

# Selection of HPLC and LC/MS Conditions for the Separation of Polar Compounds and Protein Modifications

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## Choice of Operation Mode and Examples of Conditions for Analysis

### Part 1. Examine use of HPLC for Separations of Polar Compounds

Polar Compounds refers to molecules that exhibit regions of charge (not necessarily formal charge), resulting low non-polar character, and comparatively poor solubility in non-polar solvents.

- Lower retention on hydrophobic stationary phases in RP-HPLC
- Variable solubility in organic solvent or aqueous/organic mixtures

### Part 2. Examine use of HPLC for Resolution of Polar Modifications of Peptides

- Synthetic peptides and tryptic fragments of proteins
- Glycosylation modifications (HexNAc, N-linked glycans)
- Deamidation products (Asn, Asp, isoAsp)
- Oxidations (eg., Met-SO<sub>2</sub>, Cys)

## HPLC for Separations of Polar Compounds

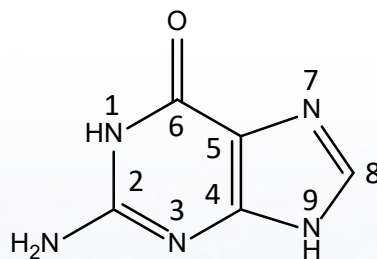
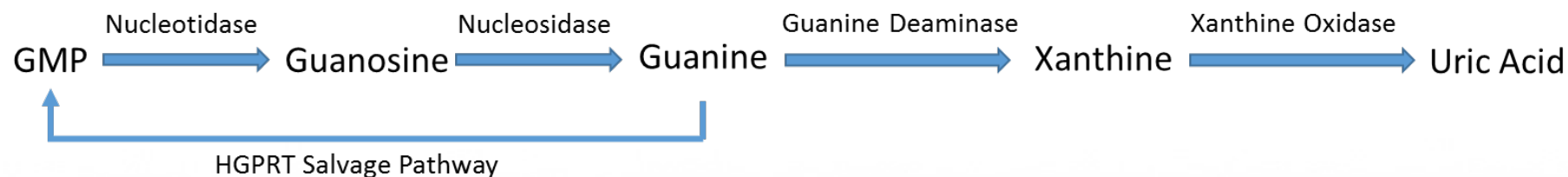
### Reversed Phase (RP)

- RP-HPLC is favored due to available high efficiency, reproducibility, robustness, and detection compatibility (all common detectors)
- Retention and selectivity can be the struggles for RP-HPLC of highly polar compounds; pH or ion pair manipulations may help, if consistent with analytical goals
- To maximize retention, low or no organic solvent conditions are favored in RP separations for polar compounds
- Under low organic conditions, RP surfaces can “De-wet” or “Phase collapse” leading to reproducibility issues and time dependent changes in analytical performance

### Hydrophilic Interaction Liquid Chromatography (HILIC)

- Many polar small molecule mixtures are effectively resolved by HILIC, through rich interactive features of contemporary columns and operating conditions. Solubility and appropriate sample management is often under-appreciated to fully utilize this mode of operation.
- As a more recently popular method, analytical utility is often underestimated.
- This is NOT your fathers Normal Phase Chromatography method.

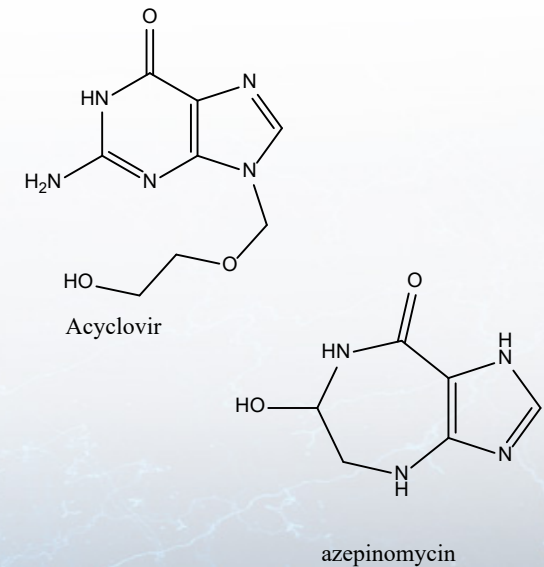
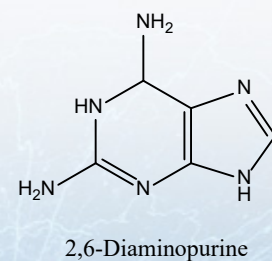
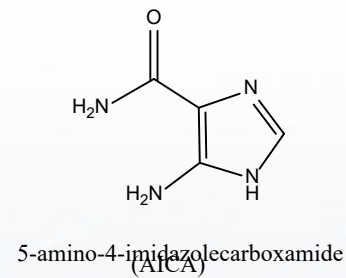
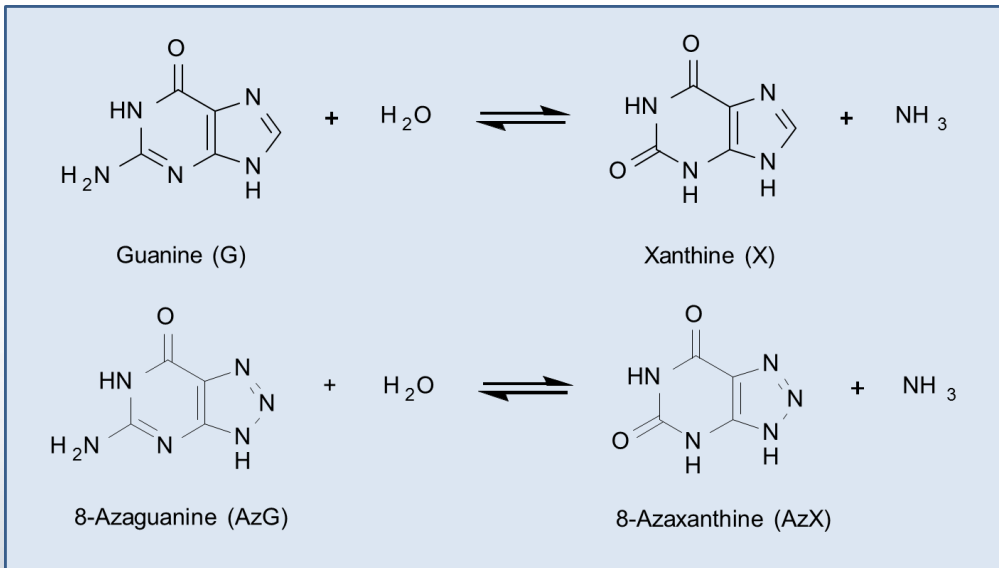
## Purine Catabolic Pathways



- Guanine is polar (Log P -0.91), poorly soluble in most organic solvents (< 100 mg/L), and almost insoluble in neutral pH water. Modestly soluble in acid, moderately soluble in base ( $\approx 2.5$  g/L)
- A particular interest in Guanine Deaminase (GD) as the metabolic “commitment” to elimination of purines, and it’s potential for control by expression levels and enzyme activity control (PTMs)

## Substrates and Inhibitors of Mammalian Guanine Deaminase

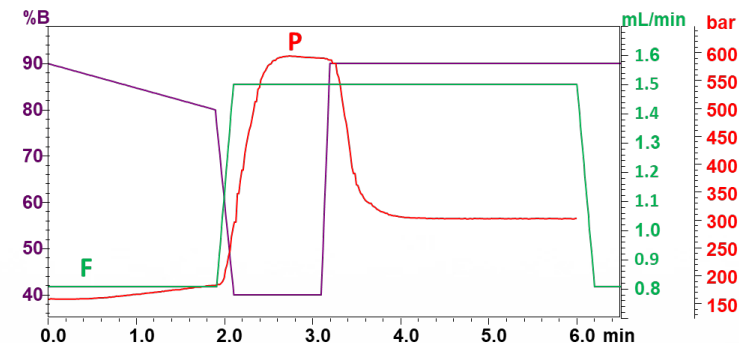
- Dimer of 50 kDa subunits with exon selection; variants - at least 4 significant forms.
- $Zn^{+2}$  dependent amino hydrolase, with TIM barrel structure.
- Interactions: tubulin, snapin, and the post-synaptic domain protein 95 (PSD-95).
- Enzymatic levels vary widely between tissues, and within CNS structures. Low levels in plasma/serum, notably altered by liver dysfunction.
- Biochemical pharmacology is not well understood (limited inhibitors are available).



# Purinergetic Compounds are Well Retained in HILIC: GD Assay Conditions

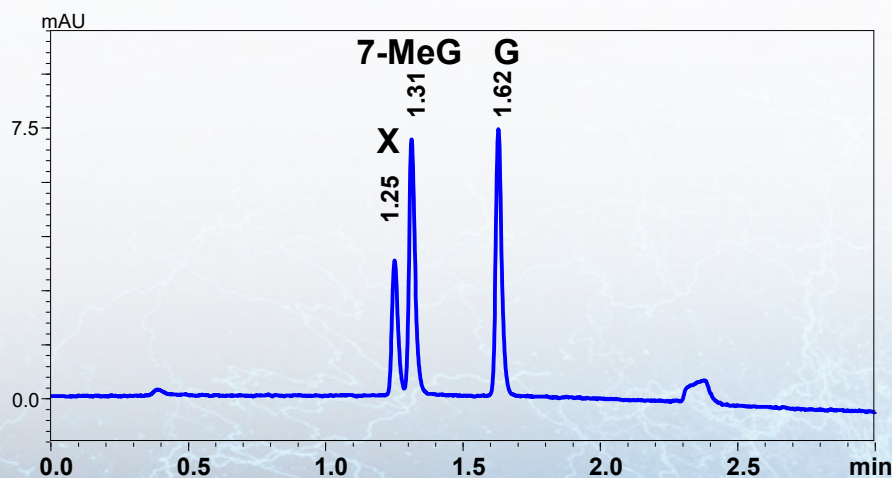
## LC Conditions:

- 3.0 x 75 mm 2.0  $\mu\text{m}$  particle size **HALO 2 Penta-HILIC**
- Shimadzu Nexera with SPD-30A DAD at 270 nm (10 nm BW)
- 35  $^{\circ}\text{C}$ , 0.8 mL/min initial flow rate; 2-20  $\mu\text{L}$  injection
- Eluents: A – 0.1 M  $\text{NH}_4\text{OAc}$  (pH 6.5); B – AcN
- For rapid equilibration, flow rate increased to 1.5 mL/min

