Mobile Phase Alternatives for Peptide and Protein LC/MS

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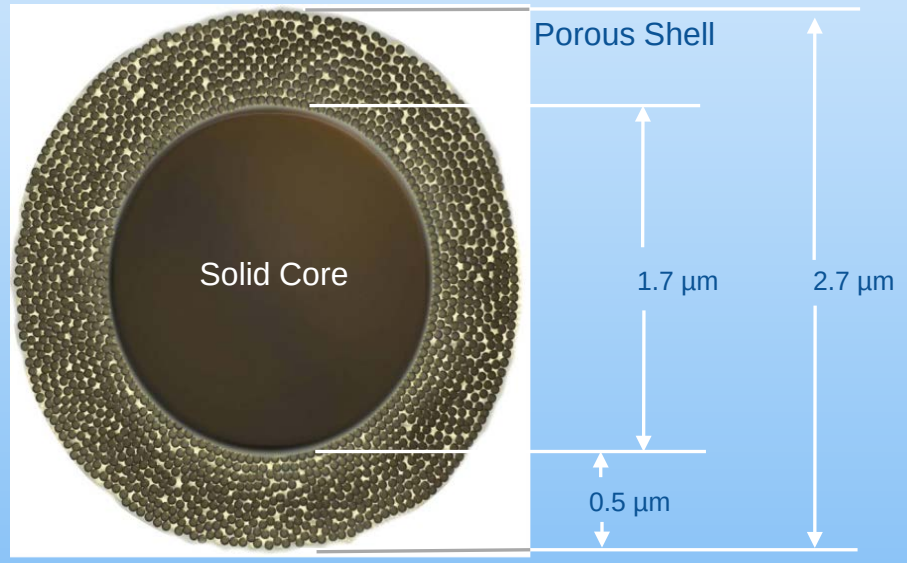
Faster or Higher Resolution HPLC Separations

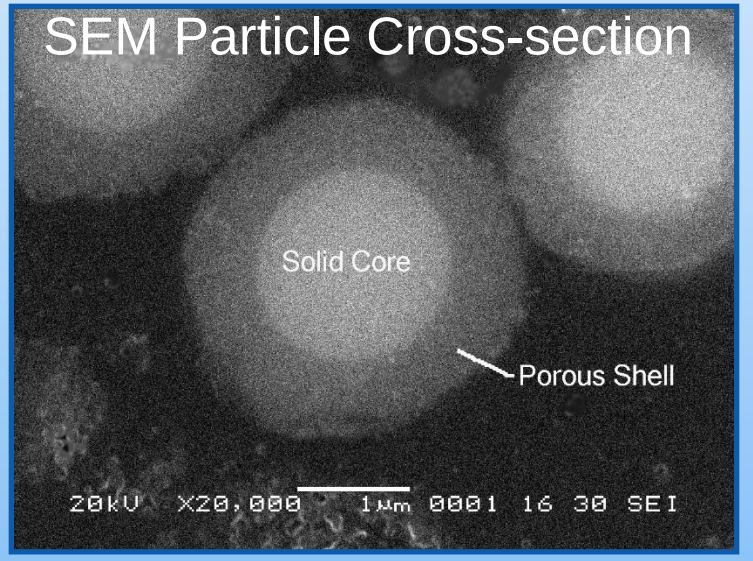
- Smaller Particle Packed Beds
  - Totally Porous
  - Not Porous (Pellicular)
  - Partly Porous (Superficially Porous) 
- Monolithic Materials
- Open Tubular Columns (channels)

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
  - Diffusion/mass transfer limitations due to size of molecules
  - Need a combination of methods/approaches
- Recent Enablers in Protein LC/MS
  - Detection Developments: MS Improves!
  - Mobile Phase Developments: Useful Newer Modifiers
  - Stationary Phase Developments: Developments in SPP for Proteins

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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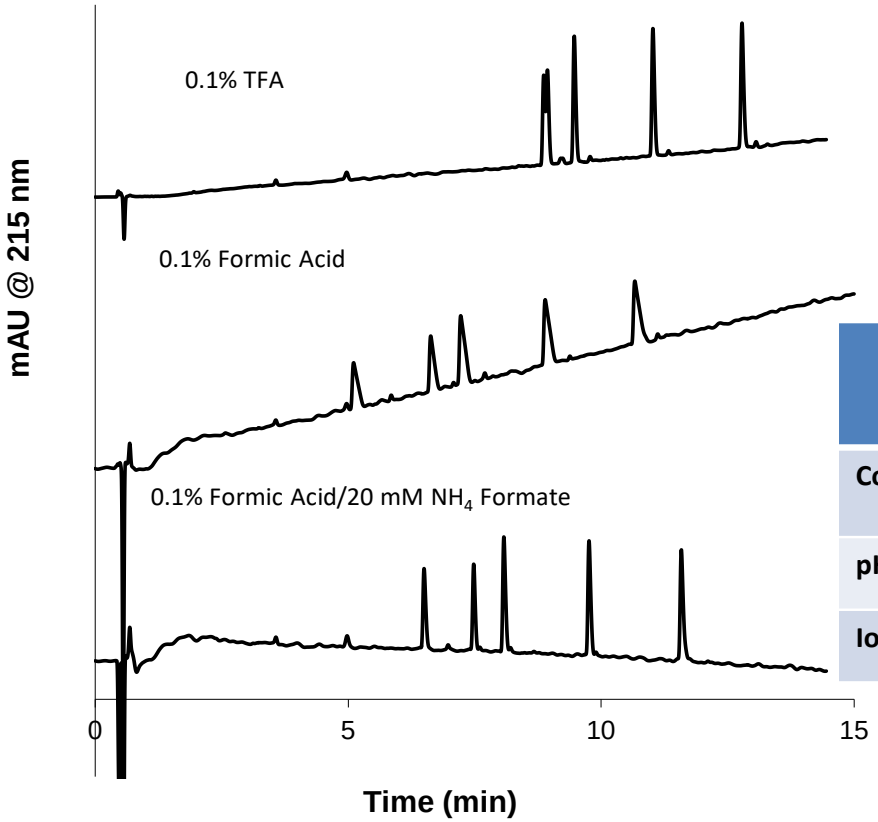
Mobile Phases for Protein and Peptide LC/MS

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
- 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:

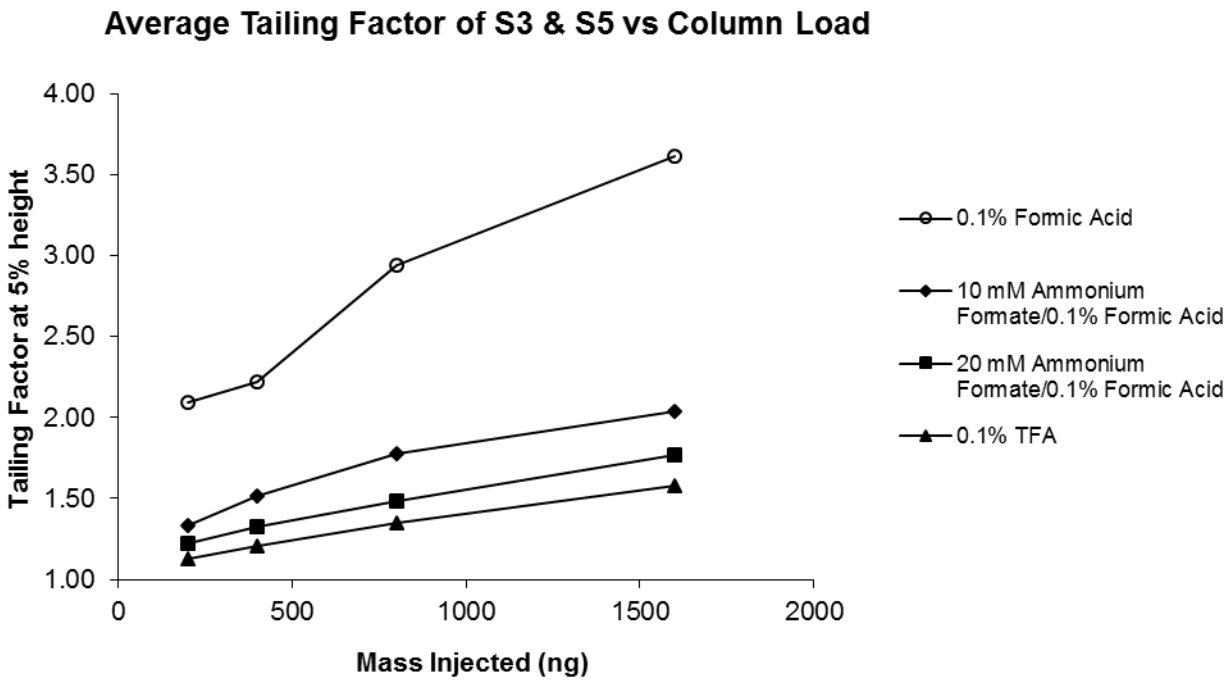
Synthetic Peptide Retention Standards:

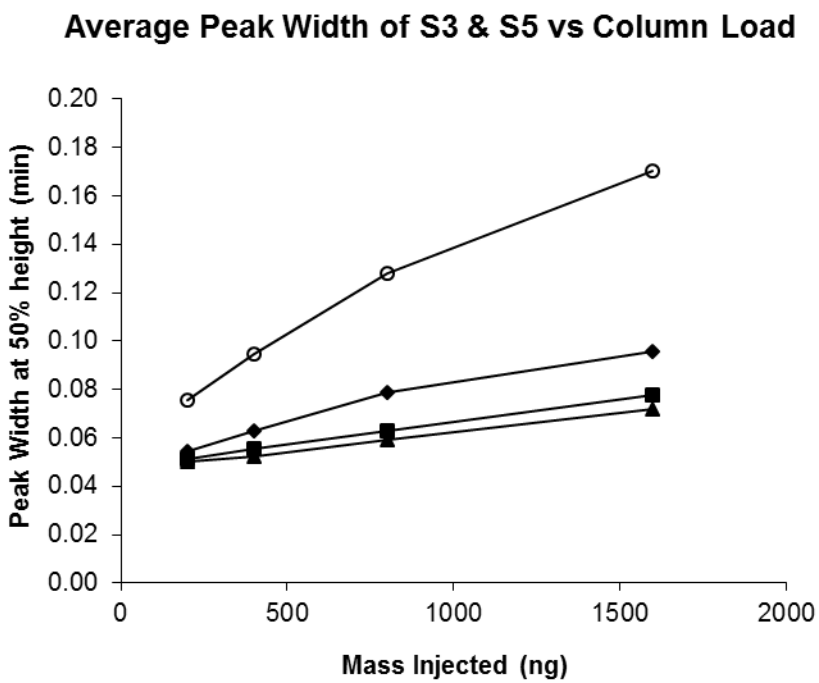
S1 RGAGGLGLGK-Am
S2 Ac-RGGGGLGLGK-Am
S3 Ac-RGAGGLGLGK-Am
S4 Ac-RGVGGLGLGK-Am
S5 Ac-RGVVGLGLGK-Am

	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.

Load Effects for Peptides Comparing FA, TFA, AFFA

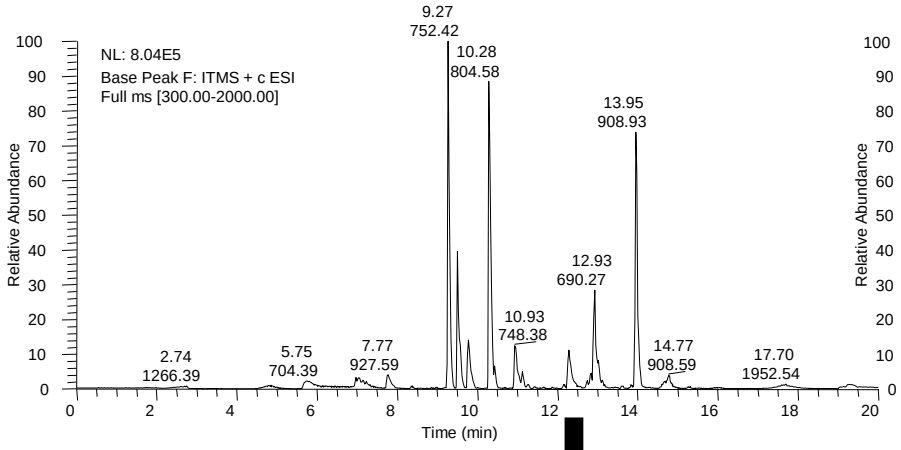


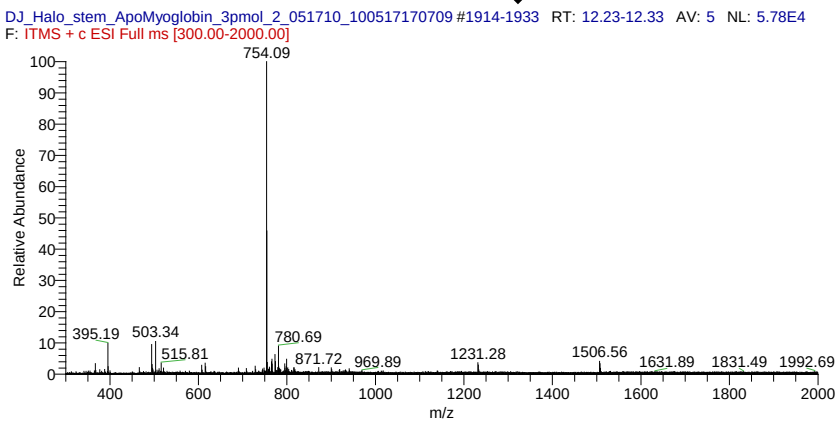


Ammonium formate as an additive for LC/MS separations

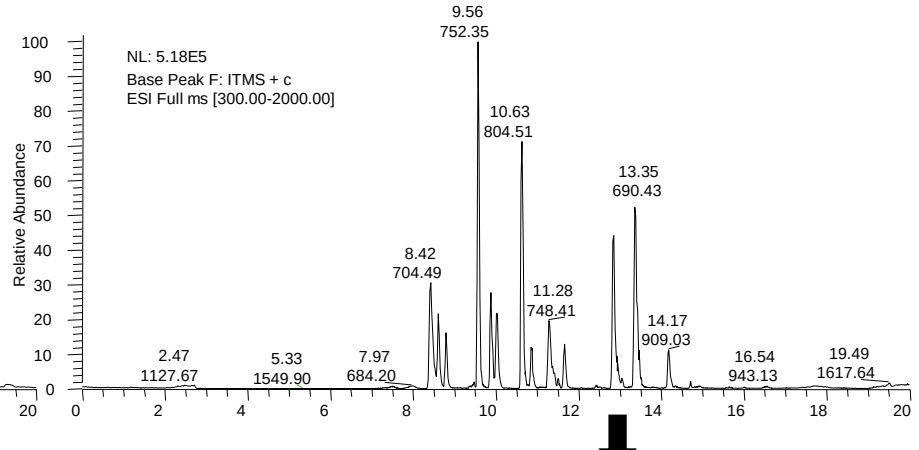
Column: 0.2 x 50 mm Halo Peptide ES-C18; Flow rate: 9 µL/min; Gradient: 2 - 45% B in 15 min; Mobile phases as shown; Sample: 2 µL (3 pmol) apomyoglobin digest.

