Combined HILIC and RPLC Peptide Mapping: Two Methods are Better (And Faster!) Than One

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Not All Methods are Equal

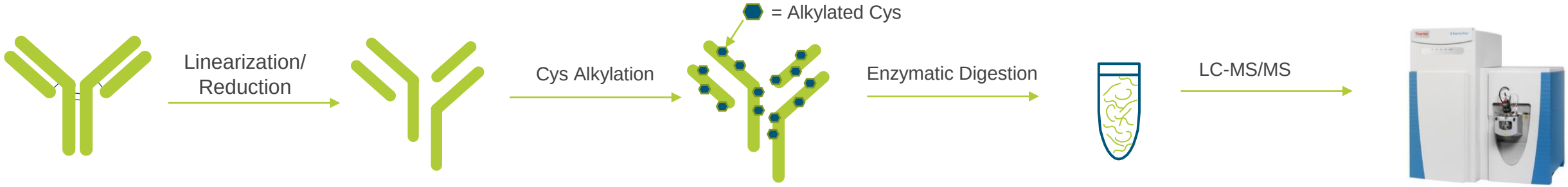
- ***Desire:***
  - *More informative data earlier and more often*
  - *Inter- and intra-program data trending*

- ***Barriers:***
  - *Involved and variable sample preparation*
  - *Extended chromatography; lengthy data processing*
  - *“Force-fitting” the legacy methods*

Solution: *Intelligently design a scalable, selective method for complete characterization*

Unifying and Platforming

Peptide mapping using LC-MS/MS is the ***only assay*** that provides information on a ***per-residue basis***

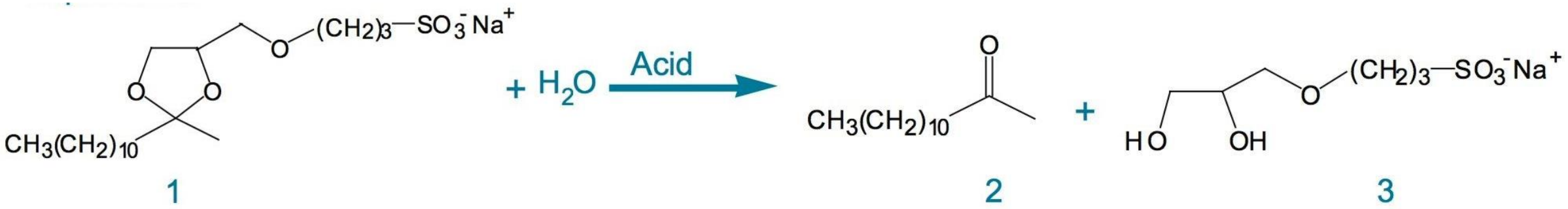


Embrace Change: Build a superior platform method from the ground up.

Three key aspects:

1. Sample Preparation
2. Sample Analysis Method
3. Data Processing and Reporting

Acid-labile Surfactant for Protein Denaturation



1

Surfactant

2

Water insoluble, removed by centrifugation

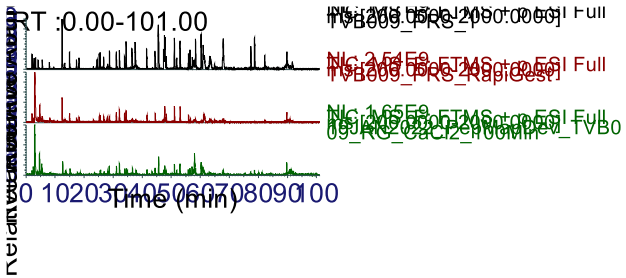
3

Water soluble, un-retained by RPLC

- *Surfactant reconstituted in 50 mM ammonium bicarbonate (AmBic)*
- *All reduction/alkylation/digestion is performed in one pot*
  - *No risk of sample loss*
- *After acidification, samples are centrifuged and decanted to remove water-insoluble product*
- ***Removes the need to perform buffer exchange step***

