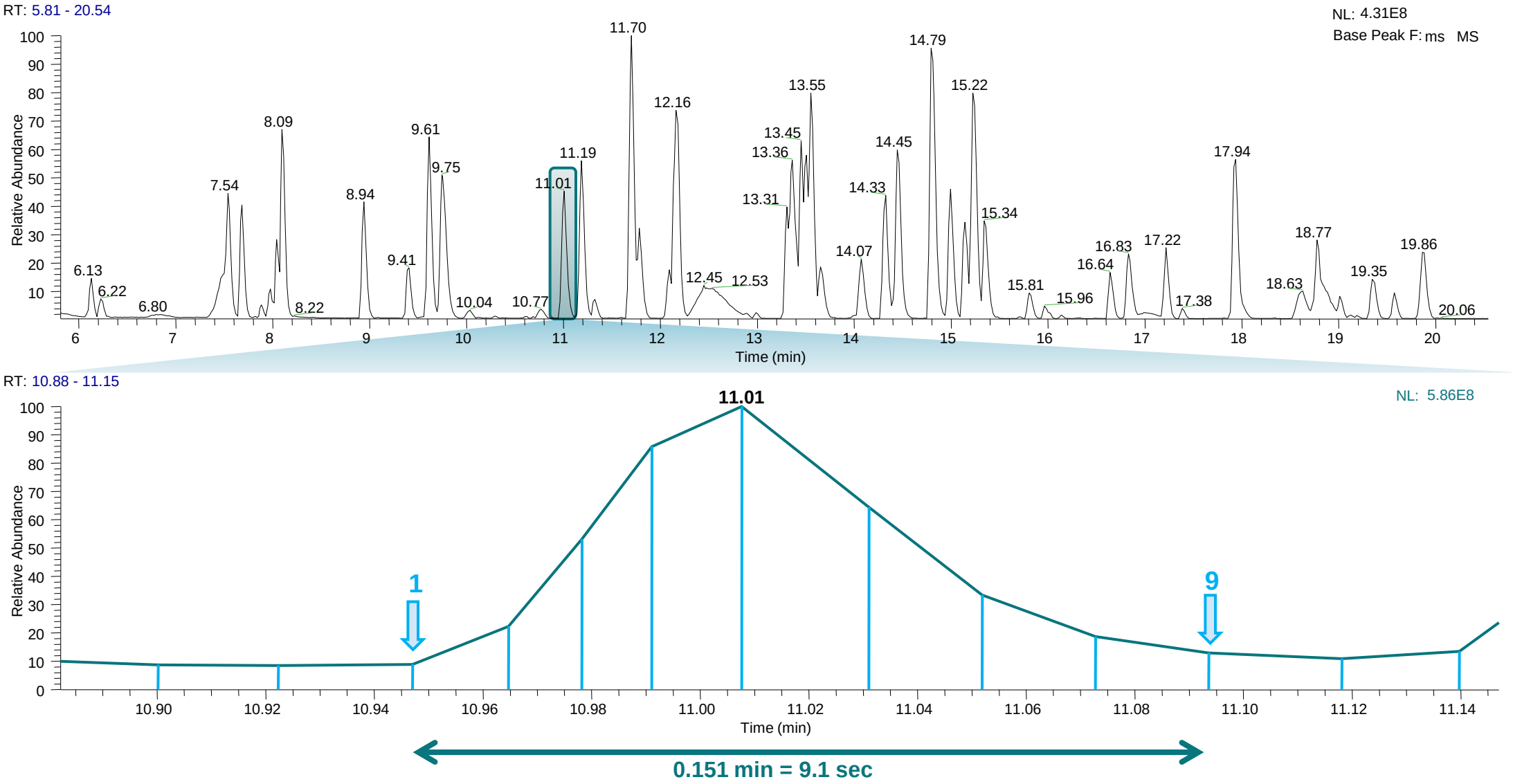
Critical Parameter 4: TopN with N=?



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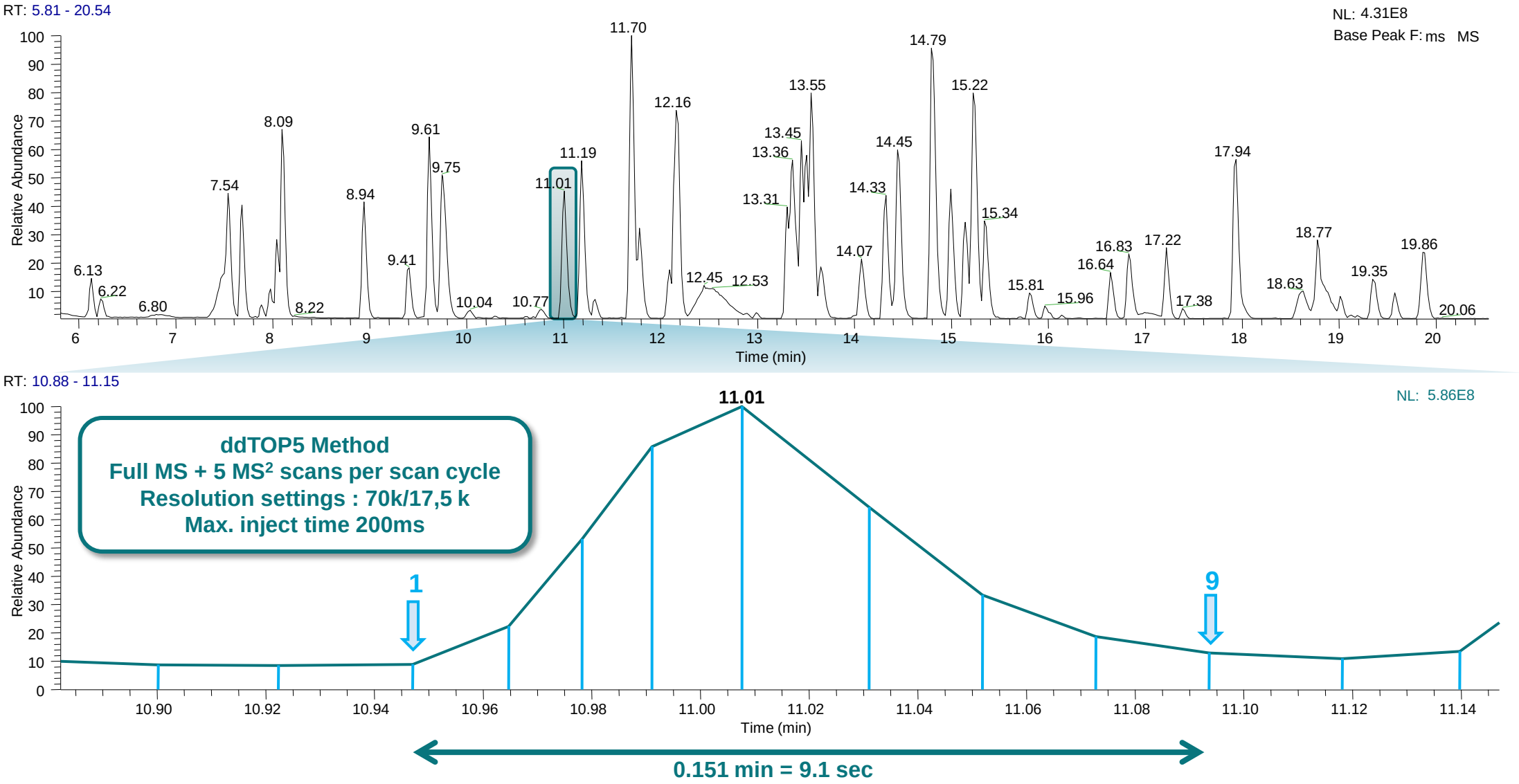
Critical Parameter 4: TopN with N=?



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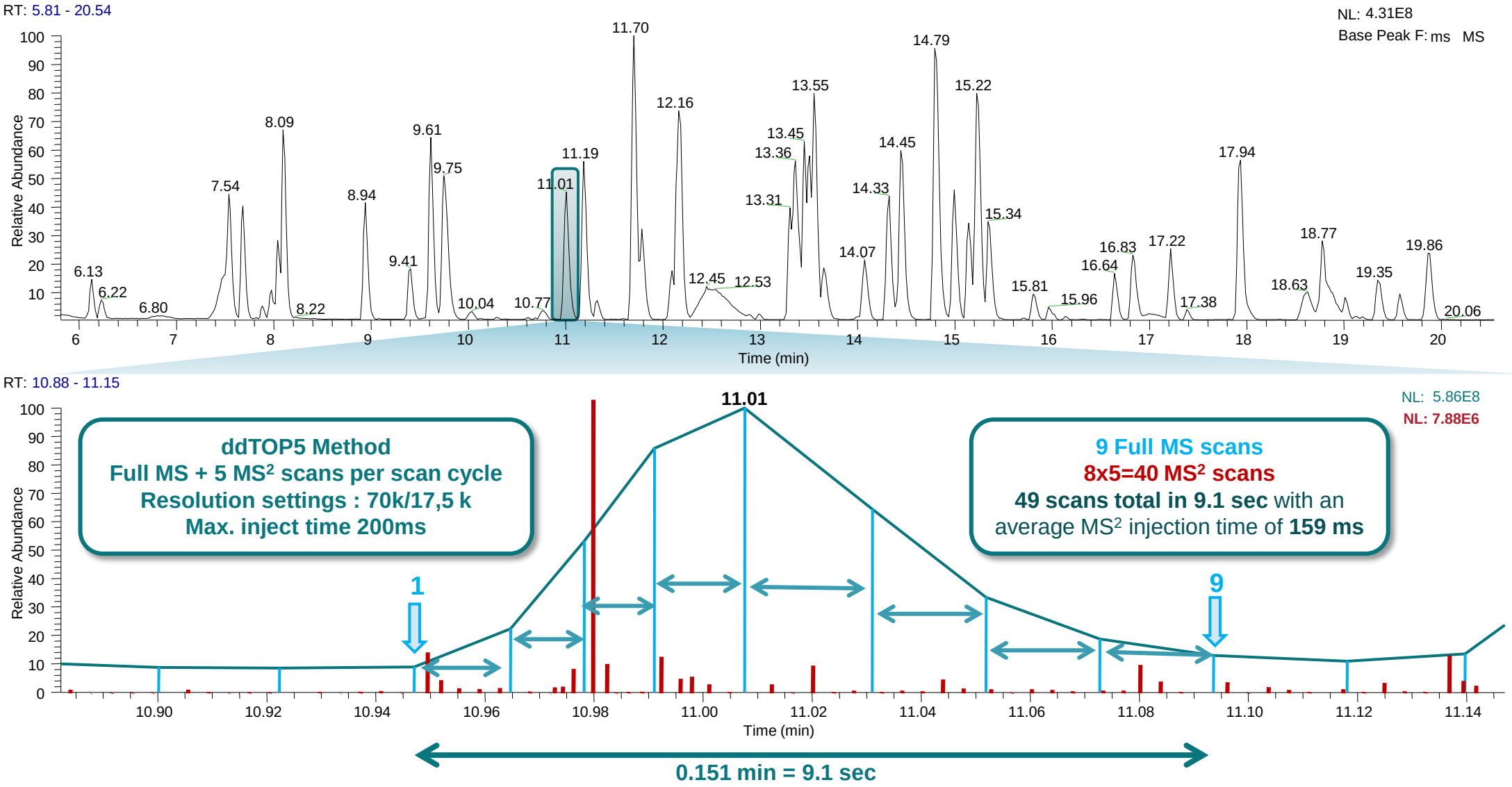
Critical Parameter 4: TopN with N=?



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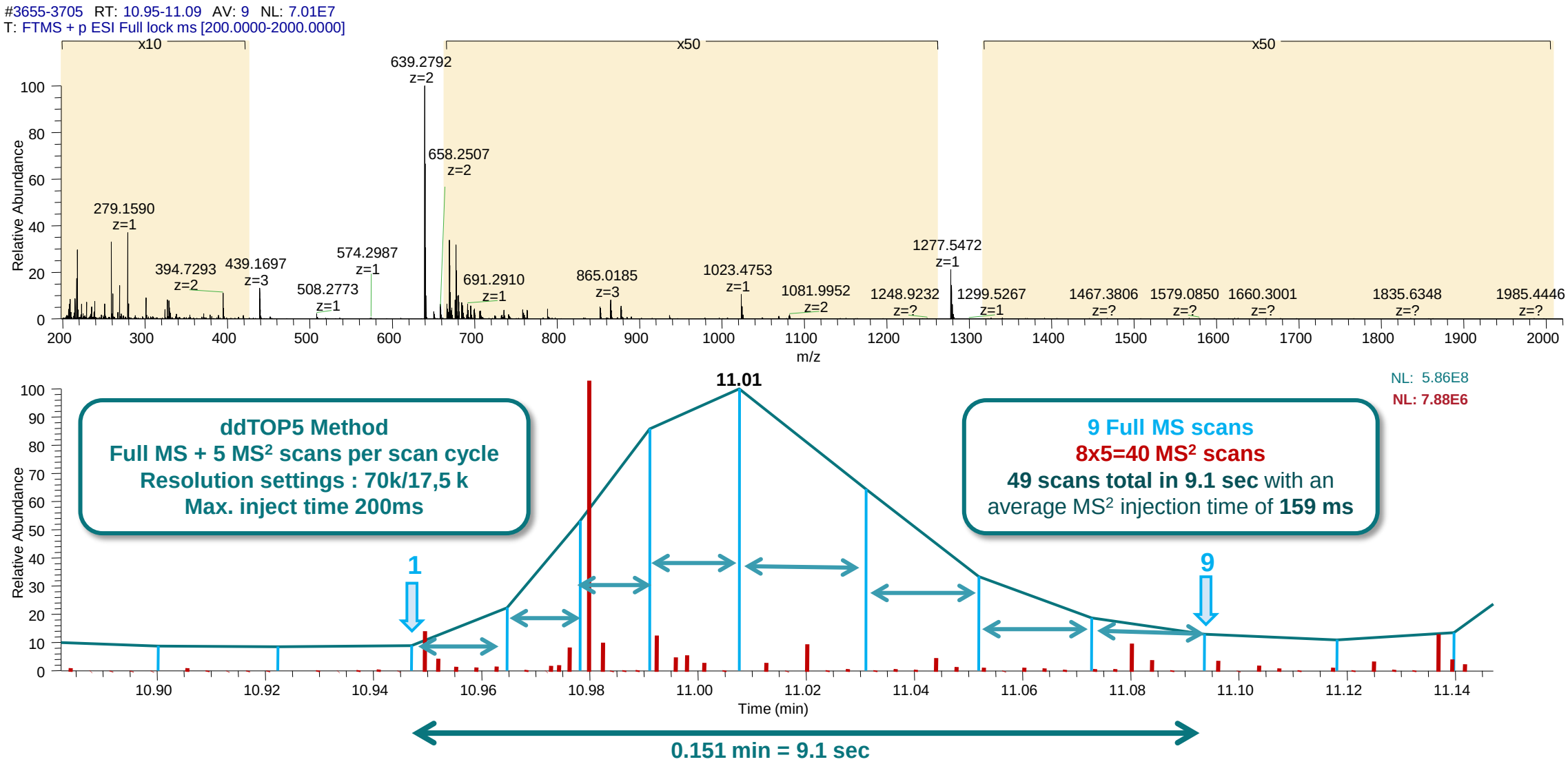

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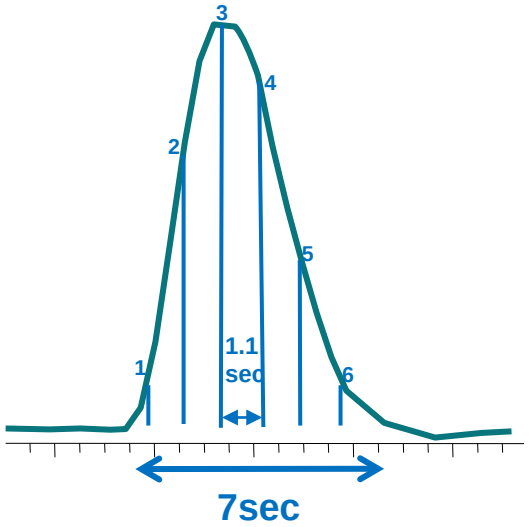
Estimation of Top N

- For the decisions on what N exactly shoud needs to be considered the average chromatographic peak width at the base and calculate the time available for each scan cycle to obtain at least ~6 Full MS scans over the peak.