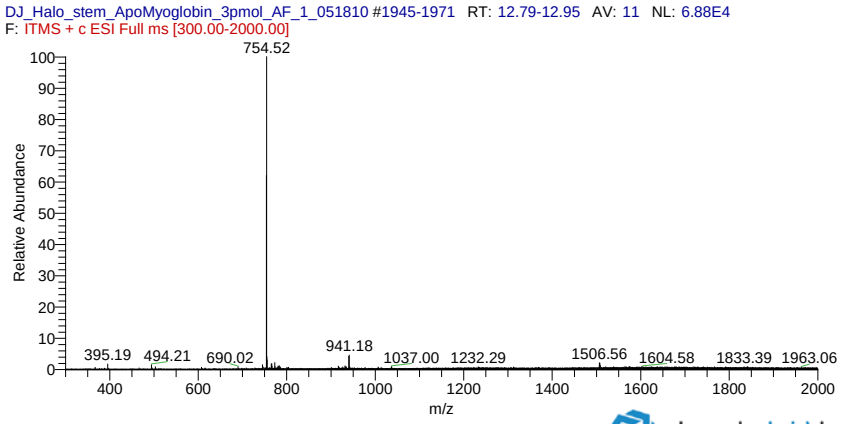
A: 0.1 % Formic Acid





B: 0.1 % Formic Acid/10 mM Ammonium Formate





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Improved Proteomic Analysis

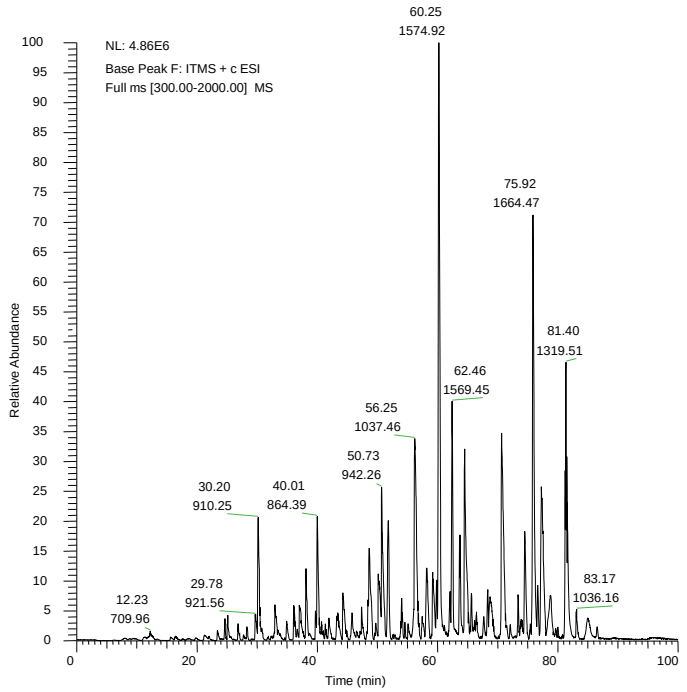


TABLE 7

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

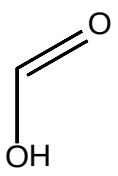
Name of Acid	MW (g/mol)	B.P. (°C)	pKa	UV Cut-off (nm)	Biological Hazard
Formic	46.02	100.8	3.8	220 +	Very low
Acetic	60.05	118-119	4.8	230 +	Very low
Fluoroacetic	78.04	165	2.6	ND	Very High
Difluoroacetic	96.03	134	1.3-1.4	205	Low
Trifluoroacetic	114.02	72.4	≤ 0.5	205	Low
Chloroacetic	94.50	189	2.8	ND	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

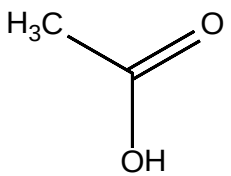
Initial selection and testing indicated some candidates with promise:

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

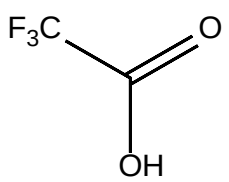
FA	HOAc	TFA	DFA	3FPA
3.8	4.8	0.3-1.6	1.3-1.4	3.1



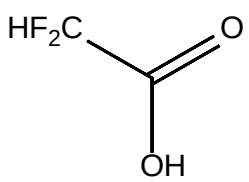
formic acid



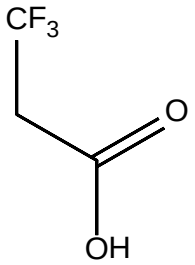
acetic acid



2,2,2-trifluoroacetic acid



2,2-difluoroacetic acid

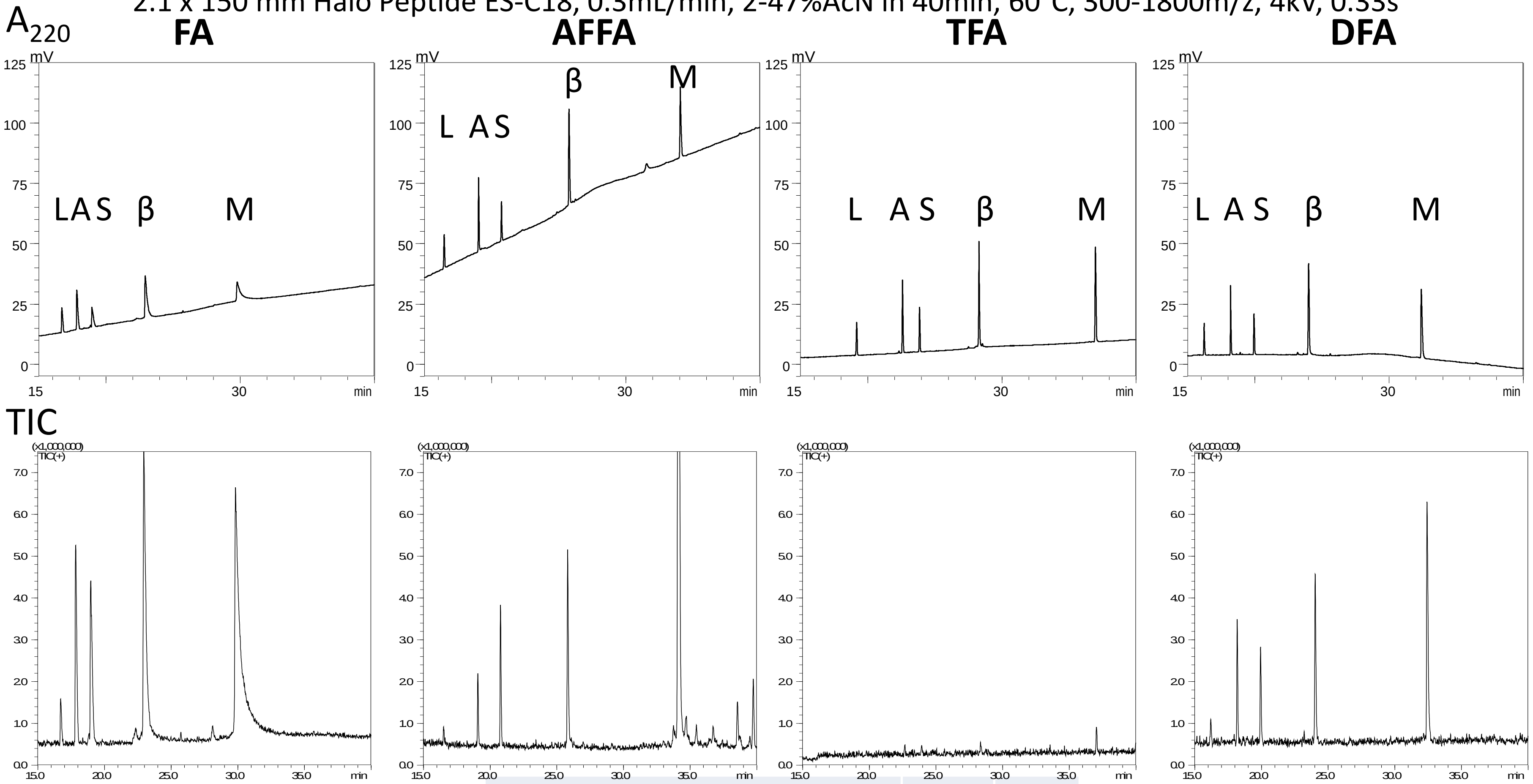


3,3,3-trifluoropropanoic acid

Synthetic Peptide Mixture LC/MS in Several Acidic Modifiers

10 mM Acid; 50pmol 5 peptide mix

2.1 x 150 mm Halo Peptide ES-C18, 0.3mL/min, 2-47%AcN in 40min, 60°C, 300-1800m/z, 4kV, 0.33s



Peptide	Abbrev.	MW
[Leu5]-enkephalin	L	555.6
angiotensin I, human acetate hydrate	A	1297
substance P acetate salt hydrate	S	1348
Melittin, honey bee venom	M	2847
beta-endorphin, human	β	3465

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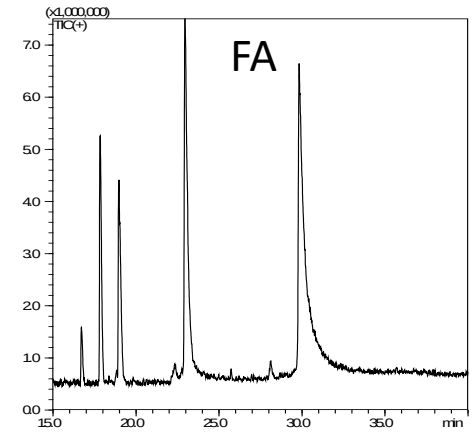
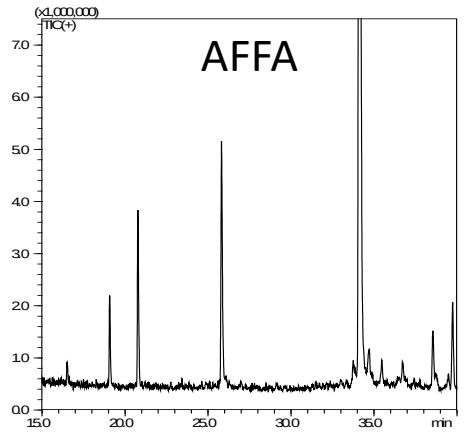
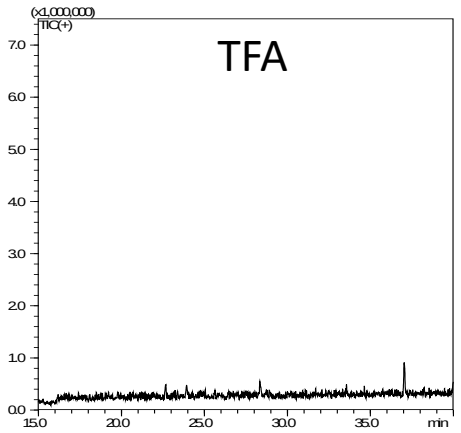
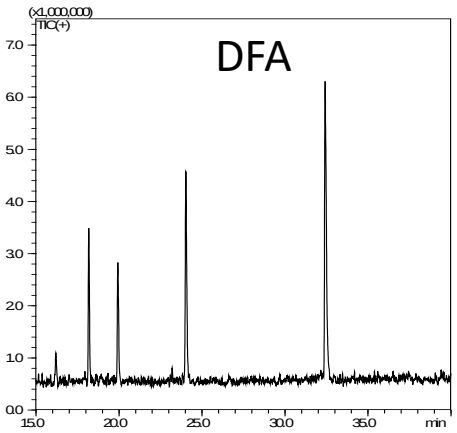
Synthetic Peptide Mixture LC/MS in Several Acidic Modifiers

10 mM acid in mobile phases; 2.1 x 150 mm Halo Peptide ES-C18, 0.3mL/min, 2-47%AcN in 40min, 60°C, 300-1800m/z, 4kV, 0.33s







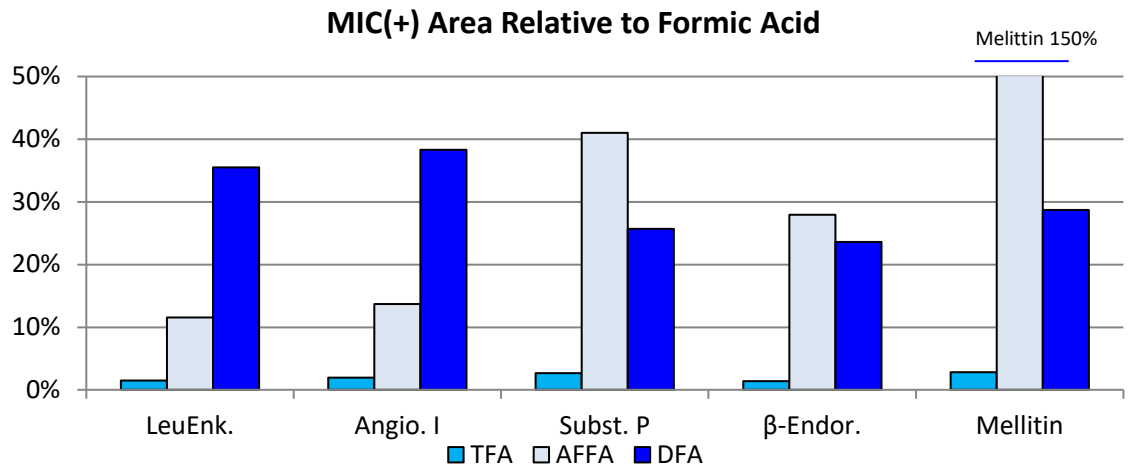


10FA	LeuEnk.	Angio. I	Subst. P	β-Endor.	Mellitin
Ret.Time	16.7	17.9	19.0	23.0	29.8
MIC(+)	9.3E+06	4.4E+07	3.0E+07	7.3E+07	1.0E+08
Relative	100%	100%	100%	100%	100%

10AFFA	LeuEnk.	Angio. I	Subst. P	β-Endor.	Mellitin
	16.5	19.1	20.8	25.8	34.1
	1.1E+06	6.1E+06	1.2E+07	2.0E+07	1.6E+08
	12%	14%	41%	28%	150%

10TFA	LeuEnk.	Angio. I	Subst. P	β-Endor.	Mellitin
	19.0	22.5	23.7	28.2	36.9
	1.4E+05	8.8E+05	8.0E+05	1.0E+06	2.9E+06
	1%	2%	3%	1%	3%

10DFA	LeuEnk.	Angio. I	Subst. P	β-Endor.	Mellitin
	16.2	18.2	20.0	24.0	32.4
	3.3E+06	1.7E+07	7.8E+06	1.7E+07	3.0E+07
	35%	38%	26%	24%	29%



Mean of triplicates for signal intensities at 50 pmol of each peptide. RSD less than 10%.