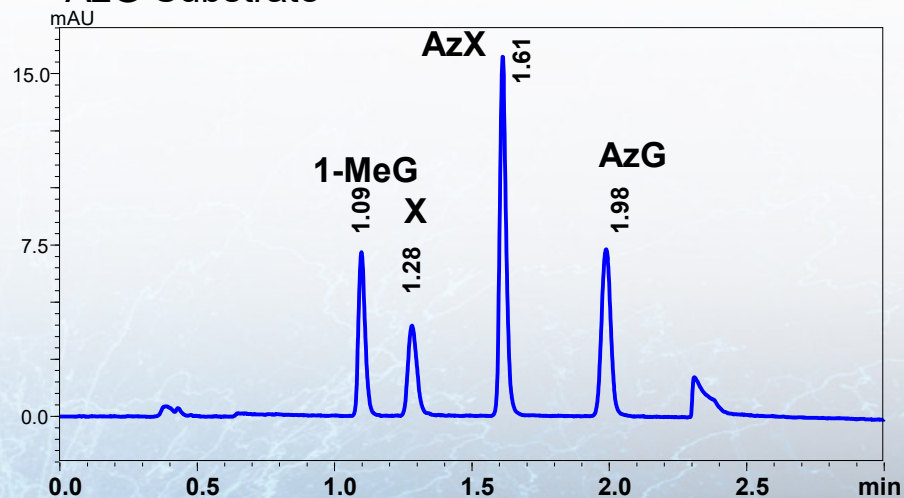


## Resolution of Standard Purines

### G Substrate



### AzG Substrate



## GD HILIC Assay Conditions Applied to Tissue Homogenates

### Enzymatic Reaction:

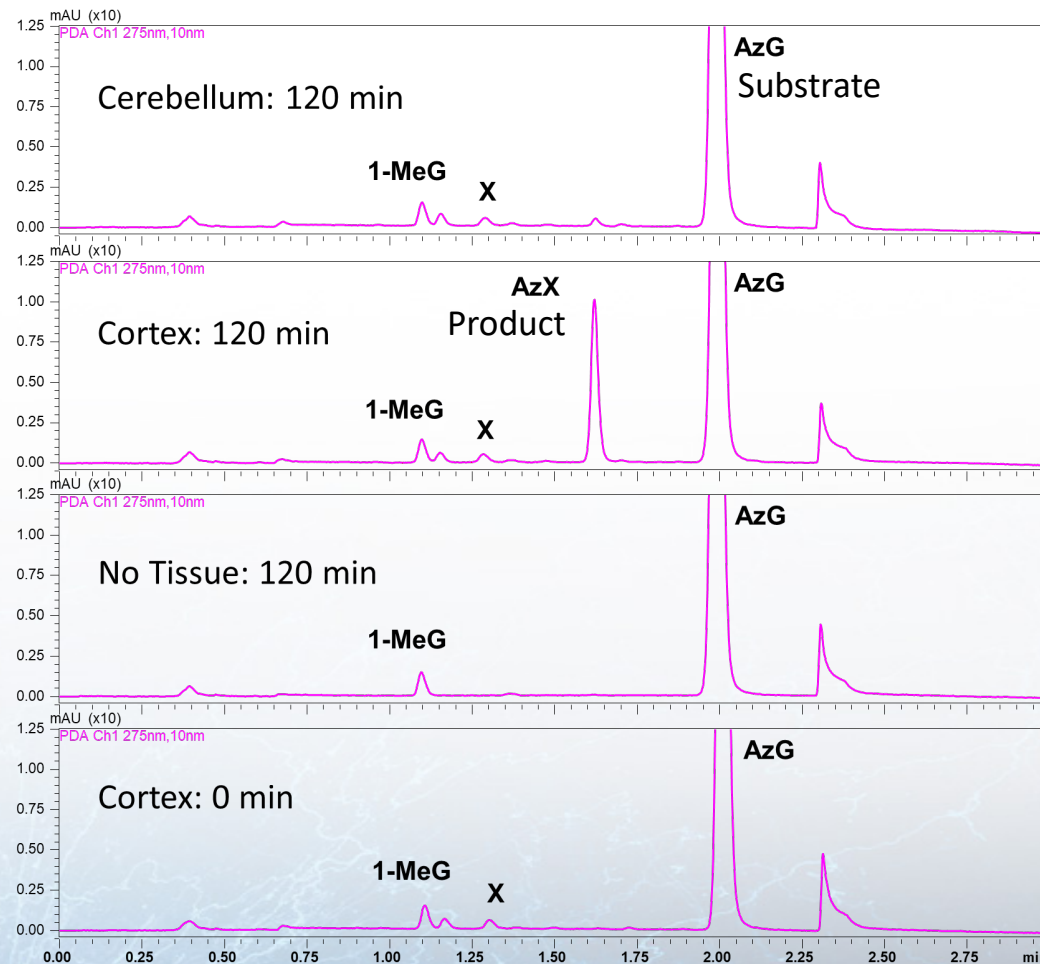
- 3.0 x 75 mm 2.0  $\mu$ m particle size **HALO 2 Penta-HILIC**
- Temp: 25  $^{\circ}$ C
- Substrate: 0.2 mM G or 0.4 mM AzG
- Buffer: 0.1 M Bicine-HCl, pH 7.8
- Internal standard: 0.5  $\mu$ M 1-methylguanine
- 10% (vol/wt) tissue homogenate
- Stop Solution: 9 Volumes of 1% HOAc/99% Acetonitrile

### Good News

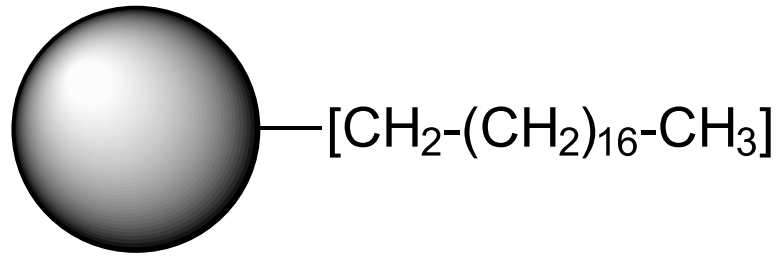
- Linear with time, reproducible, biologically relevant
- MS compatible
- Excellent selectivity (broad retention range)
- Fairly fast

### Bad News

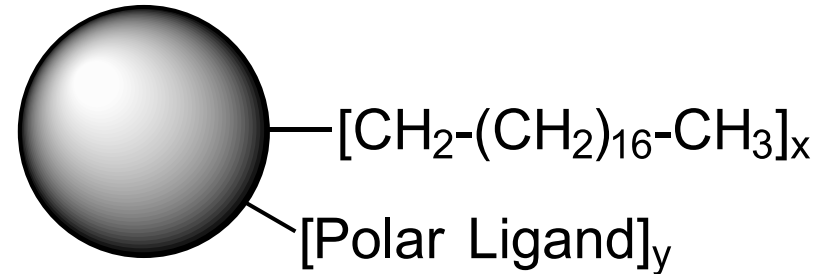
- Modest volume injection tolerance (10-25  $\mu$ L)
- Only 2% of reaction can be analyzed (STOP solution)
- Never going to get above 10-15% of reaction



# HALO AQ-C18 Bonded Phase



C18



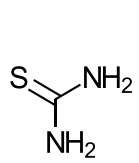
AQ-C18

- The combination of a C18 ligand and a ligand that has polar characteristics increases the wettability of HALO AQ-C18.
- Contemporary High Aqueous column packing materials have a mixture of polar functionalities and hydrocarbon bonded-phases, either as a single silane entity (polar embedded), or separate structure within the surface modified bonded phase.

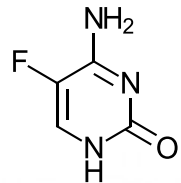


# pH 2 Dewetting results: Nucleic Acid Bases

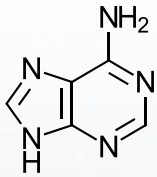
1. Thiourea
2. 5-Fluorocytosine
3. Adenine
4. Thymine



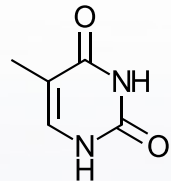
Thiourea



5-Fluorocytosine



Adenine



Thymine

Columns: HALO 90Å, AQ-C18, 2.7 μm, 4.6 x 50 mm  
 HALO 90Å, C18, 2.7 μm, 4.6 x 50 mm