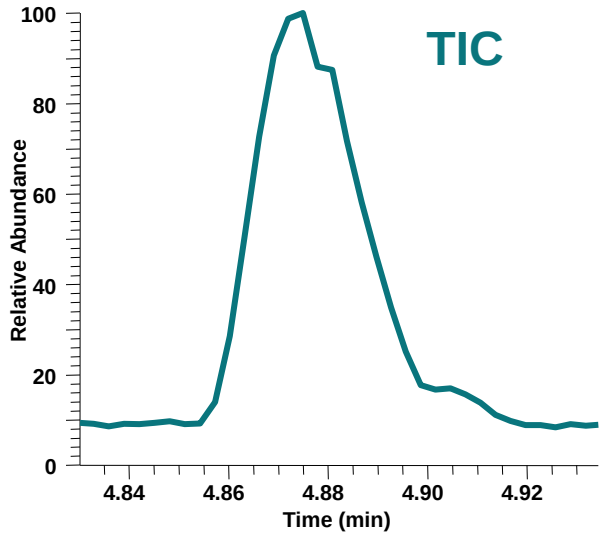
- How many MSMS-scans can fit in every scan cycle to stay within the calculated max. cycle time will depend on the maximum injection time set.

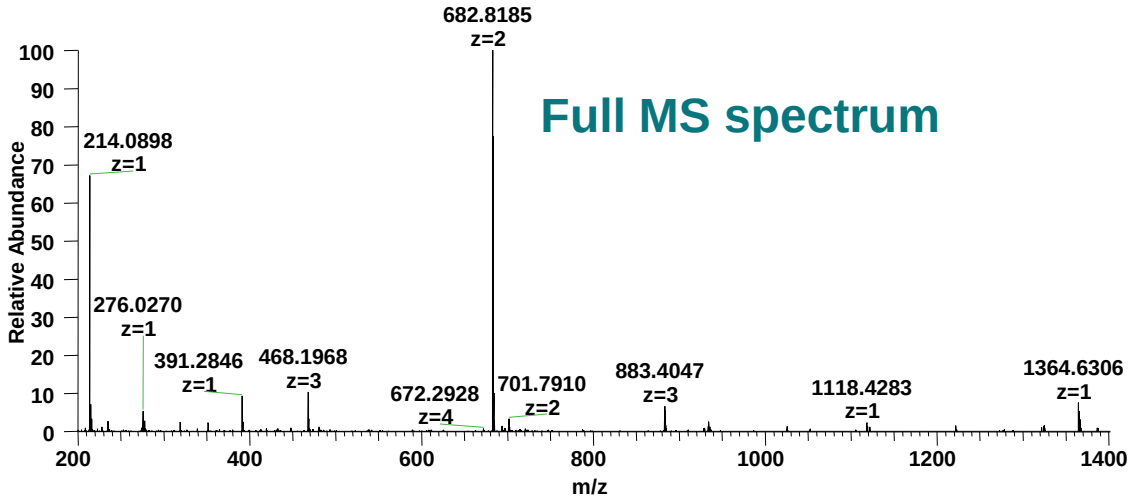
- E.g. if the peak width is 7 secs aiming at 6 full MS scans, the resulting the cycle time should not exceed 1.15 sec, and can fit
  one full MS scan + 5 MSMS scans with 200 ms max. inject time.



A **ddTop5 method** has been proven as a **very good standard method** for peptide mapping on all Q Exative MS instruments and only requires modification if extended max. injection times are required.

Critical Parameter 5: Intensity Threshold





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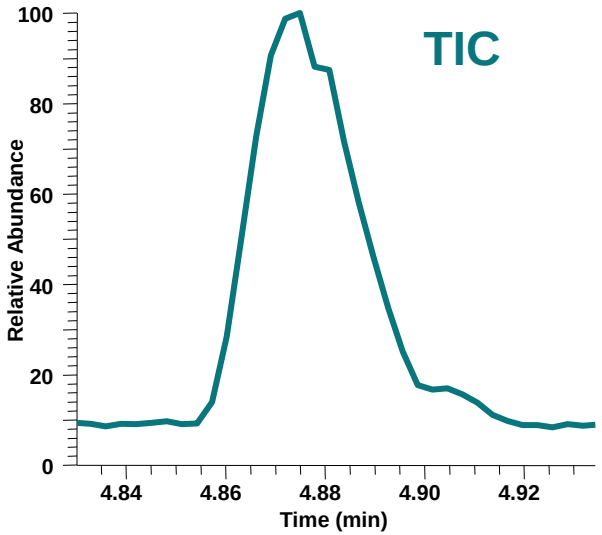

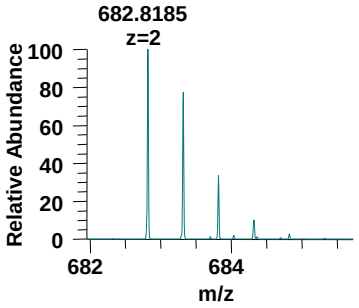

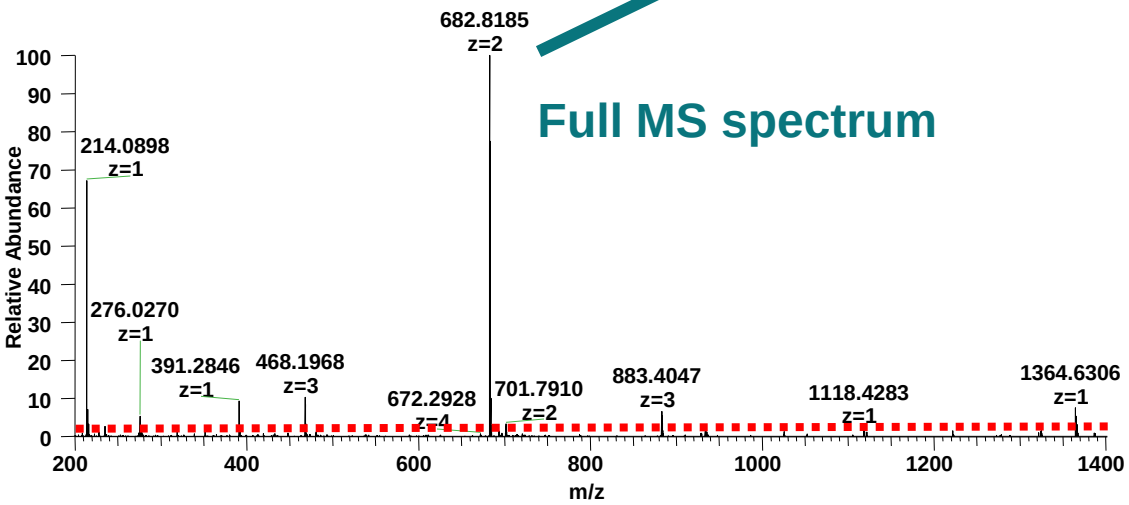

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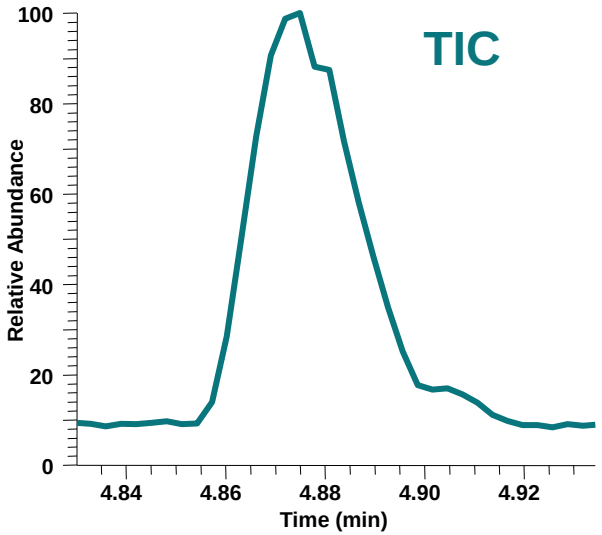