Summary

- TFA 20-50 fold lower signal
- DFA 3-4 fold lower signal
- FA wider peaks, tailing

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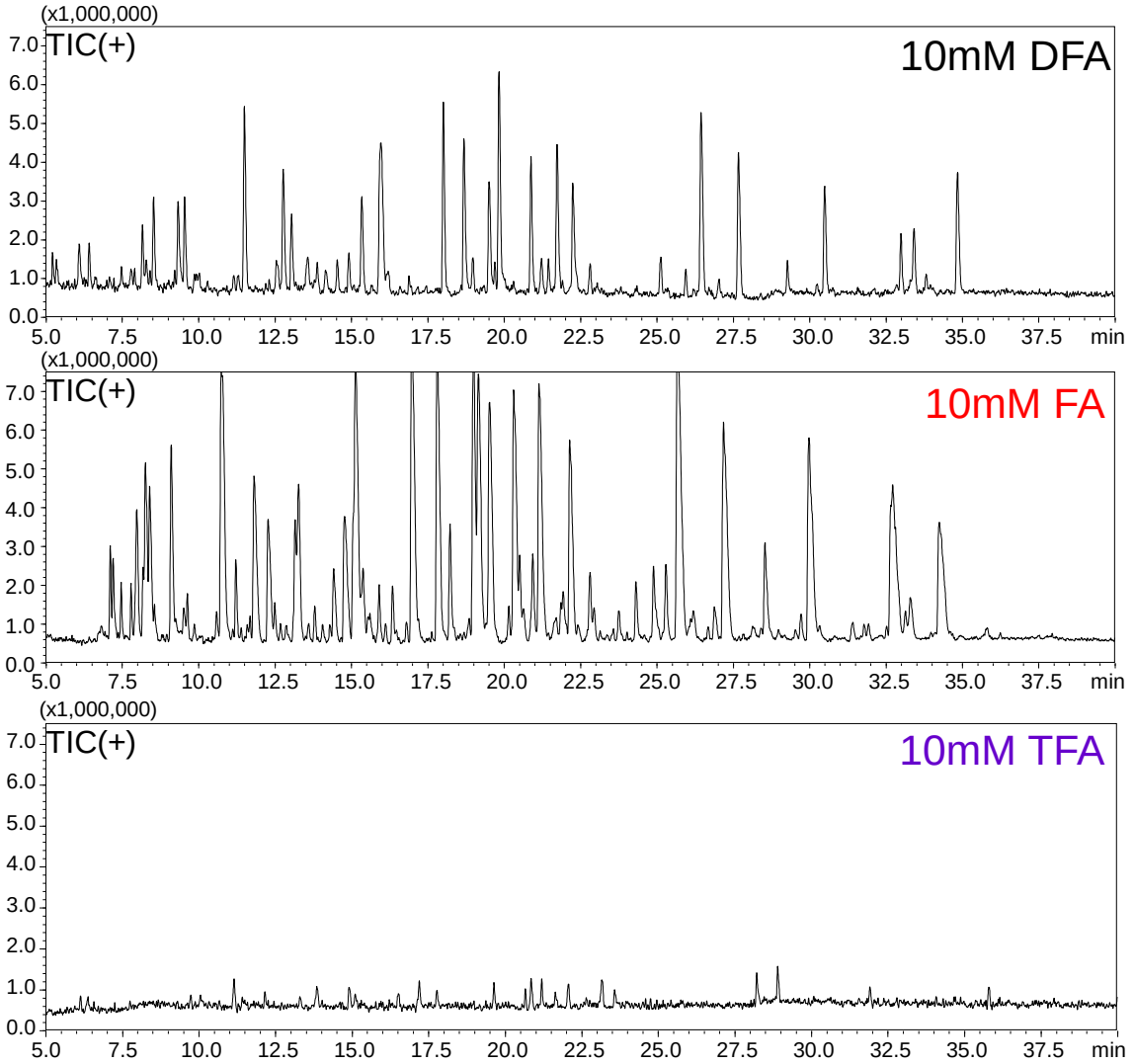
Tryptic Digest Peptide Mixture LC/MS in Several Acidic Modifiers

10 mM acid in mobile phases; 2.1 x 150 mm Halo Peptide ES-C18, 0.3mL/min, 2-47%AcN in 40min, 60°C, 300-1800m/z, 4kV, 0.33s



50pmol Reduced and Alkylated Enolase Digest Peptides

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Mobile Phases for Improved Protein LC/MS

2.1 x 100 mm
HALO Protein C4 400Å
15-55% AcN in 30 min

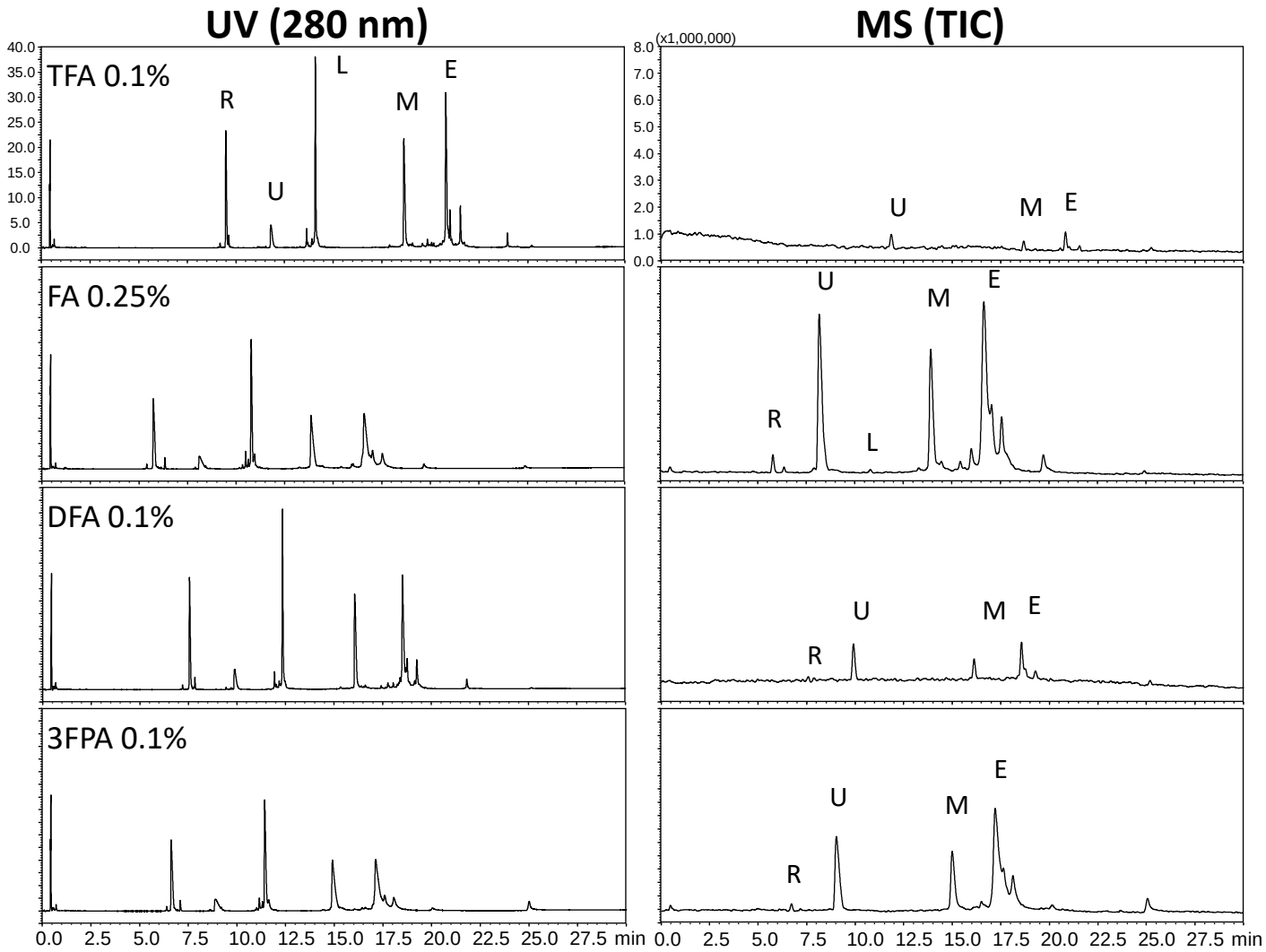
0.35 mL/min; 50ºC

25 pmol each protein

R – Ribonuclease
U – rec. Ubiquitin
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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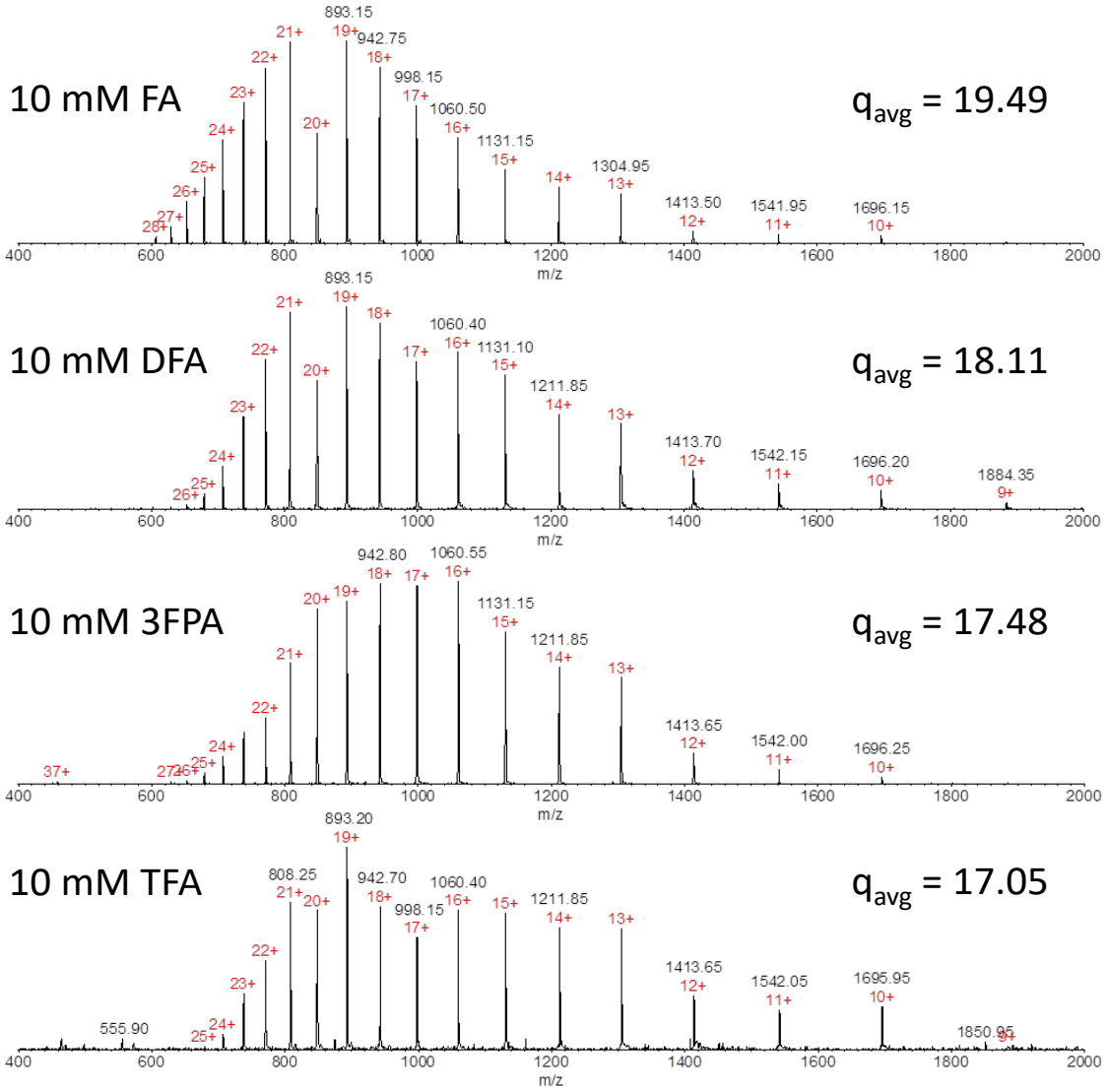
Mobile Phases for Improved Protein LC/MS



