Unraveling the Structure of Proteins, and Particularly of Monoclonal Antibodies, Utilizing HALO® BioClass Fused-Core™ Particles

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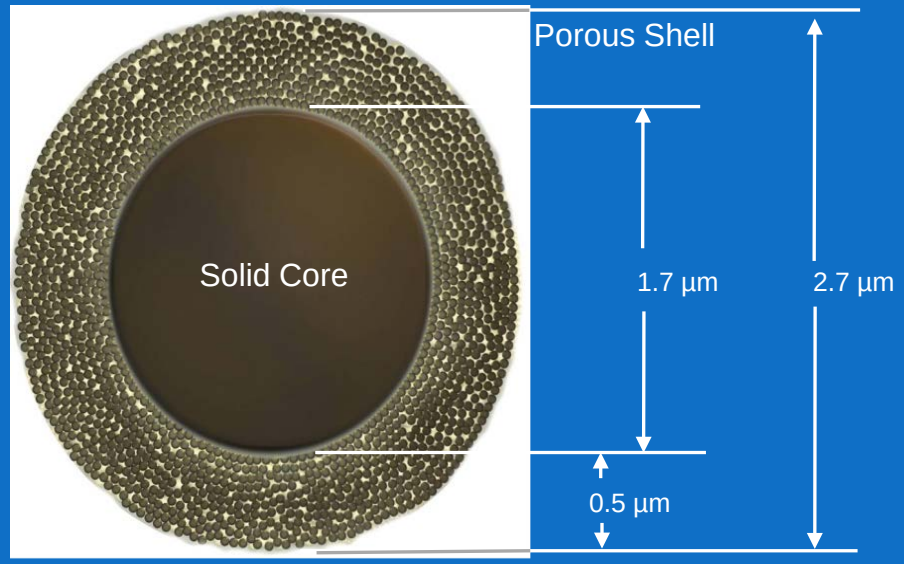
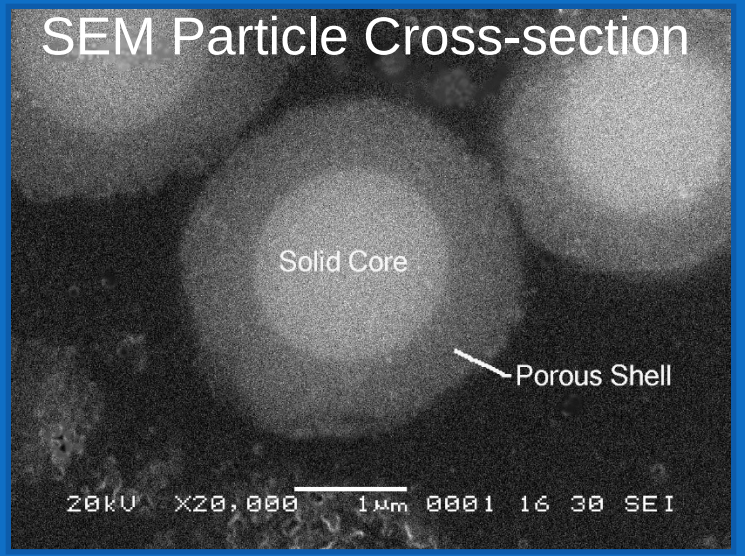
Agenda

- Detailed Analysis of Proteins can be HARD
  - High MW polyelectrolytes (peak shape, ESI issues)
  - Little to a lot of heterogeneity (PTMs, chem mods)
  - Subject to change for various environmental reasons
  - Need a combination of methods/approaches
- Recent Enablers in Protein LC/MS
  - Detection Developments: MS Improves!
  - Stationary Phase Developments: LC Improves!
  - Mobile Phase Developments: Not so much…
- AMT Focus on Biomolecule Applications
  - Further Improvements of Columns and Materials
  - Defining Useful Mobile Phases and Operating Conditions
  - Workflow View of Analytics

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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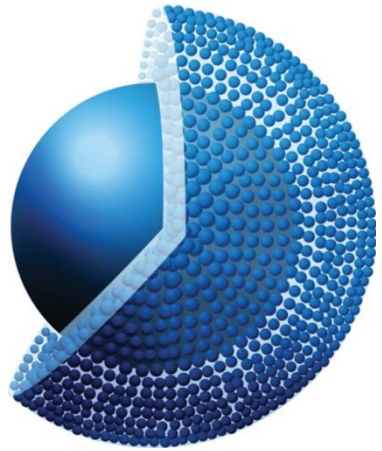
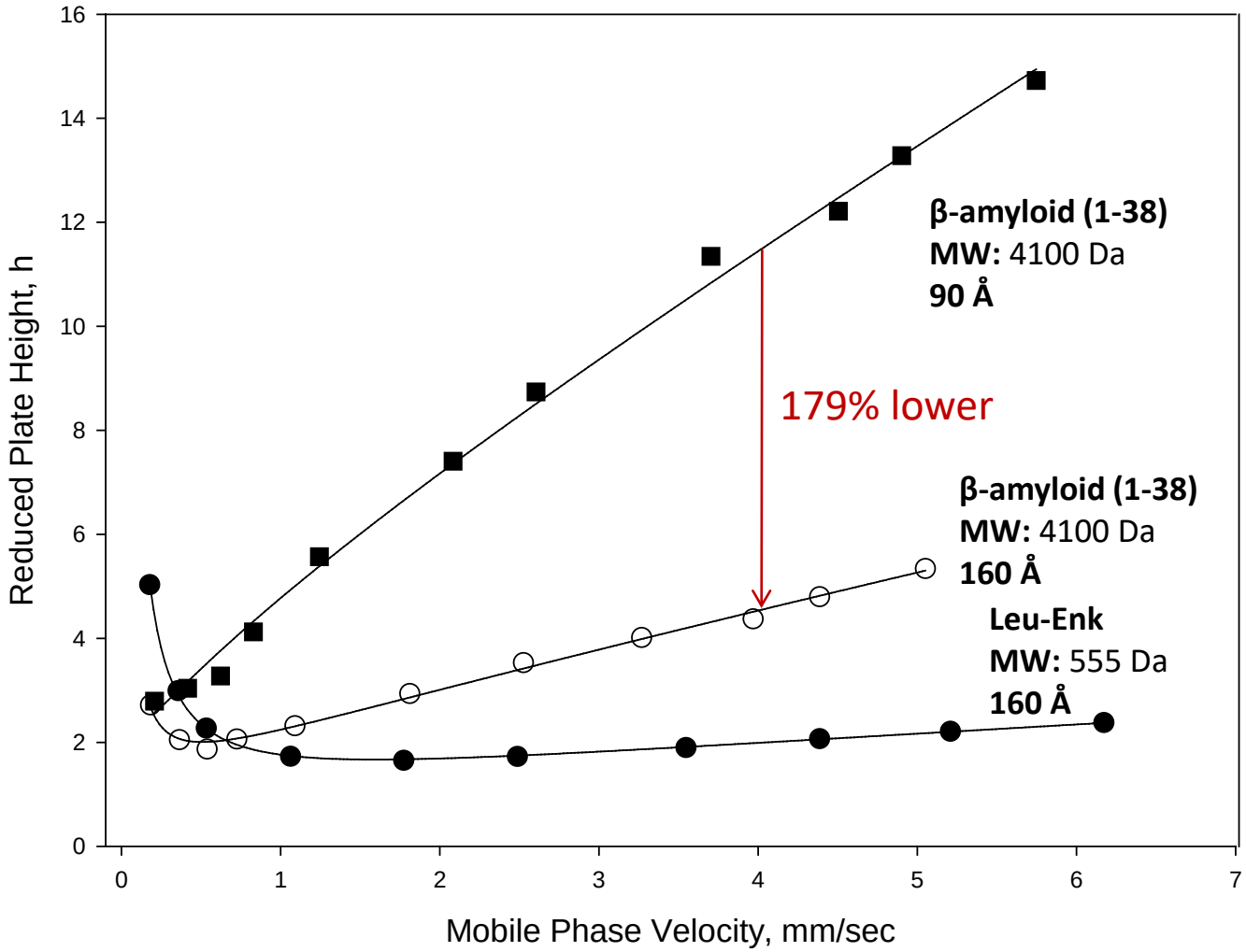
Wide Pore SPP Can Fit the Needs for Protein Science

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 limitations
- **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
- **Surface Chemistry appropriate to Samples**

SPP Pore Size on Efficiency: 90Å vs 160Å



Columns: 4.6 x 100 mm HALO C18, 2.7 µm, 90 Å
4.6 x 100 mm HALO Peptide ES-C18, 2.7 µm, 160 Å
Mobile Phase: Leu-Enk: 21% ACN/79% Water/0.1% TFA
β-APP (1-38) 160 Å : 29% ACN/71% Water/0.1% TFA
β-APP (1-38) 90 Å : 27% ACN/73% Water/0.1% TFA

Temperature: 60 °C
Detection: 215 nm

Schuster, Boyes, Wagner, Kirkland (2012)
J. Chromatogr. 1228, 232.

