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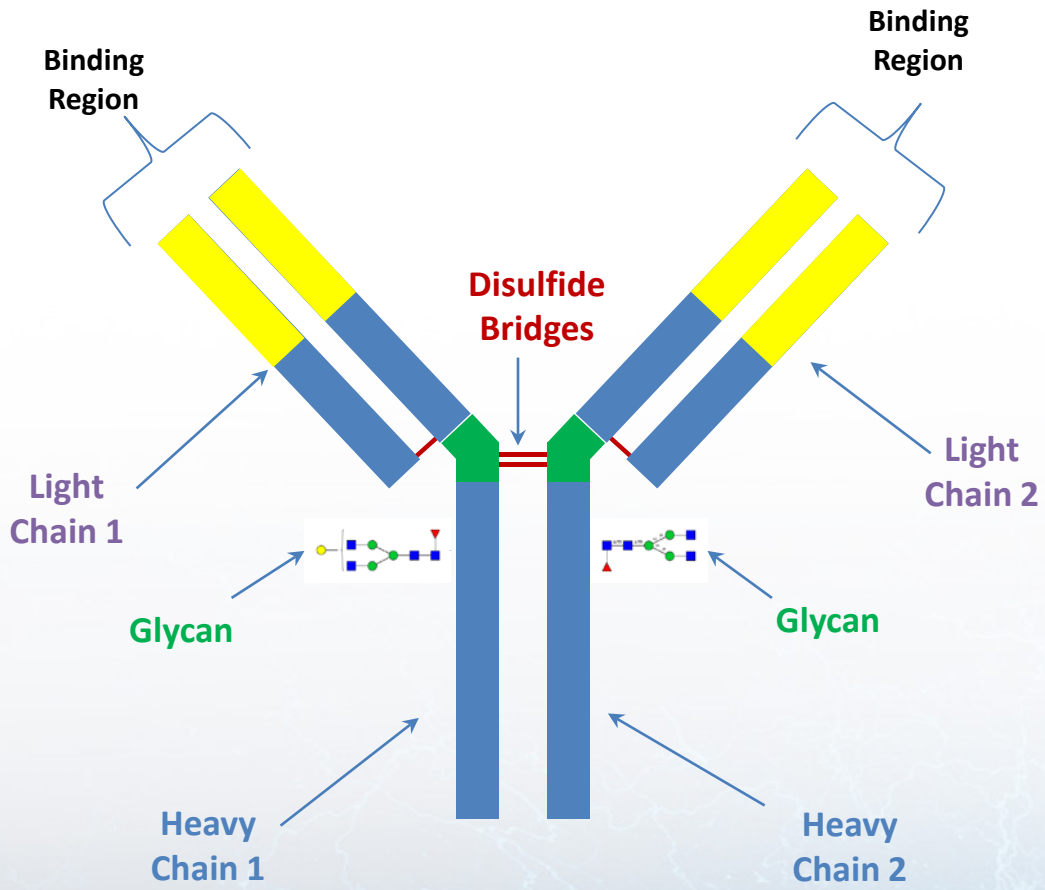
HILIC Analysis for Polar Modifications of mAbs

Barry Boyes, Ph.D.
Chief Technology Officer
Advanced Materials Technology, Inc.
Wilmington, Delaware, USA
bboyes@advanced-materials-tech.com

Outline

- Monoclonal antibody structure
- Post-translational modifications
  - Polar PTMs
    - Glycosylation
    - Deamidation
    - Oxidation
- Conclusions

IgG1 Monoclonal Antibody Structure



- ~ 150 kDa
- Two heavy chains, 50 kDa each
- Two light chains, 25 kDa each
- Disulfide bridges connect the heavy chains to the light chains and the heavy chains to each other
- Glycans attached at Asn297 of each heavy chain

Protein Polar Post Translational Modifications (PTMs)

Polar PTMs discussed today will include:

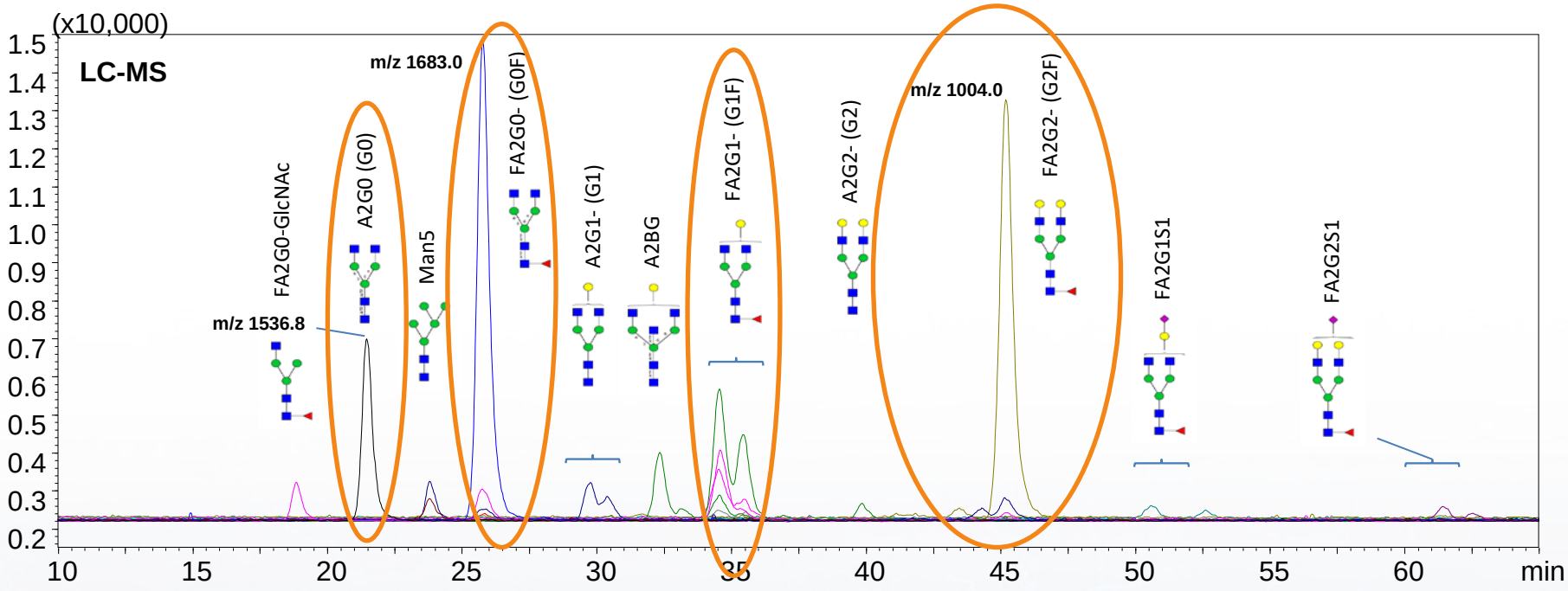
- N- and O-linked glycosylation, adding one or many carbohydrate residues to the protein
- Deamidation and isomerization at Asn and Gln
- Oxidation

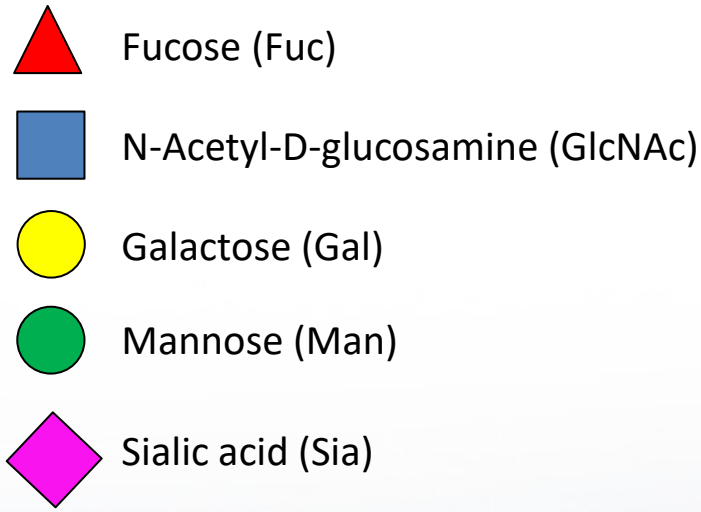
GLYCOSYLATION

Protein Glycosylation

- Carbohydrates/sugars linked to a protein
- Why is glycosylation important for mAbs?
  - Impacts the safety/immunogenicity
  - Influences the efficacy and clearance
  - Impacts the stability and solubility
  - As a critical quality attribute, must be characterized

Glycosylation Variants of Trastuzumab



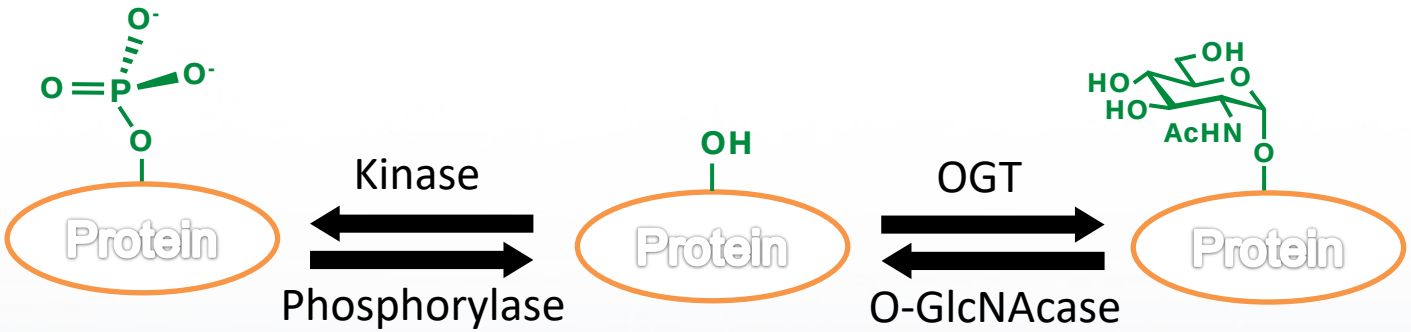


- HILIC with PNGase F released N-linked glycans reductively aminated by procainamide
- Great peak shape and great resolution of compositional isomers
- Covers the entire range of N-linked glycovariants that are present in mAbs

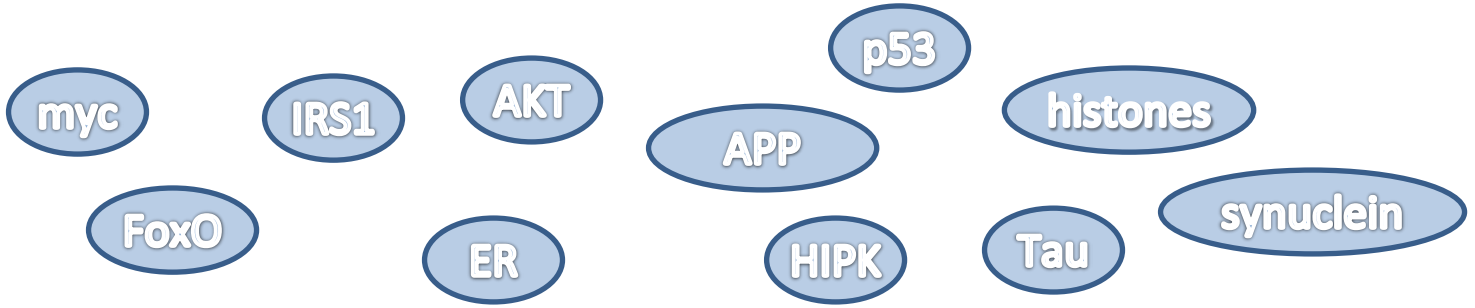
Column – HALO 90 Å Glycan, 2.7 µm, 2.1 x 150 mm; 0.35 mL/min; 40°C.
Gradient Eluents: A –50 mM ammonium formate (pH 4.5); B – ACN
Gradient: 75% to 68% ACN in 75 min

β-O-(N-acetylglucosamine) Modifications of Proteins (O-GlcNAc)

- O-GlcNAc reversibly modifies protein Ser and Thr residues.
- O-GlcNAc is a modifier of biological activity, in some cases, with competition for phosphorylation.
- Multiple independent sites on a particular protein can be modified by –P or –GlcNAc, near-by or far apart.