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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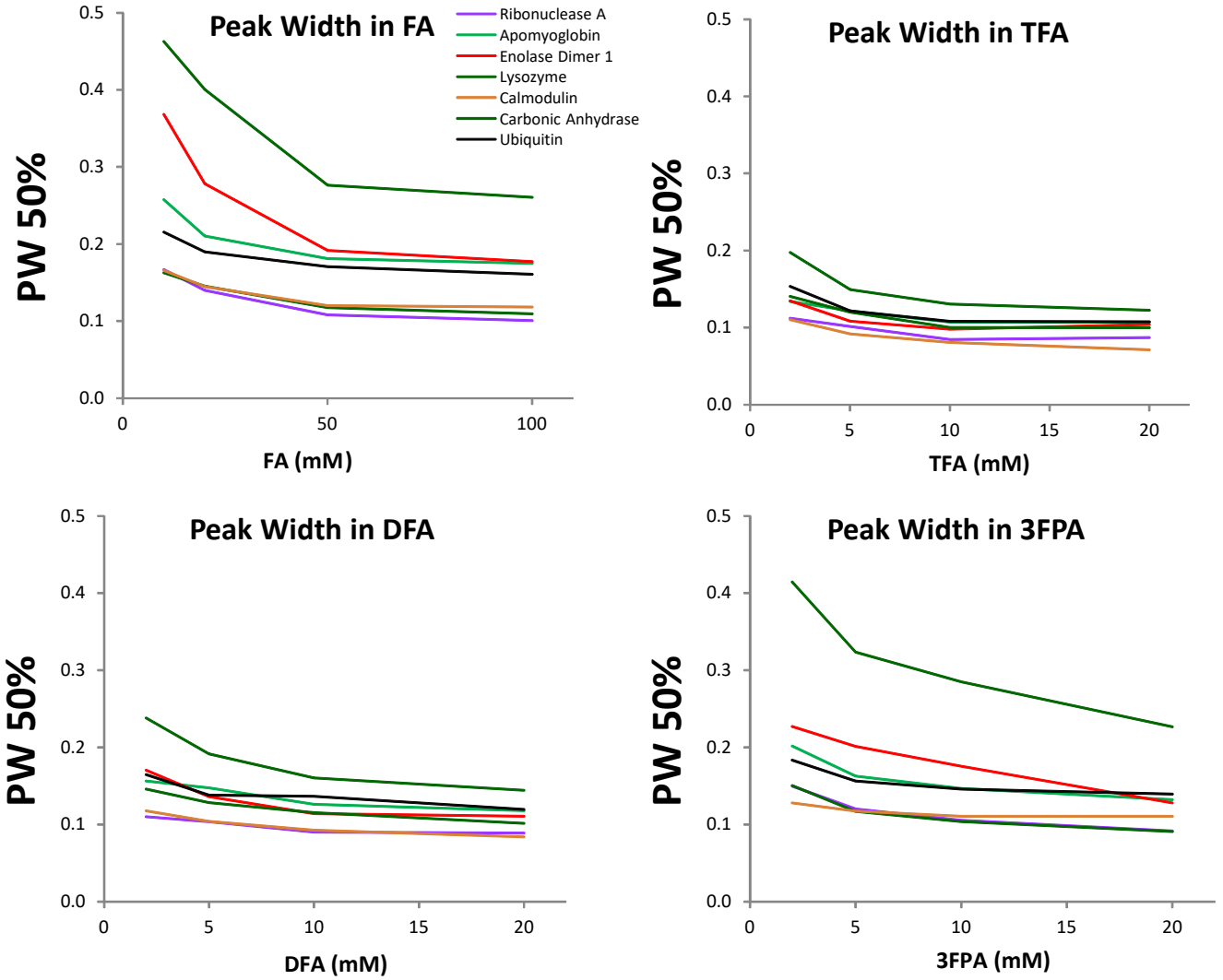
Mobile Phases for Improved Protein LC/MS



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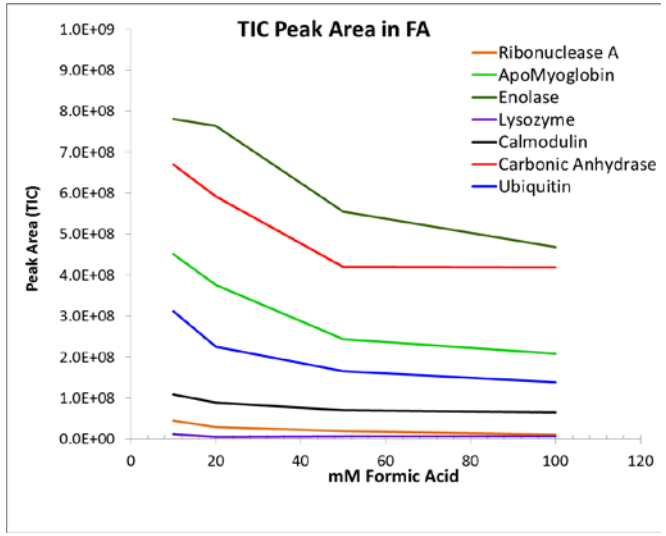

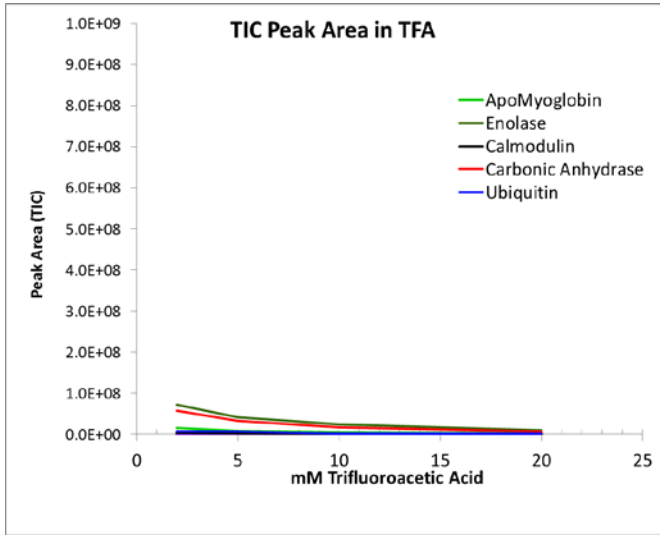

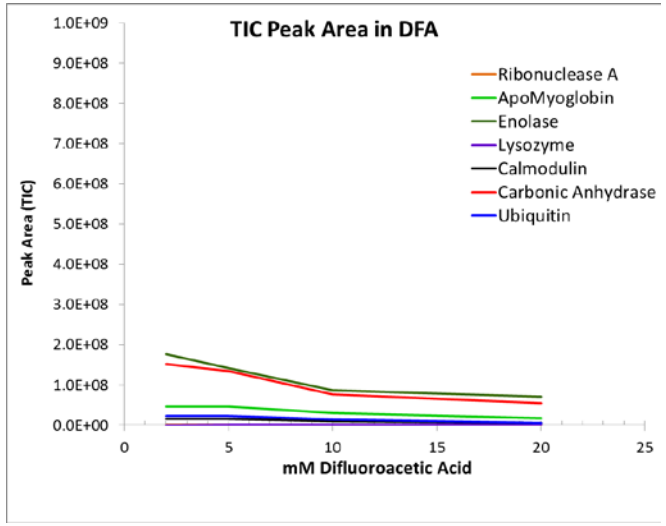

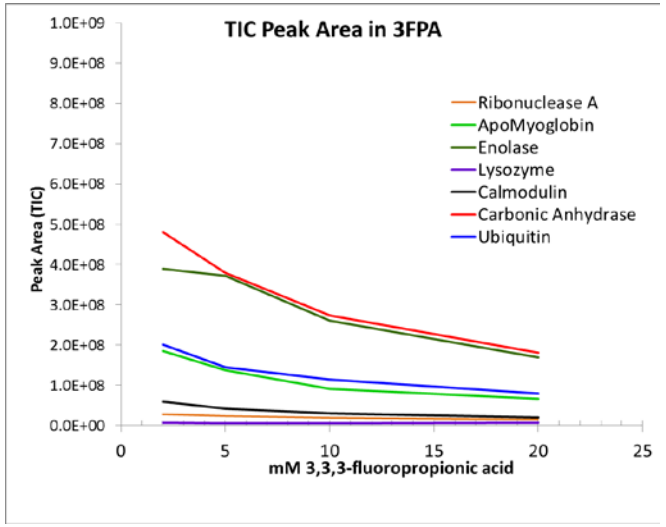

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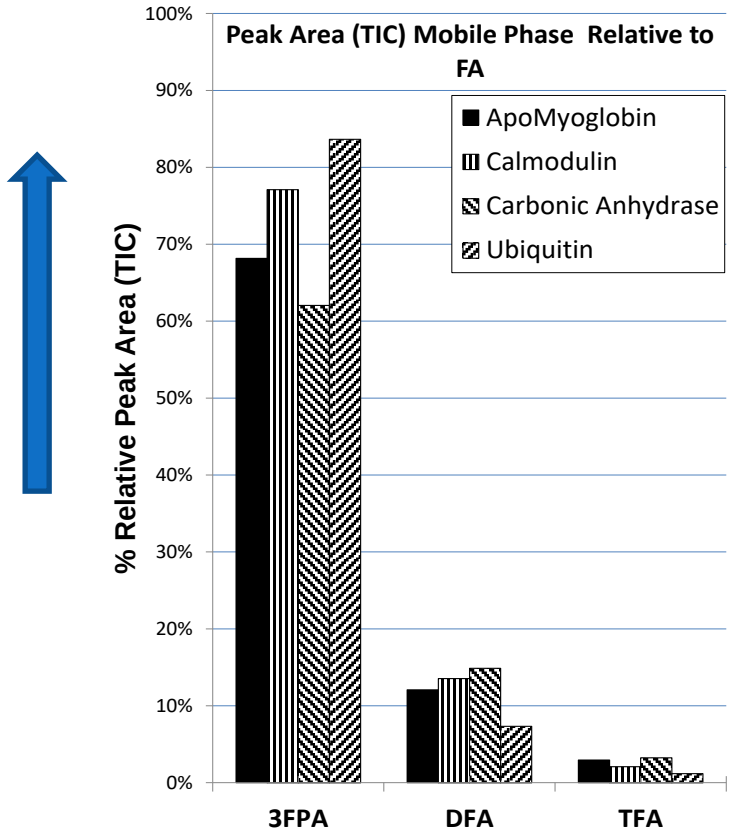
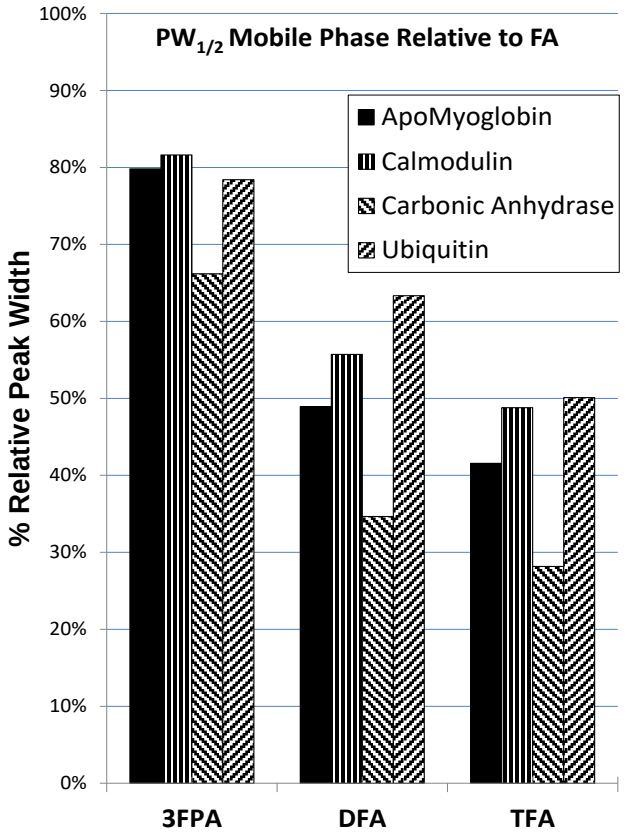
Mobile Phases for Improved Protein LC/MS

- 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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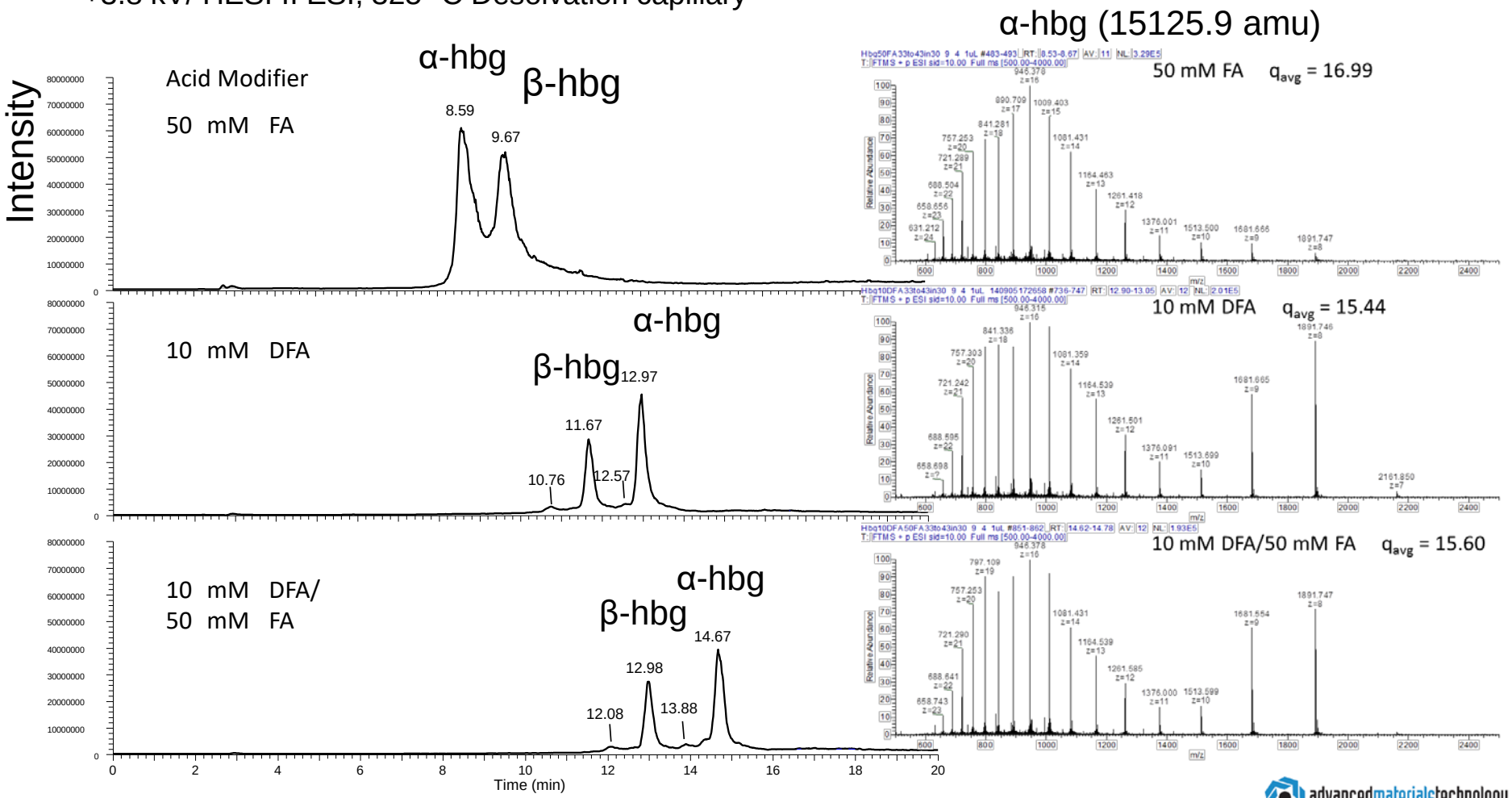
Mobile Phases for Improved Protein LC/MS

