(Digging Deeper in Every Direction)

Developing an Industrial Platform for Advanced Multi-Faceted Characterization of Emerging Complex Biologics

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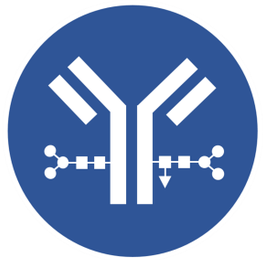
[2]Thermo Fisher Scientific, 355 River Oaks Parkway, San Jose, CA, USA, 95134

Mass Spec Services for Drug Research and Development



Intact Mass

Peptide Mapping

Biologics

Candidate Screening

Molecular Characterization

Process Development

Drug Research and Development

Discovery Proteomics

Pre-Clinical Proteomics

Clinical Proteomics

Proteomics

Comprehensive characterization needs both **peptide mapping** and **intact mass**

Q Exactive
BioPharma
Plus / HF / HF-X

QE Plus BioPharma (2016)

Began era of ‘single MS’ platform for biologics characterization

Native
LC-MS

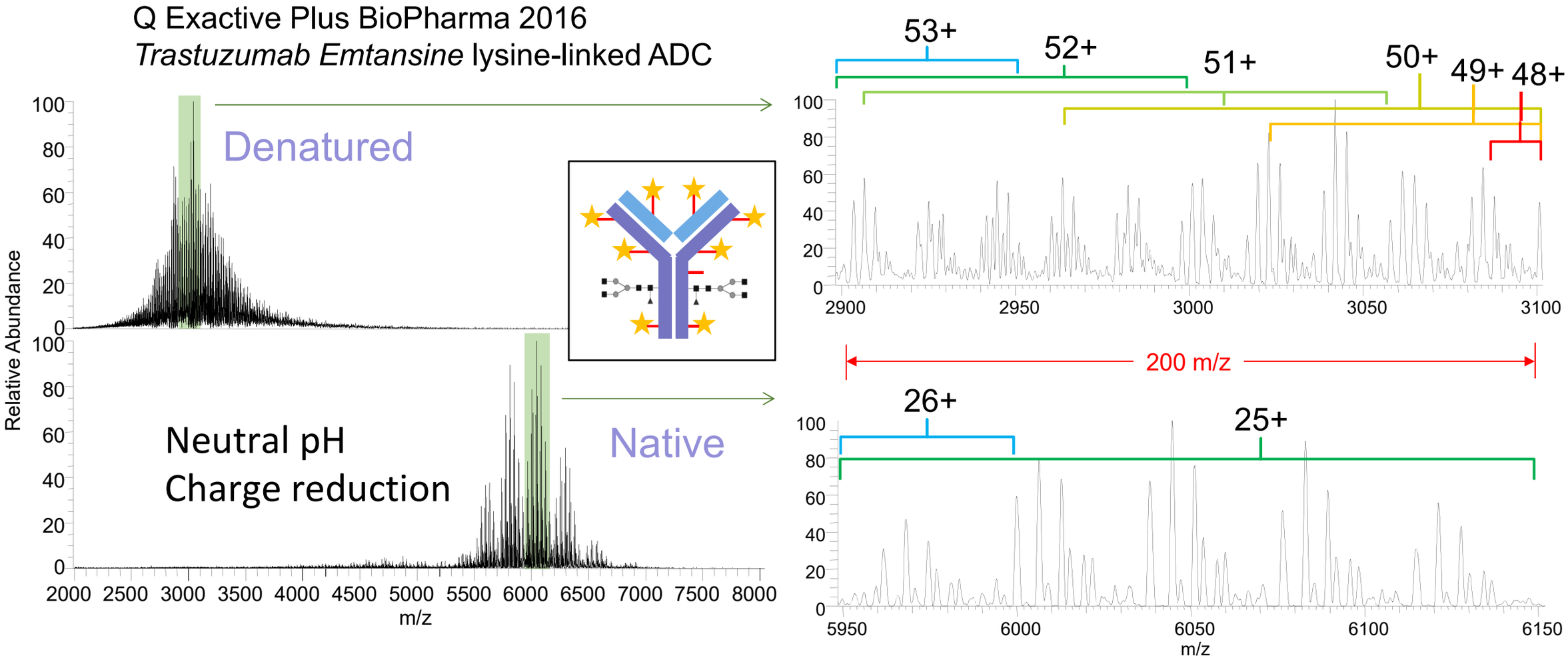
Intact Mass

HCD

Peptide Mapping

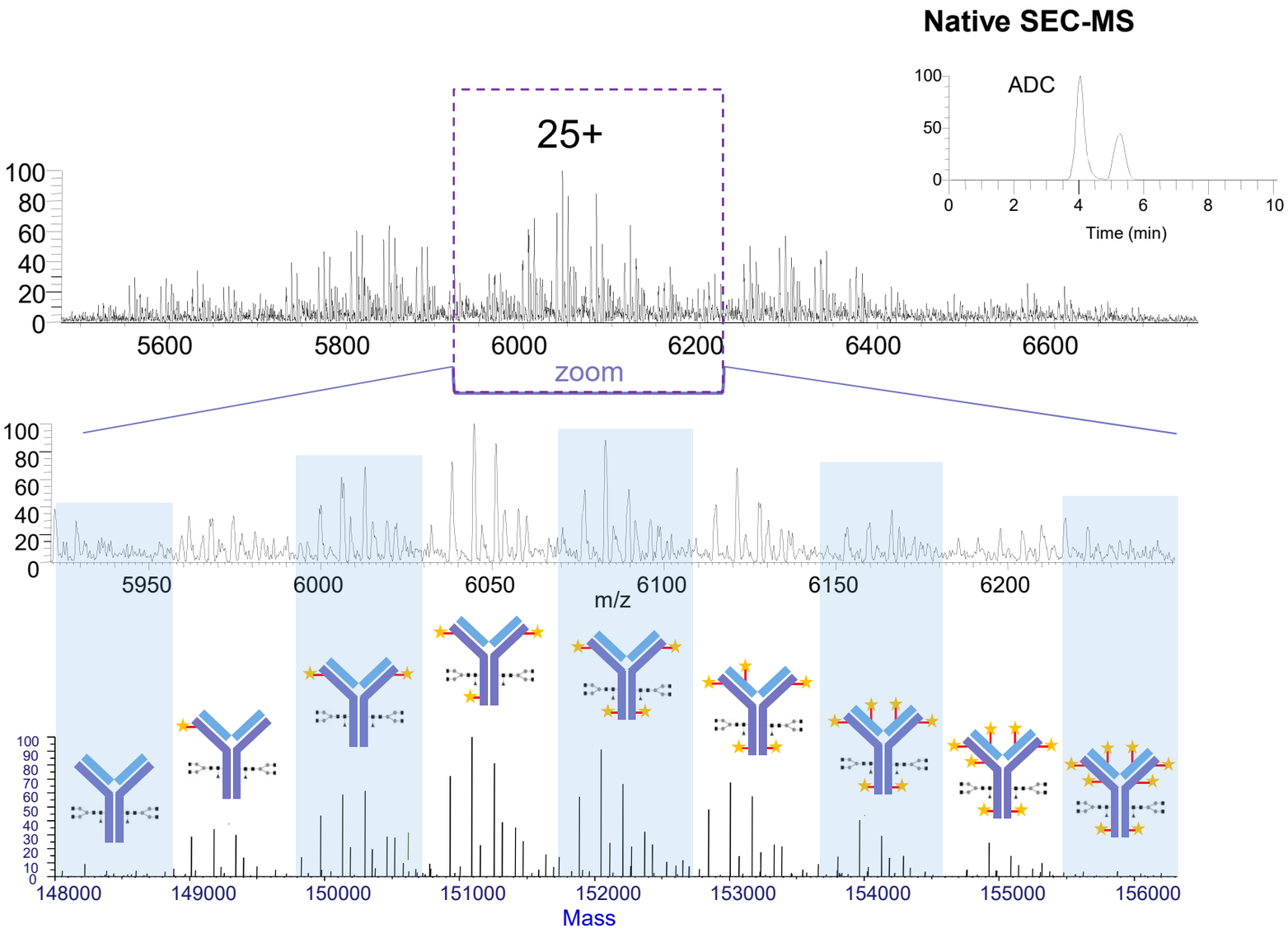
XXNXS/T

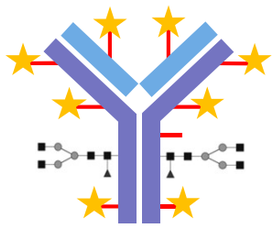
Native MS improves quality of congested intact mass spectra



- Greater m/z separation of co-eluting species' charge states
- Helpful for **microheterogenous isoform mixtures of** covalently-assembled molecules

Intact mass analysis via native SEC-MS provides accurate high throughput answer





Average Drug-to-Antibody Ratio (DAR)

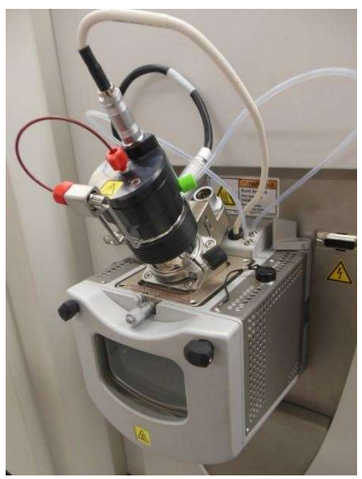
3.71

G0F/G1F DAR	Mass Accuracy (ppm)	Relative Abundance
DAR0	**6.49**	9.19
DAR1	21.69	34.26
DAR2	**0.05**	59.03
DAR3	**6.81**	100.00
DAR4	**5.17**	91.16
DAR5	**6.69**	67.42
DAR6	15.20	40.46
DAR7	**6.28**	24.28
DAR8	**3.78**	3.84

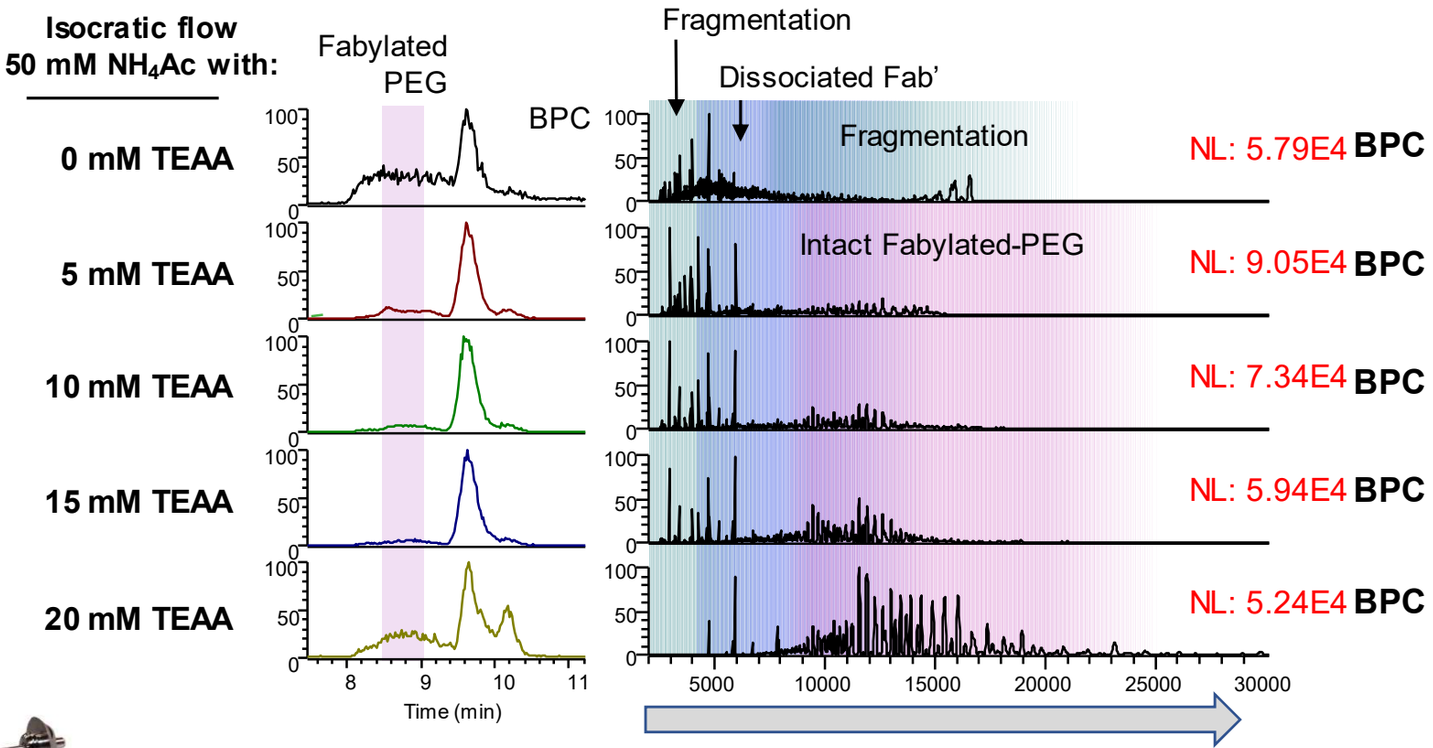
Solution phase charge reduction additives are powerful but messy

Thermo Scientific™ Q Exactive™ UHMR Hybrid Quadrupole-Orbitrap™ Mass Spectrometer

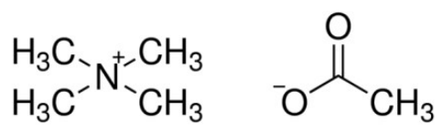
Thermo Scientific™ Vanquish™ Horizon UHPLC System



Heated capillary requires constant cleaning with many solution phase additives



Higher TEAA concentration drives further charge reduction and greater spectral separation of intact protein isoforms



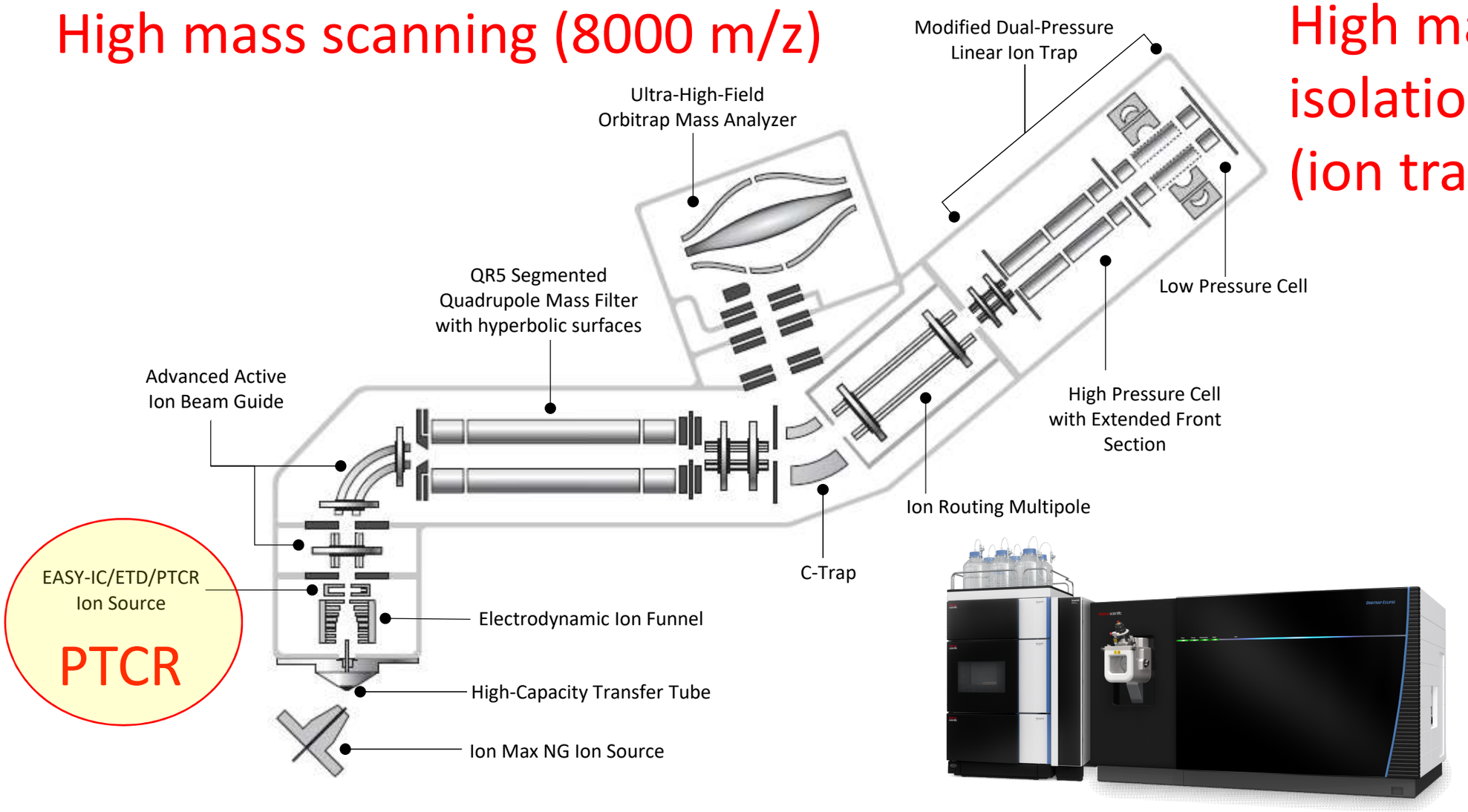
TEAA
(triethylammonium acetate)
pKa 11

Wendy Sandoval, Genentech

Testing new Orbitrap Eclipse Tribrid MS Platform (2019)

High mass scanning (8000 m/z)

High mass isolation (ion trap)



What is Proton Transfer Charge Reduction (PTCR)?