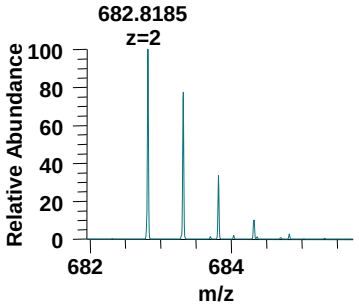

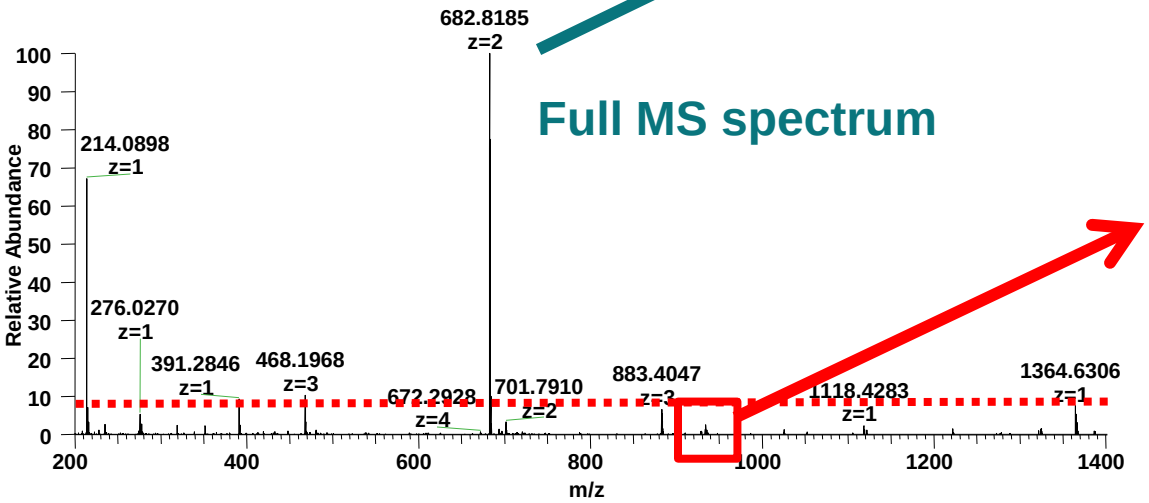

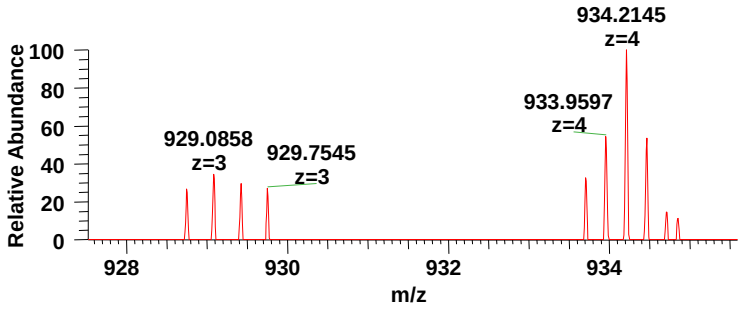

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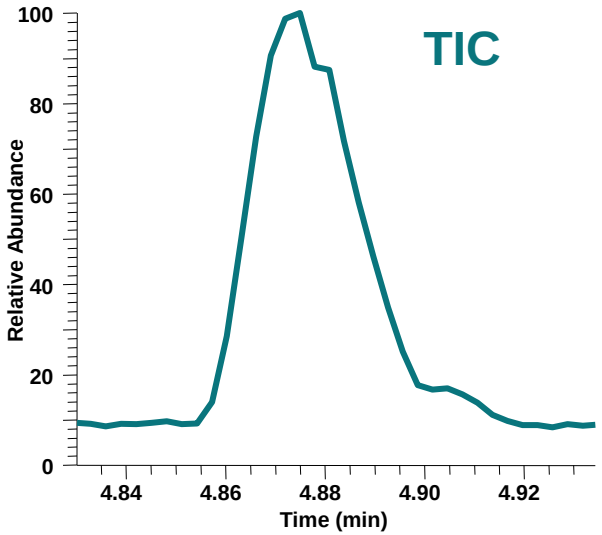
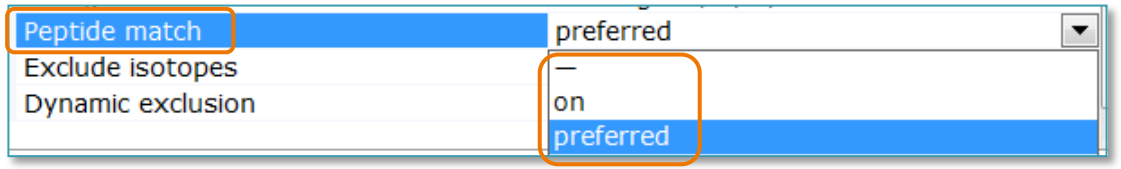
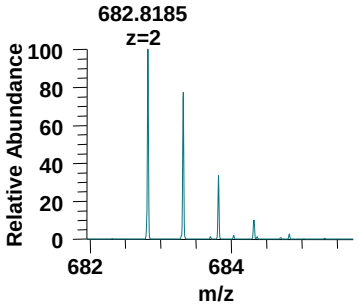
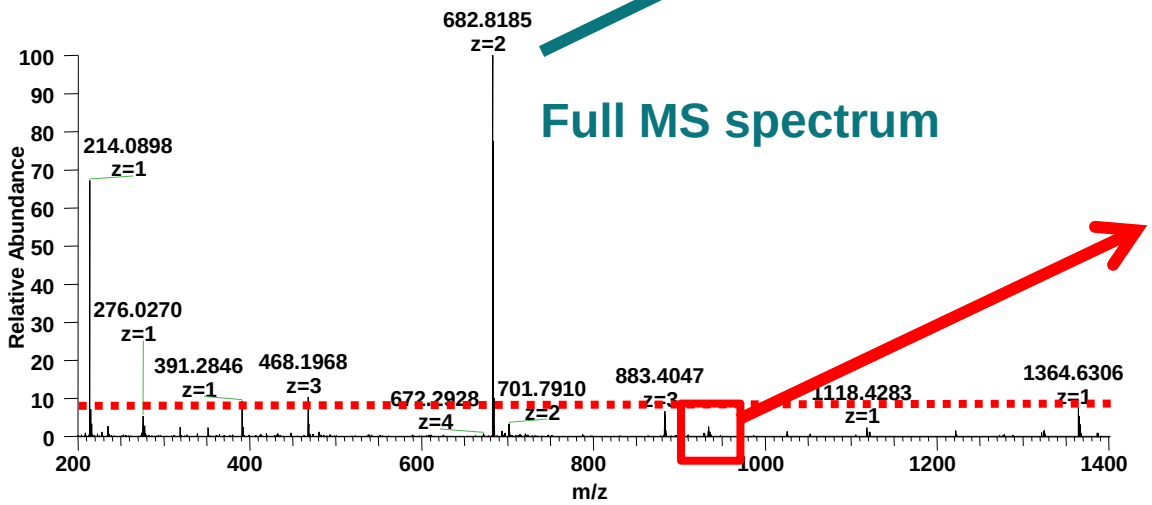


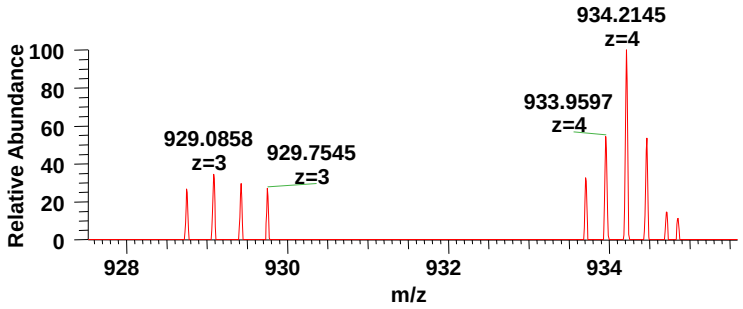
- intensity threshold
- charge state
- peptide match: on/off/preferred
- dynamic exclusion







Full MS spectrum



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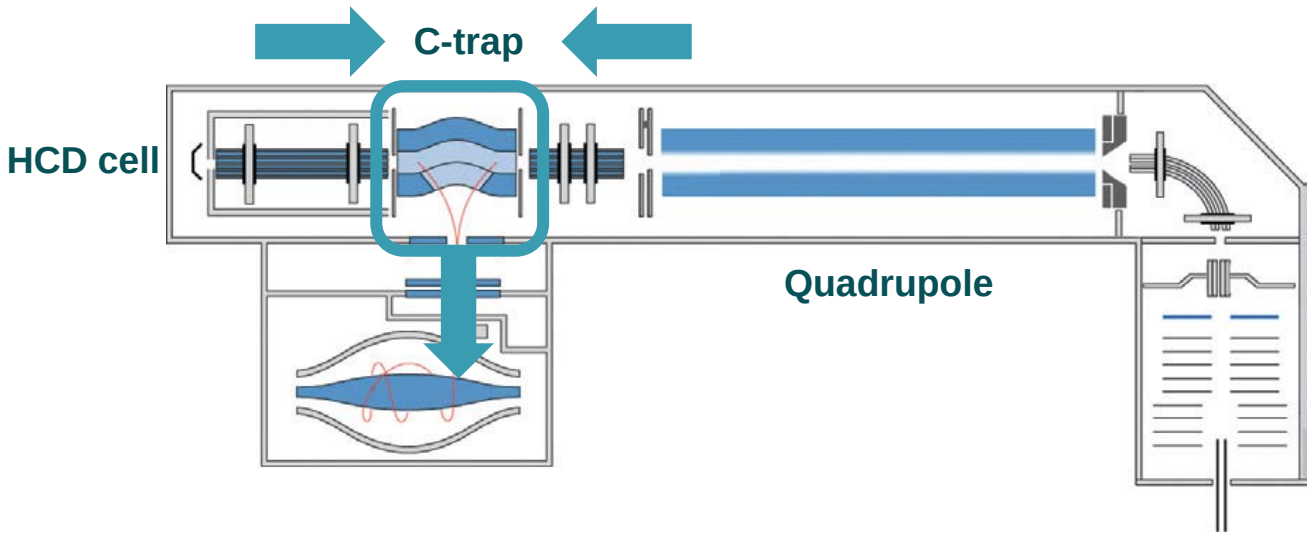
Critical Parameter 6: Fixed First Mass

Properties of Full MS / dd-MS² (TopN)

Default charge state	2
Inclusion	—
Exclusion	—
Tags	—
Full MS	
Resolution	70,000
AGC target	3e6
Maximum IT	100 ms
Scan range	200 to 2000 m/z
dd-MS² / dd-SIM	
Resolution	17,500
AGC target	1e5
Maximum IT	200 ms
Loop count	5
TopN	5
Isolation window	2.0 m/z
Fixed first mass	—
NCE / stepped NCE	28
dd Settings	
Minimum AGC target	2.00e3
Intensity threshold	1.0e4
Apex trigger	—
Charge exclusion	unassigned, 7, 8, >8
Peptide match	preferred
Exclude isotopes	on
Dynamic exclusion	10.0 s



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Default charge state	2
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Exclusion	—
Tags	—
Full MS	
Resolution	70,000
AGC target	3e6
Maximum IT	100 ms
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dd-MS² / dd-SIM	
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AGC target	1e5
Maximum IT	200 ms
Loop count	5
TopN	5
Isolation window	2.0 m/z
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NCE / stepped NCE	28
dd Settings	
Minimum AGC target	2.00e3
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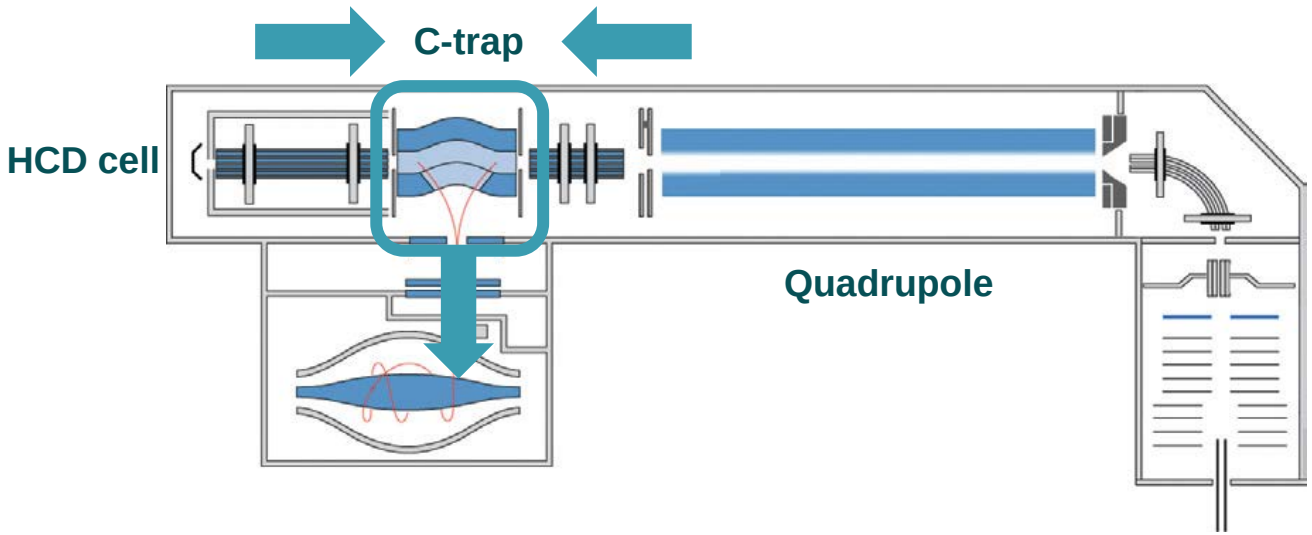
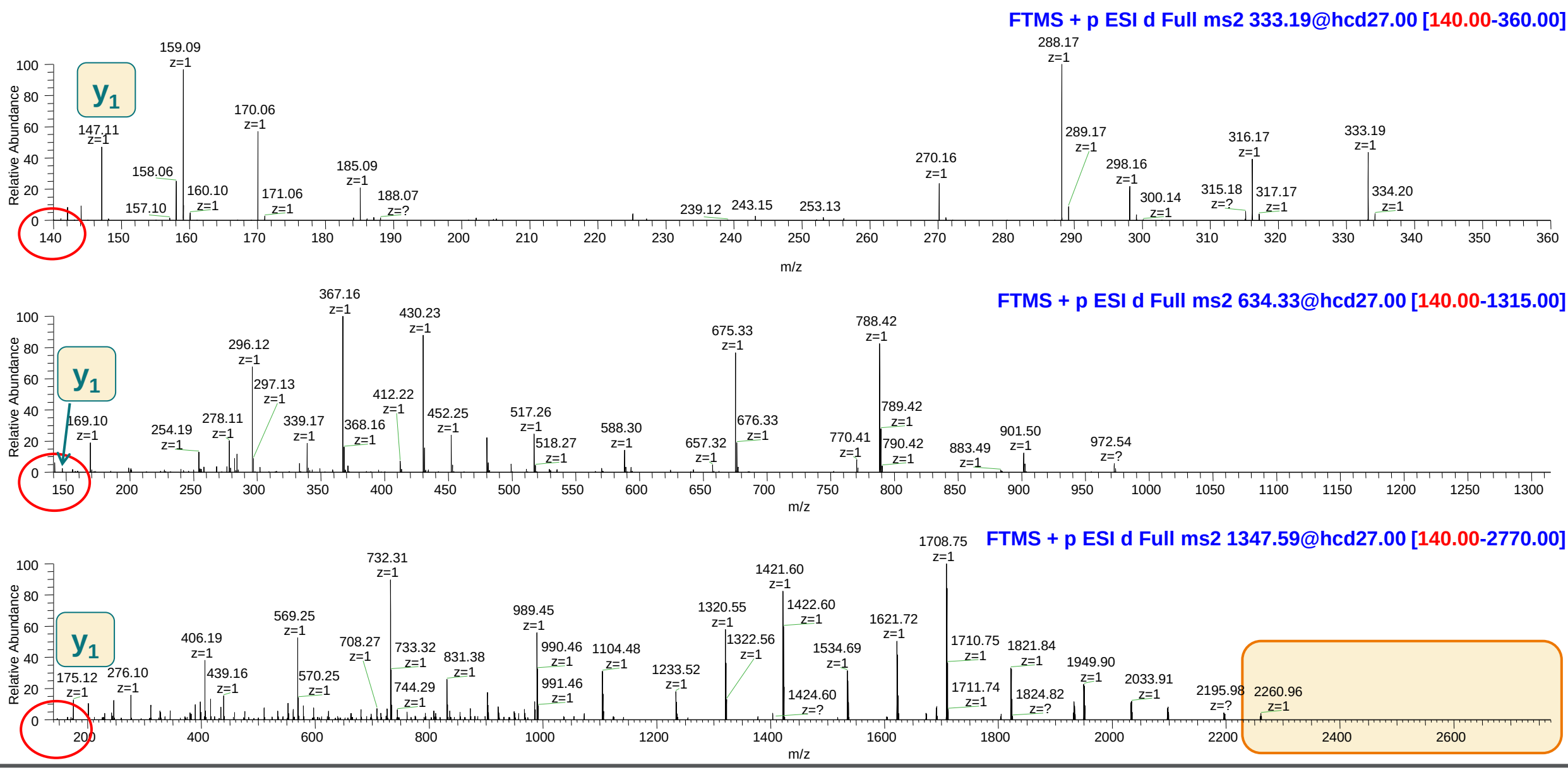


Scan range: last mass ≤ 15 × first mass

- Leaving the first mass open will automatically adjust the mass range based on m/z of the precursor ensuring that also high mass fragments ions are captured.
- Setting a fixed first mass is suggested only when certain low mass ions are desired for monitoring, e.g. 204 (oxonium ions) or for de-novo sequencing aiming e.g. at the Lys/Arg y_1 ions at m/z 147/175.