Hundreds to thousands of proteins central to biological process and diseases such as cancer, diabetes and neurodegeneration are O-GlcNAcylated.

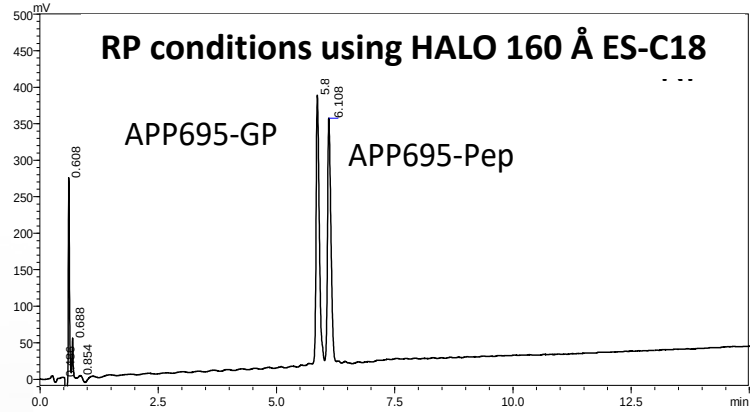


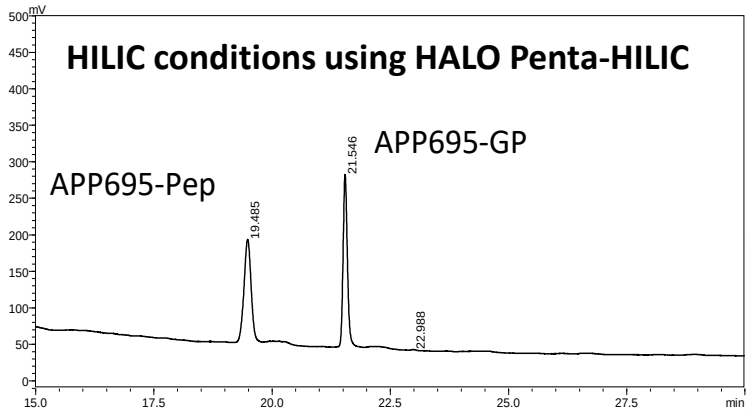
LC/MS of O-GlcNAcylated Peptides: RP vs HILIC

Conditions: Columns - 2.1 x 100 mm HALO 90 Å Penta-HILIC or HALO 160 Å ES-C18; 0.4 mL/min; 60°C.
Gradient Eluents: A – 0.1% formic acid/10 mM ammonium formate; B – 90% AcN in A.
RP Gradient - 4% to 34% AcN/30 min (1%/min); HILIC Gradient - 90% to 60% AcN/30 min (-1%/min).

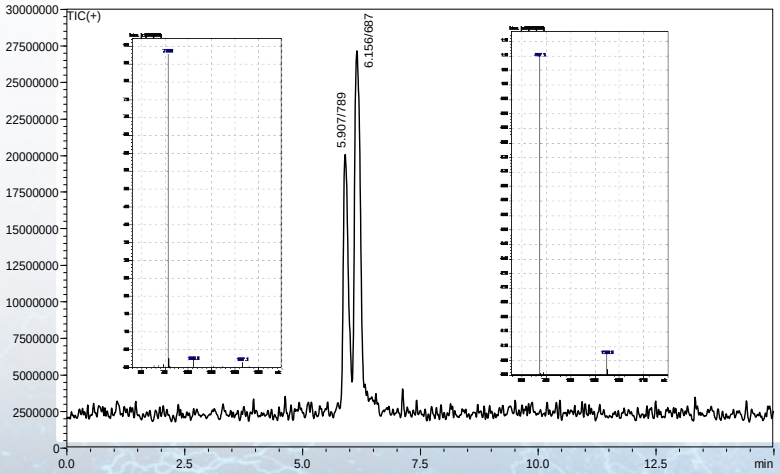
APP695-14GPep	**VPTT(OGlcNAc)AASTPDAVDK**
APP695-14Pep	**VPTTAASTPDAVDK**

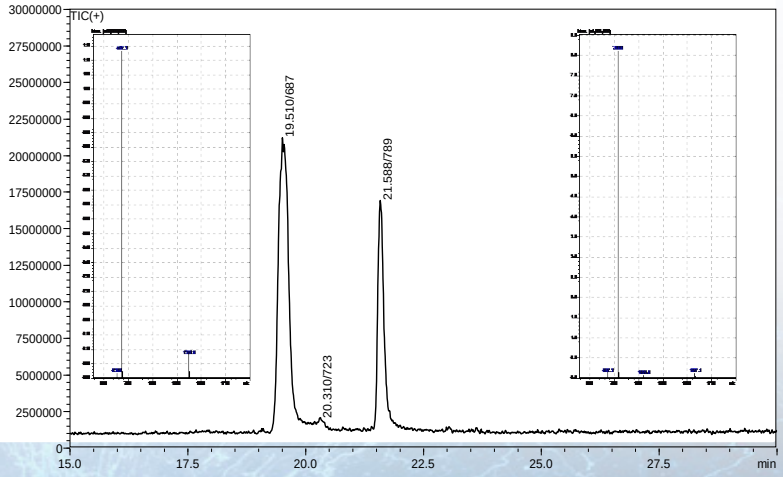
A_{210}





MS





LC/MS of O-GlcNAcylated Peptides: RP vs HILIC

			RP Results			HILIC Results		
Peptide Description	Sequence	Mass (neutral)	Rt RP (min)	Δ Rt RP (GP-P)	Rs RP	Rt HILIC (min)	ΔRt HILIC (GP-P)	Rs HILIC
APP695-14GPep	VPTT(OGlcNAc)AASTPDAVDK	1574.8	5.87			21.55		
APP695-14Pep	VPTTAASTPDAVDK	1371.7	6.11	-0.24	1.90	19.49	2.07	9.41
MUC5AC	GTTPSPVPTTSTTSAP	1501.6	9.28			16.41		
MUC5AC-3	GTT(OGalNAc)PSPVPTTSTTSAP	1704.6	8.45	-0.83	6.88	18.68	2.27	13.40
MUC5AC-13	GTTPSPVPTTSTT(OGalNAc)SAP	1704.6	8.53	-0.75	5.82	18.51	2.10	10.72
MUC5AC3/13	GTT(OGalNAc)PSPVPTTSTT(OGalNAc)SAP	1908.1	7.76	-1.52/2	11.84	20.48	4.07/2	23.35
GP-41	Ac-CSTFRPRT(OGlcNAc)SSNAST	1758.8	7.09			18.59		
P-42	Ac-CSTFRPRTSSNAST	1555.7	7.03	0.06	0.44	17.03	1.56	11.58
GP-78	Ac-CQHPPVT(OGlcNAc)NGDTVK	1639.8	6.47			20.32		
P-84	Ac-CQHPPVTNGDTVK	1436.7	6.56	-0.10	0.66	18.72	1.61	11.23
GP-79	Ac-CKIADFGLS(OGlcNAc)KIVEHQ	1932.0	19.36			19.15		
P-85	Ac-CKIADFGLSKIVEHQ	1728.9	20.80	-1.44	8.16	17.21	1.94	14.76
GP-17s	CTLHTKAS(OGlcNAc)GMALLHQ	1854.9	13.62			17.29		
P-20s	CTLHTKASGMALLHQ	1651.8	14.23	-0.61	3.06	15.15	2.14	15.38
GP-15	Ac-CFELLPT(OGlcNAc)PPLSP	1557.8	25.16			5.64		
P-18	Ac-CFELLPTPPLSP	1354.7	27.16	-2.00	8.88	2.71	2.93	20.11
GP-46	Ac-CRSSHYGGS(OGlcNAc)LPNVNQI	1975.9	12.48			17.32		
P-47	Ac-CRSSHYGGSLPNVNQI	1772.8	12.96	-0.48	3.83	15.43	1.89	13.91
GP-51	Ac-CSALNRTS(OGlcNAc)SDSALHT	1806.8	9.08			17.23		
P-52	Ac-CSALNRTSSDSALHT	1603.7	9.55	-0.47	3.85	15.55	1.69	12.42
GP-16	Ac-CKIPGVS(OGlcNAc)TPQTL	1487.7	16.41			13.27		
P-19	Ac-CKIPGVSTPQTL	1284.6	16.98	-0.58	3.74	10.59	2.68	21.63
GP-2-p53	Ac-CQLWVDS(OGlcNAc)TPPPG	1543.7	16.43			12.72		
P-3-p53	Ac-CQLWVDSTPPPG	1340.6	17.66	-1.23	7.23	10.41	2.31	10.28
GP-17r	Ac-CLHTKAS(OGlcNAc)GMALL	1488.7	16.21			10.59		
P-20r	Ac-CLHTKASGMALL	1285.6	16.98	-0.77	2.79	7.45	3.14	24.73
Average			13.01	-0.73	4.93	15.29	2.17	15.21
Standard Deviation			5.95	0.54	3.32	4.74	0.47	5.13
% RSD			45.7	74.3	67.3	31.0	21.8	33.7

This sample of 12 peptides and 14 glycopeptides reveals:

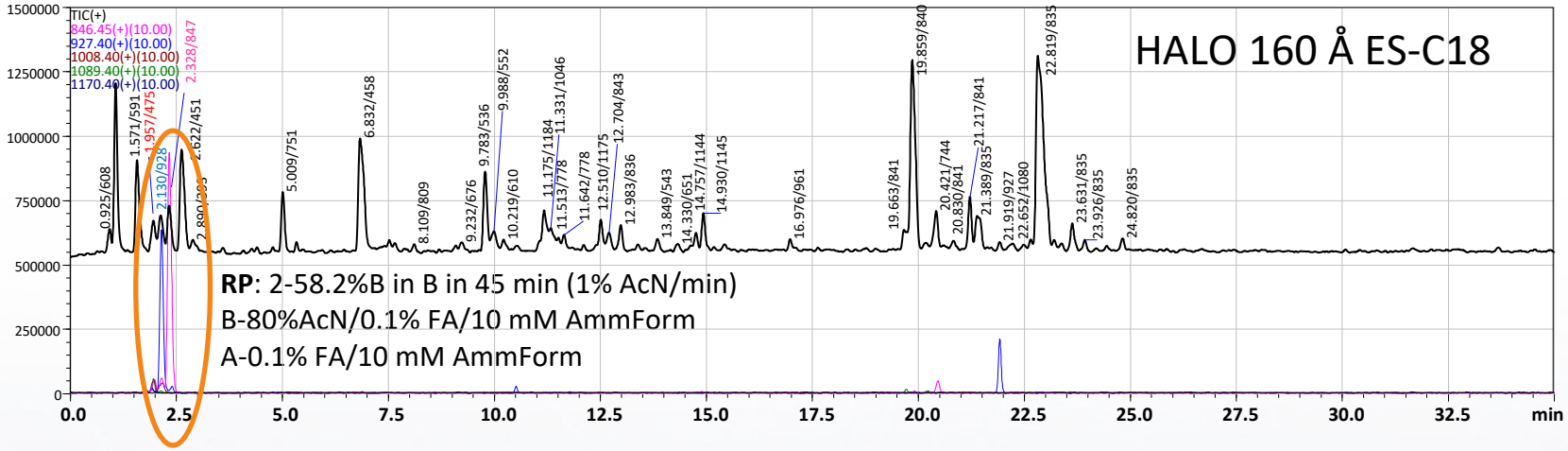
HILIC shows higher resolution (3X), at lower variance (2X), compared to RP