Isocratic: Water/0.1% TFA

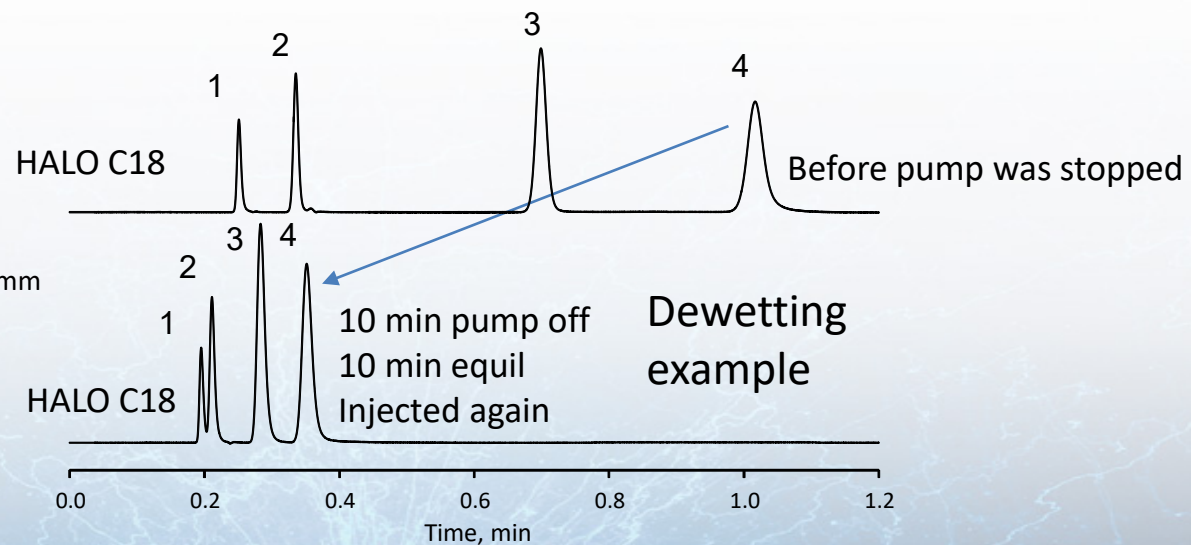
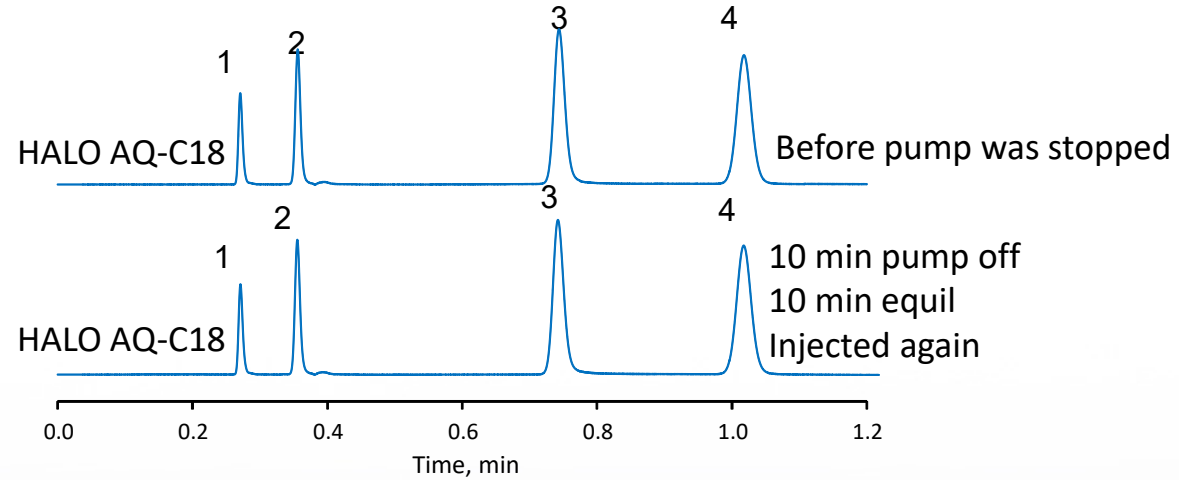
Flow Rate: 2.0 mL/min

Pressure: 290 bar

Temperature: 30 ° C

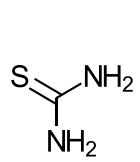
Detection: UV 254 nm, PDA

Injection Volume: 0.5 μL

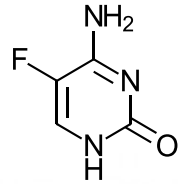


# pH 7 Dewetting results: Nucleic Acid Bases

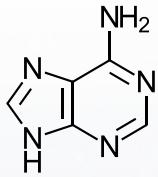
1. Thiourea
2. 5-Fluorocytosine
3. Adenine
4. Thymine



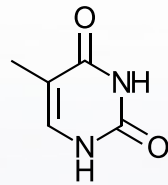
Thiourea



5-Fluorocytosine



Adenine



Thymine

Columns: HALO 90Å, AQ-C18, 2.7 μm, 4.6 x 50 mm  
 HALO 90Å, C18, 2.7 μm, 4.6 x 50 mm

Isocratic: 20 mM Phosphate buffer, pH 7

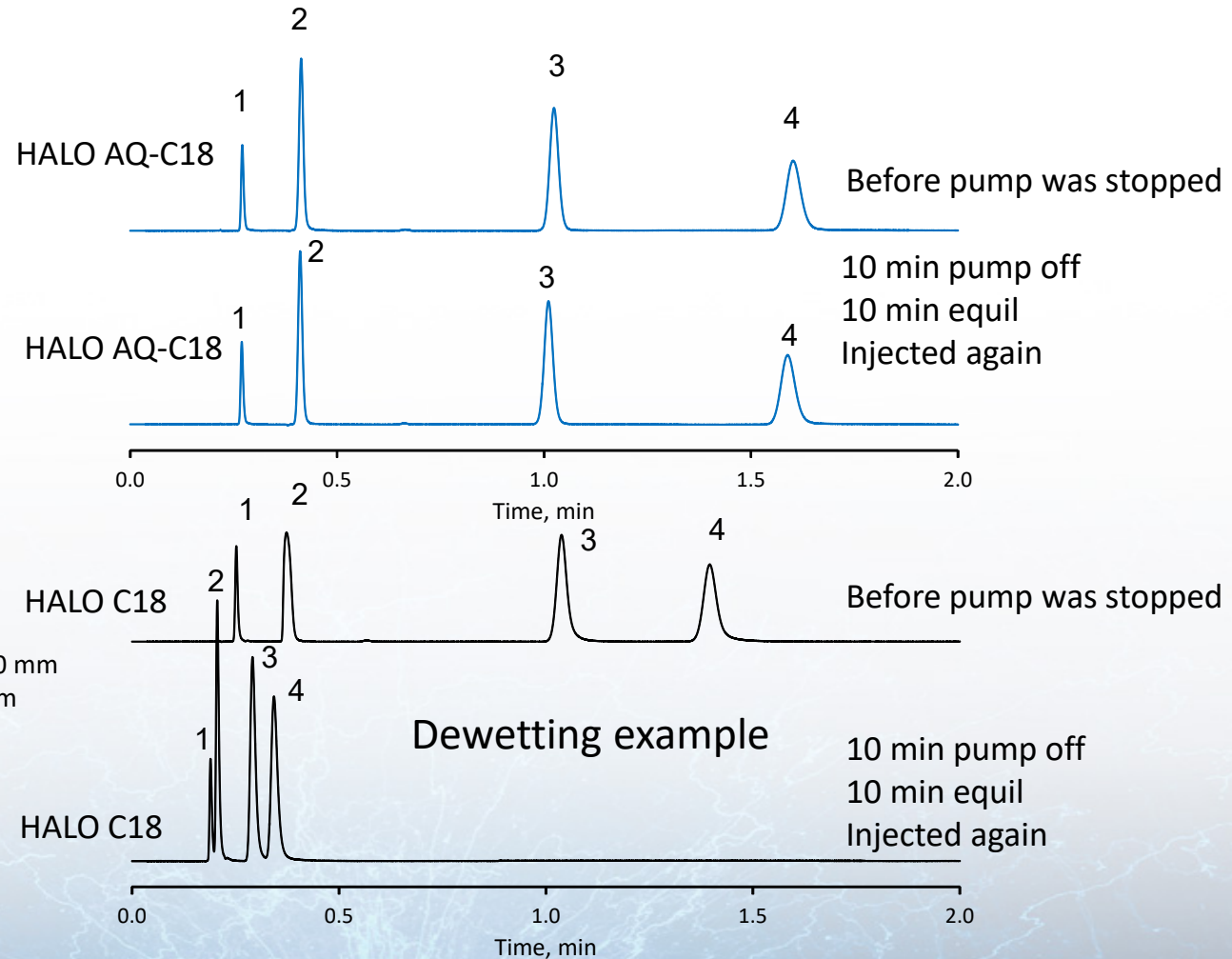
Flow Rate: 2.0 mL/min

Pressure: 290 bar

Temperature: 30 ° C

Detection: UV 254 nm, PDA

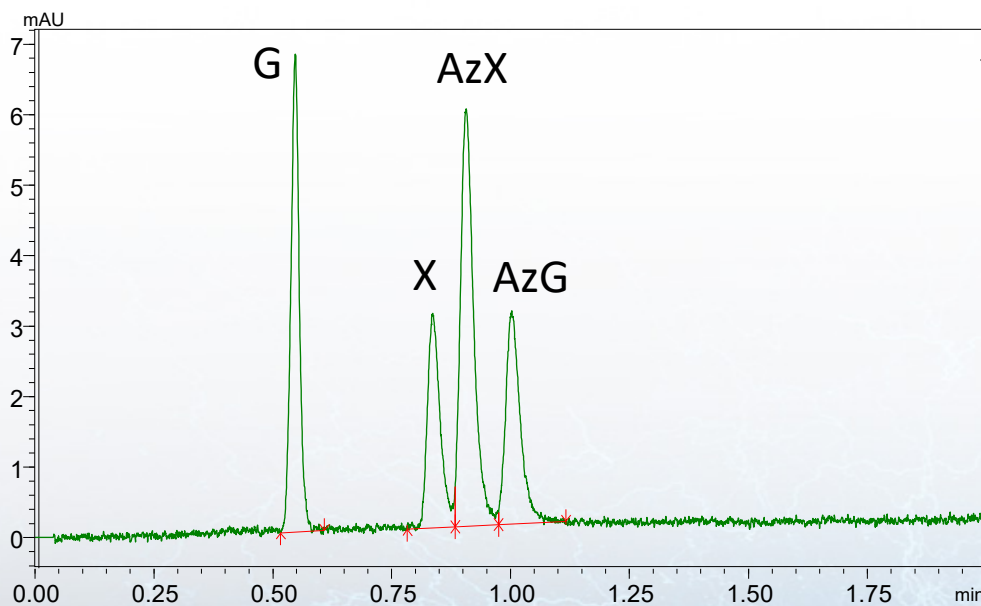
Injection Volume: 0.5 μL



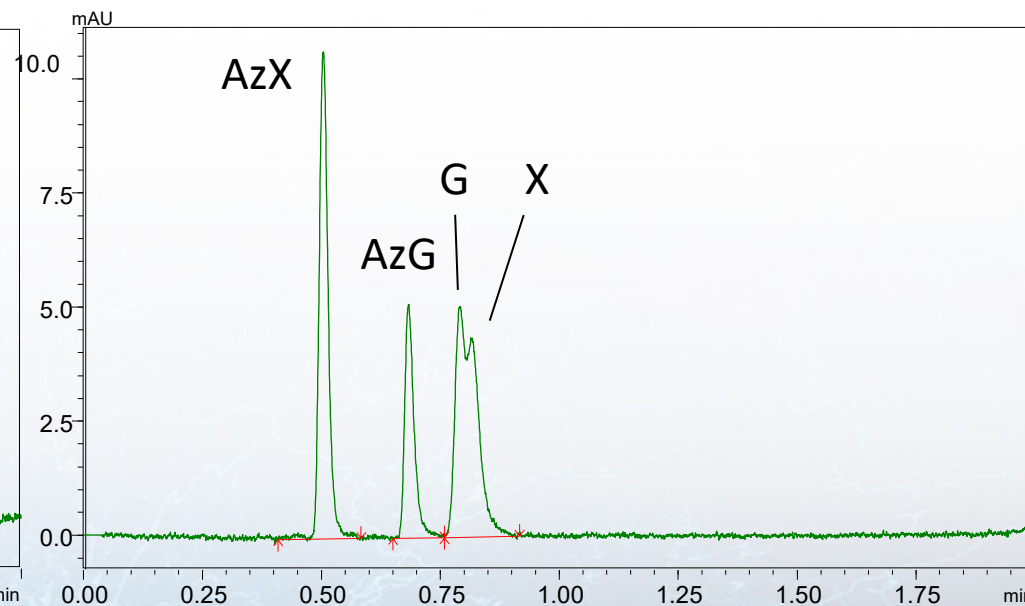
# Mobile Phase Impacts Polar Compound Separation