Critical Parameter 4: Fixed First Mass - Set to 140 m/z



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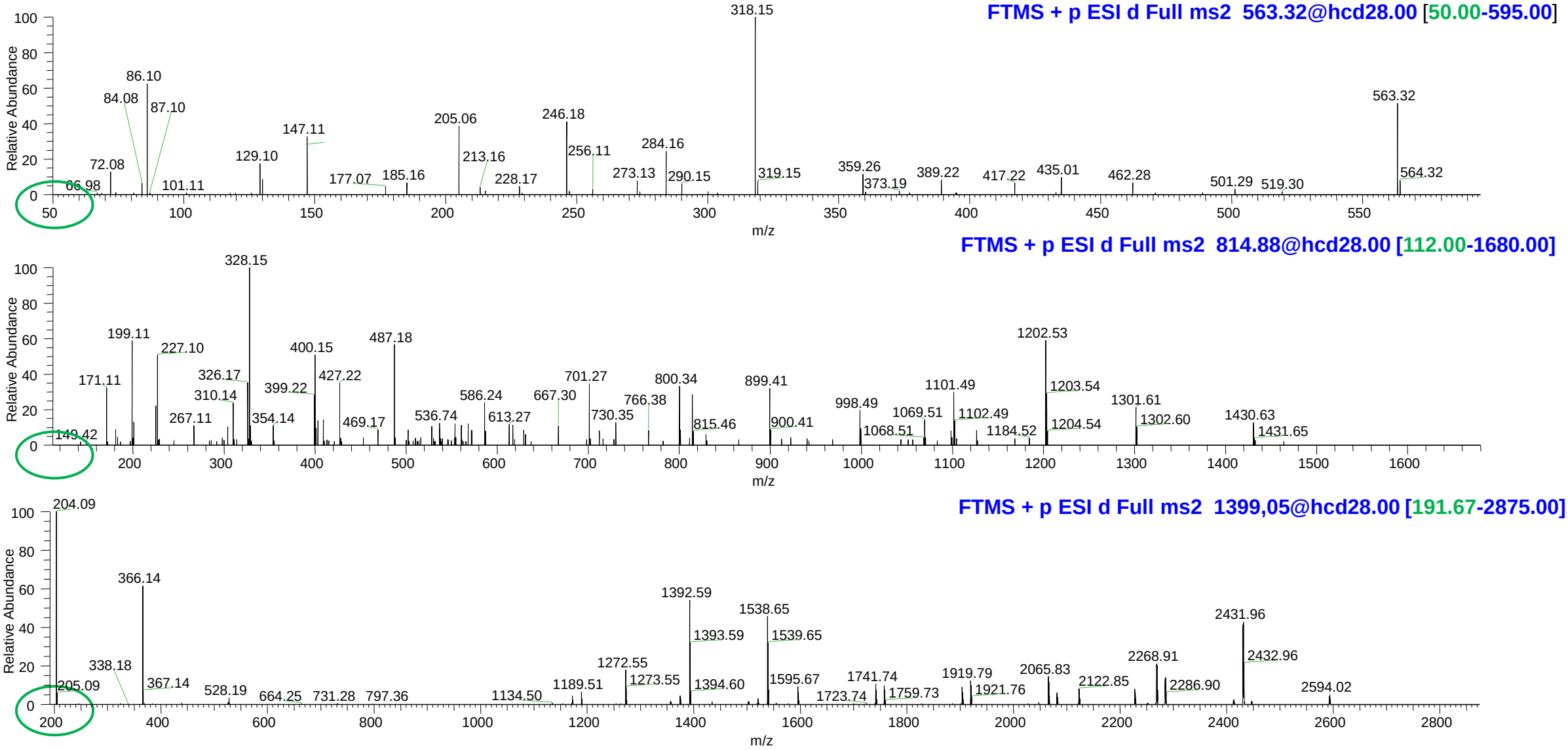
Critical Parameter 4: Fixed First Mass – Set to Auto Adjust



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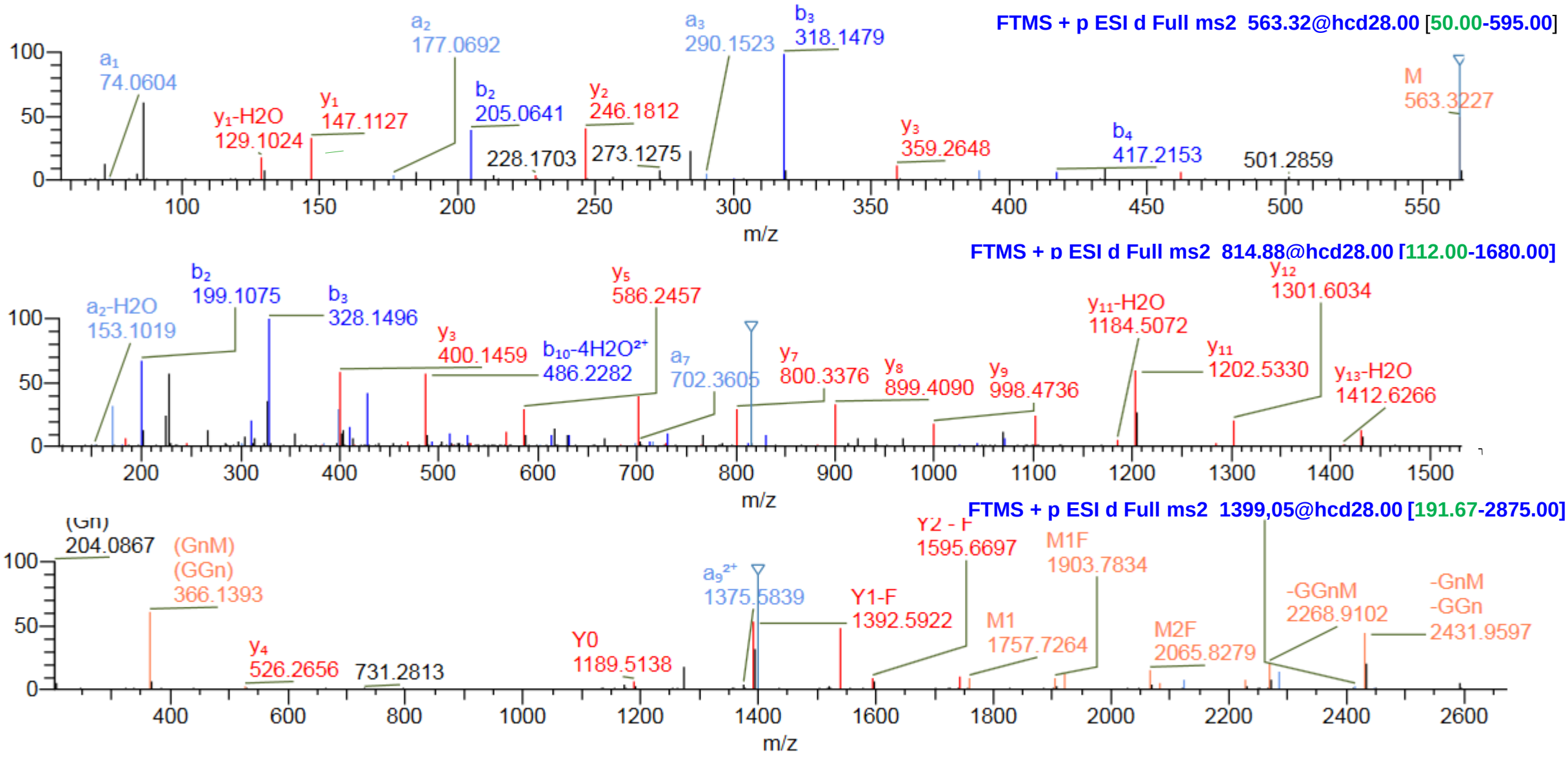
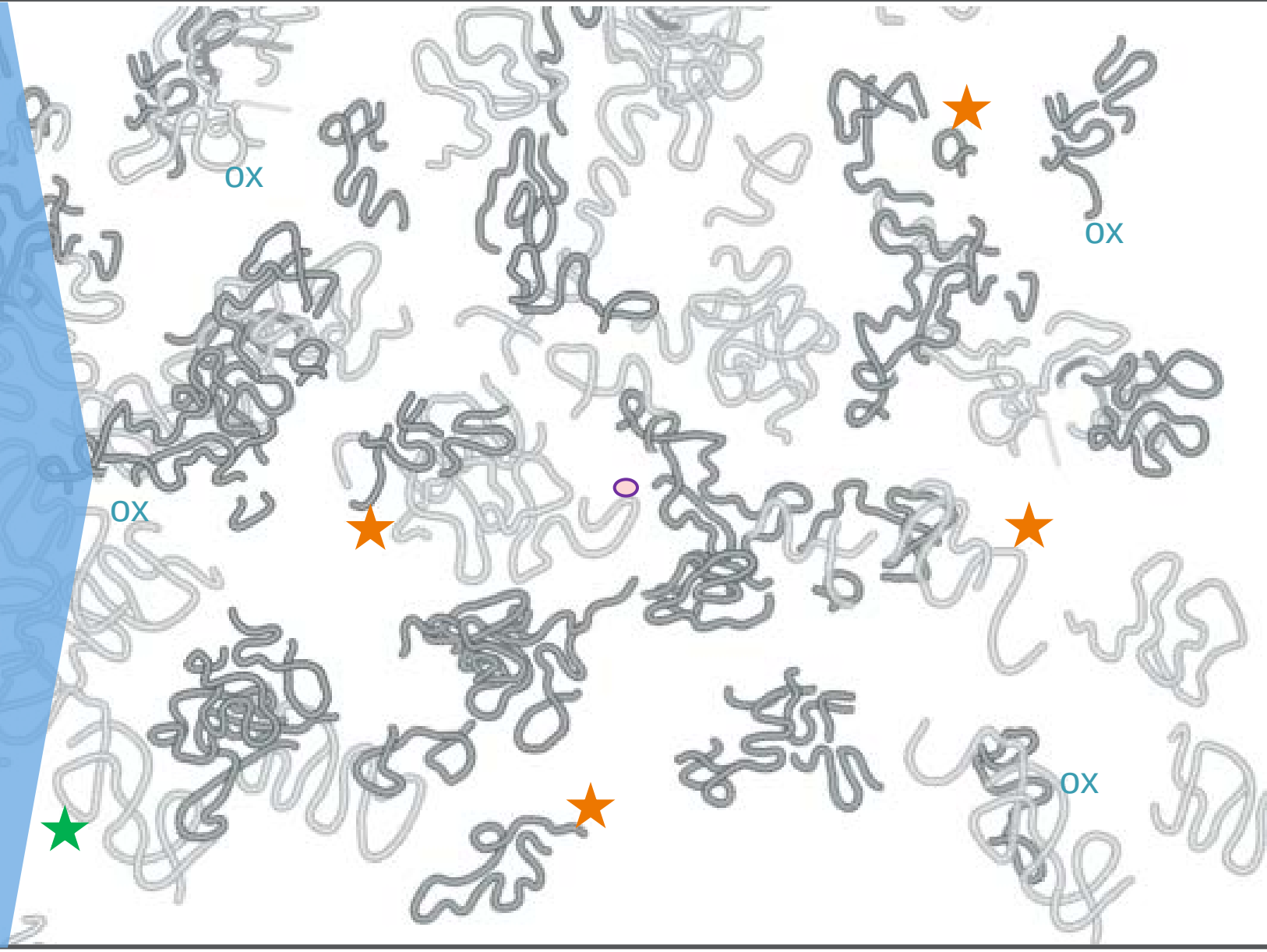
Critical Parameter 4: Fixed First Mass – Set to Auto Adjust



ThermoFisher SCIENTIFIC

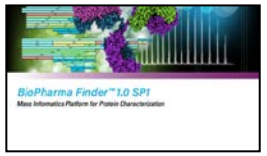
Reviewing Identified Modifications

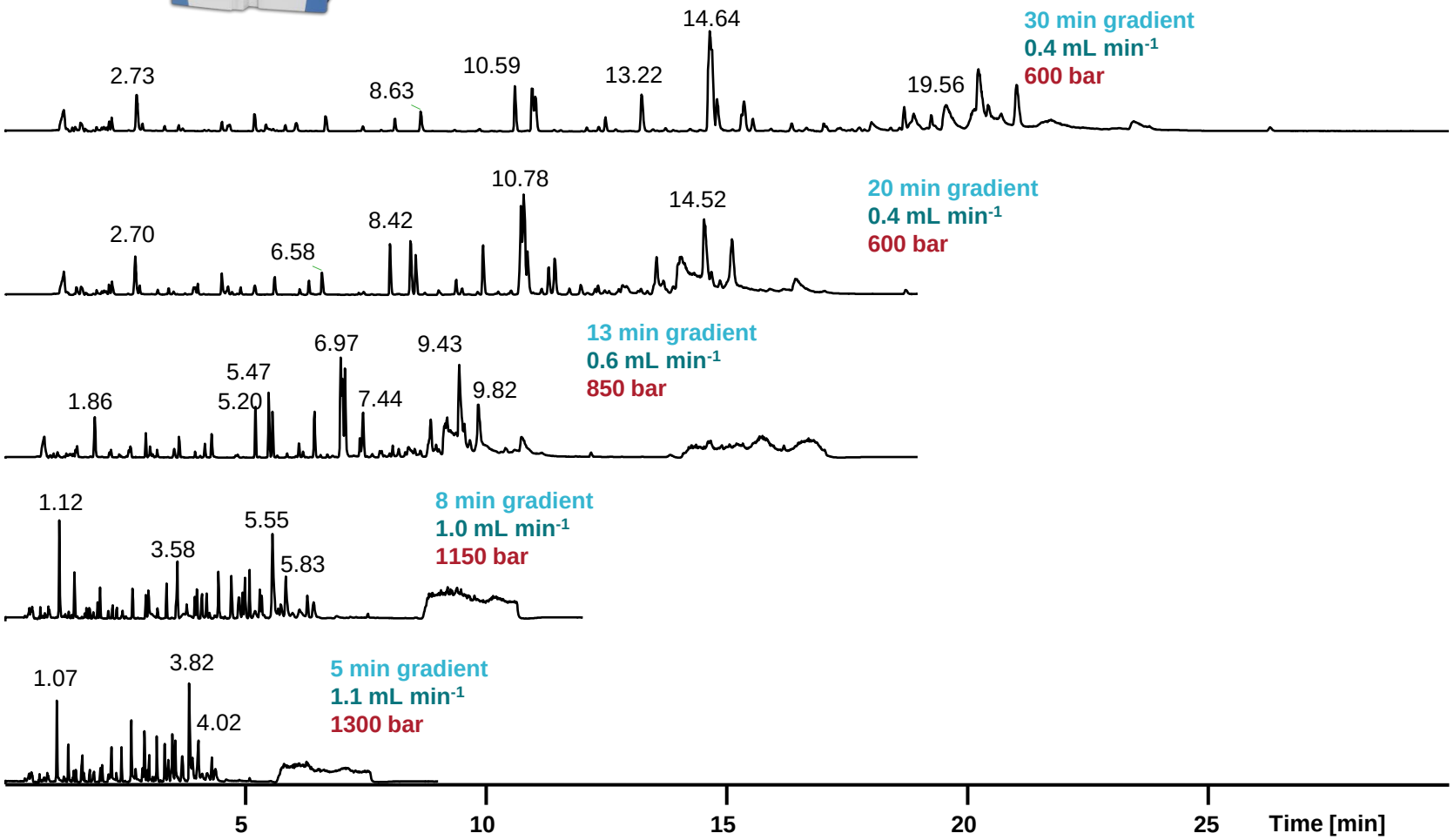


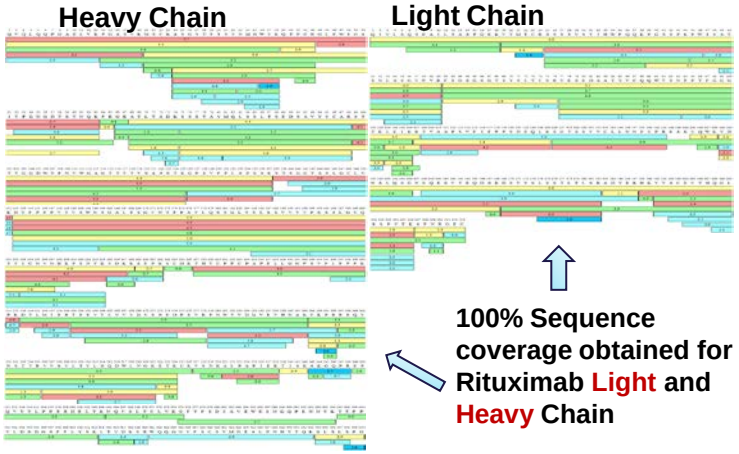
ThermoFisher SCIENTIFIC

Peptide Mapping with Short and Long Separation Times

Thermo Scientific™ Vanquish™ UHPLC Platform coupled to a Thermo Scientific™ Q Exactive™ HF MS