J. Am. Chem. Soc. **1996,** *118,* 7390−7397

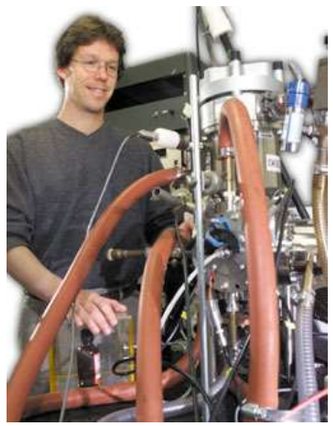
Ion/Ion Reactions in the Gas Phase: Proton Transfer Reactions Involving Multiply-Charged Proteins

James L. Stephenson, Jr., and Scott A. McLuckey*

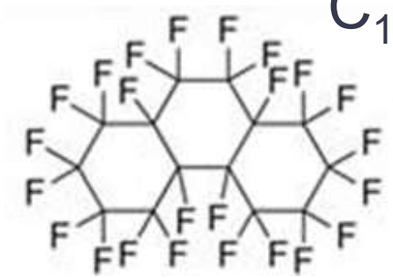
Contribution from the Chemical and Analytical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-6365

Received April 9, 1996[⊗]

Scott A. McLuckey



James L. Stephenson



$C_{14}F_{24}$

m/z 623.9

- Proton Transfer Charge Reduction (PTCR)
  - Same Technique Described by Stephenson and McLuckey.
- Ion-Ion Proton Transfer (IIPT)
  - Hunt Lab
- Proton Transfer Reaction (PTR-MS)
  - Proton Transfer Ionization

New era of ADVANCED ‘single MS’ platform for biologics characterization

Orbitrap
Eclipse
Tribrid MS

Native
LC-MS

HCD

ETD

UVPD

EXTENSIVE GLYCOSYL… …TY

EXTE…NS… …MODIFICATIONS

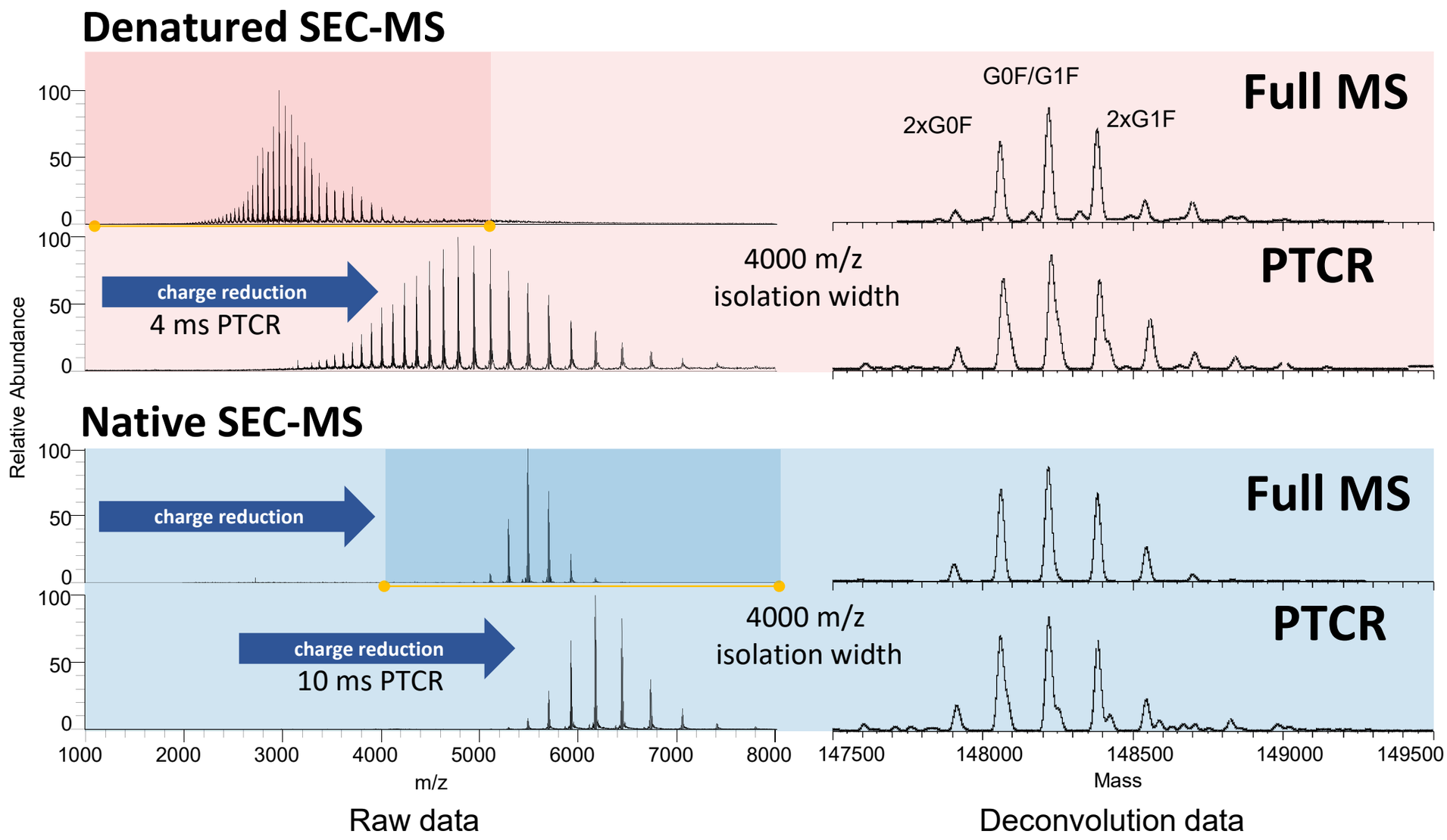
More charge reduction!

Intact Mass

Peptide Mapping

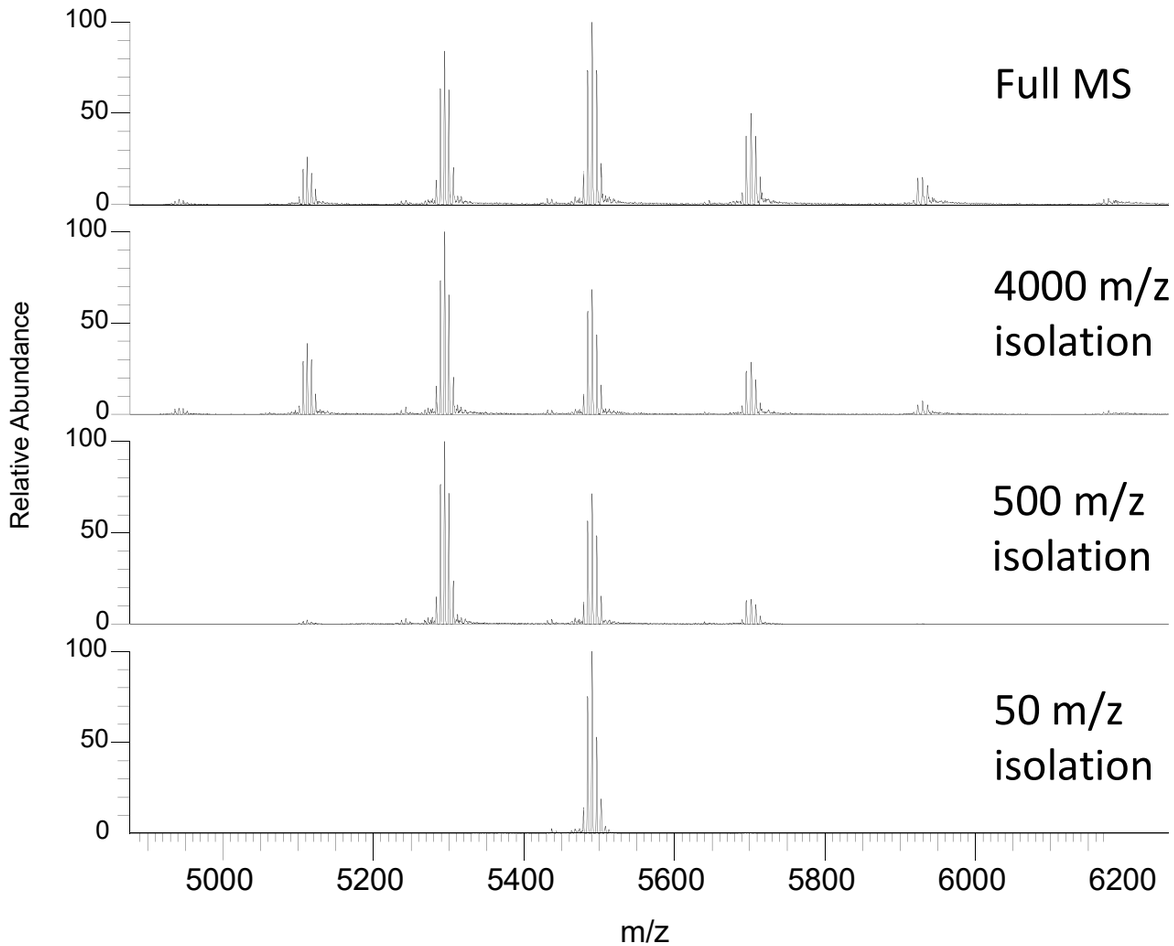
XXNXS/T

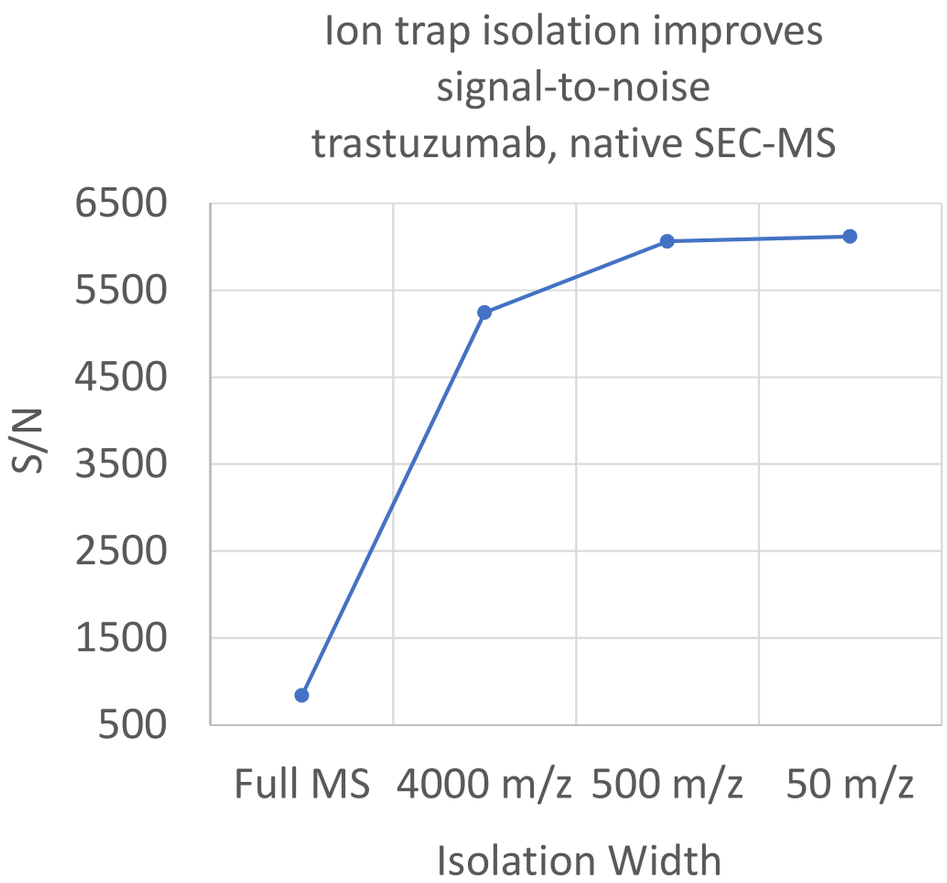
Proton Transfer Charge Reduction (PTCR)



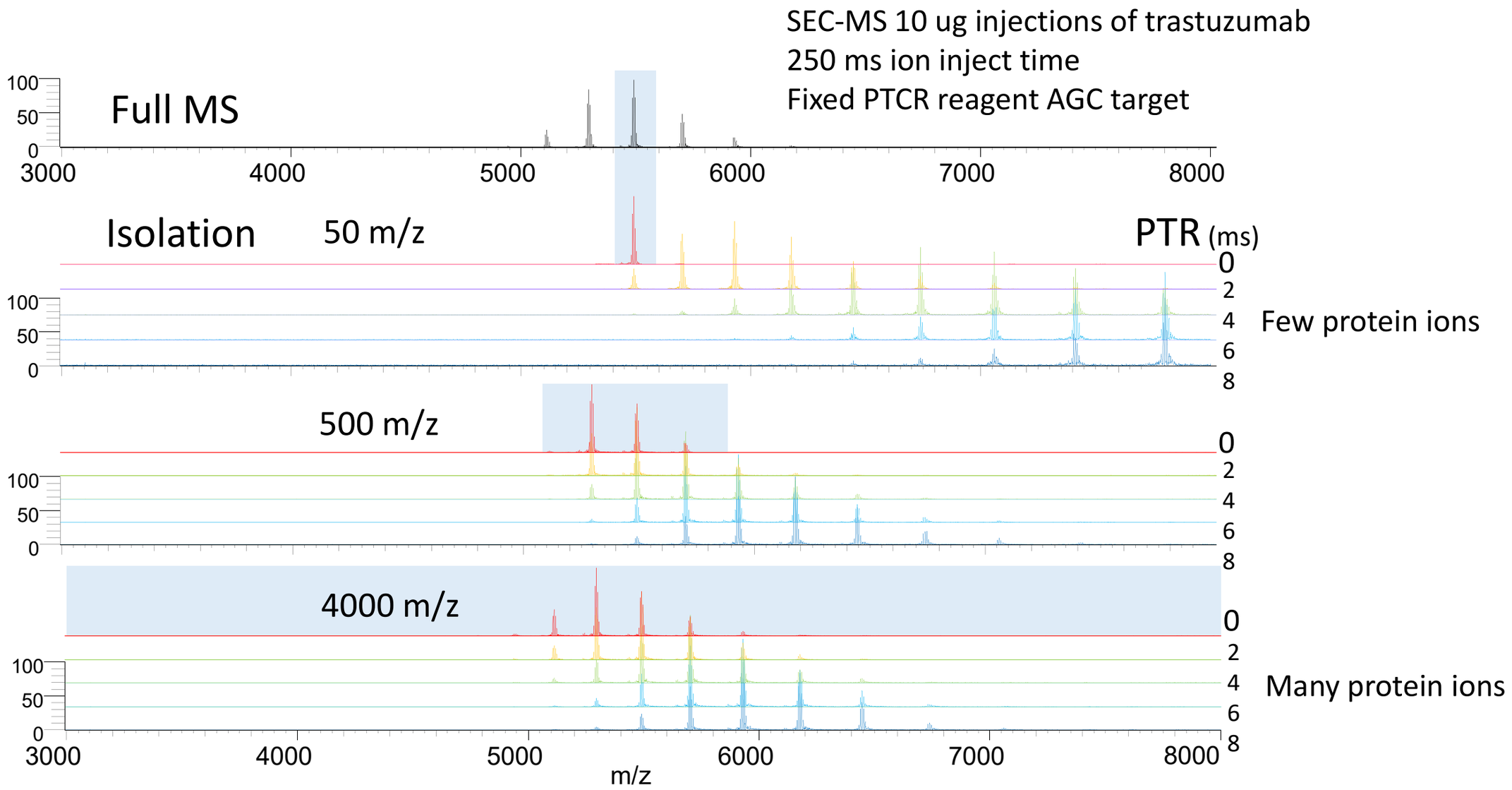
Further charge reduction of Native SEC-MS platform possible using PTCR gas phase chemistry

