Elucidation, Characterization and Monitoring of a Unique Tyrosine Sulfation Post-translational Modification During Bispecific Antibody Process Development and Scaleup

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Analytical Development and Attribute Sciences
Biologics Development

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CASSS-Mass Spectrometry
Molecular Design, Developability and Biotransformation

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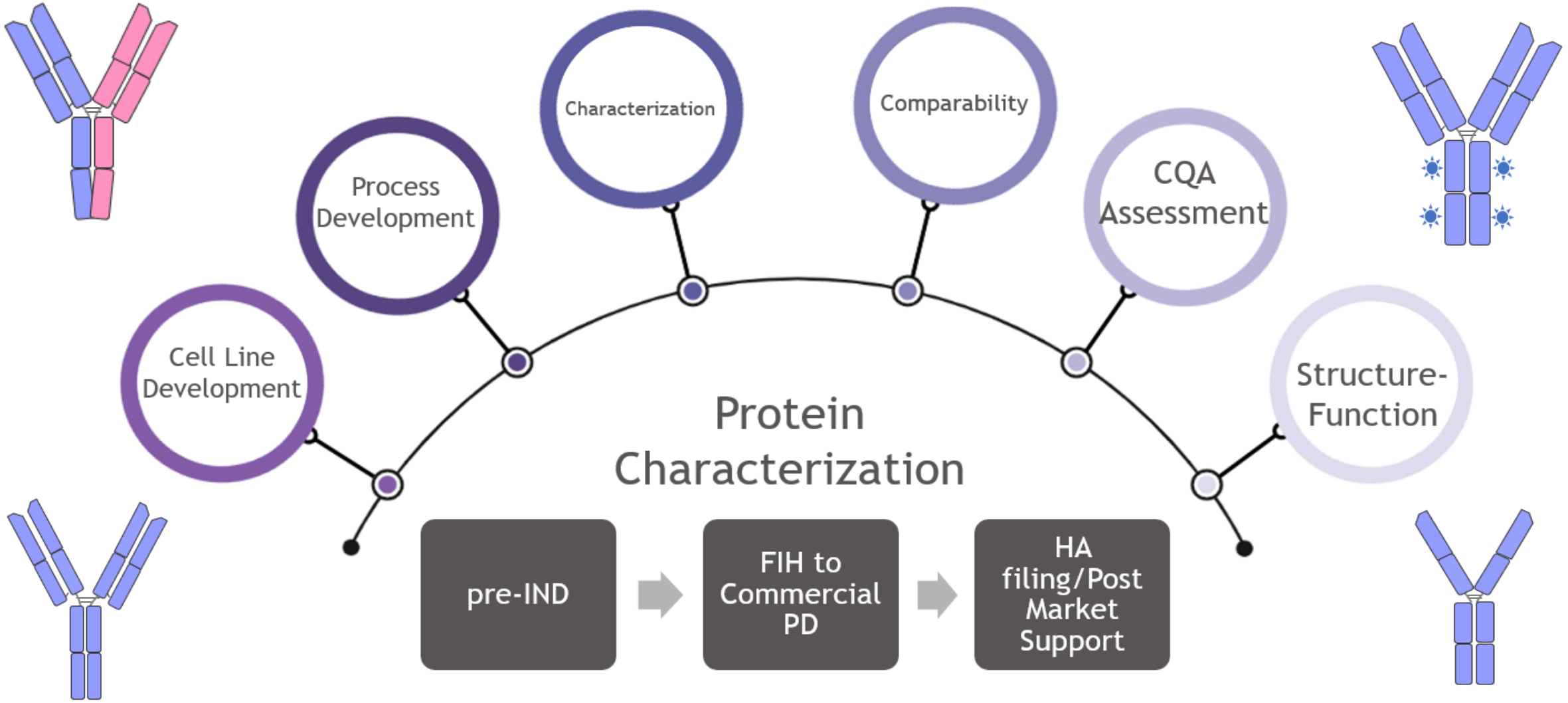
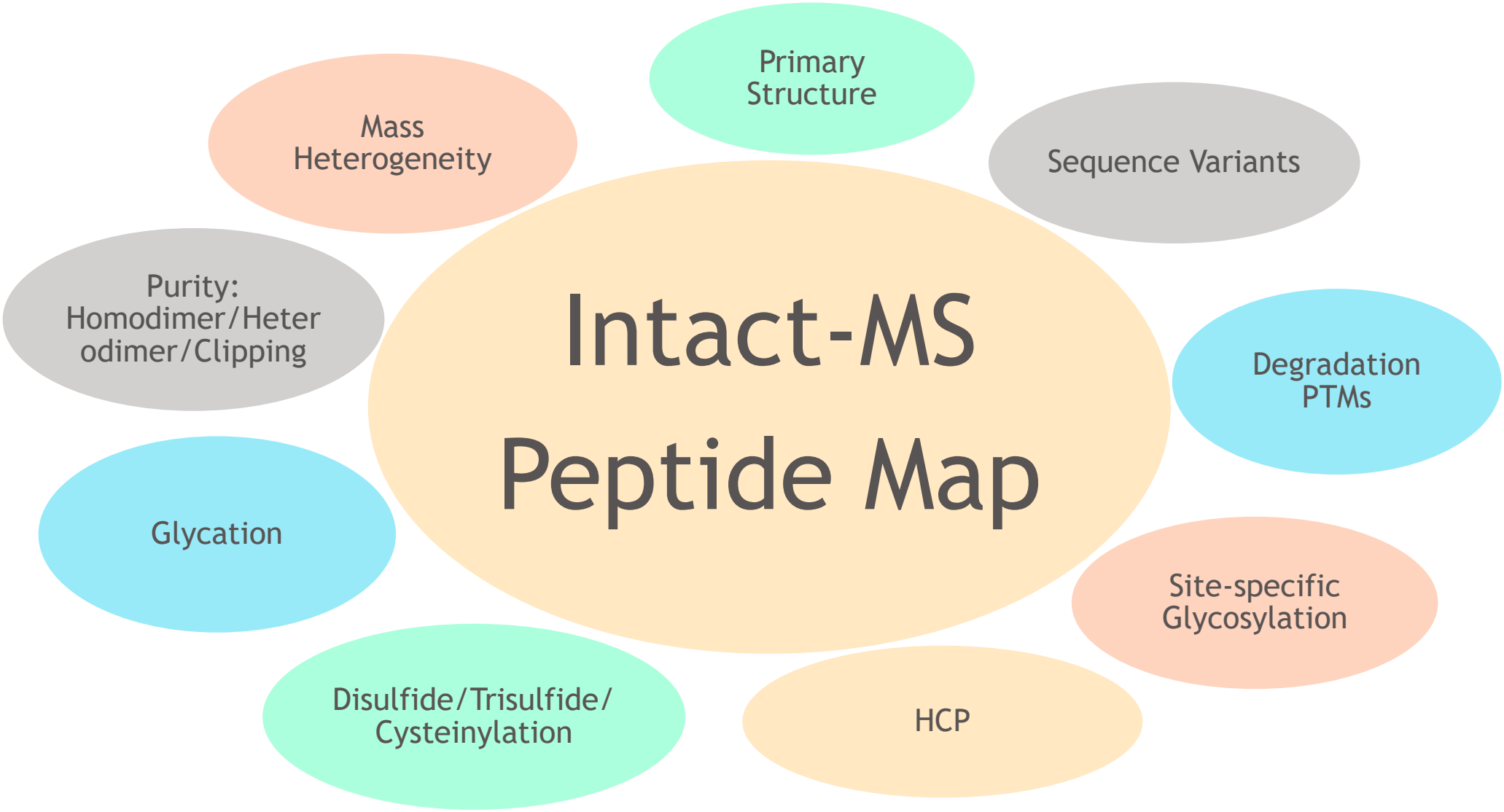
Protein Characterization Plays an Integral Role in the Development of Biologics Products