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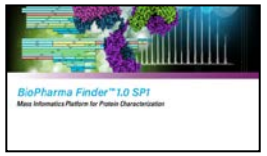

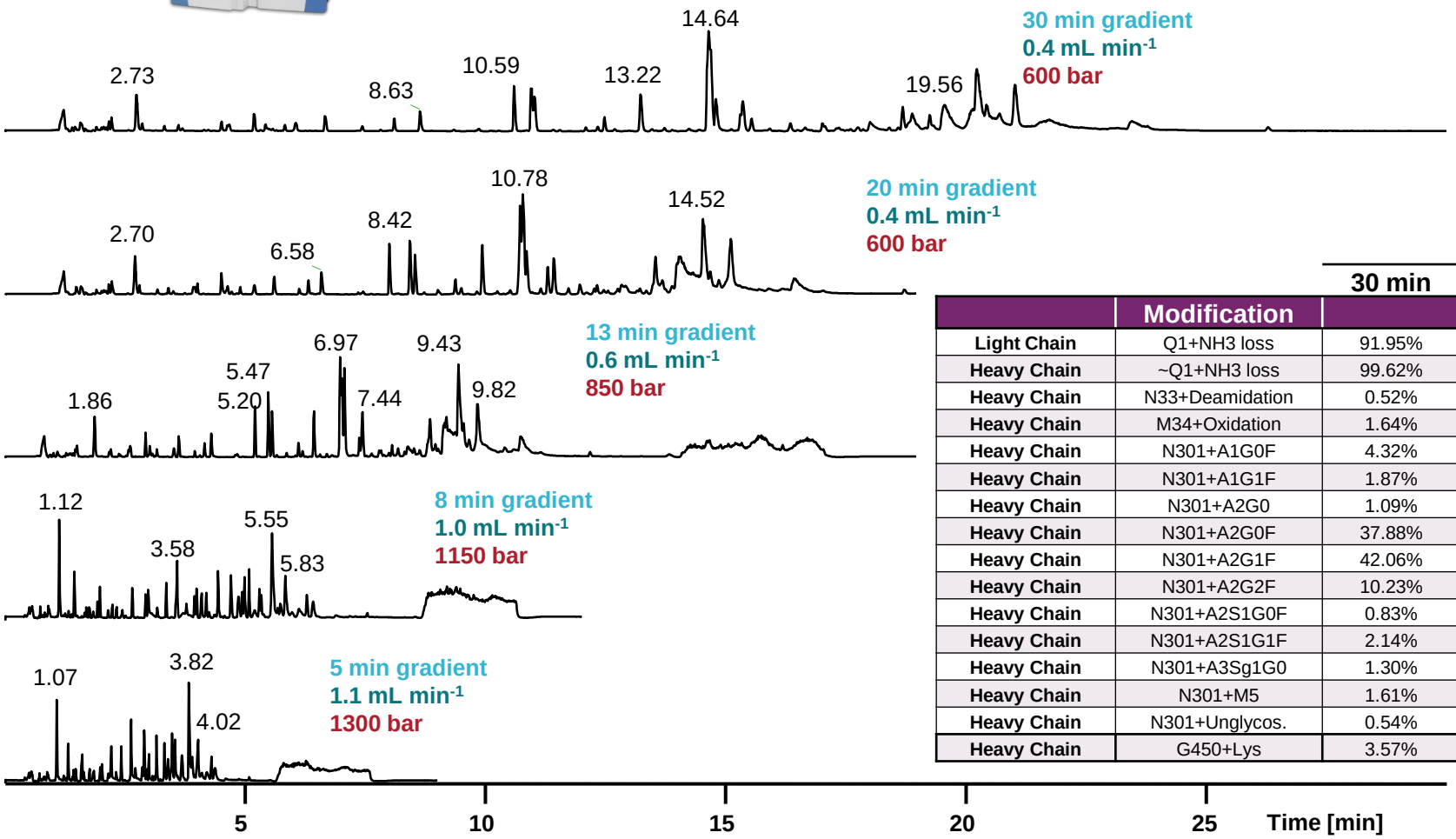

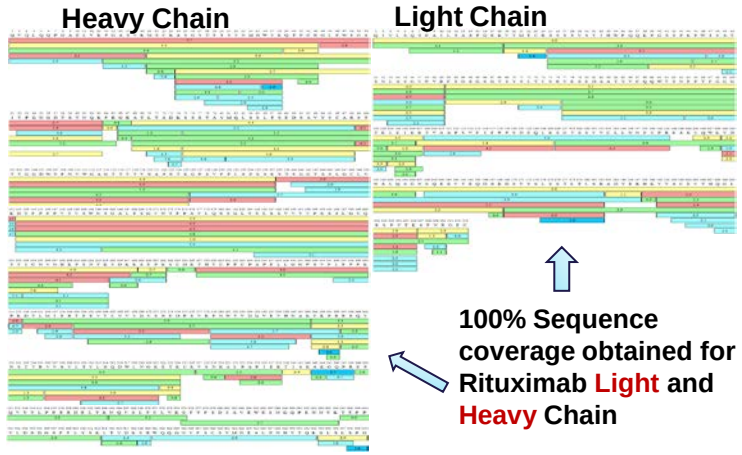

		Gradient Time					
		30 min	20 min	13 min	8 min	5 min	
	Modification	Abundance					σ
Light Chain	Q1+NH3 loss	91.95%	91.17%	89.69%	90.93%	26.57%	28.80%
Heavy Chain	~Q1+NH3 loss	99.62%	99.67%	99.61%	99.68%	99.69%	0.04%
Heavy Chain	N33+Deamidation	0.52%	0.51%	0.58%	-	0.51%	0.03%
Heavy Chain	M34+Oxidation	1.64%	1.54%	1.73%	1.42%	1.45%	0.13%
Heavy Chain	N301+A1G0F	4.32%	4.42%	3.83%	3.52%	3.38%	0.46%
Heavy Chain	N301+A1G1F	1.87%	1.91%	1.72%	3.32%	1.46%	0.73%
Heavy Chain	N301+A2G0	1.09%	1.02%	1.02%	-	0.98%	0.05%
Heavy Chain	N301+A2G0F	37.88%	37.11%	38.59%	40.48%	43.12%	2.41%
Heavy Chain	N301+A2G1F	42.06%	41.89%	43.42%	43.20%	43.35%	0.75%
Heavy Chain	N301+A2G2F	10.23%	10.17%	9.81%	10.36%	10.05%	0.21%
Heavy Chain	N301+A2S1G0F	0.83%	0.86%	-	-	-	0.02%
Heavy Chain	N301+A2S1G1F	2.14%	-	-	-	-	-
Heavy Chain	N301+A3Sg1G0	1.30%	-	-	-	-	-
Heavy Chain	N301+M5	1.61%	1.59%	1.66%	1.87%	1.86%	0.14%
Heavy Chain	N301+Unglycos.	0.54%	0.90%	0.76%	0.83%	0.97%	0.16%
Heavy Chain	G450+Lys	3.57%	3.56%	3.92%	3.40%	3.15%	0.28%
						median	0.19%

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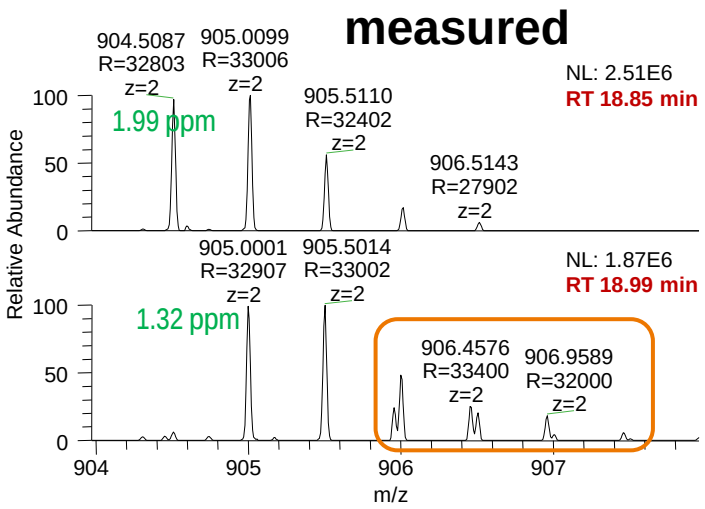
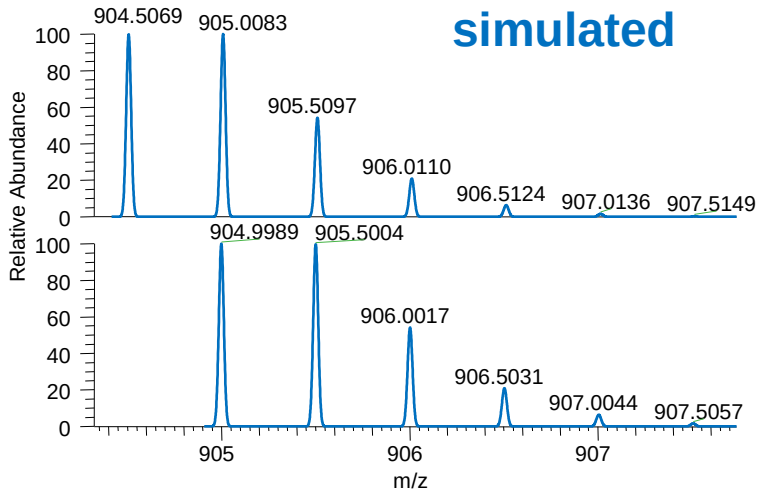
Identified Deamidation of Peptide VVSVLTVHQDWLN*GK

Intact peptide precursor





NL:
8.26E3

$c_{83}h_{134}n_{22}o_{23}$ +H:
$C_{83}H_{136}N_{22}O_{23}$
p (gss, s /p:40) Chrg 2
R: 30000 Res .Pwr . @FWHM