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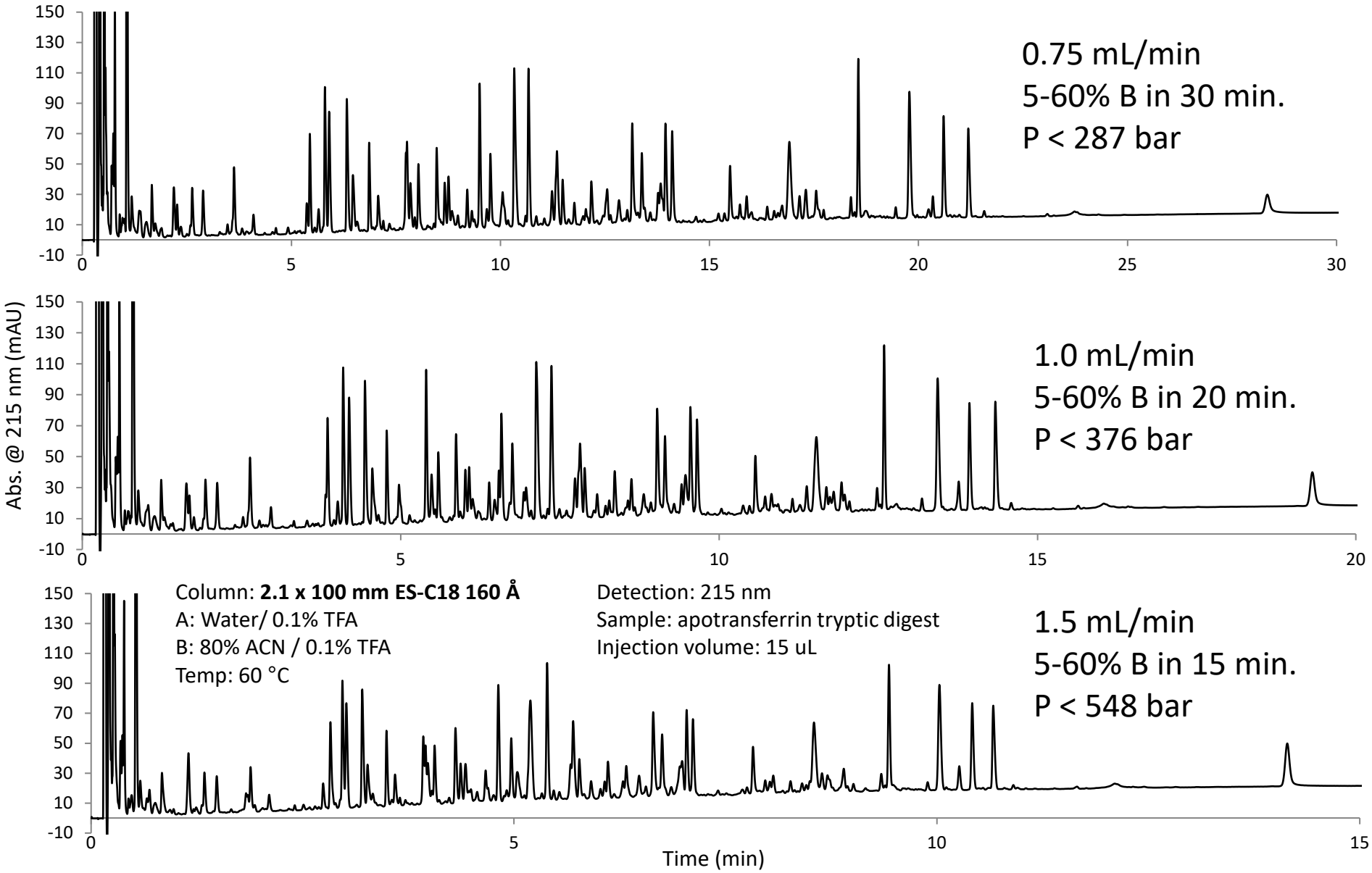
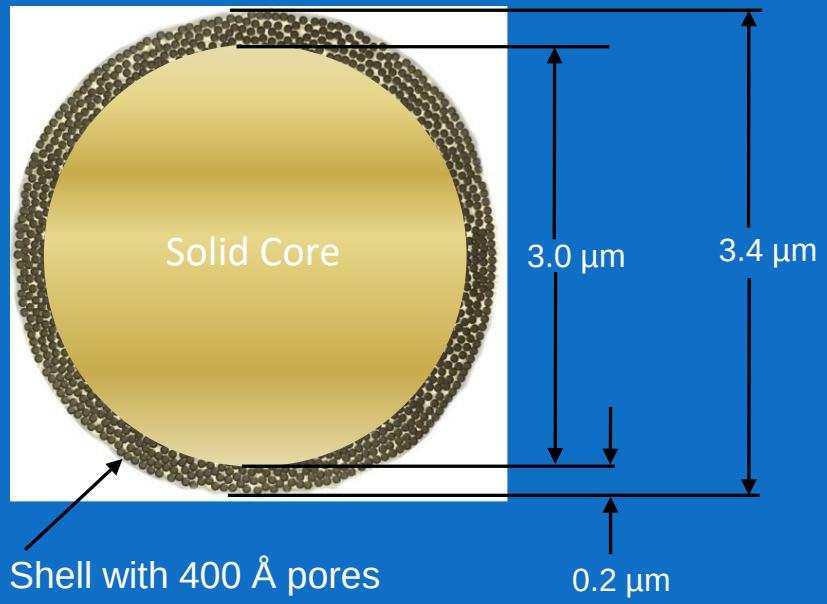
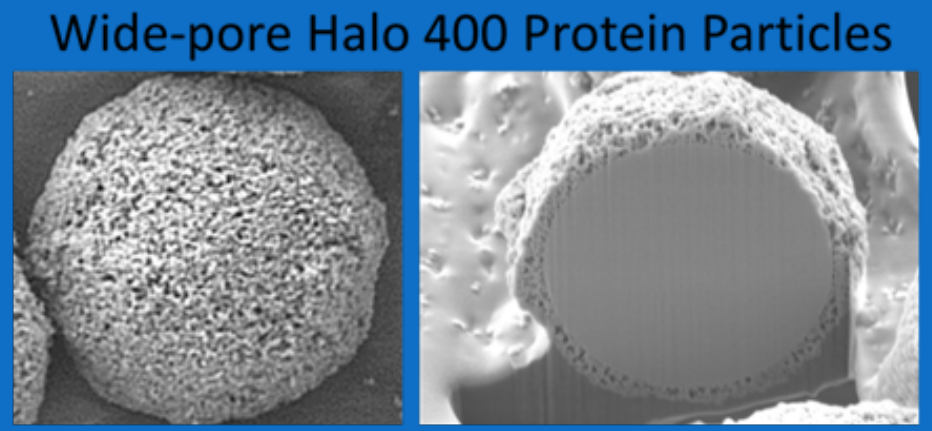
Fast High Resolution Separation of apo-Transferrin Tryptic Digest



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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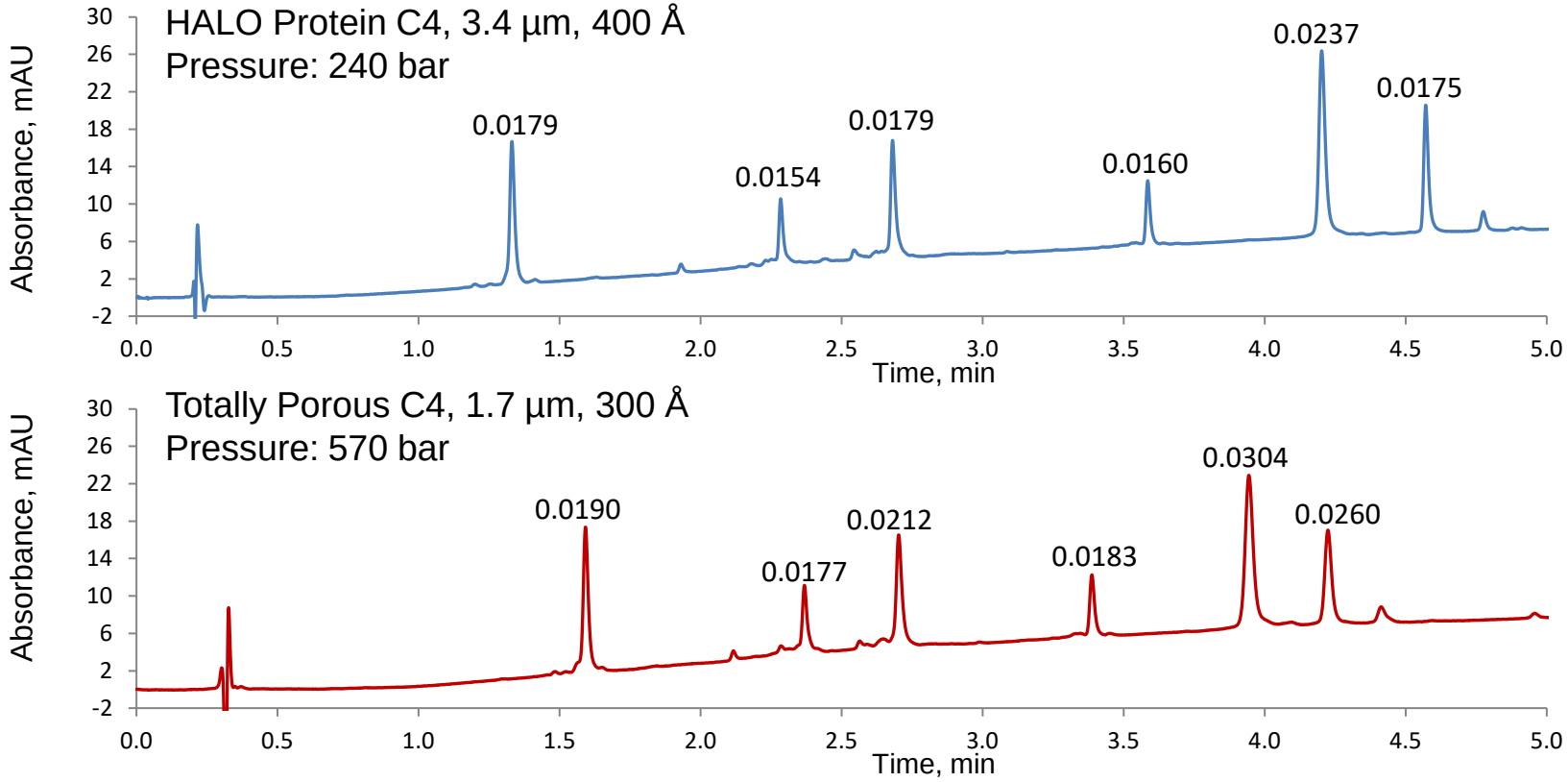
Protein Separations: SPP compared to Totally Porous

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

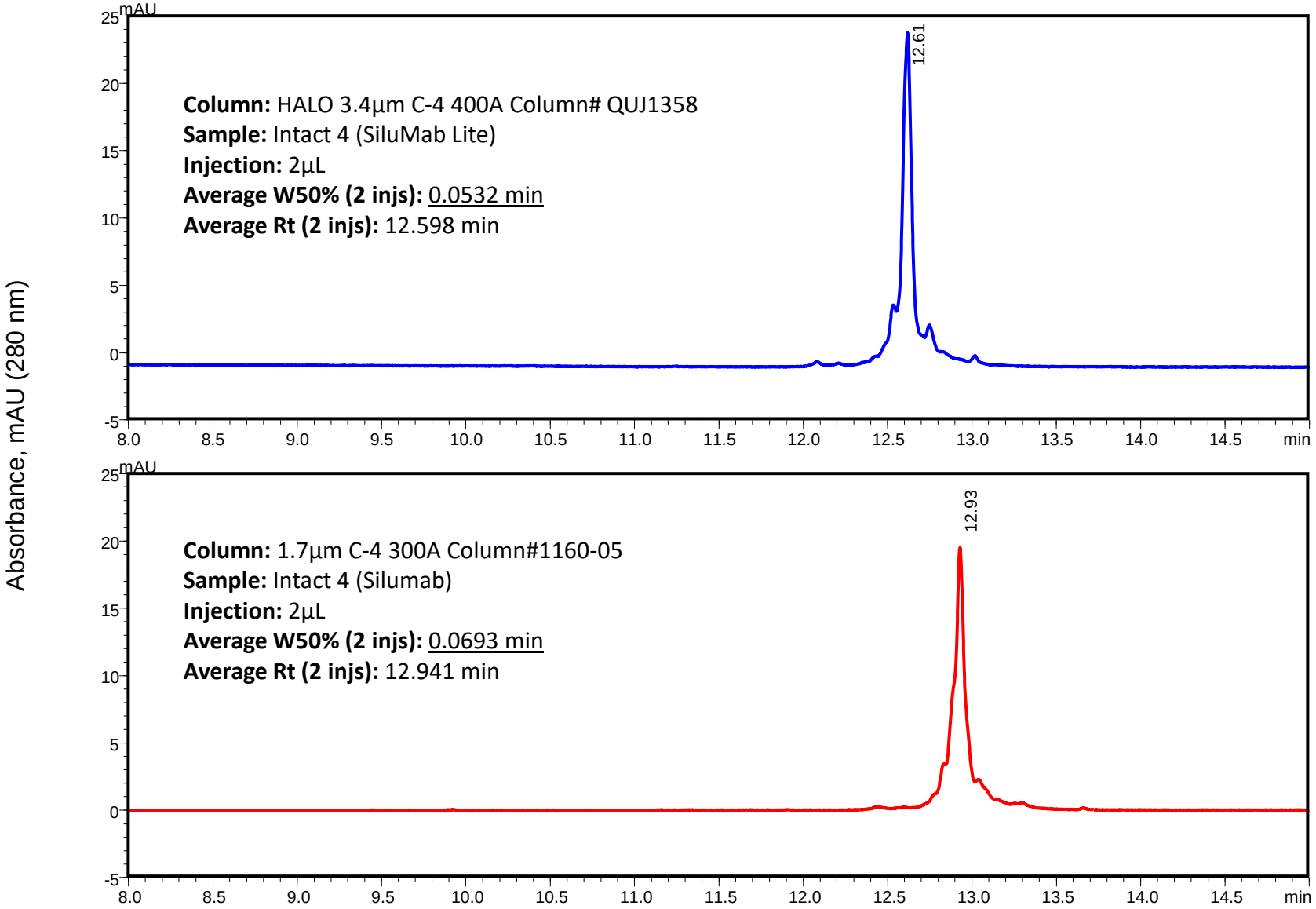
Flow rate: 1.1 mL/min
Mobile Phase A: water/0.1% TFA
Mobile Phase B: acetonitrile/0.1% TFA
Gradient: 23-52% B in 5 min