Driven by better separation selectivity of this polar modification

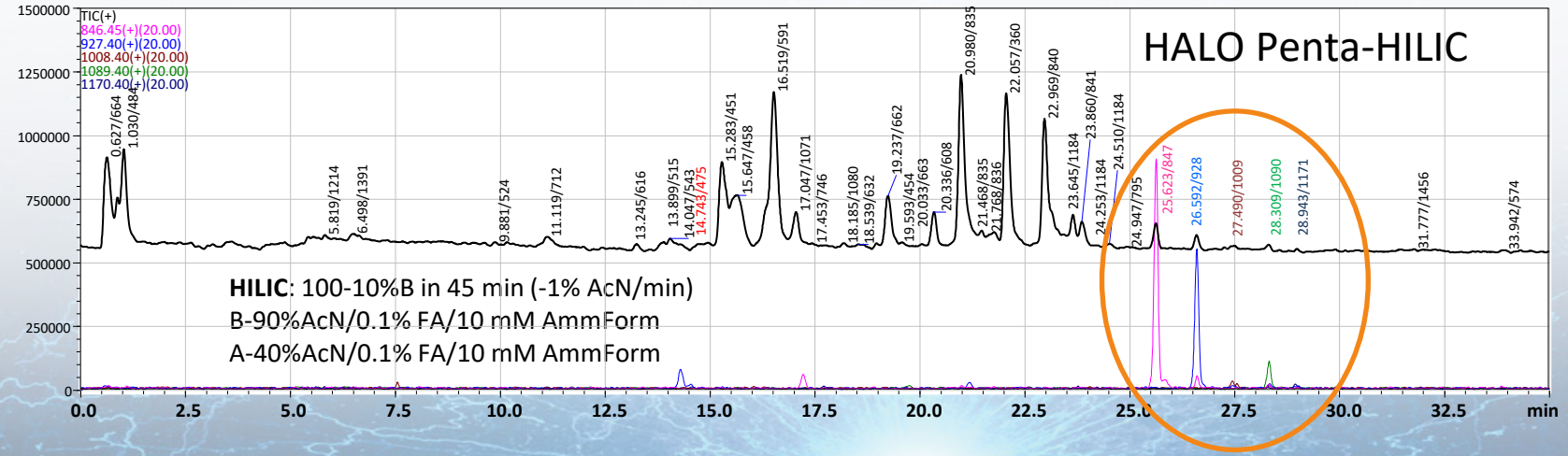
Note results for O-GalNAc in modified peptides, so these averages actually refer to O-HexNAc effects.

HILIC Strongly Retains and Resolves N-linked Glycopeptides

2.1 mm ID x 100 mm, 0.35 mL/min, 40 C, MS: SQ TIC (+ 300-2000 m/z) @ 0.35/s
20 µg Bovine Ribonuclease B tryptic digest (CAM)



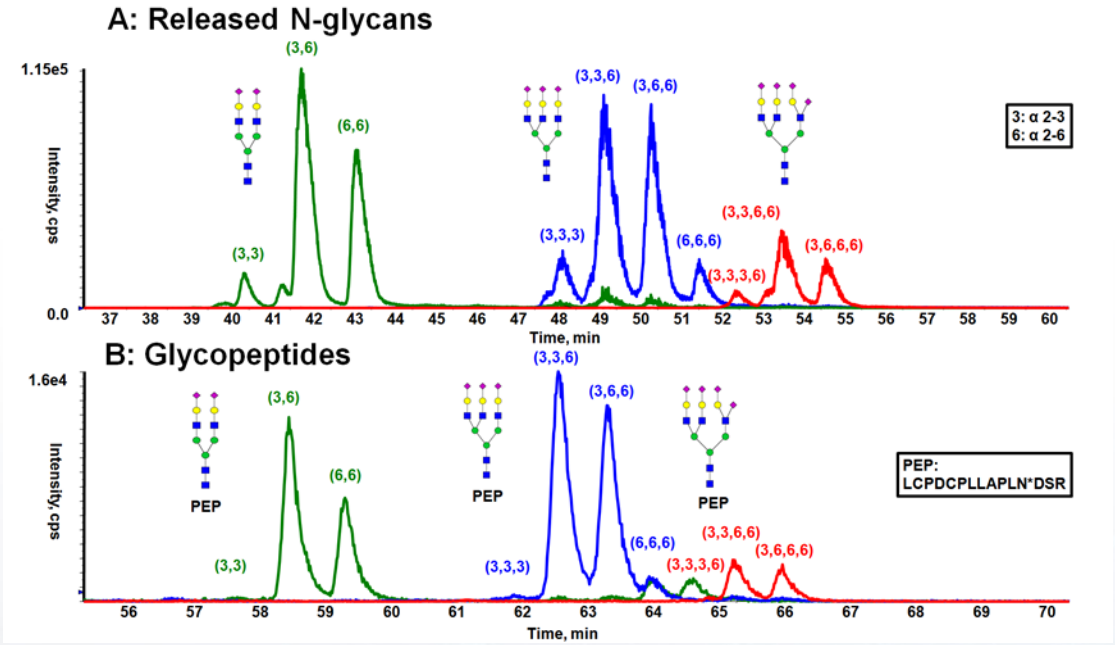
No resolution of high mannose variant glycopeptide



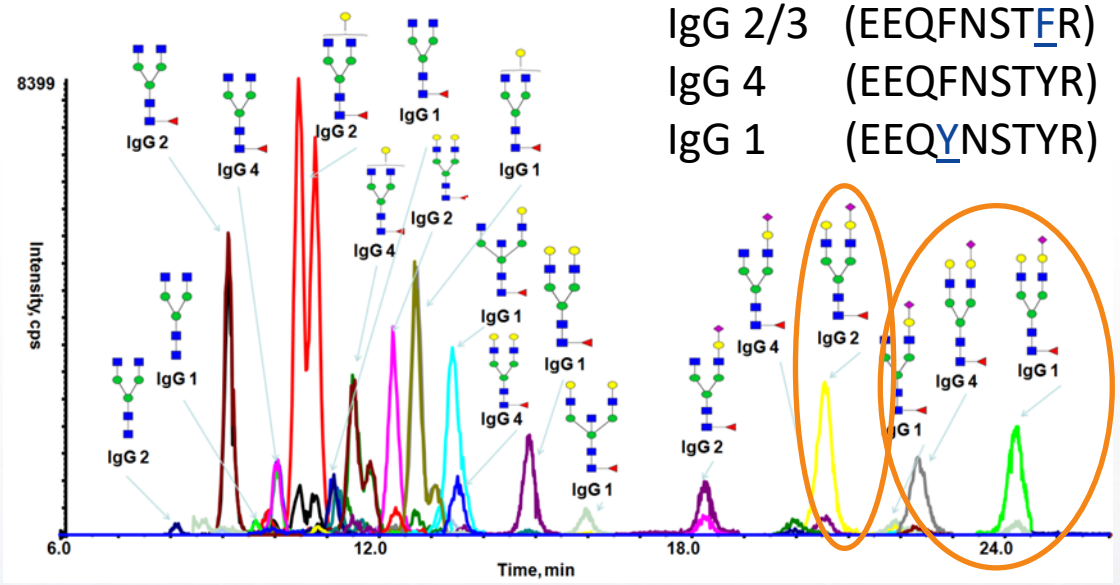
Good resolution of high mannose variant glycopeptide

LC-MS/MS of N-Linked Glycans ON and OFF Peptide by HILIC/SRM

Conditions: Column – 2.1 x 150 mm HALO Penta-HILIC; 0.4 mL/min; 60°C.
Gradient Eluents: A –50 mM ammonium formate (pH 4.4)/5% AcN; B – AcN
Gradient – RG: 80% to 62% AcN/60 min (-1%/min); GP 85% to 48%B 75 min



Comparison released N-glycans and glycopeptides of Fetuin. (A) Procainamide labeled released N-glycans. (B) Glycopeptides with the same peptide backbone.

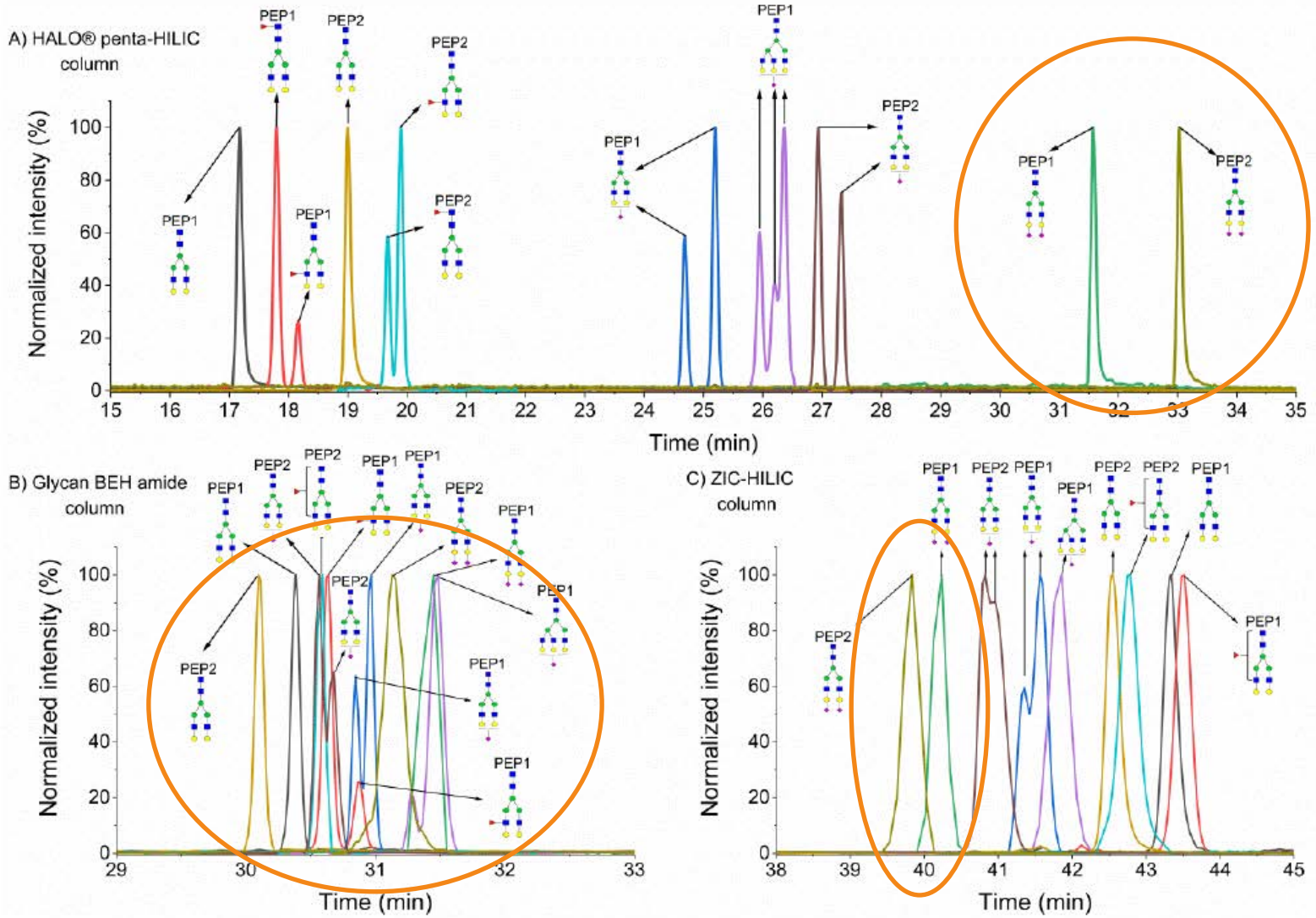


HILIC SRM analysis of human serum IgGs demonstrating the ability to resolve isomeric glycopeptide glycoforms.