Ion Trap Isolation Improves Signal-to-Noise of native intact mAb mass spectra



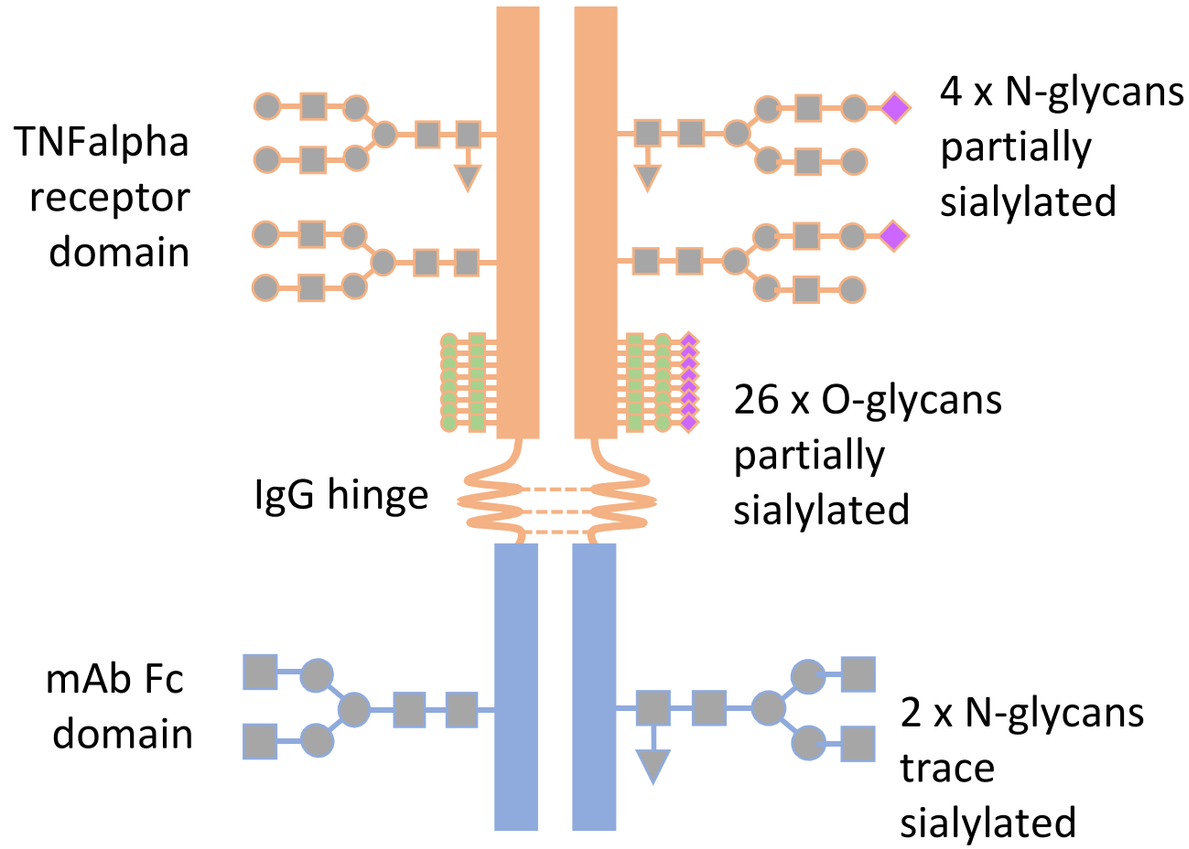


PTCR reaction influenced by available ion current, isolation window size



Etanercept: highly glycosylated Fc-fusion therapeutic protein

- Sequesters free Tumor Necrosis Factor (TNF)
- Treatment for rheumatoid arthritis
- Enbrel® is a trademark of Amgen



Etanercept: highly glycosylated Fc-fusion therapeutic protein

nature COMMUNICATIONS

ARTICLE

DOI: 10.1038/s41467-018-04061-7 OPEN

Native mass spectrometry combined with enzymatic dissection unravels glycoform heterogeneity of biopharmaceuticals

Therese Wohlschlager[1,2], Kai Scheffler[2,3], Ines C. Forstenlehner[1,2,4], Wolfgang Skala[1,2], Stefan Senn[1,2], Eugen Damoc[5], Johann Holzmann[2,4] & Christian G. Huber[1,2]

Article

pubs.acs.org/ac

ion Analysis of Etanercept Using Liquid d Quadrupole Time-of-Flight Mass Spectrometry ron-Transfer Dissociation Functionality

d,‡ Ying Qing Yu,† Niaobh McLoughlin,‡ Silvia Millan Martin,‡ Williams,§ and Weibin Chen*,†

partially sialylated

- Physicochemical Characterization, Glycosyla
- and Biosimilarity Assessment of the Fusion P

Othman Montacir[1,2] · Houda Montacir[1] · Andreas Springer[3] · Steph
Amirhossein Saadati[4] · Maria Kristina Parr[1]

analytical chemistry

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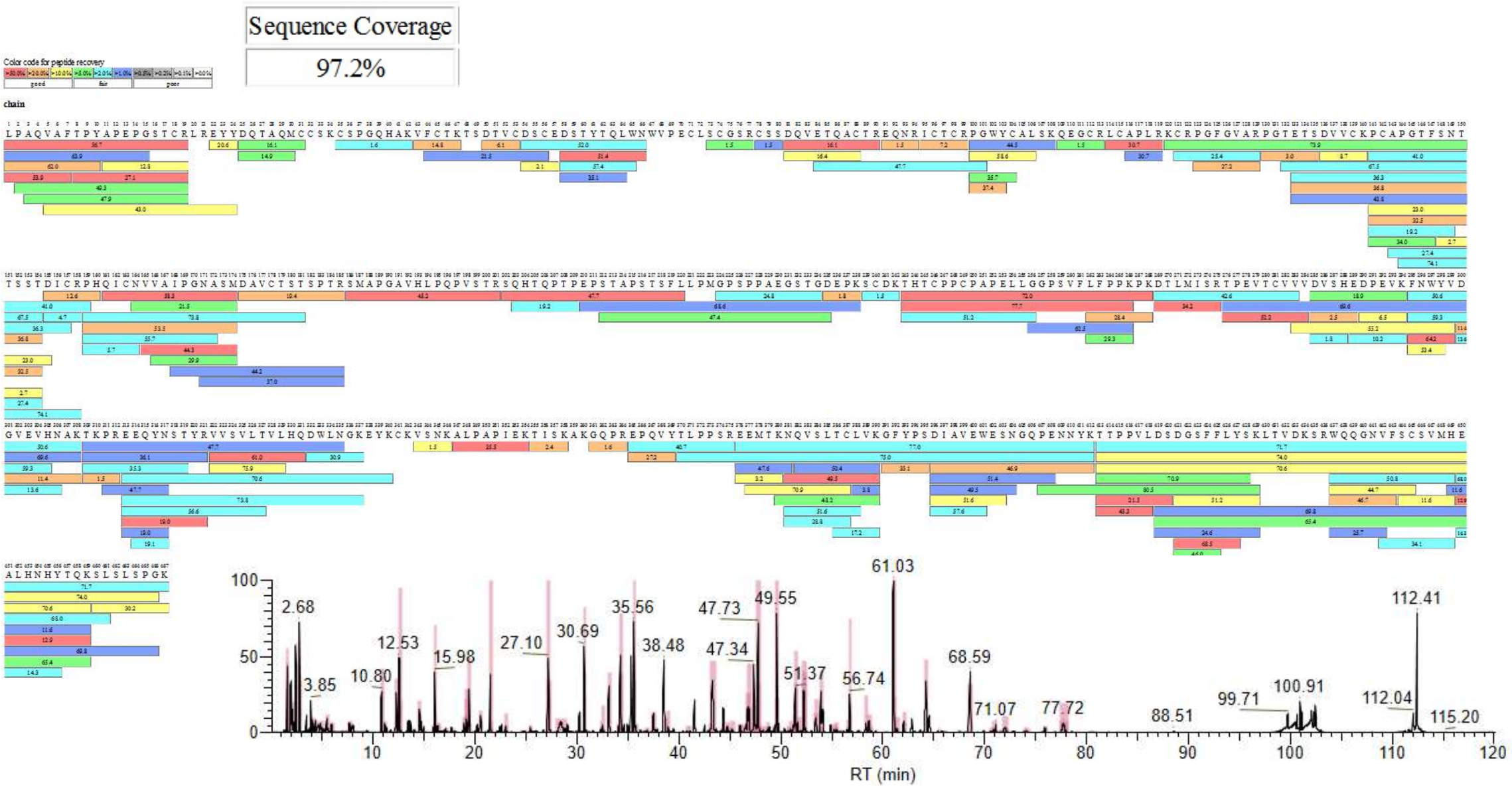
Article

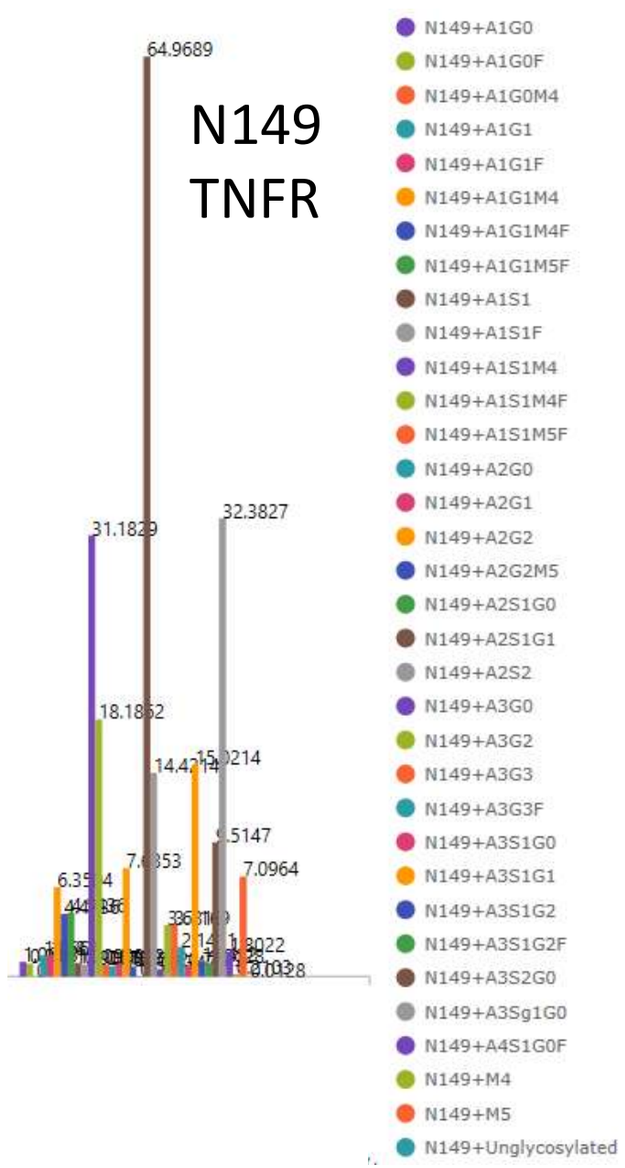
Orthogonal Middle-up Approaches for the Characterization of the Glycan Heterogeneity of Etanercept by Hydrophilic Interaction Chromatography Coupled to High Resolution Mass Spectrometry

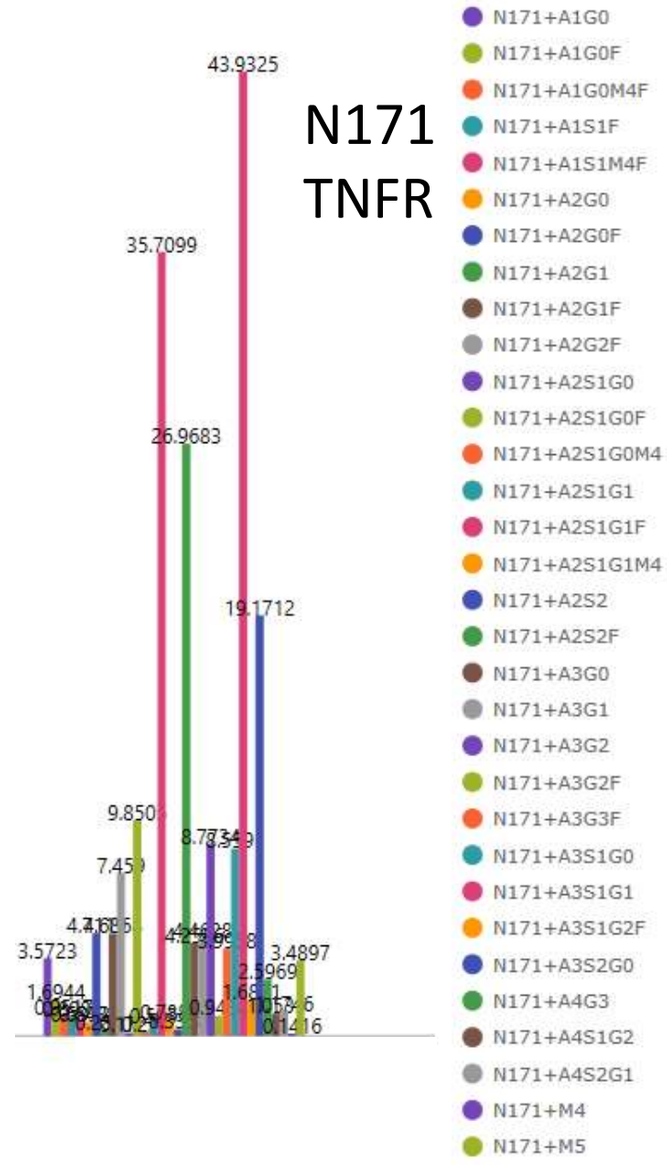
Valentina D'Atri, Lucie Nováková, Szabolcs Fekete, Dwight R Stoll, Matthew Allen Lauber, Alain Beck, and Davy Guillarme