MS Role in Protein Characterization



Presentation Outline

✓ Tyrosine Sulfation Characterization

- Tyrosine Sulfation Introduction and Molecule Background Information

- Identification Site of Sulfation
  - Multi-Enzymatic Proteolytic Digestion Approach
  - Synthetic Peptides
  - EAD Fragmentation

✓ LC-MS for Cell Line Development & Process Development Support

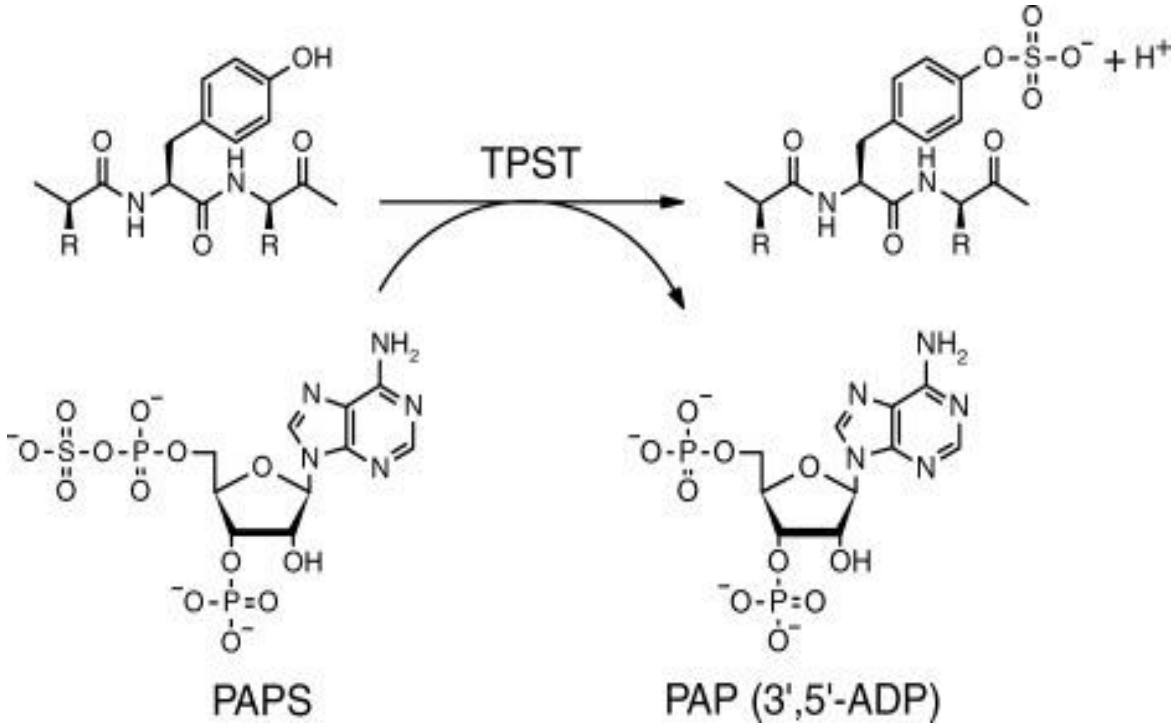
- High-throughput Sulfation LC-MS Intact Mass Method Development

- LC-MS Support of CLD and Process Development Efforts to Reduce Sulfation During Protein Therapeutic Process Development

Sulfo-Tyrosine- Background

Recent Literature

- Tyrosines are sulfated by tyrosylprotein sulfotransferase (TPST) in the Golgi
- TPST motifs are associated with adjacent E/D residues
- AEX purification step removed sulfo-tyrosine species (lower yield).
- Sodium chlorate at 16 mM inhibited tyrosine sulfation by more than 50%- no impact on antibody titer or quality.