Huang, Y., Nie, Y., Boyes, B., and Orlando, R. (2016) J. Biomol. Technol., 27, 98-104. Tao, S.J., Huang, Y., Boyes, B.E., and Orlando, R. (2014) Anal. Chem., 86, 10584-10590.

Separation of Glycopeptides of Hemopexin

2.1 mm x 150 mm, A: 0.1% formic acid in water; B: 0.1% formic acid in ACN
Gradient conditions same for Penta-HILIC and BEH Amide; shallower for ZIC-HILIC, 0.3 mL/min, 40 °C

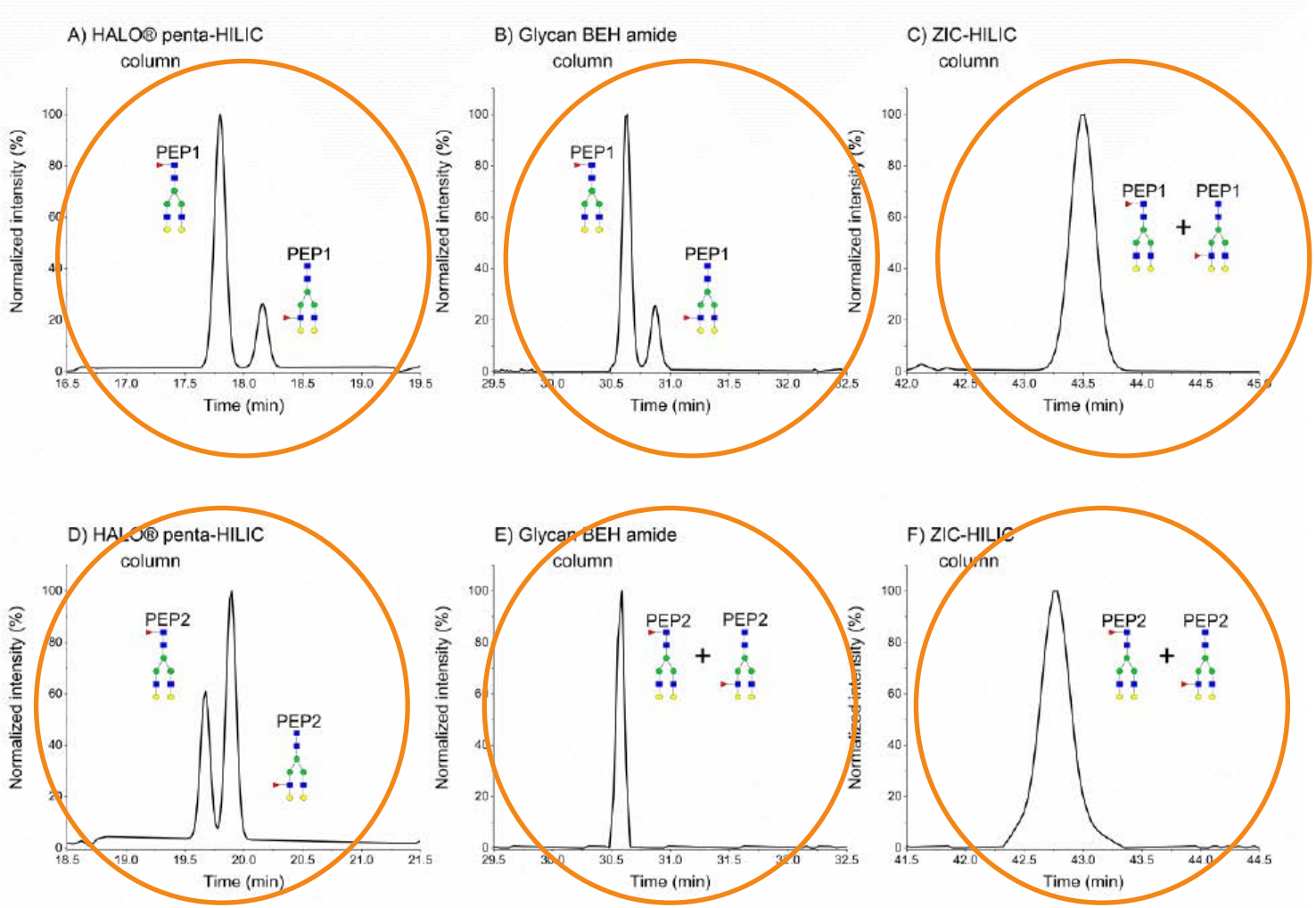


- Increased retention for Penta-HILIC for the di-sialylated glycopeptides
- Reduced retention of sialylated glycans on ZIC-HILIC may be due to ion repulsion between the negatively charged sulfobetaine functional group and the negatively charged sialic groups of the glycans
- Reduced elution range observed on the BEH amide (< 2 min)

Molecules **2020**, 25, 4655

Separation of Fucosylated Glycopeptide Isomers

2.1 mm x 150 mm, A: 0.1% formic acid in water; B: 0.1% formic acid in ACN
Gradient conditions same for Penta-HILIC and BEH Amide; shallower for ZIC-HILIC, 0.3 mL/min, 40 °C

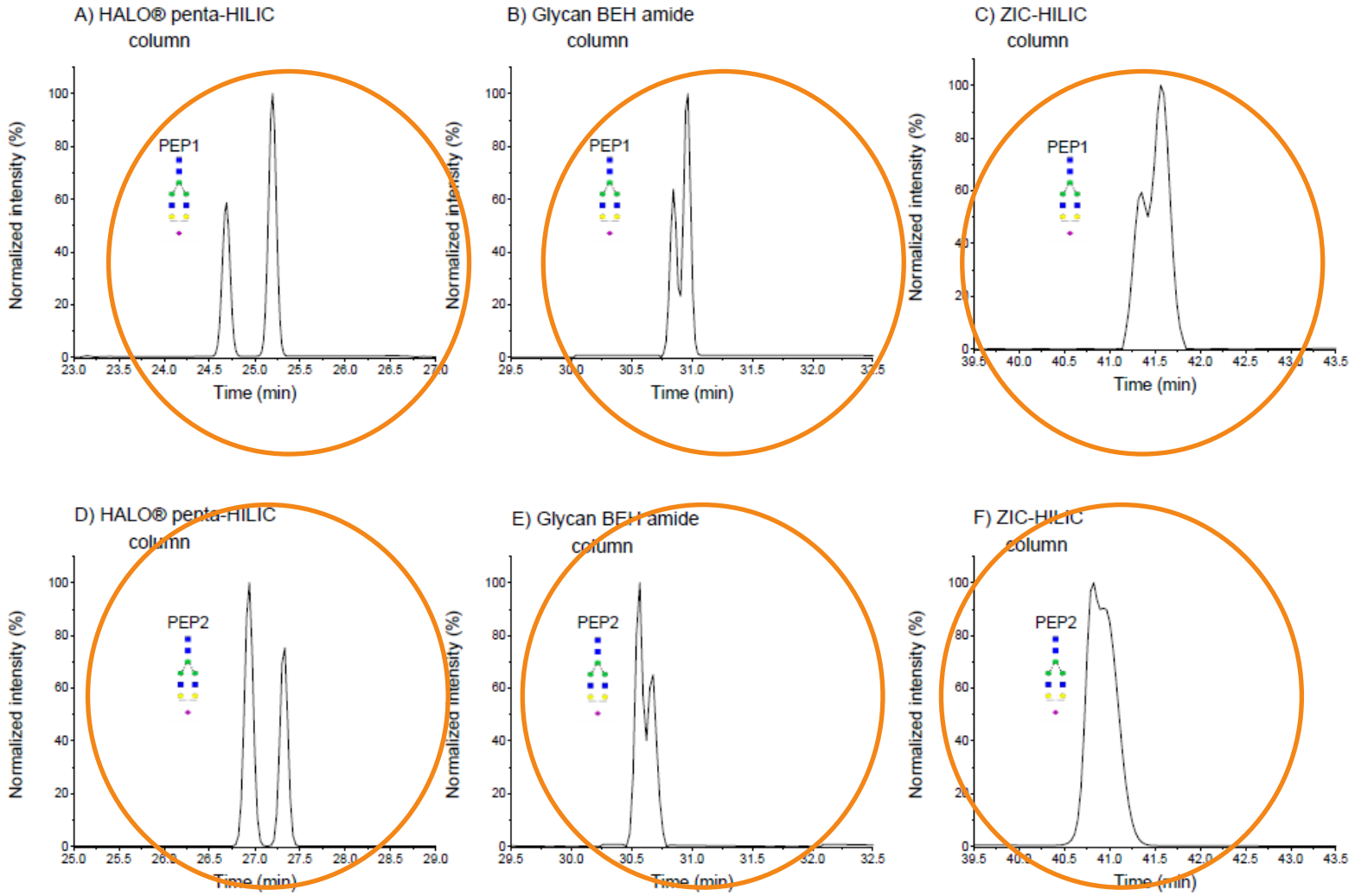


- Normalized EIC chromatograms of A2G2F1 glycoforms of SWPAVGN187CSSALR (PEP1) (A–C) and ALPQPQN453VTSLLGCTH (PEP2) (D–F) on 3 different HILIC columns
- HALO Penta-HILIC resolves the glycoforms of both PEP1 and PEP2
- BEH Amide resolves the glycoforms of PEP1, but not PEP2 while ZIC-HILIC shows complete coelution of the glycoforms of PEP1 and PEP2

Molecules **2020**, 25, 4655

Separation of Sialylated Glycopeptide Isomers

2.1 mm x 150 mm, A: 0.1% formic acid in water; B: 0.1% formic acid in ACN
Gradient conditions same for Penta-HILIC and BEH Amide; shallower for ZIC-HILIC, 0.3 mL/min, 40 °C