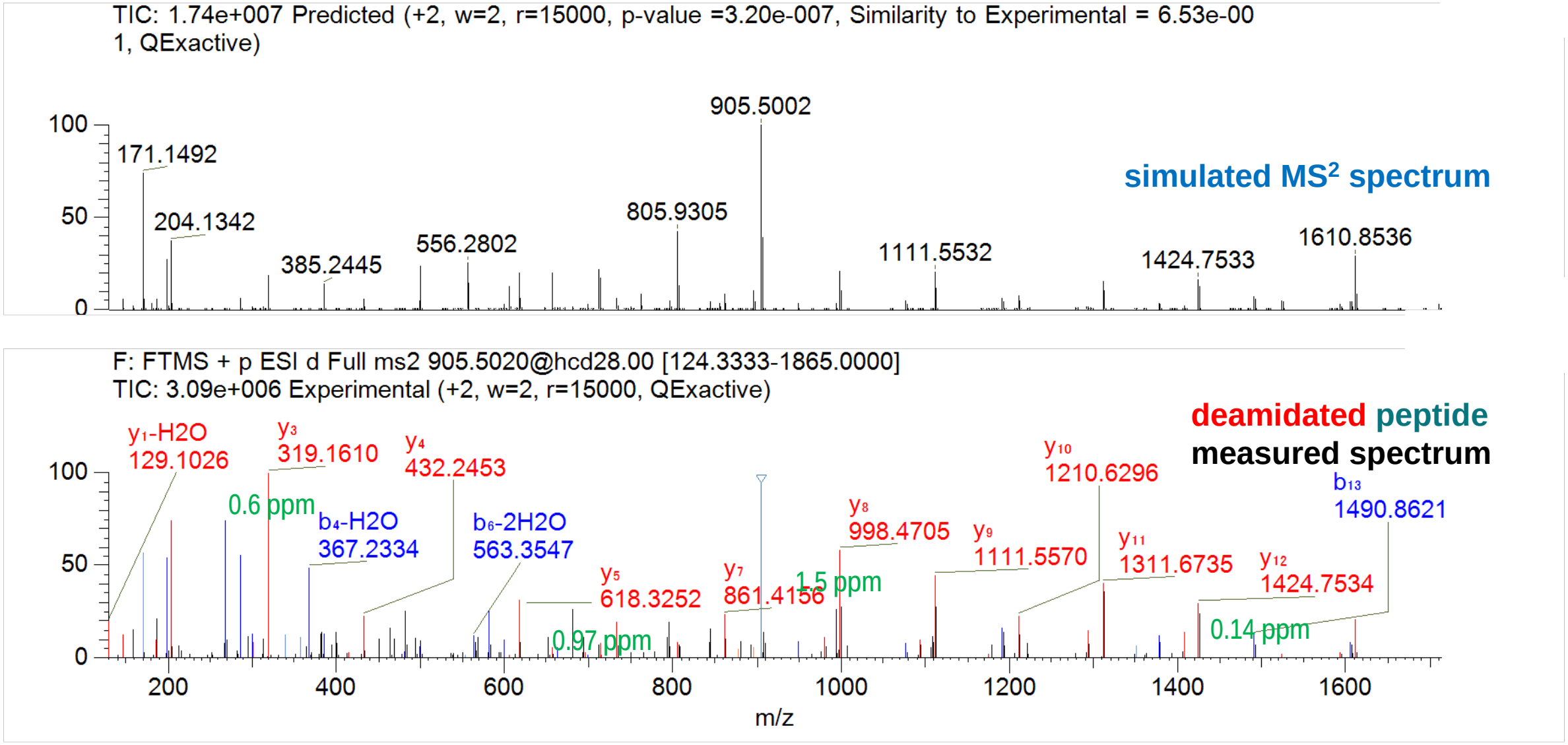
unmodified peptide

NL:
8.26E3

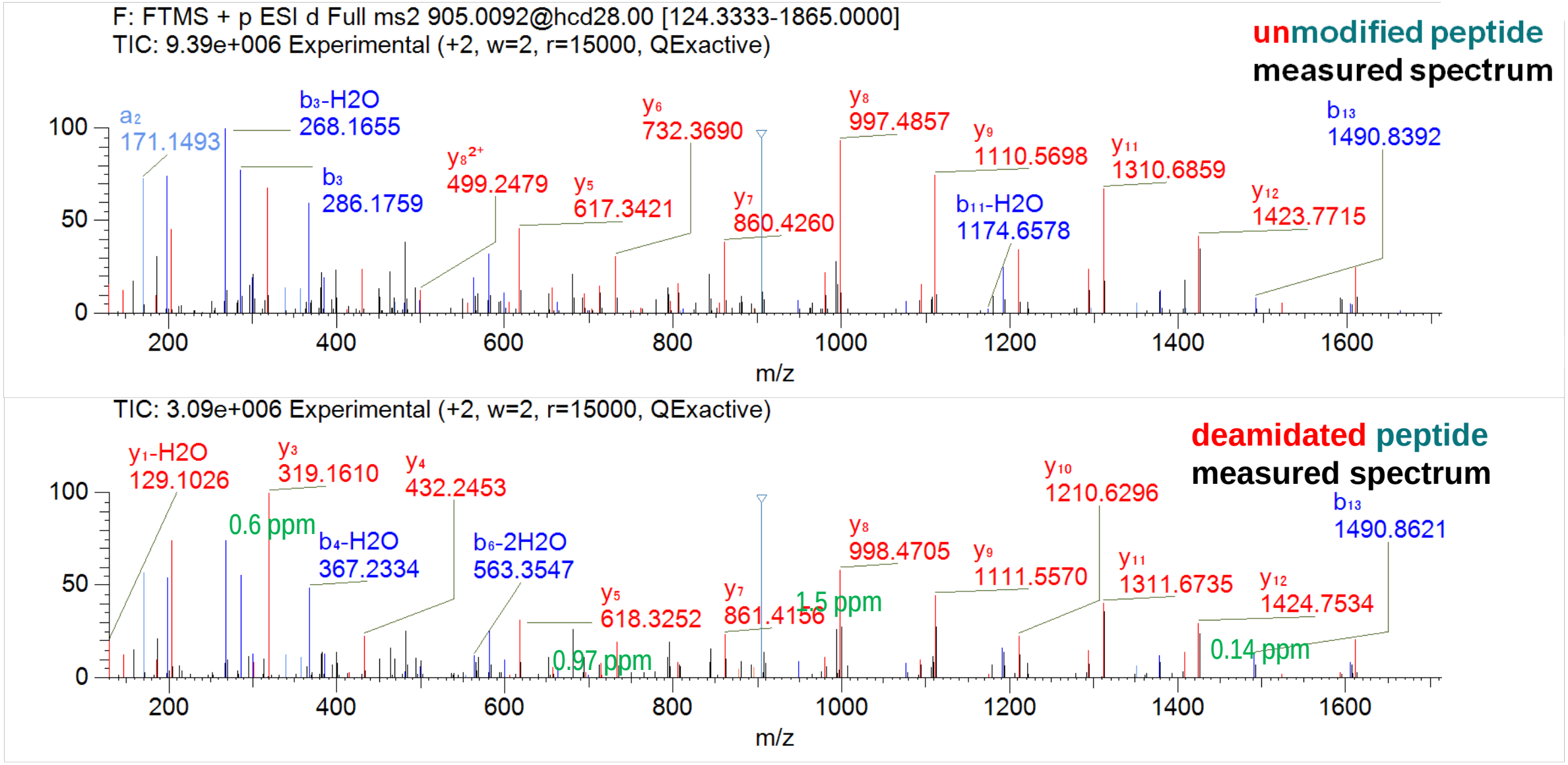
$c_{83}h_{133}n_{21}o_{24}$ +H:
$C_{83}H_{135}N_{21}O_{24}$
p (gss, s /p:40) Chrg 2
R: 30000 Res .Pwr . @FWHM

deamidated peptide

Identified Deamidation of Peptide VVSVLTVHQDWLN*GK

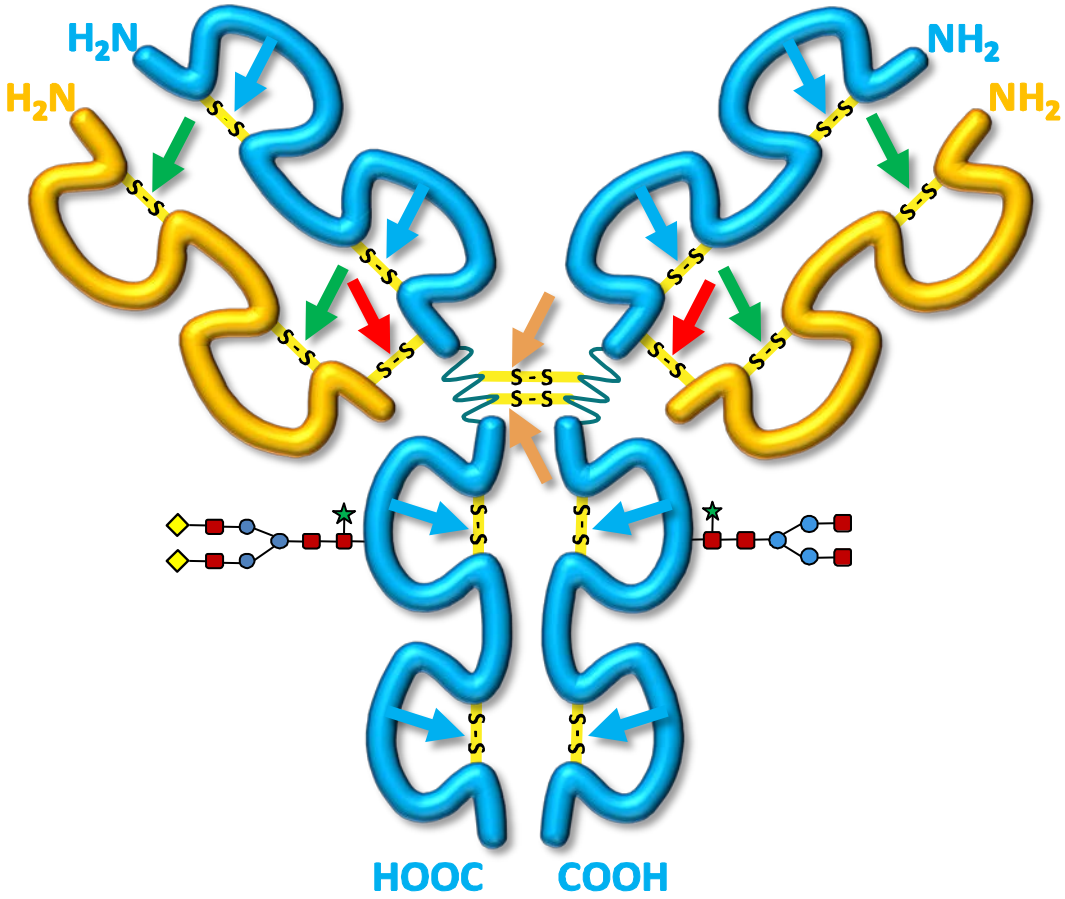


Identified Deamidation of Peptide VVSVLTVHQDWLN*GK



ThermoFisher SCIENTIFIC

Disulfide Bridge Analysis



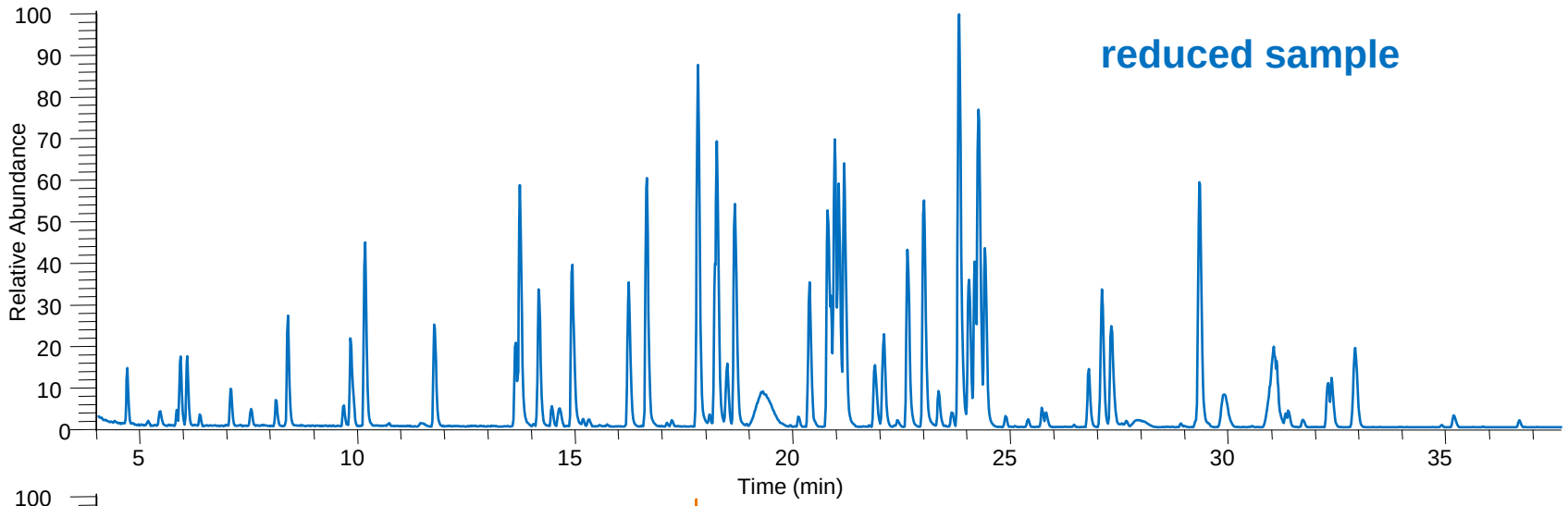
ThermoFisher SCIENTIFIC

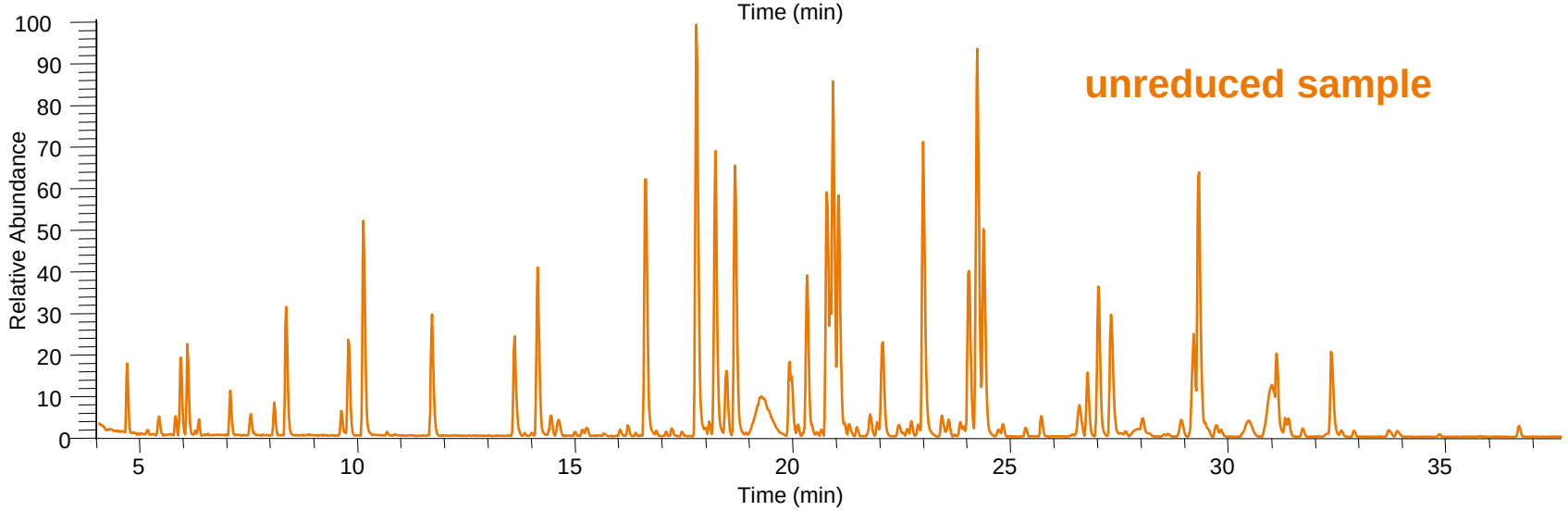
Sample Analysis by Mass Spectrometry

Q Exactive Plus

Analysis of **reduced (+alkylated)** sample

Analysis of **unreduced** sample





Method used for both samples:
data-dependent Top5 Method:
Full MS + top5 MS/MS scans per cycle