Seibert and Sakmar. Pep.Sci. ***2008****, 90, 459-477*

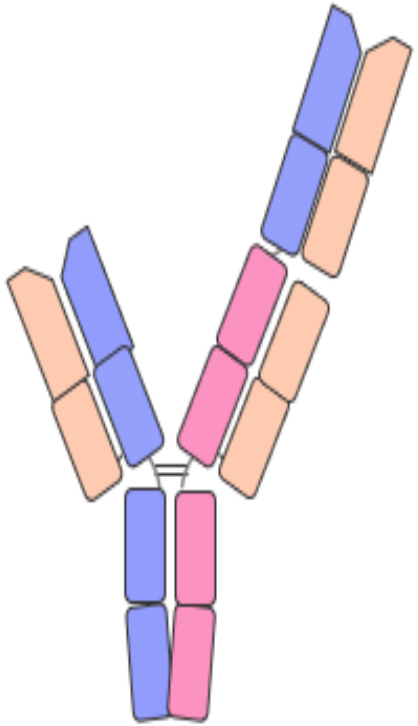
Zhao et al. Mabs, 2017, 9, 985-995

Tyshchuk et al. Mabs, 2019, 11, 1219-1232

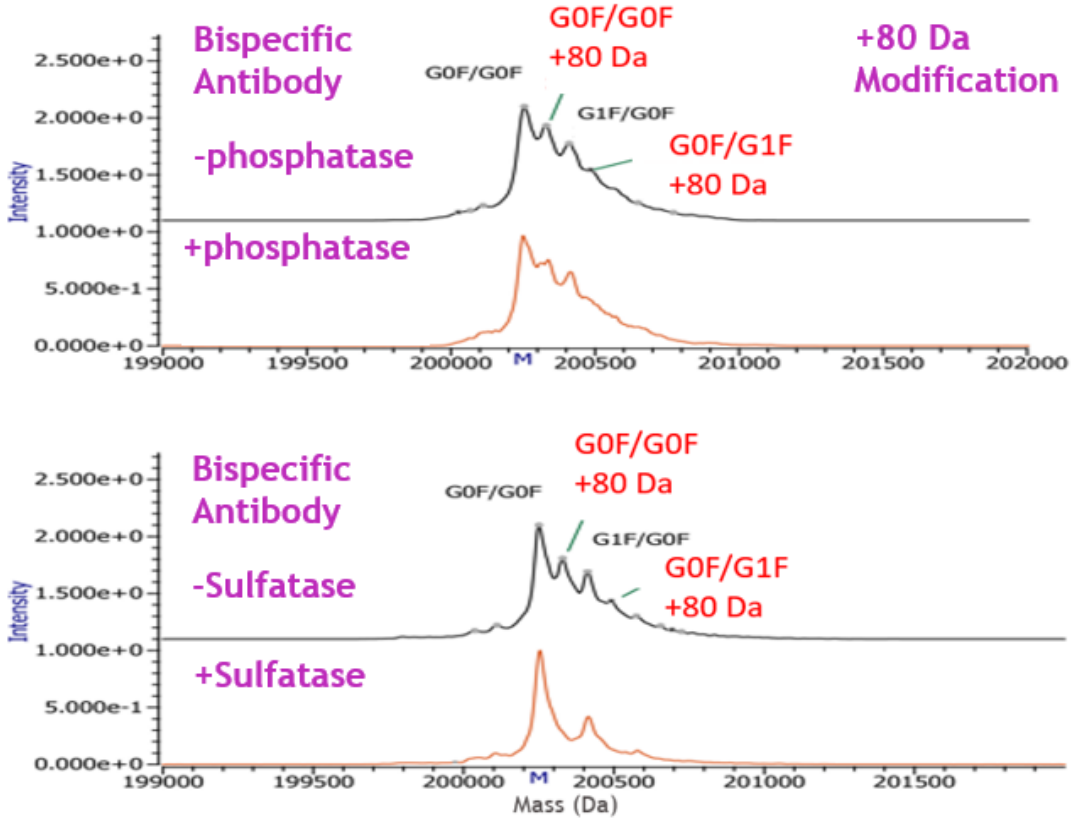
Liu et al. Biotech J. 2021

Discovery Data
Phosphatase & Sulfatase Treatment Confirms Sulfo-Tyrosine

2+1 Bispecific

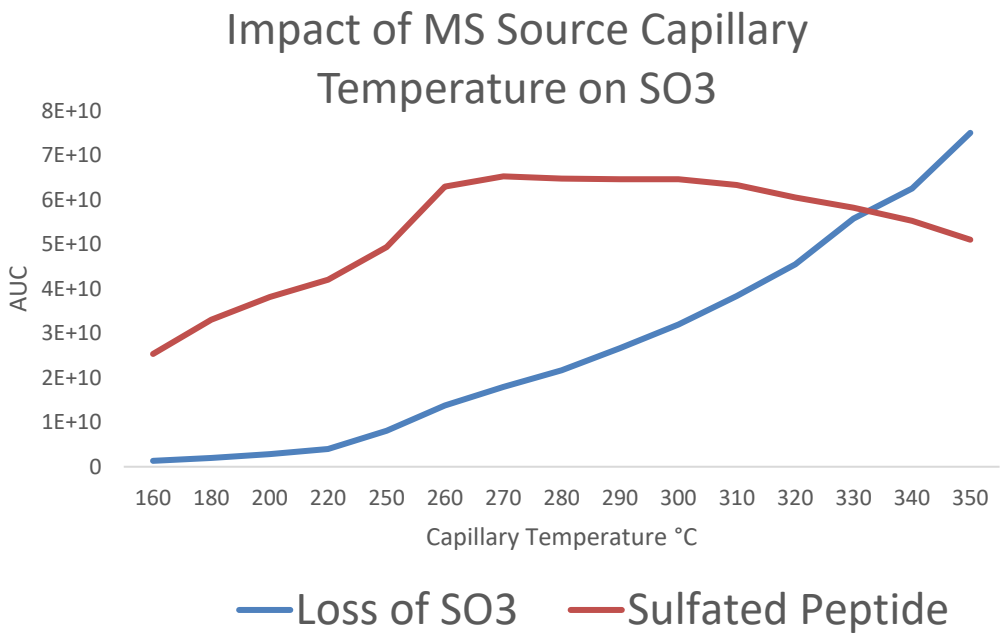


Intact Mass Analysis

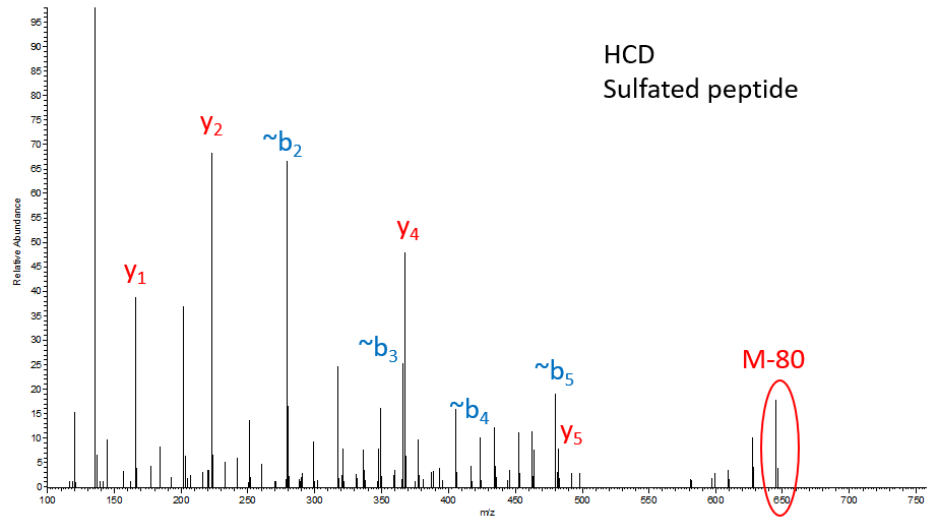


Identifying Site of Sulfation- Challenges for Quantitation and Localization Using MS

- Sulfotyrosine is highly labile: MS Source Parameters Can Induce Loss of SO3

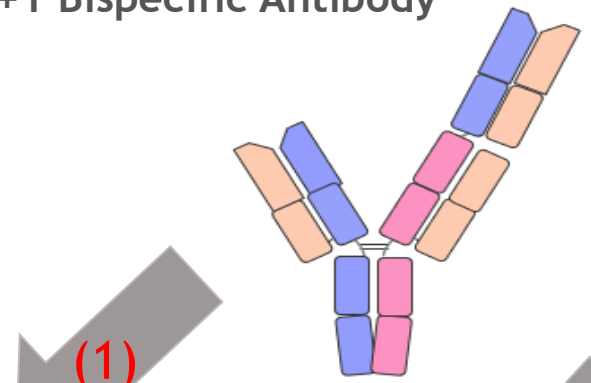


- Complete Loss of sulfate SO3 (80 Da) Prior to Fragmentation-CID or HCD.
- Impossible to Identify Site if ≥ 2 Y Present on the Peptide



Summary of Multi-Enzymatic Proteolytic Digestion Approach to Identify Site of Tyrosine Sulfation

2+1 Bispecific Antibody



(1) **Trypsin digestion** identified a +80Da modification on tryptic peptide:

R.XXXXDY^{1}Y^{2}Y^{3}DXXXXXXXY^{4}Y^{5}Y^{6}XXXXXXXXXXXXXXXK.X

Identification with ETD

(2) **Asp-N digestion** identified a +80Da modification on HC CDR peptide:

DY^{1}Y^{2}Y^{3}.D

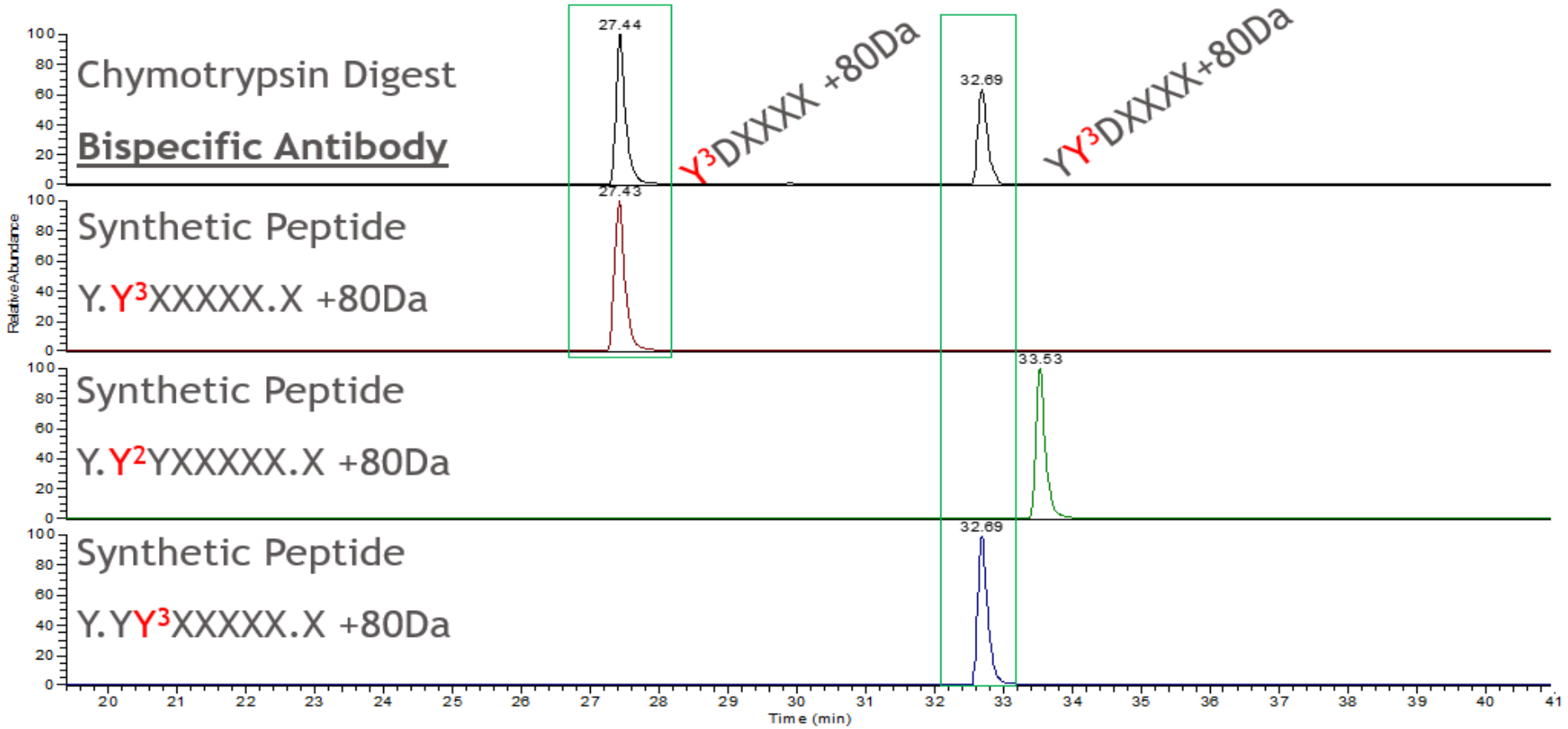
(3) **Chymotrypsin digestion** identified a +80Da modification on HC CDR peptides:

Y.Y^{3}DXXXX.X and Y.Y^{2}Y^{3}DXXXX.X

(4) **Synthetic peptides** confirmed Y^{3} on HC CDR region as site of sulfation

Synthetic Peptides Confirmed Tyrosine Sulfation on Y^3

- XIC of Chymotrypsin Peptides Y^3-X and Y^2-X +80Da

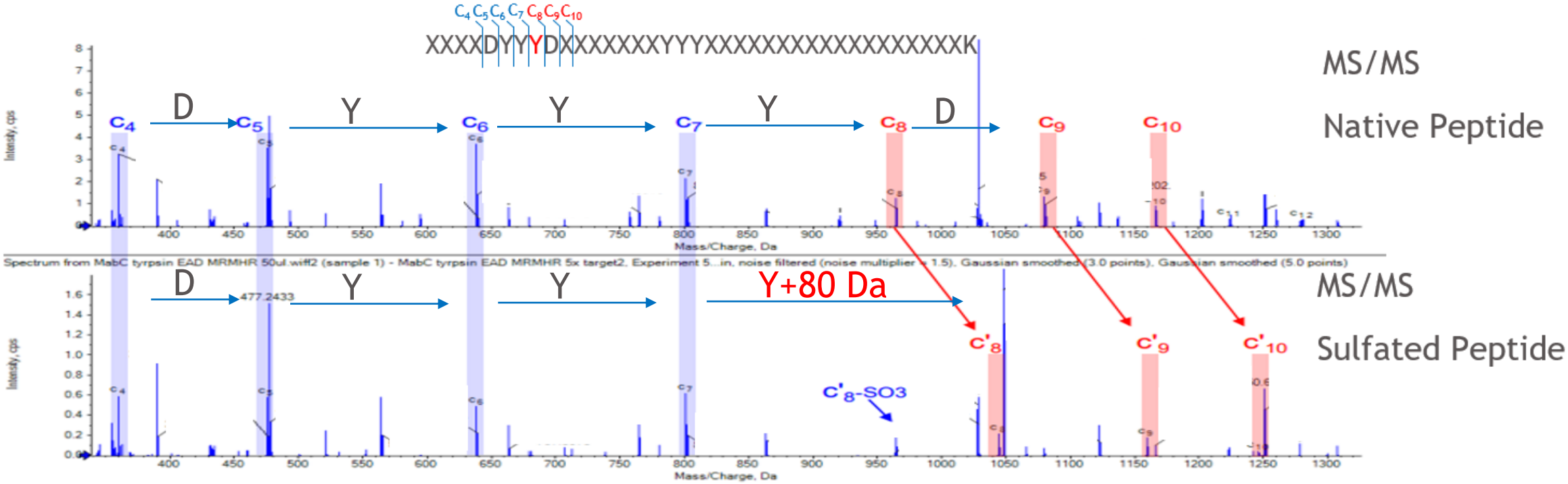


- Sulfated Peptide in Sample Digest Elute at the Same Retention Time as Y^3 Sulfated Synthetic Peptides

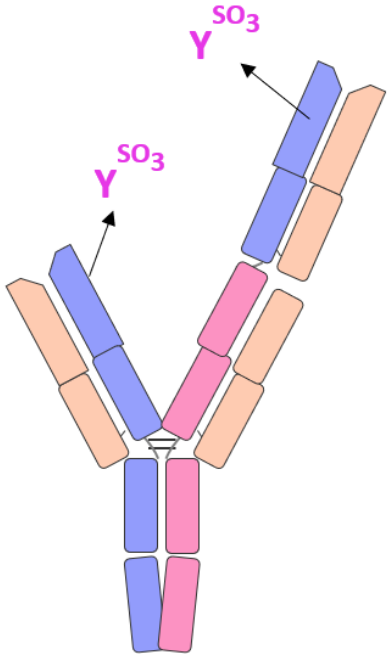
Orthogonal Electron Activated Dissociation (EAD) Confirmation: Site of Sulfation on Y^3

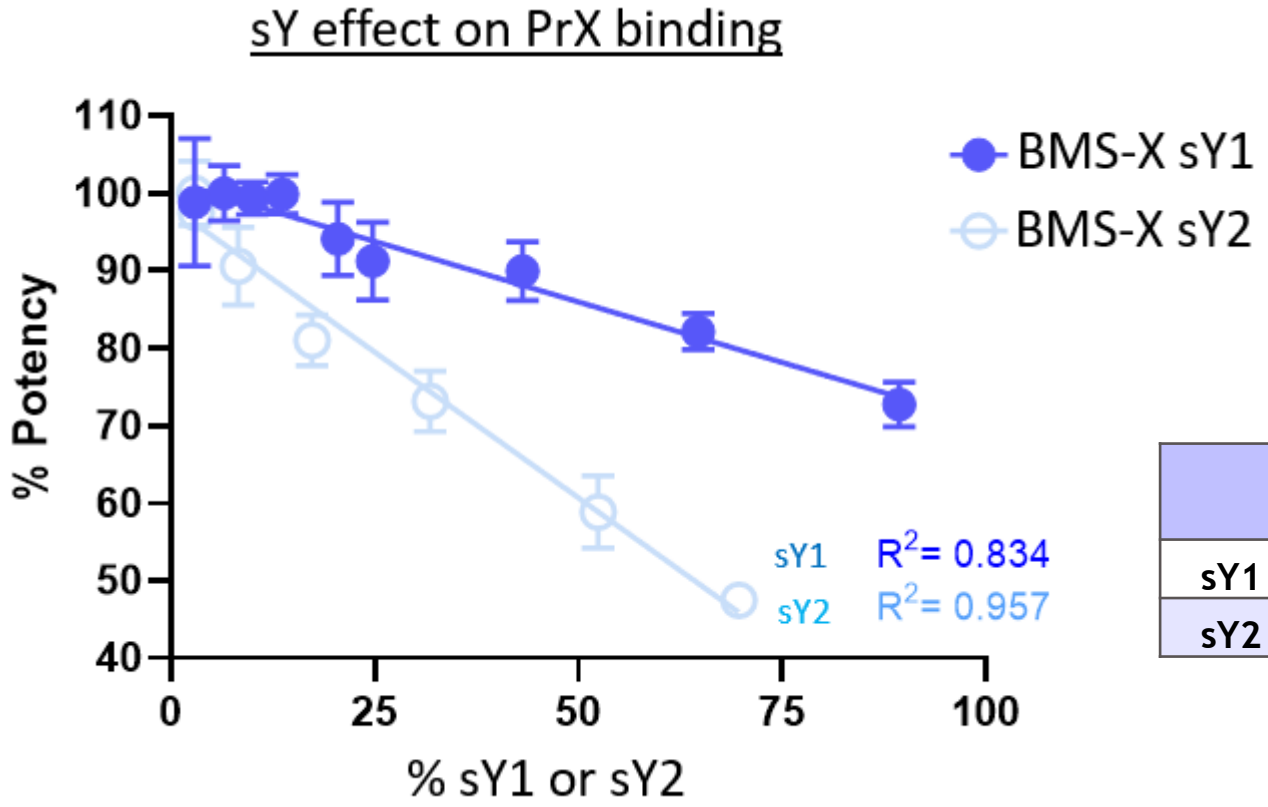
- Alternative MS/MS fragmentation such as Electron-transfer dissociation (ETD) and Electron-capture dissociation (ECD) are milder fragmentation techniques compared to CID and HCD.