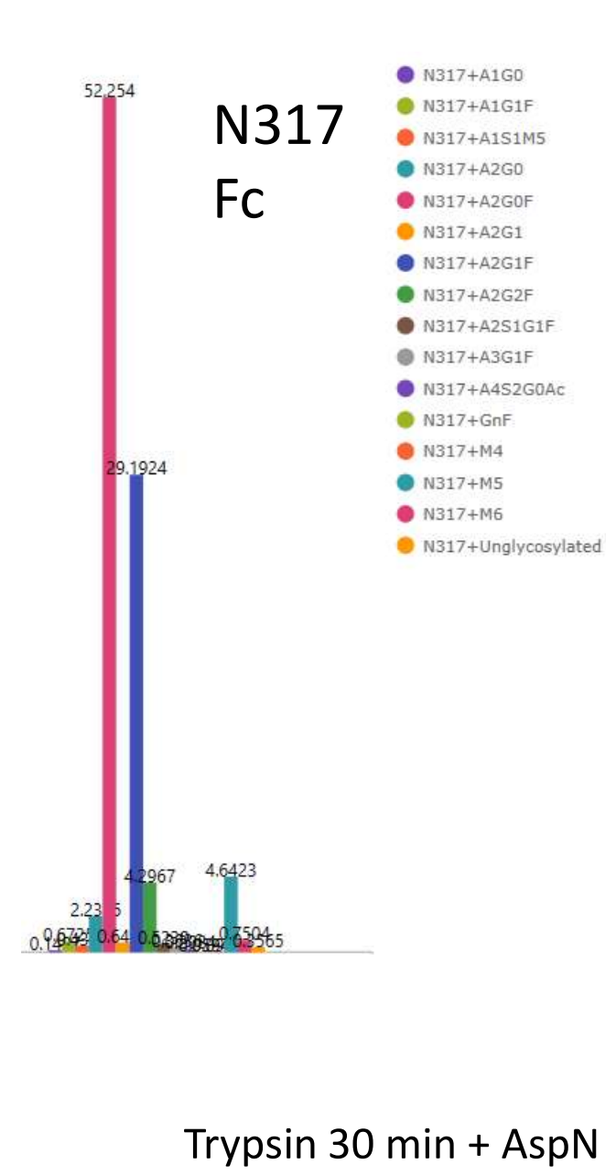
HCD+ETD peptide mapping results: Trypsin 30 min + AspN 18hrs



HCD+ETD N-glycopeptide mapping







Trypsin 30 min + AspN 18hrs

HCD+ETD peptide mapping results: Preliminary O-glycan summary

Trypsin 30 min

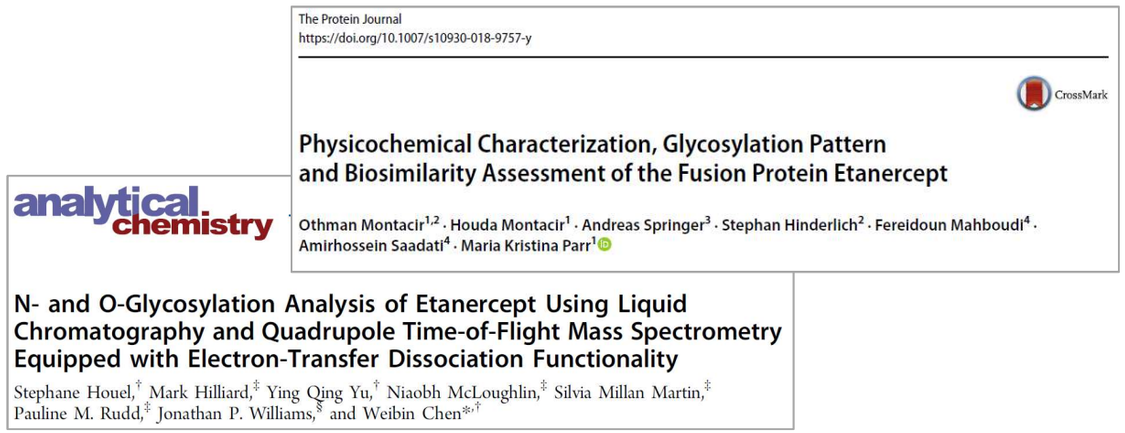
186	S186+GalNAc-3G	O-Glycan	22.0349
186	S186+GalNAc-6GGn-3G	O-Glycan	56.7437
186	~S186+GalNAc-6GGn-3SG	O-Glycan	7.7420
200	T200+GalNAc-6GGn-3SG	O-Glycan	27.9970
200	~T200+GalNAc-3SG	O-Glycan	27.7357
200	~T200+GalNAc-6Gn-3G	O-Glycan	10.3988
200	~T200+GalNAc-6Gn-3SG	O-Glycan	36.3837
200	~T200+GalNAc-6S-3SG	O-Glycan	1.0282
202	~S202+GalNAc-6S-3SG	O-Glycan	100.0000
226	S226+GalNAc-3SG	O-Glycan	100.0000
309	~T309+GalNAc-3SG	O-Glycan	70.4285
309	~T309+GalNAc-6S-3SG	O-Glycan	29.5715

Trypsin 18 hrs

186	S186+GalNAc-3SG	O-Glycan	45.9604
186	S186+GalNAc-6S-3SG	O-Glycan	12.2392
199	~S199+GalNAc-6Gn-3SG	O-Glycan	55.7023
200	~T200+GalNAc-6GGn-3SG	O-Glycan	34.0920
200	~T200+GalNAc-6Gn-3G	O-Glycan	26.9155
213	~T213+GalNAc-6S-3SG	O-Glycan	99.5643
217	~T217+GalNAc-3SG	O-Glycan	100.0000
218	~S218+GalNAc-3G	O-Glycan	0.5719
226	S226+GalNAc-3SG	O-Glycan	89.4899

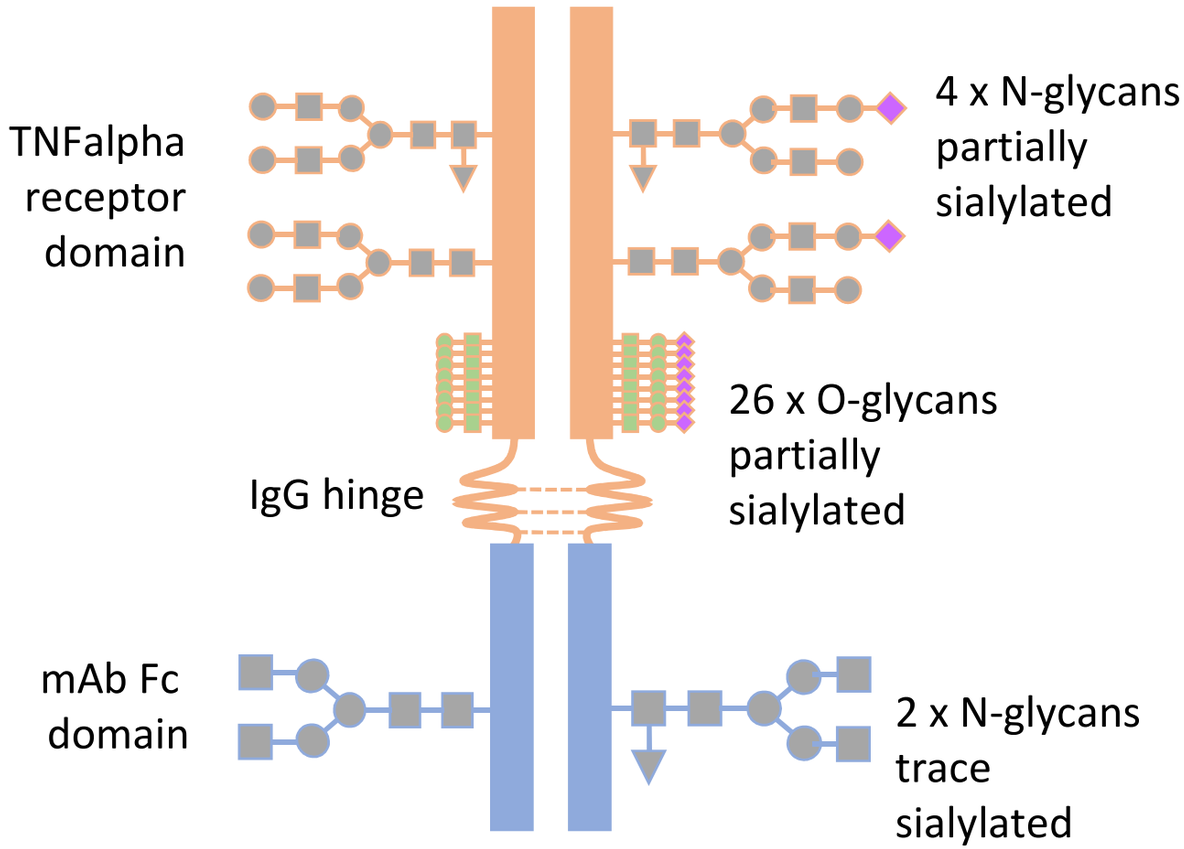
Trypsin 30 min + AspN 18hrs

186	S186+GalNAc-6G...	O-Glycan	6.4239
186	S186+GalNAc-6G...	O-Glycan	74.3229
199	~S199+GalNAc-3SG	O-Glycan	42.2293
199	~S199+GalNAc-6...	O-Glycan	40.2466
200	~T200+GalNAc-3G	O-Glycan	0.8195
200	~T200+GalNAc-6G...	O-Glycan	22.8685
200	~T200+GalNAc-6Gn	O-Glycan	0.6567
200	~T200+GalNAc-6S...	O-Glycan	2.1785
213	~T213+GalNAc-6S...	O-Glycan	100.0000
226	~S226+GalNAc-3SG	O-Glycan	100.0000
232	~S232+GalNAc-6S	O-Glycan	100.0000
287	S287+GalNAc-3G	O-Glycan	2.7387
287	S287+GalNAc-3SG	O-Glycan	54.0540
287	S287+GalNAc-6S-...	O-Glycan	48.0036



Initial methods identified 11 (of 13) known O-glycan sites:
S186, S199, T200, S202, T213, T217, S218, S226, S232, S287, T309

Etanercept structure and enzymatic tools for intact characterization

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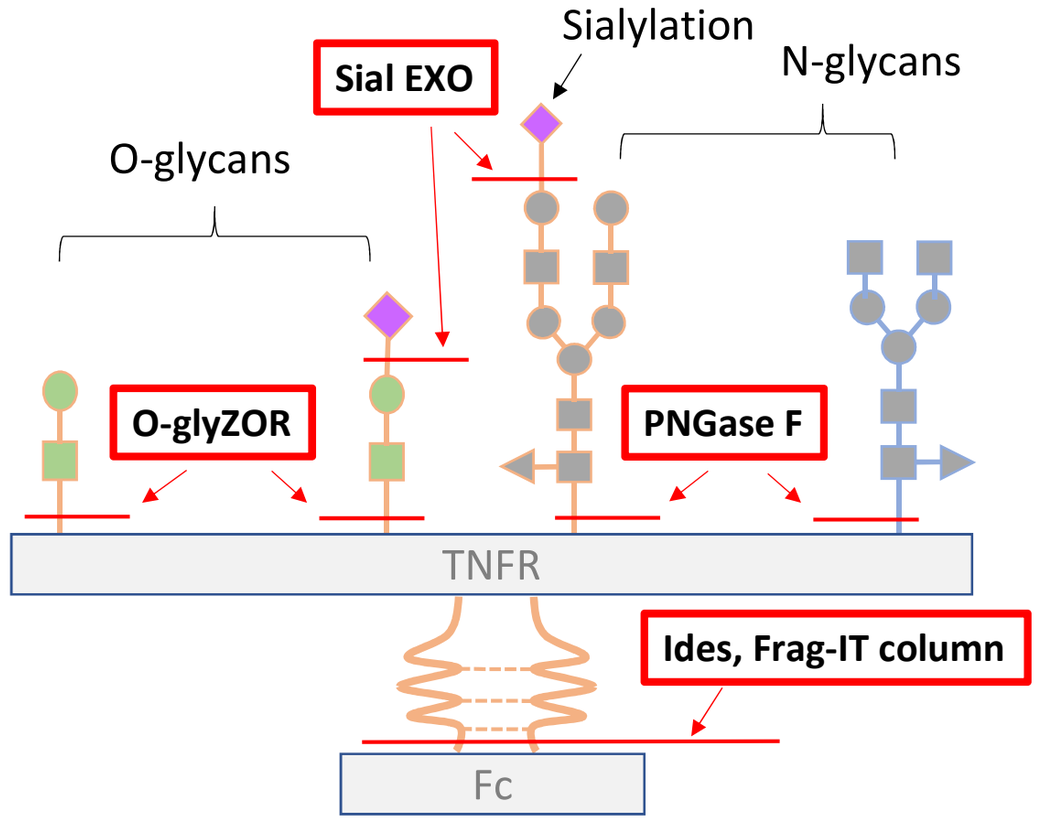
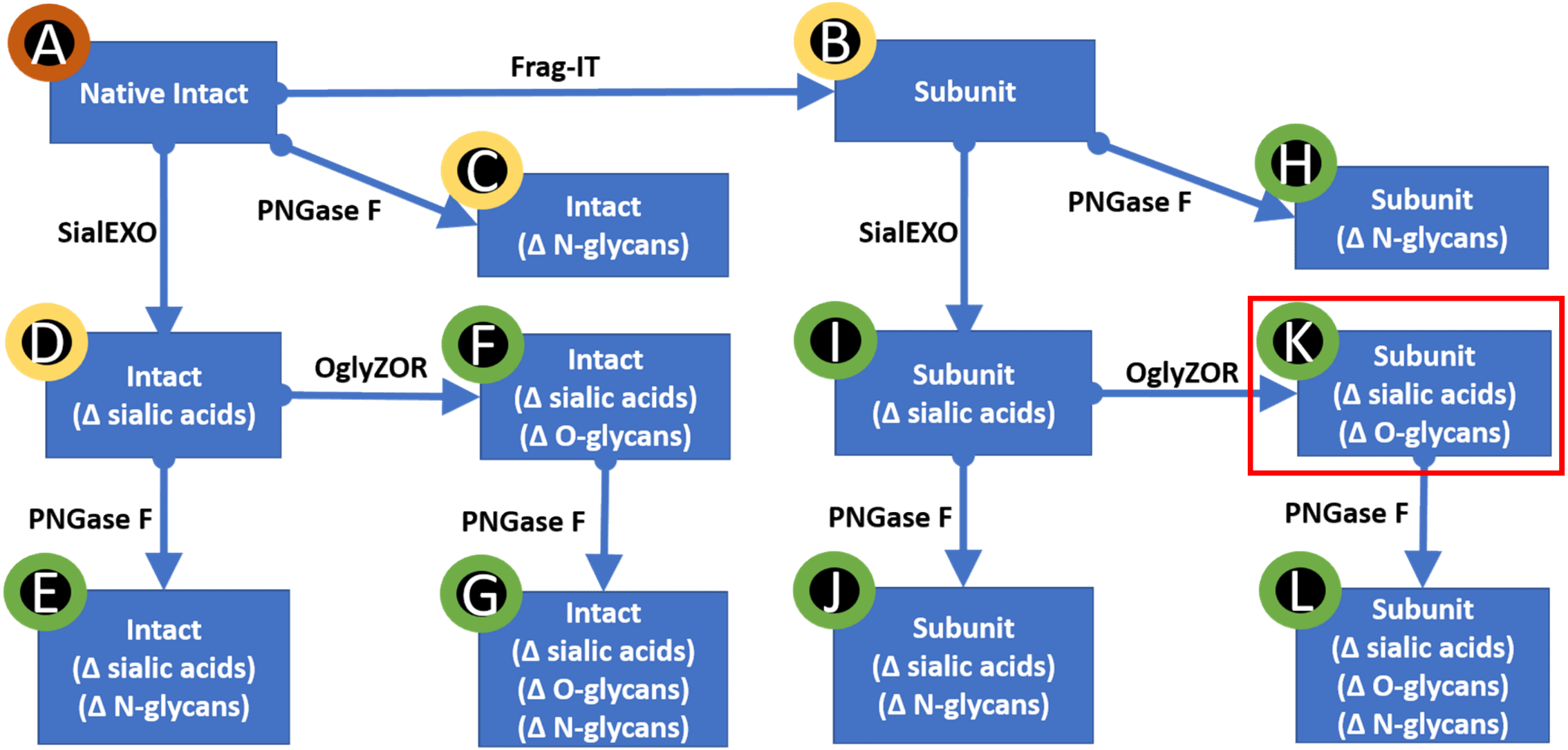
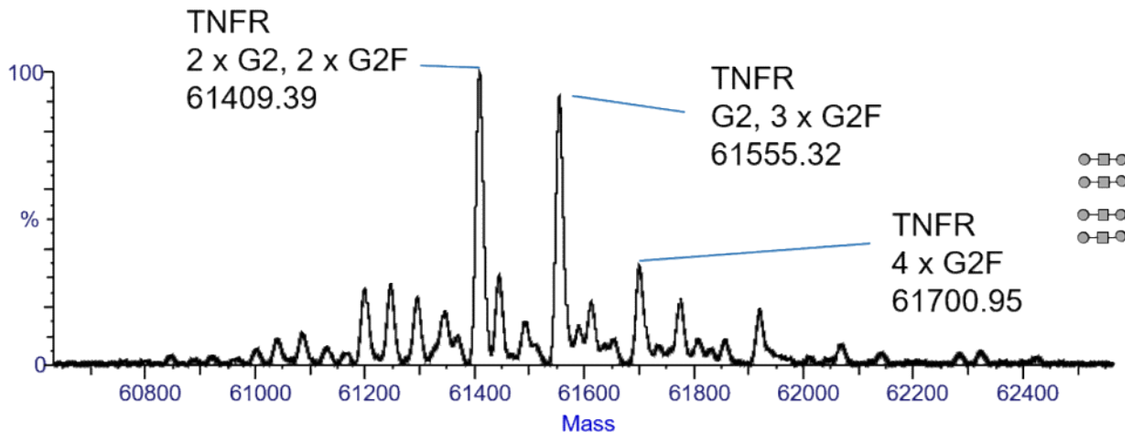