Image: Waters Application Note 720003102, June 2009

Full Teva-mAb Sequence Covered in Surfactant Method



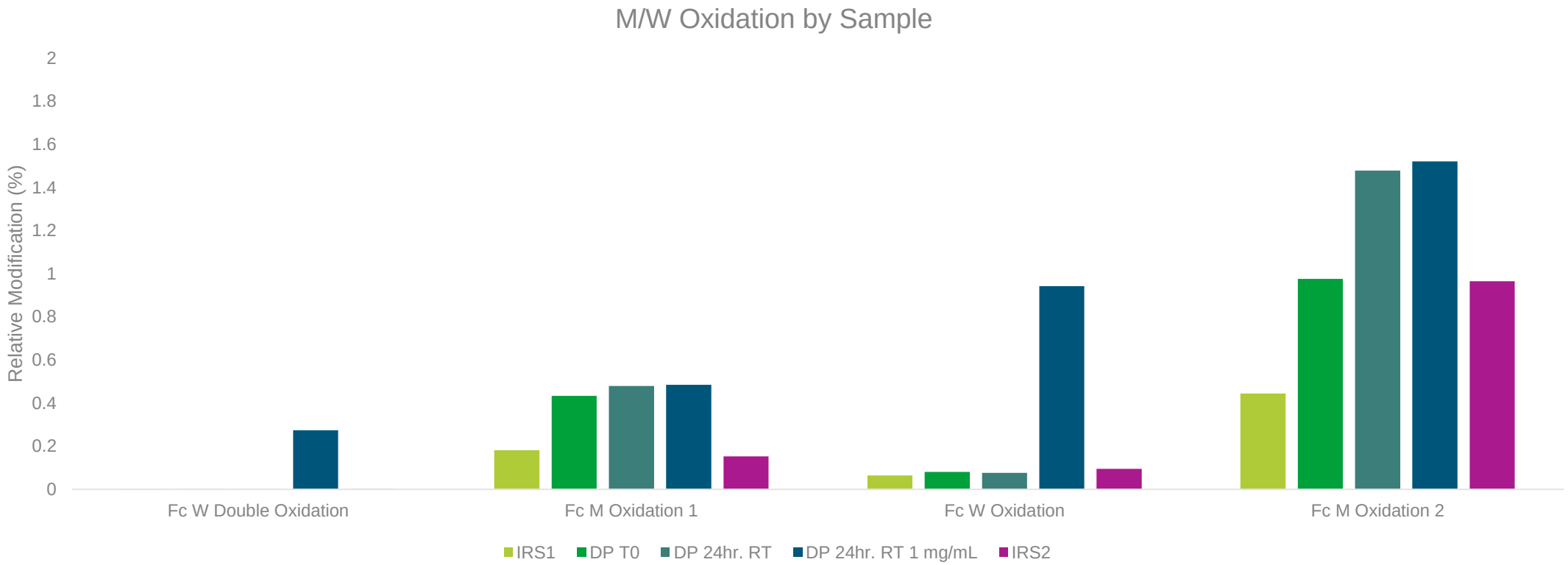
Legacy Preparation

Surfactant

Proteins	Number of MS Peaks	MS Peak Area	Sequence Coverage	Abundance (mol)
1:LC	117	60.6%	100.0%	60.41%
2:HC	181	39.4%	94.2%	39.59%
Unidentified	0	0.0%		

Small, hydrophilic peptides not retained

Scalability



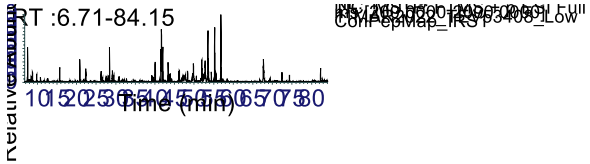
- *Surfactant platform method was used to characterize **low concentration** drug product*
  - *136 pmol (20 µg) starting material used*
- *Obtained full sequence coverage and maintained ability to quantify critical PTMs*

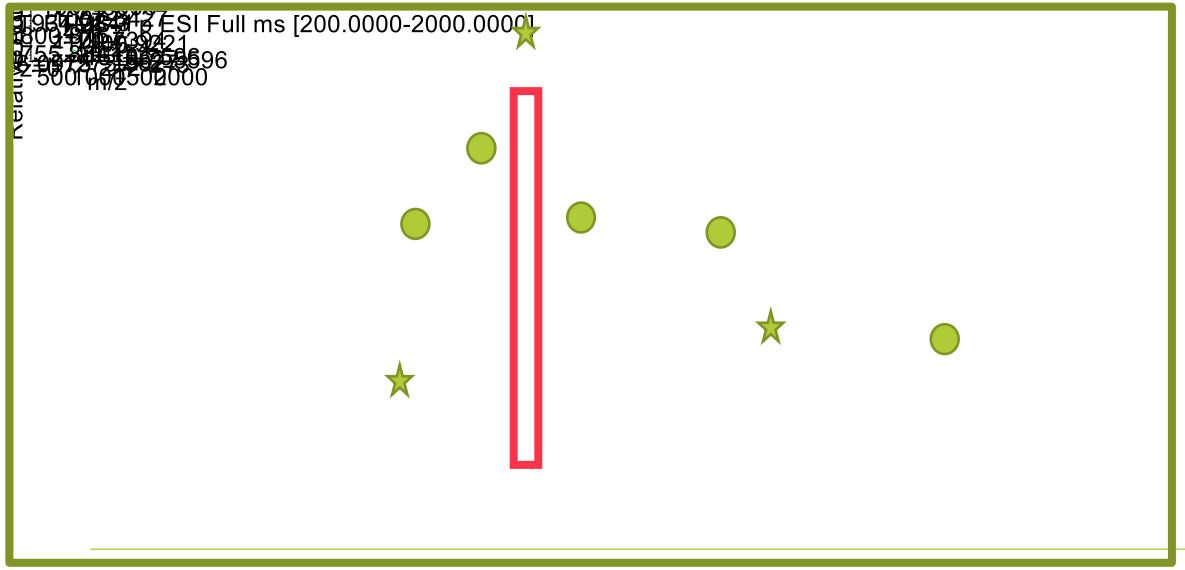
Next Steps

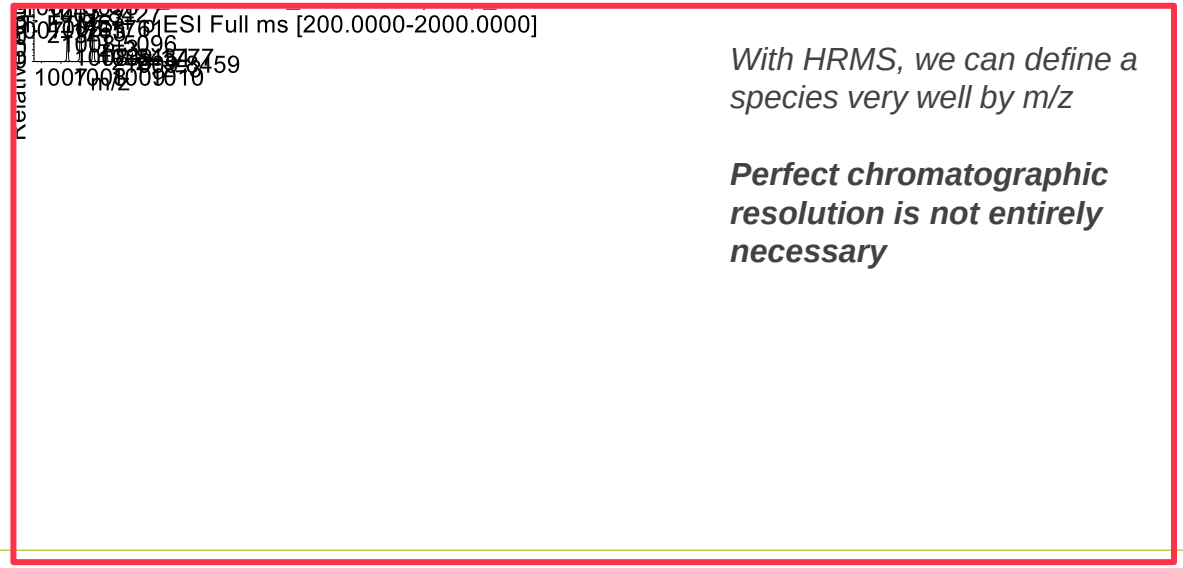
- A simplified, platform, scalable digestion protocol has been developed
  - Only 1/3 of the story. . .