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



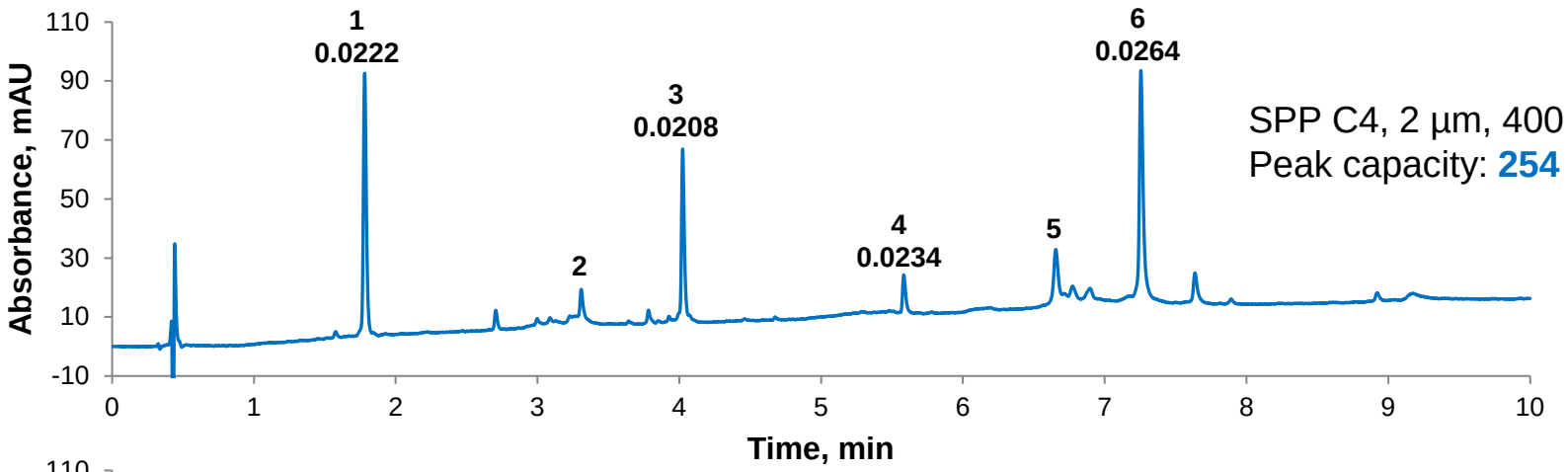
Protein Separations: Smaller SPP compared to sub-2 µm

Conditions***

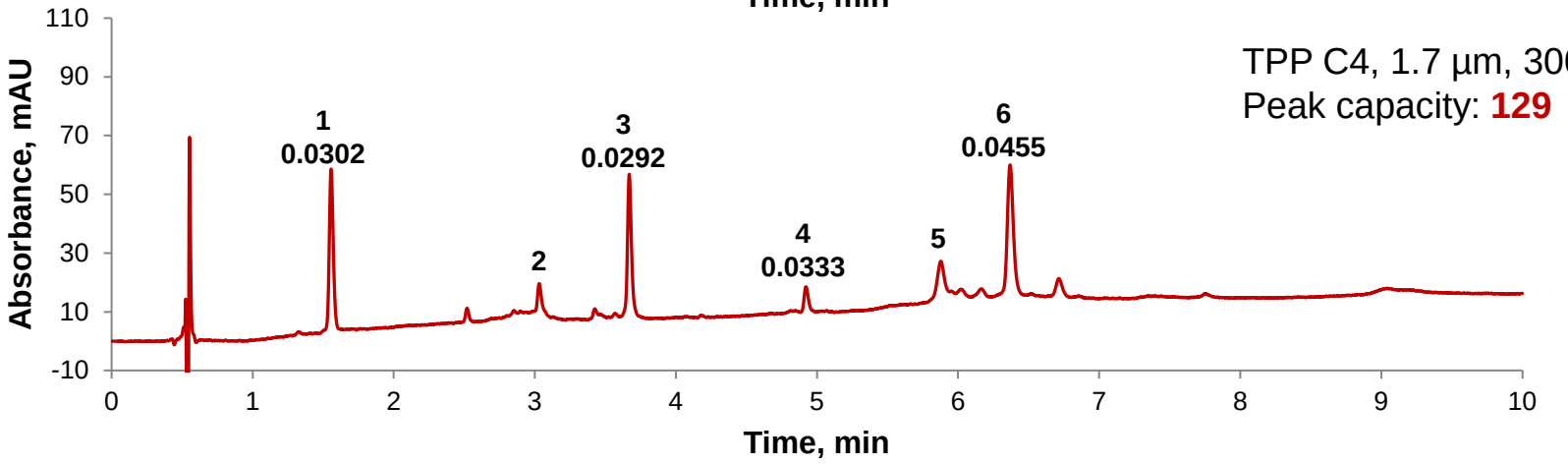
Peak capacity: $P_{C,4\sigma} = 1 + \left[\frac{2.35}{4} \left(\frac{t_G}{w_{h,avg}} \right) \right]$

Peak Identities:

1. Ribonuclease A 13.7 kDa
2. Cytochrome *c* 12.4 kDa
3. Lysozyme 14.3 kDa
4. α-Lactalbumin 14.2 kDa
5. Catalase 250 kDa total; tetramer of ~60 kDa each
6. Enolase 46.7 kDa monomer



SPP C4, 2 µm, 400 Å
Peak capacity: **254**



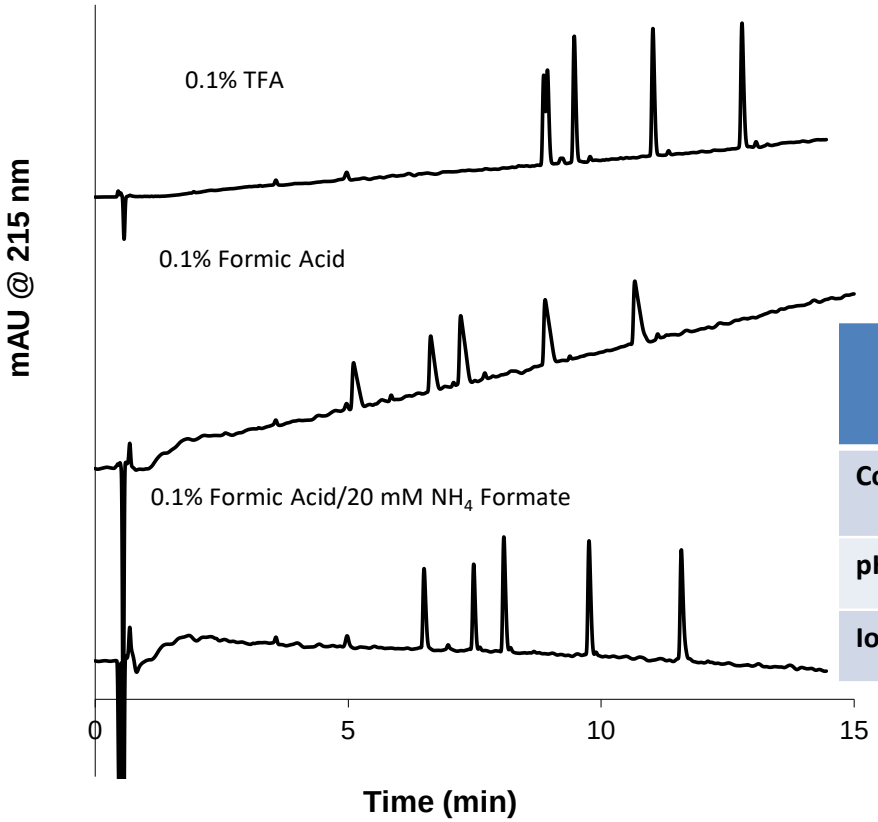
TPP C4, 1.7 µm, 300 Å
Peak capacity: **129**

Mobile Phases for Protein and Peptide LC/MS

Successful LC/MS depends on chemistry 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
- Variants of organic modifier have been reported, but comparatively little drive from current conditions
- Use of elevated temperature (>60°C) is much more common for proteins than in the past; thank goodness.

Improving Retention and Peak Shape Using Ammonium Formate



Column: Halo Peptide ES-C18, 4.6 x 100
A: Water/acid modifier; B: ACN
Gradient: 1.5% to 26% B in 15 min.; Injection:

Synthetic Peptide Retention Standards:

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

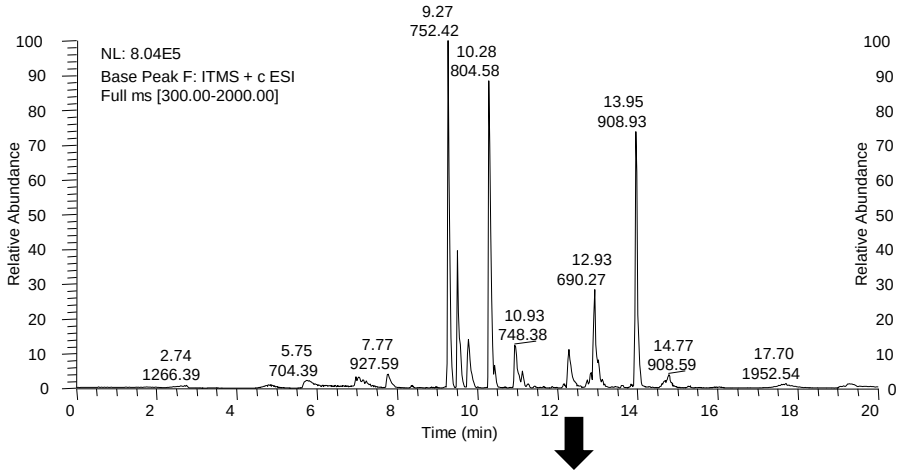
	TFA 0.1%	Formic Acid 0.1%	Ammonium Formate	AF/FA
Concentration	**0.013 M**	**0.026 M**	**0.020 M**	**20 mM AF 26 mM FA**
pH	**1.8**	**2.7**	**c. 7**	**3.3**
Ionic strength	**26 mM**	**4.4 mM**	**40 mM**	**c. 44 mM**

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.