- ECD/EAD generated signature ion (C_8-C_{10}) to locate sulfation on Y^3 of tryptic peptide XXXXDYYY^3DXXXXXXXYYYXXXXXXXXXXXXXXXXXK.X.



Tyrosine Sulfation Impacts Binding of Bispecific to Target





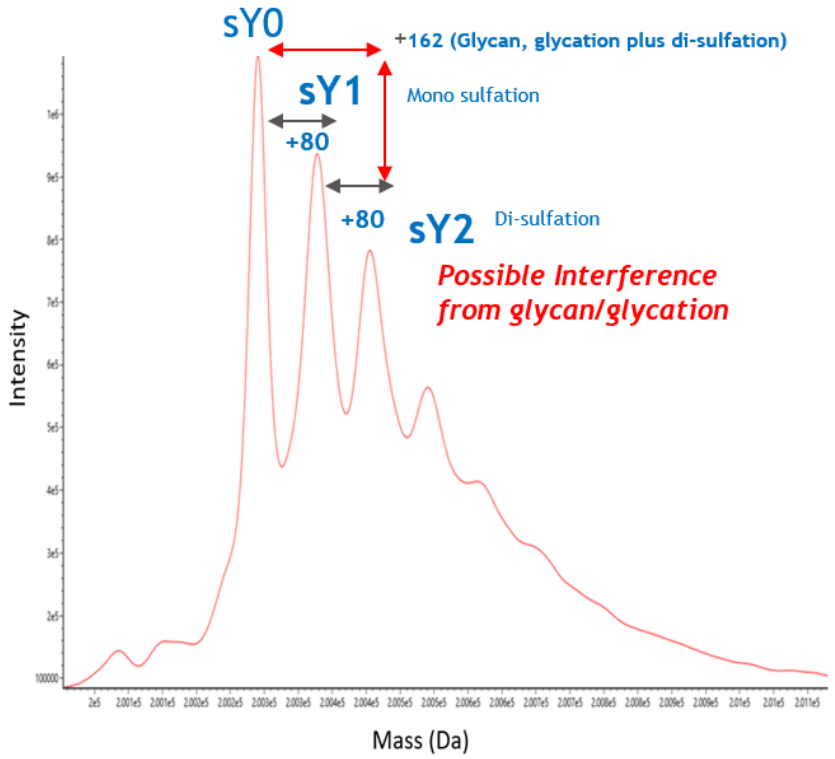
	1% sY change corresponds to change in potency of y% (slope):
sY1	-0.312
sY2	-0.752

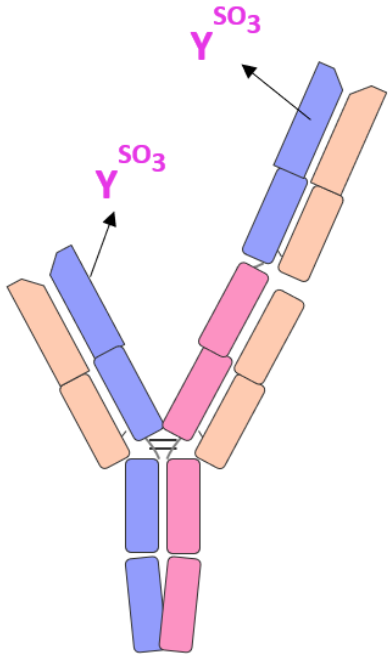
- Tyrosine Sulfation Determined to be a CQA

LC-MS Cell Line Development & Process Development Support

High-throughput Sulfation LC-MS Intact Mass Method Development for CLD and PD Support

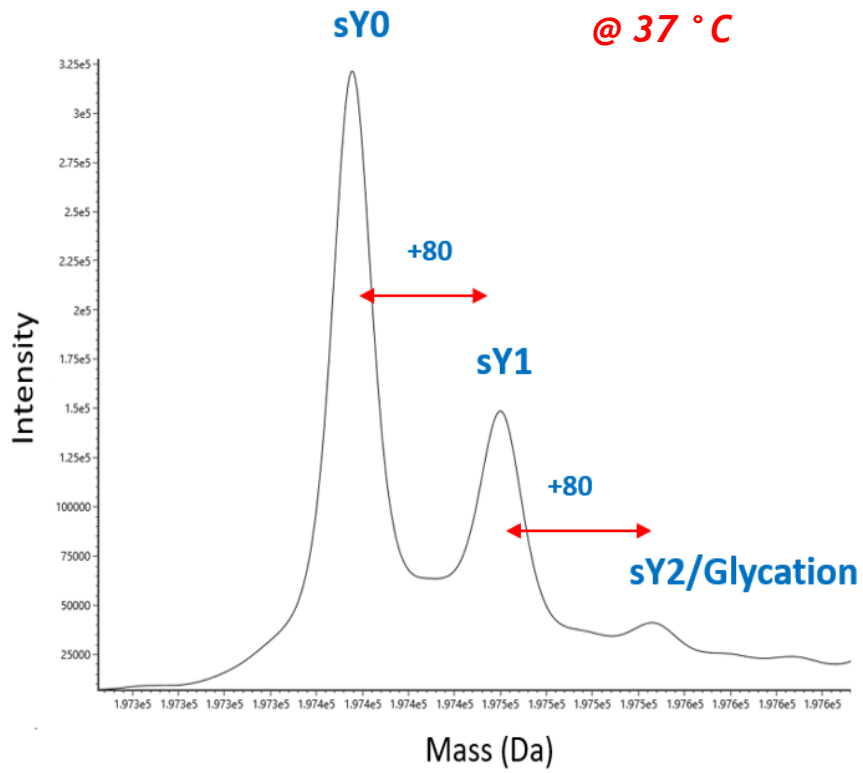
A: Glycosylated Intact





B: De-glycosylated Intact

PNGaseF 2 hr @ 37 °C

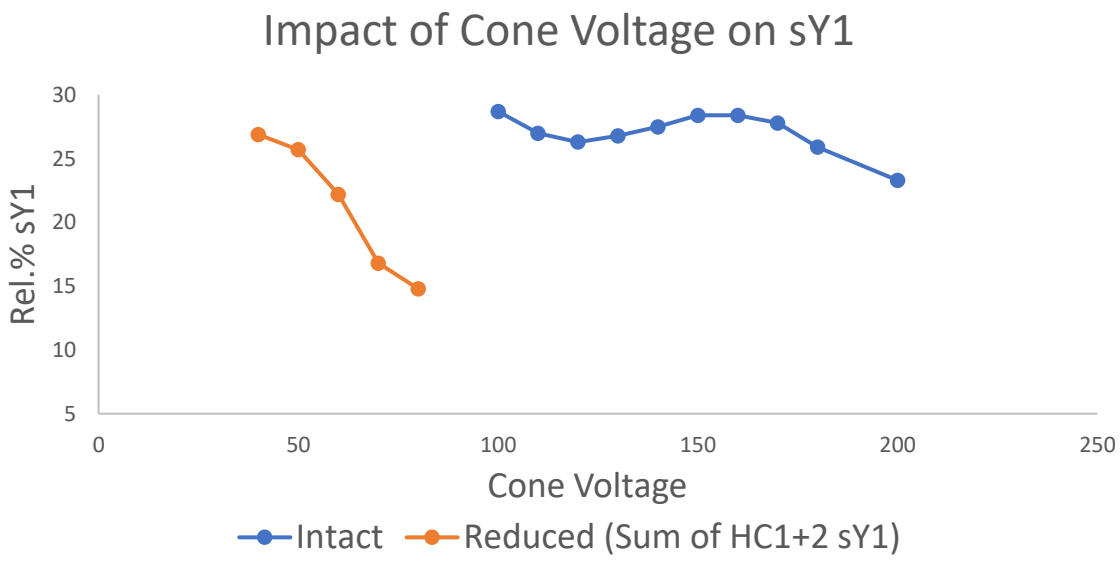


Sample Preparation: PNGaseF reduces sample complexity and possible interference from glycans