Conditions: 2.1 mm x 75 mm AQ-C18, 0.5 mL/min., 35°C, 2  $\mu$ L injected

0.1% Formic Acid (pH  $\approx$  3.8)

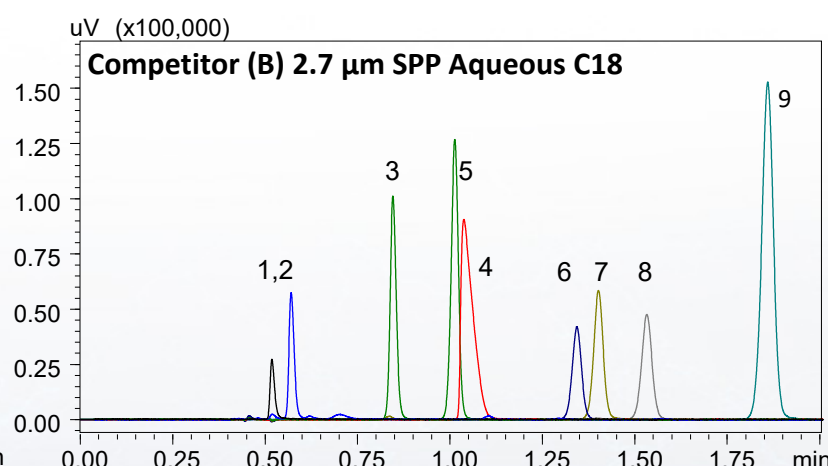
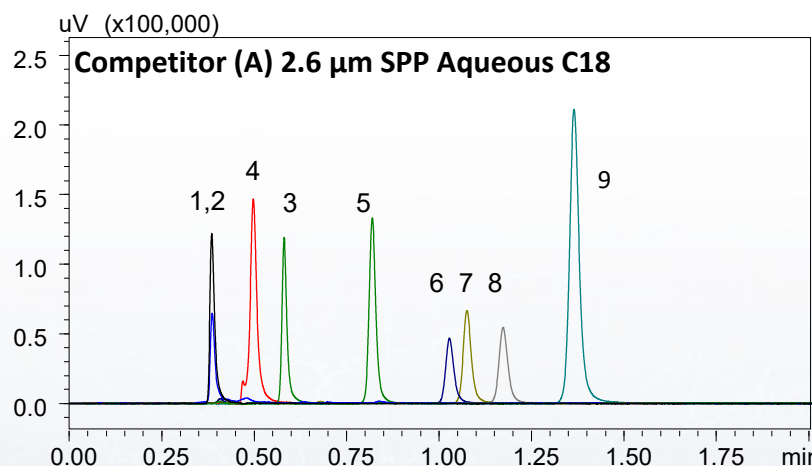
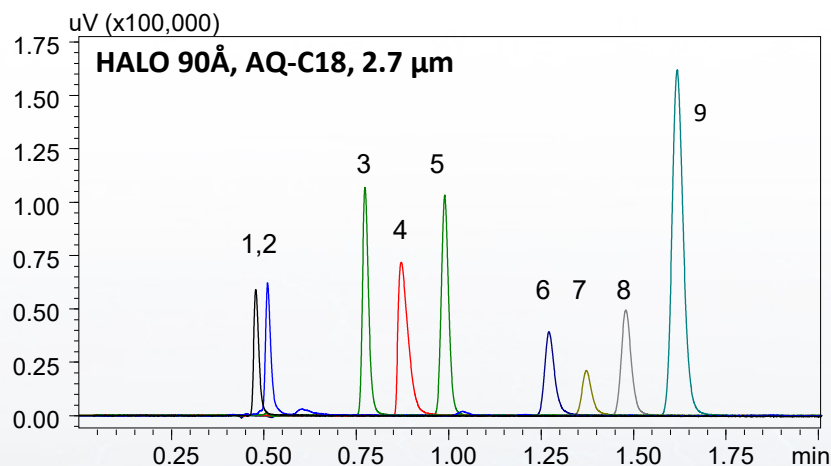


0.02M Ammonium Acetate (pH = 6.4)



# Aqueous C18 Bonded Phases for Analysis of Purinergic Compounds

Conditions: Columns (SPP-type): 2.1 x 100 mm; 0.1% Formic Acid; 0.5 mL/min; 35 °C  
 Detection: UV 254 nm; Injection: 1 µL  
 Instrument: Shimadzu Nexera

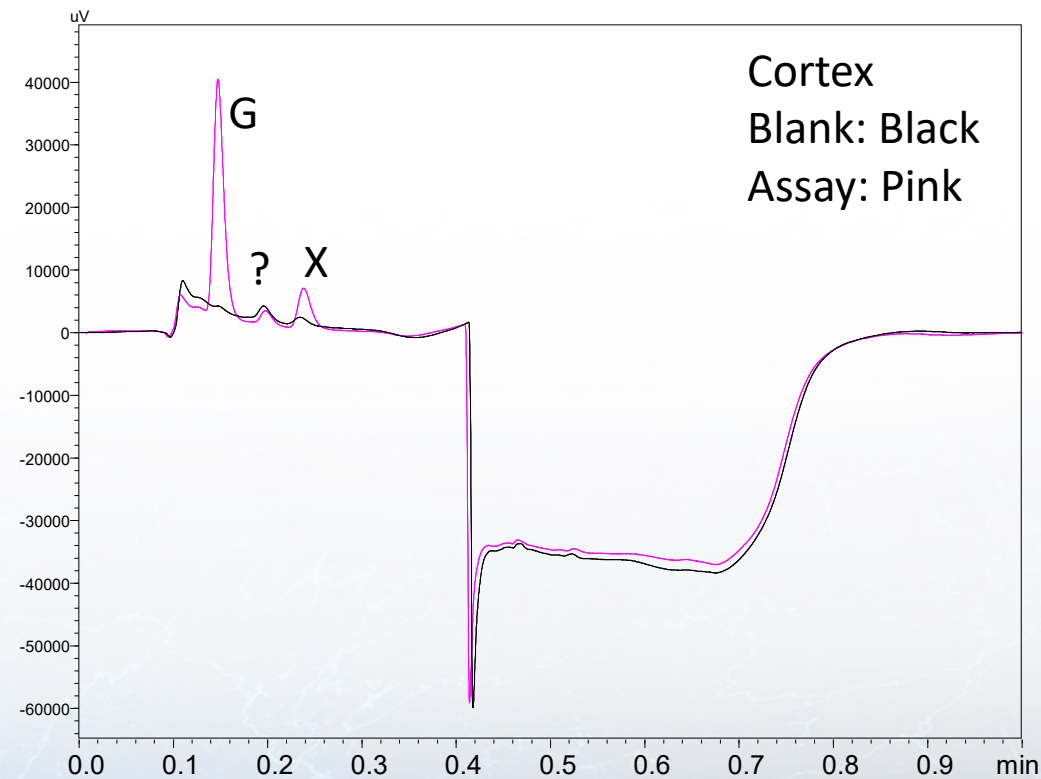
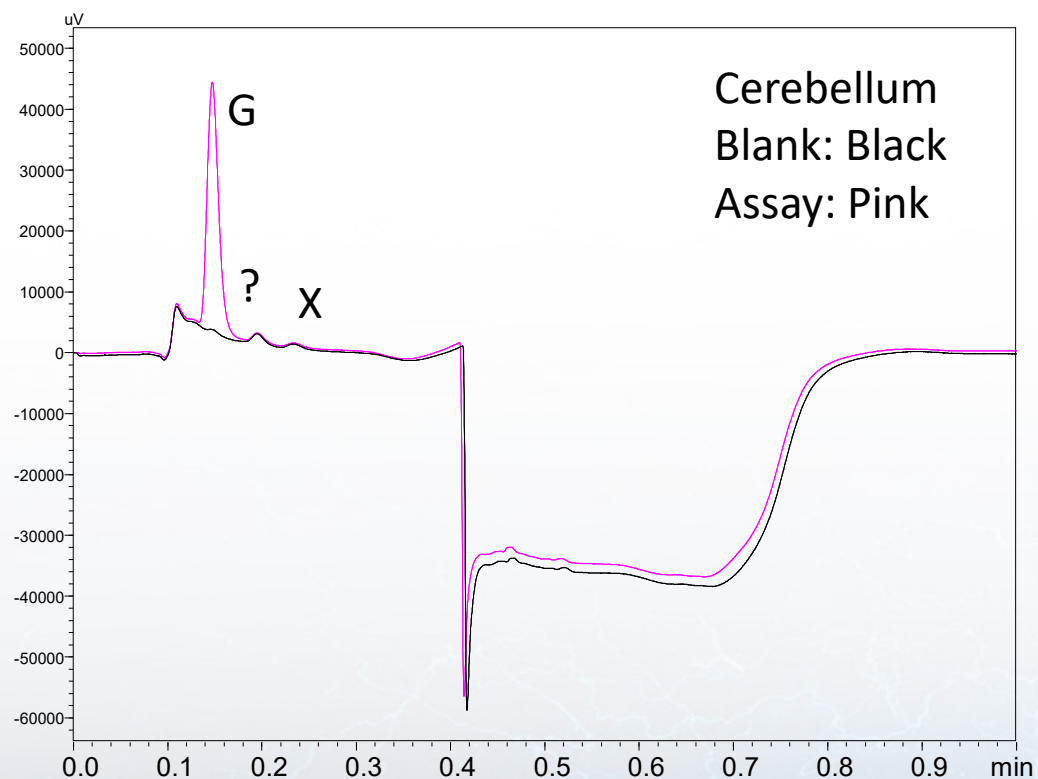


### Peak Identities

- |   |                       |
|---|-----------------------|
| 1: 5-Amino-imidazole-4-carboxamide (AICA) | 5: Uric Acid          |
| 2: Azepinomycin                           | 6: Xanthine - Product |
| 3: Guanine - Substrate                    | 7: 8-Azaxanthine      |
| 4: 2,6-Diaminopurine                      | 8: 8-Azaguanine       |
|   | 9: Allopurinol        |

The “Aqueous Type” dewetting-resistant bonded-phases exhibit good stability in NO organic conditions, with acceptable retention of polar compounds, varying in selectivity and peak shapes between different manufacturers.