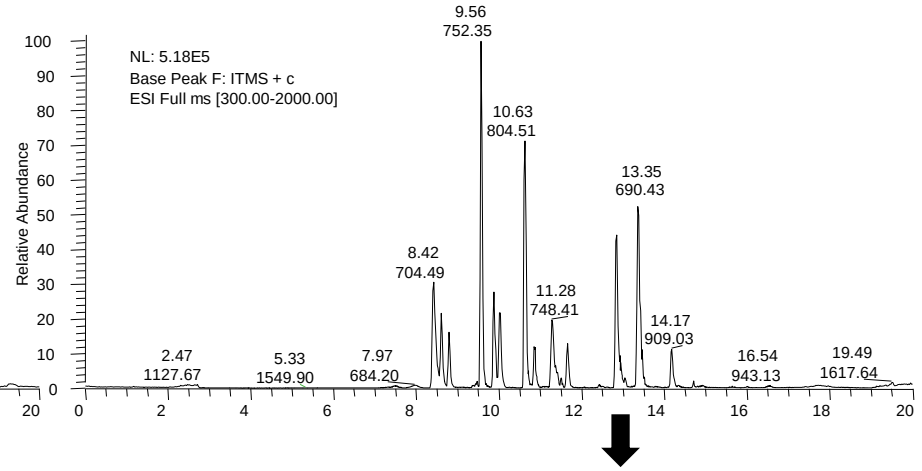
Ammonium formate as an additive for LC/MS separations

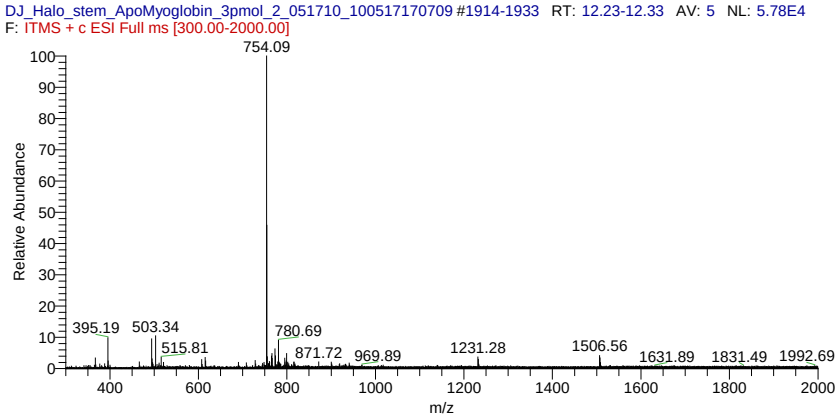
Column: 0.2 x 50 mm Halo Peptide ES-C18; Flow rate: 9 µL/min; Gradient: 2 - 45% B in 15 min; Mobile phases as shown; Sample: 2 µL (3 pmol) apomyoglobin digest.

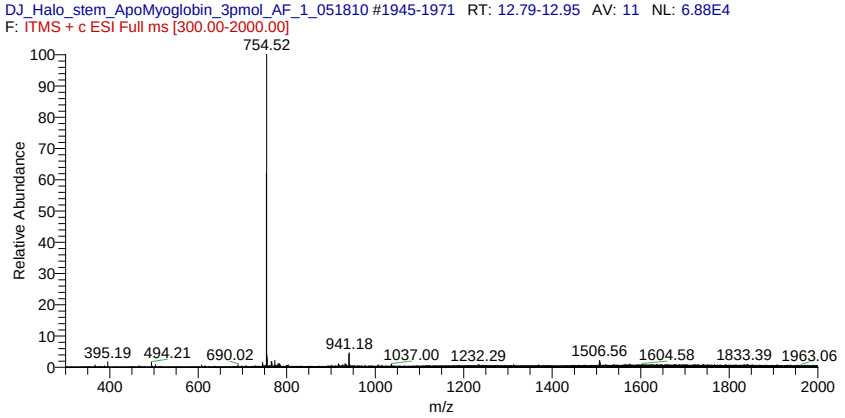
A: 0.1 % Formic Acid



B: 0.1 % Formic Acid/10 mM Ammonium Formate







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Improved Proteomic Analysis

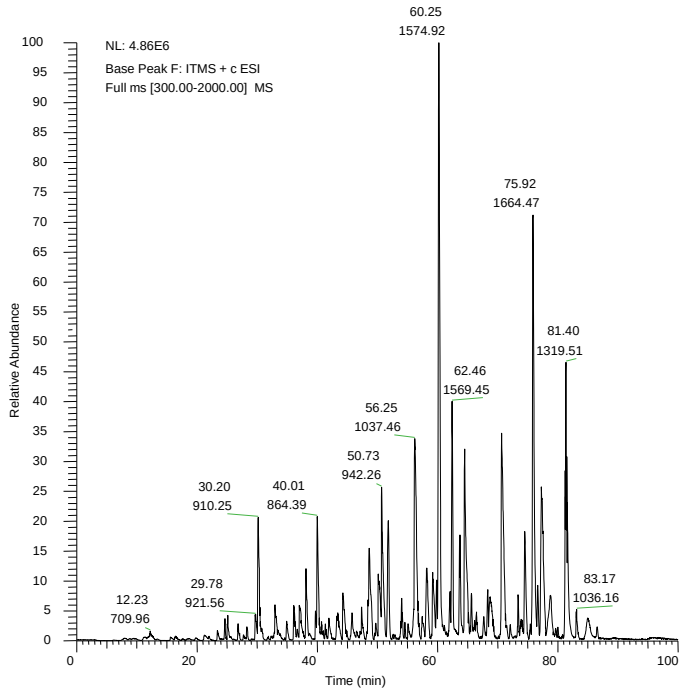


TABLE 7

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.

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.

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

Volatility

- **Necessary but not sufficient for additives. Must NOT plug our ESI interface and capillary ion entrance path!**
- **Henry's Law Coefficients (Hcc): A higher value of the coefficient indicates ease of transfer of the protonated acid from the idealized aqueous phase of the mobile phase mixture. Not readily available, and not certain to predict partitioning from organic aqueous mixtures.**

Low pKa

- **Low pH and dissociation of acid; sufficient ionic strength appears beneficial for separation needs, while effect on ESI suppression must be managed**

Favor Peptide and Protein Solubility

- **Acidic (usually). Fluorinated? Polar? Chaotropic?**

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

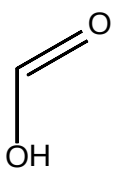
Name of Acid	MW (g/mol)	B.P. (°C)	pKa	UV Cut-off (nm)	Biological Hazard
Formic	46.02	100.8	3.8	220 +	Very low
Acetic	60.05	118-119	4.8	230 +	Very low
Fluoroacetic	78.04	165	2.6	ND	Very High
Difluoroacetic	96.03	134	1.3-1.4	205	Low
Trifluoroacetic	114.02	72.4	≤ 0.5	205	Low
Chloroacetic	94.50	189	2.8	ND	Moderate
Dichloroacetic	128.95	192-193	1.3	ND	Low
Trichloroacetic	163.40	197.6	0.7	ND	Low
Chlorofluoroacetic	112.49	162	(1.3-1.4)	ND	Unknown
Chlorodifluoroacetic	130.48	122	≤ 0.5	ND	Low
Propanoic acid	74.08	141	4.9	ND	Very low
(3,3,3)-trifluoropropanoic (3FPA)	128.05	146	3.1	ND	Unknown
Pentafluoropropanoic	164.03	96-98	≤ 0.5	220 +	Low
n-Butanoic	88.11	164	4.8	ND	Very low
i-Butanoic	88.11	155	4.9	ND	ND
2H-(3,3,3,2,2,2)- Hexafluoro-i-butanoic	196.05	125	(2.5-3.0)	210+	Unknown
n-Heptafluorobutanoic	214.04	120	≤ 0.5	230+	Low

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

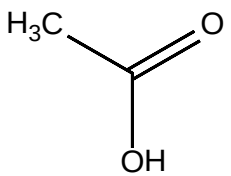
Initial selection and testing indicated some candidates with promise:

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

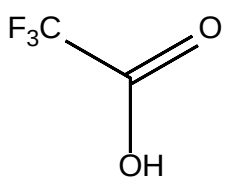
FA	HOAc	TFA	DFA	3FPA
3.8	4.8	0.3-1.6	1.3-1.4	3.1



