LC/MS Analysis of Monoclonal Antibody Structure Utilizing HALO® BioClass Fused-Core® Particles; Multilevel Analysis for Proteins and Glycovariants

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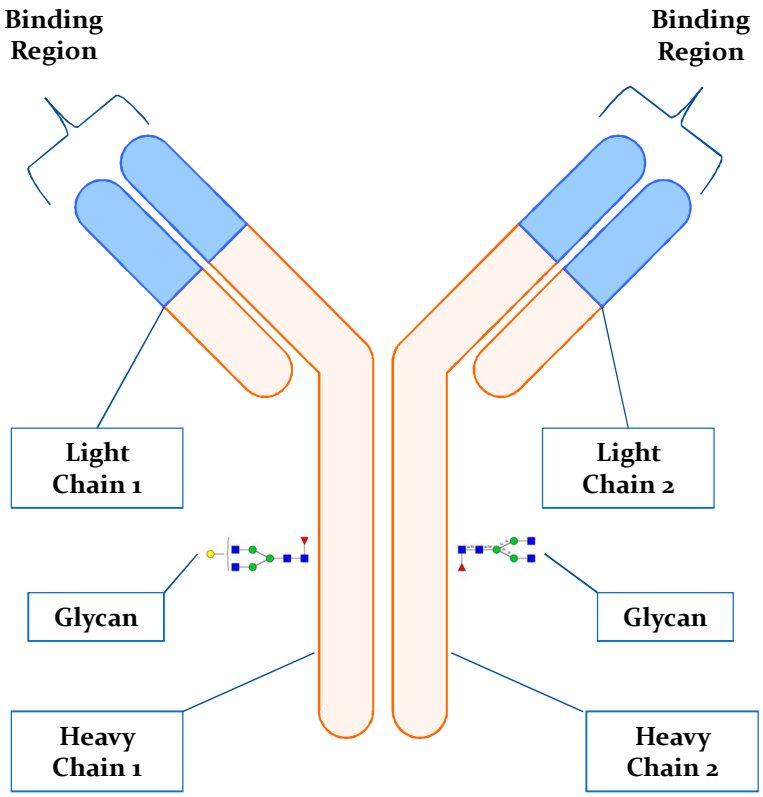
Herceptin (Trastuzumab)



Trastuzumab is the chemical name of one of more than 30 monoclonal antibody drugs that have been approved for clinical applications.

It was originally developed and commercialized by Genentech (Herceptin®) for the treatment of a specific type of metastatic breast cancer, and was approved by the U.S. FDA in 1998.

Trastuzumab was the first monoclonal antibody targeted for a cancer-related biomarker to obtain approval by the FDA.

Trastuzumab consists of two light chains, two heavy chains, and has an ensemble of N-linked glycans attached to Asn 297 of each heavy chain.

Biotechnology and Applied Biochemistry

Industry and regulatory experience of the glycosylation of monoclonal antibodies

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Abstract.

We surveyed 23 antibody-related marketing applications for glycoform analytical and functional information. Our database analysis shows a clear trend of increasing sophistication of analytical methods used to identify and quantify glycans. These have revealed a high degree of complexity and heterogeneity of glycans attached to antibody products. The nature of the complexity is influenced by product type and expression system, and may be associated with functional consequences in some but not all cases.

Published 2011. This article is a U.S. Government work and is in the public domain of the USA. Volume 58, Number 4, July/August 2011, Pages 213–219 •
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Keywords: glycosylation, monoclonal antibodies

1. Introduction

The number of licensed therapeutic monoclonal antibodies (mAbs) has been increasing over the past few years, with hundreds more already undergoing clinical study for indications for a variety of therapeutic applications, including cancer and inflammatory diseases [1]. Most of these products are produced in conventional bioreactor-based mammalian cell culture [*e.g.*, Chinese hamster ovary (CHO) or murine myeloma transfectomas], although a few are produced by other expression systems (*e.g.*, *Escherichia coli*) [2]. Therapeutic antibodies must be demonstrated to meet applicable quality requirements to ensure continued safety, purity, and potency to convince regulators to allow marketing as a drug product. Part of the demonstration of product quality is an intensive biochemical characterization of the antibody itself, which includes a thorough examination of glycan distribution and potential impacts of glycoform on function [3]. This characterization is conducted in two major stages, (a) a complete glycan distribution characterization of reference standard or conformance lots of the antibody glycoprotein and (b) abbreviated testing of all subsequent batches to establish manufacturing consistency and comparability with the reference material. The tests used in these analyses span a wide range of analytical methodologies, which have grown more sophisticated over the years [4].

For the most part, glycans on commercial antibodies are attached at asparagine residues at or near position 297 (N297) within the Fc portion of the protein [5]. Mammalian cell culture-produced antibodies typically possess N-linked complex biantennary structures, with heterogeneous levels of terminal galactosylation and fucosylation of the core N-acetylglucosamine [6]. To a lesser degree, terminal sialylation and bisecting N-acetylglucosamine are also present. Although these glycans do not directly impact the antigen-binding function of the antibody protein, they can impact effector functions such as antibody-dependent cellular cytotoxicity (ADCC) or complement binding and activation (also known as CDC or complement-dependent cytotoxicity) [7]. Examples of documented impacts of glycosylation on antibody functionality include, but are not limited to, (a) an inverse correlation between ADCC activity on core fucosylation [8], (b) an increase in CDC activity with increased galactosylation [9], and (c) a positive correlation between anti-inflammatory activity and increased sialylation [10]. A subset of antibody-like products, Fc fusion proteins, possesses more complex glycan distributions, including O-linked glycans. Thus, glycoform variation can impact the potency or *in vivo* distribution/clearance of therapeutic antibodies and needs to be characterized and controlled. As part of glycan characterization, the impact of glycan distribution on the product mechanism of action (MoA; *e.g.*, cancer cell destruction, down-modulation of inflammatory activity) is commonly evaluated by firms wishing to market antibody-based medicinal products.

Over the past 25 years, almost 40 antibody products have been approved for marketing by US Food and Drug Administration (FDA). The licensure decision is based on information submitted in the marketing dossier including the above

Abbreviations: α-gal, α-galactosyl residues; ADCC, antibody-dependent cellular cytotoxicity; BLAs, Biological License Applications; CE, capillary electrophoresis; CHO, Chinese hamster ovary; CDC, complement-dependent cytotoxicity; exo, exoglycosidase; MS, mass spectrometry; MoA, mechanism of action; mAbs, monoclonal antibodies; OP, oligosaccharide profiling; GoF, G1F and G2F, outer arm non, mono or bi-β-galactosylated variant of core fucosylated biantennary N-linked glycans; FDA, US Food and Drug Administration.

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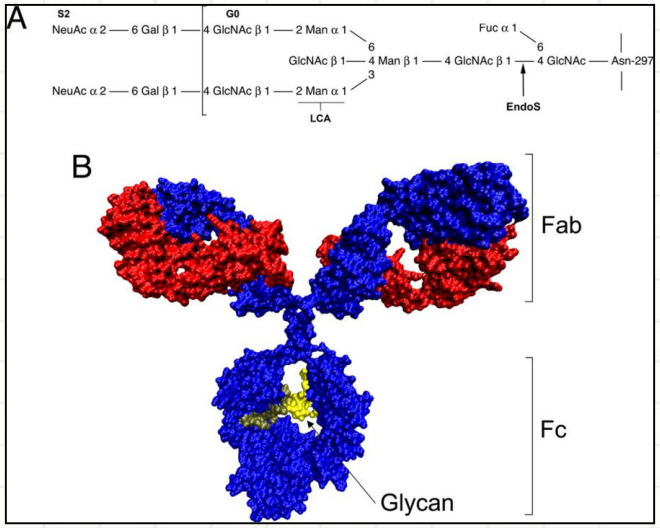
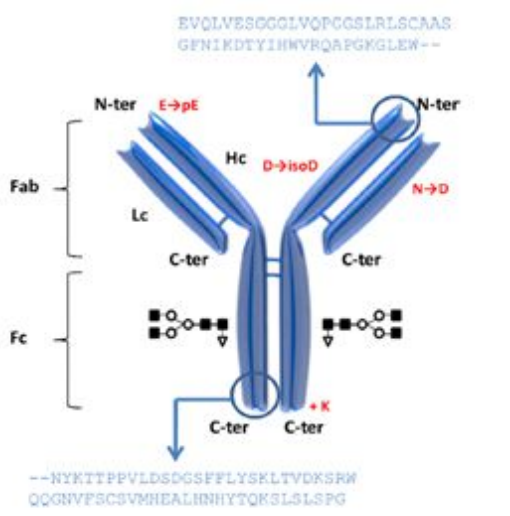
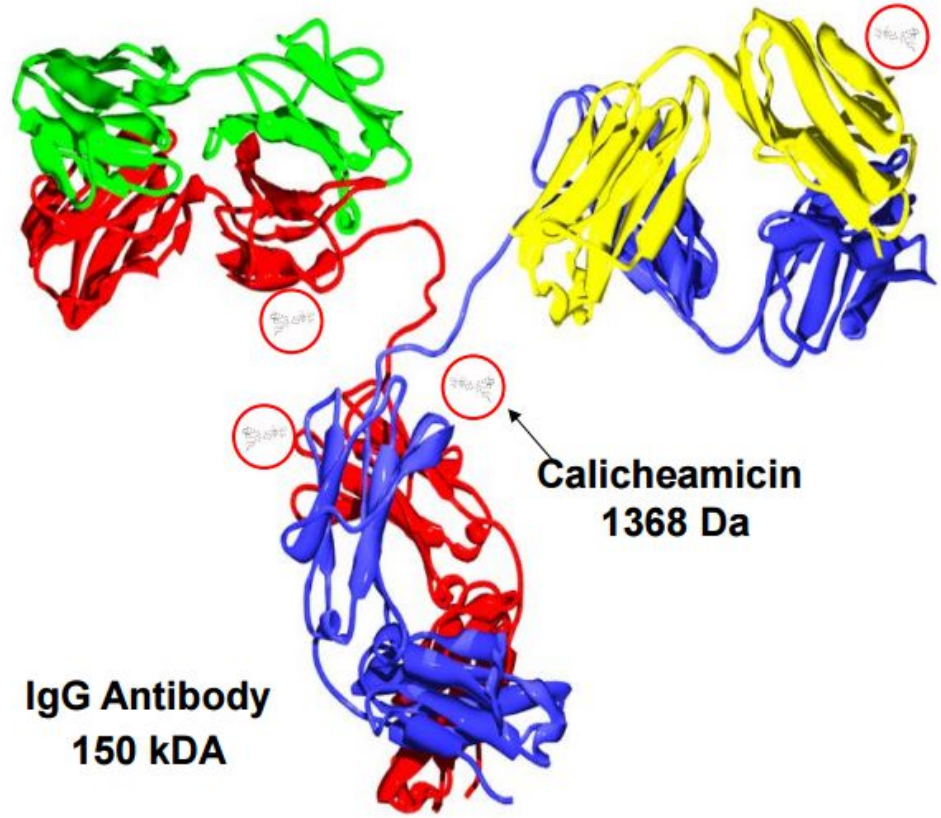


Mattias Collin et al. PNAS 2008;105:4265-4270



6/10 top sellers; > 400 mAbs in trials

Monoclonal antibodies



Regulatory Considerations for Antibody Drug Conjugates Sarah Pope Miksinski, Ph.D. 2013 DIA 49TH meeting

Antibody Drug Conjugates

Single Domain Antibody

Fragment Antibody

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HALO® BioClass Fused-Core: Particle Design Improvements

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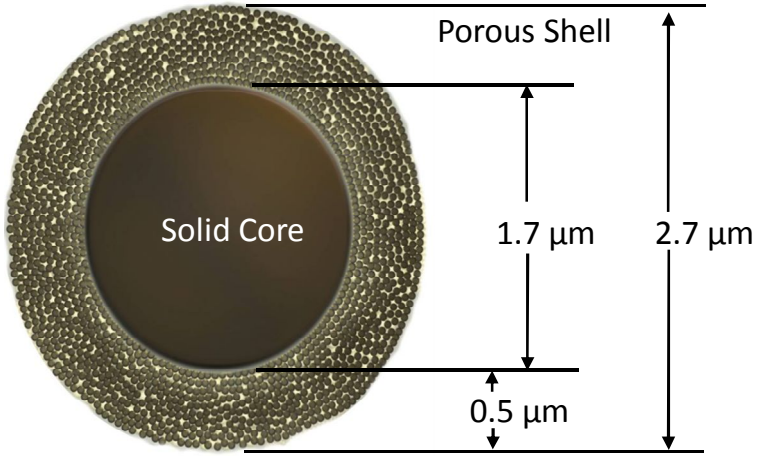
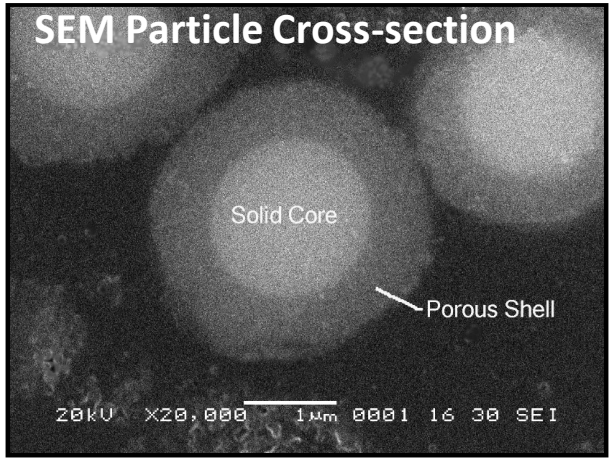
Original Halo Superficially Porous Particles Fused-Core®





- Low back pressure due to the particle design (solid core with a porous shell)
- No need for specialized HPLC equipment
- Not necessary to filter samples and mobile phase since frits are not as small as needed for sub-2-µm
- High resolution is maintained at high flow rates (flat C-term in van Deemter plot)

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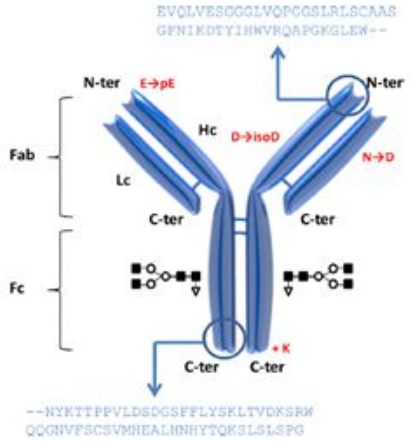
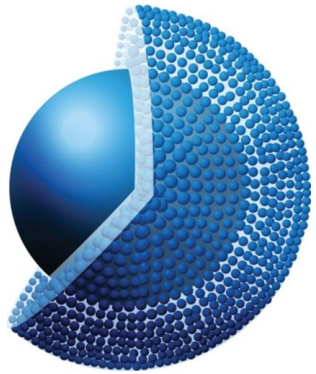
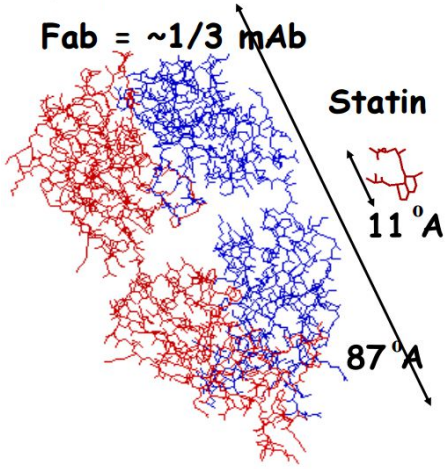
Wide Pore SPP Can Fit the Needs for Protein Science



What is Needed for High Performance Separations of Larger (Bio) Molecules?