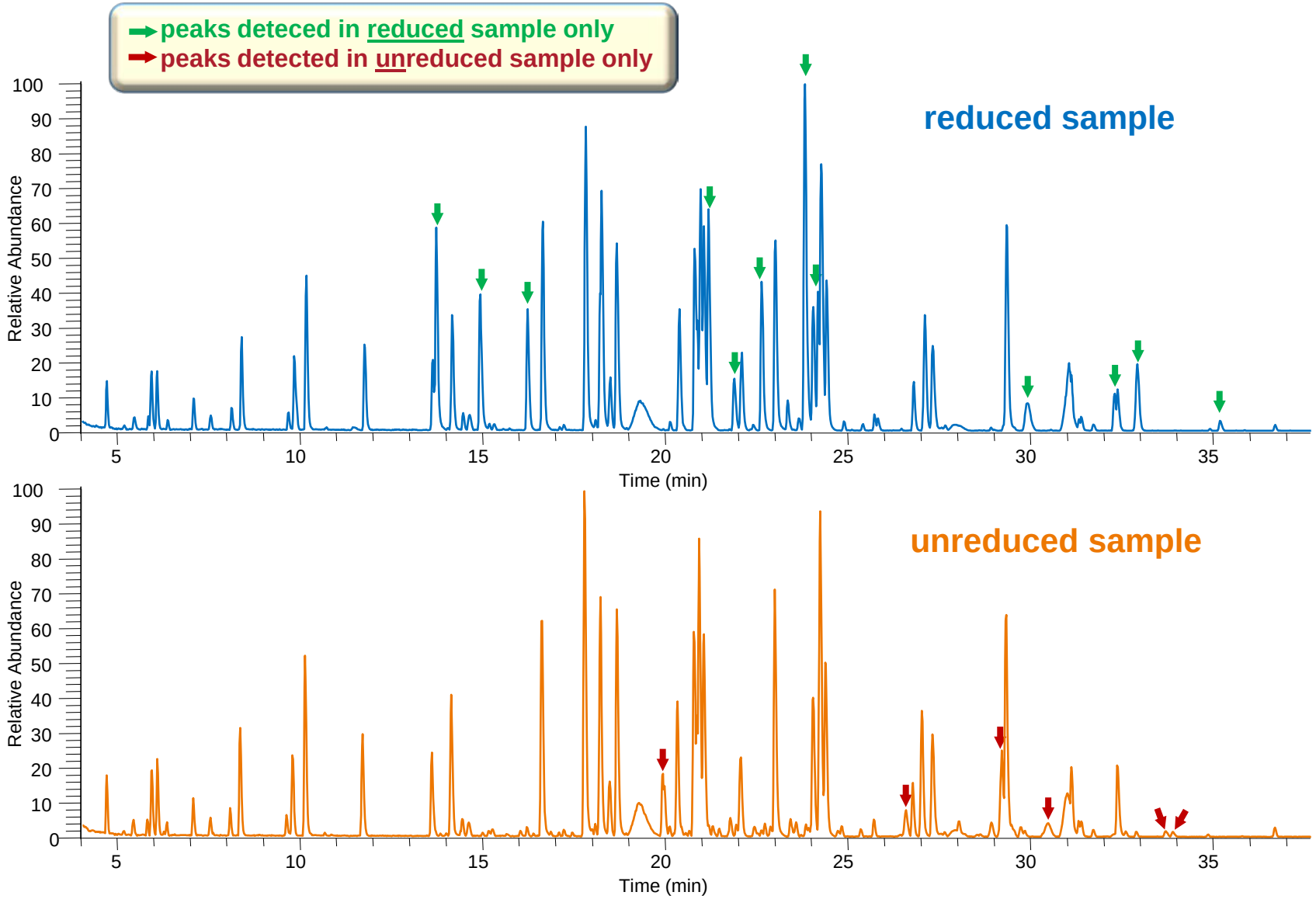
ThermoFisher SCIENTIFIC

Sample Analysis by Mass Spectrometry

Q Exactive Plus

Analysis of **reduced (+alkylated)** sample

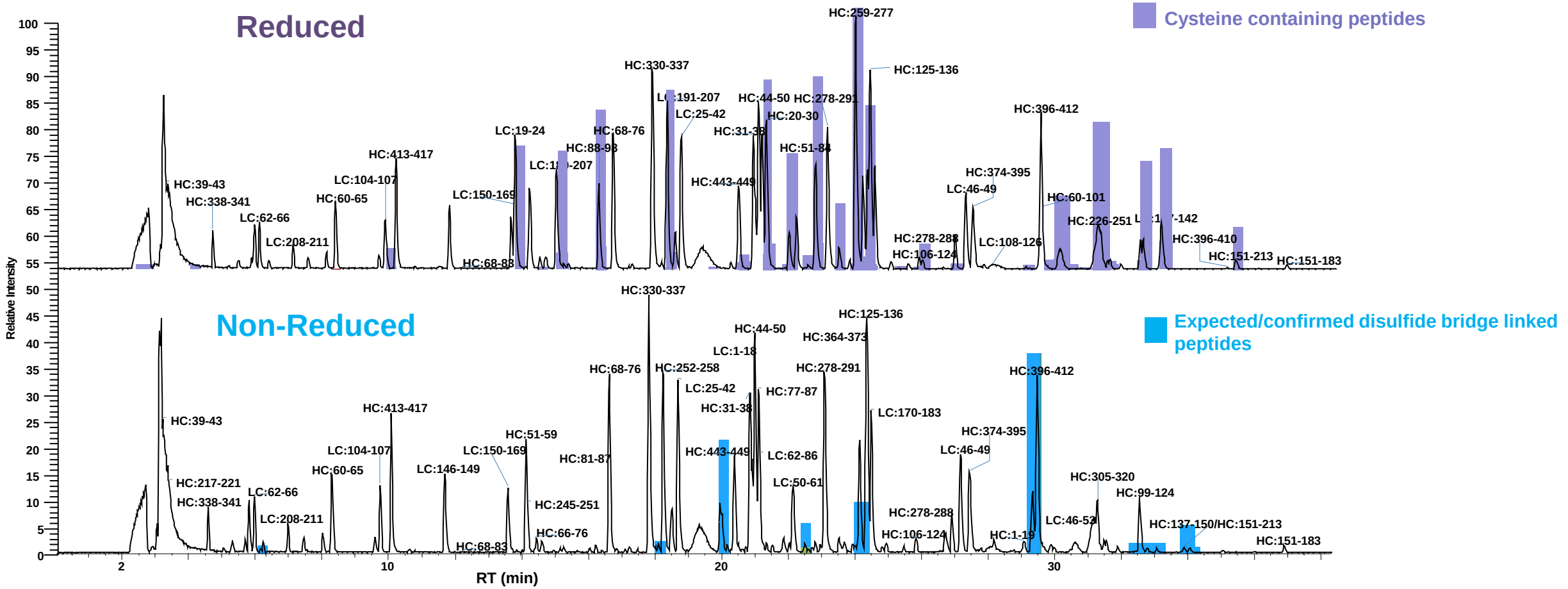
Analysis of **unreduced** sample



Method used for both samples:
data-dependent Top5 Method:
Full MS + top5 MS/MS scans per cycle

ThermoFisher SCIENTIFIC

Disulfide Bridge Analysis - Result Review



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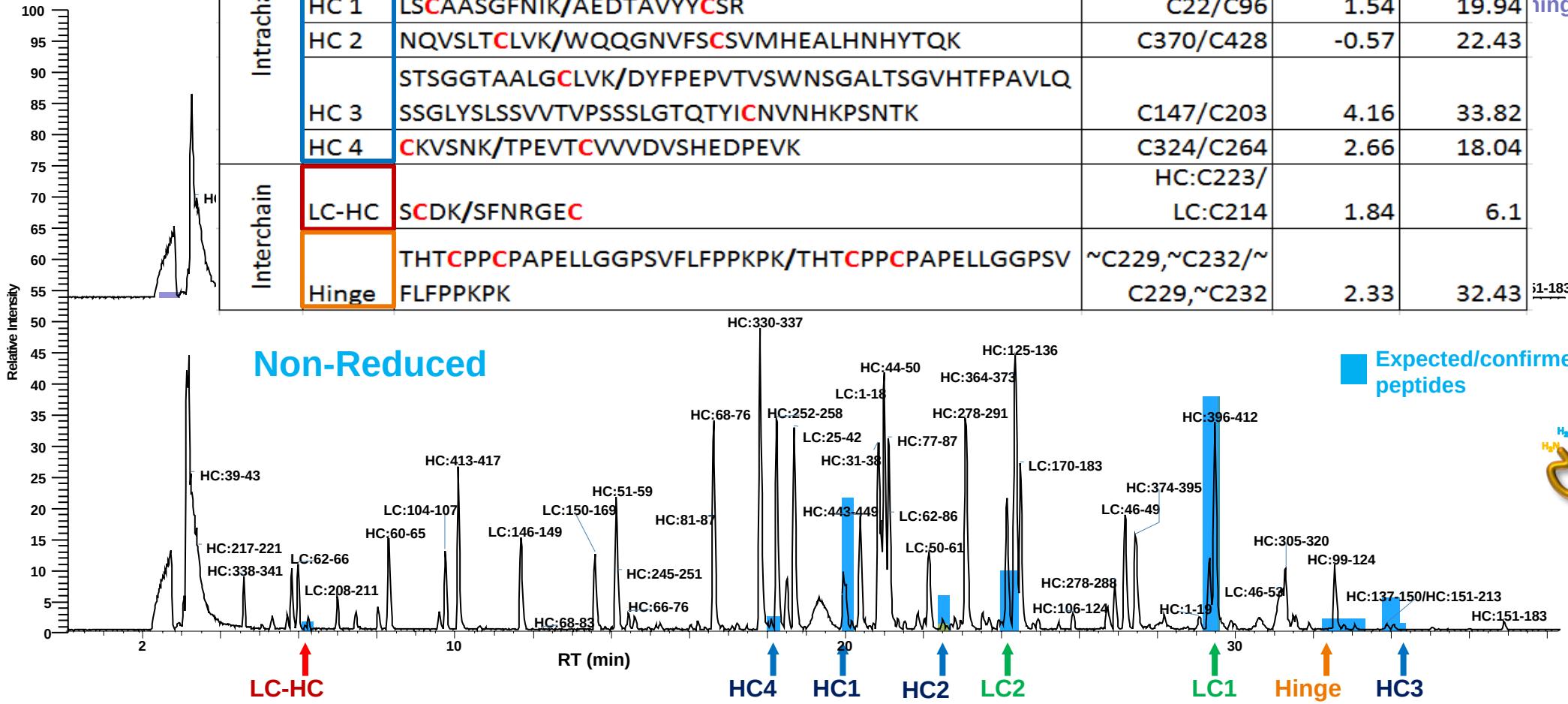
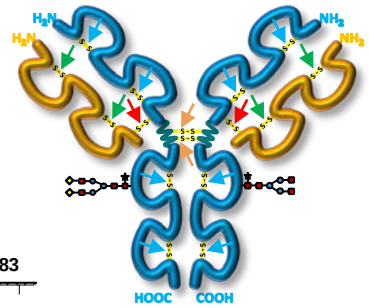
Disulfide Bridge Analysis - Result Review

		Peptide Sequence	Site	Delta (ppm)	RT
Intrachain	LC 1	VTITCR/SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTK	C23/C88	1.42	29.28
	LC 2	SGTASVVCLLNNFYPR/HKVYACEVTHQGLSSPVTK	C134/C194	1.98	24.05
	HC 1	LSCAASGFNIK/AEDTAVYYCSR	C22/C96	1.54	19.94
	HC 2	NQVSLTCLVK/WQQGNVFSCSVMHEALHNHYTQK	C370/C428	-0.57	22.43
	HC 3	STSGGTAALGCLVK/DYFPEPVTVSWNSGALTSGVHTFPAVLQ SSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTK	C147/C203	4.16	33.82
	HC 4	CKVSNK/TPEVTCVVVDVSHEDPEVK	C324/C264	2.66	18.04
Interchain	LC-HC	SCDK/SFNRGEC	HC:C223/ LC:C214	1.84	6.1
	Hinge	THTCPPCPAPELLGGPSVFLFPPKPK/THTCPPCPAPELLGGPSV FLFPPKPK	~C229,~C232/~ C229,~C232	2.33	32.43

ing peptides



Expected/confirmed disulfide bridge linked peptides



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Details for Expected Light Chain S-S-Bond: C134-C194

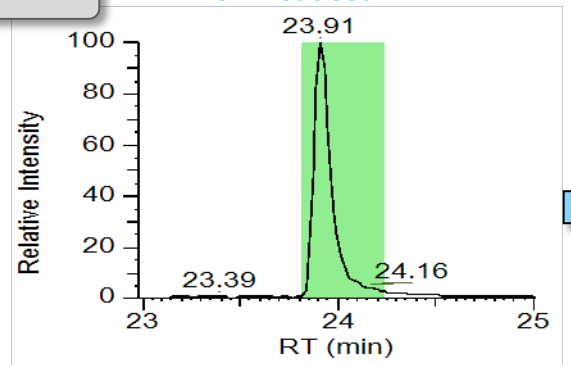
1:S127-R142/1:V191-K207 = 3555.749m[1ss]	SGTASVVCLLNNFYPR/VYACEVTHQGLSSPVTK	1ss		0.55	99.9%	MS2/Full

Light chain amino acid sequence

```
                10                  20                  30                  40                  50                  60                  70
DIQMTQSPSS  LSASVGDRVT  ITCRASQDVN  TAVAWYQQKP  GKAPKLLIYS  ASFLYSGVPS  RFSGSRSGTD

                80                  90                 100                 110                 120                 130                 140
FTLTISSLQP  EDFATYYCQQ  HYTTPPTFGQ  GTKVEIKRTV  AAPSVFIFPP  SDEQLKSGTA  SVVCLLNNFY

               150                 160                 170                 180                 190                 200                 210
PREAKVQWKV  DNALQSGNSQ  ESVTEQDSKD  STYSLSSTLT  LSKADYEKHK  VYACEVTHQG  LSSPVTKSFN

               220
RGEC
```