- High-resolution mass spectrometry provides additional mode of "separation" with *near infinite* signal-to-noise
- *Opportunity to significantly shorten analytical method without sacrificing data quality*

Leveraging HRMS to Increase Throughput







With HRMS, we can define a species very well by m/z

Perfect chromatographic resolution is not entirely necessary

Achieving Complete Characterization

- Tryptic peptide maps of IgGs contain both very hydrophobic and very hydrophilic sequences

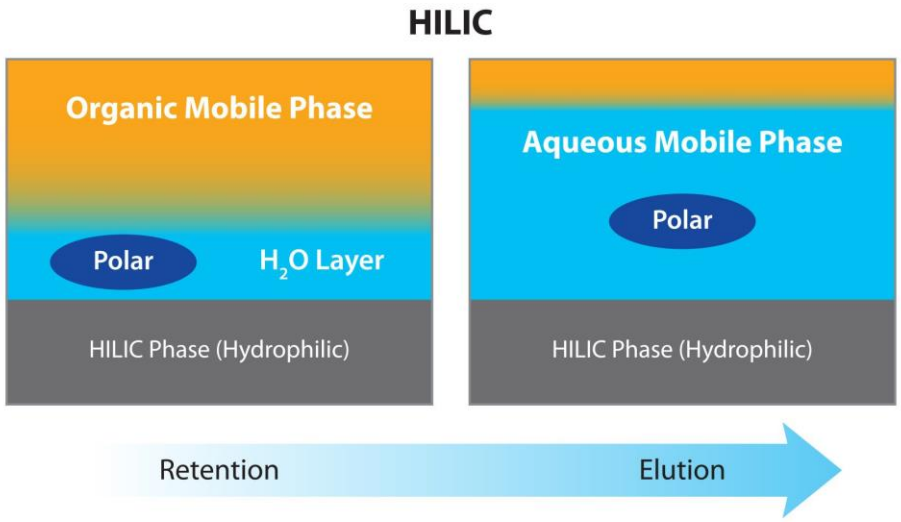
- 100% sequence coverage is desirable, but not always feasible by a single technique

Proteins	Number of MS Peaks	MS Peak Area	Sequence Coverage	Abundance (mol)
1:LC	117	60.6%	100.0%	60.41%
2:HC	181	39.4%	94.2%	39.59%
Unidentified	0	0.0%		

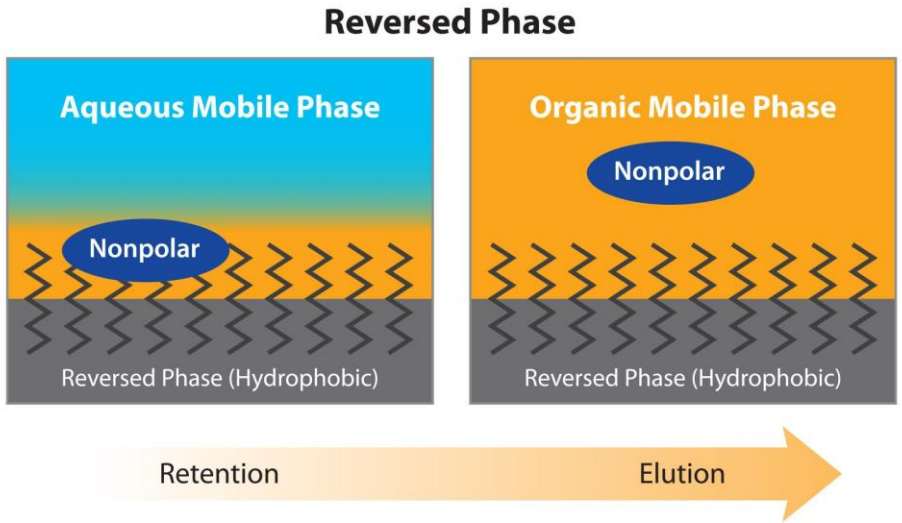
- Some CQAs, such as glycoforms and deamidations are difficult to resolve, so others have chosen longer reversed-phase gradients or alternative enzymatic digestion

What if we could do two gradients that employ different phase characteristics to provide complete characterization in a shorter amount of time?