- **Pore Size must "fit" molecule size**
  - Restricted diffusion limits efficiency and load capacity
  - Peak capacity effects by kinetic and retention properties



- **Particle Geometry must Optimize Surface Area/Volume**
  - Shell thickness determines diffusion path and Surface Area
  - Core Size (a determinant of particle size must match application needs)
  - Must have "right" size AND desirable particle distribution

- **Chemistry/Conditions appropriate to Samples**

Fab Image Regulatory perspectives on the manufacture and characterization of biotechnology products during pharmaceutical Development. Richard Ledwidge, Ph.D. FDA.gov

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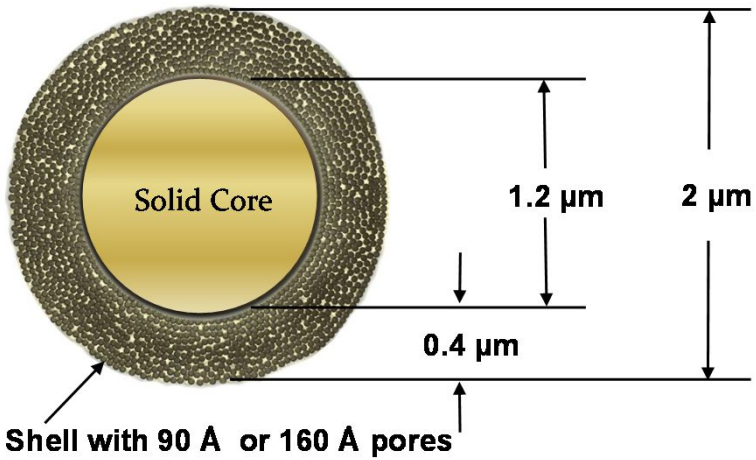
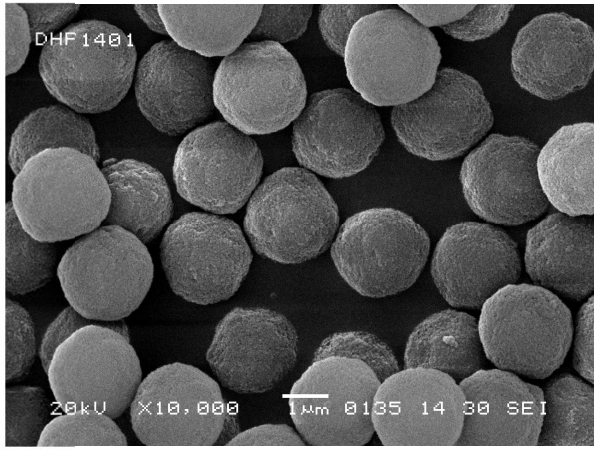
HALO 2 Particle Design

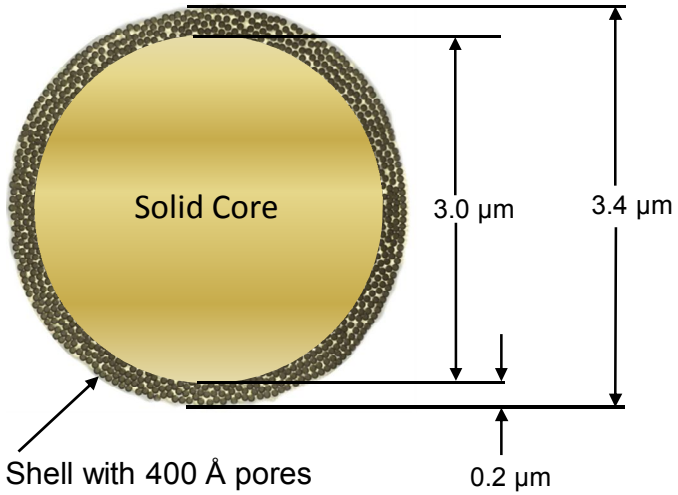


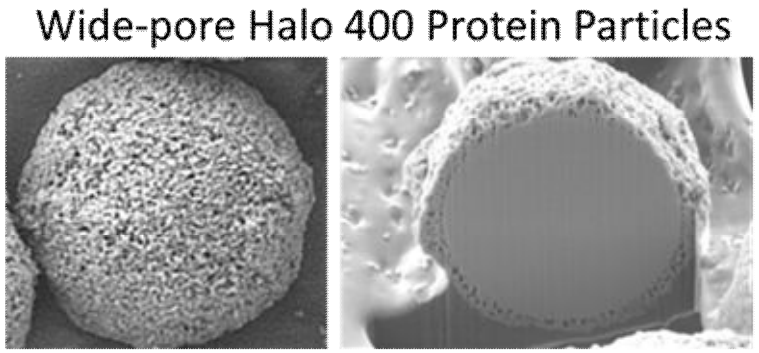
SEM image of 2 µm HALO particles



- ~300K plates/meter efficiency: Speed and High Resolution
- Robustness and Ruggedness for UHPLC Analyses
  - larger porosity 1 micron frits on the column inlet
  - Less likely to be plugged compared to 0.2–0.5 µm frits used on sub-2-um non-core columns
  - **Lower back pressure (~20%)** than most commercially available non-core, sub-2 µm columns
- 1000-bar pressure maximum

Superficially Porous (Fused-Core®) Wide Pore Particles (400 Å)





- Low back pressure due 3.4 µm particle diamater
- No need for specialized HPLC equipment
- Not necessary to filter samples and mobile phase since frits are not as small as needed for sub-2-µm
- Shortest practical diffusion path for high MW molecules (to maintain small C-term)

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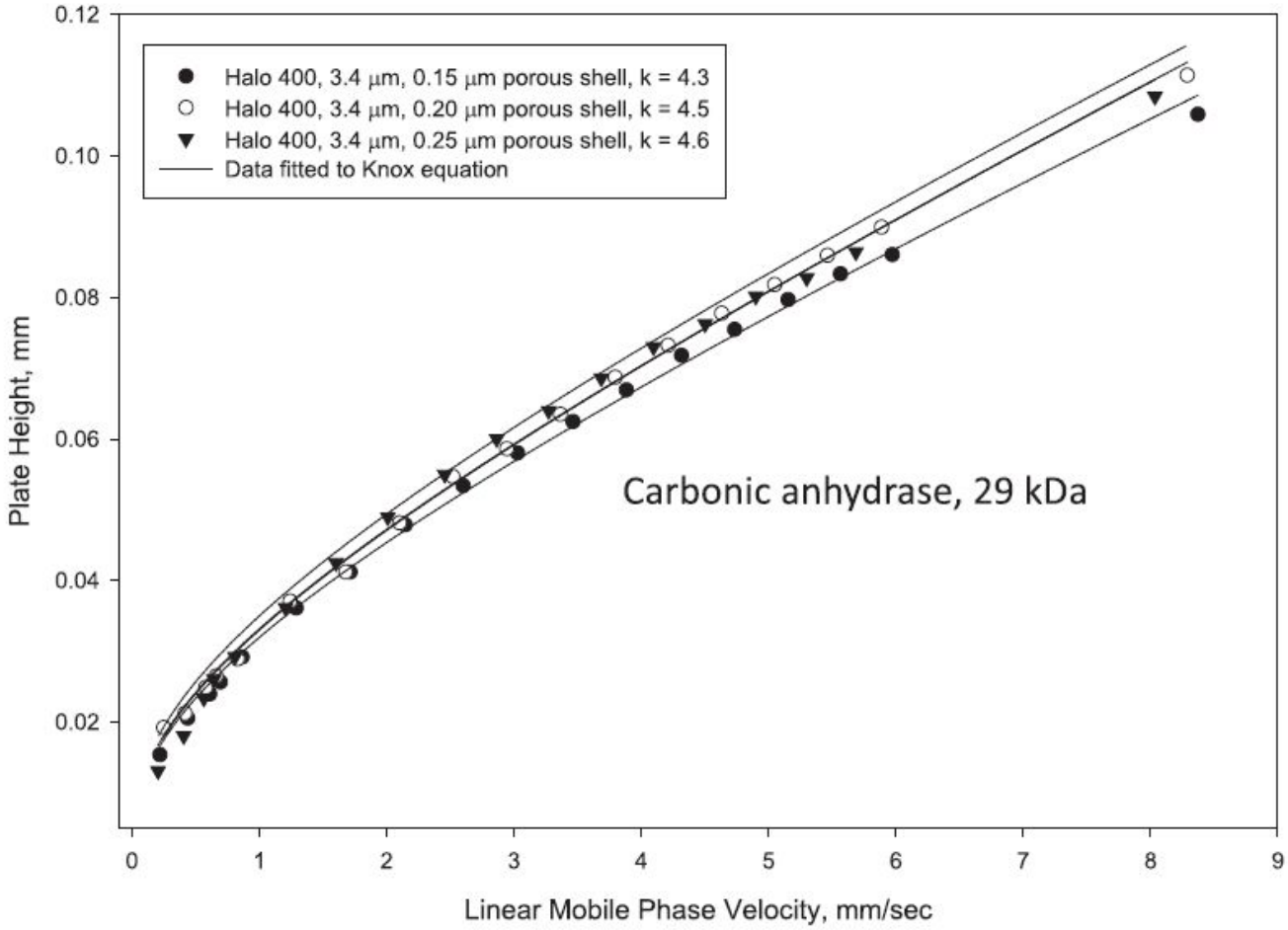
Effect of Porous Shell Thickness



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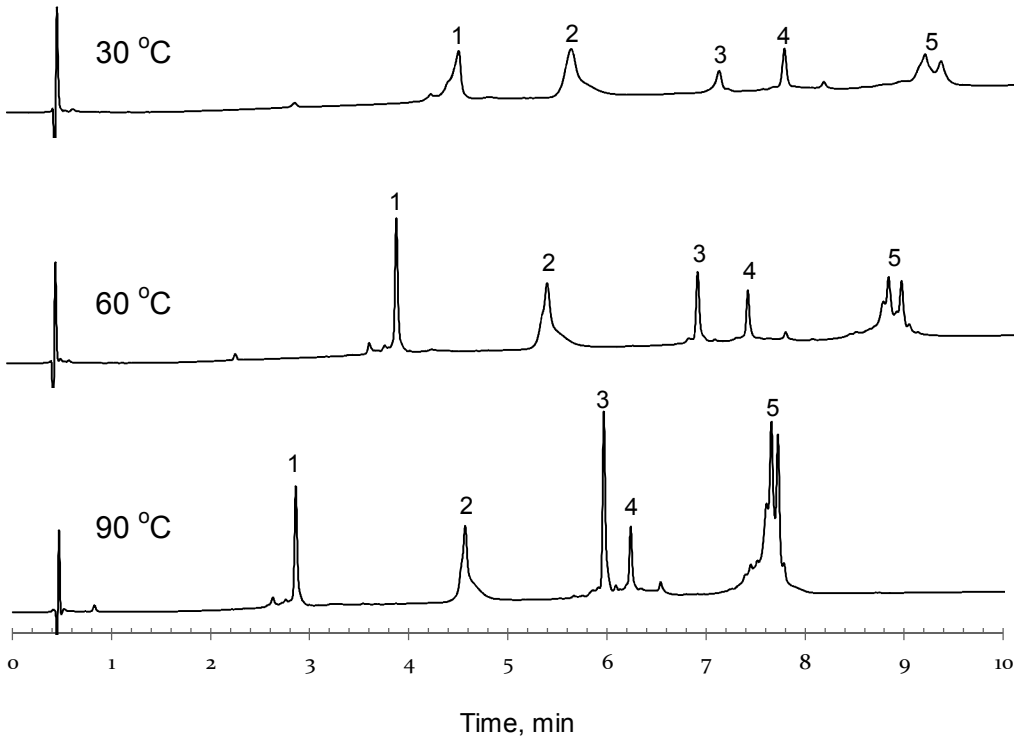
Operational Variables: Temperature

Column: 2.1 x 100 mm HALO Protein C4
Instrument: Agilent 1200 SL
Injection Volume: 2 µL
Detection: 215 nm
Temperature: as indicated

Mobile Phase A: water/0.1% TFA
Mobile Phase B: acetonitrile/0.1% TFA
Gradient: 28-58% B in 10 min.
Flow rate: 0.45 mL/min

Peak Identities (in order):

1.	Lysozyme	14.3 kDa
2.	BSA	66.4 kDa
3.	α-Chymotrypsinogen A	25.0 kDa
4.	Enolase	46.7 kDa
5.	Ovalbumin	44.0 kDa



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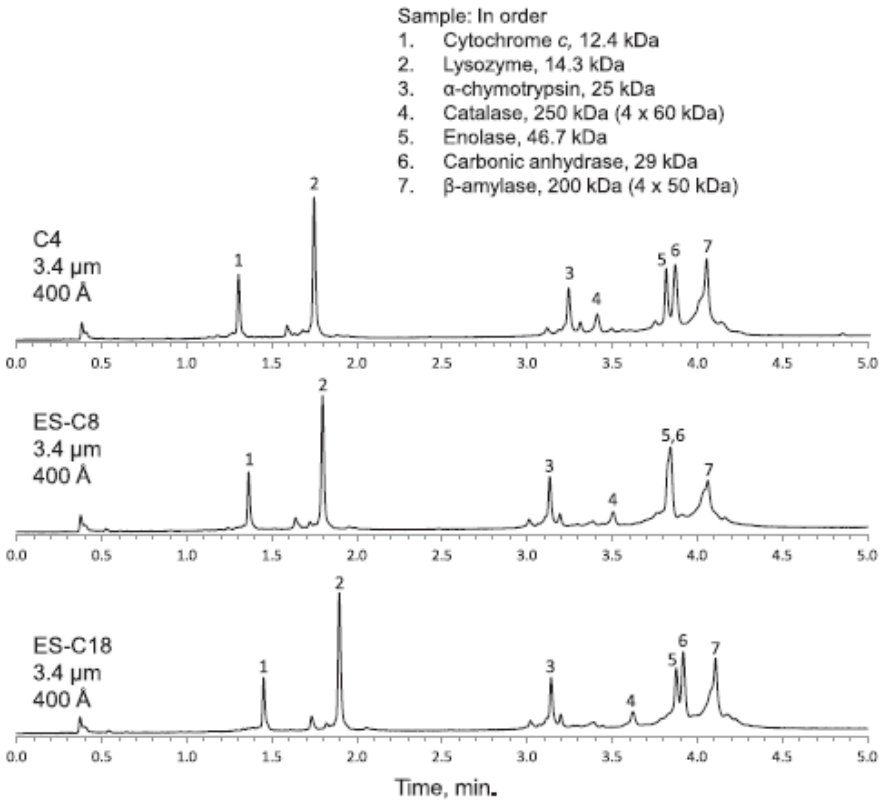
Surface Functionality: Resolution/Selectivity



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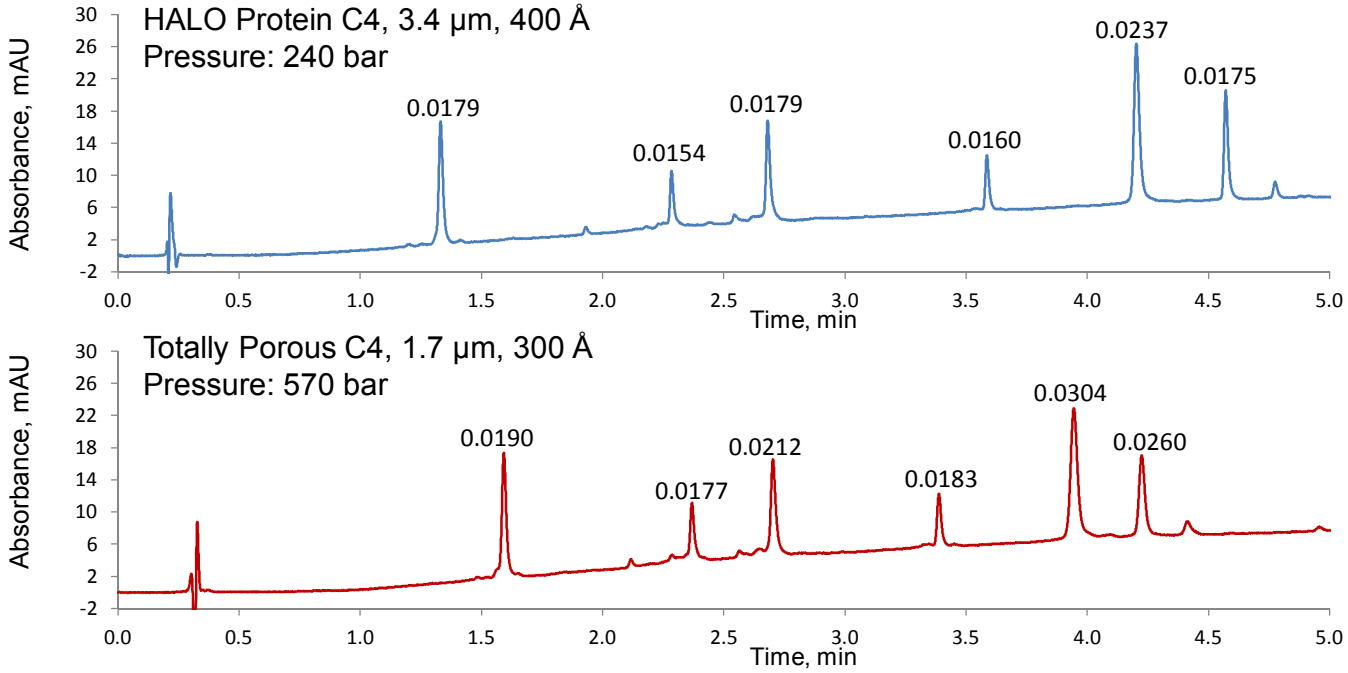
Protein Separations: SPP compared to Totally Porous

Columns: 2.1 x 100 mm
Instrument: Agilent 1200 SL
Injection Volume: 1 µL
Detection: 215 nm
Temperature: 60 °C