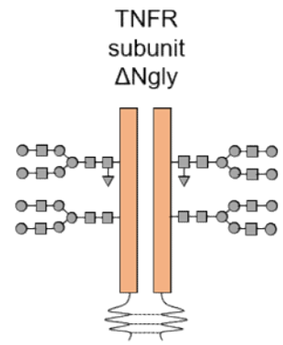
Sample Prep for Native Intact Analysis of Etanercept

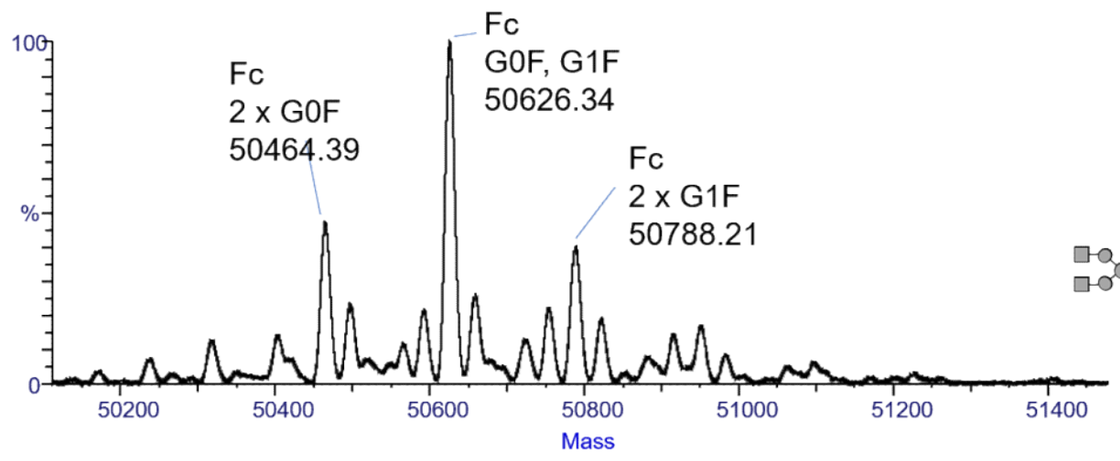


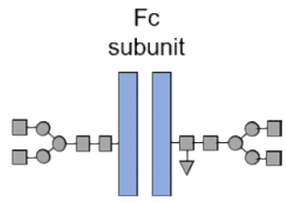
Prep K – native SEC-MS

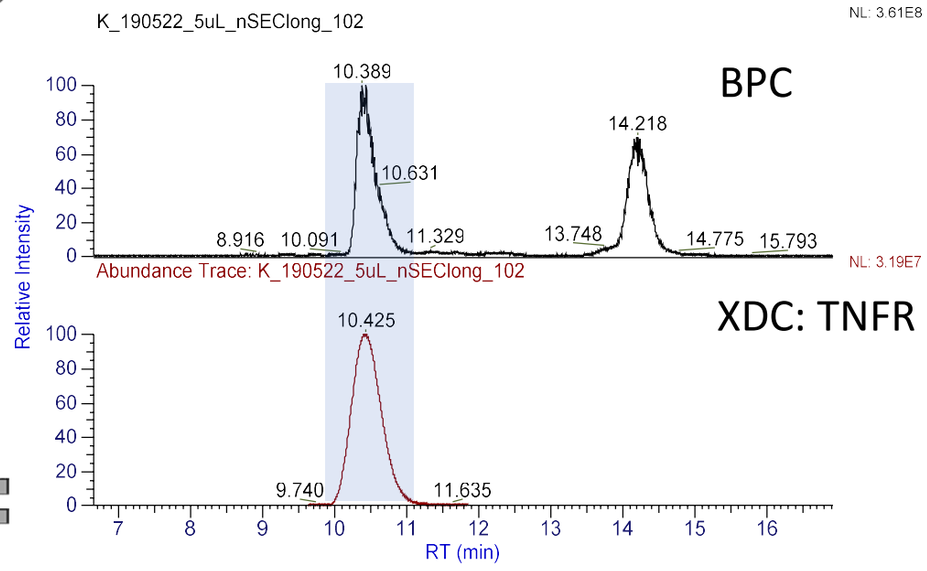
K **Subunit (Δ sialic acids) (Δ O-glycans)**