Reversed-Phase and HILIC as Complementary Techniques



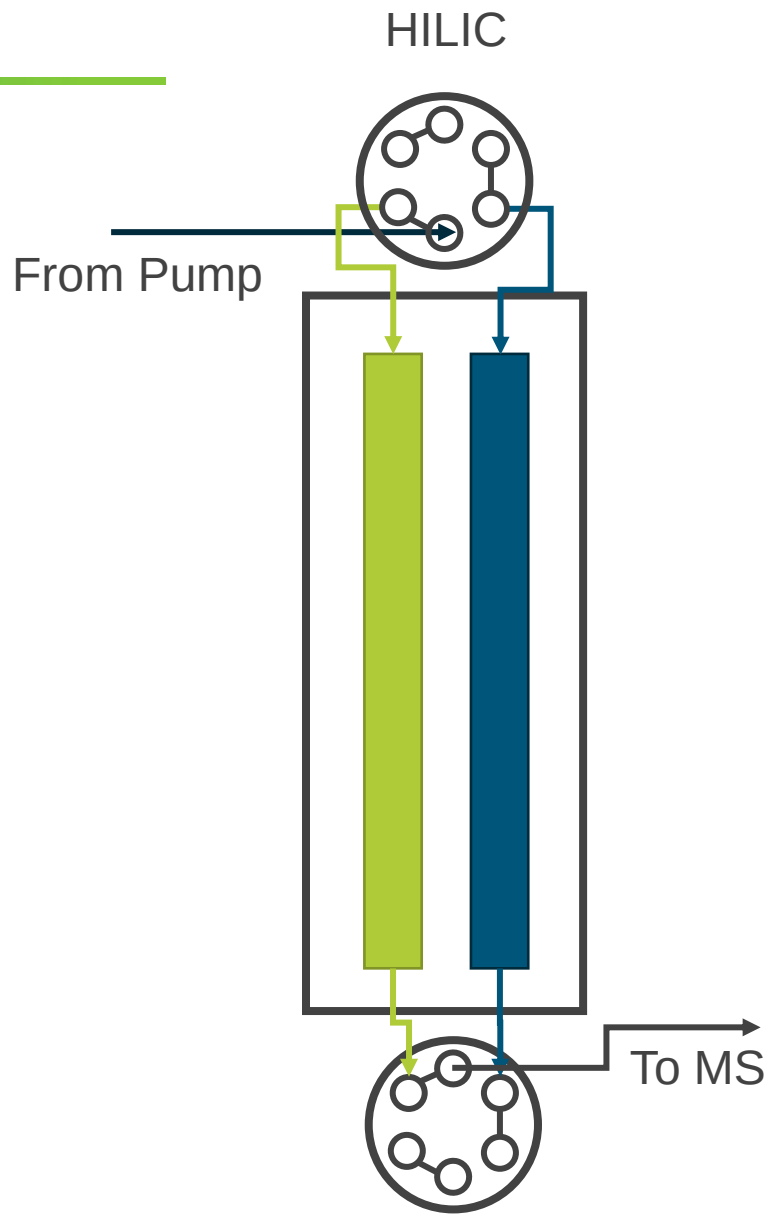
- Polar (amide) stationary phase
- Separation achieved by hydrophilic partitioning into surface water layer
- Good for retention of *polar* analytes
- Mobile Phases:
  - A: water, 10 mM ammonium formate, 0.05% formic acid, pH 4
  - B: acetonitrile, 0.1% formic acid

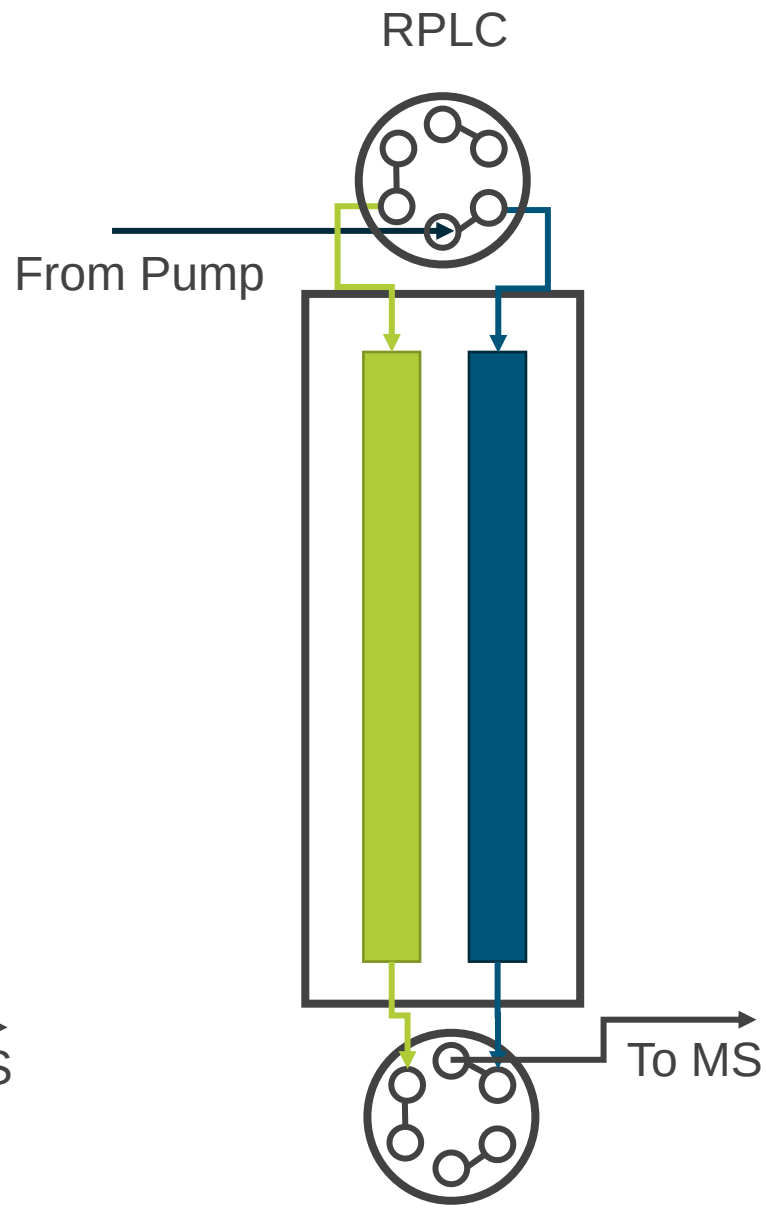


- Non-polar (C18) stationary phase
- Separation achieved by partition into stationary phase with organic elution
- Good for retention of *non-polar* analytes
- Mobile Phases:
  - A: water, 0.1% formic acid
  - B: acetonitrile, 0.1% formic acid