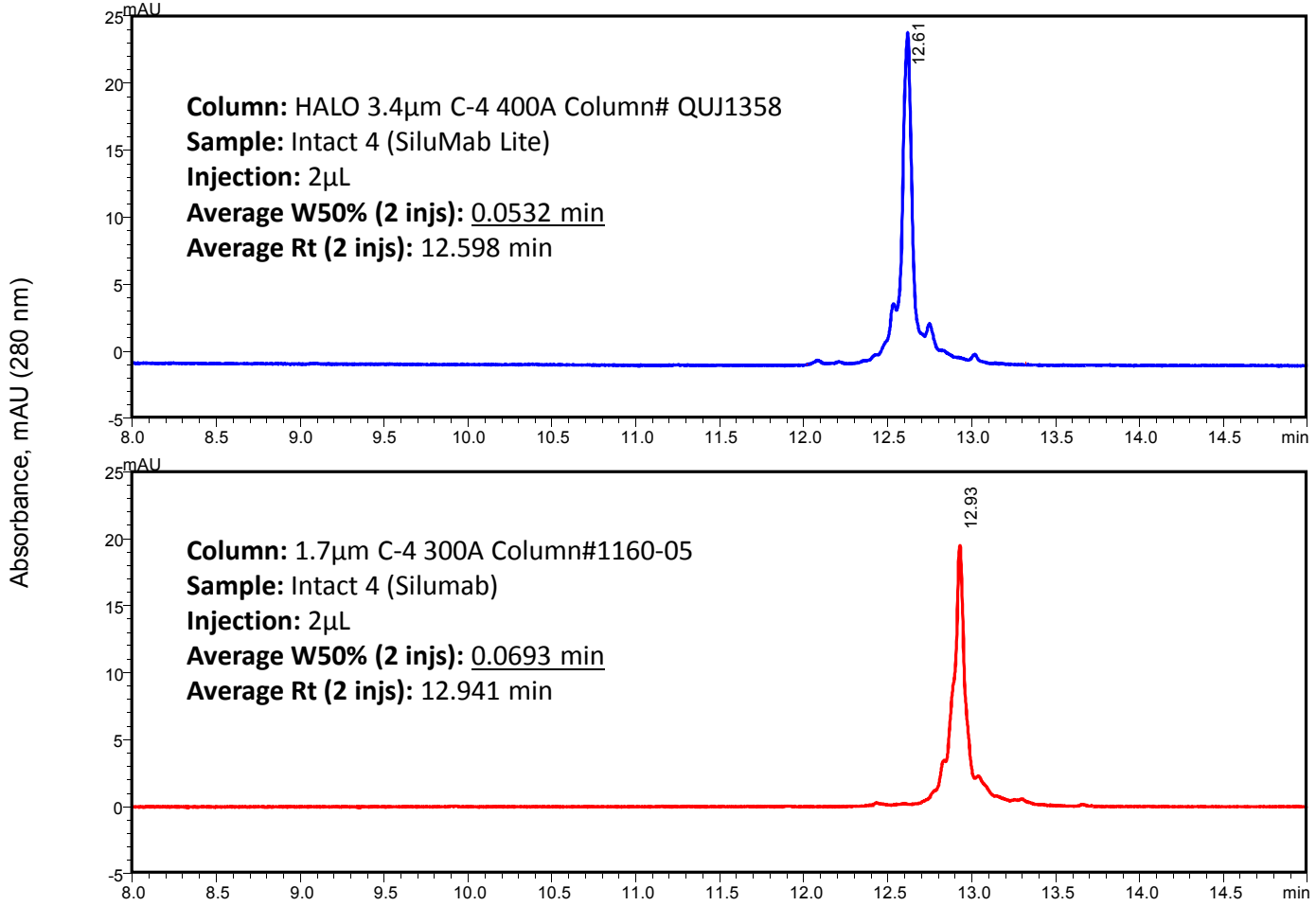
Flow rate: 1.1 mL/min
Mobile Phase A: water/0.1% TFA
Mobile Phase B: acetonitrile/0.1% TFA
Gradient: 23-52% B in 5 min

Peak Identities:
1. Ribonuclease A
2. Cytochrome *c*
3. Lysozyme
4. α-Lactalbumin
5. Catalase
6. Enolase



mAb Separation: SPP compared to Totally Porous

2.1 x 150 mm columns; 0.35 mL/min; 90°C;
A – 0.1% TFA in water; B- 0.1% TFA in AcN



HALO BioClass Columns for Characterization of Monoclonal Antibodies (mAbs)

Monoclonal Antibody Characteristics	Technique(s)	Applicable HALO BioClass Column	Pore Size (Å)	Particle Size
Purity, impurities, post-translational modifications, molecular weight	Reversed-phase LC-MS	HALO Protein C4	400	3.4
Identity, purity, impurities, site-specific modifications	Reversed-phase LC-MS RPLC-UV	HALO Peptide ES-C18	160	2.0, 2.7, 5
Glycosylation (sequence, composition, linkage, branching)	HILIC-MS HILIC-FLD	HALO Glycan	90	2.0, 2.7

HALO® BioClass Fused-Core:
Mobile Phases for Improved Protein LC/MS

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Mobile Phases for Protein and Peptide LC/MS

Successful LC/MS depends on Stationary Phase, Mobile Phase and Instrument fitness to task

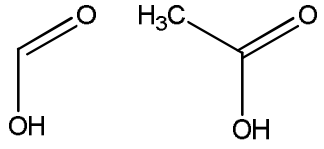
- TFA is the acidic mobile phase modifier of choice for protein and peptide separations, showing good peak shape and high column efficiency
- Formic acid (and acetic) has been widely adopted for LC/MS applications, with (mostly) reasonable LC performance and excellent MS compatibility
- TFA is widely considered a bad choice for LC/MS, largely due to ESI suppression (low signal), and perhaps due to background problems, and system persistence after use
- The vast majority of protein LC/MS examples use FA or TFA

Mobile Phases for Improved Protein LC/MS
Properties That May Help

Initial selection and testing indicated some candidates with promise:

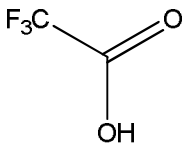
Share required features of volatility, lower pKa, but variable protein solubility

FA	HOAc	TFA	DFA	3FPA
3.8	4.8	0.3-1.6	1.3-1.4	3.1

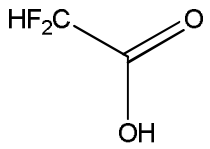


formic acid

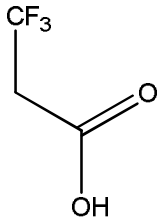
acetic acid



2,2,2-trifluoroacetic acid

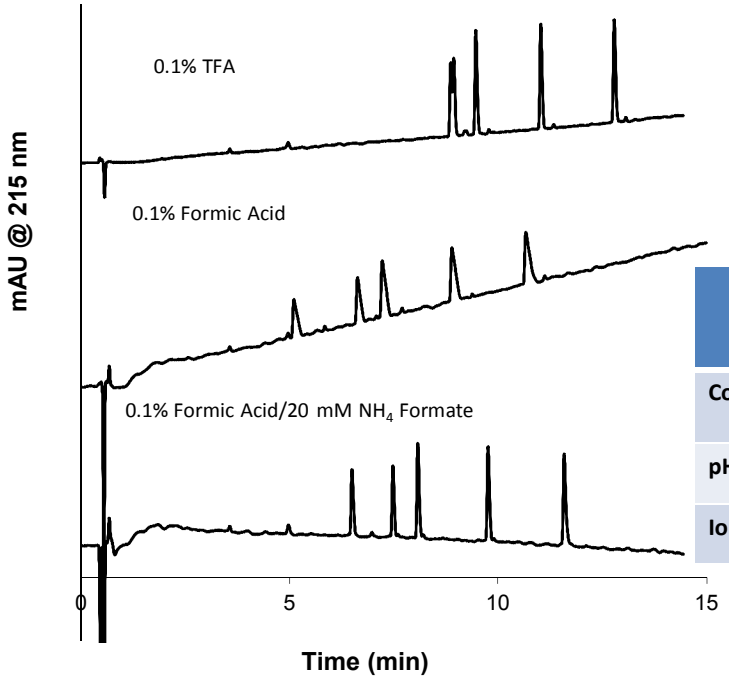


2,2-difluoroacetic acid



3,3,3-trifluoropropanoic acid

Improving Retention and Peak Shape Using Ammonium Formate



Column: Halo Peptide ES-C18, 4.6 x 100 mm; Flow rate: 2.0 mL/min; T= 30 C;
A: Water/acid modifier; B: ACN/0.1% TFA or Formic Acid
Gradient: 1.5% to 26% B in 15 min.; Injection: 8 µL (800 ng) of synthetic peptides S1-S5

Synthetic Peptide Retention Standards:

S1 RGAGGLGLGK-Am
S2 Ac-RGGGGLGLGK-Am
S3 Ac-RGAGGLGLGK-Am
S4 Ac-RGVGGLGLGK-Am
S5 Ac-RGVVGLGLGK-Am

	TFA 0.1%	Formic Acid 0.1%	Ammonium Formate	AF/FA
Concentration	**0.013 M**	**0.026 M**	**0.020 M**	**20 mM AF 26 mM FA**
pH	**1.8**	**2.7**	**c. 7**	**3.3**
Ionic strength	**26 mM**	**4.4 mM**	**40 mM**	**c. 44 mM**

McCalley, D. V., Effect of buffer on peak shape of peptides in reversed -phase high performance liquid chromatography. *J Chromatogr* **2004,** *1038* (1-2), 77-84.
Schuster, S. A.; Boyes, B. E.; Wagner, B. M.; Kirkland, J. J., Fast high performance liquid chromatography separations for proteomic applications using Fused-Core® silica particles. *J Chromatogr* **2012,** 1228, 232-241.

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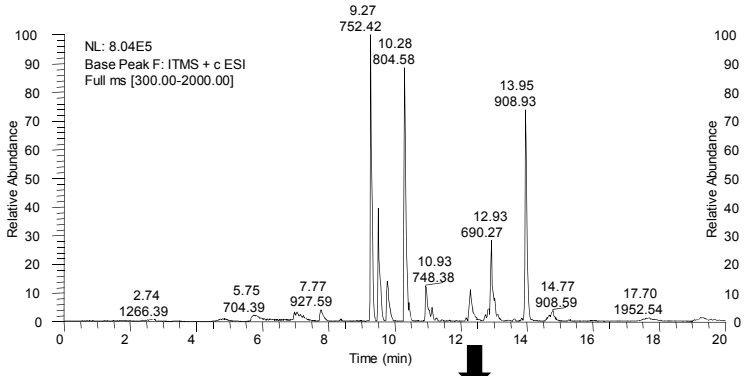
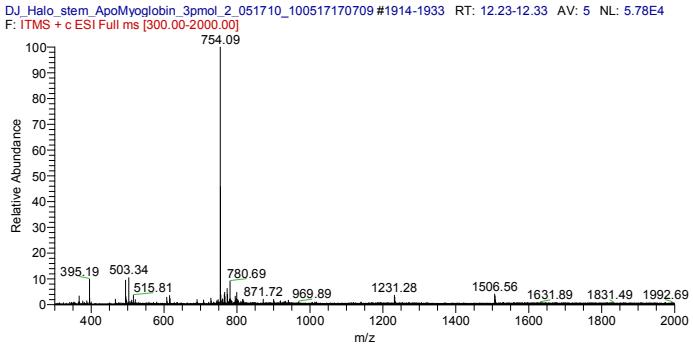
Ammonium formate as an additive for LC/MS separations

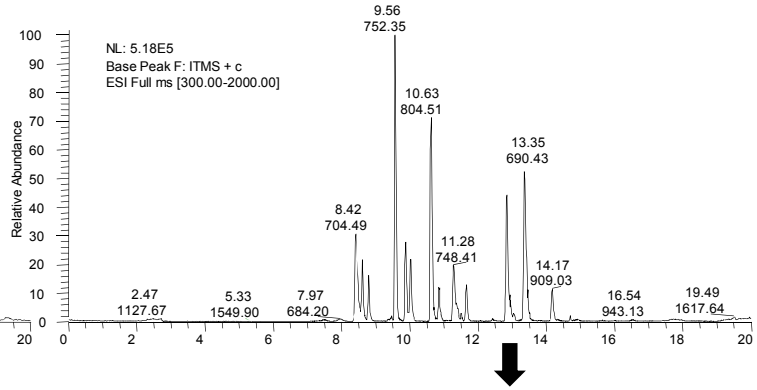
Column: 0.2 x 50 mm Halo Peptide ES-C18; Flow rate: 9 µL/min; Gradient: 2 - 45% B in 15 min; Mobile phases as shown; Sample: 2 µL (3 pmol) apomyoglobin digest.

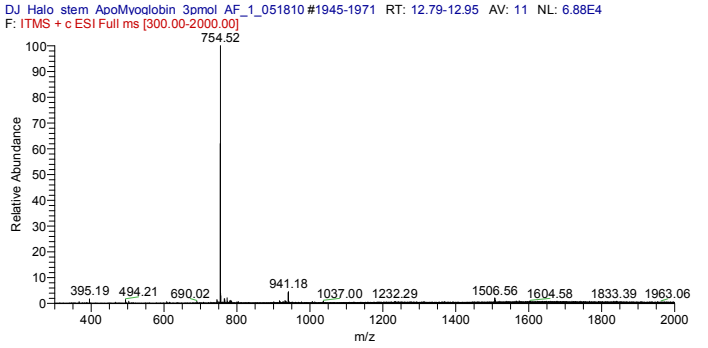
A: 0.1 % Formic Acid





B: 0.1 % Formic Acid/10 mM Ammonium Formate





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Improved Proteomic Analysis

Column: 0.2 ×150 mm HALO Peptide ES-C18;
flow rate: 4 L/min; gradient: 2–45% B in 85 min; A: 0.1% formic acid/water; B: acetonitrile/0.1% formic acid; maximum pressure: 320 bar; sample: mixed protein digest (5 pmol total of transferrin, carbonic anhydrase, and apomyoglobin

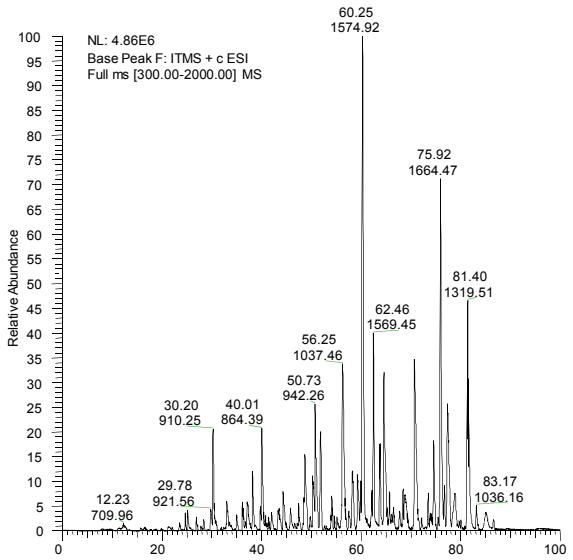


TABLE 7

Proteomic Results from Canine Prostate Carcinoma Analysis Under Various Chromatographic Conditions for Each Mobile-Phase Modifier

Column length (mm)	Flow rate (μL/min)	Experiment time (min)	Mobile-phase modifier	Protein IDs[a]	Matched MS/MS spectra	Peptide IDs[a]	Spectra/peptide ID[b]
50	9	21	0.1% FA	44	455	196	2.32
50	9	21	0.1% FA, 10 mM AF	60	697	255	2.73
150	4	140	0.1% FA	70	1142	359	3.18
150	4	140	0.1% FA, 10 mM AF	118	2028	538	3.77

[a]Results for each mobile-phase modifier generated from duplicate sample analysis with protein and peptide identifications validated using a 5% false discovery rate.
[b]Total number of database-matched MS/MS spectra, divided by the total number of peptide identifications for each condition from triplicate sample analysis.