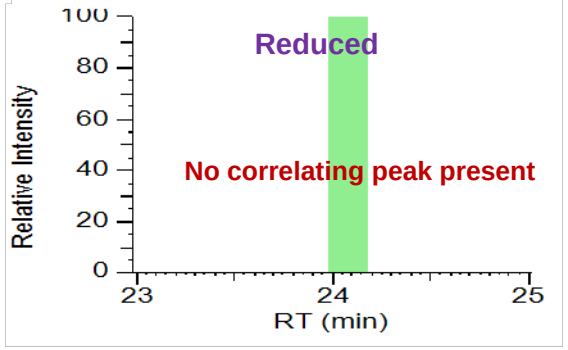
XICs

Non-Reduced

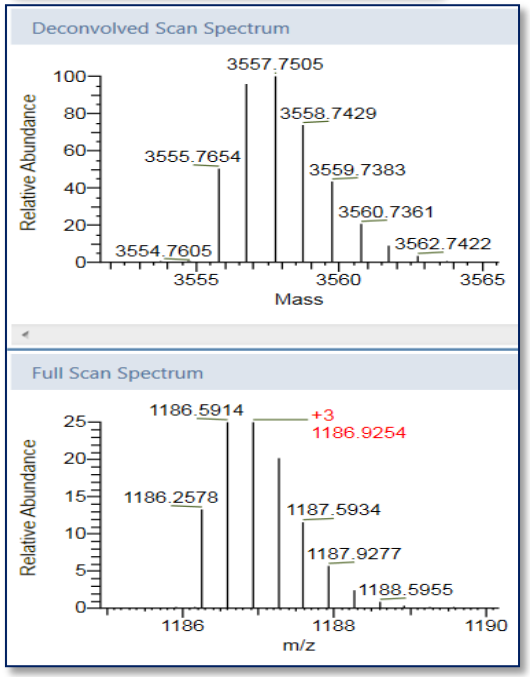


Reduced

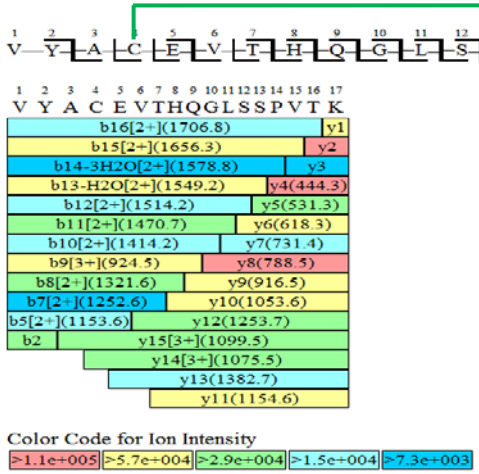
No correlating peak present

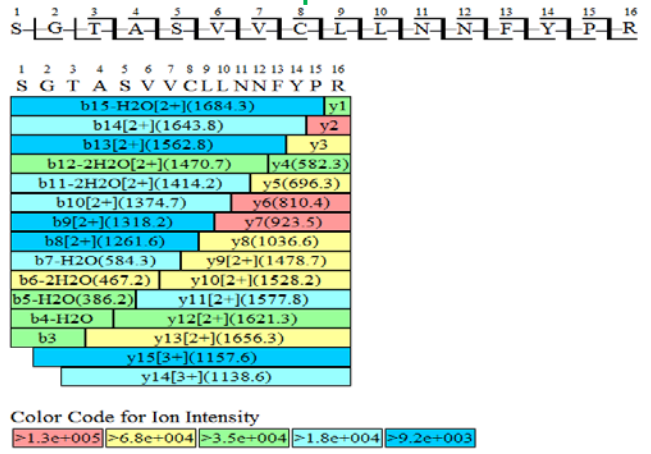


Precursor isotope pattern



Fragment ion coverage maps





ThermoFisher SCIENTIFIC

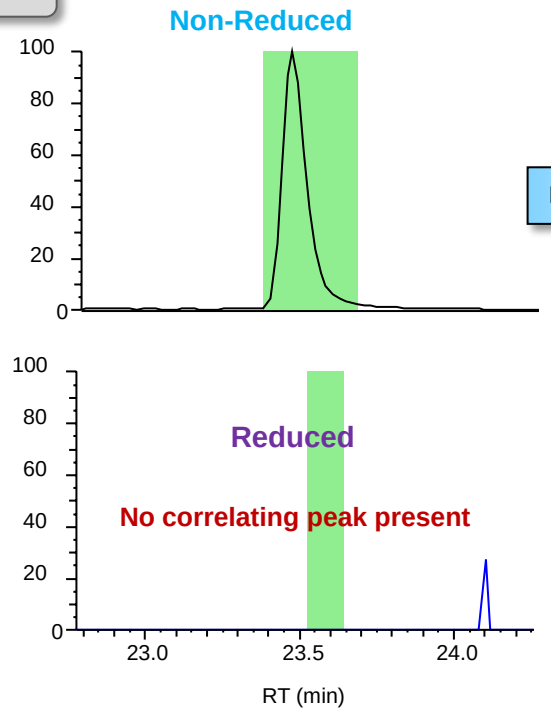
Details for Unexpected Heavy Chain S-S-Bond: C22-C264

Heavy chain amino acid sequence

```
          10                  20                  30                  40
EVQLVESGGG  LVQPGGSLRL  SCAASGFNIK  DTYIHWVRQA
          90                 100                 110                 120
LQMNSLRAED  TAVYYCSRWG  GDGFYAMDYW  GQGTLVTVSS
         170                 180                 190                 200
WNSGALTSGV  HTFPAVLQSS  GLYSLSSVVT  VPSSSLGTQT
         250                 260                 270                 280
PSVFLFPPKP  KDTLMISRTP  EVTCVVVDVS  HEDPEVKFNW
```

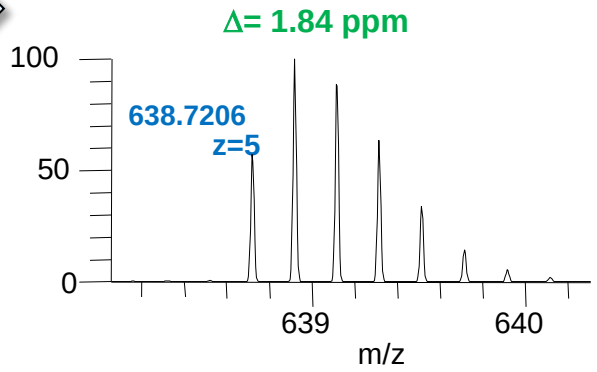
location of scrambled disulfide bridge in the trastuzumab heavy chain sequence

XICs

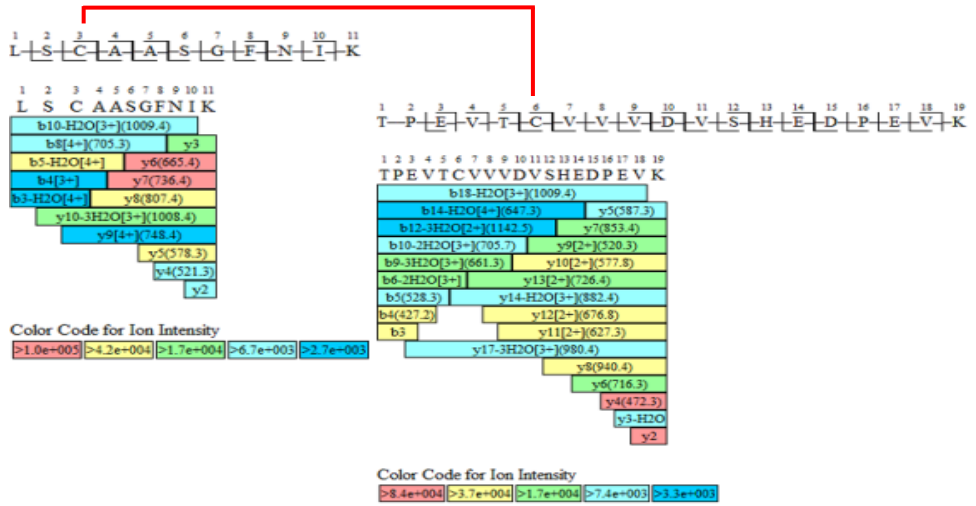


MS **pattern**

Precursor isotope pattern



Fragment ion coverage maps



ThermoFisher SCIENTIFIC

Benefits of ETD: Distinguising Leu/Ile Based on Diagnostic ETD-HCD MS^3 ions

analytical chemistry

Article

pubs.acs.org/ac

Discrimination of Leucine and Isoleucine in Peptides Sequencing with Orbitrap Fusion Mass Spectrometer

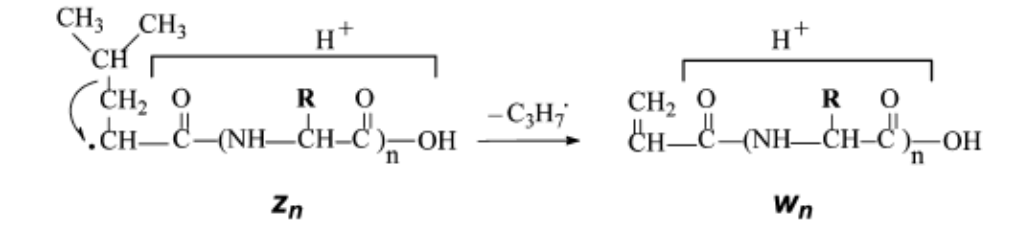
Albert T. Lebedev,[*,†] Eugen Damoc,[‡] Alexander A. Makarov,[‡] and Tatiana Yu. Samgina[†]

[†]Chemistry Department, M.V. Lomonosov Moscow State University, Leninskie Gory 1/3, Moscow, 119991, Russia

[‡]ThermoFisher Scientific (Bremen) GmbH, Hanna-Kunath Strasse 11, 28199, Bremen, Germany

Supporting Information

Scheme 1. Formation of w and d Ions



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analytical chemistry

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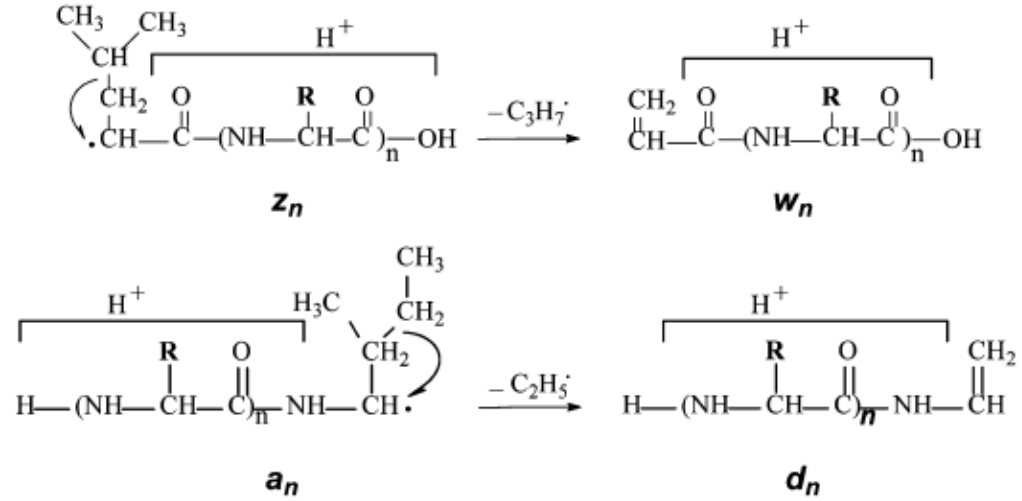
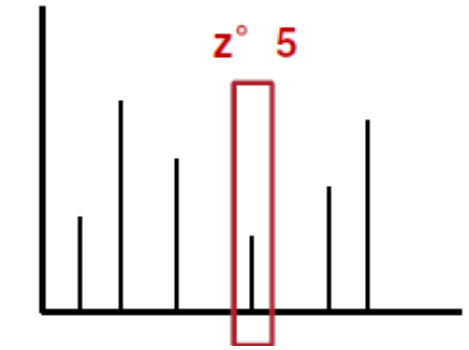
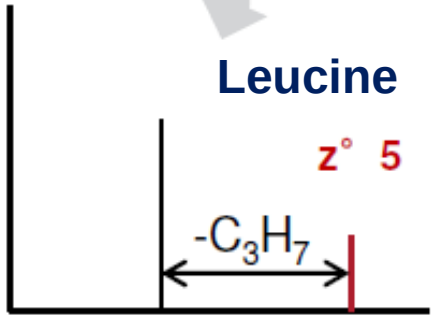


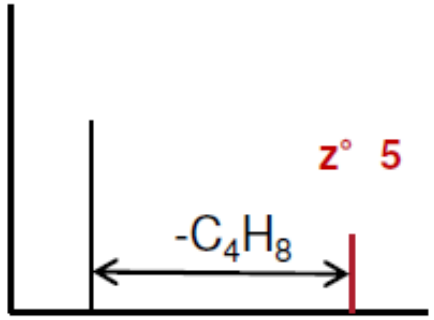


ETD MS^2 spectrum

HCD fragmentation ->MS^3 spectrum

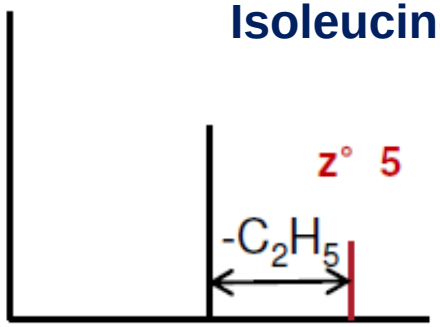
Leucine





or/ and

Isoleucine



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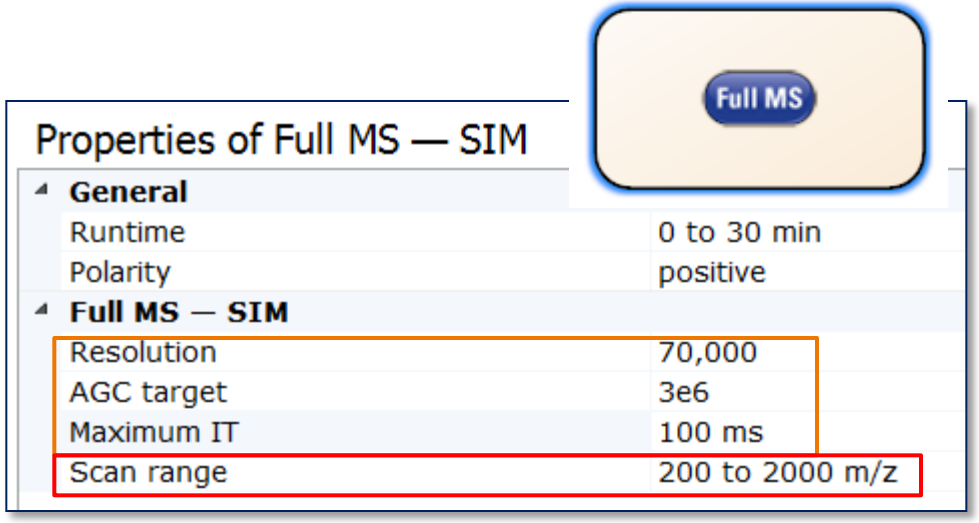
What we Learned

What we Learned



What we Learned

Properties of Full MS / dd-MS² (TopN)	
Default charge state	2
Inclusion	—
Exclusion	—
Tags	—
Full MS	
Resolution	70,000
AGC target	3e6
Maximum IT	100 ms
Scan range	200 to 2000 m/z
dd-MS² / dd-SIM	
Resolution	17,500
AGC target	1e5
Maximum IT	200 ms
Loop count	5
TopN	5
Isolation window	2.0 m/z
Fixed first mass	—
NCE / stepped NCE	28
dd Settings	
Minimum AGC target	2.00e3
Intensity threshold	1.0e4
Apex trigger	—
Charge exclusion	unassigned
Peptide match	preferred
Exclude isotopes	on
Dynamic exclusion	10.0 s