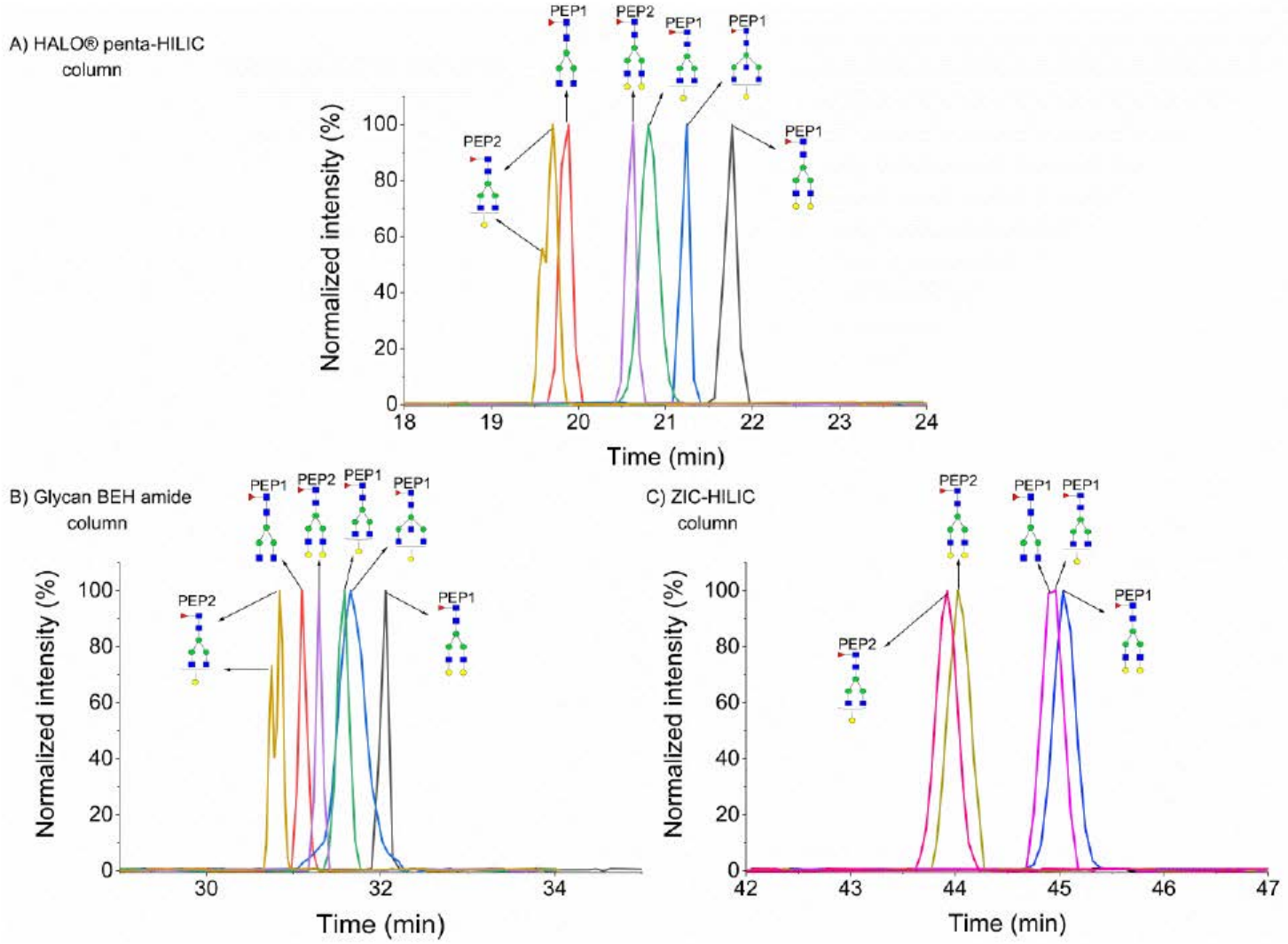


- Normalized EIC chromatograms of A2G2F1 glycoforms of SWPAVGN187CSSALR (PEP1) (A–C) and ALPQPQN453VTSLLGCTH (PEP2) (D–F) on 3 different HILIC columns
- HALO Penta-HILIC shows baseline resolution of the glycoforms of both PEP1 and PEP2
- BEH Amide is starting to resolve the glycoforms of PEP1 and PEP2 while ZIC-HILIC shows coelutions of the glycoforms of PEP1 and PEP2

Molecules **2020**, 25, 4655

Separation of Glycopeptides of IgG

2.1 mm x 150 mm, A: 0.1% formic acid in water; B: 0.1% formic acid in ACN
Gradient conditions same for Penta-HILIC and BEH Amide; shallower for ZIC-HILIC, 0.3 mL/min, 40 °C



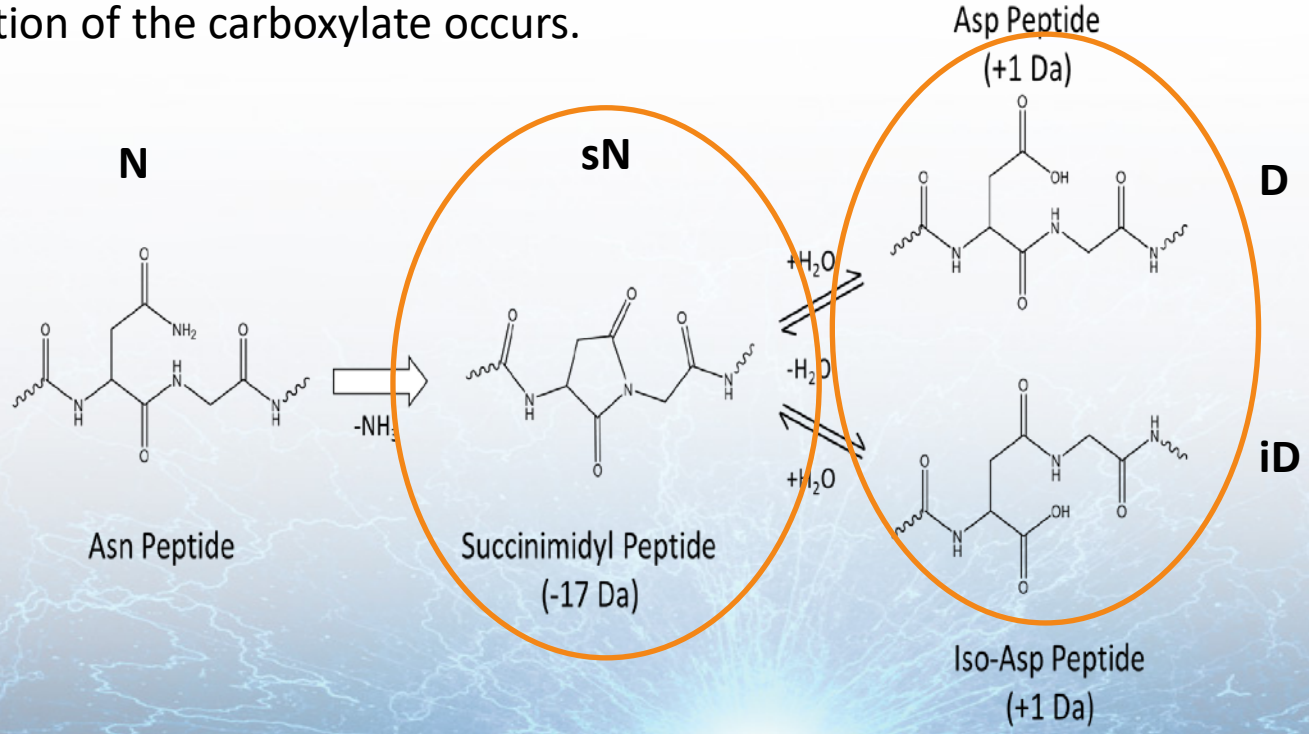
- Different selectivity observed across the 3 HILIC phases studied
- Increased resolution for Penta-HILIC compared to the other 2 HILIC phases

Molecules **2020**, 25, 4655

DEAMIDATION

Deamidation/Isomerization of Asparagine

- Deamidation of asparagine (N) and glutamine (Q) residues occurs in both peptides and in intact proteins
- Rate of these deamidation reactions is strongly condition (pH, T) and sequence dependent (C-terminal G)
- Mechanism of *ammonium loss* is understood to occur as an irreversible reaction through a 5 membered (N) or 6 membered (Q) cyclic intermediate – shown below for Asn with intermediate cyclic succinimidyl structure (sN)
- Symmetrical intermediate will *hydrolyze* to Asp (D) or, via polypeptide backbone rearrangement, to iAsp (iD)
- Asp/iAsp dehydration to the cyclic intermediate is reversible, resulting in an equilibrium distribution of Asp/iAsp containing peptides or polypeptides
- Formation of Asp or iAsp results in a *more polar modified* peptide or polypeptide, amenable to HILIC resolution. At pH >3 ionization of the carboxylate occurs.



Badgett, M.J., Boyes, B.E., Orlando, R.C. Am. Soc. Mass Spectrom. 28: 818 (2017)

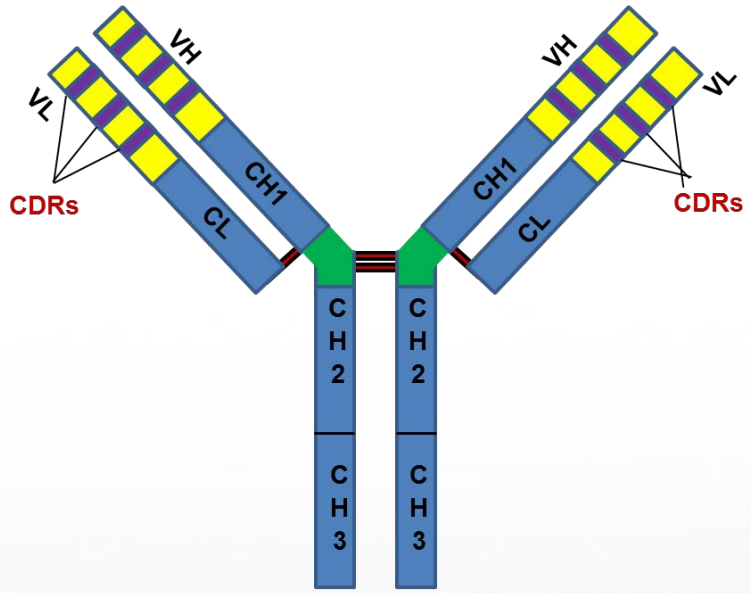
Why is Deamidation of mAbs Important?

- Could alter the mAb structure and function
  - Reduced bioactivity
  - Change to pharmacokinetics
  - Change to antigenicity
- Could change the stability, leading to degradation

Selected Tryptic Peptides from Trastuzumab

IgG Peptides Studied

Trastuzumab Light Chain			
25	ASQDVNTAVAWYQQKPGK	42	$N^{30}T$
Trastuzumab Heavy Chain			
76	NTAYLQMNSLR	86	$N^{83}S$
99	WGGDGFYAMDYWGQGTLVTVSSASTK	124	$D^{102}G$
279	FNWYVDGVEVHNAK	292	$D^{284}G$
306	VVSVLTVLHQDWLNGK	321	$N^{319}G$
375	GFYPSDIAVEWESNGQPENNYK	396	$N^{388}G$--$NN^{393}N^{394}Y$
421	WQQGNVFSCSVMHEALHNHYTQK	443	$N^{425}V$--$N^{438}H$



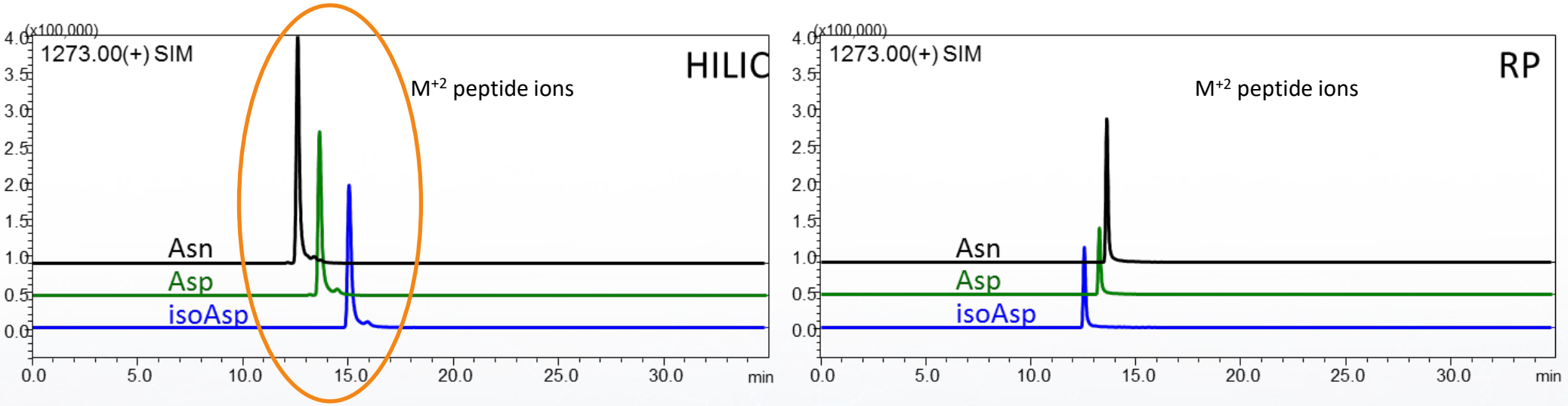
IgG Peptide with Multiple Asn as a Model System:

GFYPSDIAVEWESN^{388}GQPEN^{393}N^{394}YK

Comparison of HILIC and RP for Resolving Deamidated and Isomerized Asn Peptides

GFYPSDIAVEWESNGQPENNYK
GFYPSDIAVEWESDGQPENNYK
GFYPSDIAVEWESiDGQPENNYK

Three peptides from IgG1 – variant at position 14 (IgG N^{388})

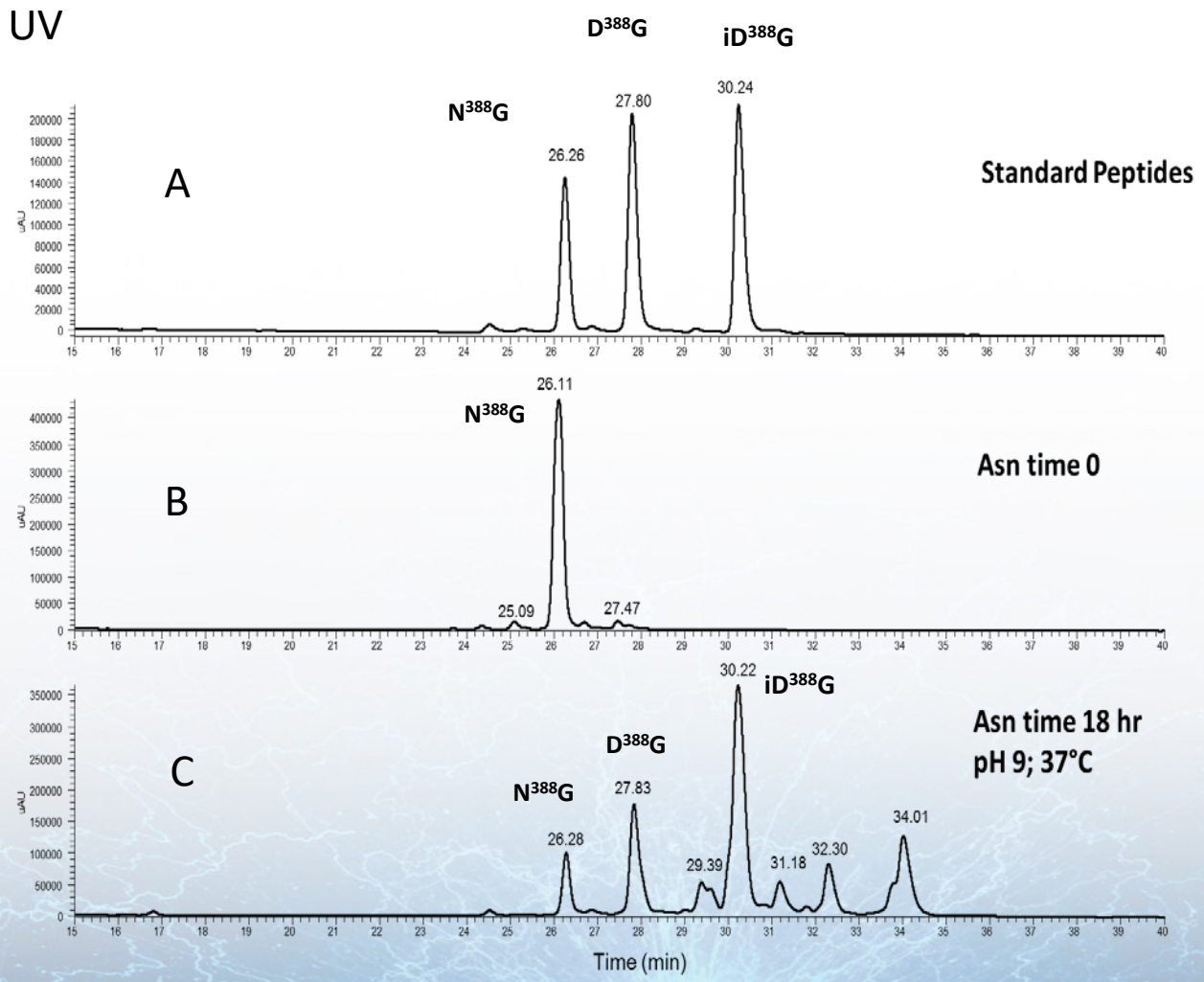


- The same mobile phase conditions and temperature were employed, reversing the direction of the acetonitrile gradient to effect elution on RP and HILIC columns.
- *Note the greater selectivity difference for resolving these peptides in HILIC, compared to RP.*
- *Unlike* RP, HILIC *reliably* resolves Asn/Asp/iAsp, with the retention order shown above

Columns: 2.1 x 150 mm HALO 160 Å ES-C18, 2.7 µm or HALO 90 Å Penta-HILIC, 2.7 µm; Flow rate: 0.4 mL/min; Temp: 60 °C;
Mobile Phase A: water/50 mM Ammonium Formate, pH 4.4; Mobile Phase B: acetonitrile/0.1% Formic acid
Gradient: HILIC – 80%-46.2% in 60 min.; RP - 10-70% B in 60 min; Injection Volume: 4 µL (0.1 µg)

Deamidation/Isomerization of Asparagine at pH 9, 37 °C

Column: 0.5 x 150 mm Halo Penta-HILIC; Flow rate: 12 µL/min; Temp: 60 °C; Detection: Abs (220 nm) or Orbitrap Velos Pro MS Mobile Phase A: water/50 mM Ammonium Formate, pH4.4; Mobile Phase B: acetonitrile/0.1% Formic acid; Gradient: Hold 80%B for 4 min.; 80%-48% in 64 min.



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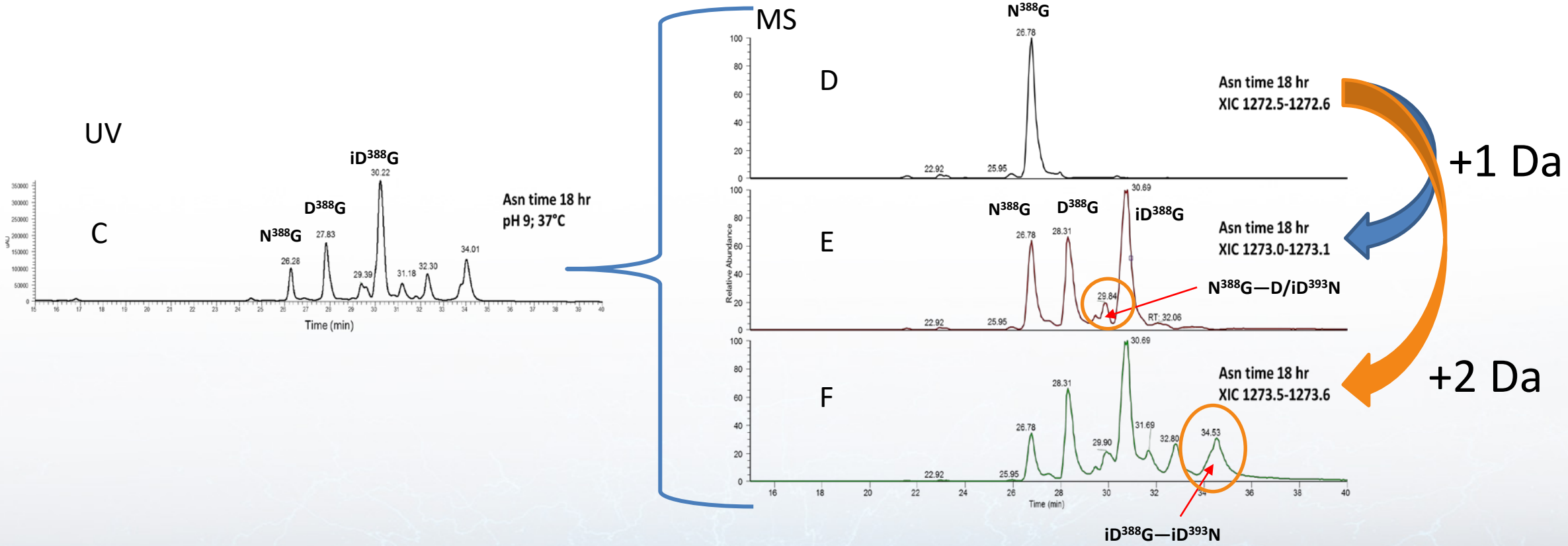
Column: 0.5 x 150 mm Halo Penta-HILIC; Flow rate: 12 µL/min; Temp: 60 °C; Detection: Abs (220 nm) or Orbitrap Velos Pro MS Mobile Phase A: water/50 mM Ammonium Formate, pH4.4; Mobile Phase B: acetonitrile/0.1% Formic acid; Gradient: Hold 80%B for 4 min.; 80%-48% in 64 min.



- Mass analysis and MS/MS fragmentation identifies the $N^{388}G—(D/iD)^{393}N$ peptide at 29.8 min.
- Degradation of $iD^{388}G—N^{393}N$ rapidly formed two peptides with 1 Da shift at 31.69 and 32.80 min, confirmed as deamidations to D or iD at position 393, and eventually all peptides degraded to predominantly 34.53 minutes, the $iD^{388}G—iD^{393}N$ peptide. Supported by mass analysis, using CID and ETD fragmentation.
- No evidence of the formation of a triple deamidation was obtained (N^{394}).