TABLE 8

Analysis of the 61 Proteins Commonly Identified Using Both Mobile-Phase Modifier Conditions from LC-MS/MS Analysis Canine Prostate Carcinoma Using a 0.2 × 150-mm Column

Mobile-phase modifier	Average peptide IDs/protein[a]	Average spectral count/protein ID[b]	Single-spectrum protein IDs[c]
0.1% FA	6.60	20.71	3
0.1% FA, 10 mM AF	9.64	28.56	0

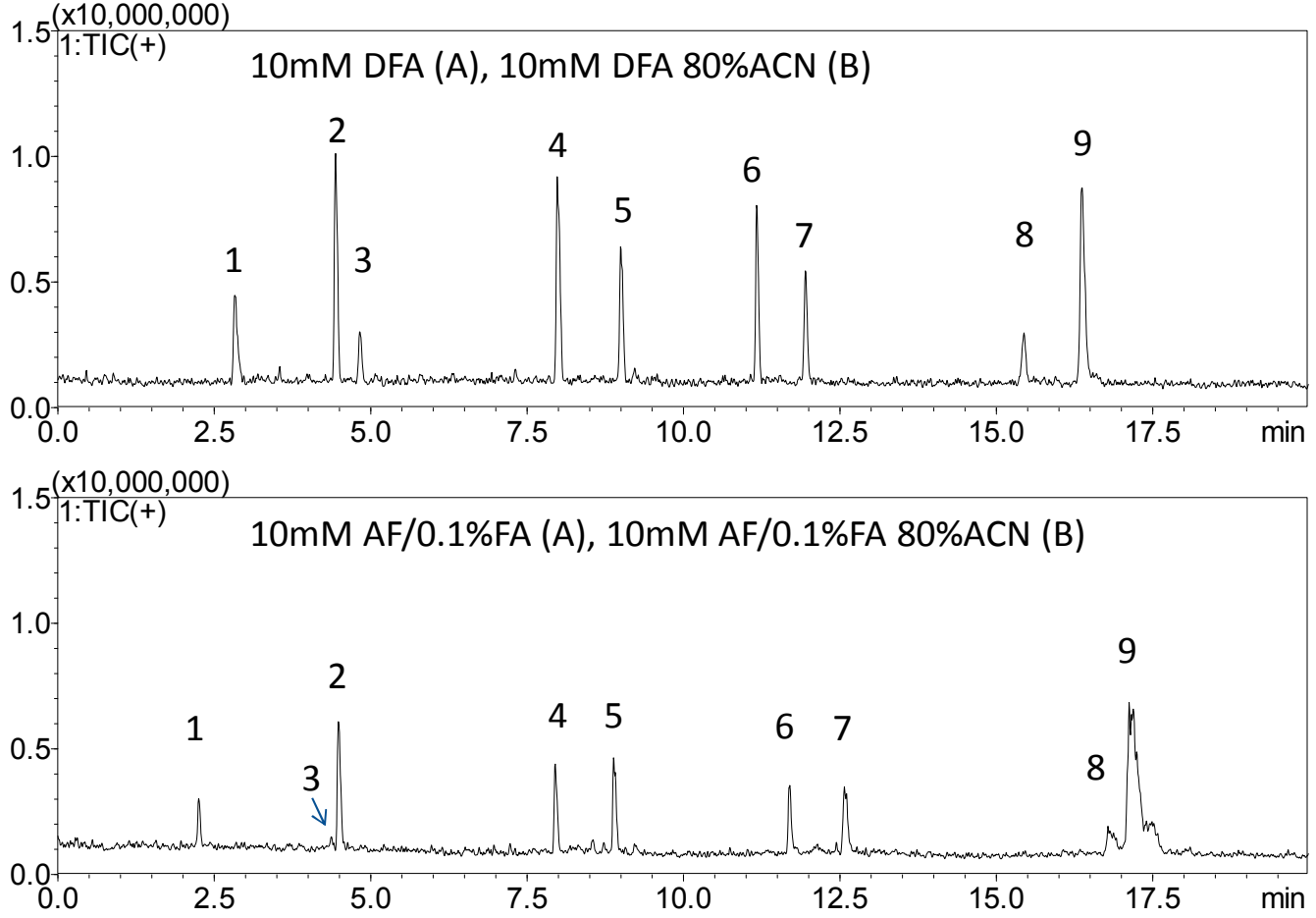
[a]The number of peptides identified from the 61 common identification proteins, divided by the number of common protein identifications.
[b]The total number of database-matched MS/MS spectra from the 61 common identification proteins, divided by total of common protein identifications.
[c]Protein identifications from only one single MS/MS spectra after application of a 5% false discovery rate.

Schuster, S.A, Boyes, B.E., Wagner, B.M., Kirkland J.J. Fast high performance liquid chromatography separations for proteomic applications using Fused-Core® silica particles. **2012** *J. Chromatogr A*, 1228, 232– 241.
Johnson, D.J., Boyes, B.E., Orlando, R.C. The Use of Ammonium Formate as a Mobile-Phase Modifier for LC-MS/MS Analysis of Tryptic Digests. **2013** *J. Biomol.Tech.*, 24, 187-197.

DFA as an additive for LC/MS separations

Column: 2.1 x 150 mm Halo Peptide ES-C18; Flow rate: 0.4 mL/min;
Gradient: 5 - 60% B in 20 min; Mobile phases as shown; Sample: 5 µL (250µg).

DFA improved MS sensitivity!



1. Asp-Phe
2. Angiotensin(1-7)amide
3. Tyr-Tyr-Tyr
4. Angiotensin(II)
5. Neurotensin
6. Angiotensin(1-2)human
7. β-endorphin
8. Sauvagine
9. Mellitin

Mobile Phases for Improved Protein LC/MS

2.1 x 100 mm
Protein 400 C4

15-55% AcN 30 min

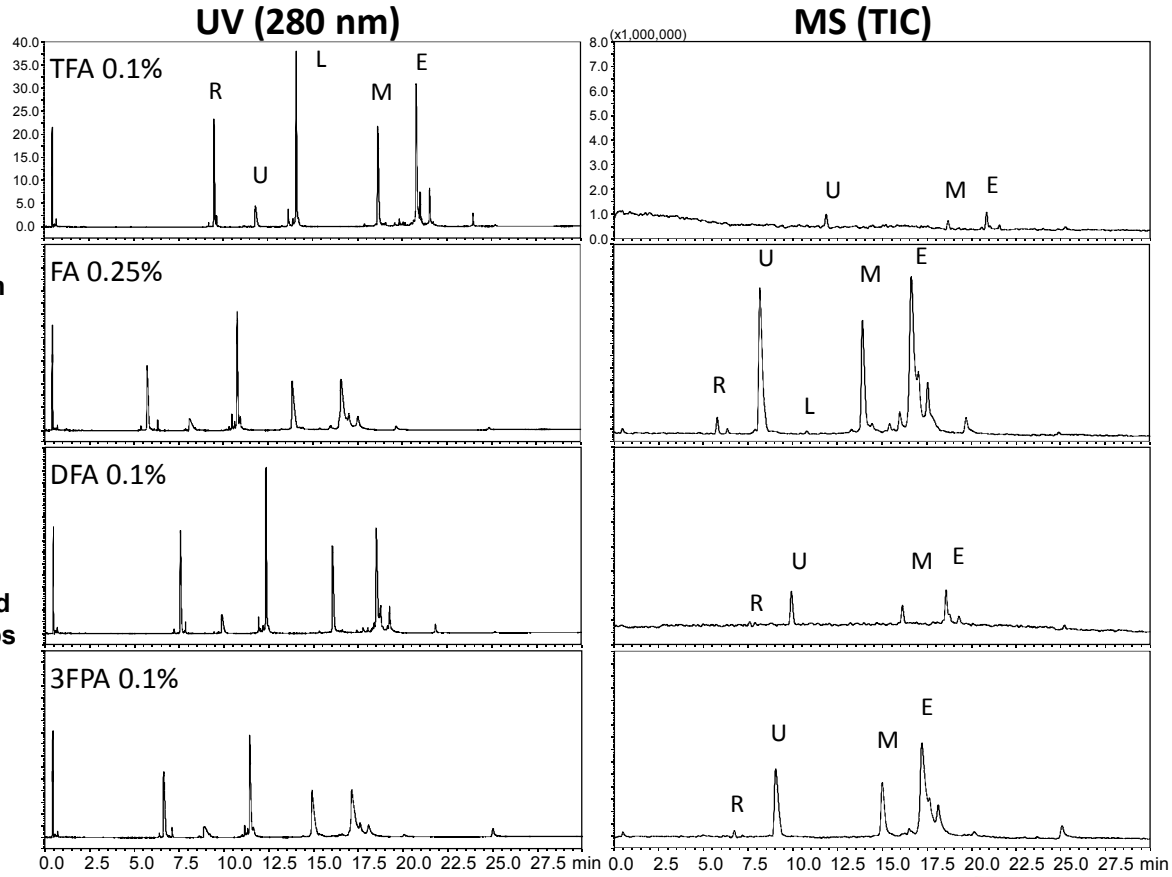
0.35 mL/min; 50ºC

25 pmol each protein

R – Ribonuclease
U – rec. Ubiqutin
L – Lysozyme
M – apo-Myoglobin
E – Enolase

Nexera LC system

MS-2020 Single Quad
400 – 2000 m/z 3 pps
3.8 kV ESI



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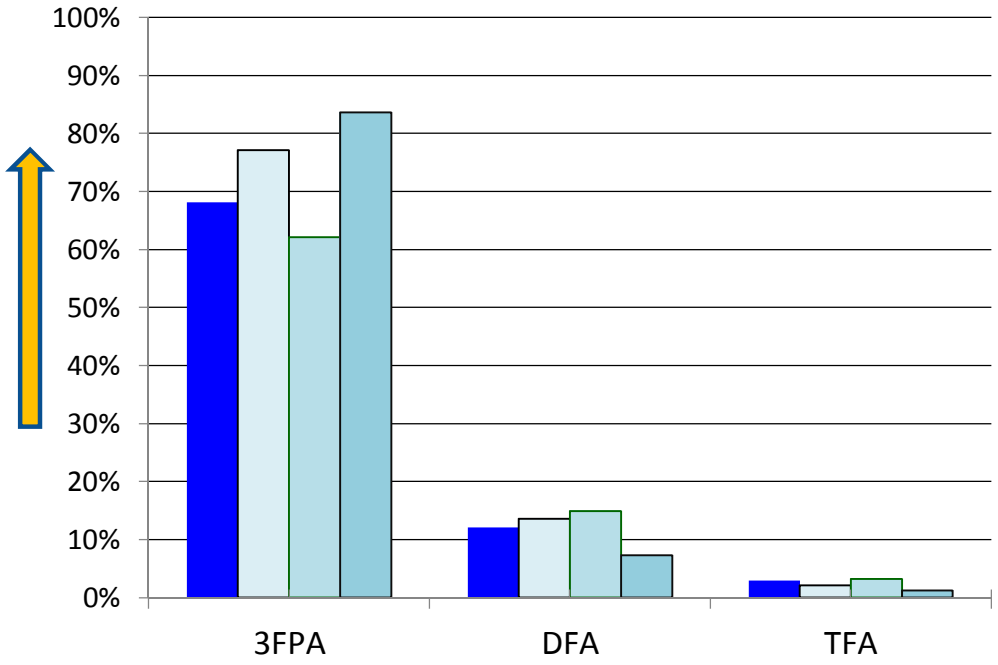
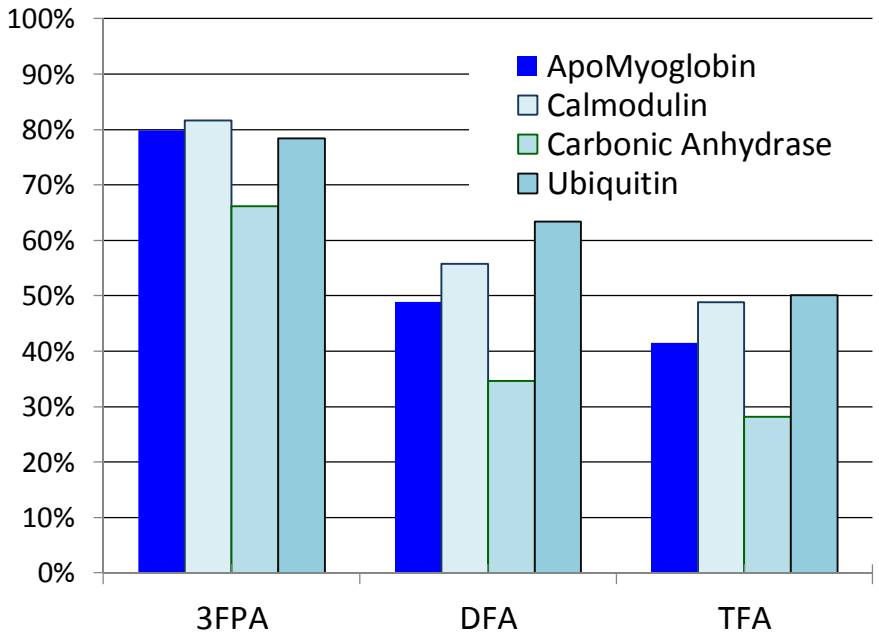
Mobile Phases for Improved Protein LC/MS

Peak Area (TIC) Mobile Phase Relative to FA



$PW_{1/2}$ Mobile Phase Relative to FA



MS and LC performance for 10 mM of each acid modifier relative to 10 mM Formic Acid.
3FPA = 3,3,3,-trifluoropropanoic acid, DFA = 2,2-difluoroacetic acid

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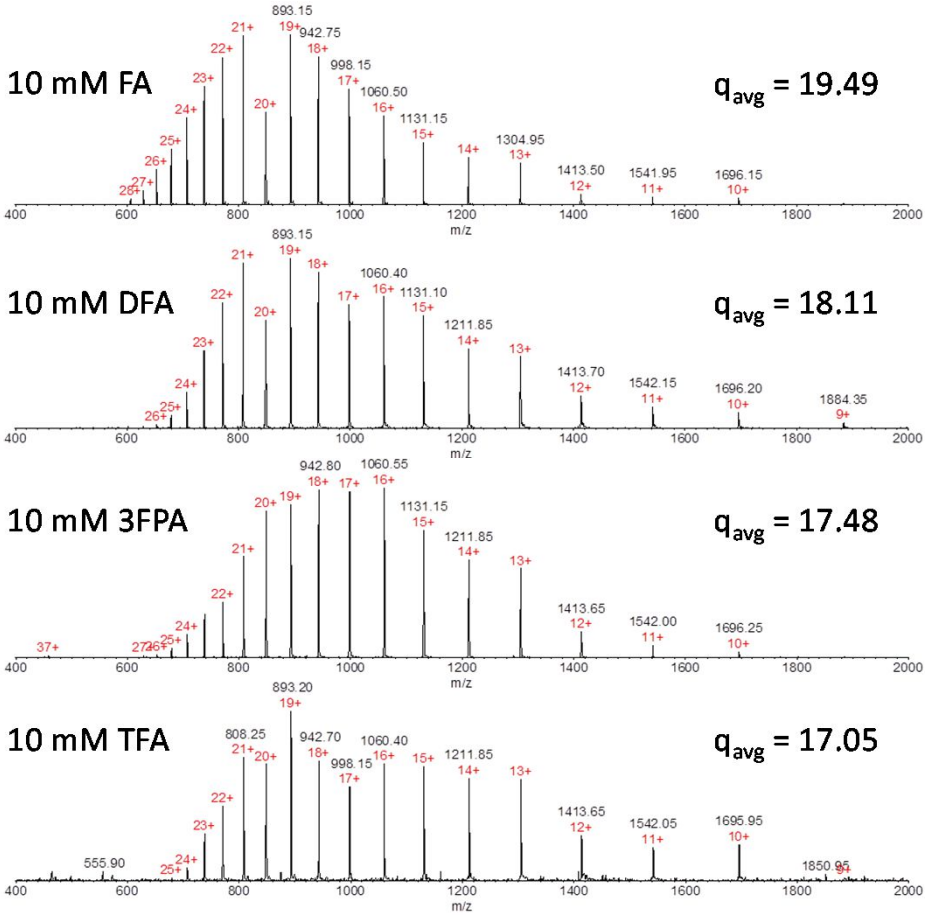
Mobile Phases for Improved Protein LC/MS



apo-Myoglobin
MS spectra average
Ionization state

$$q_{avg} = \frac{\sum_{i=1}^{N} q_i * w_i}{\sum_{i=1}^{N} w_i}$$

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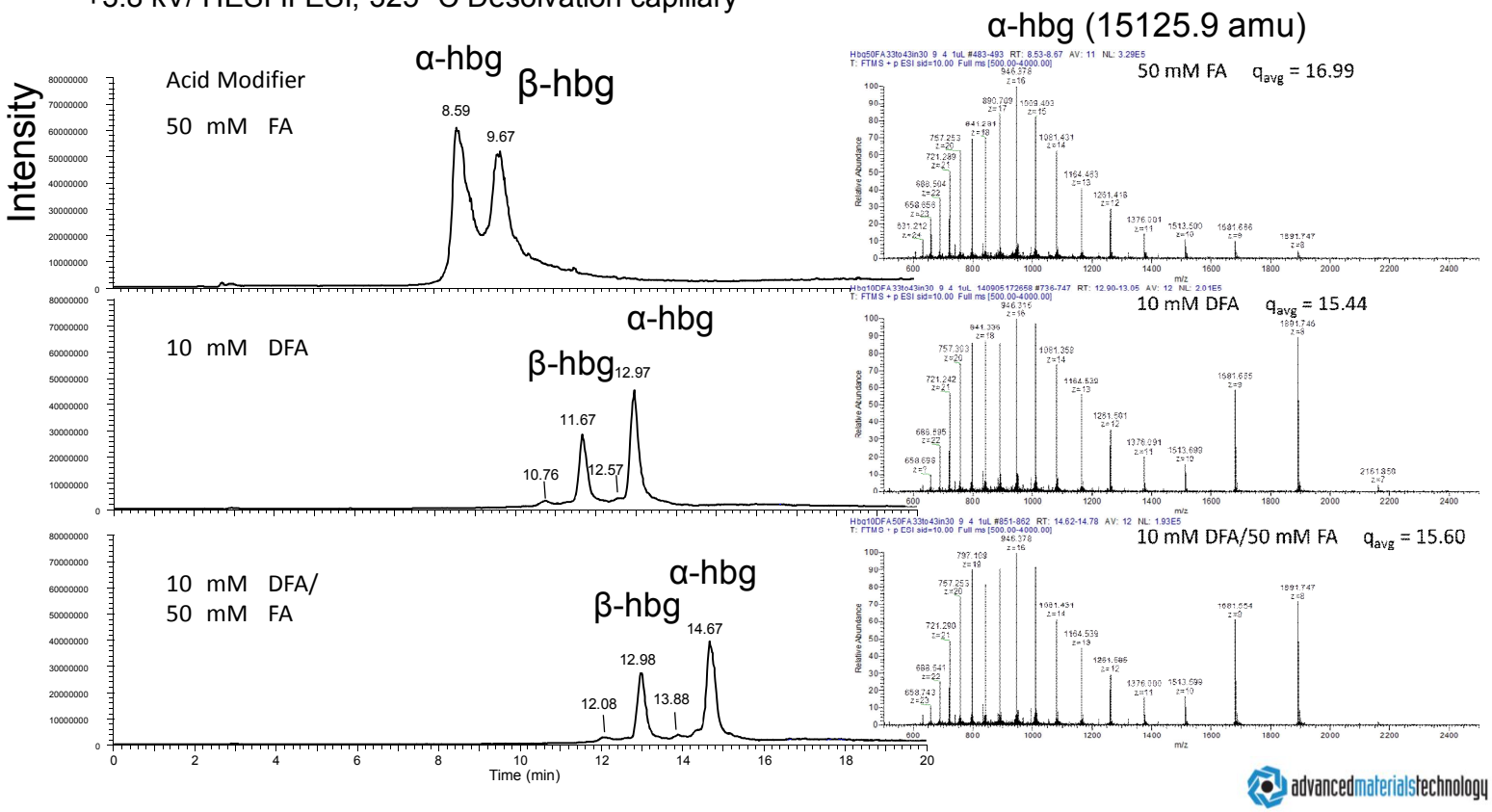
Mobile Phases for Improved Protein LC/MS