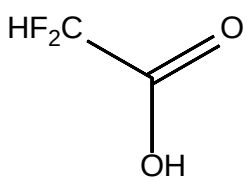
formic acid



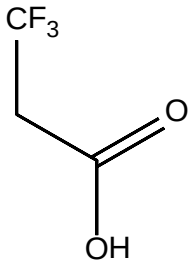
acetic acid



2,2,2-trifluoroacetic acid



2,2-difluoroacetic acid



3,3,3-trifluoropropanoic acid

Mobile Phases for Improved Protein LC/MS

2.1 x 100 mm
Protein 400 C4

15-55% AcN 30 min

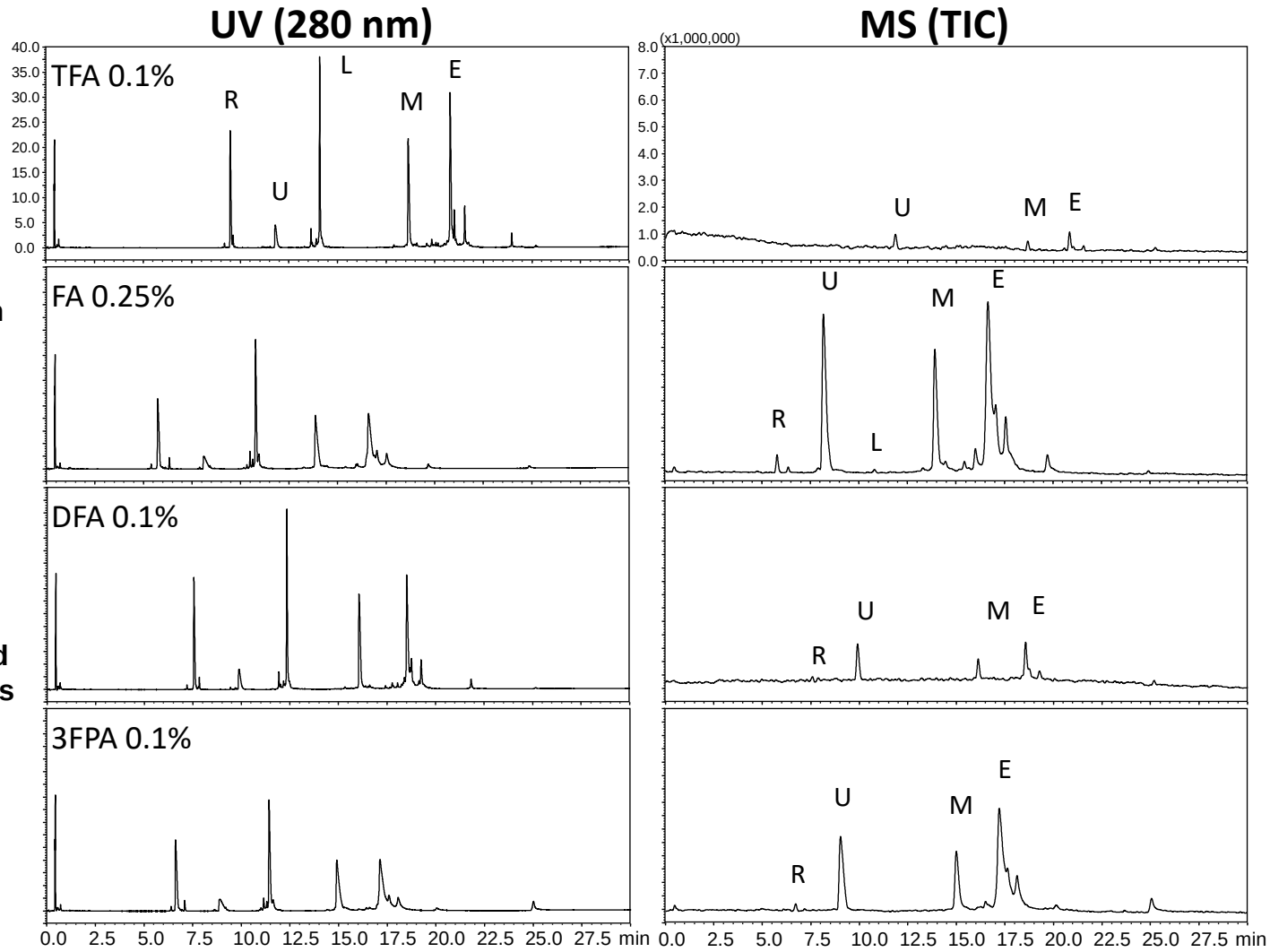
0.35 mL/min; 50°C

25 pmol each protein

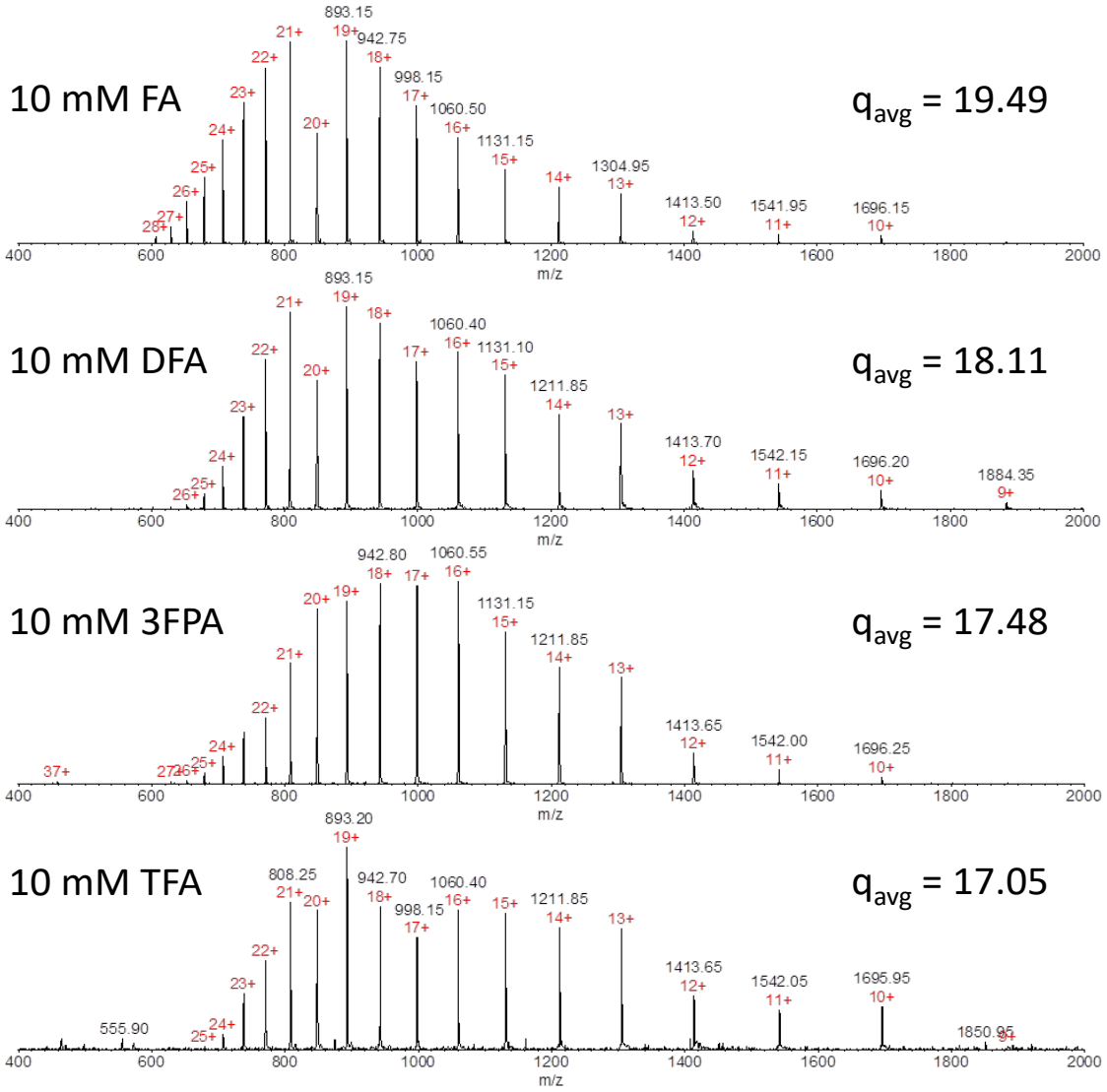
R – Ribonuclease
U – rec. Ubiqutin
L – Lysozyme
M – apo-Myoglobin
E – Enolase

Nexera LC system

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



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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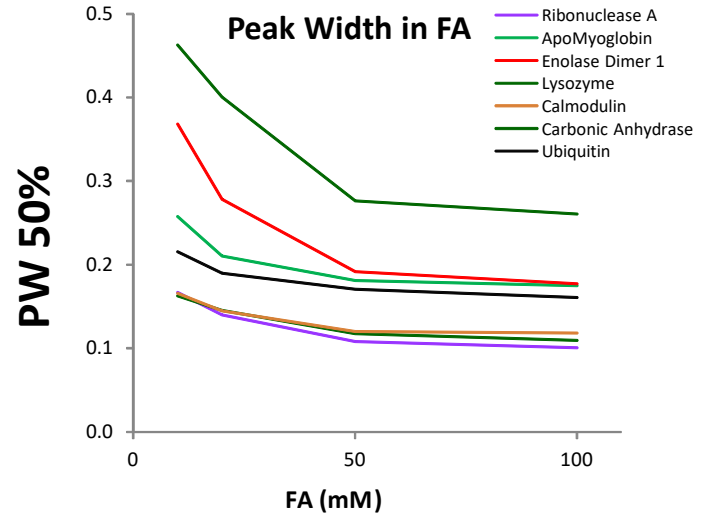
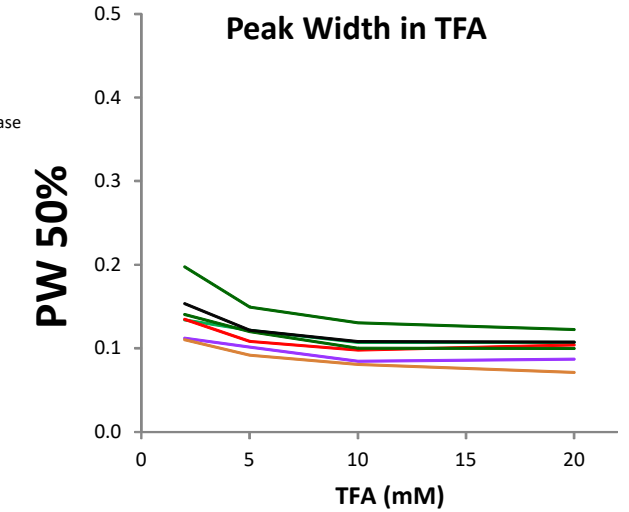
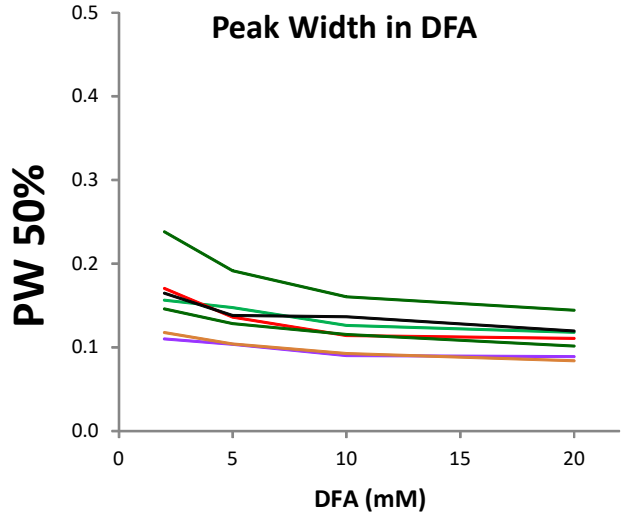
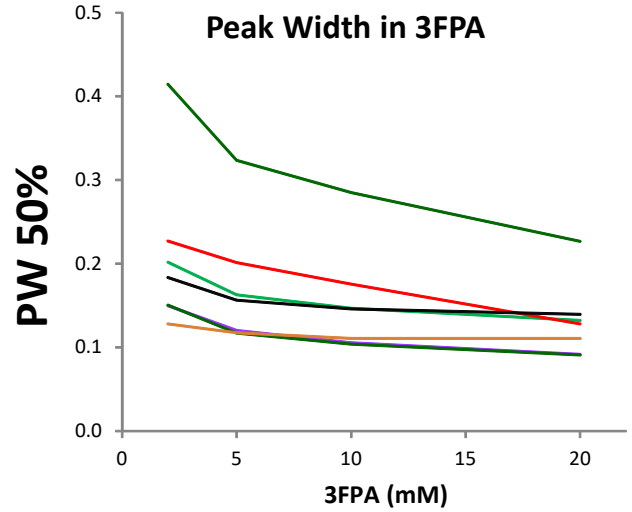
Mobile Phases for Improved Protein LC/MS









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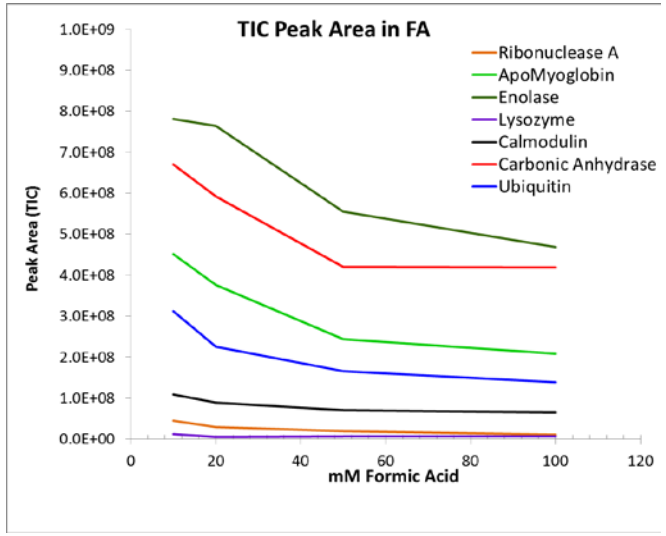
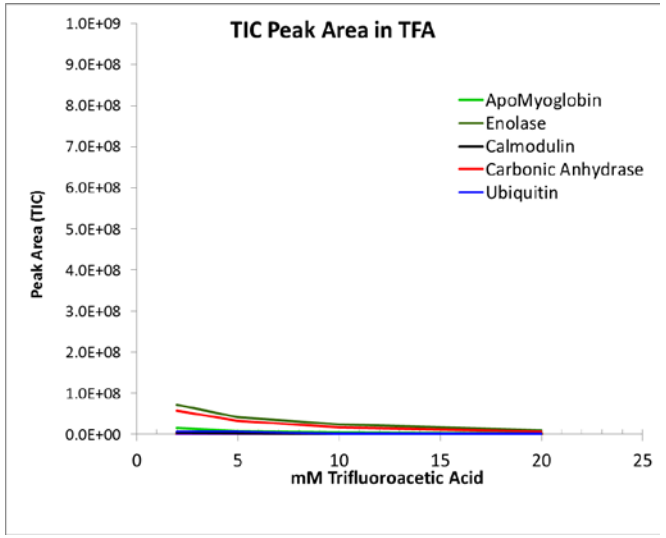
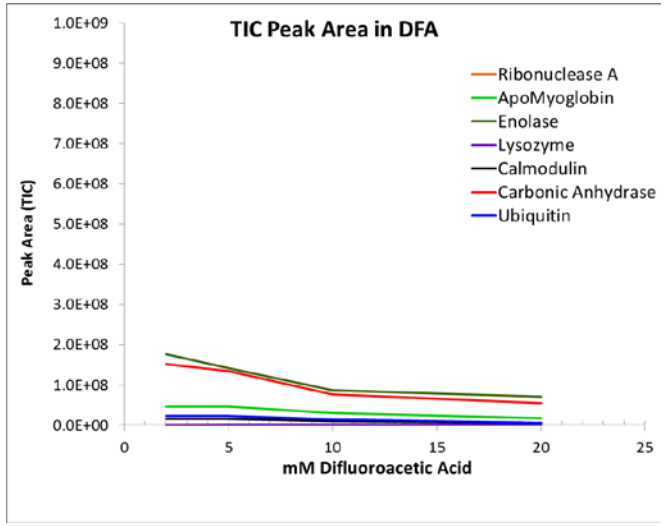
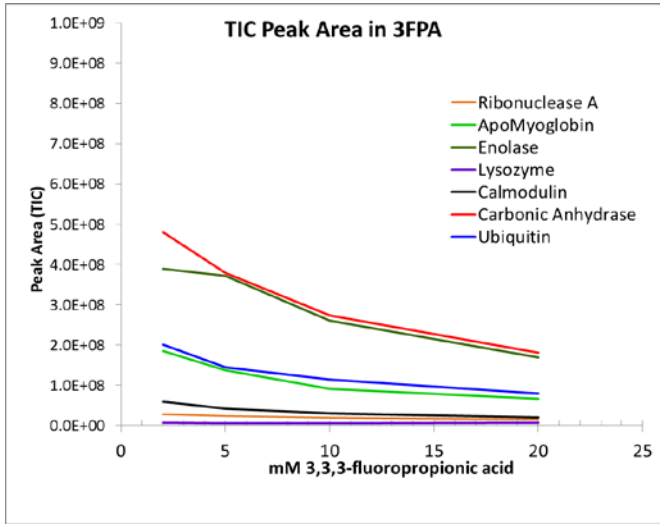
Mobile Phases for Improved Protein LC/MS