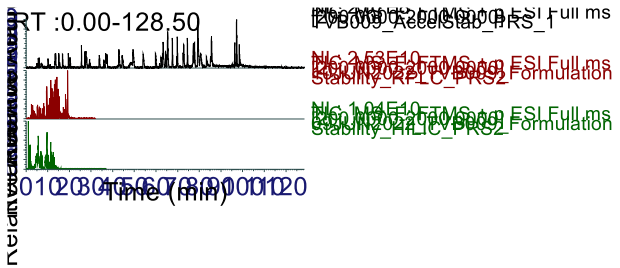
Image: Restek, Inc. How to Avoid Common Problems with HILIC Methods. Lit. Cat.# GNAR2716-UNV. 2017

Science Plumbing!





Two Rapid, Complementary Gradients Provide Full Coverage in Half the Time



Teva mAb
Legacy Method
10 µg injection
128 min. run time
95.8% Seq. Covg.

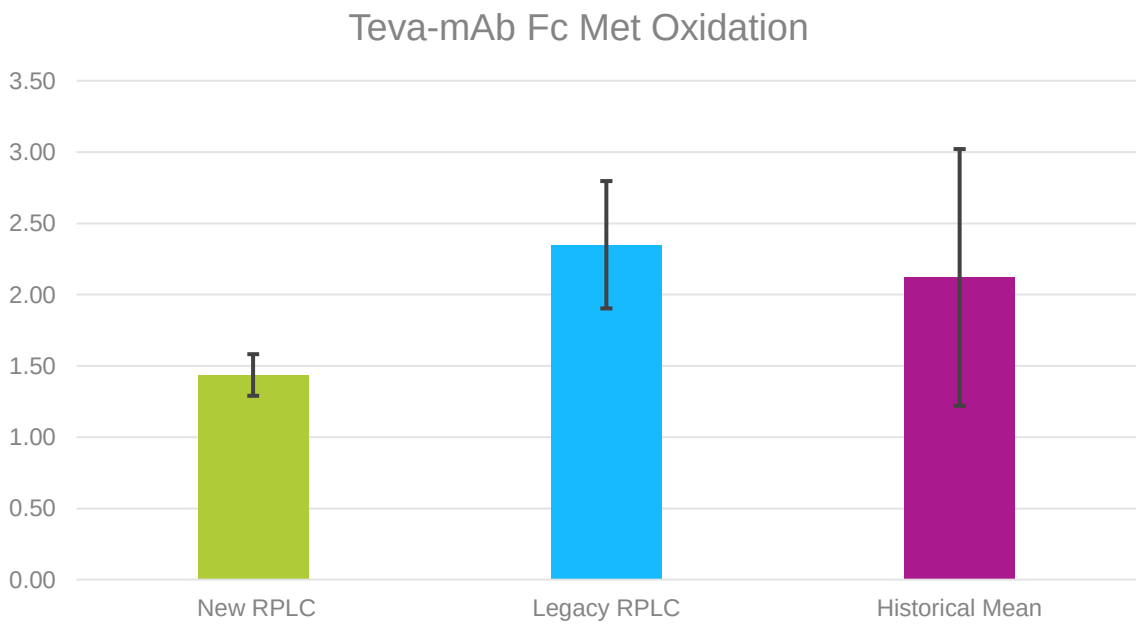
Teva mAb
New RPLC
5 µg injection
32 min. run time
100% Coverage

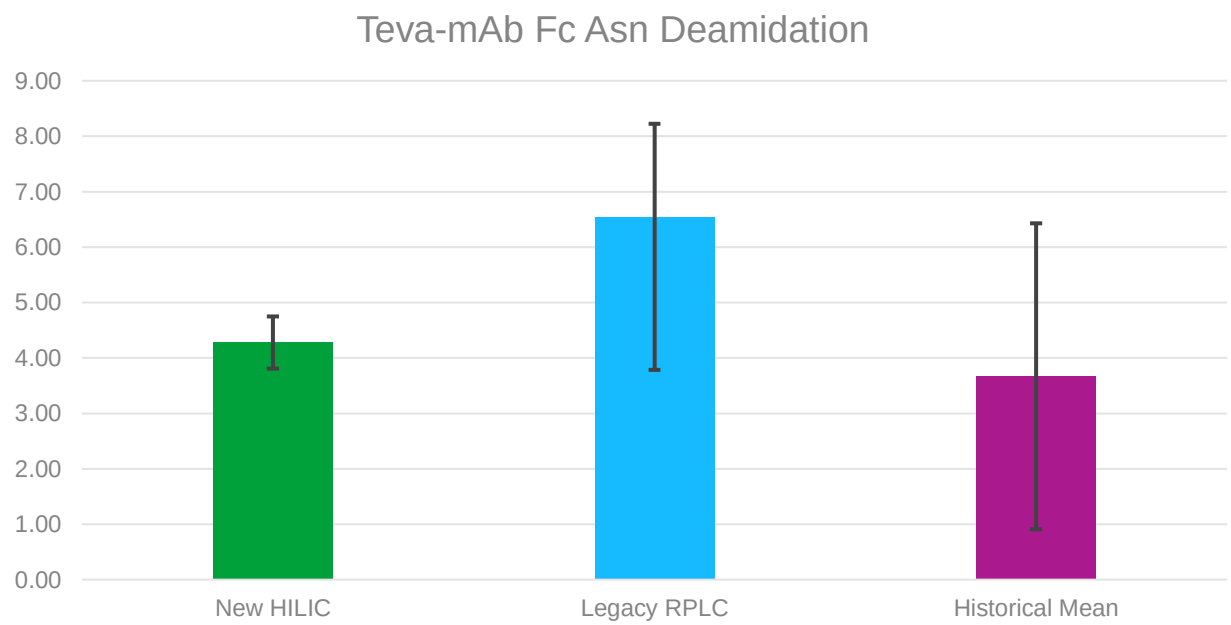
Teva mAb
New HILIC
5 µg injection
37 min. run time
98% Coverage