Halo Protein C4 0.3 mm ID x 100 mm PeekSil Capillary Column; 0.68 uL StemTrap
33-45% AcN in 20 min; 8.0 µL/min, 50ºC; Orbitrap Velos Pro (60,000 Res) 500-2500 m/z
+3.8 kV/ HESI II ESI, 325 ºC Desolvation capillary



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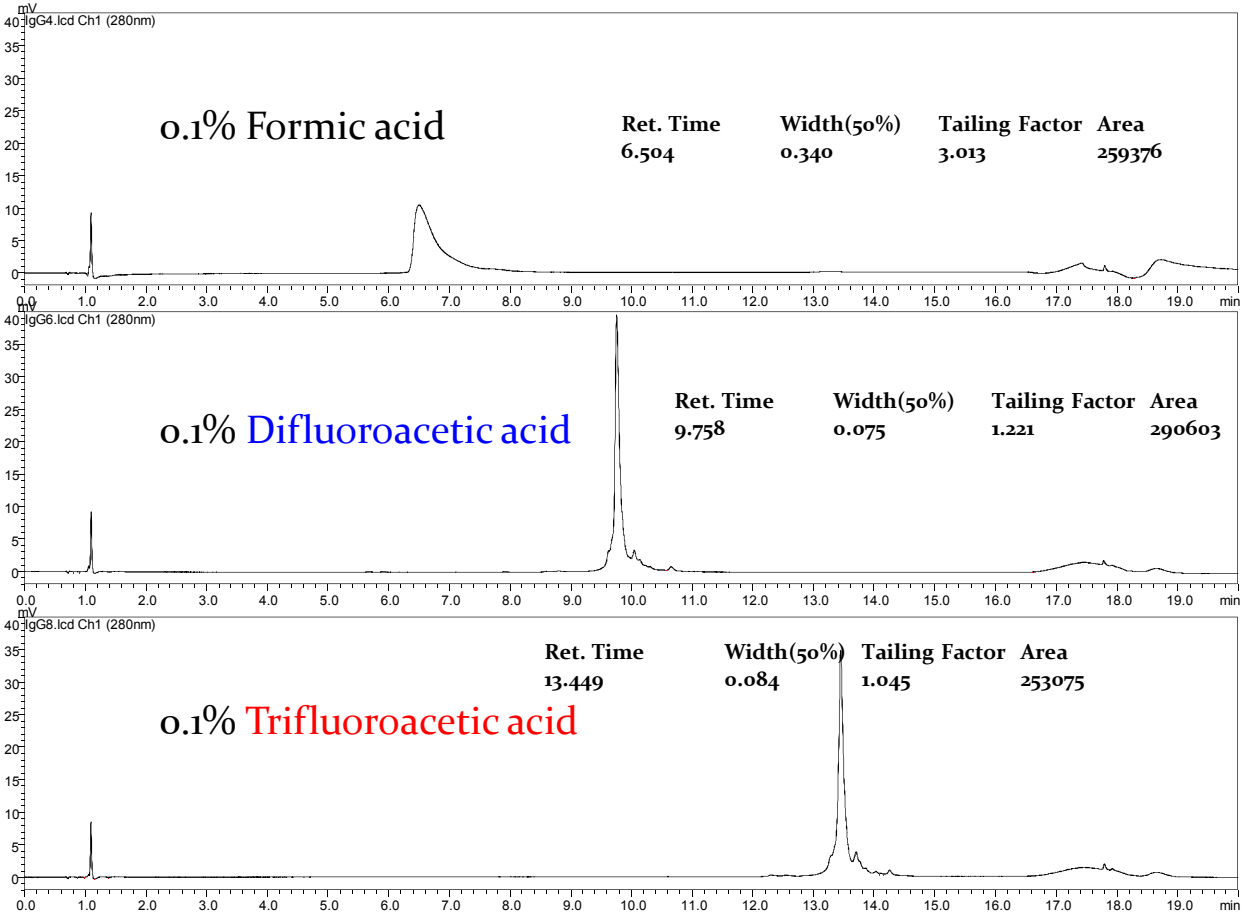
Effect of Acid Modifier on Intact Antibody Separation

Column: 2.1 x 150 mm HALO Protein C4 400 Å
Detection: 280 nm
Temperature: 80 °C
Flow rate: 0.3 mL/min
Gradient: 28-38% ACN/0.1% acid as indicated in 15 min
Sample: 2 µL of Intact SILu™ Lite SigmaMAb - 0.5 µg/µL (H_2O) ~ 150 kDa



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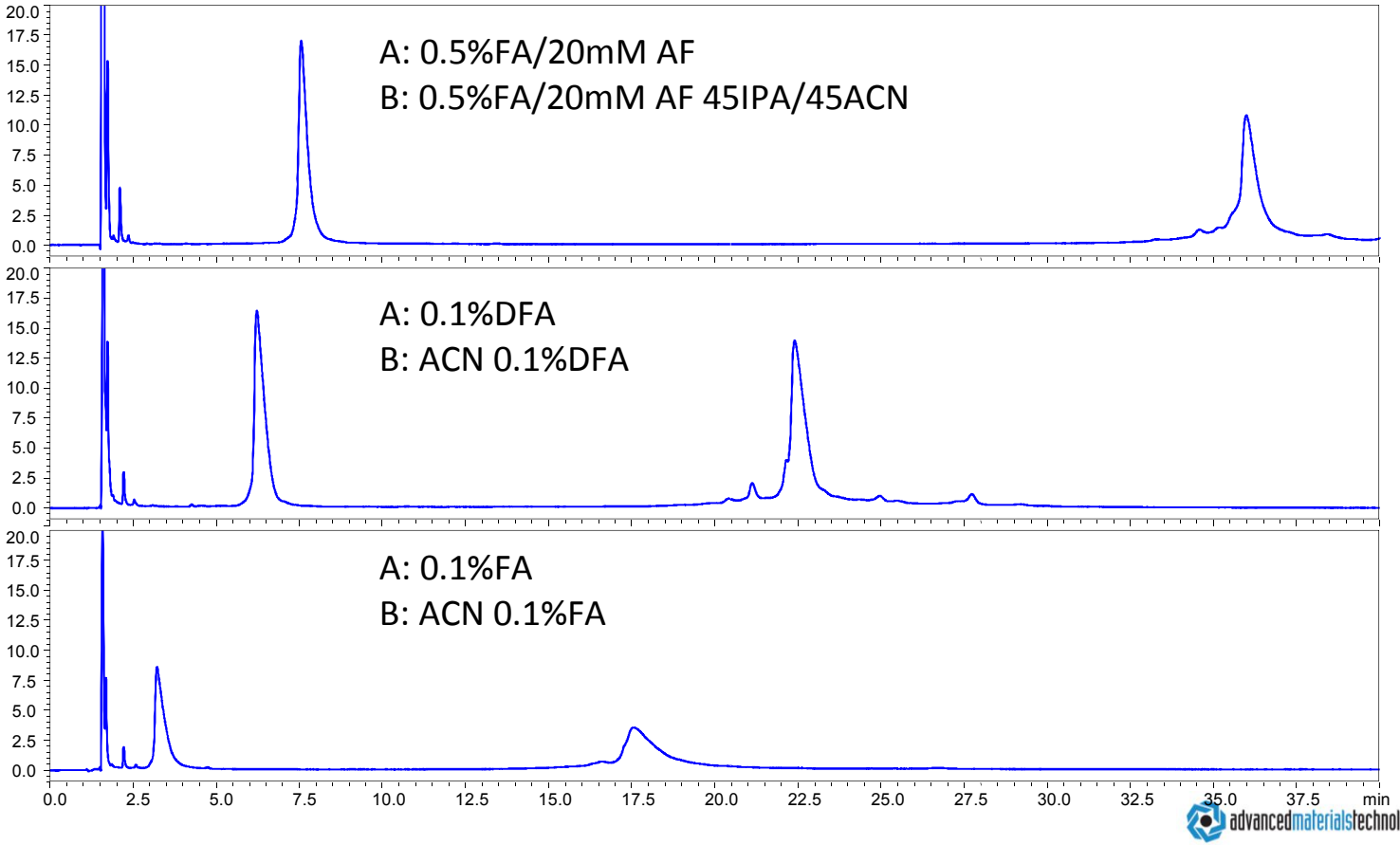
Reduced mAb Chain Separation: MP Effect

Reduced SiluMab

2.1 x 150 mm HALO Protein C4; 30-35%B; 40min ; 0.2mL/min; 80°C



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LCMS Analysis of mAb:
Intact, Component (LC/HC), Digest

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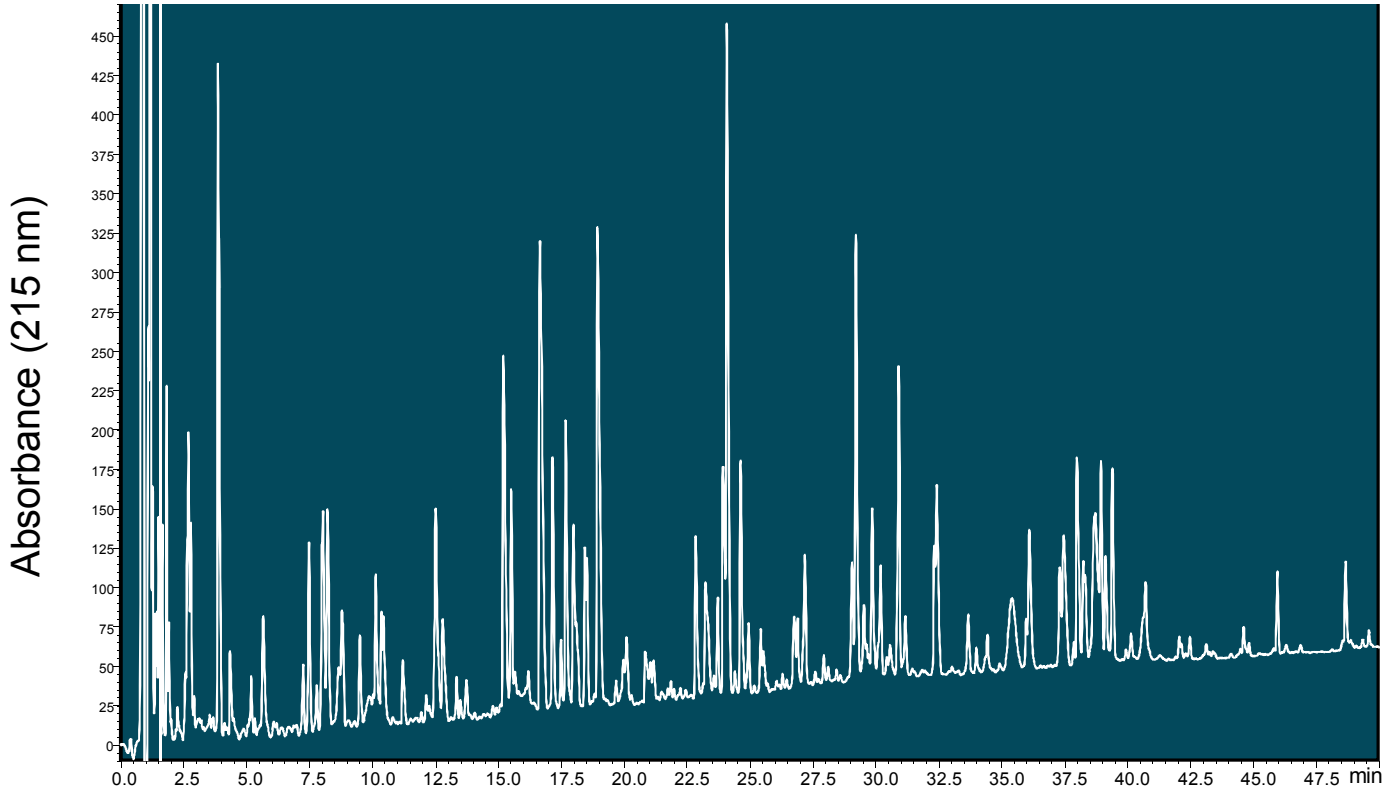
RPLC Analysis of Herceptin Tryptic Digest

***Column*:** **HALO Peptide ES-C18, 2.1 x 150 mm, 2.7 μm**

***Mobile Phase A*:** 0.1% formic acid/10 mM ammonium formate

***Mobile Phase B*:** Acetonitrile with 0.1% formic acid

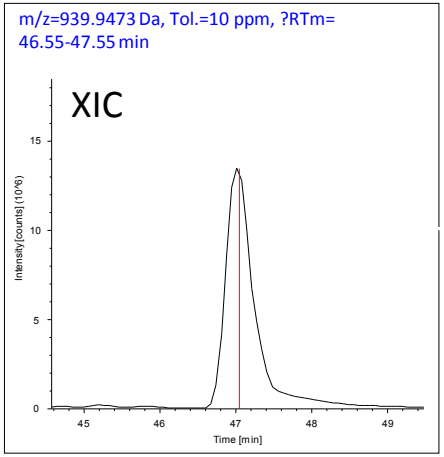
0.4 mL/min; 5–40% B in 60 min.; 60 °C; 50 µL of 2 μg/μL reduced and alkylated, trypsin digested Herceptin