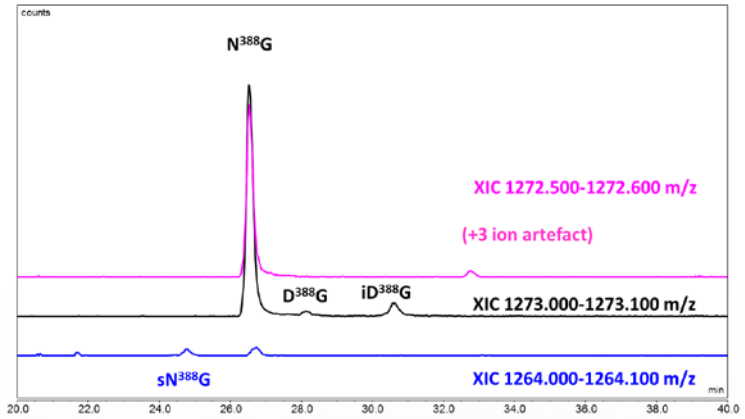
Method Conditions for IgG Tryptic Digests

- Trastuzumab
  - Tryptic digest of both native and stressed by incubation in Tris-HCl pH 9.0 for 7 days at 4 mg/mL
    - Reduced and alkylated proteins digested 4 hrs in 50 mM Tris-HCl (pH 7.8)/1.5 M Guanidine-HCl
  - Analysis via HILIC capillary LC/MS using the Orbitrap/IT
    - Extracted ions at the monoisotopic masses of the target sequences were integrated
    - Reported sequences were confirmed by CID MS/MS fragmentation

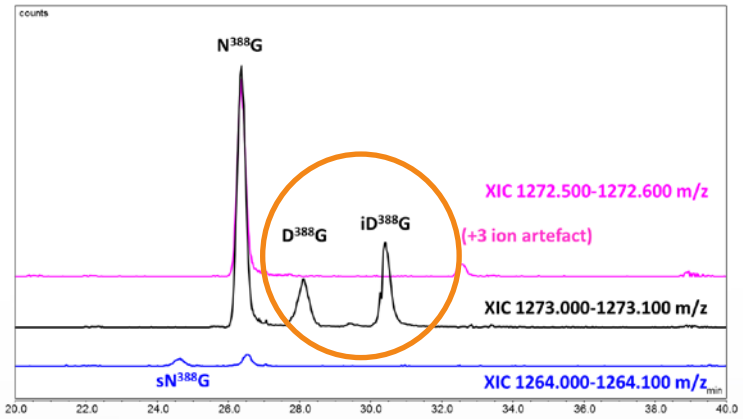
Deamidation and Isomerization in IgG Tryptic Digests

GFYPSDIAVEWESNGQPENNYK in Trastuzumab Digest

Native



Stressed



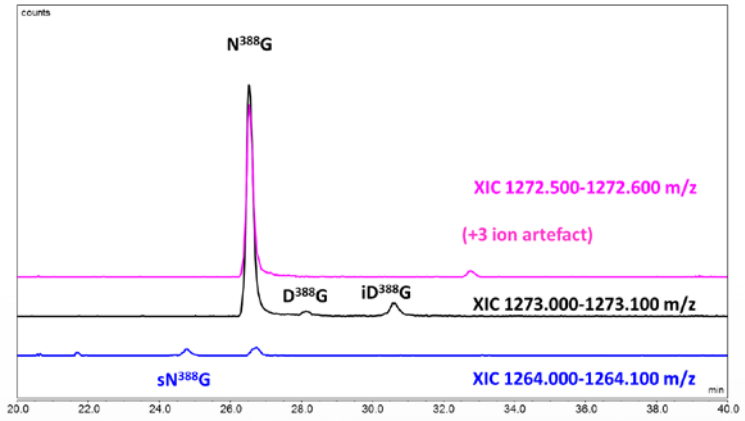
- 9 sites of potential modification were analyzed
  - 3 Asn sites with significant deamidation, and subsequent isomerization.

*JASMS, 28 (2017) 818-826
J Chromatogr A, 1537 (2018) 58-65

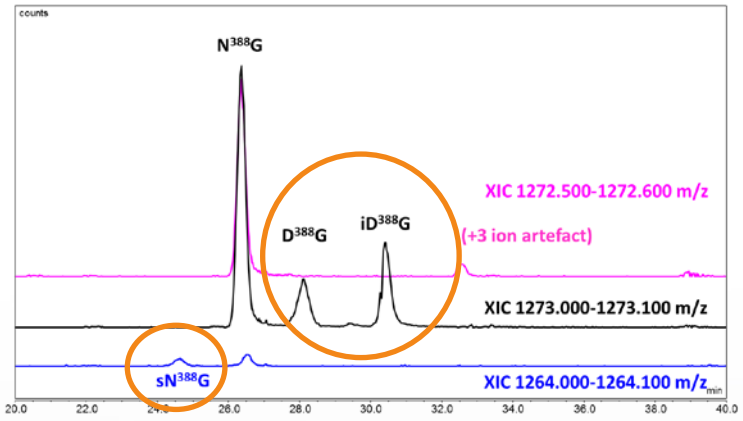
Deamidation and Isomerization in IgG Tryptic Digests

GFYPSDIAVEWESNGQPENNYK in Trastuzumab Digest

Native

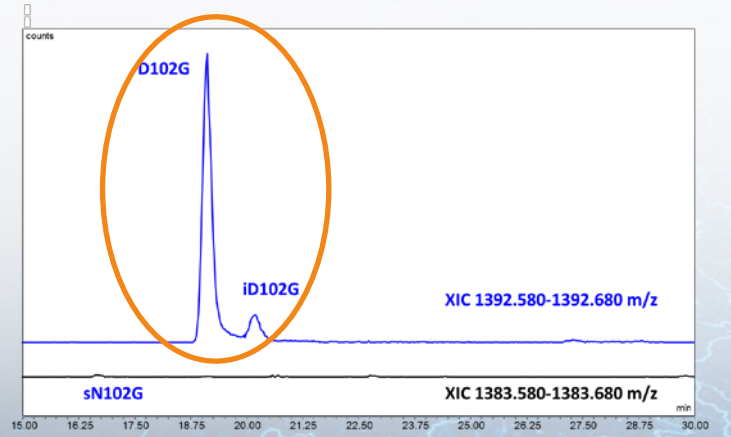


Stressed

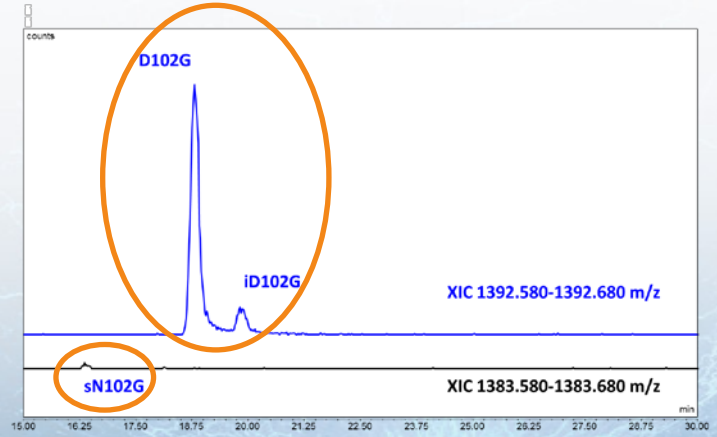


WGGDGFYAMDYWGQGTLVTVSSASTK in Trastuzumab Digest

Native



Stressed

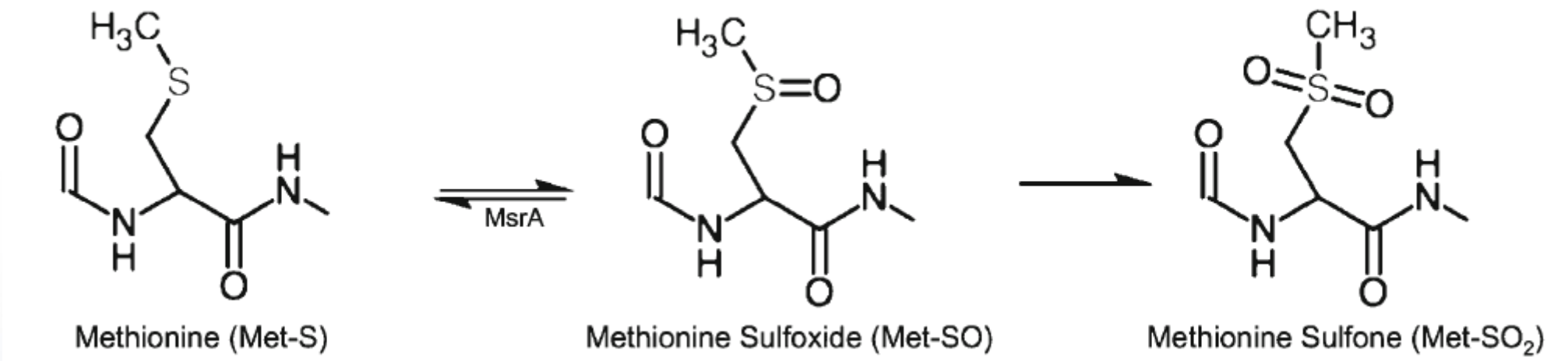


- 9 sites of potential modification were analyzed
  - 3 Asn sites with significant deamidation, and subsequent isomerization.
  - 2 sites showed presence of D/iD pairs, neither were strongly affected by “stress”
- All susceptible sites exhibited the cyclic intermediate sN, whether formed from D or N in the native sequence.

JASMS, 28 (2017) 818-826
J Chromatogr A, 1537 (2018) 58-65

OXIDATION

Oxidation of Methionine Mechanism

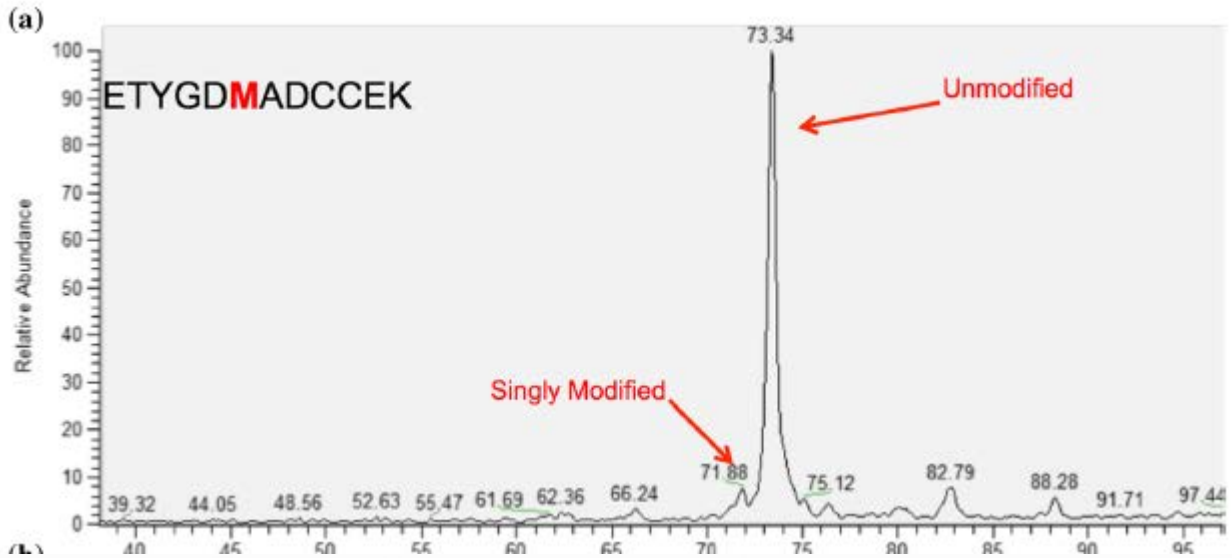


- Methionine (Met-S) oxidizes to form methionine sulfoxide (Met-SO)
- Methionine sulfoxide (Met-SO) can further oxidize to form methionine sulfone (Met-SO_2)
- Met-SO can be reduced back to methionine using methionine sulfoxide reductase A (MsrA)

Why is Oxidation of mAbs Important?