Combined method covers 100% of the Teva mAb sequence in ~54% of the time as the previous method

Ensuring Continuity Between Methods

- Method bridging study
  - N = 6 preparations of Teva-mAb reference standard prepared by the legacy method and surfactant method with combined RPLC/HILIC analysis
  - Samples prepared using the same reagents, where possible, and analyzed on the same instrument within 24 hours of each other
  - All regularly-reported PTMs are covered by combined RPLC/HILIC approach with similar results to legacy average





**Error bars represent ±3 s.d. around mean*

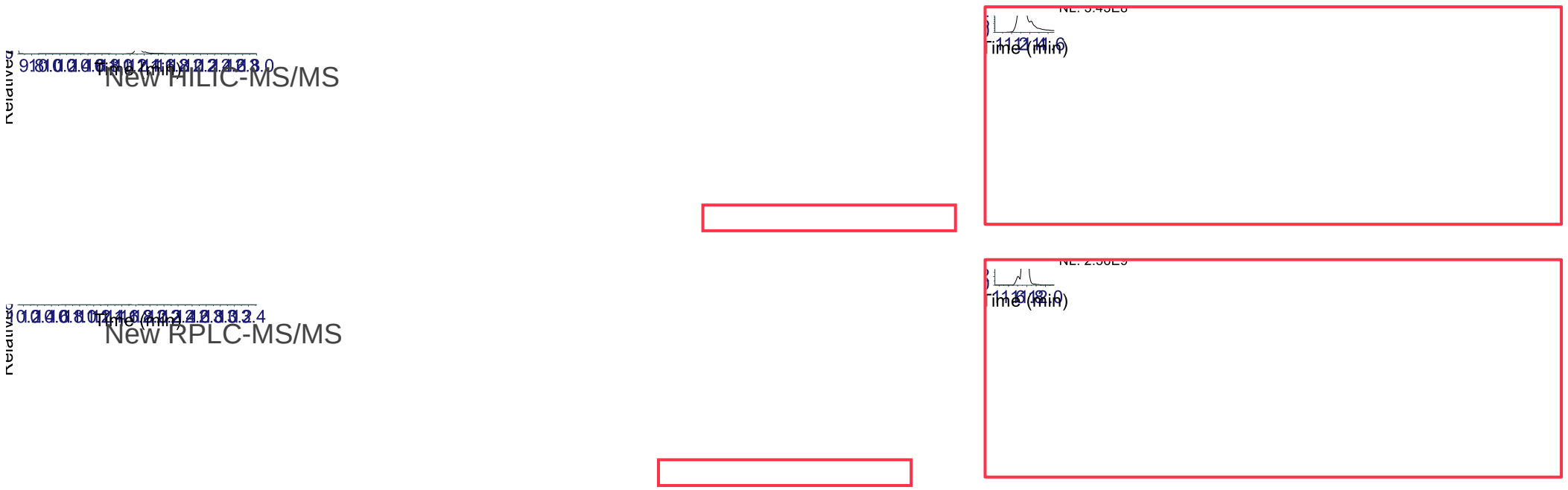
New Method Maintains Ability to Monitor Isomerization CQA in CDR