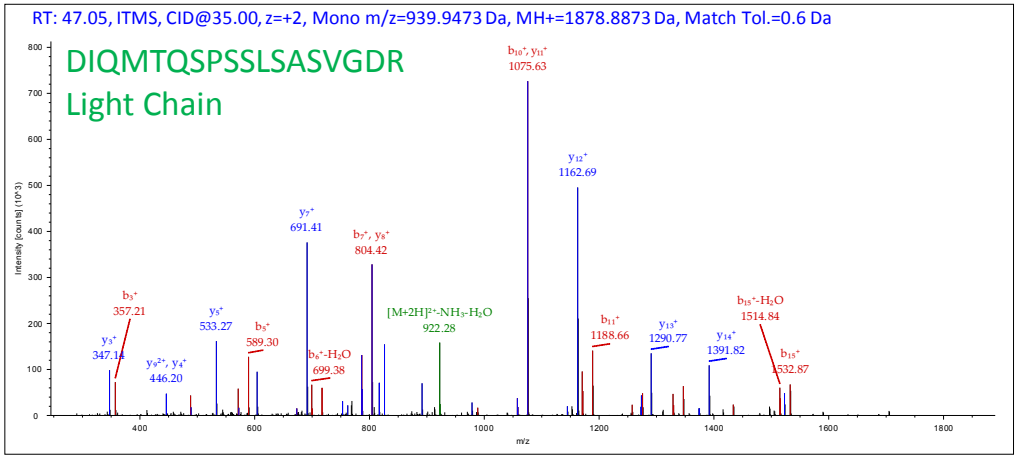


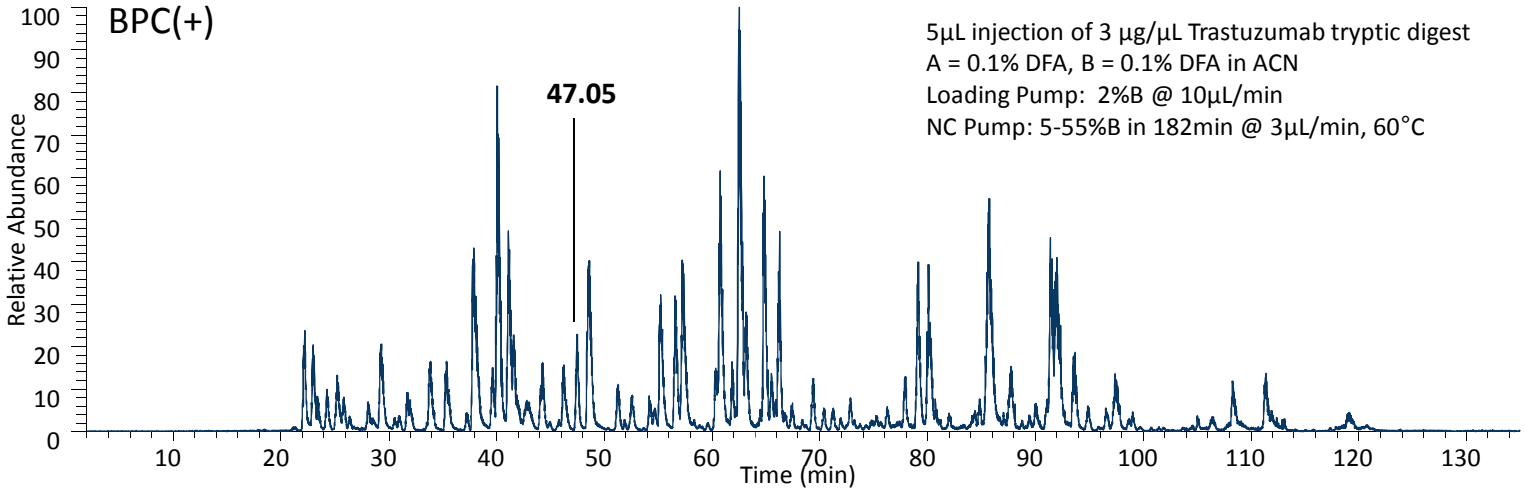
High Resolution Trastuzumab Digest

HALO 5 µm Peptide ES-C18 250 x 0.2mm (2x column in series)

Thermo Orbitrap Velos Pro/Dionex Ultimate 3000 UHPLC





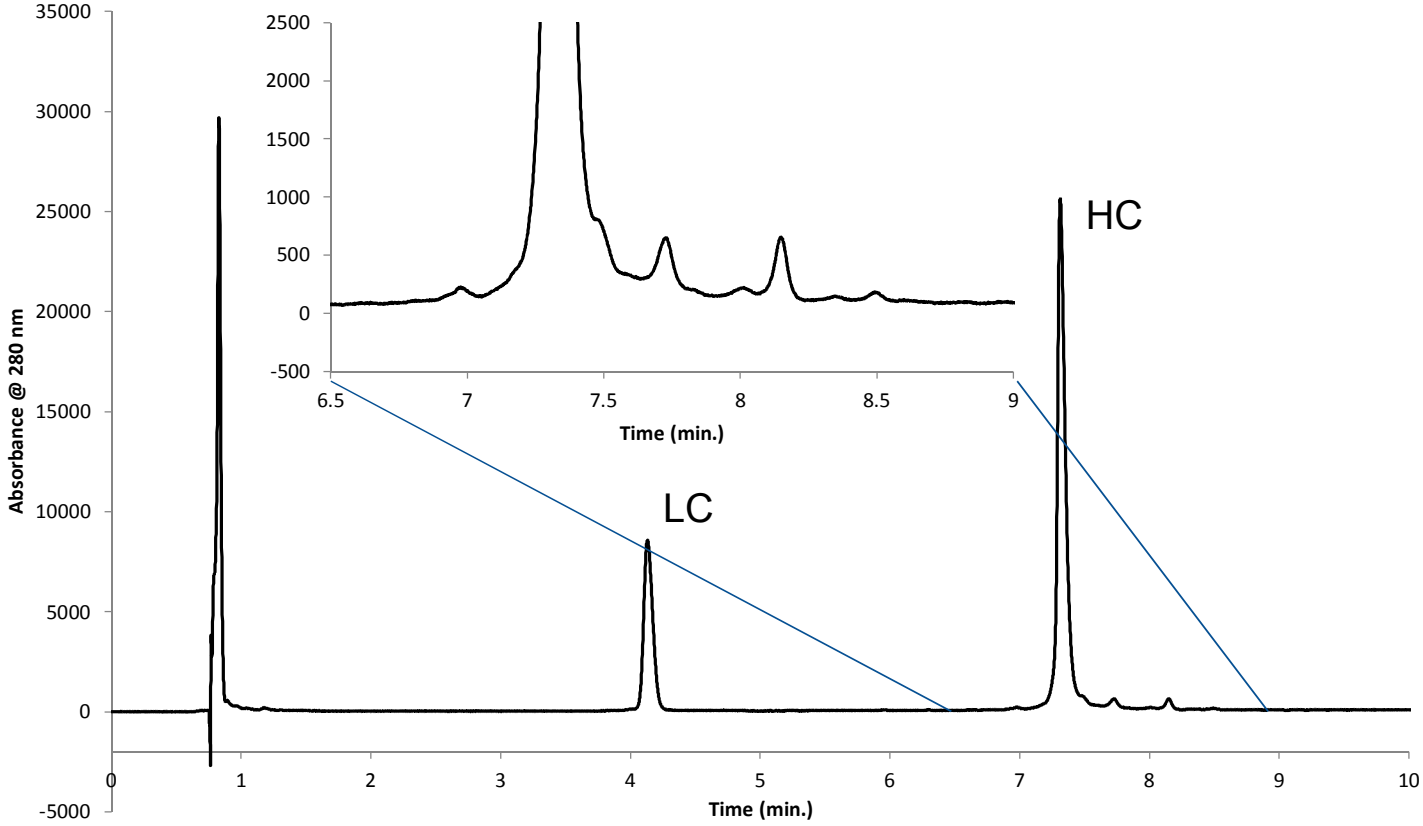


Reduced IgG2-B in TFA mAb Separation

Column: 2.1 x 100 mm HALO Protein C4
Instrument: Shimadzu Nexera
Injection Volume: 1 µL
Detection: 280 nm

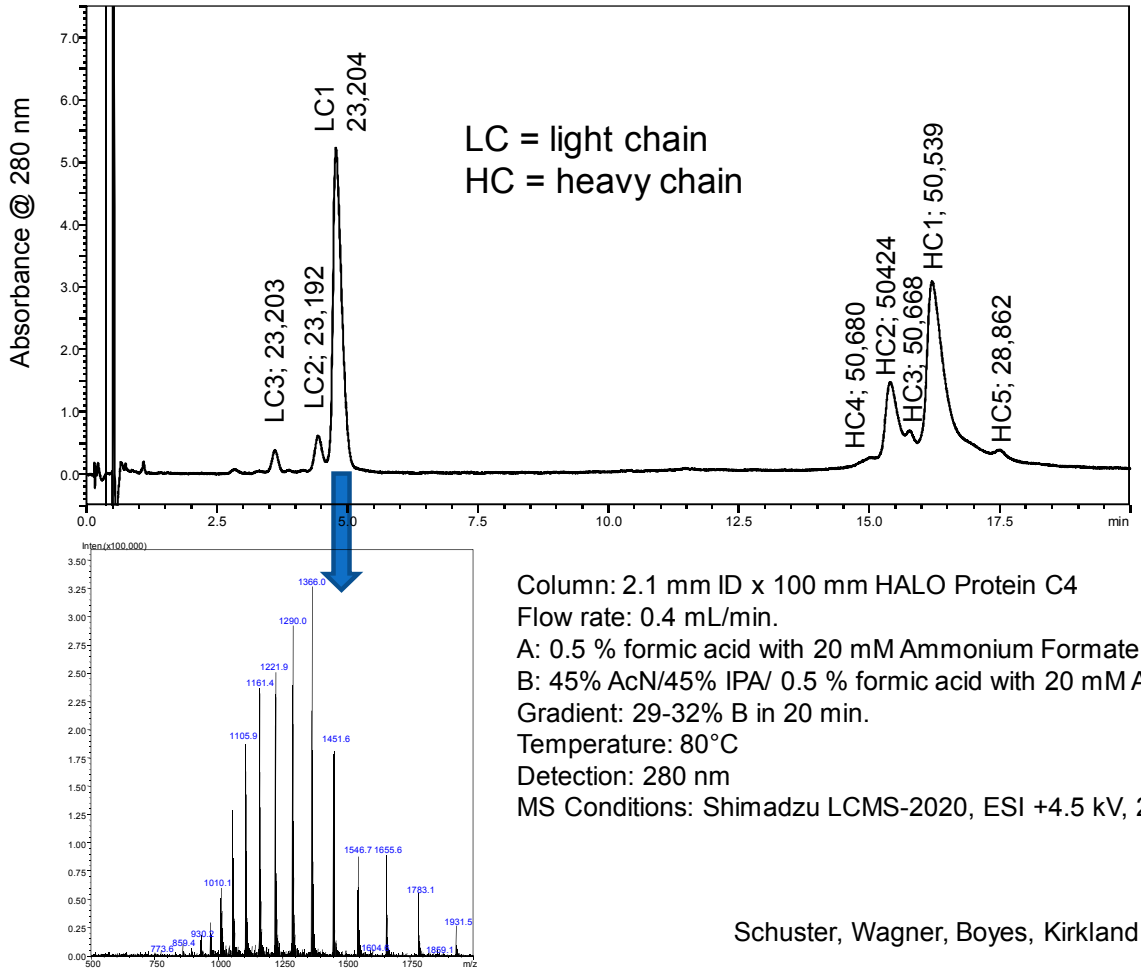
Mobile Phase A: water/0.1% TFA
Mobile Phase B: 80/20 ACN/water/0.1% TFA
Gradient: 33-40% B in 10 min.
Flow rate: 0.25 mL/min
Temperature: 80 °C

Sample: 0.5 mg/mL IgG2-B treated with 100 mM DTT in 8 M Guanidine HCl at 50 °C for 35 min.



Schuster, Wagner, Boyes, Kirkland (2013) J. Chromatogr. 1315, 118.

LC/MS Analysis of IgG1 mAb Polypeptide Chains



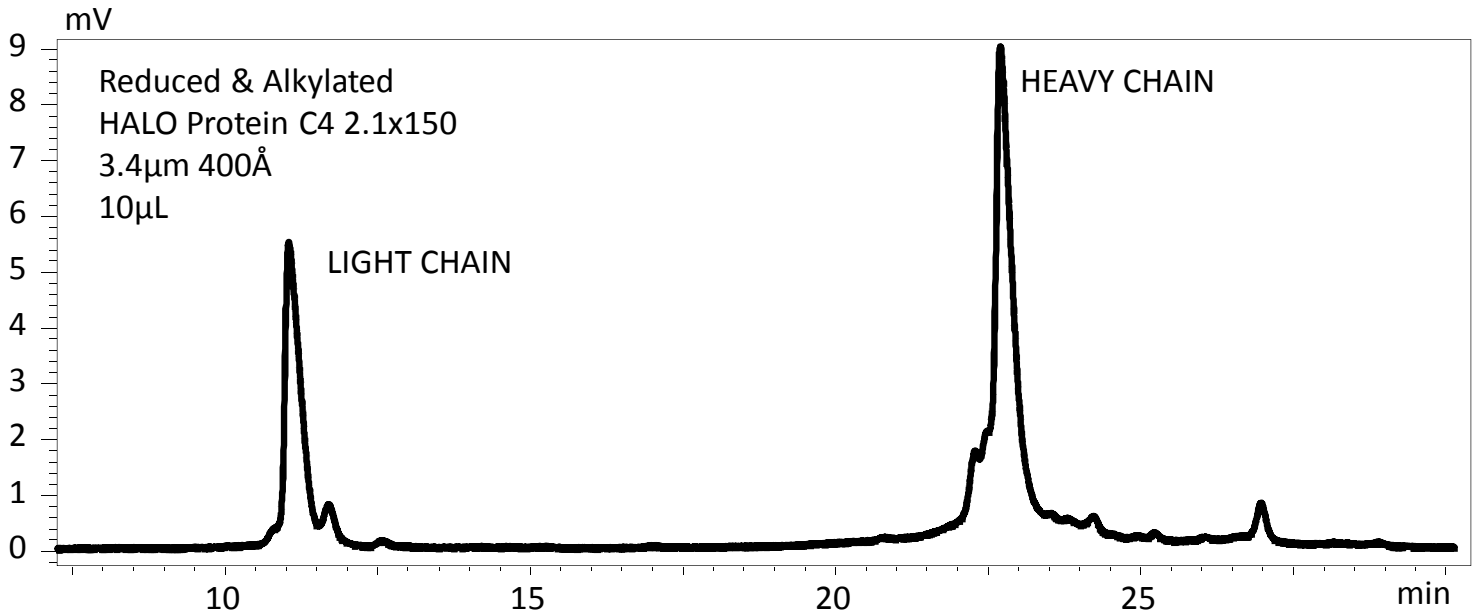
Sample Preparation

IgGs were reduced and alkylated by sequential treatment with 10 mM DTT, 15 mM iodoacetamide, then quenched with an additional 10 mM DTT, all in 6 M guanidine HCl/20 mM Tris-HCl buffer at pH 7.8. Reduced and alkylated IgG solutions were buffer exchanged into 0.1% TFA using VivaSpin (Sartorius Stedim Biotech, Goettigen, Germany) centrifugal concentrators with 5 kDa cut-off HY polymeric membranes. The reduced and alkylated IgGs were adjusted to 2 mg/mL protein in 0.1% TFA and stored at -25 °C until use.

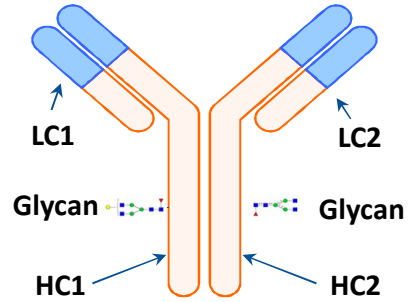
Column: 2.1 mm ID x 100 mm HALO Protein C4
Flow rate: 0.4 mL/min.
A: 0.5 % formic acid with 20 mM Ammonium Formate
B: 45% AcN/45% IPA/ 0.5 % formic acid with 20 mM Ammonium Formate
Gradient: 29-32% B in 20 min.
Temperature: 80°C
Detection: 280 nm
MS Conditions: Shimadzu LCMS-2020, ESI +4.5 kV, 2 pps, 500-2000 m/z

Schuster, Wagner, Boyes, Kirkland (2013) J. Chromatogr. 1315, 118.