Halo Protein C4 400Å : 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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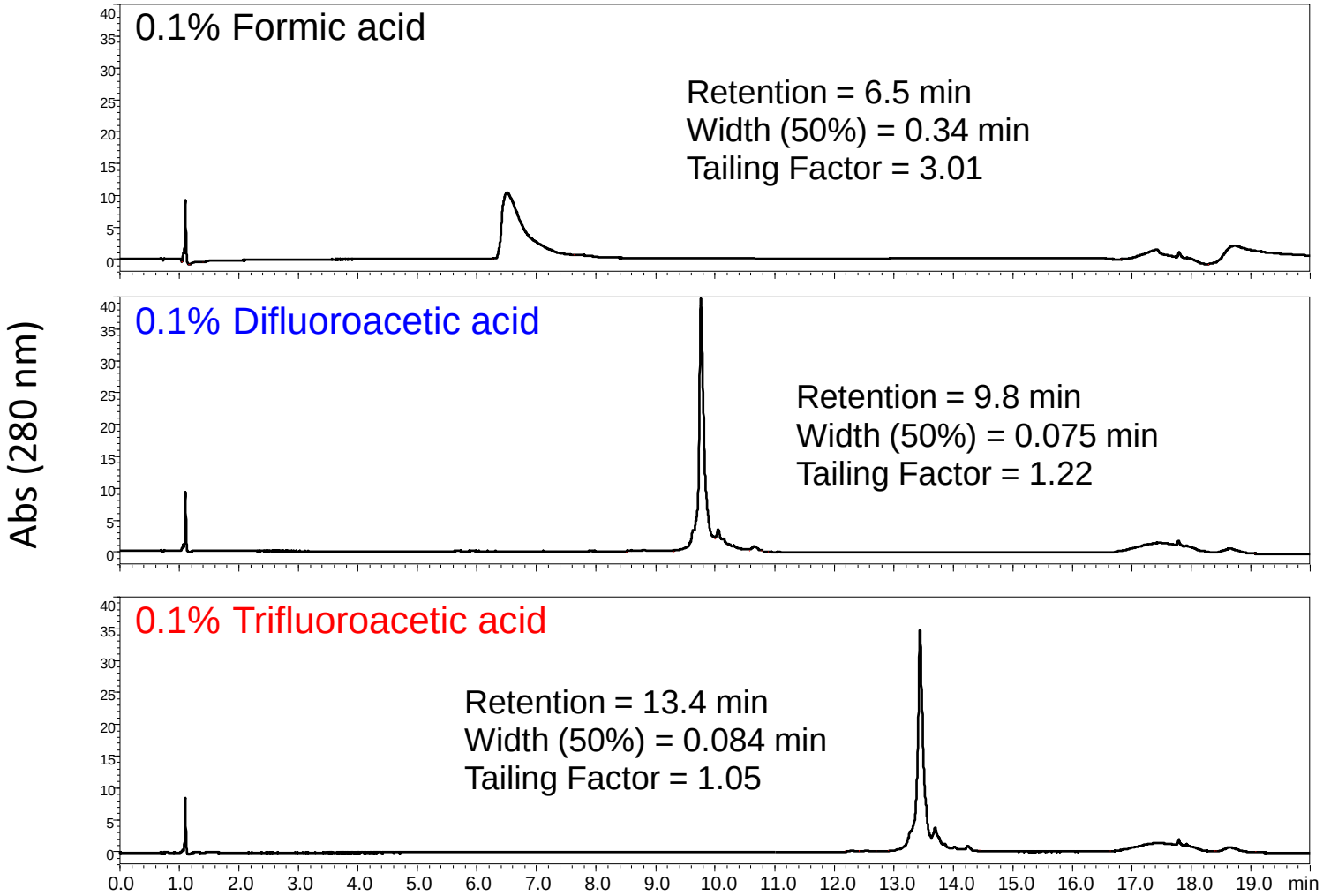
Mobile Phases for Improved mAb LC

2.1 x 150 mm Halo Protein C4 400Å; Gradient: 28–38% AcN/0.1% acid as indicated in 15 min
Flow: 0.3 mL/min; Temp: 80ºC; Sample: 2 µL of Intact SILu™Lite SigmaMAb - 0.5 µg/µL (H_2O)



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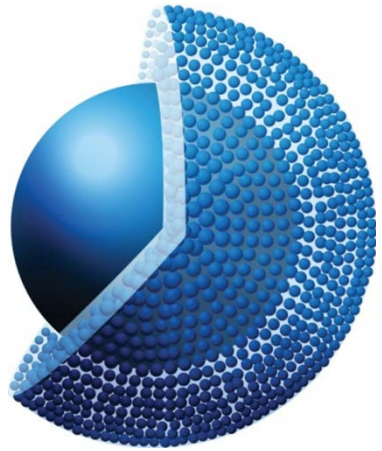
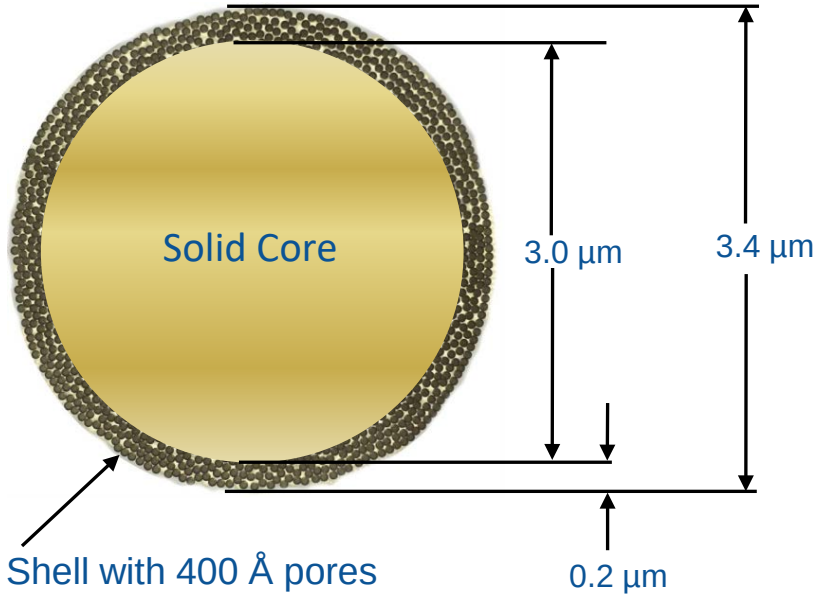
Wide Pore SPP Can Fit the Needs for Protein Science

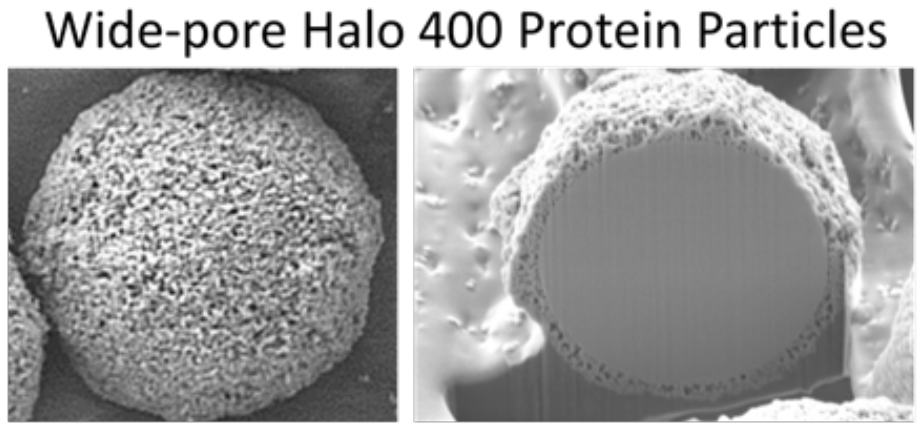
What is Needed for High Performance Separations of Larger (Bio) Molecules?



- **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**
  - **Must have "Right" size AND desirable particle distribution**
- **Surface Chemistry appropriate to Samples**

Superficially Porous (Fused-Core®) Wide Pore Particles: 160 Å, 400 Å, 1000 Å (Q4 2016)





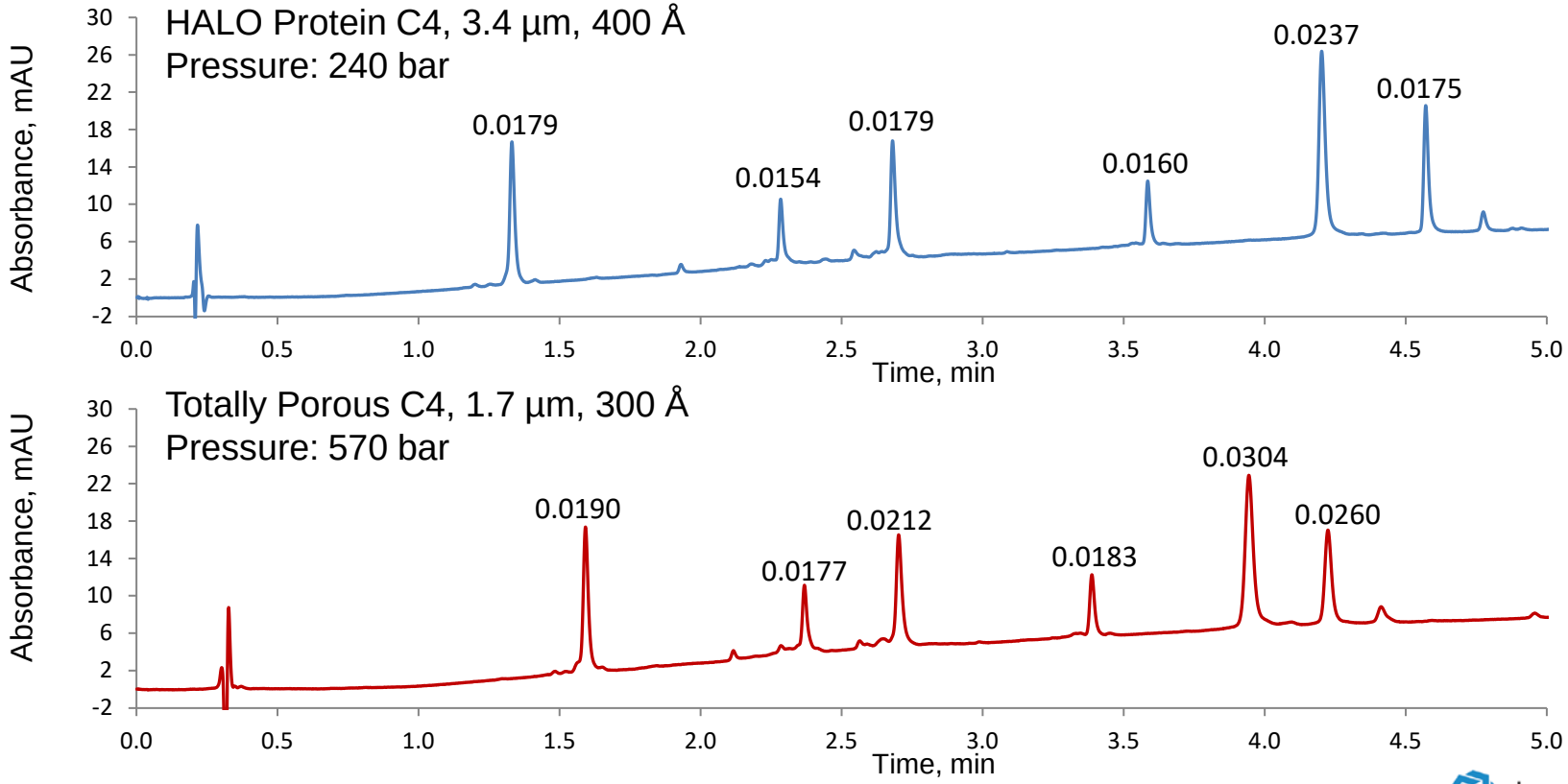
- Example above is 3.4 µm particle/400 Å pore size
- Many variations in shell thickness, pore size and particle size have been studied
- Theory to support “best properties” is complex, with limited tests using proteins, particularly with larger proteins
- Look for compromise in diffusion path for high MW molecules (to maintain small C-term), load tolerance, usability, speed and efficiency