- Met oxidation in mAbs linked to
  - Function loss
  - Folding stability decrease
  - Increase in propensity to aggregate
- Could happen at various development stages
  - Production
  - Formulation
  - Storage

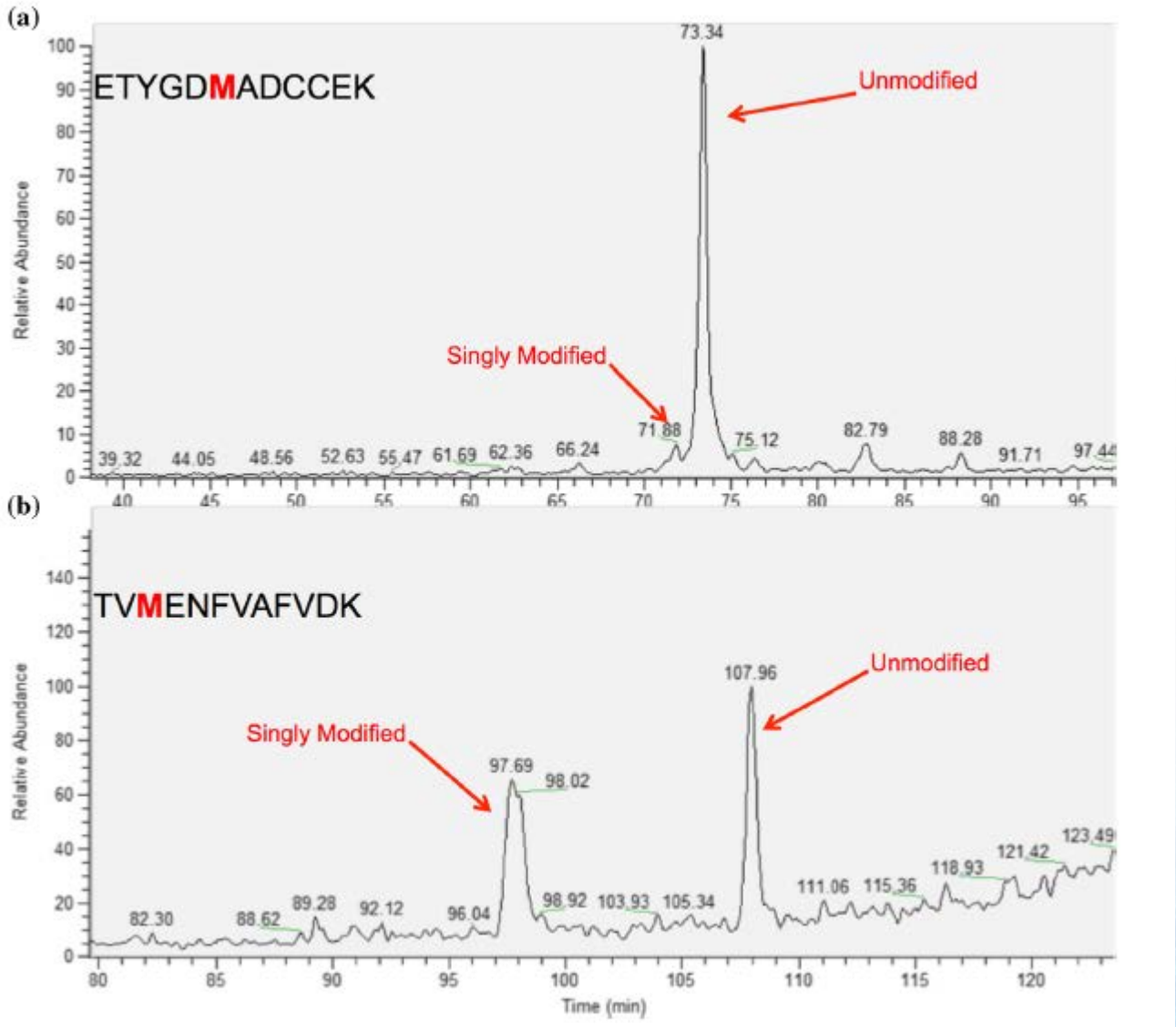
Reversed-Phase Separations of Oxidized Peptides



- Tryptic peptides from BSA
- Example in A is not resolved well enough for quantitation

J. Am. Soc. Mass Spectrom. (2017) 28:818-826

Reversed-Phase Separations of Oxidized Peptides



- Tryptic peptides from BSA
- Example in A is not resolved well enough for quantitation
- Example in B is resolved well enough for quantitation
- No consistent resolution with separation using reversed-phase so cannot be easily predicted

Column: 0.2 x 150 mm HALO 160 Å ES-C18, 5 µm; Flow rate: 2 µL/min; Temp: room temperature; Mobile Phase A: water/0.1% formic acid and 10 mM ammonium formate; Mobile Phase B: acetonitrile/0.1% formic acid and 10 mM ammonium formate; Gradient: 5%-75% B in 120 min.

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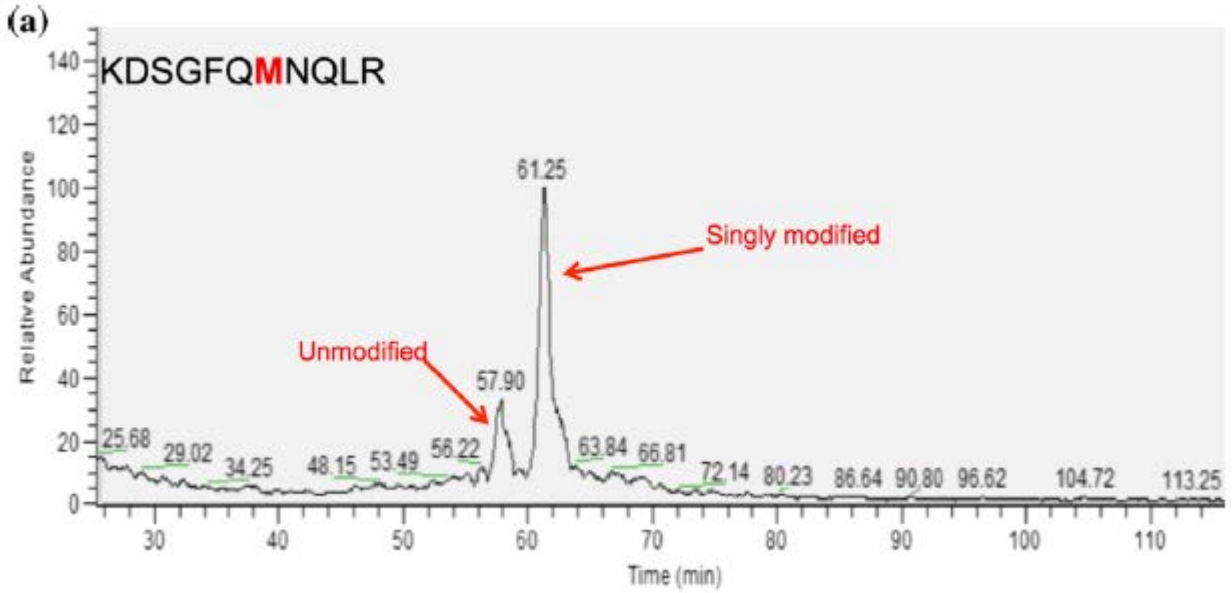
HILIC Separation of IgG Oxidized Peptide



- IgG oxidized peptide is well resolved from the unmodified form and can easily be quantified

Column: 0.2 x 150 mm HALO 90 Å Penta-HILIC; Flow rate: 2 µL/min; Temp: room temperature;
Mobile Phase A: 50 mM Ammonium Formate/0.1% formic acid; Mobile Phase B: acetonitrile/0.1% formic acid
Gradient: 95%-30% B in 90 min.

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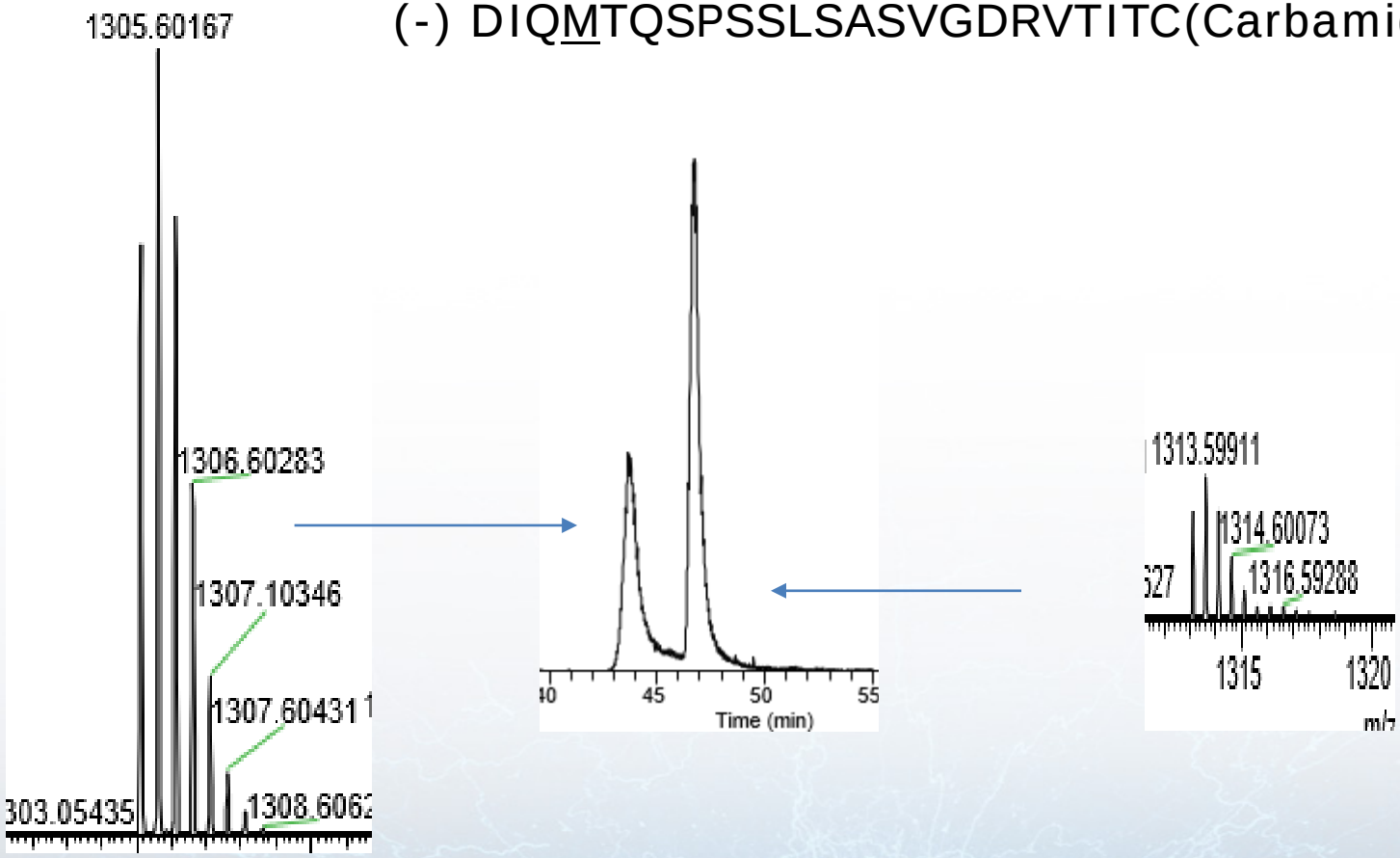
HILIC Separation of IgG Oxidized Peptide from NISTmAb digest



Column: 0.5 x 150 mm HALO 90 Å Penta-HILIC; Flow rate: 50 µL/min; Temp: 60 °C;
Mobile Phase A: 50 mM Ammonium Formate, pH 4.4; Mobile Phase B: acetonitrile/0.1% formic acid
Gradient: 80%-48% B in 55 min.

Conclusions

- Detailed characterization of PTMs continues to grow, particularly for therapeutic mAbs
- HILIC separations show increased resolution of polar PTMs of mAbs compared to reversed-phase separations
- HALO Penta-HILIC is well suited for separations of glycovariants and analysis of site occupancy
- HALO Penta-HILIC can yield quantitative details on protein deamidations and other chemical modifications

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Questions