Reduced, and Alkylated Trastuzumab Analysis



Reduced Trastuzumab 1mg/mL in 0.1%TFA, 0.2mL/min, gradient 28-34% in 40 min, 80˚C, 280nm, Mobile phase A: 0.1%DFA, B: 0.1%DFA in ACN



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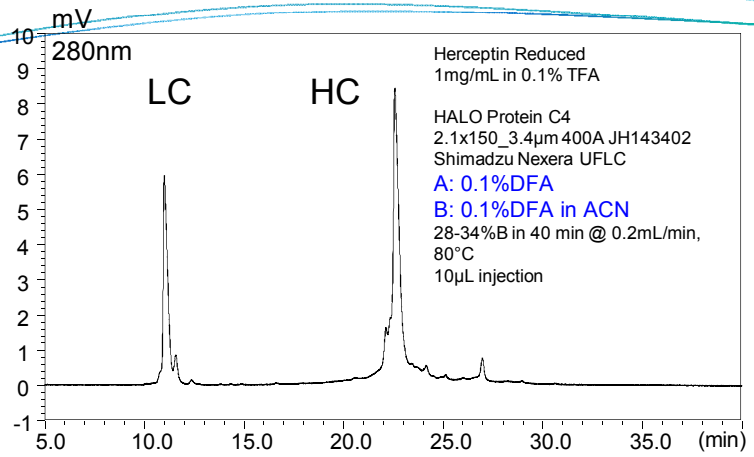
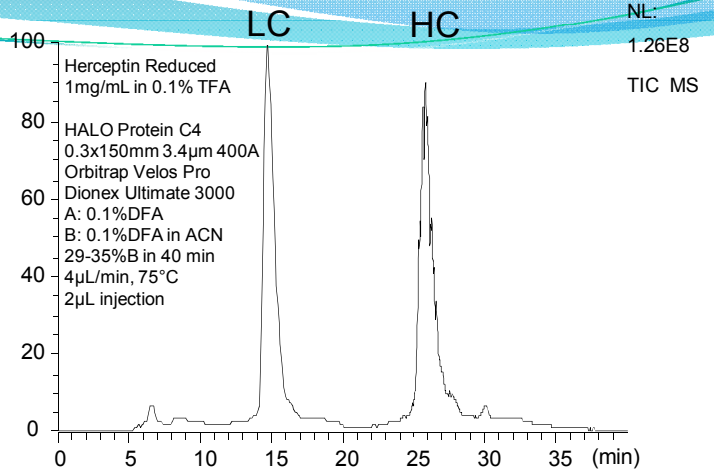
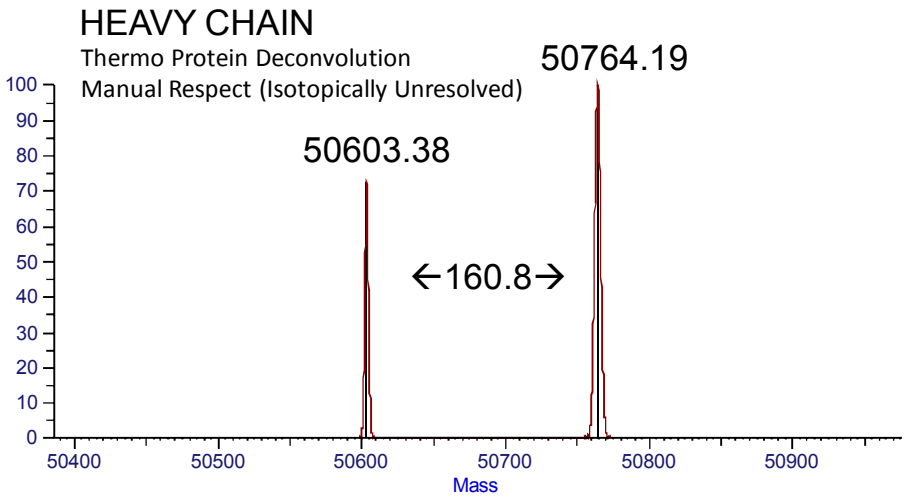
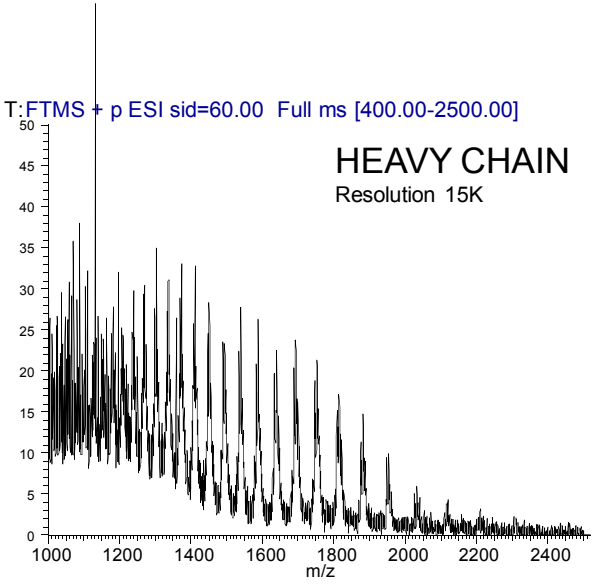
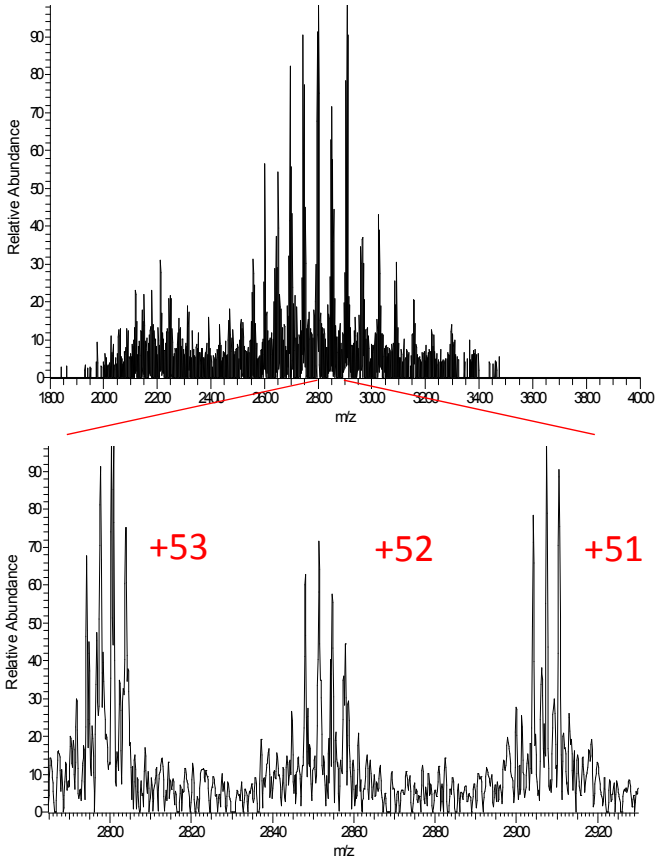

mV
280nm
LC
HC
Herceptin Reduced
1mg/mL in 0.1% TFA
HALO Protein C4
2.1x150_3.4µm 400A JH143402
Shimadzu Nexera UFLC
A: 0.1%DFA
B: 0.1%DFA in ACN
28-34%B in 40 min @ 0.2mL/min,
80°C
10µL injection
(min)


LC
HC
NL:
1.26E8
TIC MS
Herceptin Reduced
1mg/mL in 0.1% TFA
HALO Protein C4
0.3x150mm 3.4µm 400A
Orbitrap Velos Pro
Dionex Ultimate 3000
A: 0.1%DFA
B: 0.1%DFA in ACN
29-35%B in 40 min
4µL/min, 75°C
2µL injection
(min)

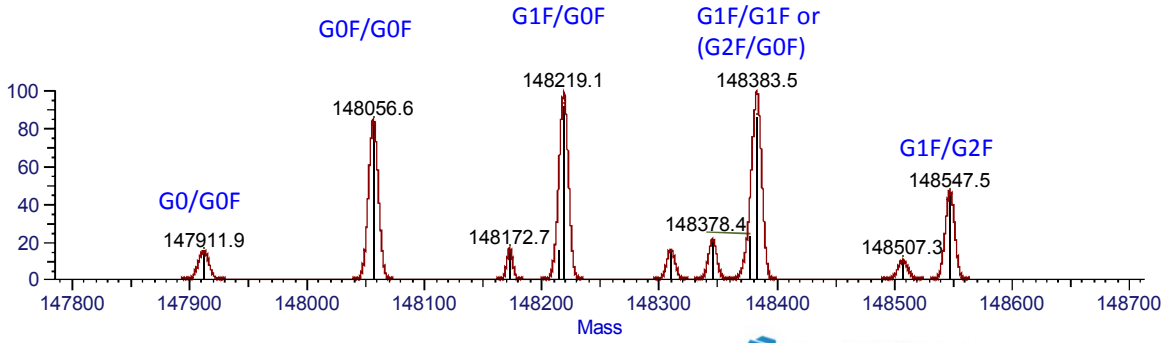

HEAVY CHAIN
Thermo Protein Deconvolution
Manual Respect (Isotopically Unresolved)
50764.19
50603.38
←160.8→
Mass


T:FTMS + p ESI sid=60.00 Full ms [400.00-2500.00]
HEAVY CHAIN
Resolution 15K
m/z

Herceptin: Infusion



Manual RESPECT
Isotopically unresolved
Min Adj Charge: 3-6
Noise Rej: 95% confidence
Rel. Abund Thres: 2
Mz Range 1.8 - 4.0
Output range: 147700-148800K
Mass tolerance: 20ppm
Target mass: 150k
Charge state range 1 to 150
Peak model: intact protein



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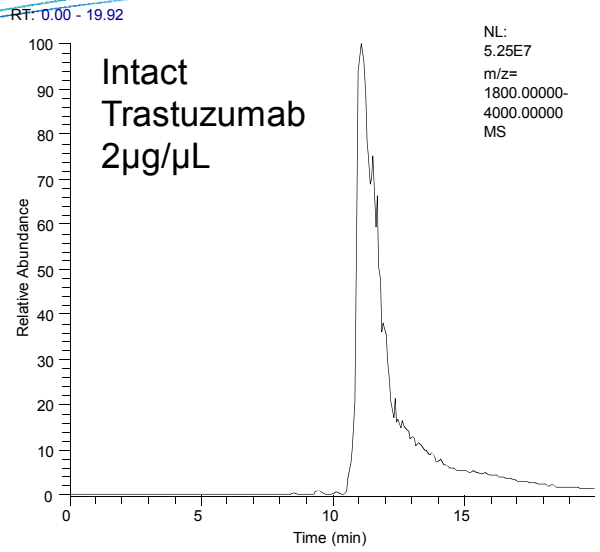
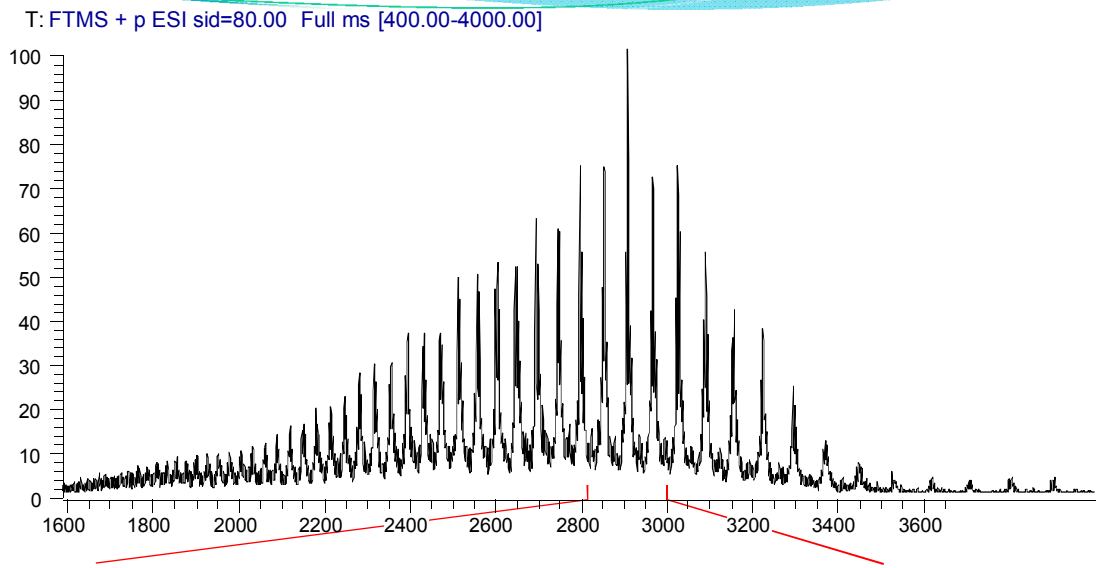
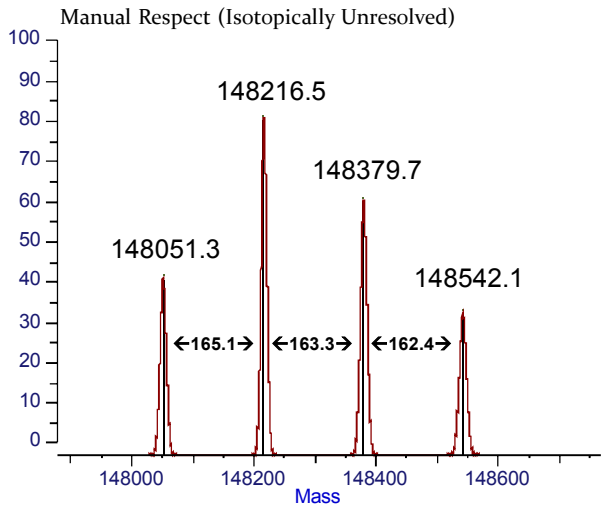
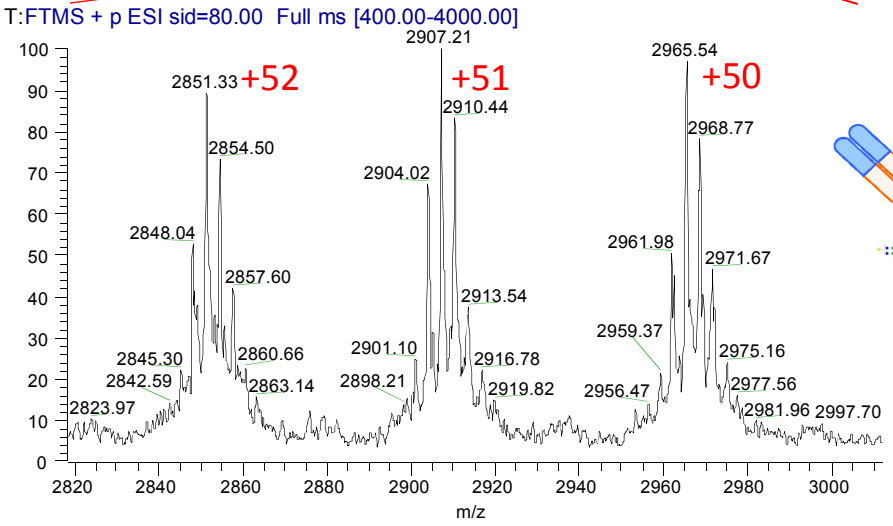
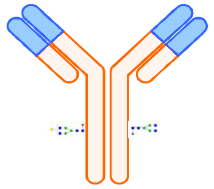
HALO Protein C4 150x0.3mm 3.4µ 400A



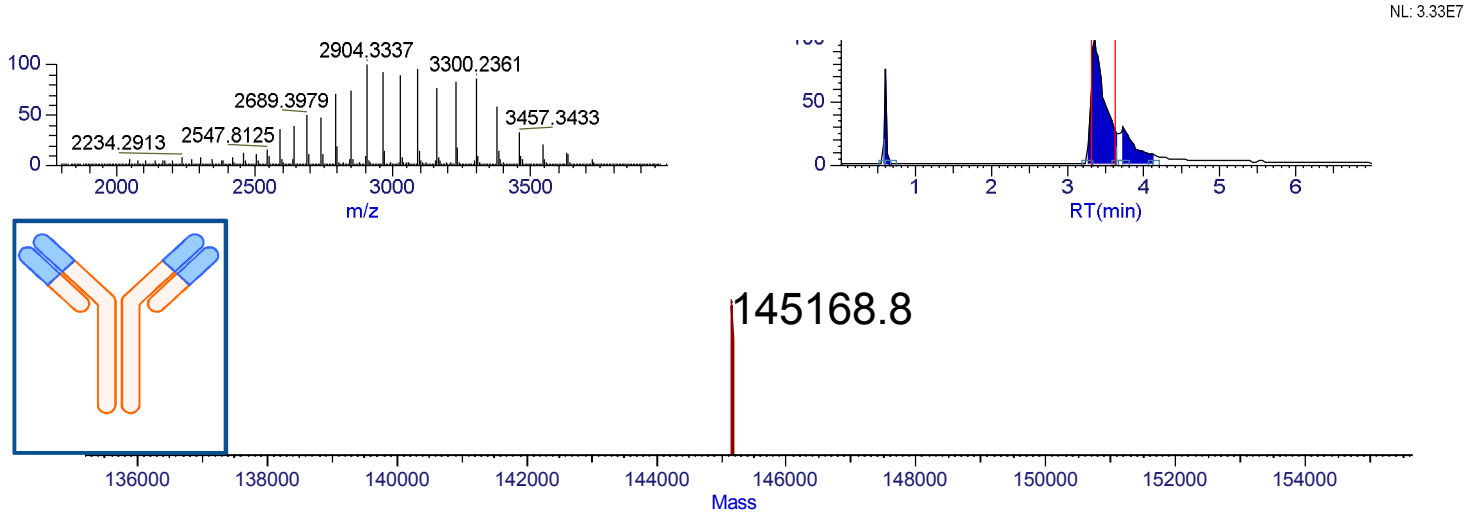




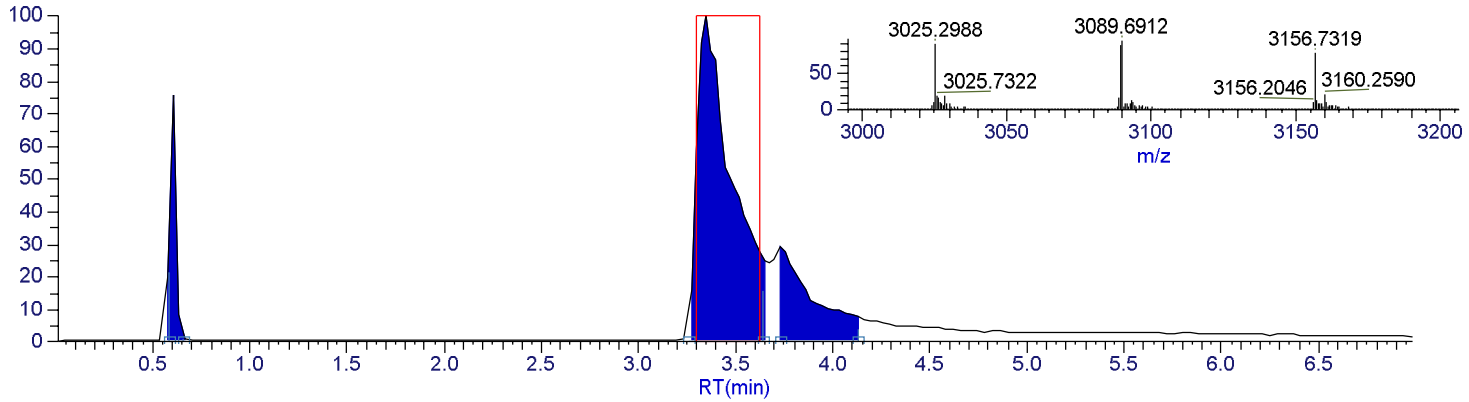




DEGLYCOSYLATED TRASTUZUMAB (HERCEPTIN)