## Analysis of RP-HPLC of Polar Compounds: GD Analysis HALO AQ-C18



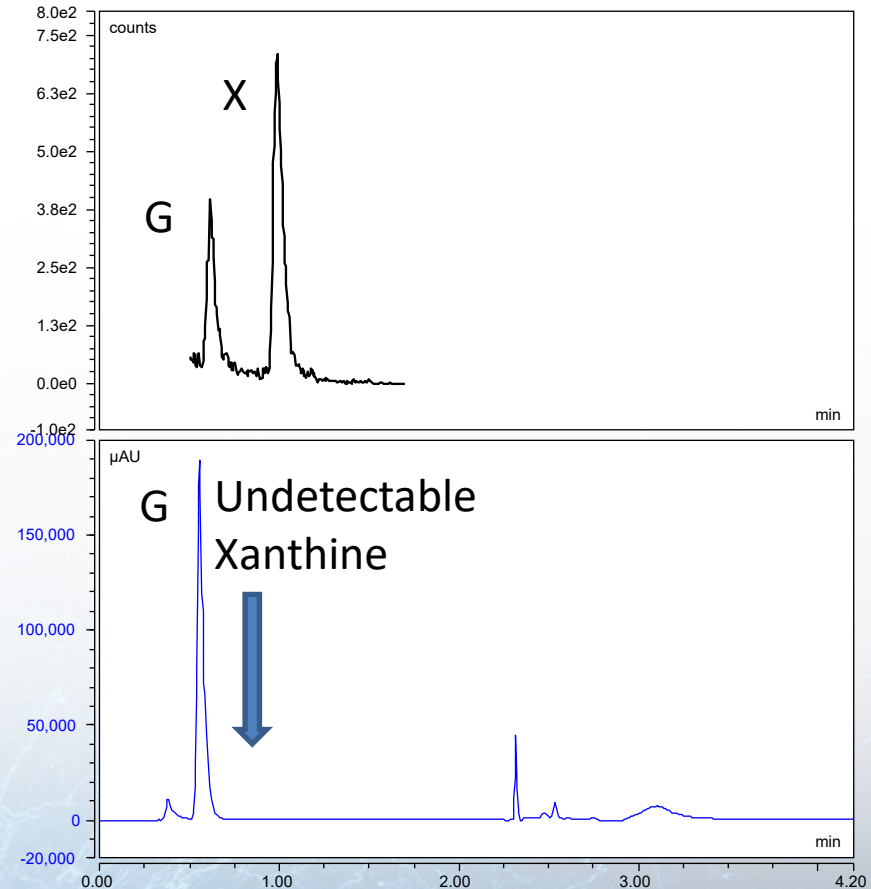
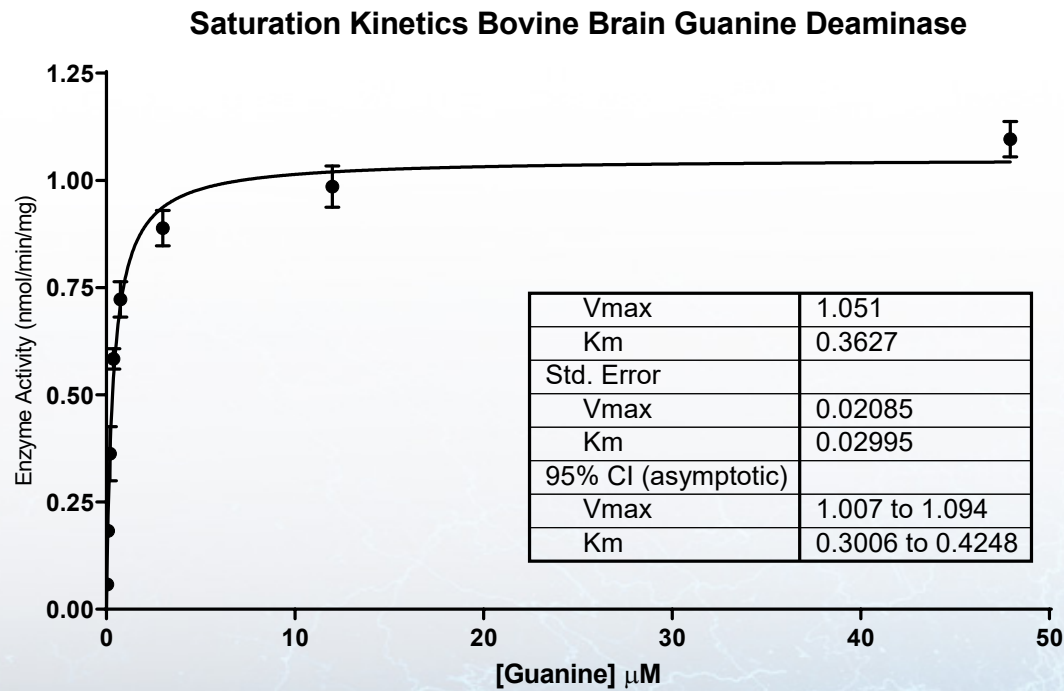
2.1 x 30 mm AQ-C18  
0.75 mL/min  
1  $\mu$ L injections

265 m  
35  $^{\circ}$ C

# Analysis of RP-HPLC/MS of Polar Compounds: GD Analysis HALO AQ-C18

2.1 x 75 mm AQ-C18; 0.5 mL/min; 10 µL injections

Using RP-LC allows for 1- 10 µL injections of 100% aqueous injections



RP-HPLC  
MS/MS

RP-HPLC  
In-line UV

## Current Considerations for HPLC of Polar Compounds

- Newer RP bonded phase C18 materials may offer enough retention for successful analysis.
  - With ionizable compounds, evaluation using several pH modifiers is advised.
  - Ion pairing may be considered, if consistent with application.
  - Well tolerate highly aqueous samples.
- Modern bonded-phase HILIC columns offer high retention and selectivity for very polar compounds, and should be considered.
  - As with RP, operating conditions for HILIC should be optimized considering pH and ionic strength of the mobile phase.
  - Gradient operation is very accessible, and bonded-phase materials favor fast re-equilibration, compared to bare silica HILIC (Seidl, Bell and Stoll (2019), J Chromatogr. A 1604, 460484).
  - Context in use should include the known effects of water on peak volume tolerance.

## Resolution of Protein Polar Post Translational Modifications (PTMs)

PTMs occur for proteins destined for both intracellular and extracellular functions. In addition, changes in specific amino acid side chains can occur, by both reversible and irreversible enzymatic and chemical modifications.

Well known PTMs include;

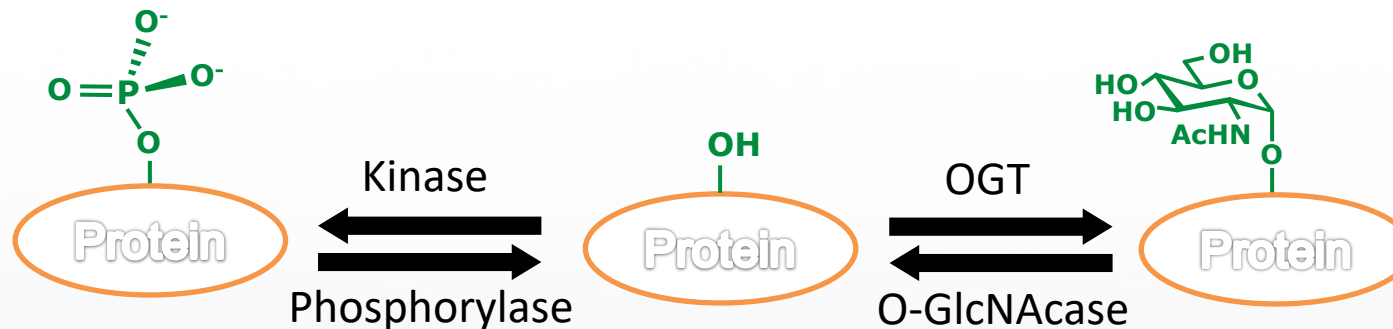
- Proteolysis
- N- and O-linked glycosylation, adding one or many carbohydrate residues to the protein
- N-terminal and lysine side chain acetylations
- Deamidation and isomerization at Asn and Gln
- O-linked phosphorylations (Tyr, Ser, Thr)
- Lipid modification (acetylation, eg., myristoylation)
- Oxidation (methionine, cysteine, tryptophan, histidine)
- Many others, both enzymatic and spontaneous

Many of these modifications have direct and significant biochemical consequences and functional purpose. Modifications occur against the context of existing polypeptide structure, and can represent a small relative proportion of the sequence present in a sample, and can also be heterogeneous in composition.