High-throughput Sulfation LC-MS Intact Mass Method Development: Comparison of Intact MS Approaches

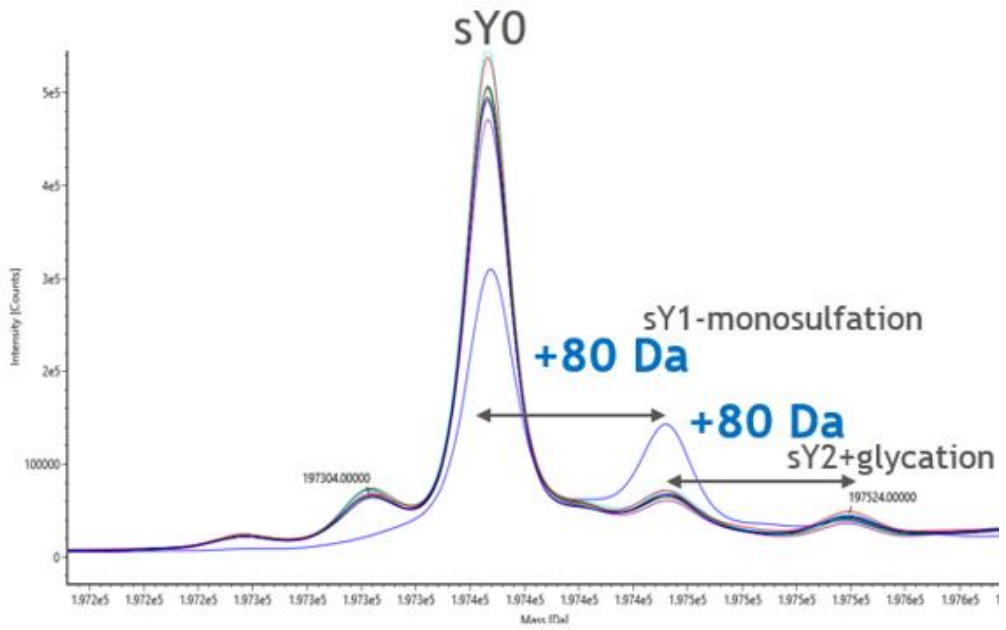
Sample Preparation	Type of Analysis	Reason
✖ De-Glycosylated	Reduced	• Better resolution. • No interference from glycosylation • More sensitive to MS conditions. • Additional data analysis Vs intact-MS
✓ De-Glycosylated (PNGaseF, 2hr, optimized)	Non-reduced	• Better resolution • No interference for sY1, Interference from glycation for sY2 quantification. • Less sensitive to MS conditions

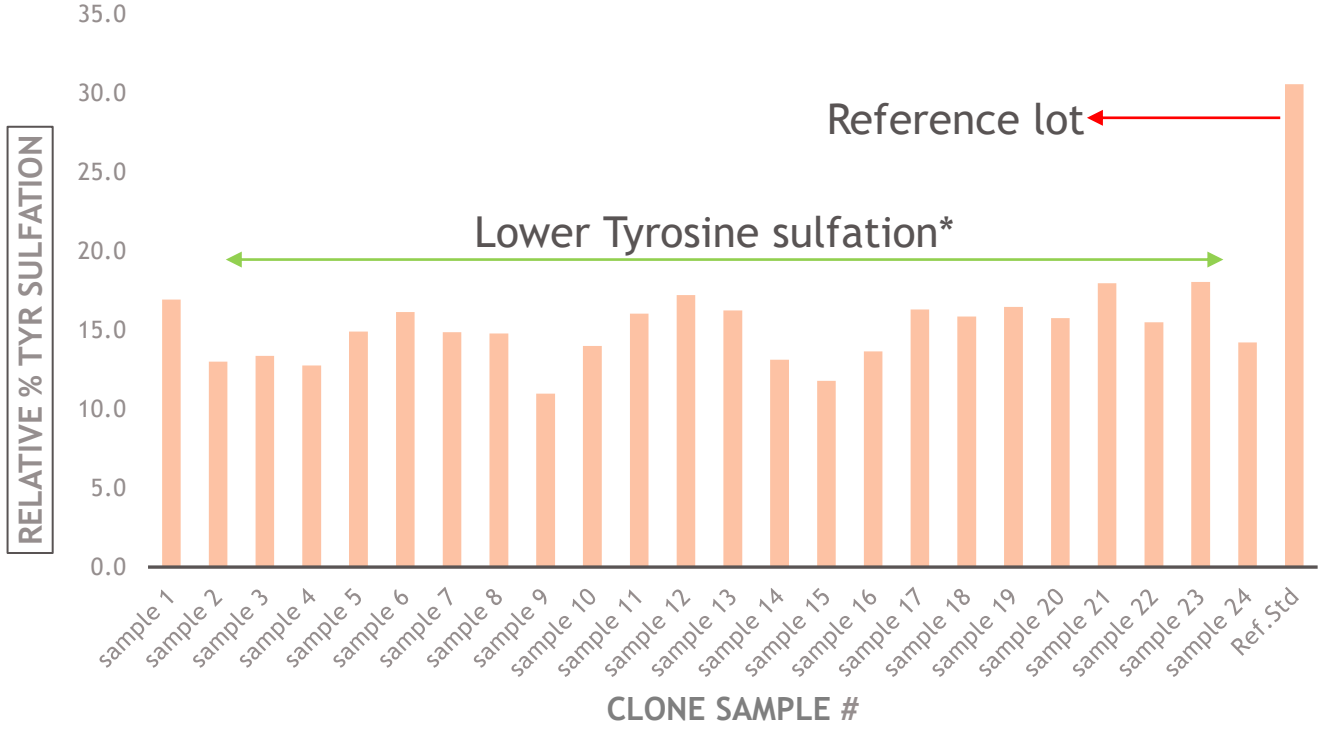
MS Parameters Impact on sY1 quantification		
MS Parameters	De-glycosylated Non reduced	De-glycosylated Reduced, HC1 & HC2
Cone Voltage	Low	High
Capillary Voltage	Low	Low
Capillary Temperature	Low	Low



Relative Quantitation of Sulfo-Tyrosine During Clone Selection

Goal: Selected a clone with relative percentage lower than reference lot.