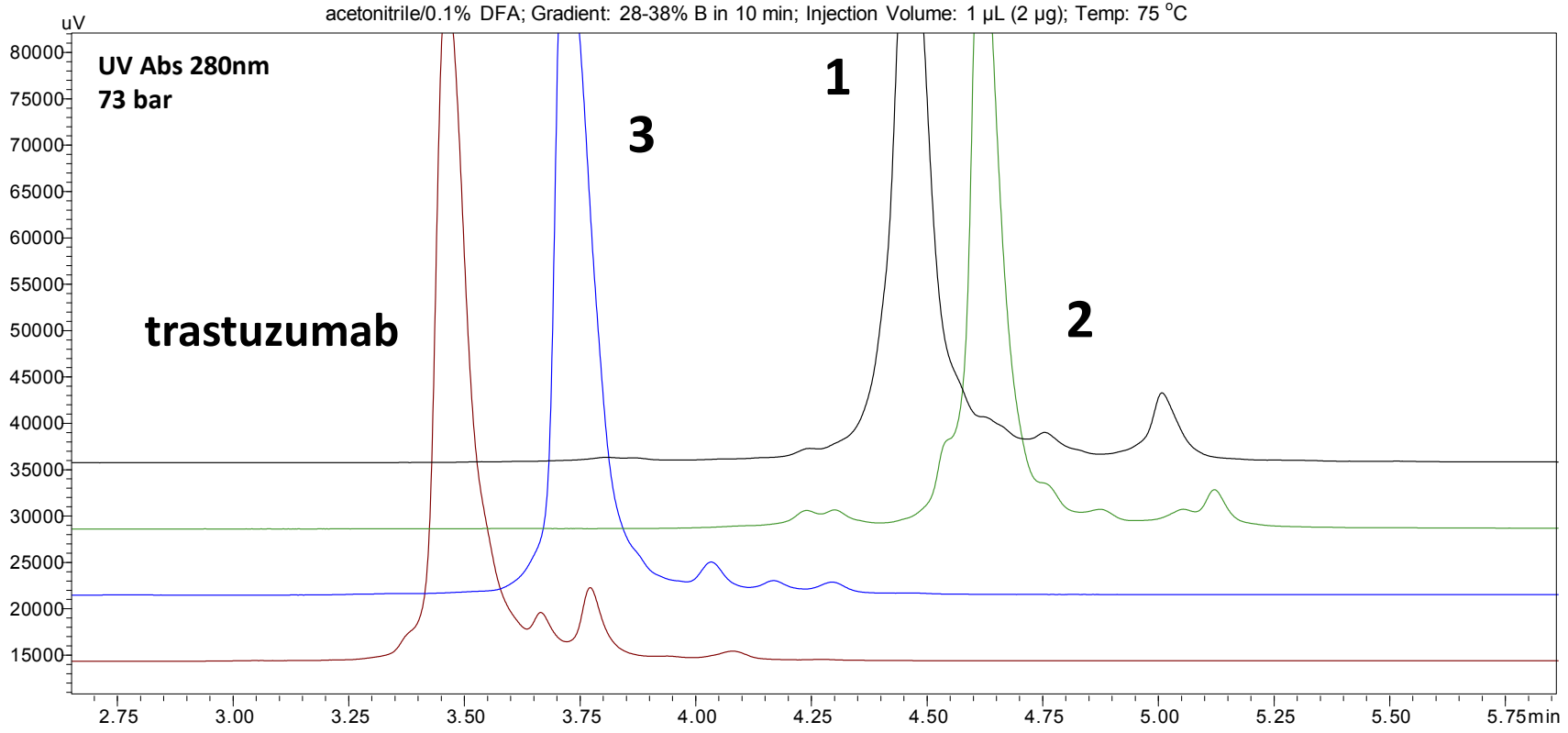


HALO_3-4_trastuz-deglycosyl2



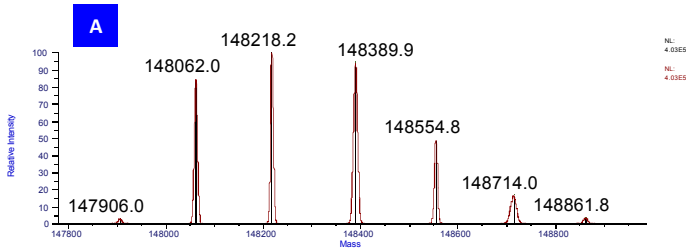
MAb Gradient Separation:
HALO 3.4µm, C-4, 400Å

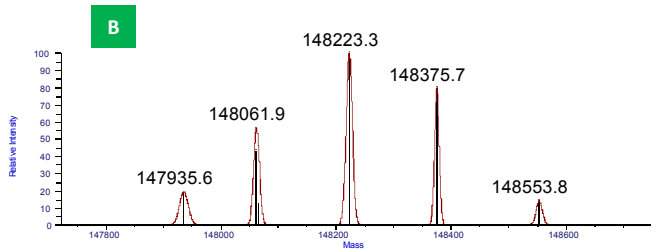
Column: 2.1 x 100 mm; Flow rate: 0.35 mL/min; Mobile Phase A: water/0.1% DFA; Mobile Phase B: acetonitrile/0.1% DFA; Gradient: 28-38% B in 10 min; Injection Volume: 1 µL (2 µg); Temp: 75 °C

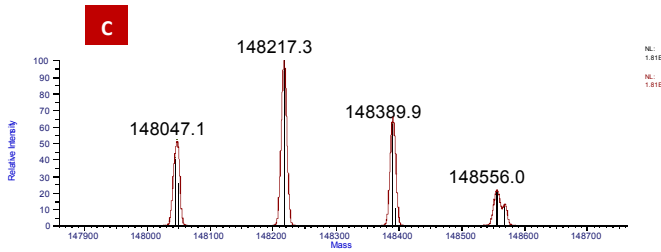


Trastuzumab compared to 3 other mAbs in development

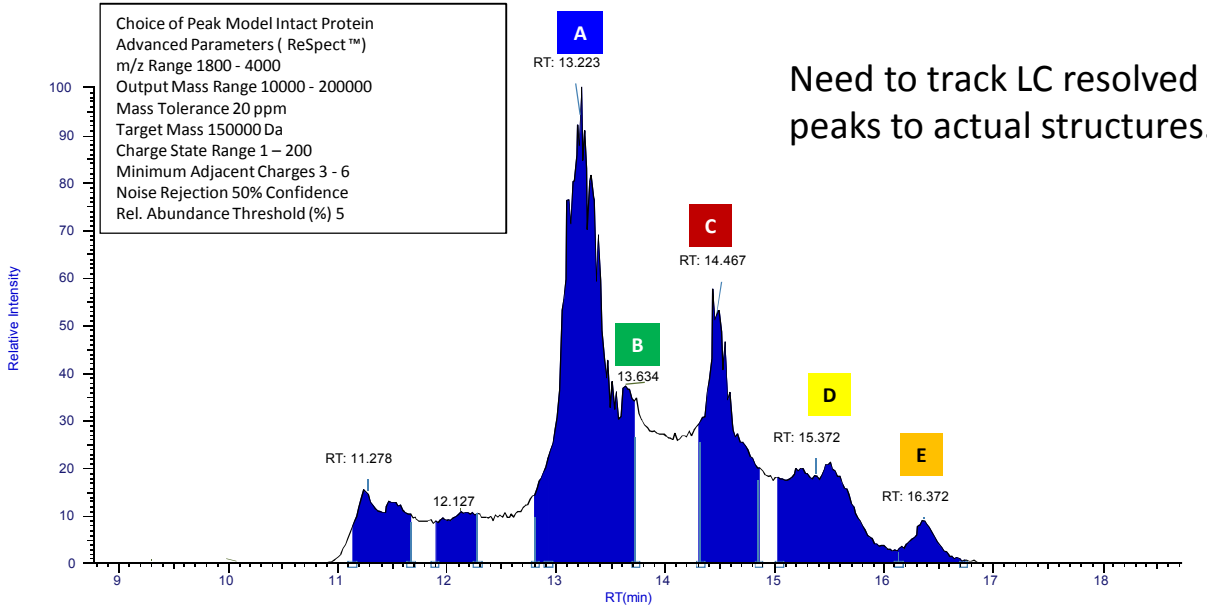
Deconvoluted Intact Trastuzumab



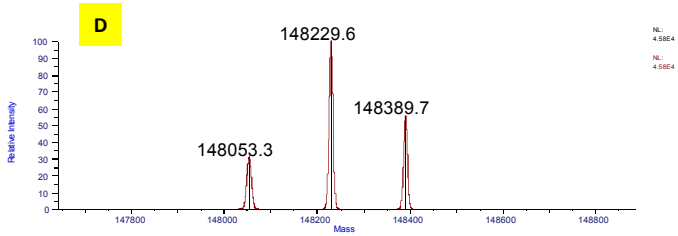


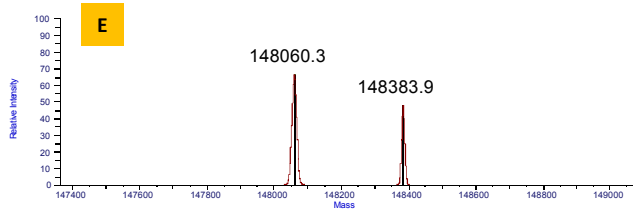


Choice of Peak Model Intact Protein
Advanced Parameters (ReSpect™)
m/z Range 1800 - 4000
Output Mass Range 10000 - 200000
Mass Tolerance 20 ppm
Target Mass 150000 Da
Charge State Range 1 – 200
Minimum Adjacent Charges 3 - 6
Noise Rejection 50% Confidence
Rel. Abundance Threshold (%) 5



Need to track LC resolved peaks to actual structures…





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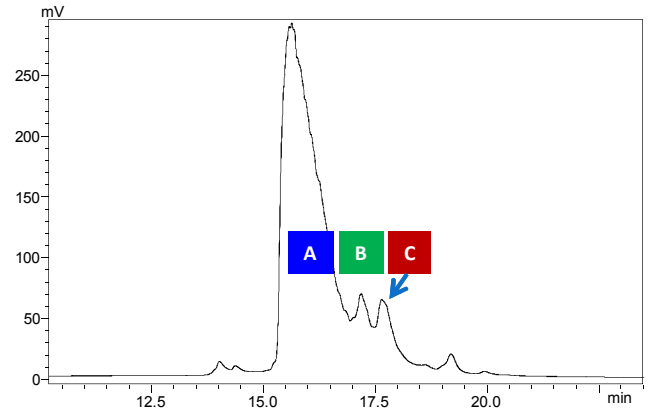
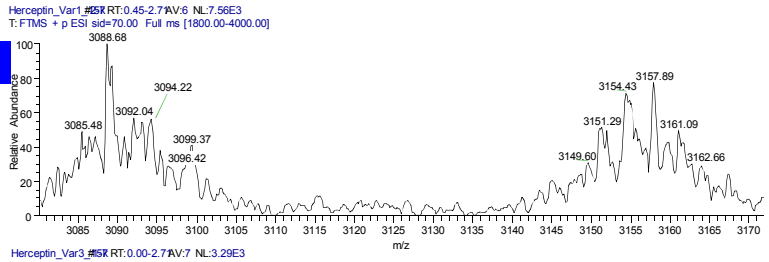
Deconvoluted Intact Trastuzumab: Variant Fractions

Fraction Collection

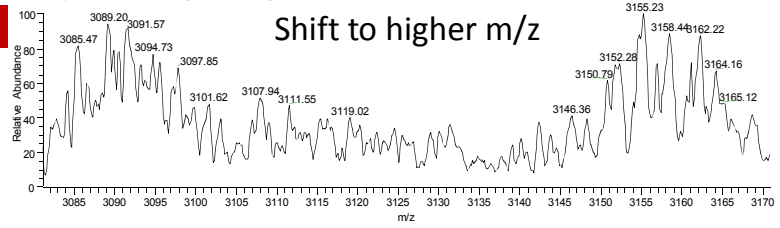
HALO Protein C4 4.6 x 150mm 3.4µ 400A



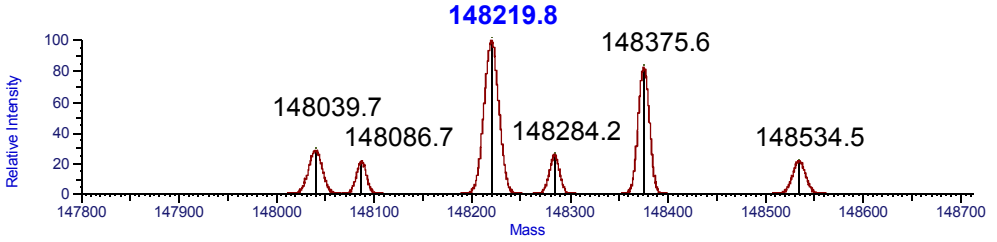
A



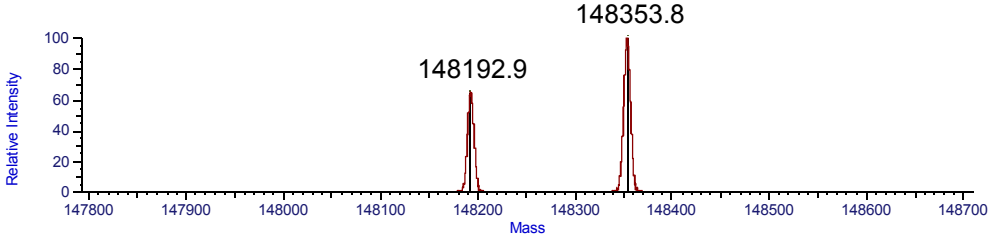
C



A

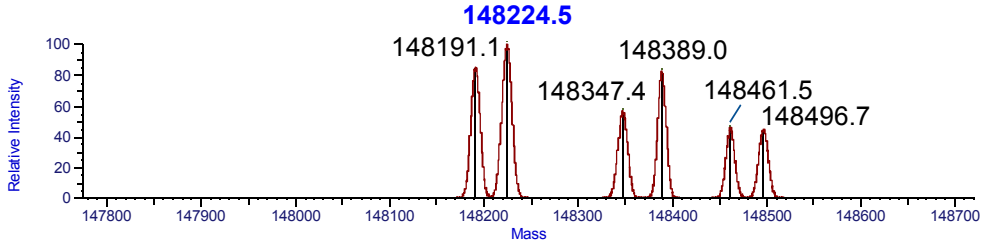


B



+4 (2 x thiol)

C



Observations for mAbs and Subunits

- Clear that TFA is not REQUIRED for higher resolution separations
- DFA Appears to be a reasonable alternative
- ESI is moderately reduced with DFA, in line with other proteins
- With Higher Resolution LC and MS:
  - What Are We Separating??? Is More is Better???
- How much of the deconvolution results are “actual”
  - Need to track LC resolved peaks to actual structures
- LC/HRAM MS is permitting evaluation of structures that are resolved, but many biotherapeutics exhibit structures not completely resolved.
- Protein heterogeneity (eg., glycosylation) observed by LC/MS of proteins or subunits, is not fully appreciated without component analysis.

Conclusions

- Bottlenecks in protein LC/MS have been improved by newer application-directed SPP material developments.
- A wider range of useful operating conditions could take advantage of improvements in column and MS capabilities. Some suggestions are made.
- Effort will be required to understand retention and resolution of larger proteins and fragments. This is already the case for common current MP additives. Understanding variant resolution will require top-down, middle-down and bottom-up approaches.
- The range of HALO Fused-Core materials continues to expand, with an expanding range of surface chemistries.

Thank You

Barry Boyes, Tim Langlois, Joe DeStefano, Jack Kirkland, Stephanie Schuster, Will Miles, Robert Moran, Thomas J. Waeghe, and Mary Ellen McNally.

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D.J. Johnson, Shujuan Tao, Yining Huang, CCRC, University of GA.
Prof. Bob Hodges and Colin Mant, U. Colorado
Some Biopharma sample contributors

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