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



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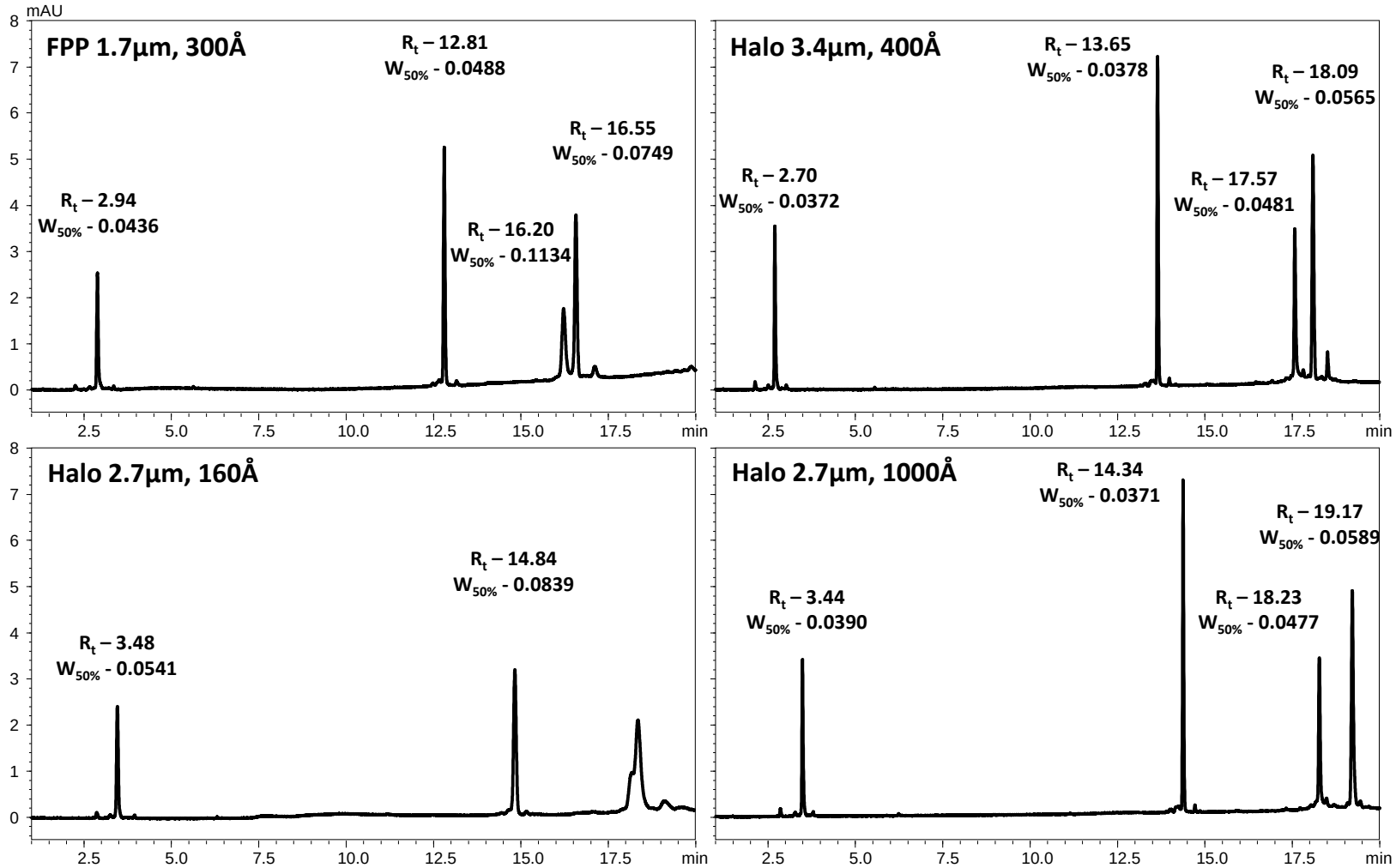
Protein Separation on Wide Pore SPP vs FPP

2.1 mm ID x 150 mm C4 columns
20-50% AcN/0.1% DFA in 24 min
Flow: 0.5 mL/min
Temp: 60°C
1.5 µL (0.15-0.2 ug each)

1. RNase A 13.7 kDa
2. α-Lactalbumin 14.2 kDa
3. Enolase 93.1 kDa
4. Carbonic Anhydrase 30.0 kDa



- Improvement in Peak Width and Retention with Larger Pore SPP
- As protein size increases, peak widths decrease with increasing pore size
- Similar results in TFA and DFA as mobile phase acidic modifiers

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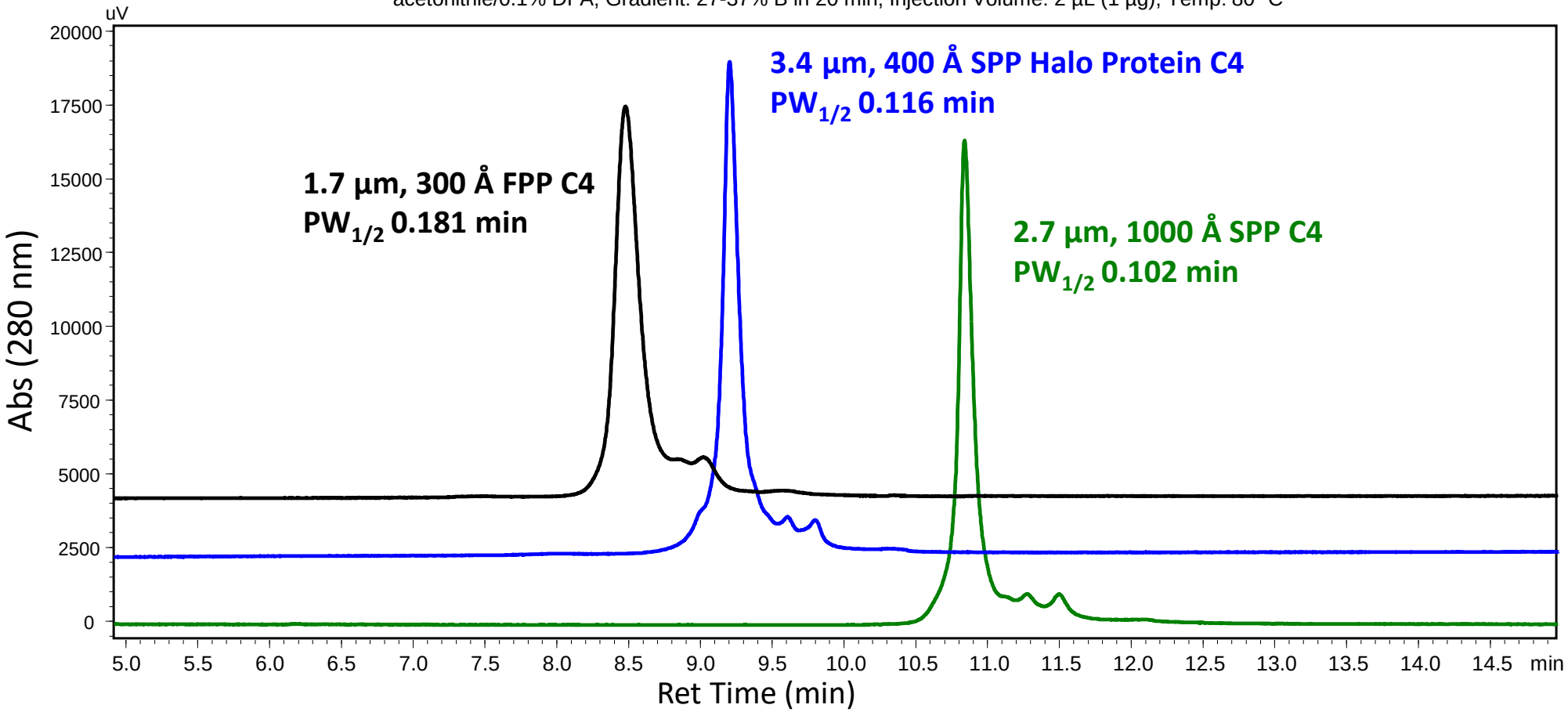
mAb IgG Separation on Wide Pore SPP vs FPP

High Efficiency Separation of Trastuzumab

Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: water/0.1% DFA; Mobile Phase B: acetonitrile/0.1% DFA; Gradient: 27-37% B in 20 min; Injection Volume: 2 µL (1 µg); Temp: 80 °C



- Large improvement in Peak Width and *increased* Retention with Larger Pore SPP, moderate additional improvement in Peak Width with Larger Pores

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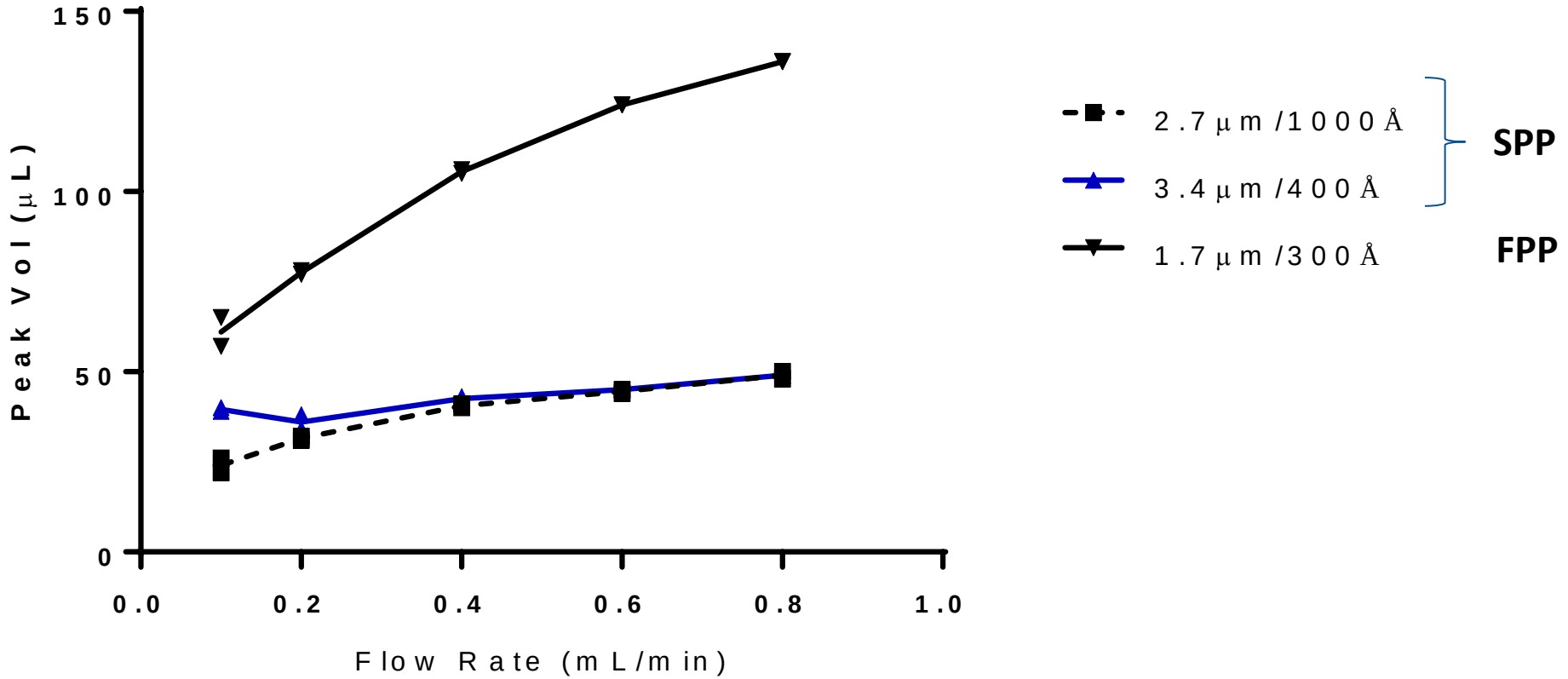
Flow Rate Effects on Peak Volume for mAb IgG

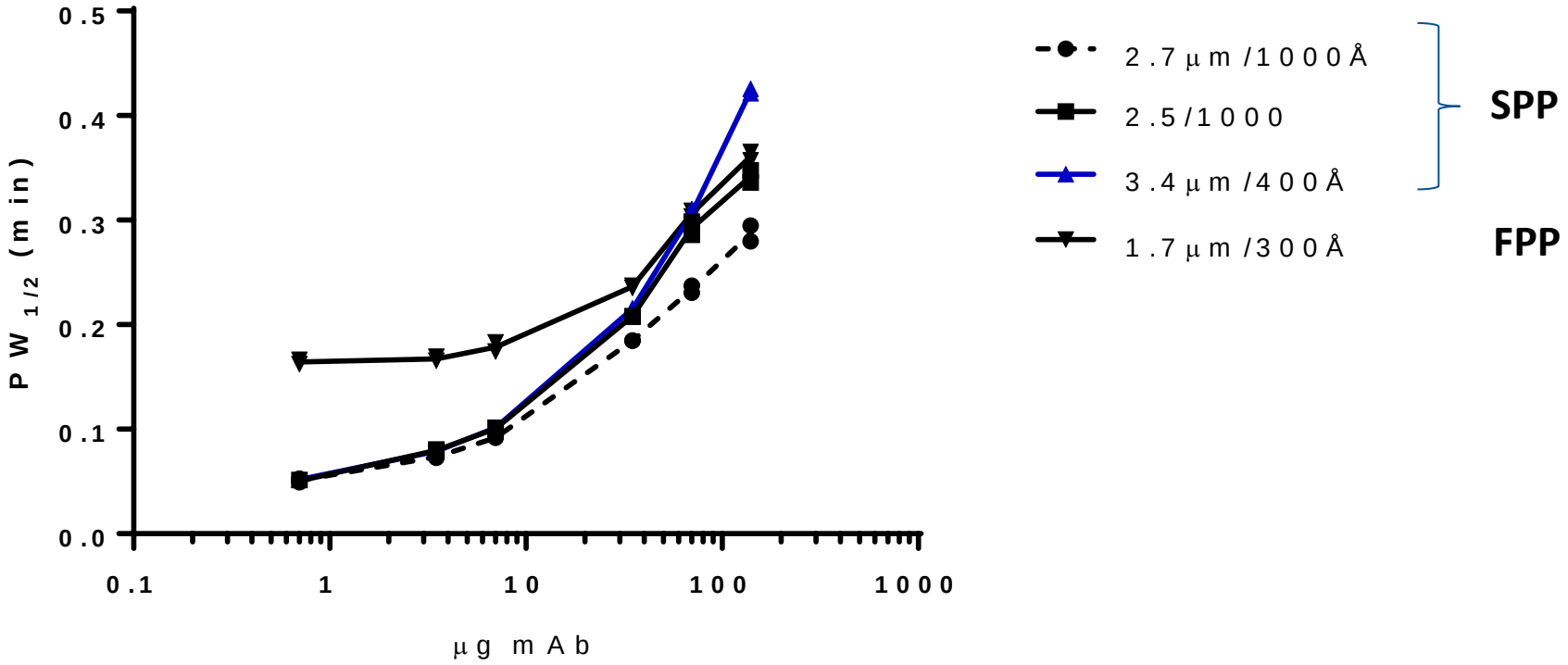
Fixed Volume Gradient Conditions (4.8 mL); Peak Volume = $PW_{1/2}$ x Flow Rate
Trastuzumab 0.5 µg; 29-35% AcN in 0.1% DFA; 80°C;