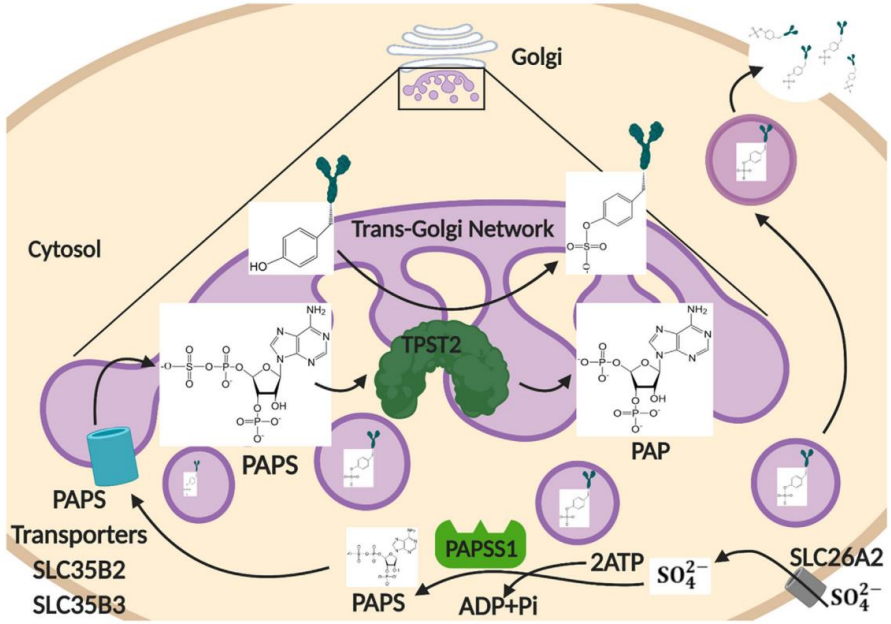


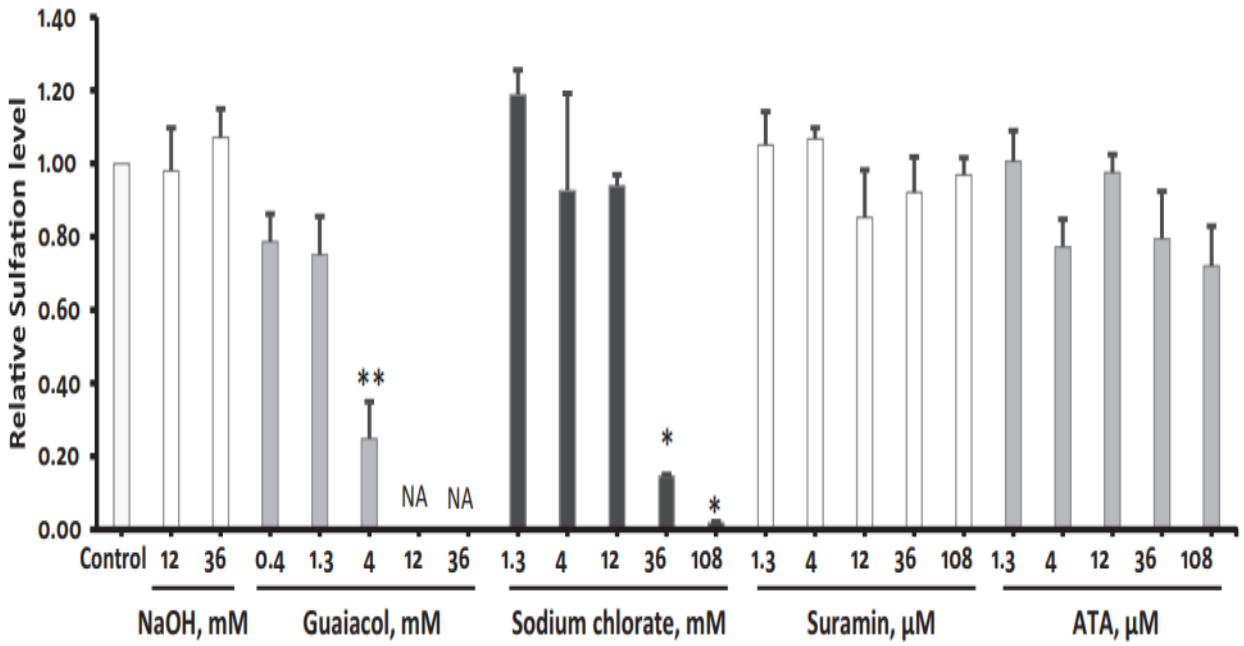


- **LC-MS based intact mass method to quantify Tyrosine sulfation trends during CLD**

Previous Study: Chemical Inhibitors to Reduce Tyrosine Sulfation

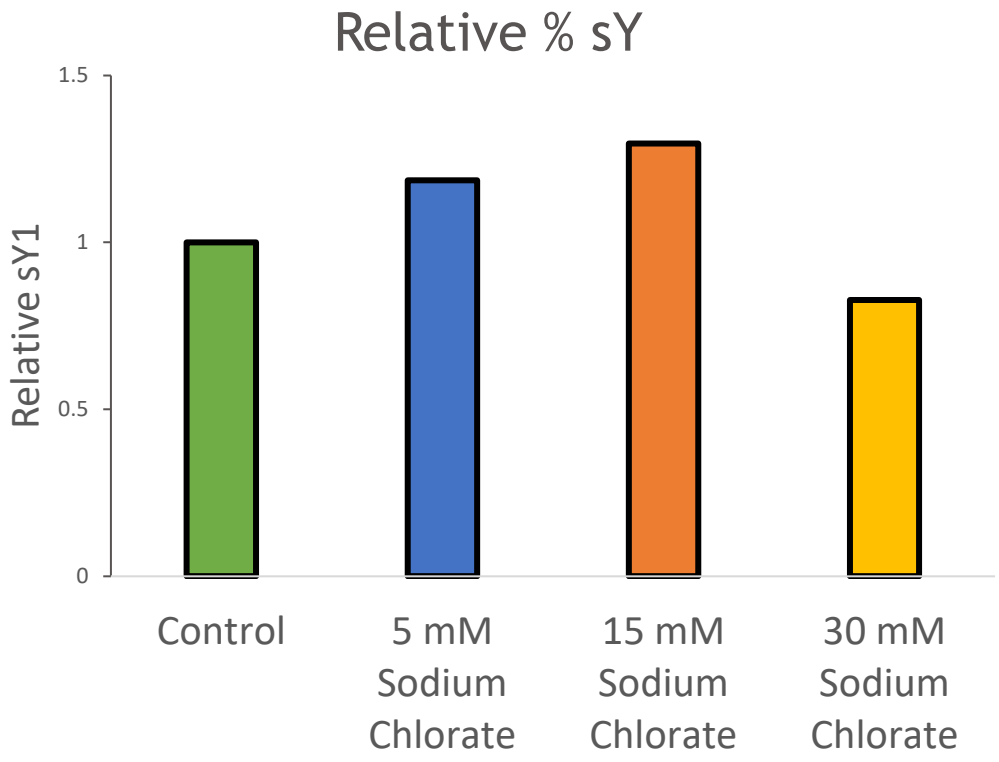
Conclusion: Sodium chlorate at 16 mM inhibited tyrosine sulfation >50% with no major impact on antibody titer or quality

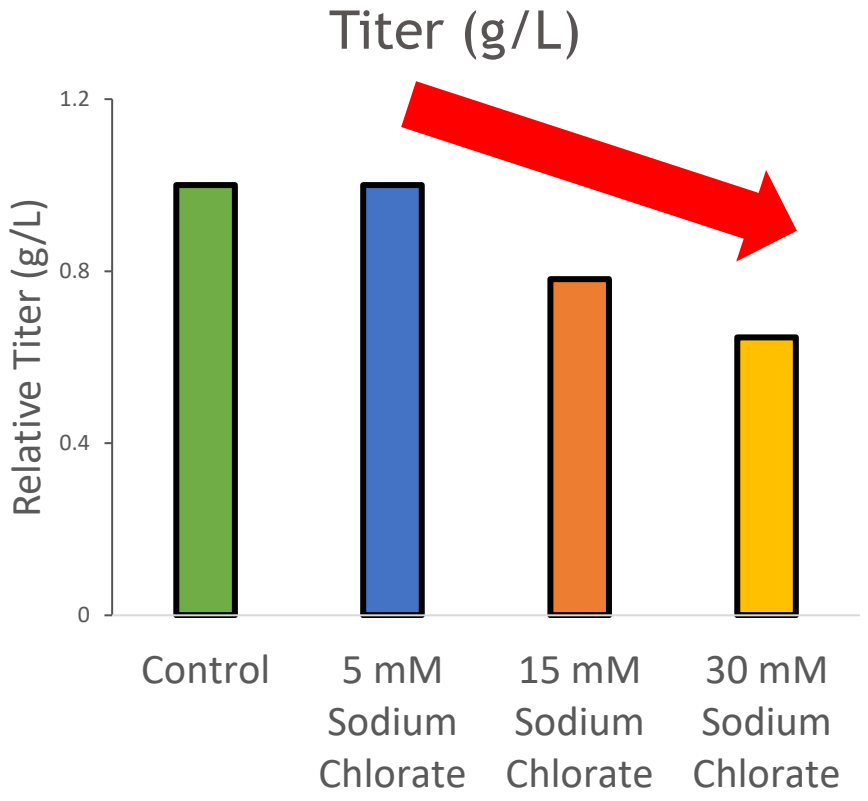




Liu, R., Zhang, Y., Kumar, A., Huhn, S., Hullinger, L., & Du, Z. (2021).
Modulating tyrosine sulfation of recombinant antibodies in CHO cell culture by host selection and sodium chlorate supplementation. Biotechnol. J