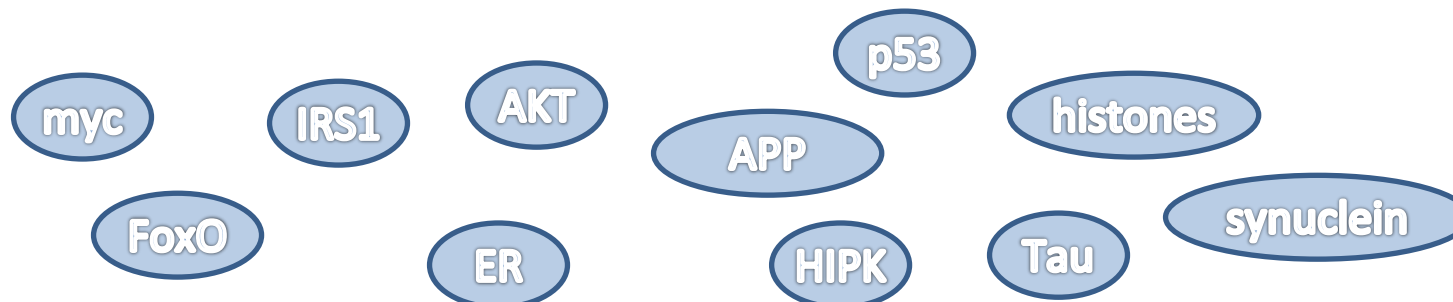


## β-O-(N-acetylglucosamine) Modifications of Proteins (O-GlcNAc)

- 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

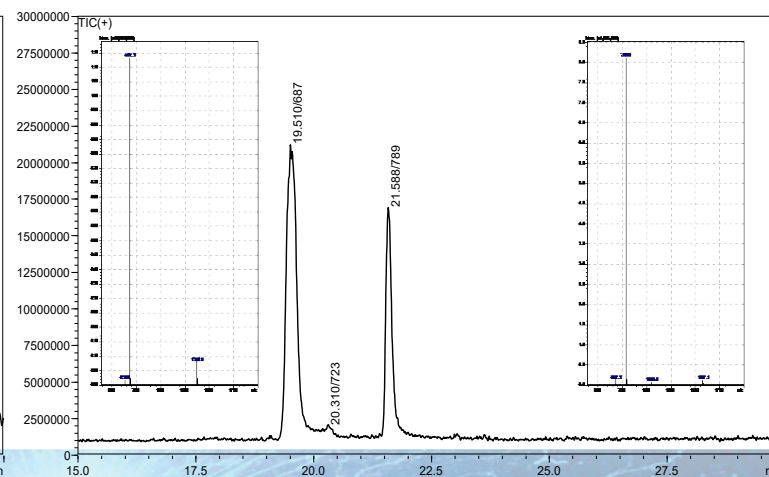
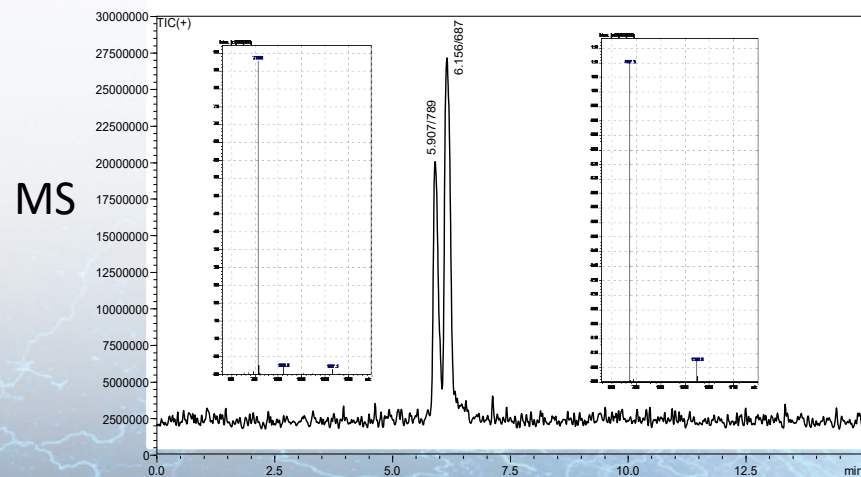
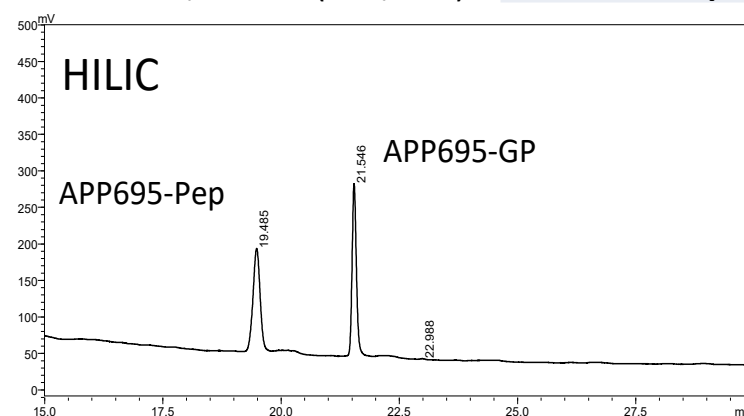
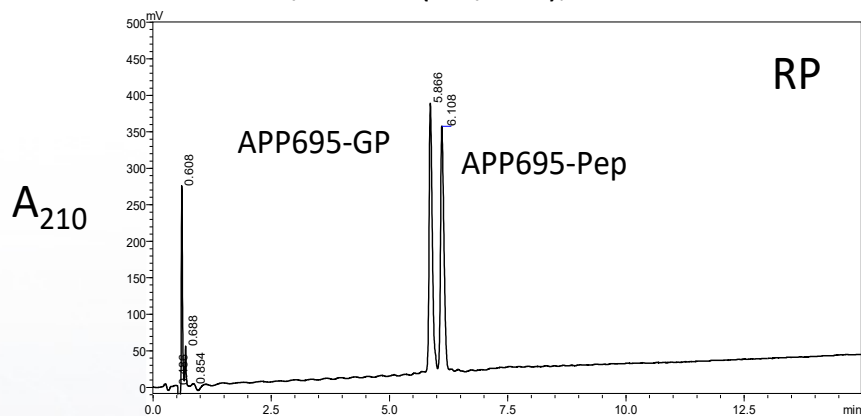
Conditions: Columns - 2.1 x 100 mm Halo Penta-HILIC or Peptide ES-C18; 0.4 mL/min; 60°C.

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**



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

Peptide Description	Sequence	Mass (neutral)	Rt RP (min)	$\Delta$ Rt RP (GP-P)	Rs RP	Rt HILIC (min)	$\Delta$ Rt HILIC (GP-P)	Rs HILIC
APP695-14Gep	VPTT(OGlcNAc)AASPDAVDK	1574.8	5.87			21.55		
APP695-14Pep	VPTTAASPDAVDK	1371.7	6.11	-0.24	1.90	19.49	2.07	9.41
MUC5AC	GTTSPVPTTSTTSAP	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	GTTSPVPTTSTT(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-CRSSHYGGS LPNVNQI	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-CKIPGVSt(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

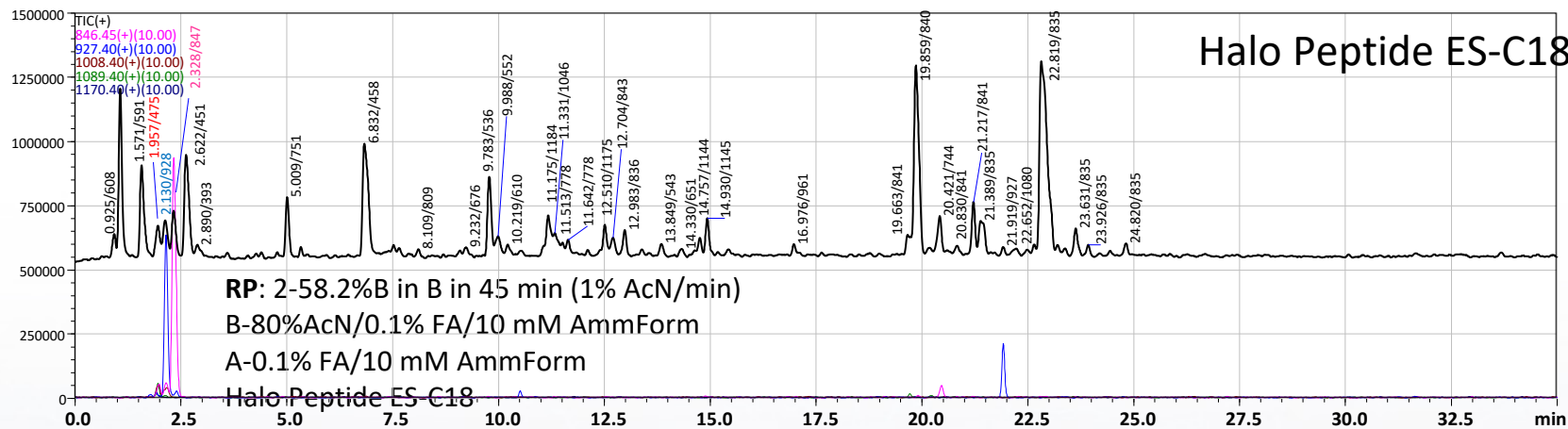
Driven by better separation selectivity of this polar modification

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

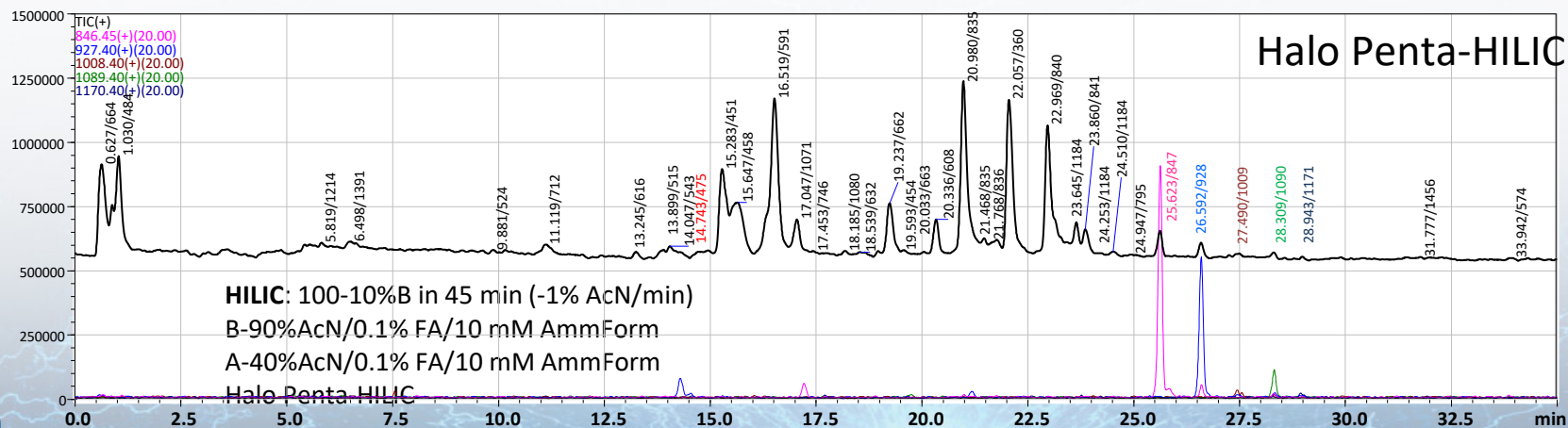
## HILIC Strongly Retains and Resolves N-linked Glycopeptides