- Mass Transfer is improved for the large pore SPP particles with higher MW protein
- Trastuzumab and Silumab exhibited similar results
- Retention time matching across columns (gradient shift) exhibited similar results

Load Effects on Peak Width for SPP and FPP for mAb IgG

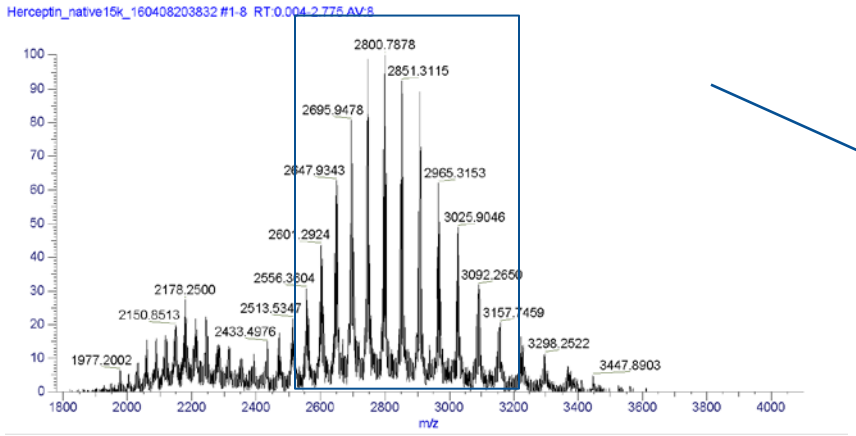
2.1 mm ID x 150 mm C4 columns; Trastuzumab 0.7 – 140 µg;
27-37% AcN (0.1% DFA) in 10 min; 80°C

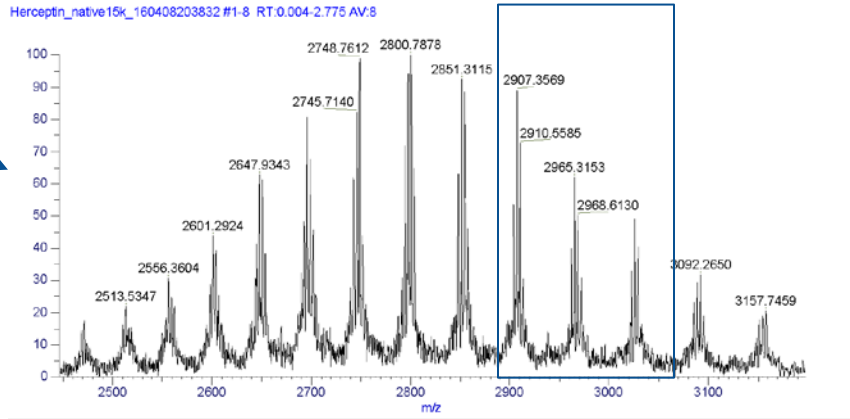


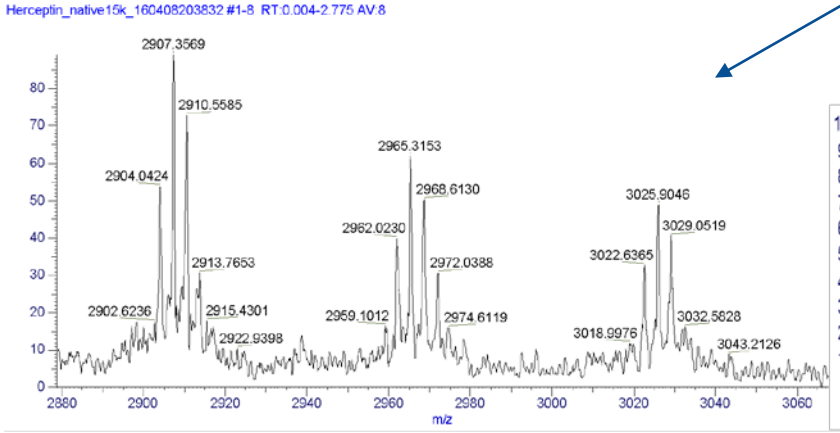
- For larger molecules, large pore SPP particles tolerate large sample masses effectively
- Performance loss is progressive, occurring around 20-50 µg on column
- At all load levels 1000Å pore size SPP performed best for this mAb

Protein Fragment (IgG) Typical Lab Spectrum

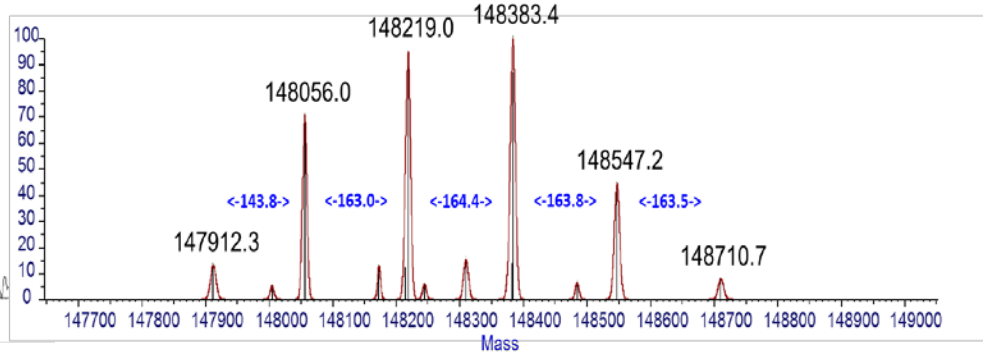
Glycoform Mixture MS







After Deconvolution



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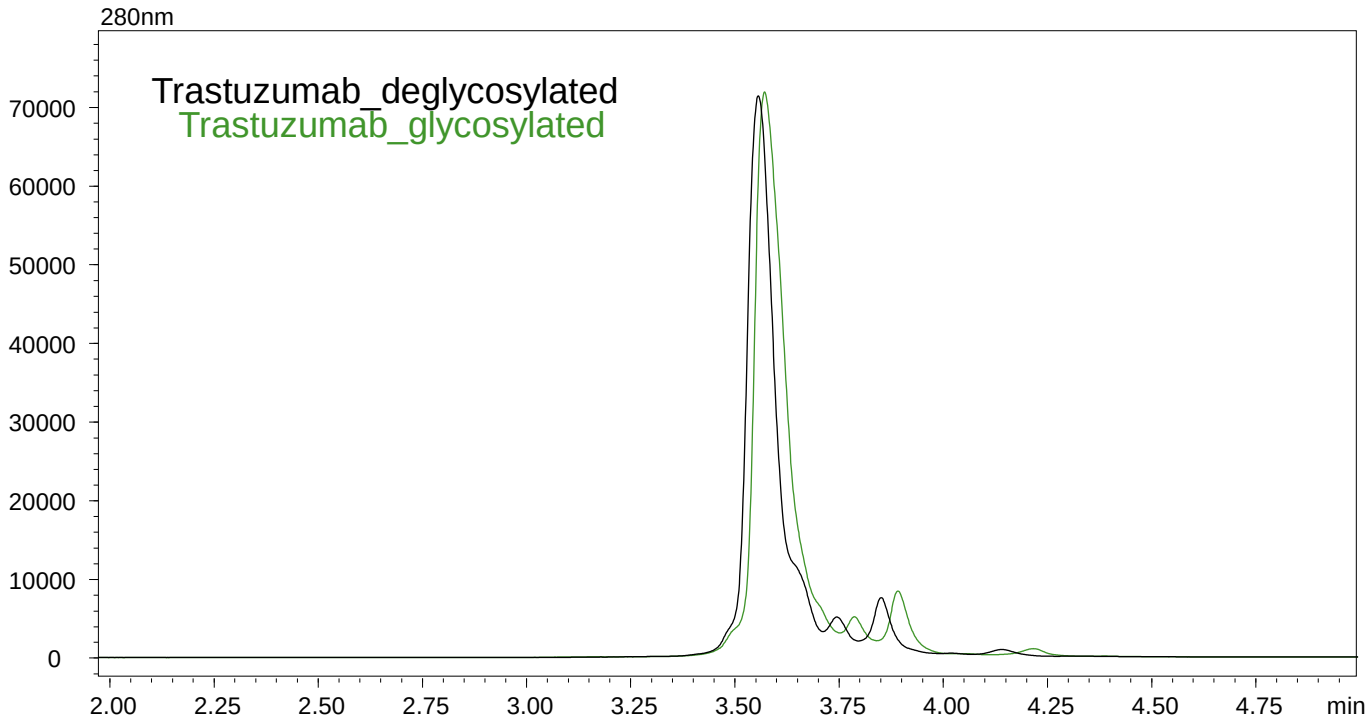
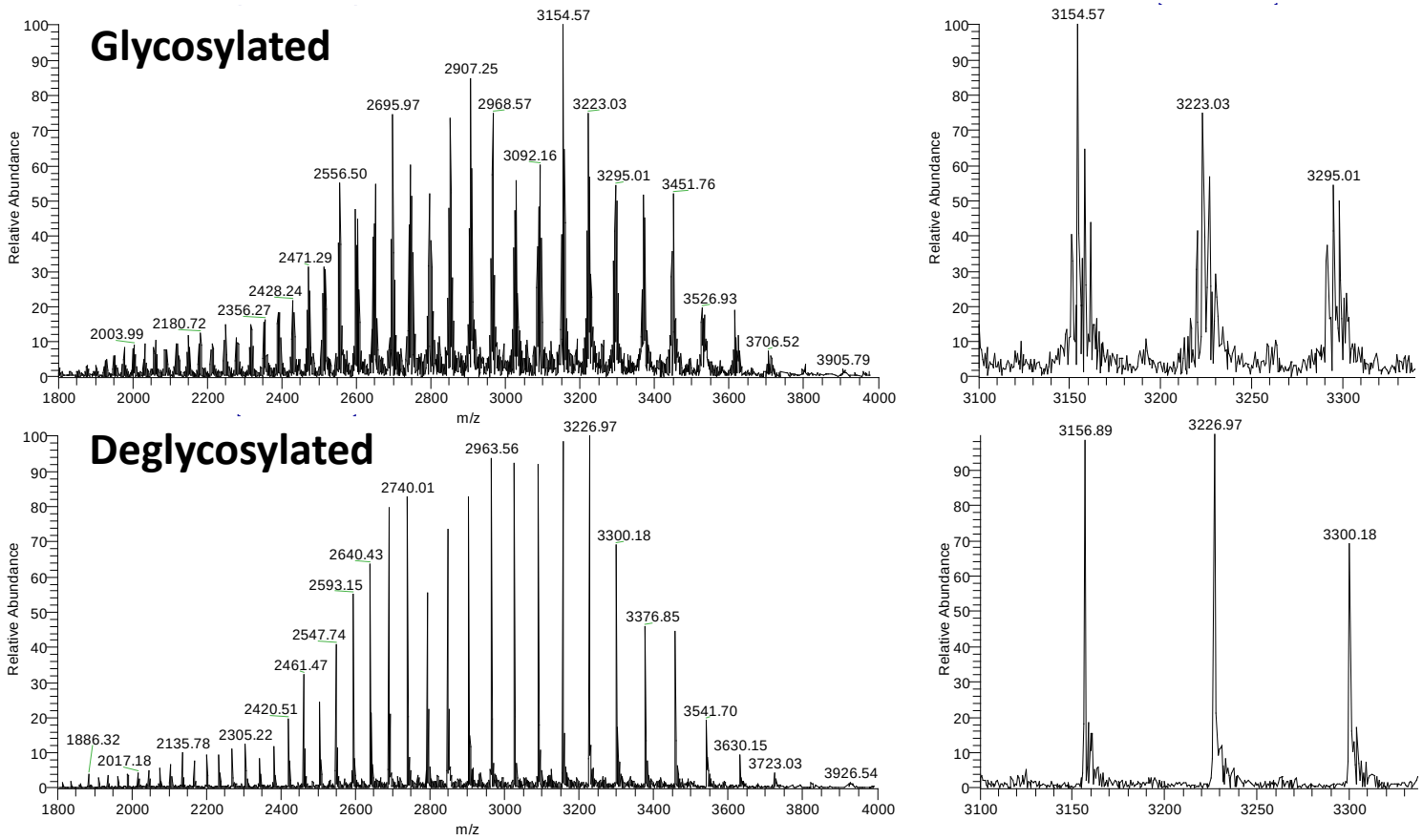
Glycosylated vs. Deglycosylated mAbs RP-LC/MS

Column: 2.1 x 150 mm; Halo Protein C4 400 Å; Flow rate: 0.4 mL/min; Mobile Phase A: water/0.1% DFA; Mobile Phase B: acetonitrile/0.1% DFA; Gradient: 27-37% B in 10 min; Injection Volume: 2 µL (1 µg); Temp: 80 ^{o}C