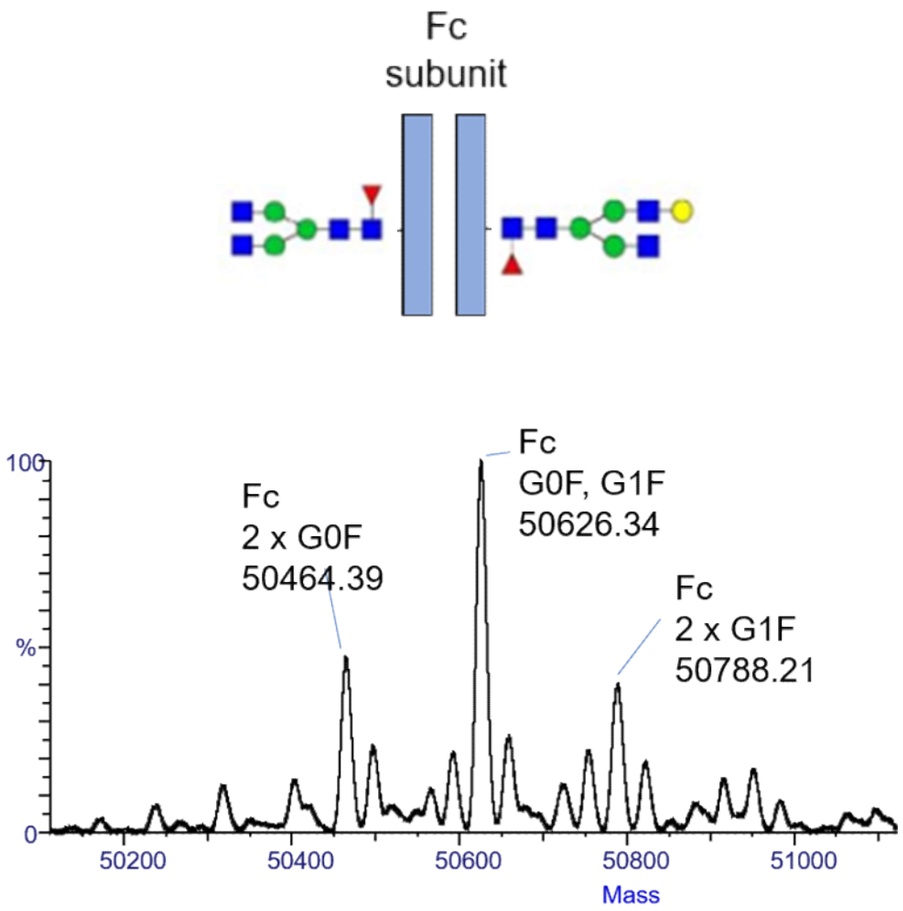




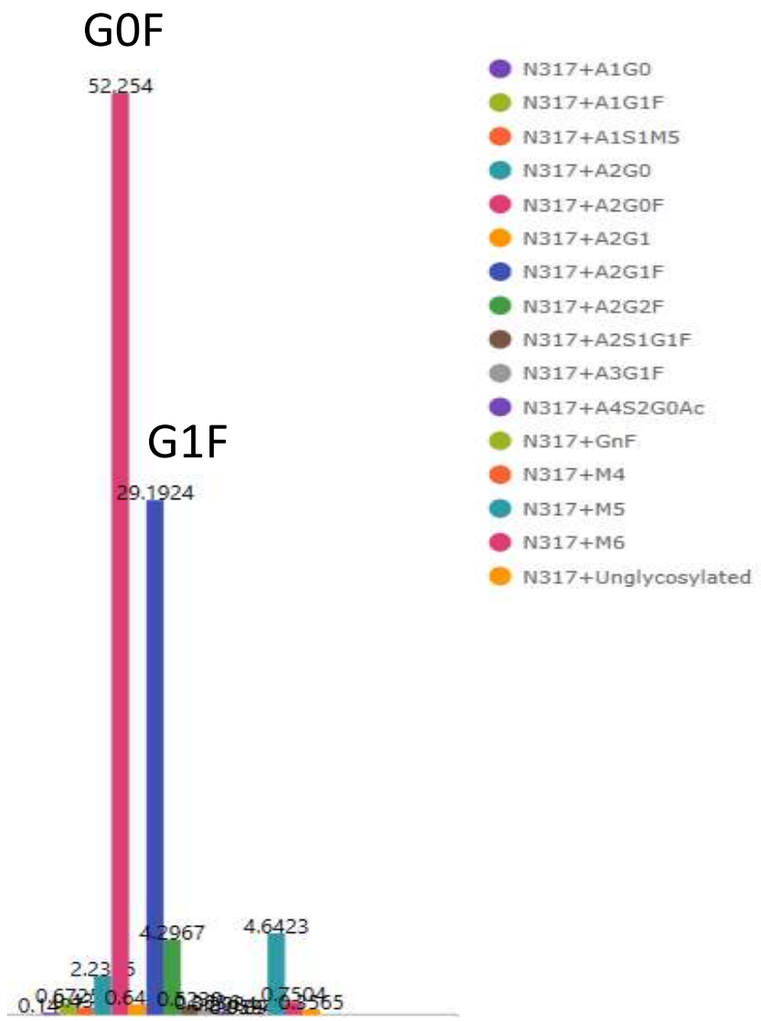


HCD+ETD peptide mapping results: N-glycans

IdeS/Fabricator digest



Trypsin 30 min + AspN 18hrs



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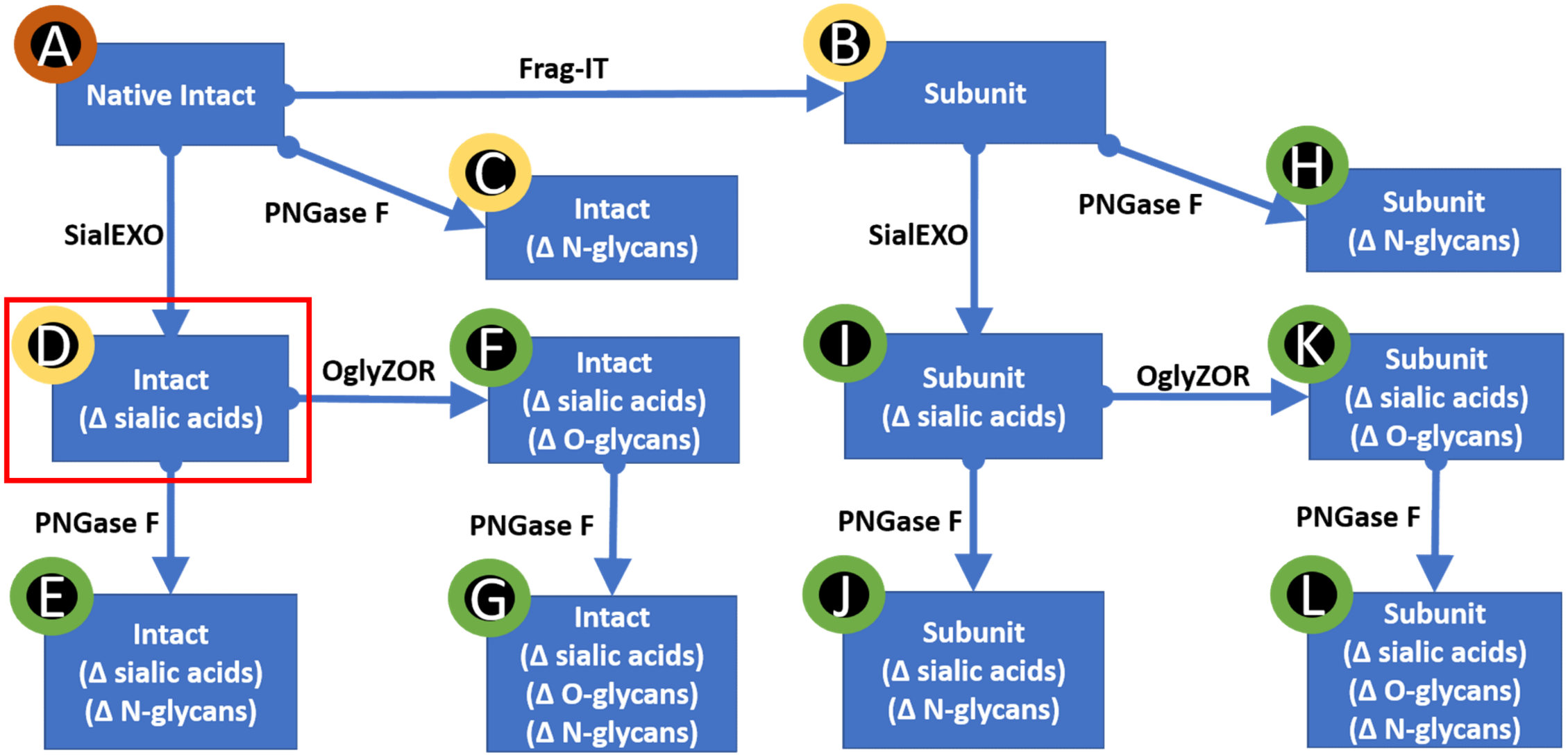
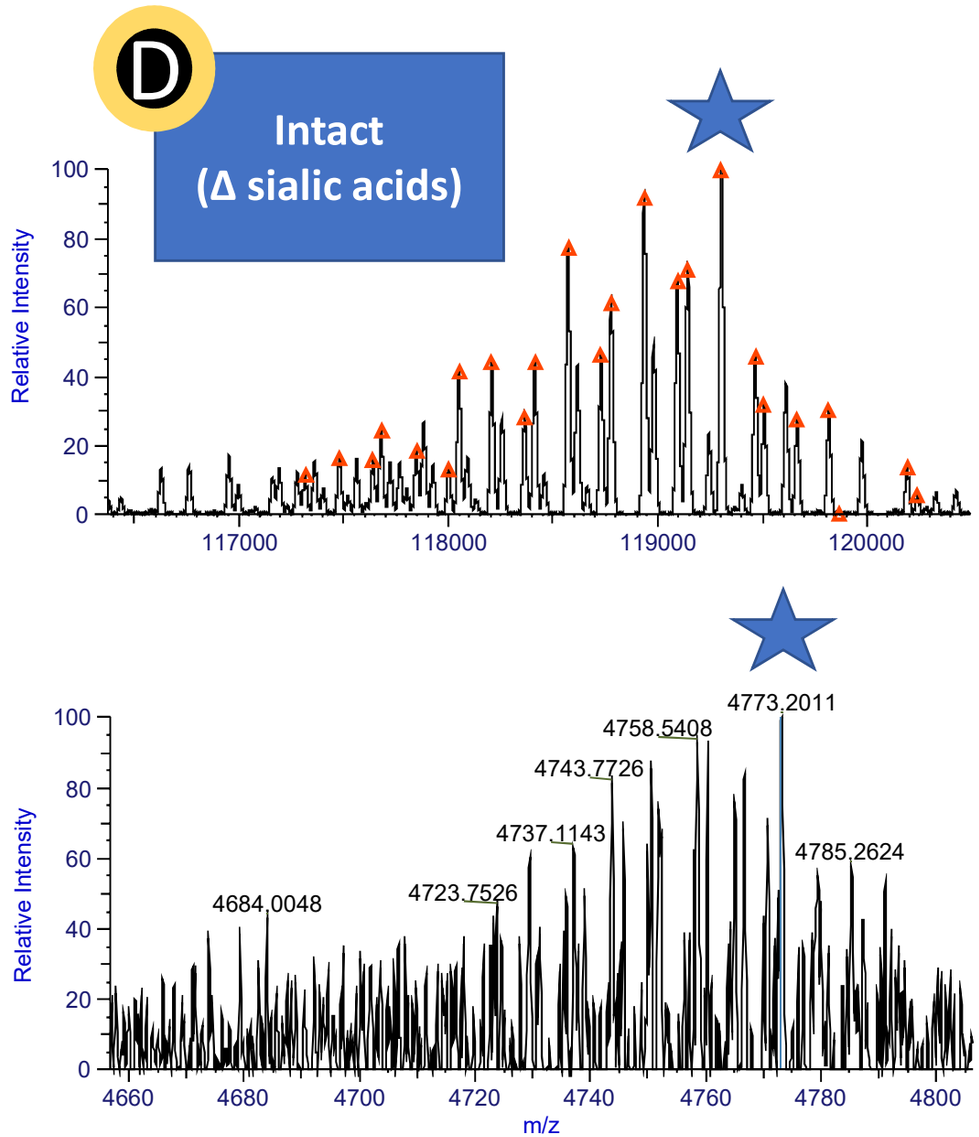
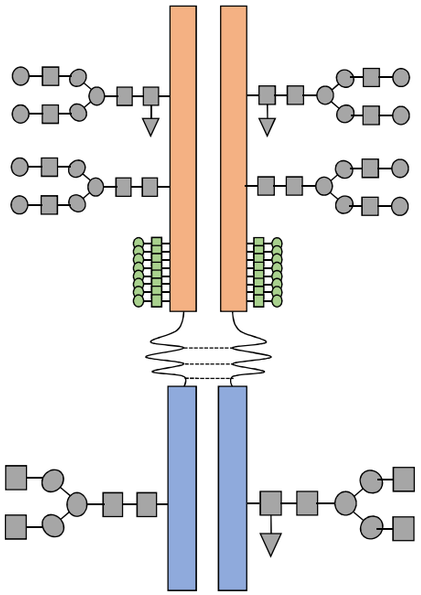
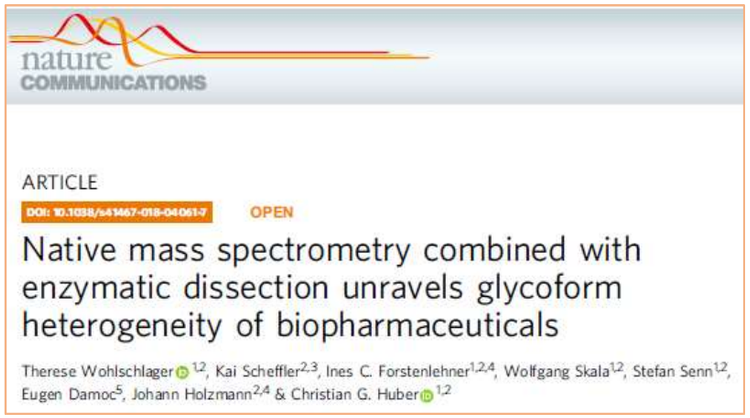
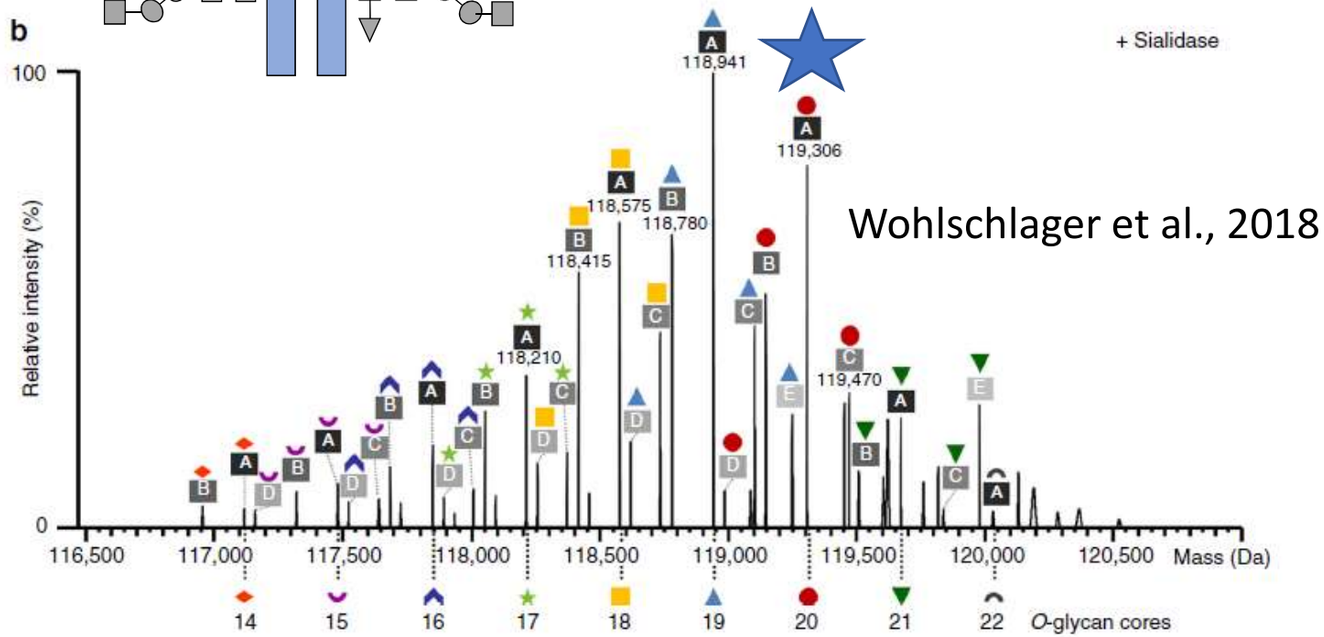


Prep D – Autosampler loop inject (native nano-ESI)







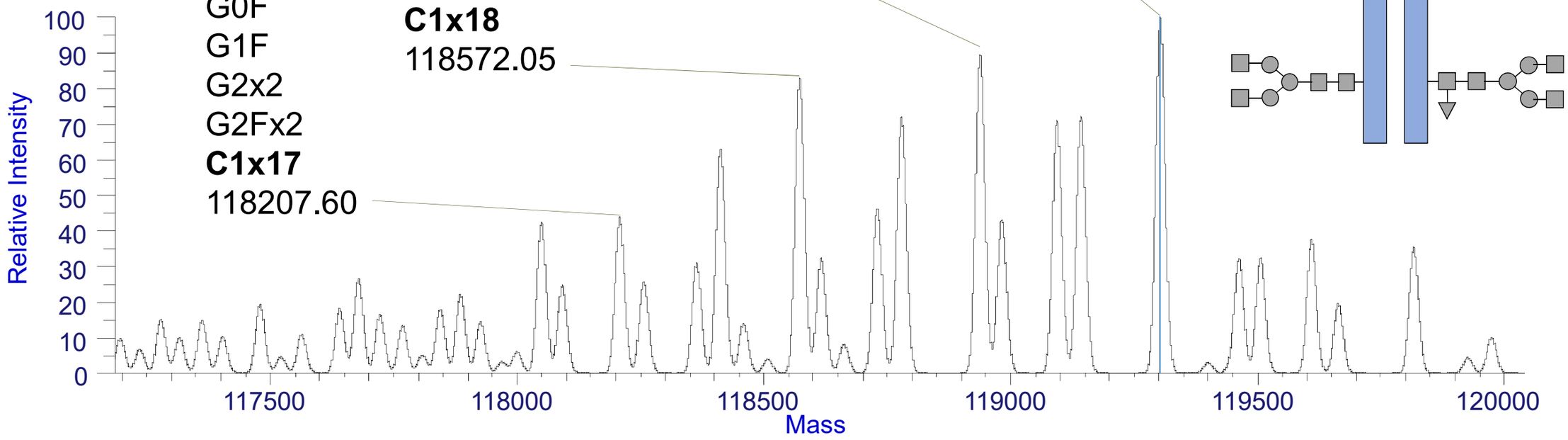


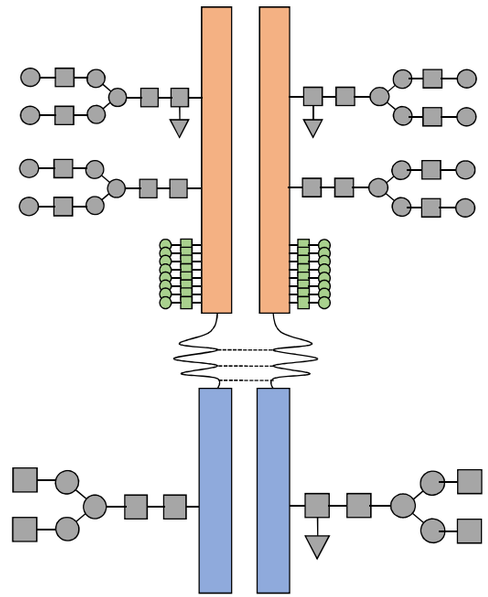
Wohlschlager et al., 2018

Prep D – Autosampler loop inject (native nano-ESI)

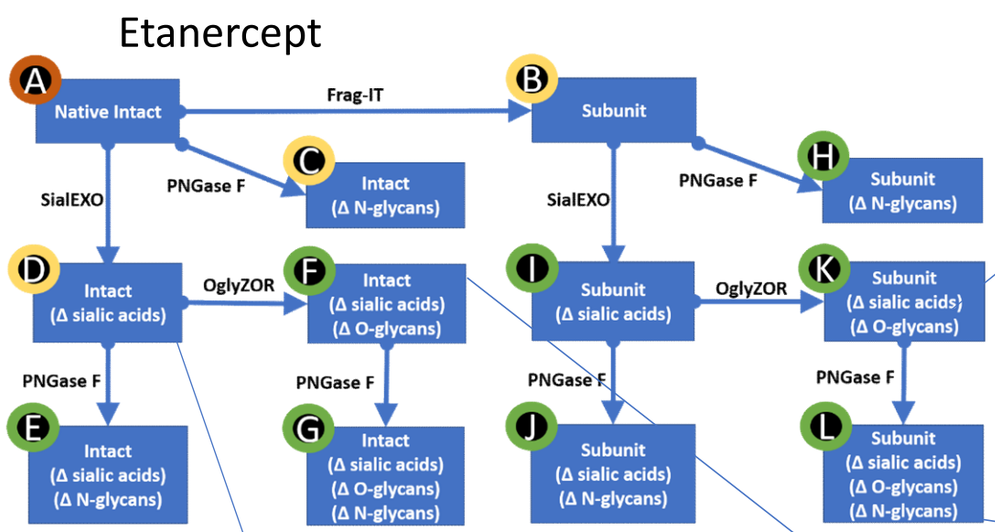
D

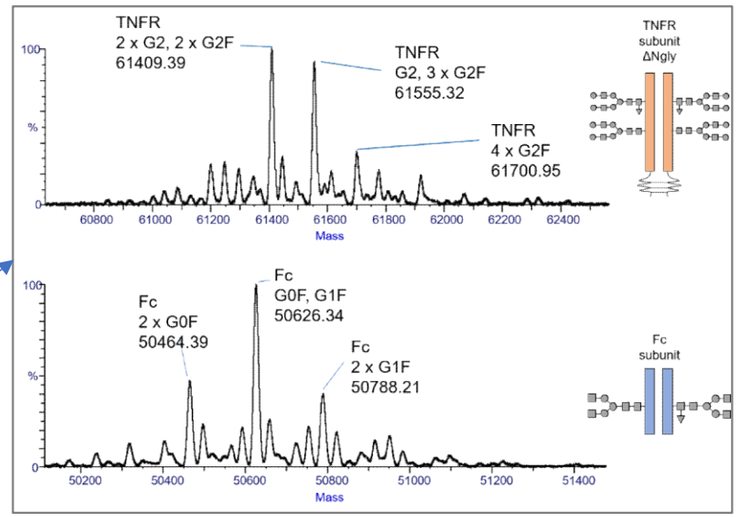
Intact
(Δ sialic acids)

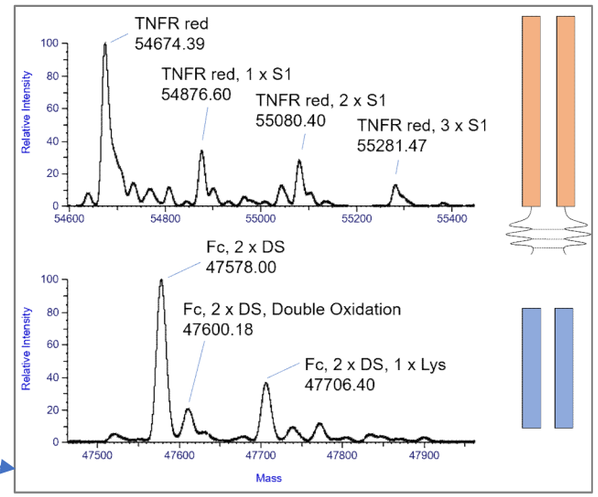


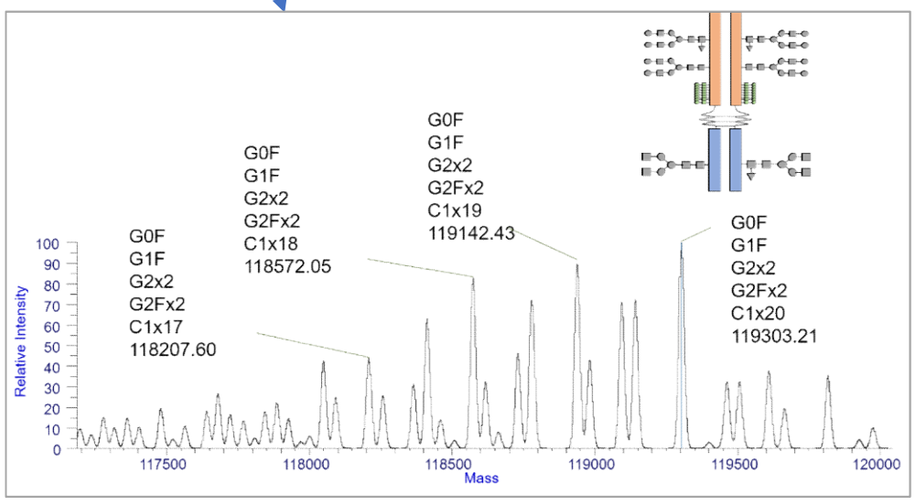


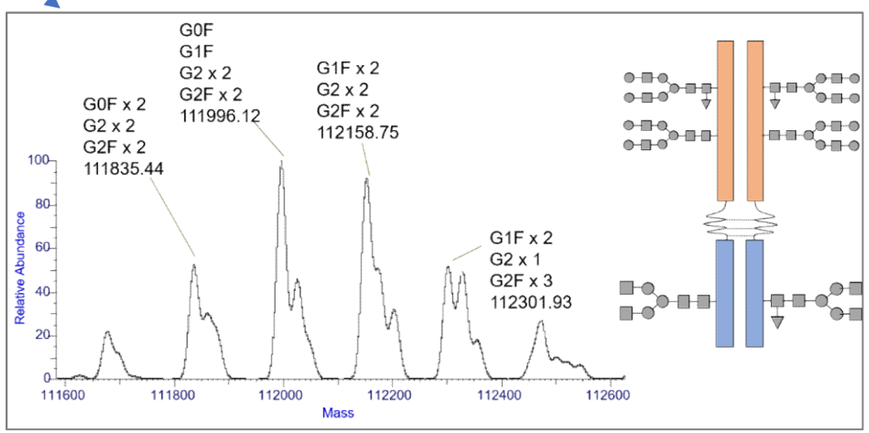
Automated native LC-MS (tested both SEC and nano loop inject)