CDR Asp-Gly Peptide; 22 Residues

D

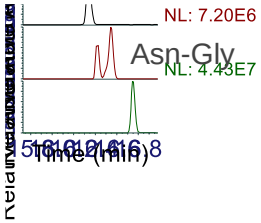
Legacy RPLC-MS/MS

Iso-D

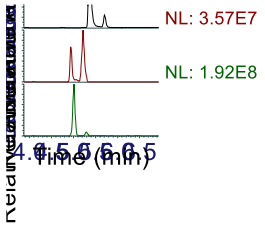


Deamidation Resolution in Fast HILIC

RPLC-MS/MS



Fc Asn-Gly Peptide; 16 Residues



HILIC-MS/MS

Possible D isomerization

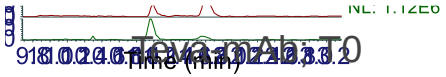
Asp/Iso-Asp-Gly

HILIC MS/MS provides more informative data in < 7 minutes

Asn(-NH_3)-Gly

Heat Stress – Fc Deamidation Uncovered in Small Peptide by HILIC

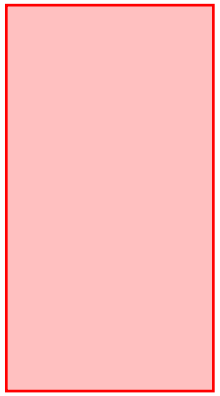
Asn-Lys Peptide; 6 residues



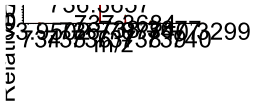
Teva-mAb; 2 Wk, 37 °C

Teva-mAb; 8 Wk, 37 °C

Asp-Lys, Modified Peptide

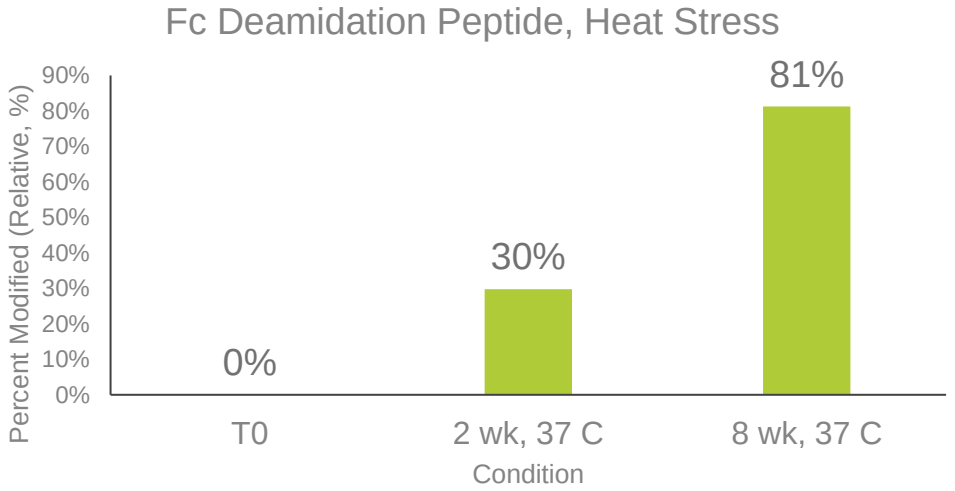


Asn-Lys, Native Peptide

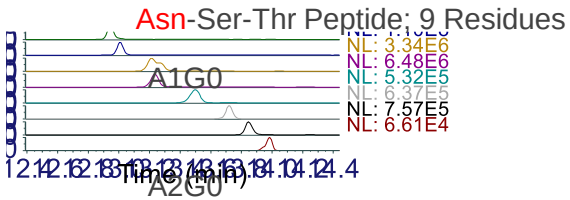


Asn-Lys, Native Peptide

Asp-Lys, Modified Peptide



Glycopeptide Resolution in HILIC-MS/MS



A2G0

A1G0F

A2G0F

A2G1F

M5

M6

M7

M8

M9

HILIC-MS/MS can be used to routinely monitor glycosylation profile

High-mannose species detected and resolved

Shortened Gradient with Targeted Analysis

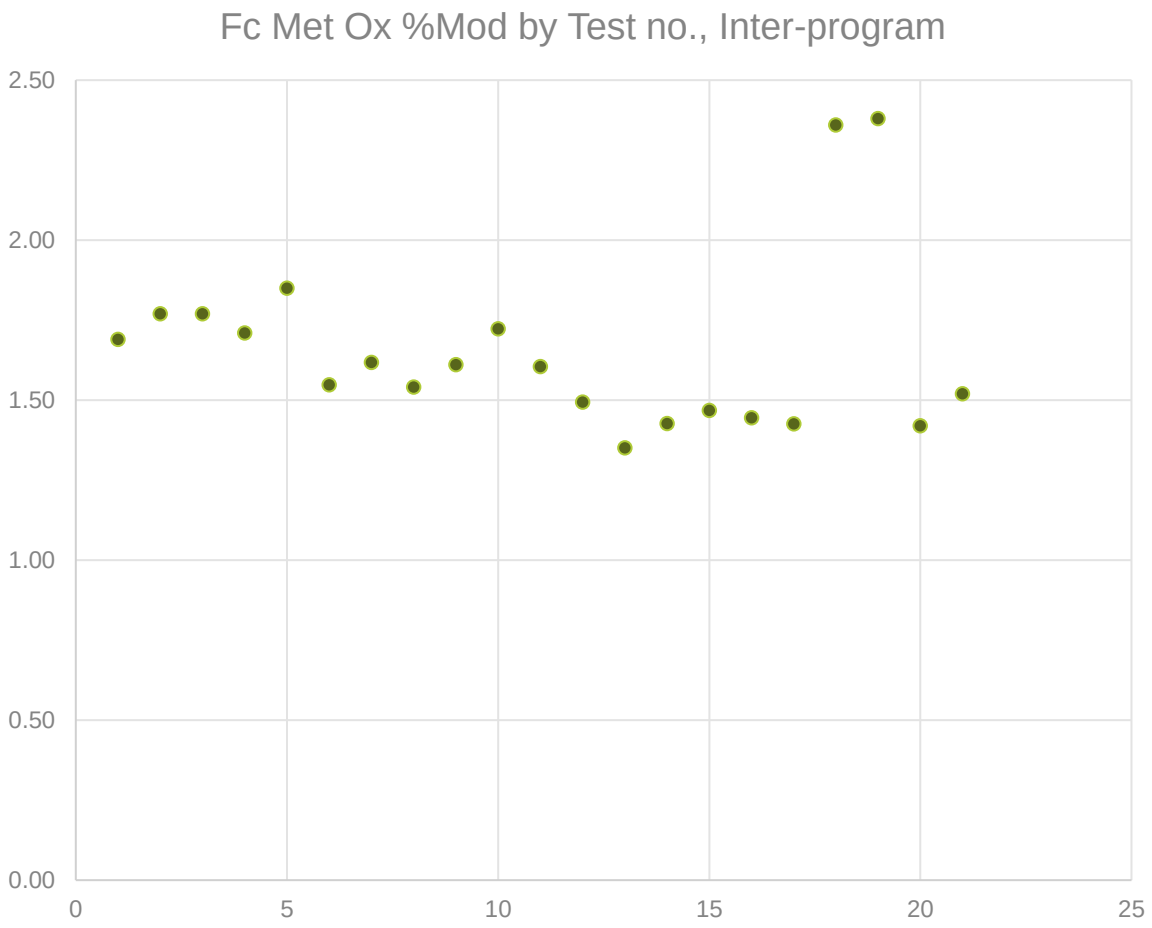
- Late-stage products have well-defined list of PTMs
  - Control chart