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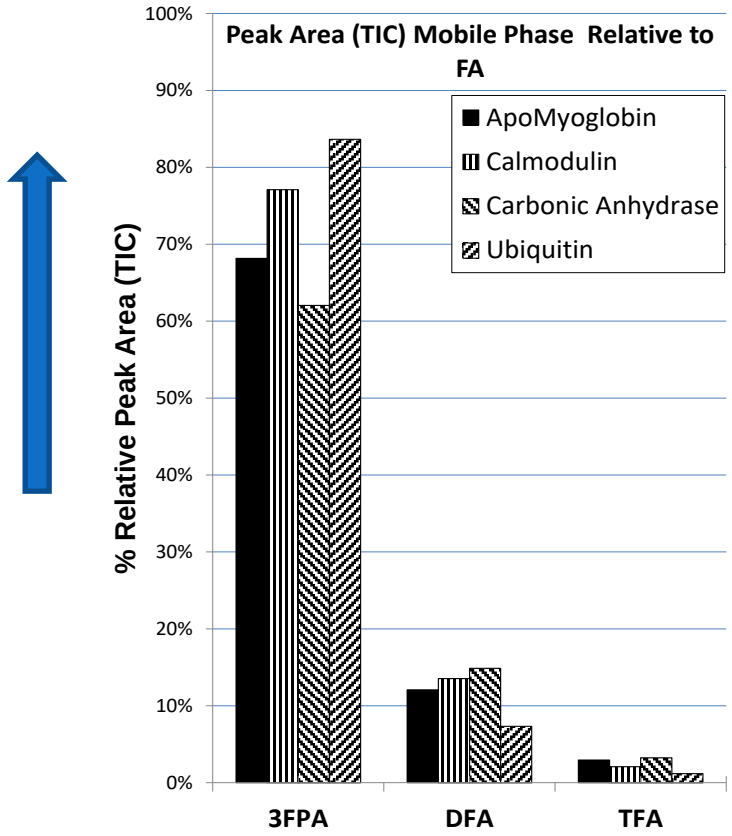
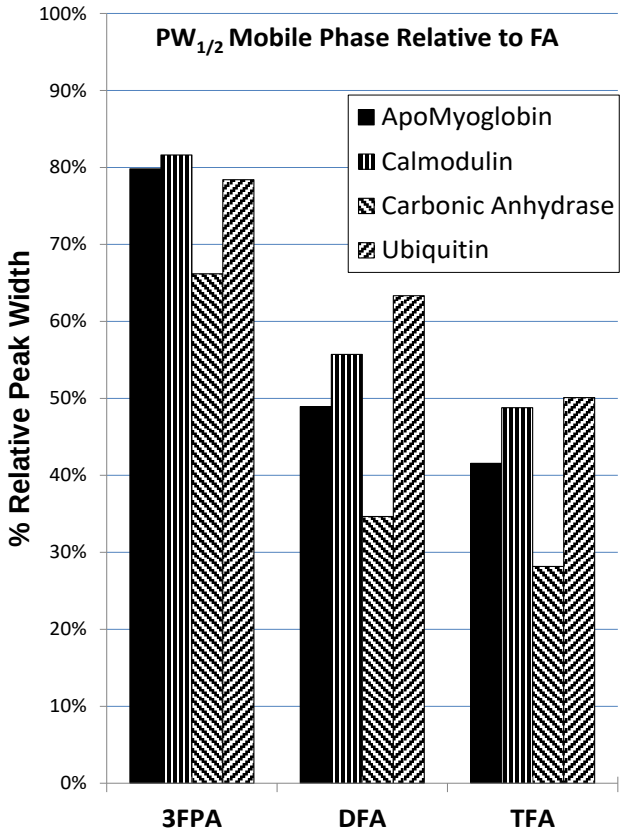
Mobile Phases for Improved Protein LC/MS

- Titration of each acid established suppression of ESI signal as a function of concentration: plateau for FA – 50 mM, others at 10-20 mM
- Graph compares 10 mM of each ion pair reagent to 10 mM FA





Better

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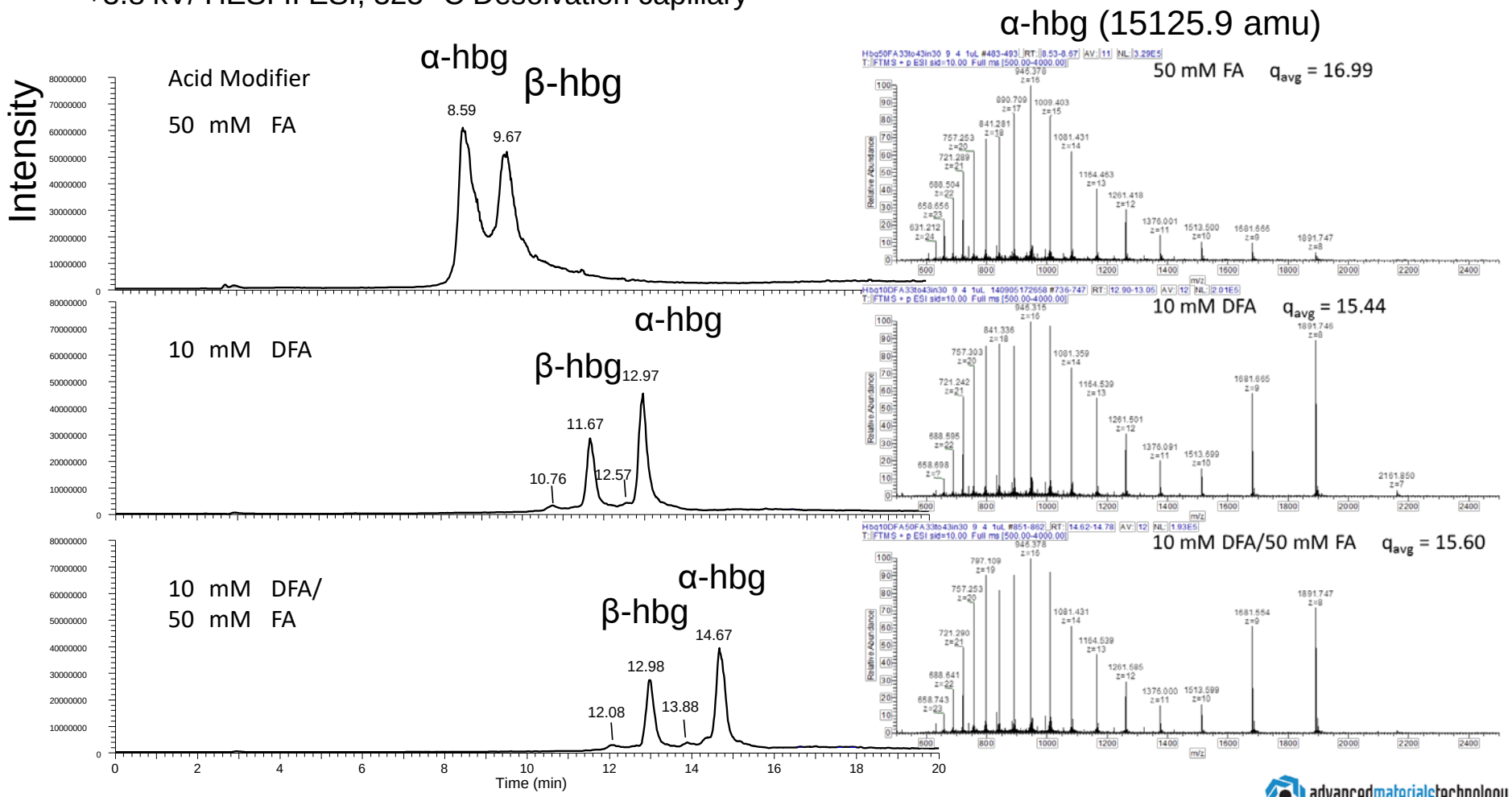
Mobile Phases for Improved Protein LC/MS

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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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.