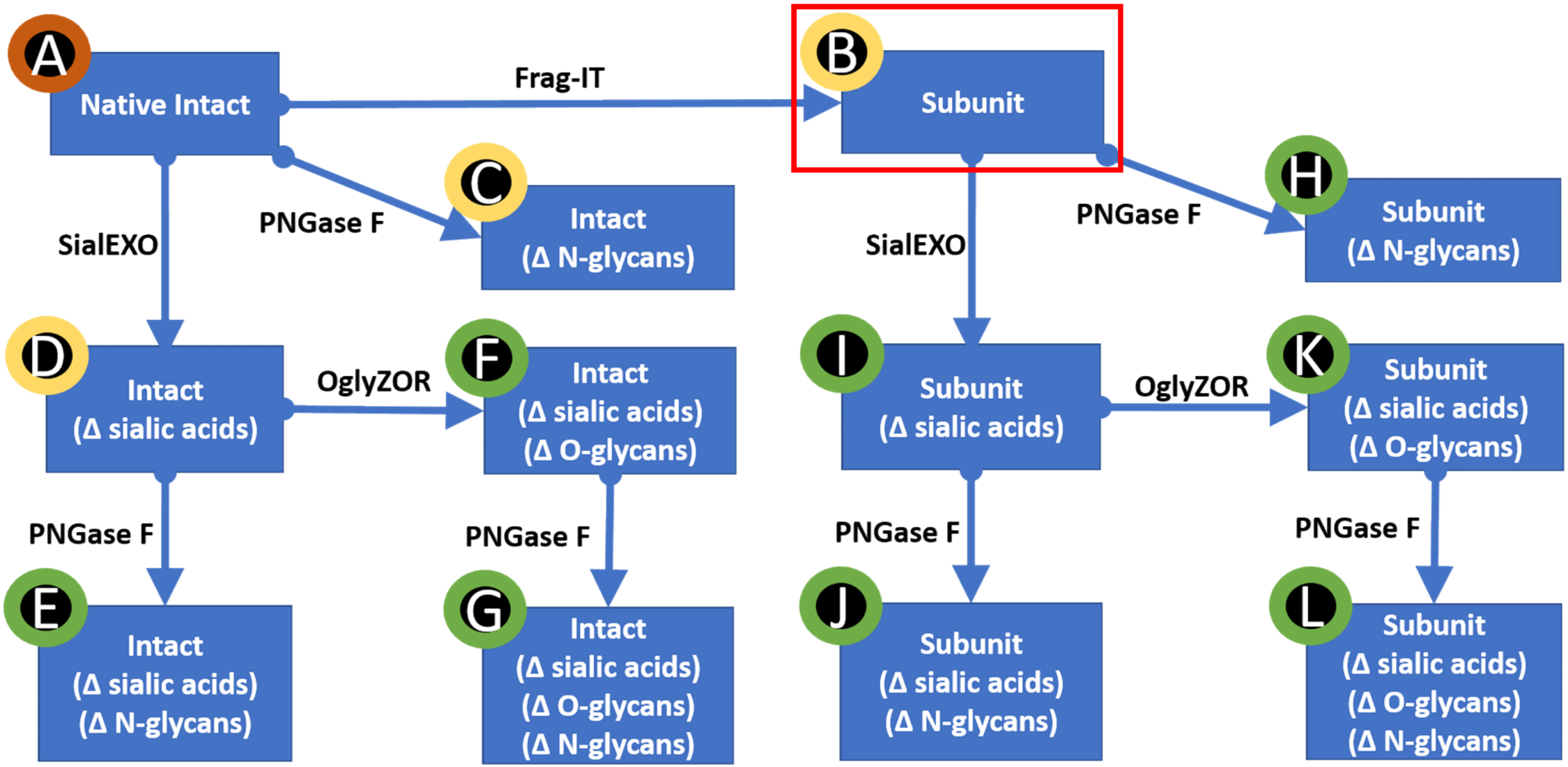




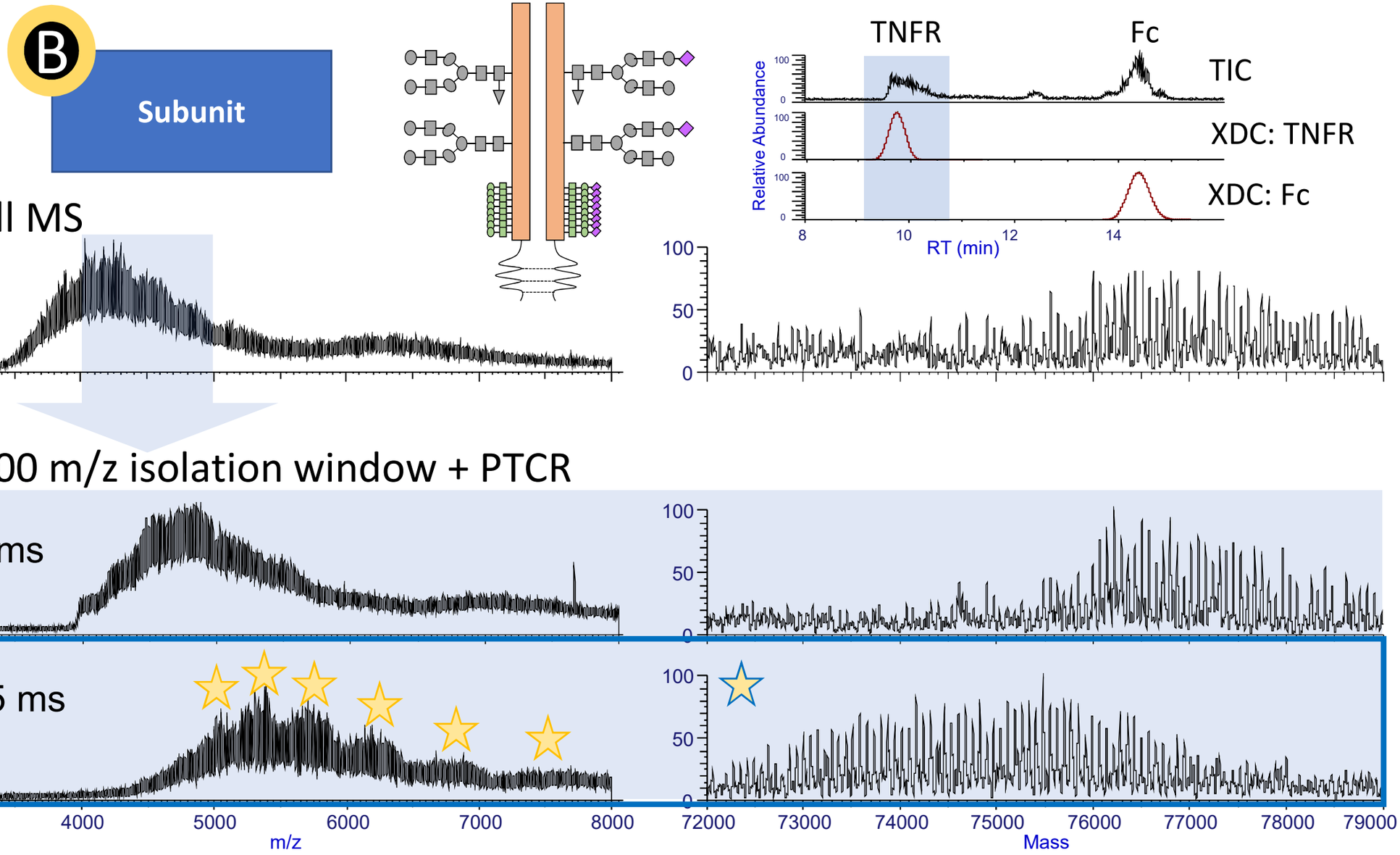




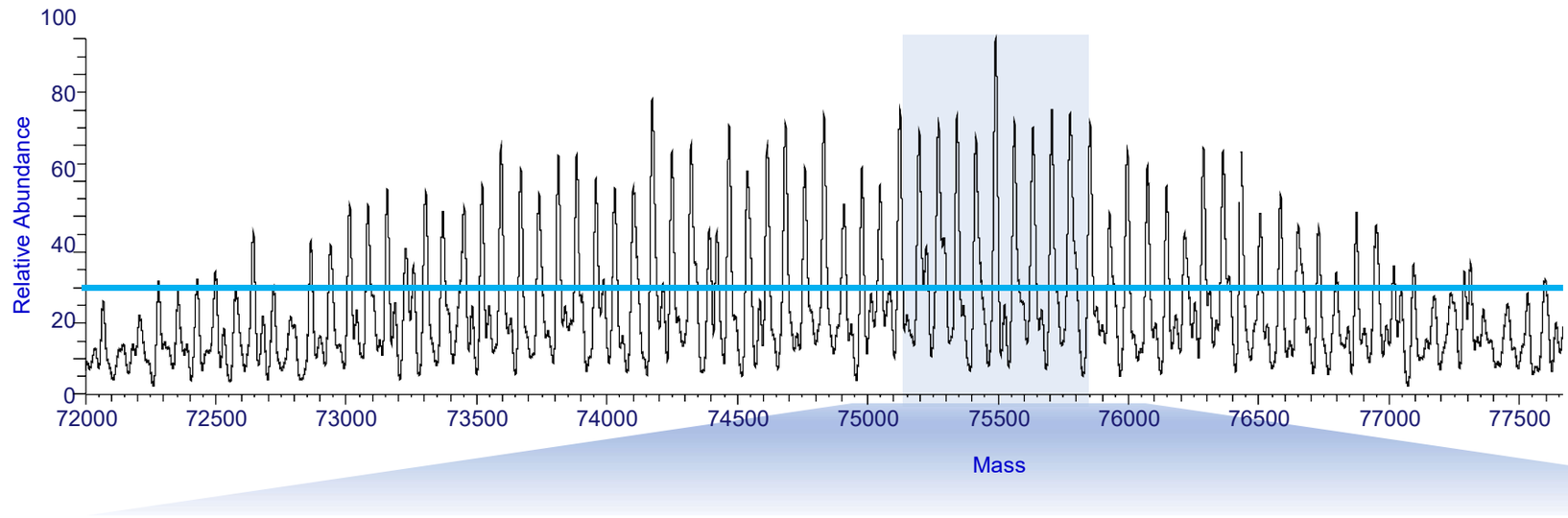
Sample Prep for Native Intact Analysis of Etanercept