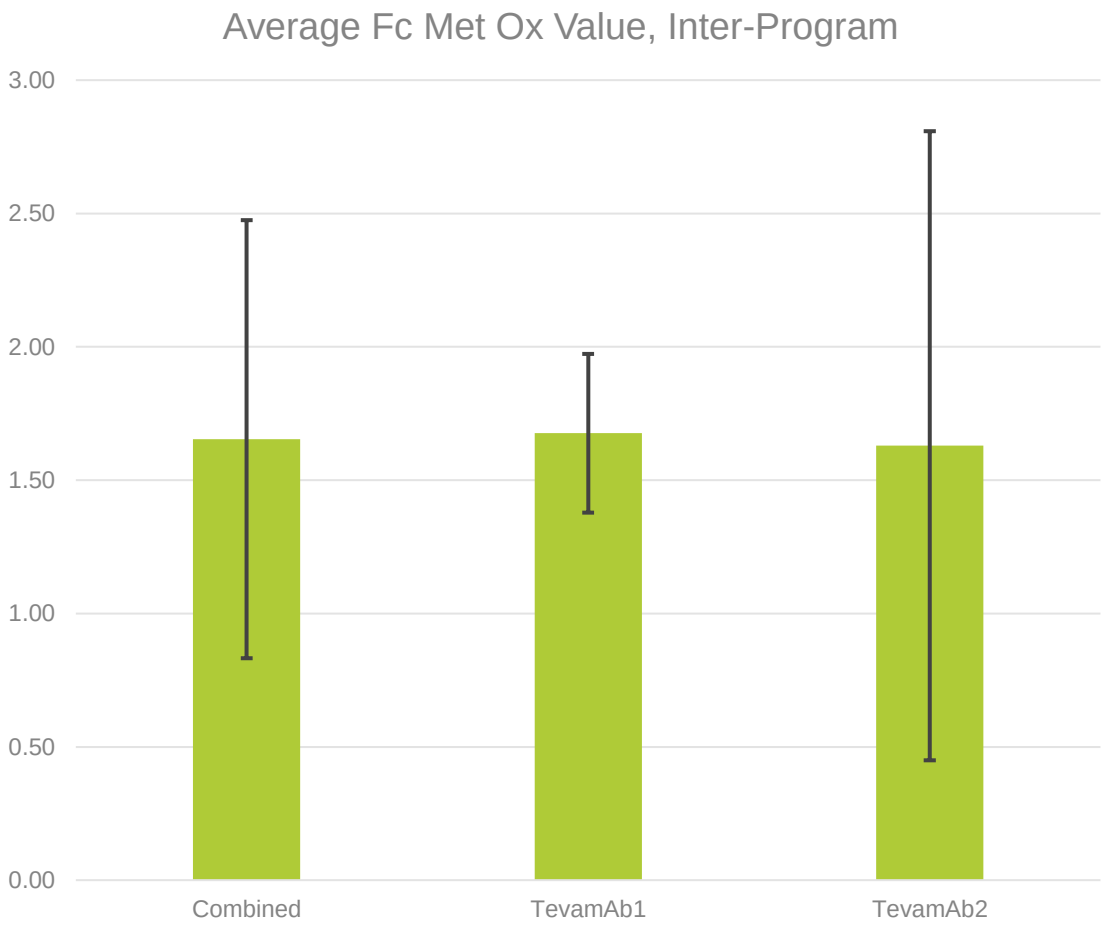
	HC PyroE	LC PyroE	HC Fc Deamidation (%)	HC Fc Aglycosylation (%)	HC CDR Ox	HC Fc Ox
Average	1.06	0.27	4.52	0.61	4.86	1.63
SD	0.09	0.03	0.41	0.07	2.04	0.39
%RSD	8.1%	12.8%	9.1%	11.4%	42.1%	24.1%
3SD	0.3	0.1	1.2	0.2	6.1	1.2
Upper Limit (Avg+3SD)	1.3	0.4	5.8	0.8	11.0	2.8
Lower Limit (Avg-3SD)	0.8	0.2	3.3	0.4	-1.3	0.4

- Use of mAbs with conserved sequences lead to the same peptides

- *Build a fully targeted data processing method that can be used across all programs*
  - *Screening assay for early-phase programs*
  - *Routine monitoring assay for late-phase programs*

Inter- and Intra-Program Trending





Where do we go from here?

- More informative data, *globally*

- Peptide mapping is considered a “Multi-Attribute Method” (MAM)
  - Has the potential to replace multiple assays in a single experiment
  - Most specific assay

- Barrier to adoption has been sample preparation, data analysis, and instrument maintenance
  - One-pot surfactant method significantly decreases sample preparation difficulty
  - Fast gradients and targeted data processing methods enable near hands-off data processing
  - Method is completely scalable to meet varying sample concentration needs
  - Still room for optimization

This work has laid the foundation to take characterization assays outside of the AD lab and into the hands of our global colleagues on a single, harmonized platform

Acknowledgements and Thanks

- AD Characterization Team
  - Mark Platt
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  - Ozlem Onder

- AD Forced Degradation Group
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Thank you.