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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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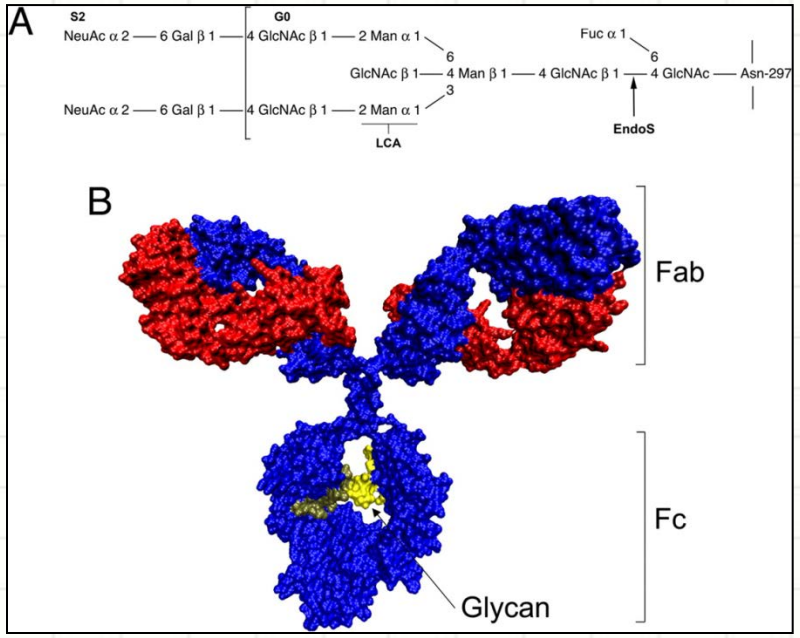
6/10 top sellers; > 400 mAbs in trials



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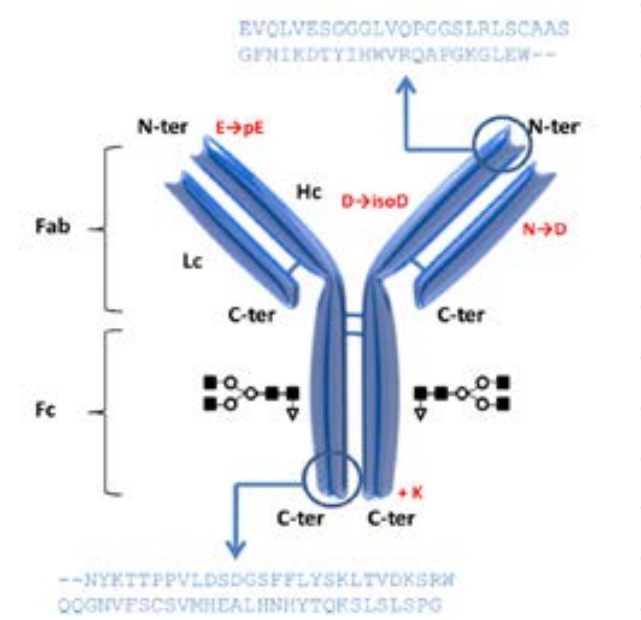
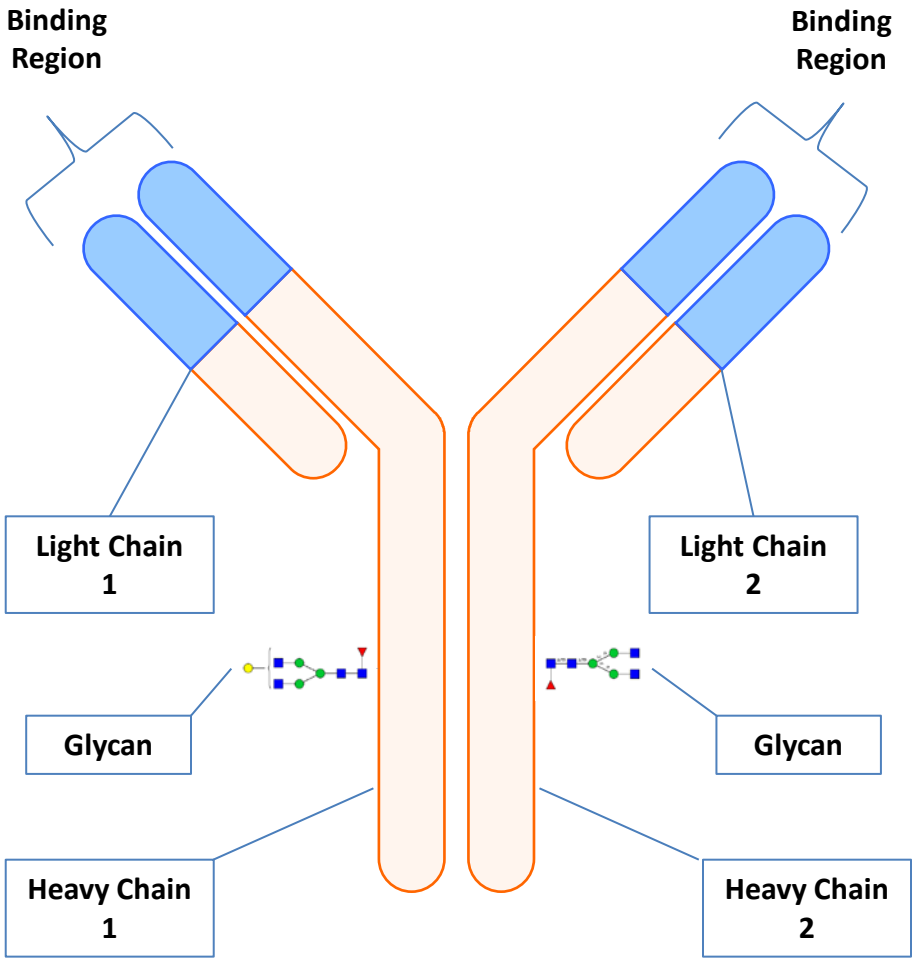


Trastuzumab



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 287 of each heavy chain.

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	2.7, 5
Glycosylation (sequence, composition, linkage, branching)	HILIC-MS HILIC-FLD	HALO Glycan	90	2.7

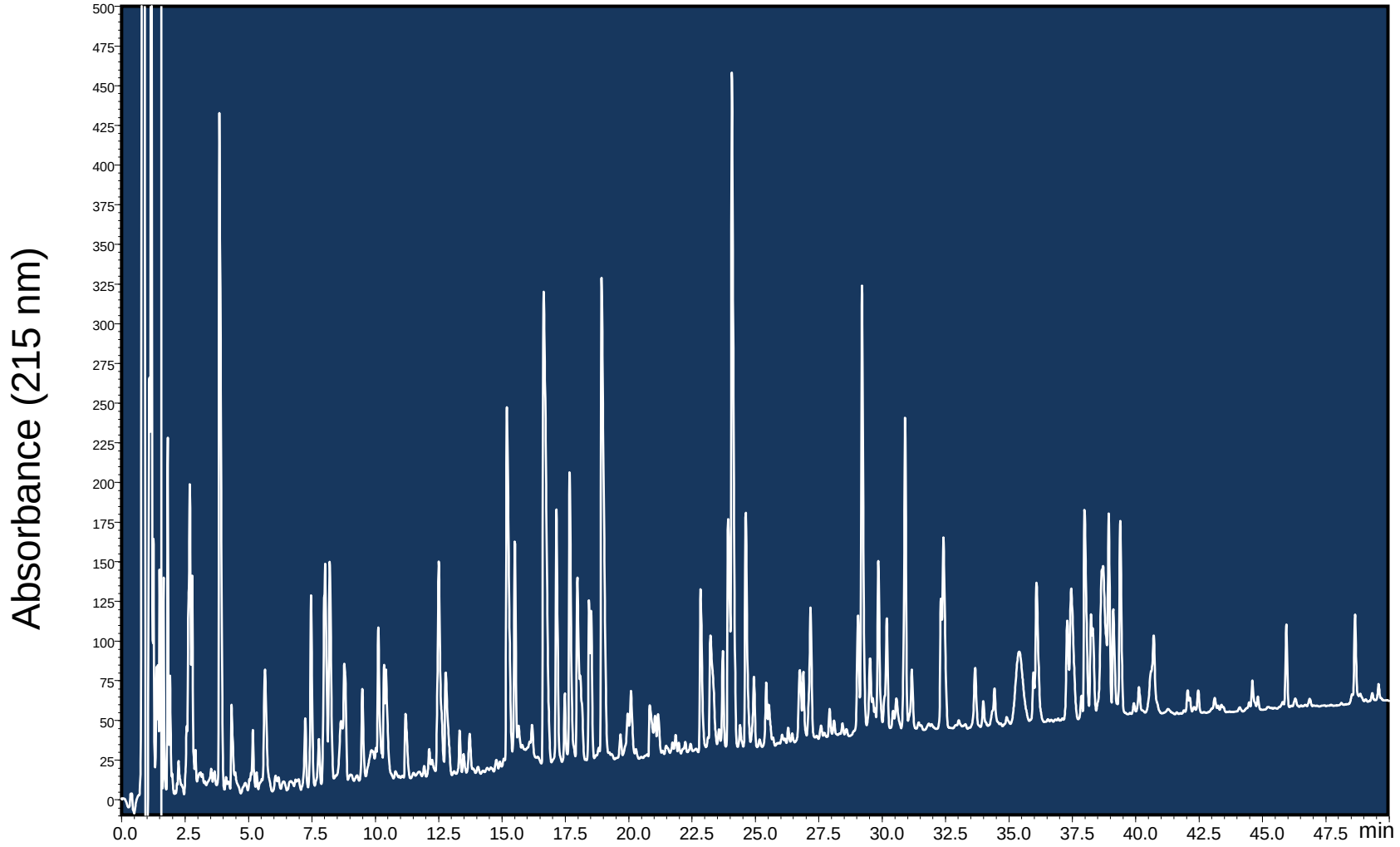
RPLC Analysis of Herceptin Tryptic Digest

***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

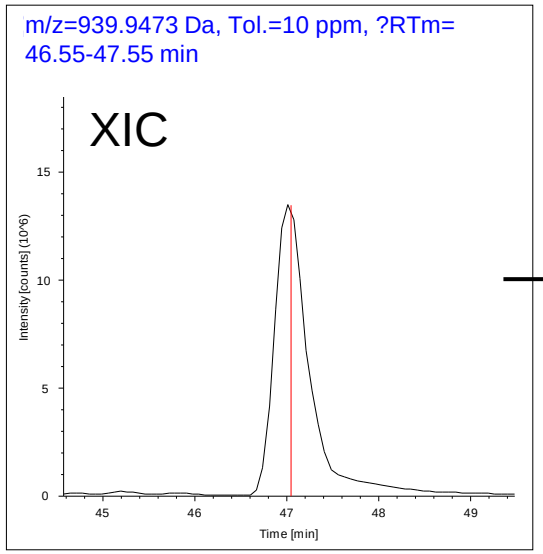
0.4 mL/min; 5–40% B in 60 min.; 60 °C; 50 µL of 2 µg/µL
reduced and alkylated, trypsin digested Herceptin