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



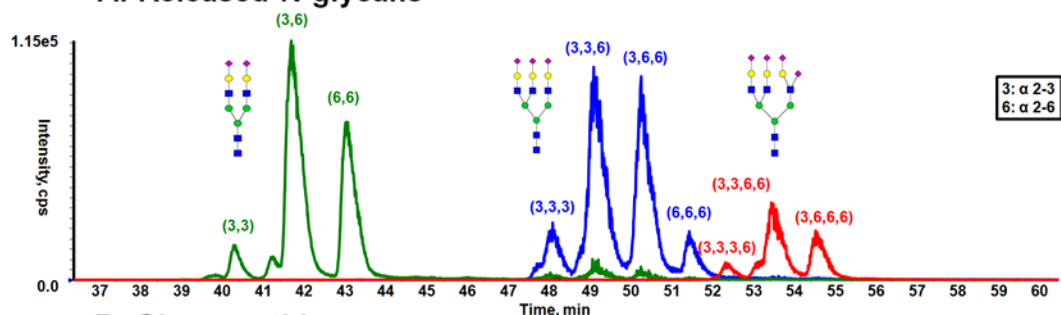
Good resolution of high mannose variant glycopeptide

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

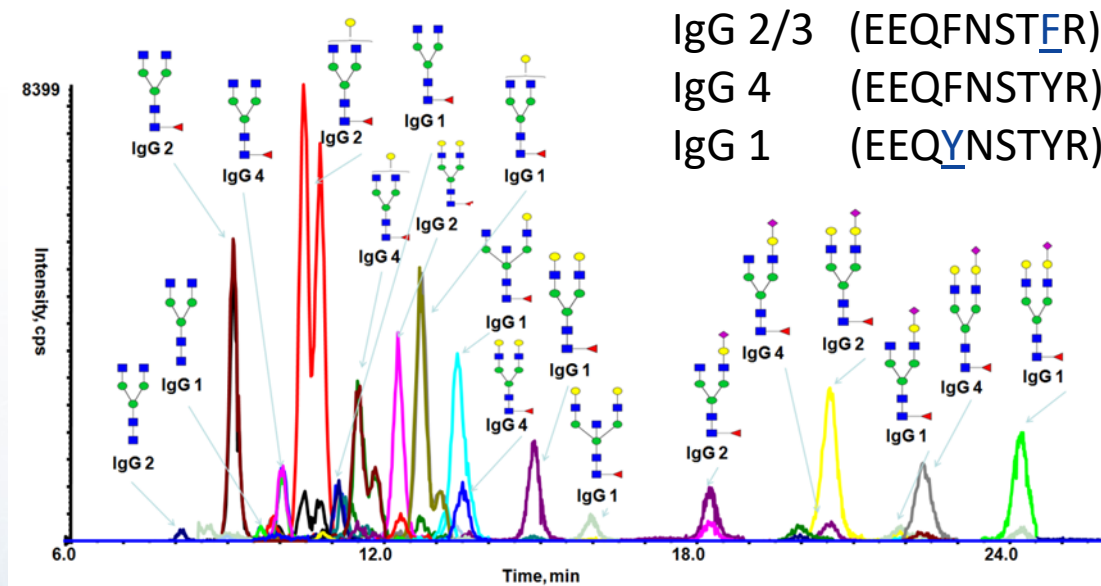
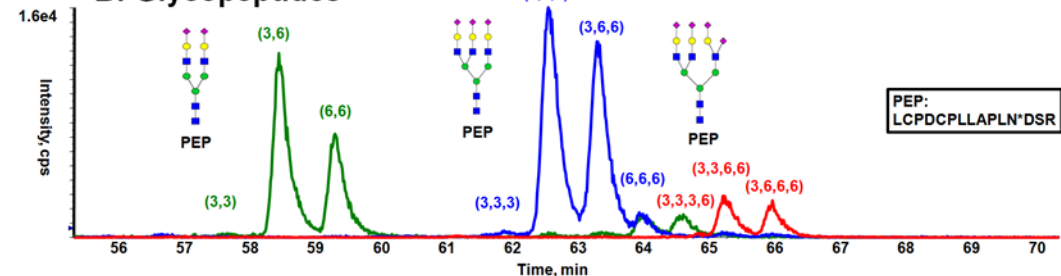
Conditions: Columns – 2@ 2.1 x 150 mm Halo Glycan; 0.4 mL/min; 55°C.  
 Gradient Eluents: A – 50 mM ammonium formate (pH 4.4)/5% AcN; B – AcN  
 Gradient – RG: 80% to 62% AcN/60 min (-1%/min); GP 85% to 48%B 75 min

Huang, Y., Nie, Y., Boyes, B., and Orlando, R. (2016) J. Biomol. Technol., 27, 97-104. Tao, S.J., Huang, Y., Boyes, B.E., and Orlando, R. (2014) Anal. Chem., 86, 10584-10590.

**A: Released N-glycans**



**B: Glycopeptides**

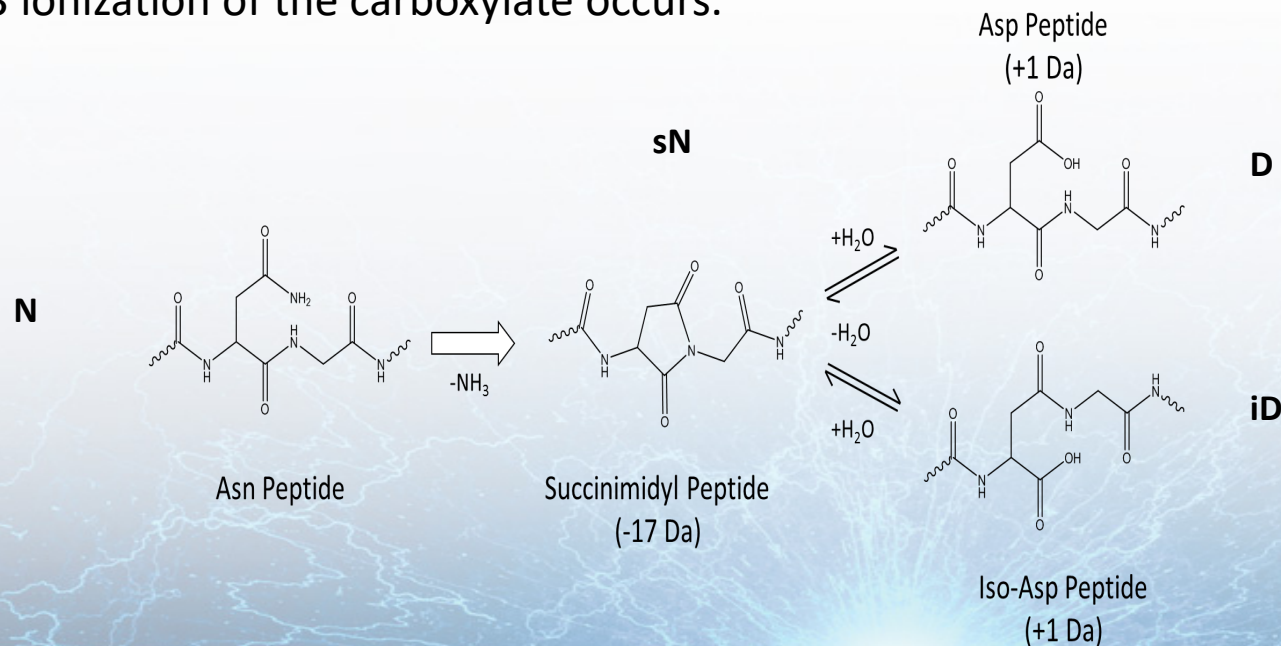


Comparison released N-glycans and glycopeptides of Fetuin. (A) Procainamide labeled released N-glycans. (B) Glycopeptides with the same peptide backbone.

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

# 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)
- The 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.



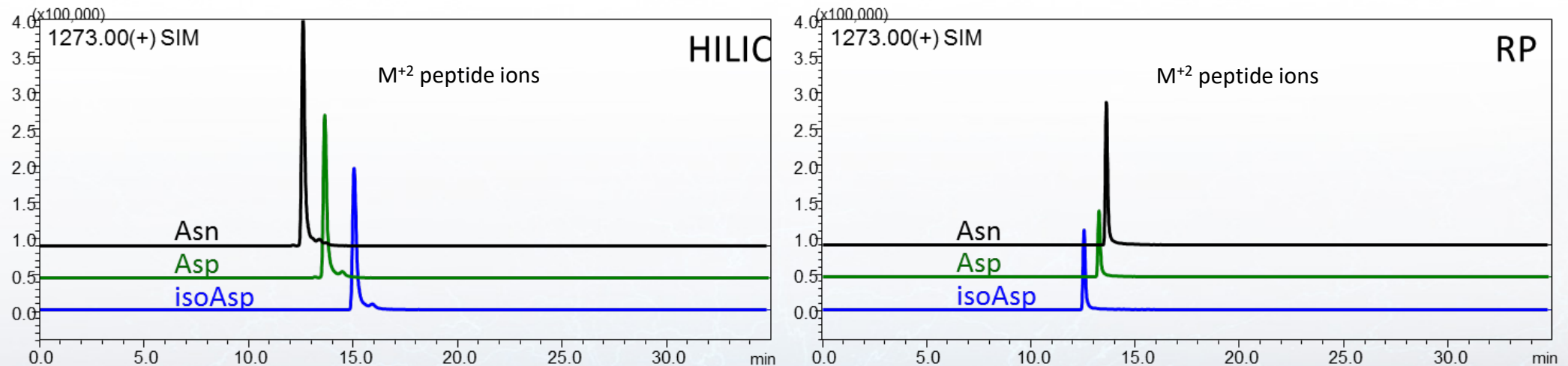
# Deamidation/Isomerization of Asparagine

GFYPSDIAVEWESNGQPENNYK  
GFYPSDIAVEWESDGQPENNYK  
GFYPSDIAVEWESiDGQPENNYK

Three peptides from IgG1 – variant at position 14 (IgG N<sup>388</sup>)

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

Columns: 2.1 x 150 mm Peptide Halo ES-C18 or Halo Penta-HILIC; Flow rate: 0.4 mL/min; Temp: 60 °C;  
Mobile Phase A: water/50 mM Ammonium Formate, pH4.4; 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);