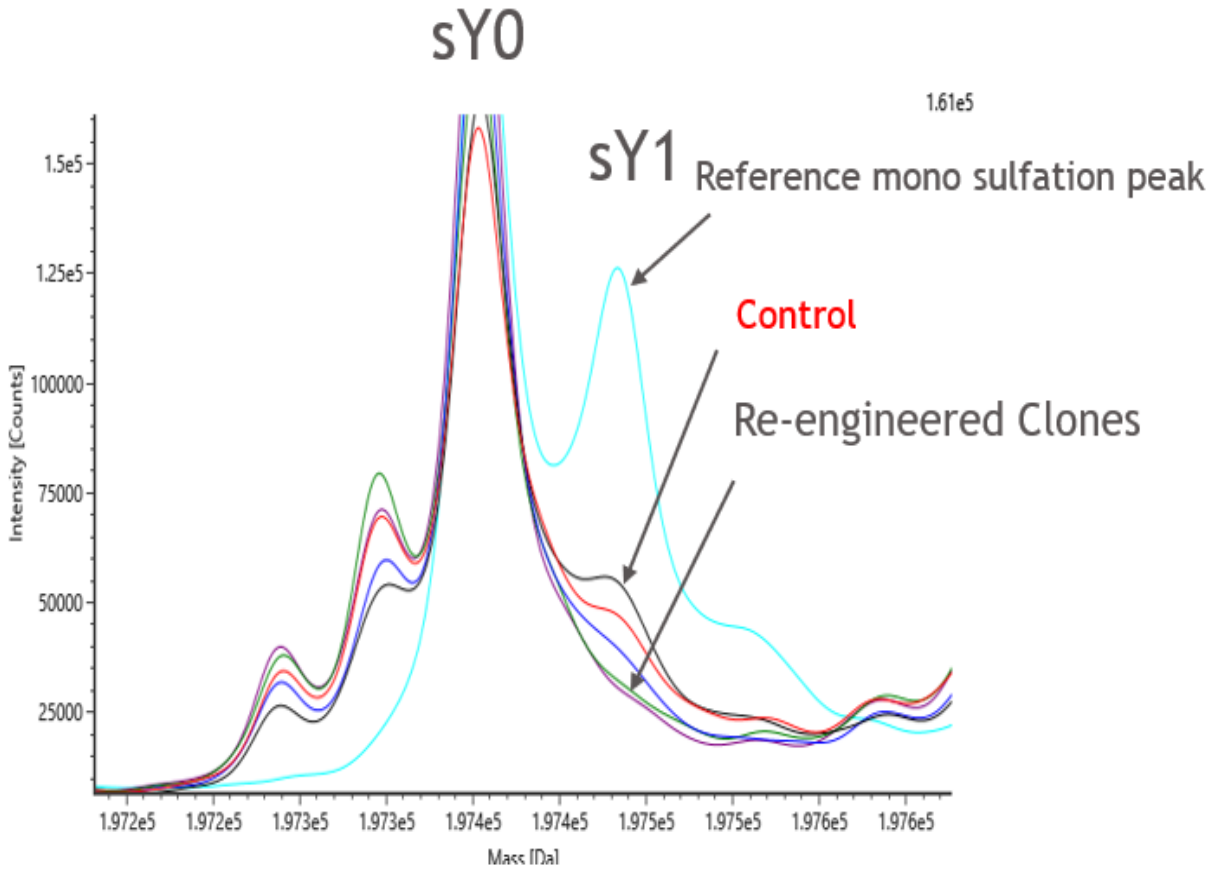
Sodium Chlorate Sulfation Inhibition Study

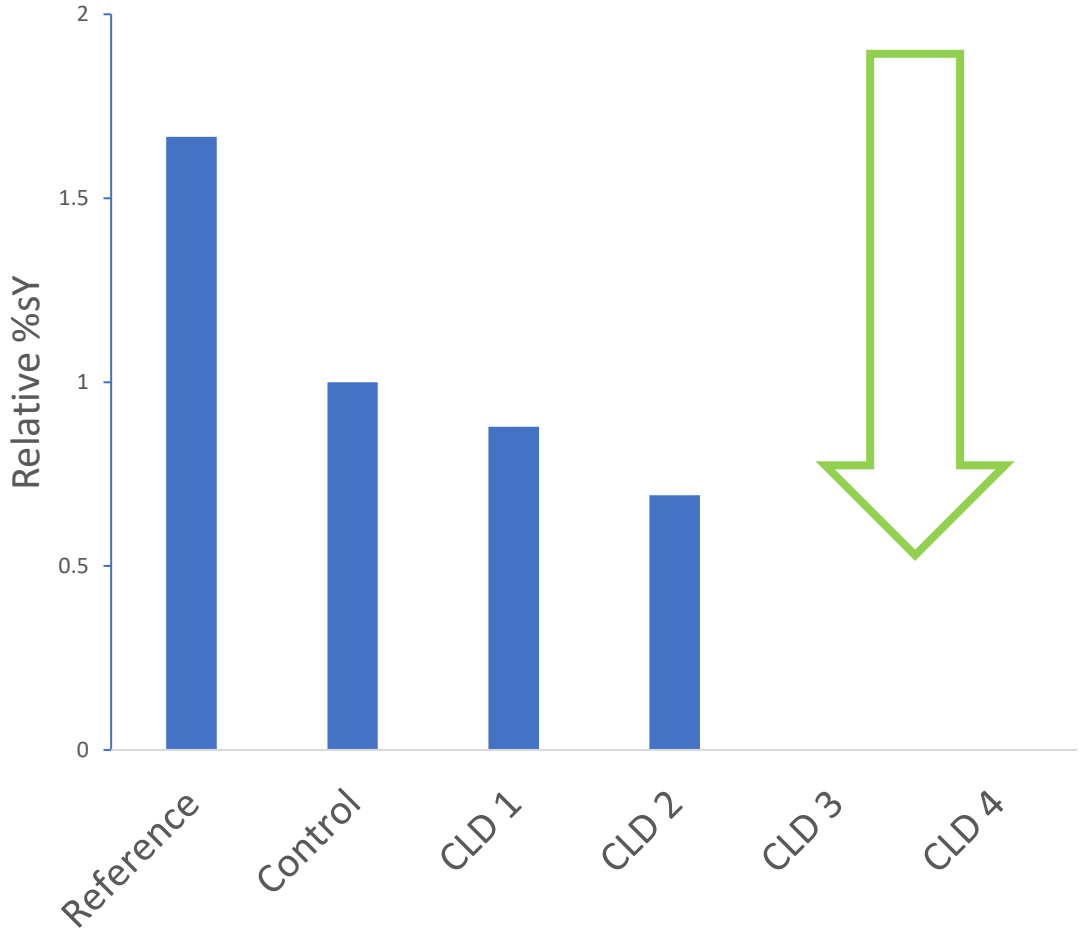




- Minimal impact on the reduction of sulfation with the addition of 30mM sodium chlorate.
- Sodium chlorate impacted titer.

CLD Re-engineered Cell Line Prevented Tyrosine Sulfation





Summary

- ✓ Tyrosine sulfation presented multiple analytical challenges for identification and quantitation.
  - ✓ Identification Site of Sulfation:
    - Multi-Enzymatic Proteolytic Digestion Approach
    - Synthetic Peptides
    - EAD Fragmentation
- ✓ Structure/Function Characterization of Sulfated Species Including Assessment of their Impact on Potency
- ✓ LC-MS Intact Mass Analysis to Support CLD and PD Efforts to Reduce Sulfation During Protein Therapeutic Process Development.
  - — Sodium Chlorate and CLD Study
- ✓ Establishment of Fundamental Guidelines for Identification and Quantification of Tyrosine Sulfation PTM in Therapeutic Proteins

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