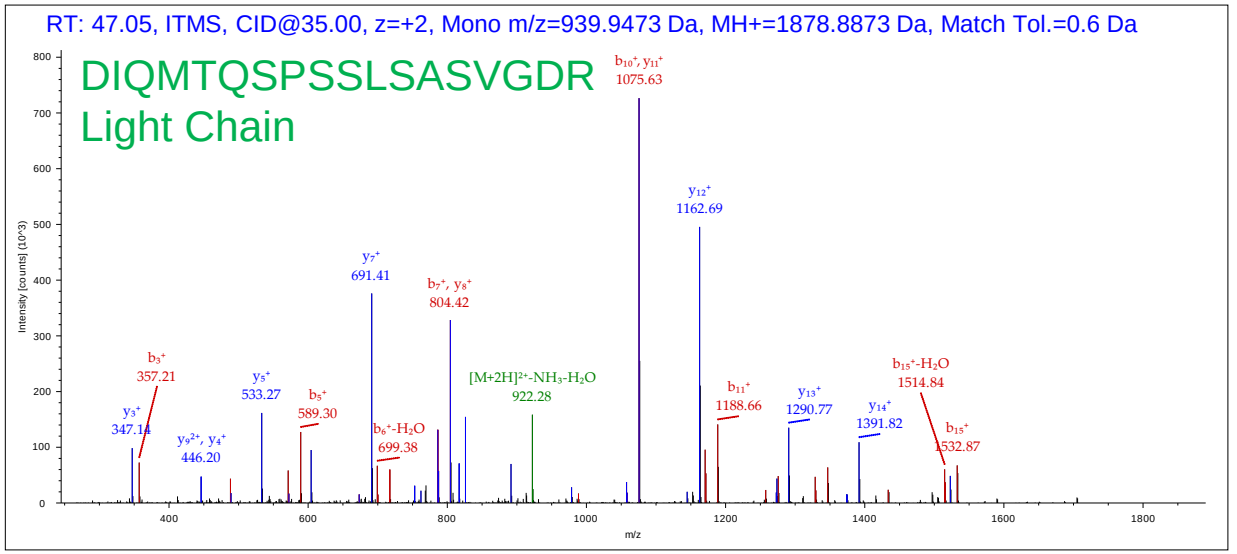


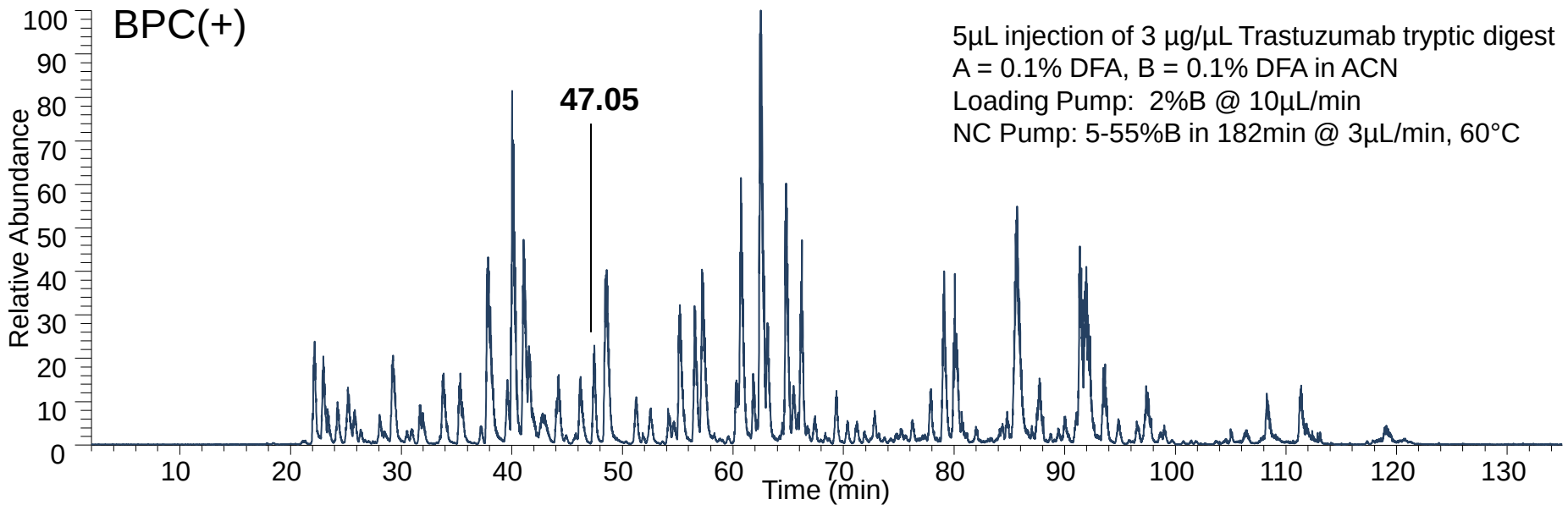
High Resolution Trastuzumab Digest

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

Thermo Orbitrap Velos Pro/Dionex Ultimate 3000 UHPLC







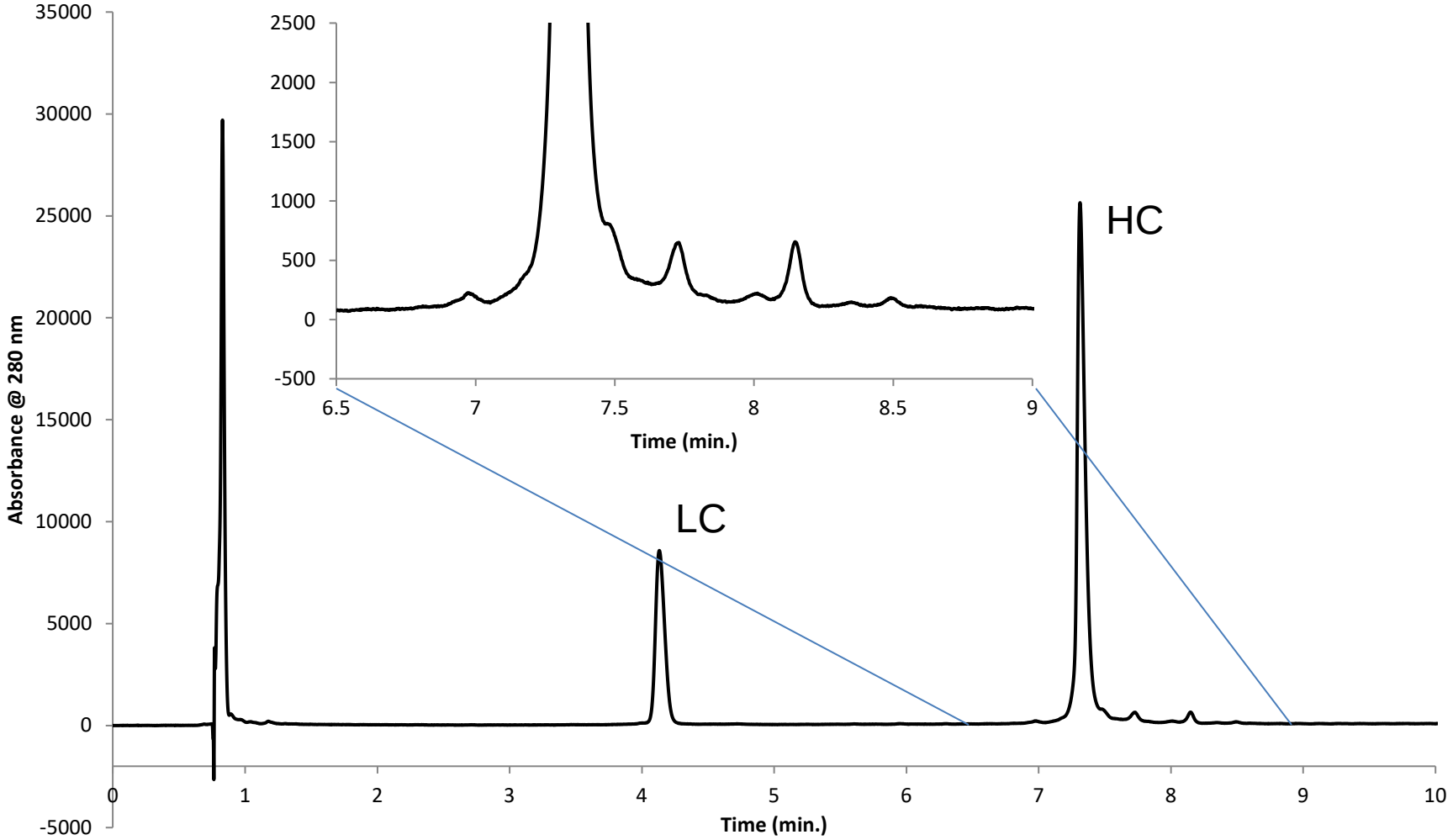
5µL injection of 3 µg/µL Trastuzumab tryptic digest
A = 0.1% DFA, B = 0.1% DFA in ACN
Loading Pump: 2%B @ 10µL/min
NC Pump: 5-55%B in 182min @ 3µL/min, 60°C

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

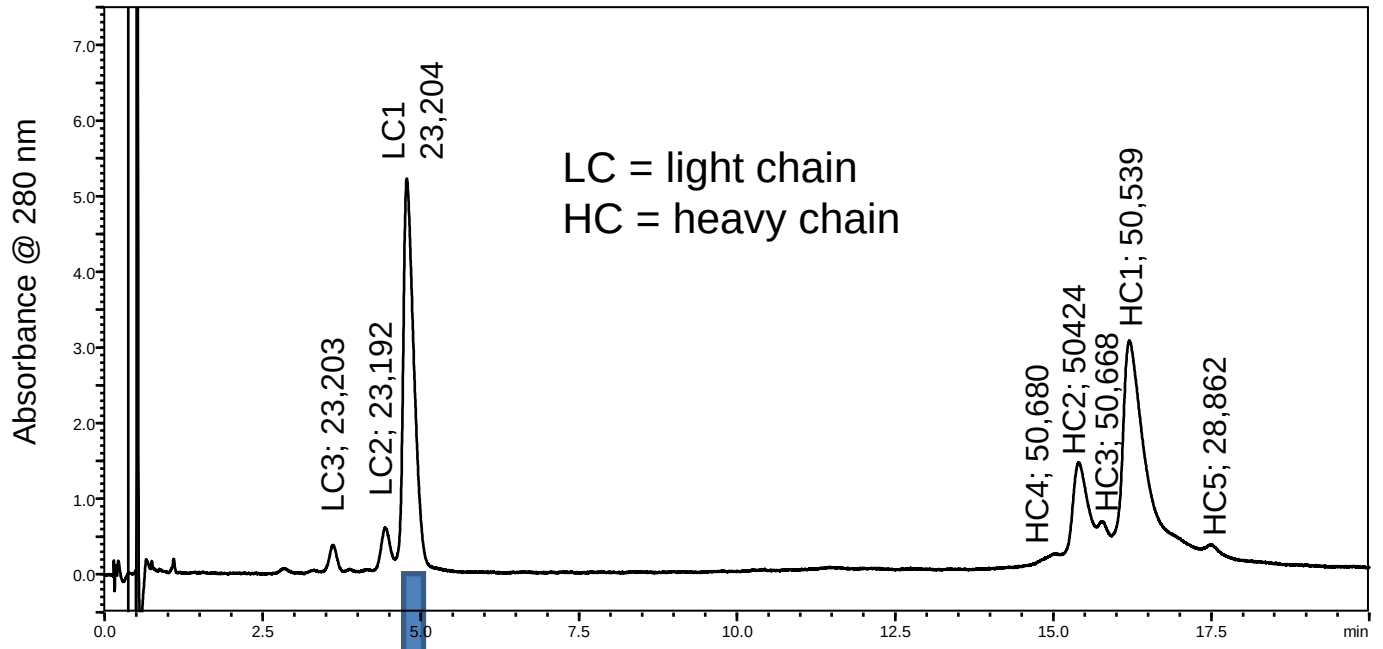
Mobile Phase A: water/0.1% TFA
Mobile Phase B: 80/20 ACN/water/0.1% TFA
Gradient: 33-40% B in 10 min.
Flow rate: 0.25 mL/min
Temperature: 80 °C

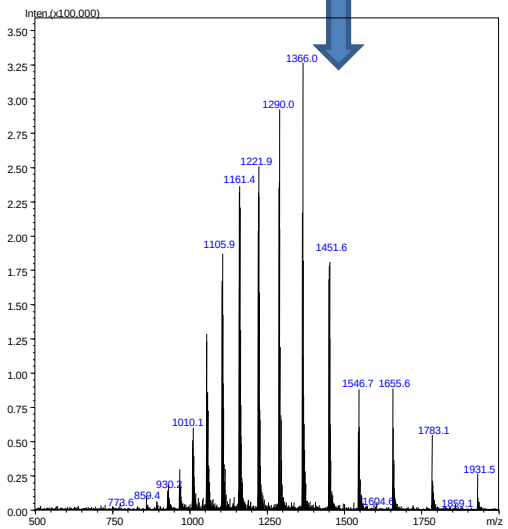
Sample: 0.5 mg/mL IgG2-B treated with 100 mM DTT in 8 M Guanidine HCl at 50 °C for 35 min.



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

LC/MS Analysis of IgG1 mAb Polypeptide Chains





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

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 ^{o}C until use.

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