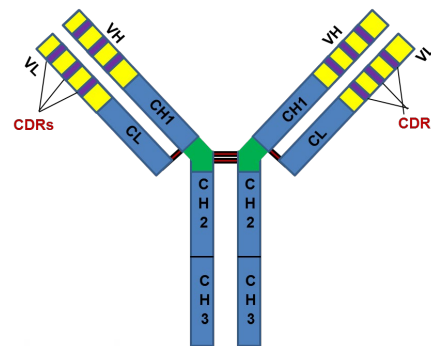


- The same mobile phase conditions and temperature were employed, reversing the direction of the acetonitrile gradient to effect elution on RP and HILIC columns.
- Note the greater selectivity difference for resolving these peptides in HILIC, compared to RP.
- Unlike RP, HILIC reliably resolves Asn/Asp/iAsn,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

# Deamidation/Isomerization of Asparagine

## IgG Peptides Studied

Trastuzumab Light Chain		
25	ASQDVNTAVAWYQQKPGK	42 N <sup>30</sup> T
Trastuzumab Heavy Chain		
76	NTAYLQMNSLR	86 N <sup>83</sup> S
99	WGGDGFYAMDYWGQGLTVSSASTK	124 D <sup>102</sup> G
279	FNWYVDGVEVHNAK	292 D <sup>284</sup> G
306	VVSVLTVLHQDWLNGK	321 N <sup>319</sup> G
375	GFYPSDIAVEWESNGQPENNYK	396 N <sup>388</sup> G--NN <sup>393</sup> N <sup>394</sup> Y
421	WQQGNVVFSCVMHEALHNHYTQK	443 N <sup>425</sup> V--N <sup>438</sup> H



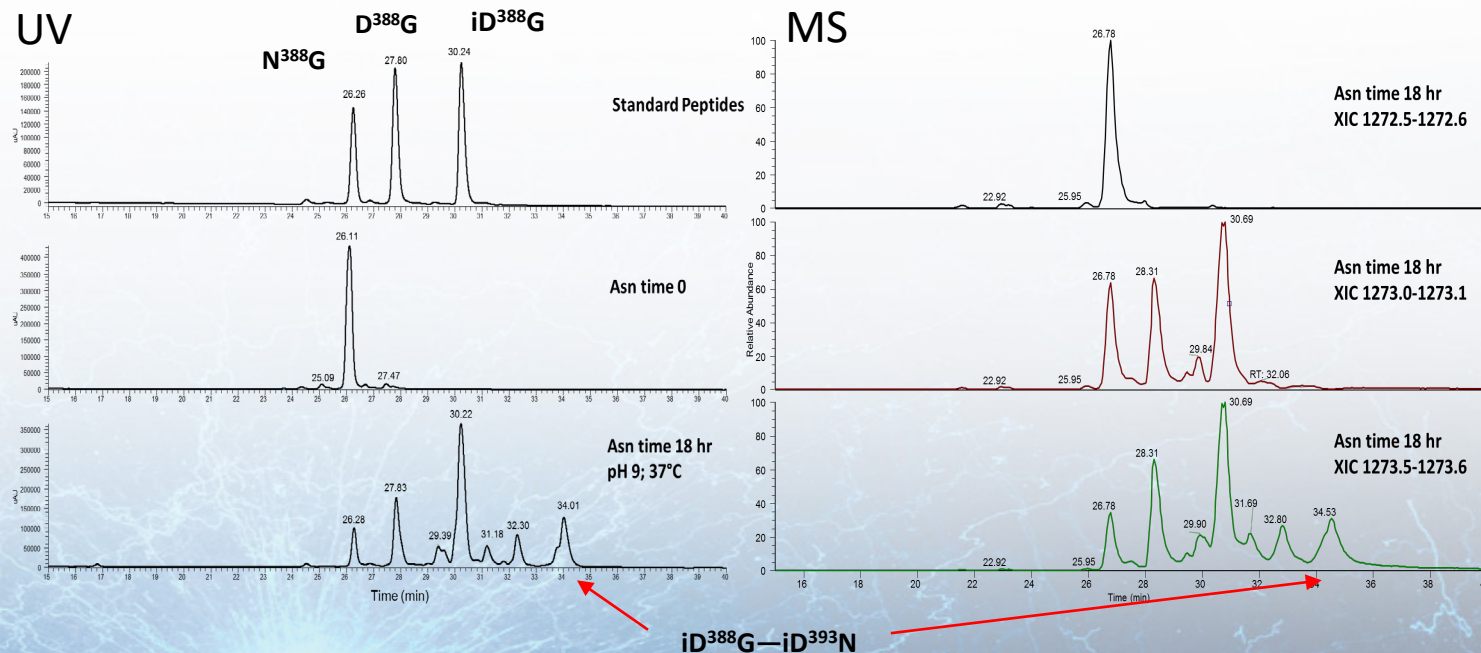
IgG Peptide with Multiple Asn as a Model System:



Deamidation/Isomerization at pH 9, 37°C

Mass analysis and MS/MS fragmentation identifies the N<sup>388</sup>G—(D/iD)<sup>393</sup>N peptide as eluting at 29.4 min. Degradation of iD<sup>388</sup>G—N<sup>393</sup>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<sup>388</sup>G—iD<sup>393</sup>N peptide. Supported by mass analysis, using CID and ETD fragmentation. No evidence of the formation of a triple deamidation was obtained (N<sup>394</sup>).

Excellent resolution of complex mixtures in HILIC, compatible with high resolution MS analysis.



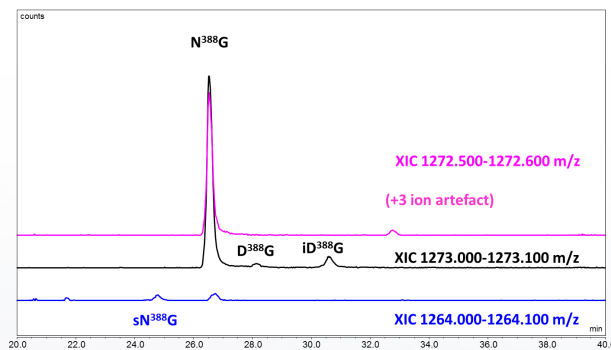


# Deamidation and Isomerization in IgG Tryptic Digests

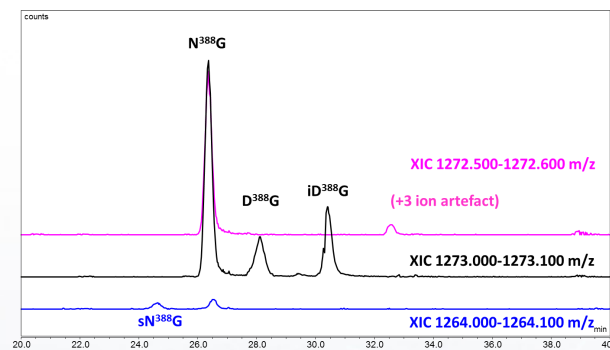
- Trypsin digestion of the mAb IgG for native, and samples “stressed” by incubation in Tris-HCl pH 9.0 for 7 days at 4 mg/mL. Reduced and alkylated proteins digested 4hrs in 50 mM Tris-HCl (pH 7.8)/1.5M Guanidine-HCl.
- Digests were analyzed by HILIC capillary LC/MS using the Orbitrap/IT. Extracted ions at the monoisotopic masses of the target sequences were integrated; the reported sequences were confirmed in CID MS/MS fragmentation

## **GFYPSDIAVEWESNGQPENNYK** in Trastuzumab Digest

### Native



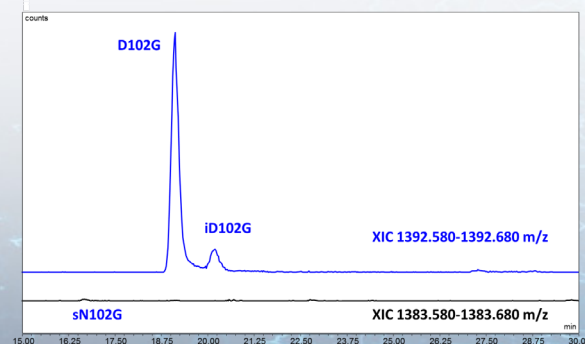
### Stressed



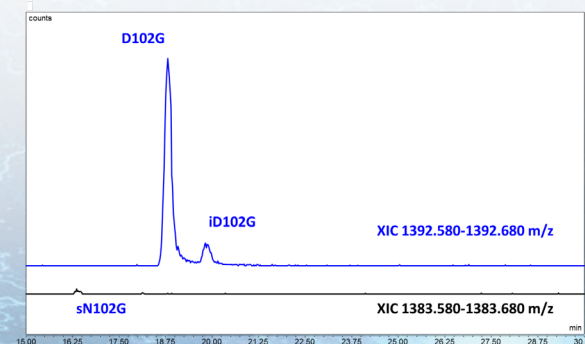
- 9 sites of potential modification were analyzed, revealing;
- 3 Asn sites with significant deamidation, and subsequent isomerization.
- 2 sites showed presence of D/iD pairs, neither were strongly effected by “stress”
- All susceptible sites exhibited the cyclic intermediate sN, whether formed from D or N in the native sequence.
- A survey of 37 additional tryptic fragments with N, D, iD, in some cases with multiple deamidations and isomers, are accurately predicted for HILIC retention using our model\*.

## **WGGDGFYAMDYWGQGLTVSSASTK** in Trastuzumab Digest

### Native



### Stressed



## Conclusions

For separations of polar compounds, recent improvement in RP have addressed the problem of phase dewetting, and irreproducible analysis in low or no organic co-solvent situations. In certain circumstances the polar selectivity differences can take advantage of HILIC mode of separations, which accentuate these polar compound structures, but the analytical purpose may make other modes (eg., RP) a better fit for the purpose.

Separations of complex biomolecule mixtures that have a collection of polar modifications are well suited to the use of HILIC separations, which better resolve the modifications, depending in a predictable way on the structure differences that are targeted. In combination with high resolution MS analysis, subtle, biologically significant features can be resolved.

Applying both RP and HILIC modes of separation to analysis of polar compounds are valid and useful approaches.

## Acknowledgements

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\*The Separation and Quantitation of Peptides with and without Oxidation of Methionine and Deamidation of Asparagine Using Hydrophilic Interaction Liquid Chromatography with Mass Spectrometry (HILIC-MS). M. J. Badgett, B. Boyes, R. Orlando (2017) JASMS 28, 818-826.;

Peptide retention prediction using hydrophilic interaction liquid chromatography coupled to mass spectrometry. (2018) J. Chromatogr. A 1537, 58-65.

Thank You for Your Attention!