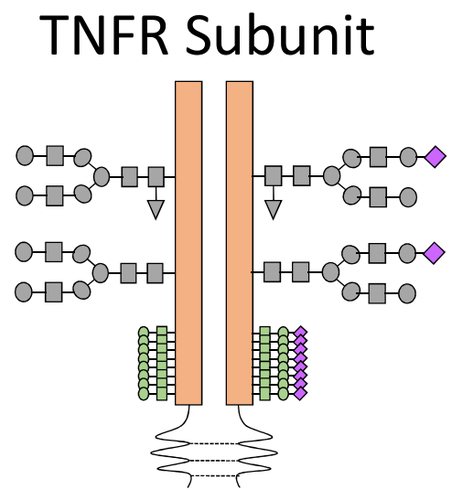


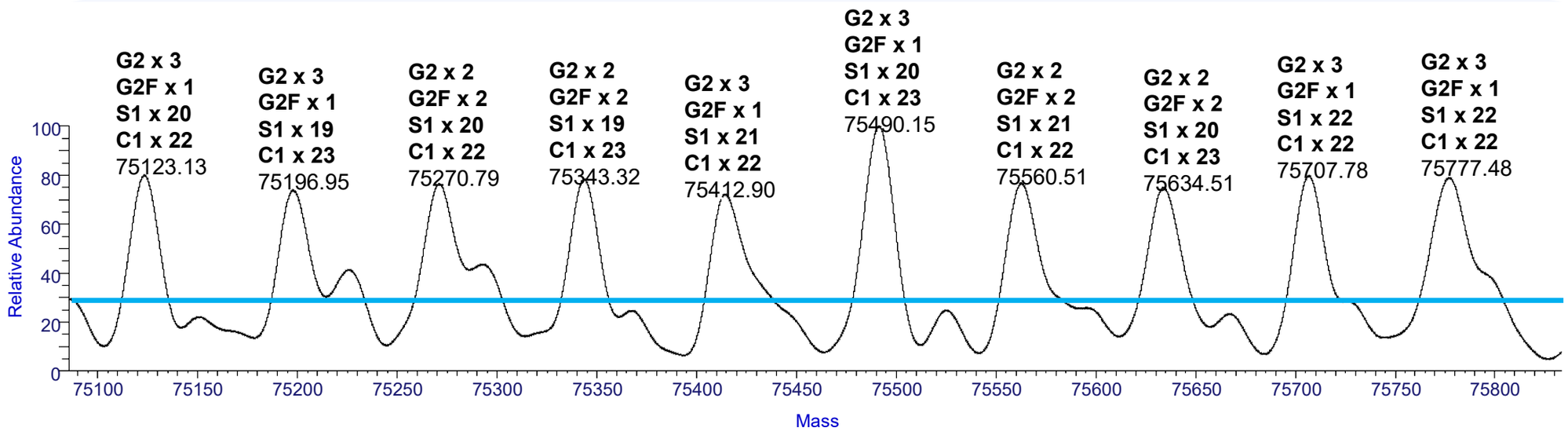
Prep B – TNFR Subunit



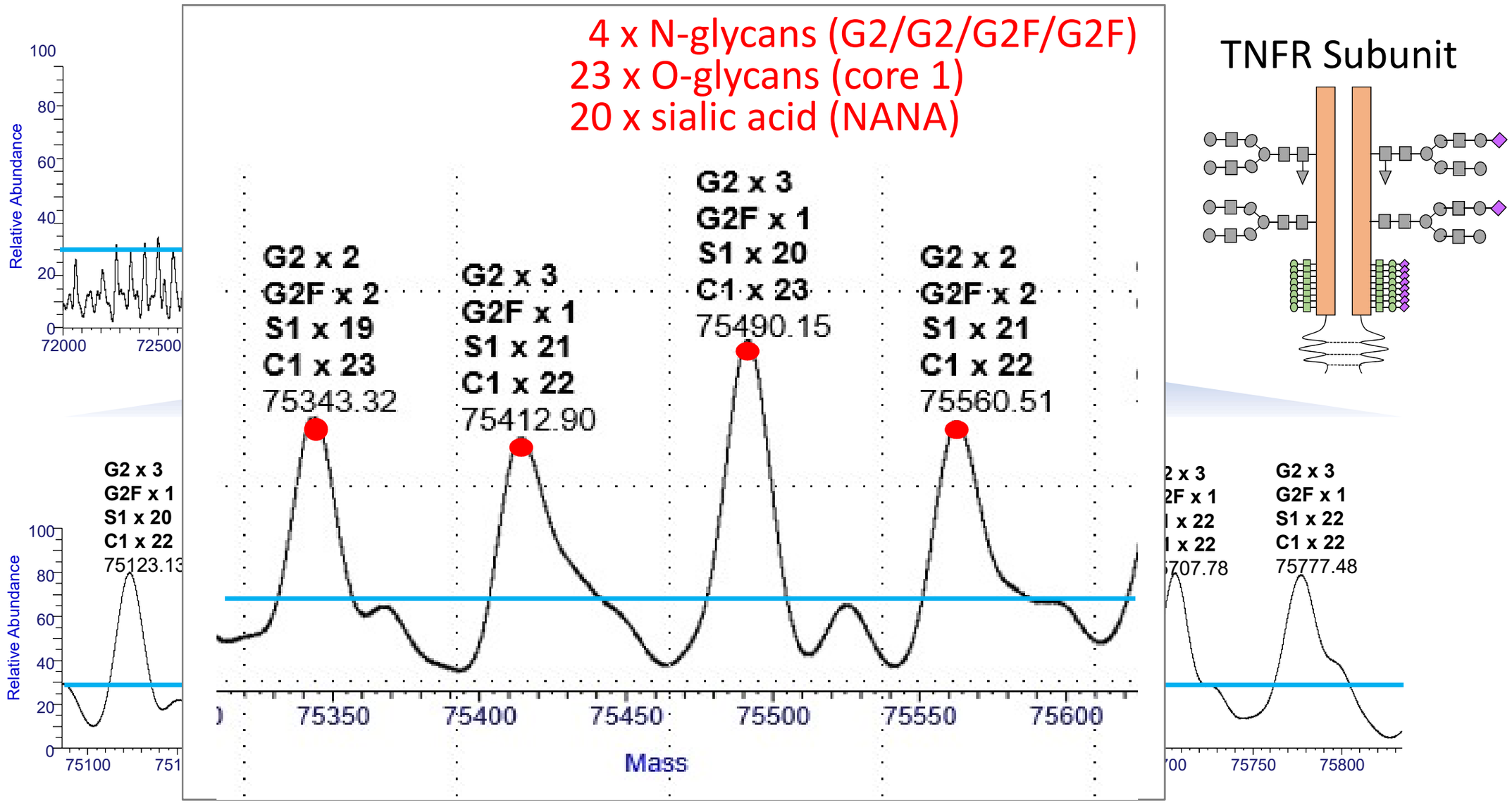
94 isoforms identified above 30% abundance level, within 50 ppm mass accuracy





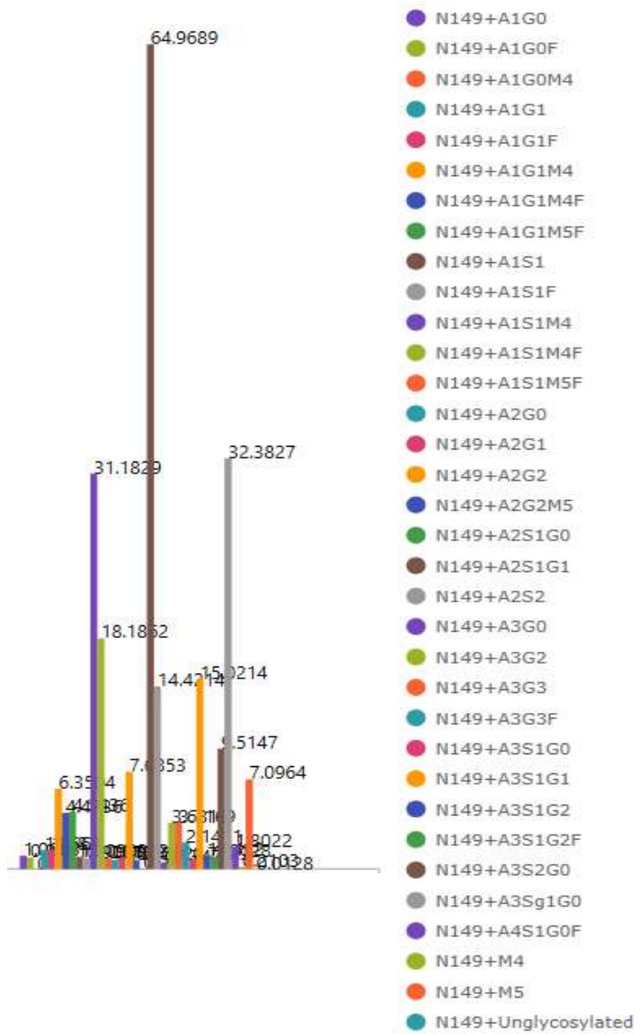


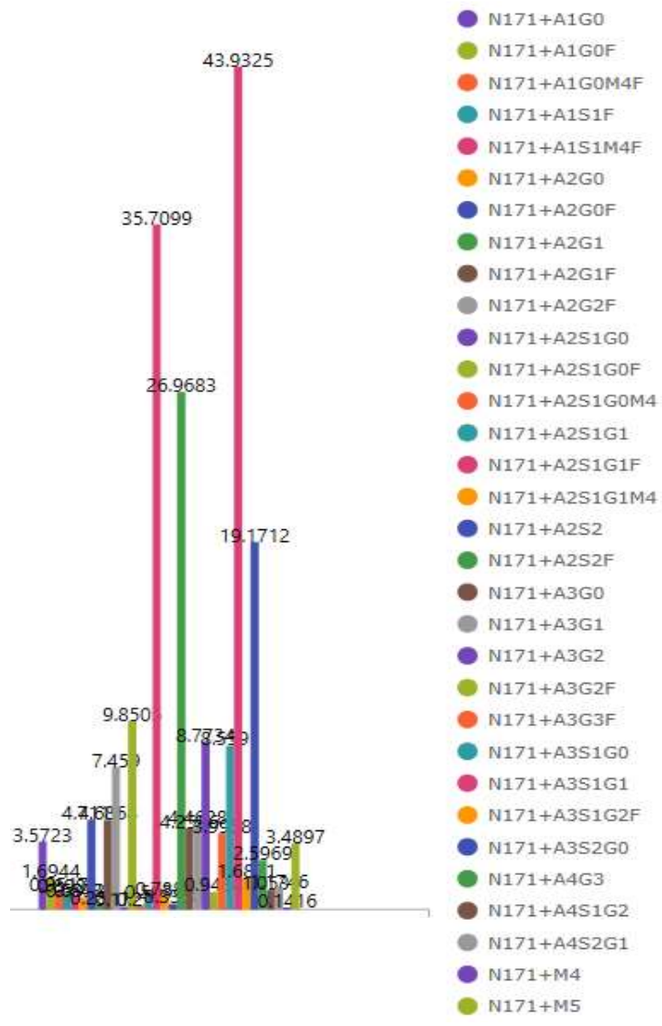
94 isoforms identified above 30% abundance level, within 50 ppm mass accuracy



End goal is to align intact data with highly complex glycopeptide map data

TNFR N-glycan sites



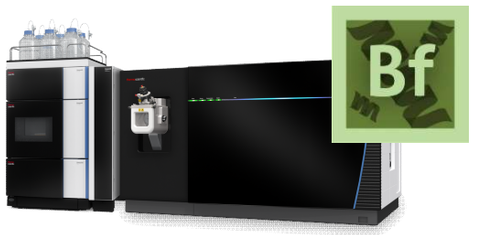


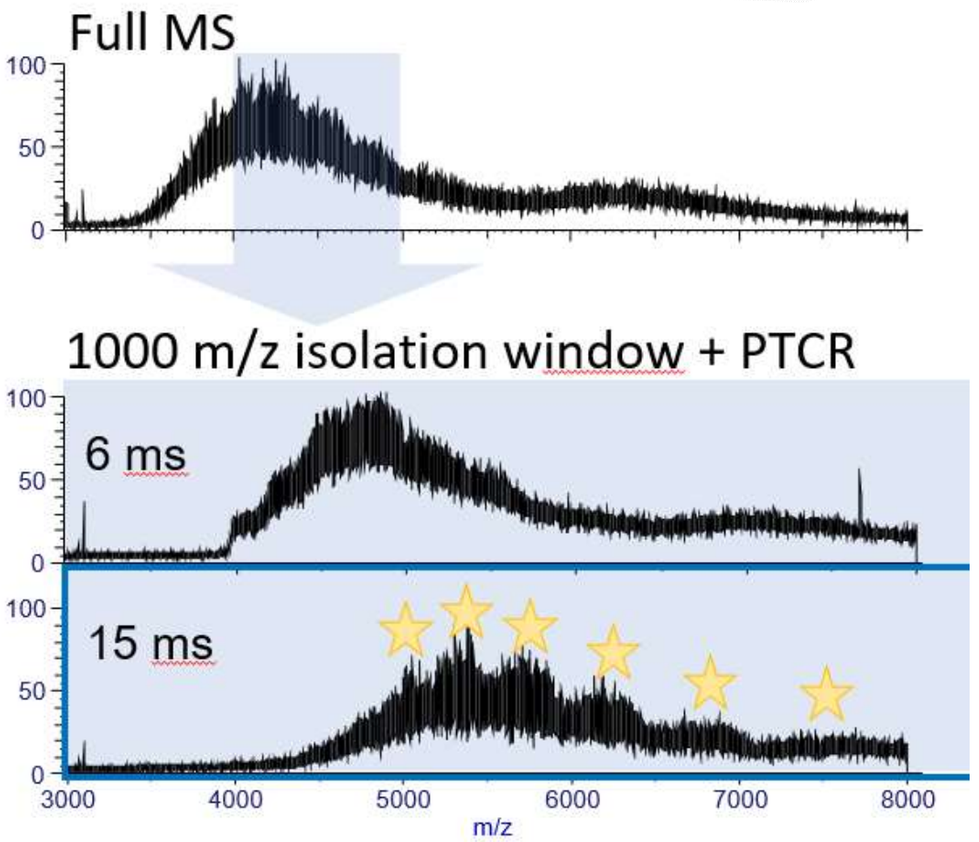
TNFR O-glycan sites

186	S186+GalNAc-3SG	O-Glycan	45.9604
186	S186+GalNAc-6S-3SG	O-Glycan	12.2392
199	~S199+GalNAc-6Gn-3SG	O-Glycan	55.7023
200	~T200+GalNAc-6GGn-3SG	O-Glycan	34.0920
200	~T200+GalNAc-6Gn-3G	O-Glycan	26.9155
213	~		
217	~		
218	~		
226	S		
186	S186+GalNAc-3G	O-Glycan	22.0349
186	S186+GalNAc-6GGn-3G	O-Glycan	56.7437
186	~S186+GalNAc-6GGn-3SG	O-Glycan	7.7420
200	T200+GalNAc-6GGn-3SG	O-Glycan	27.9970
200	~T200+GalNAc-3SG	O-Glycan	27.7357
200	~T200+GalNAc-6Gn-3G	O-Glycan	10.3988
200	~T200+Ga		
200	~T200+Ga		
202	~S202+Ga		
226	S226+Gal		
309	~T309+Ga		
309	~T309+Ga		
186	S186+GalNAc-6G...	O-Glycan	6.4239
186	S186+GalNAc-6G...	O-Glycan	74.3229
199	~S199+GalNAc-3SG	O-Glycan	42.2293
199	~S199+GalNAc-6...	O-Glycan	40.2466
200	~T200+GalNAc-3G	O-Glycan	0.8195
200	~T200+GalNAc-6G...	O-Glycan	22.8685
200	~T200+GalNAc-6Gn	O-Glycan	0.6567
200	~T200+GalNAc-6S...	O-Glycan	2.1785
213	~T213+GalNAc-6S...	O-Glycan	100.0000
226	~S226+GalNAc-3SG	O-Glycan	100.0000
232	~S232+GalNAc-6S	O-Glycan	100.0000
287	S287+GalNAc-3G	O-Glycan	2.7387
287	S287+GalNAc-3SG	O-Glycan	54.0540
287	S287+GalNAc-6S-...	O-Glycan	48.0036

Thoughts on using PTCR for measuring isoform distributions

- PTCR method requires that m/z window is **isolated** prior to reaction
  - Ion trap-based isolation (not MS1)
  - Concentrated ion population → improved ion statistics
- *Best way to avoid skewed isoform distribution?*
  - Need to measure 'true' isoform profile
  - ***How many charge states should we ideally isolate?***
- Rapid, confident methods create greater need for aligning intact data with peptide map data



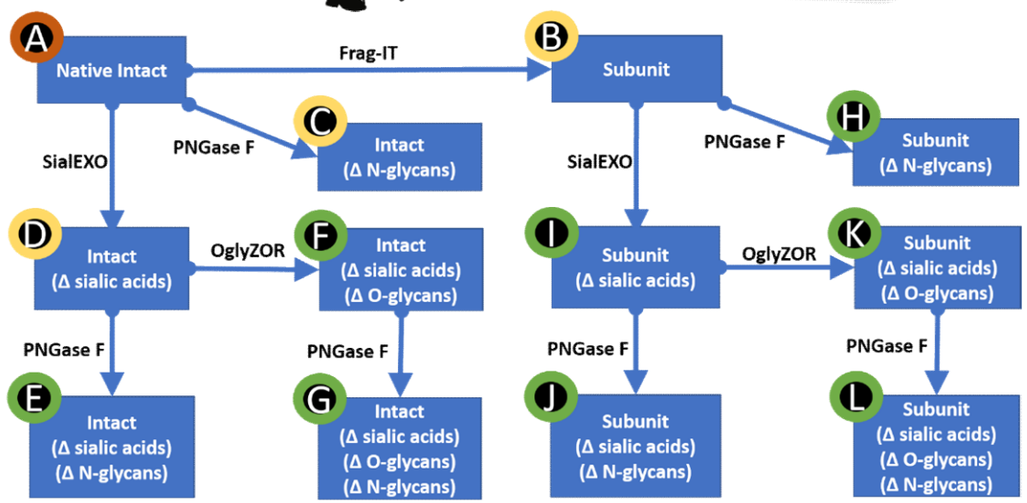


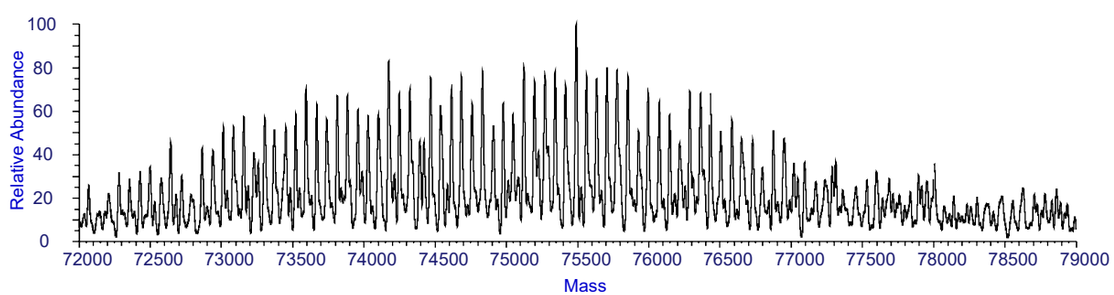
Summary

- Native SEC-MS + PTCR charge reduction is an automatable, state-of-the-art approach for intact mass analysis of highly complex biologic drugs

- Orbitrap Eclipse MS provides new means to analyze intact microheterogeneous protein isoform mixtures on a single MS platform

- Please feel free to contact us for questions or project inquiries





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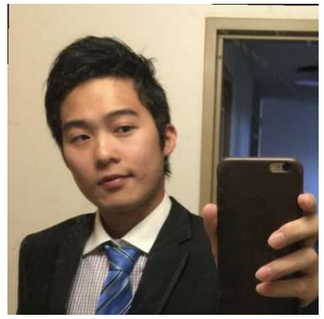

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