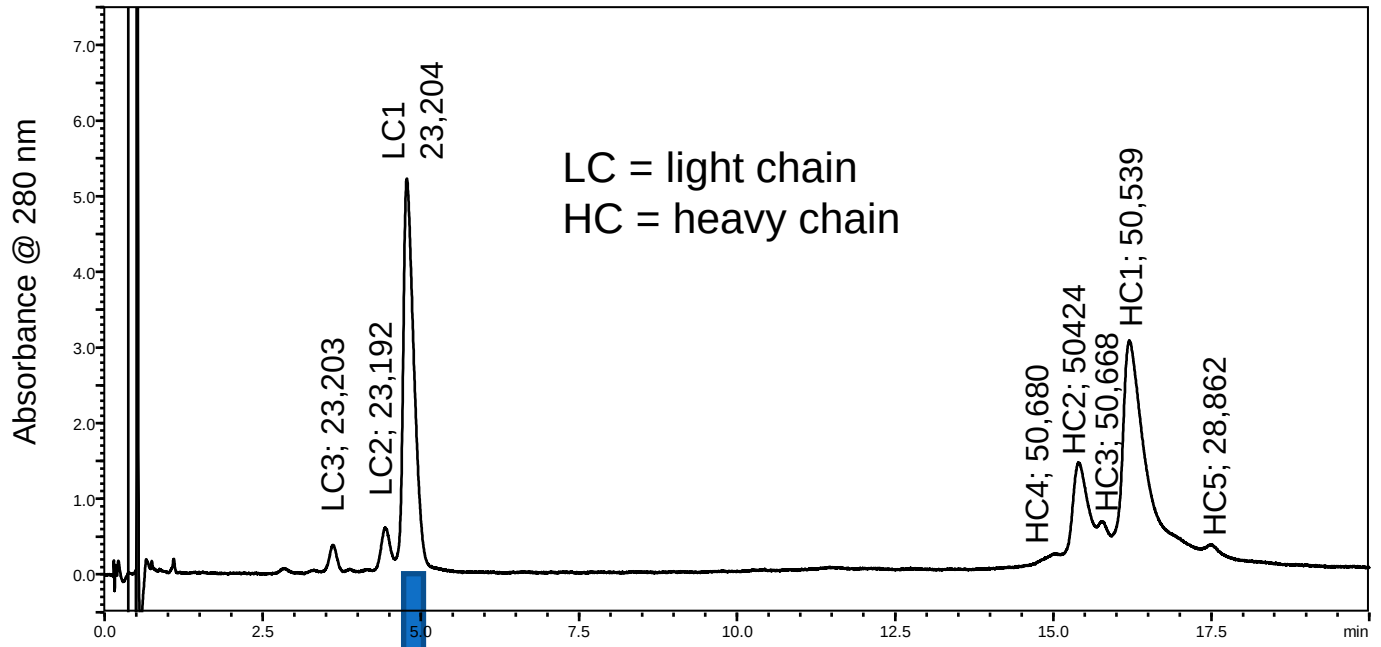


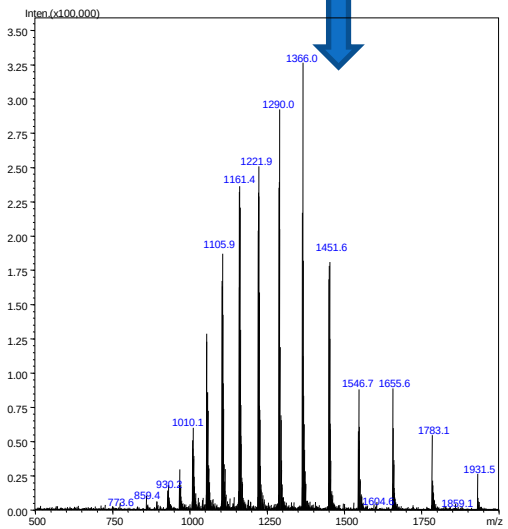
Glycosylated vs. Deglycosylated mAbs MS



	Go/GoF		GoF/GoF		G1F/GoF		G1F/G1F, G2F/GoF		Deglycosylated PNGase F	
	Theoric (Da)	Measured (Da)	Theoric (Da)	Measure (Da)	Theoric (Da)	Measured (Da)	Theoric (Da)	Measured (Da)	Theoric (Da)	Measured (Da)
Trastuzumab	147911	147909	148057	148056	148219	148218	148381	148385	145167	145173
ΔMass (glyc) Trastuzumab	2744	2736	2890	2883	3052	3045	3214	3212		6

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.

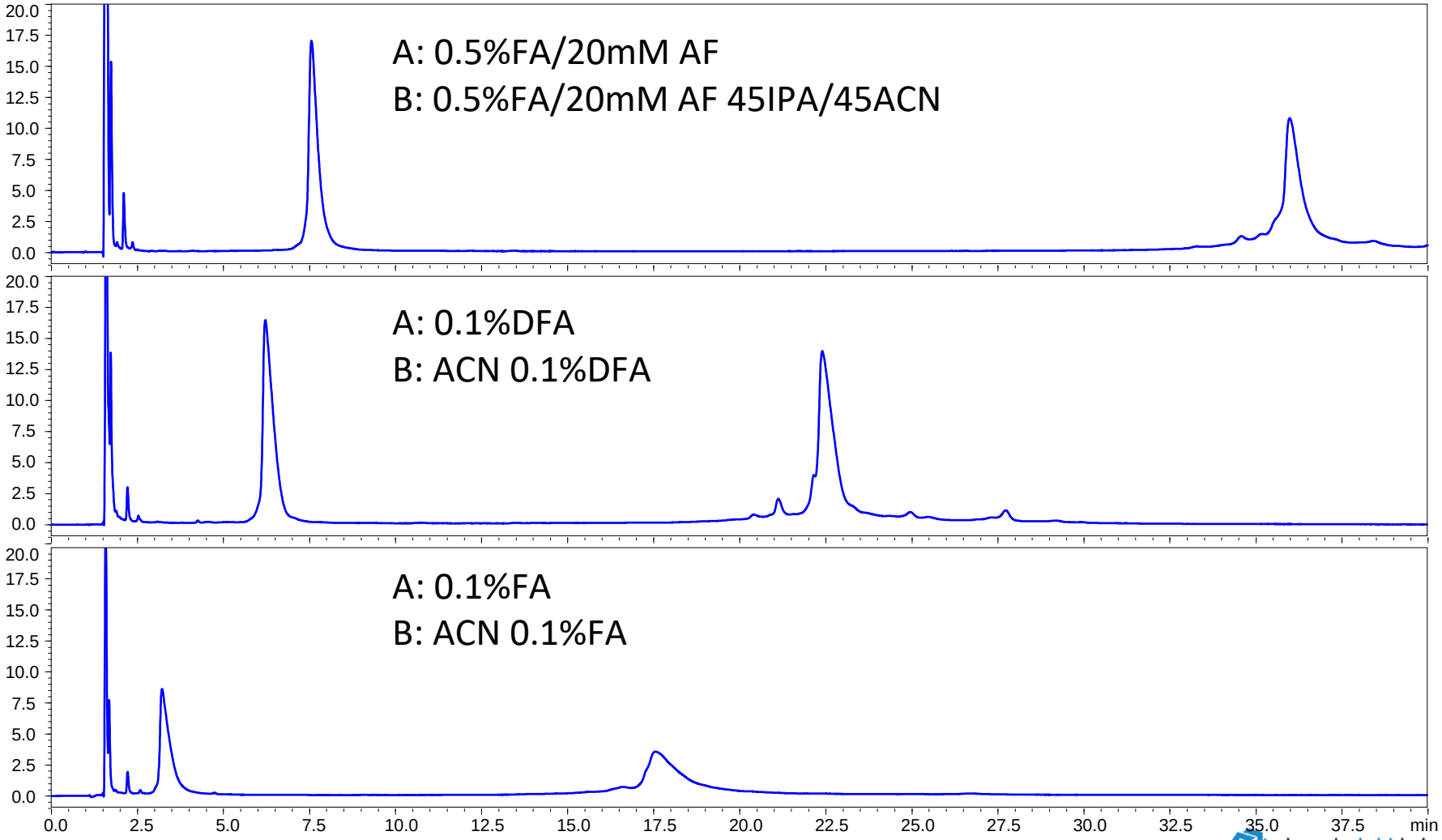
Column: 2.1 mm ID x 100 mm HALO Protein C4 400 Å
Flow rate: 0.4 mL/min.
A: 0.5 % formic acid with 20 mM Ammonium Formate
B: 45% AcN/45% IPA/ 0.5 % formic acid with 20 mM Ammonium Formate
Gradient: 29-32% B in 20 min.
Temperature: 80°C
Detection: 280 nm
MS Conditions: Shimadzu LCMS-2020, ESI +4.5 kV, 2 pps, 500-2000 m/z

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

Reduced mAb Chain Separation: MP Effect

Reduced SiluMab

2.1 x 150 mm HALO Protein C4 400 Å; 30-35%B; 40min ; 0.2mL/min; 80°C



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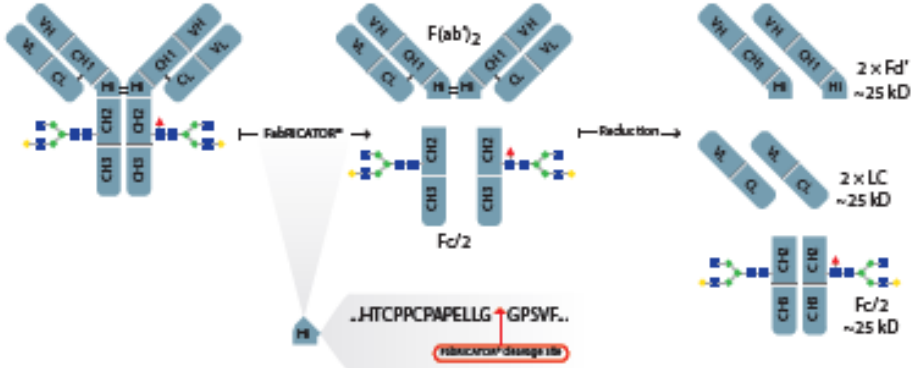
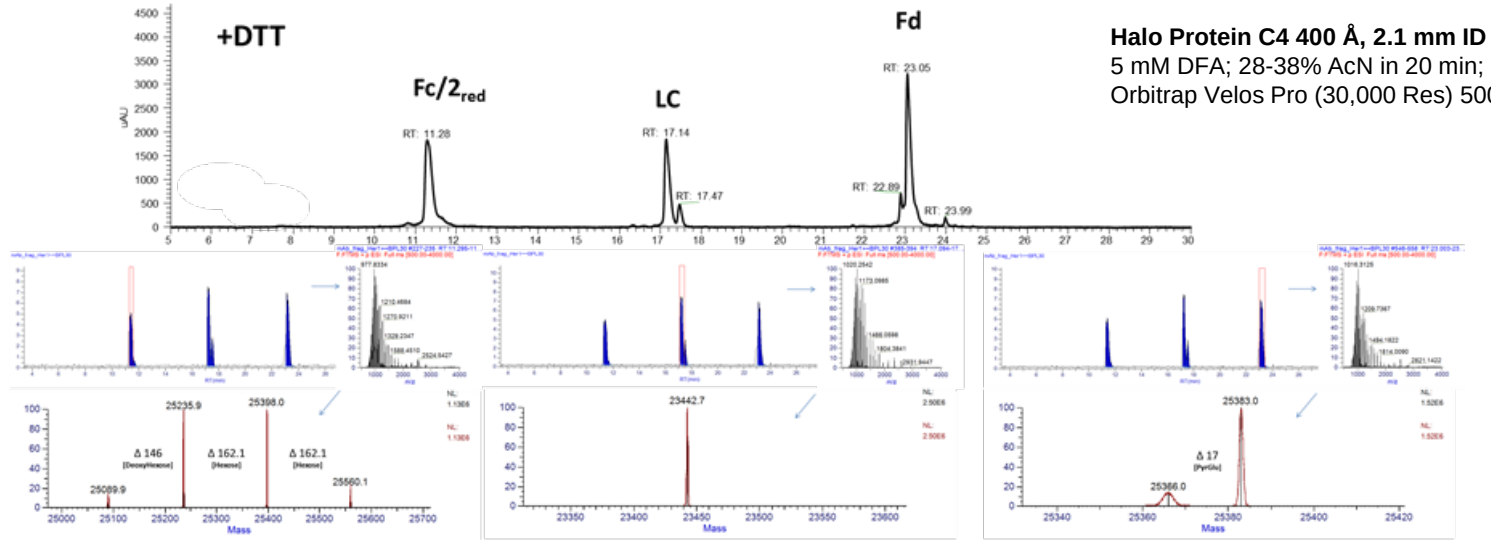
Fragments for mAb Structure: IdeS

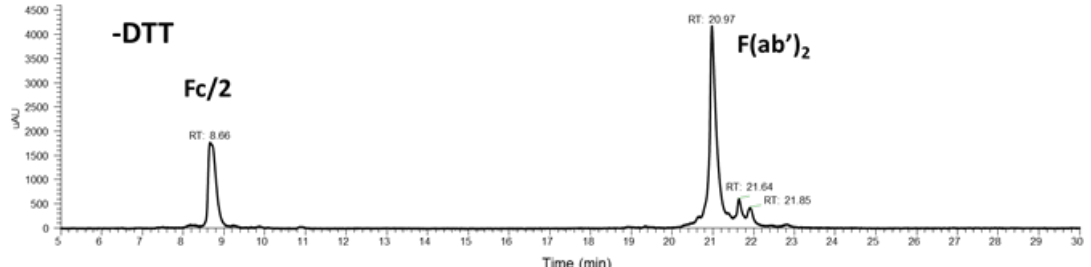


http://www.genovis.com/fabricator

An, Zhang, Mueller, Shameem & Chen (2014) A new tool for monoclonal antibody analysis, mAbs, 6:4, 879-893,
DOI: 10.4161/mabs.28762



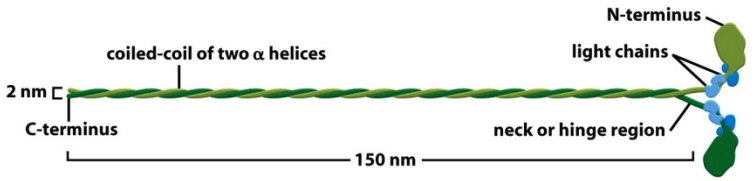
Halo Protein C4 400 Å, 2.1 mm ID x 150 mm;
5 mM DFA; 28-38% AcN in 20 min; 0.35 mL/min, 80 ºC;
Orbitrap Velos Pro (30,000 Res) 500-4000 m/z, +3.8 kV, 275 ºC desolvation capillary



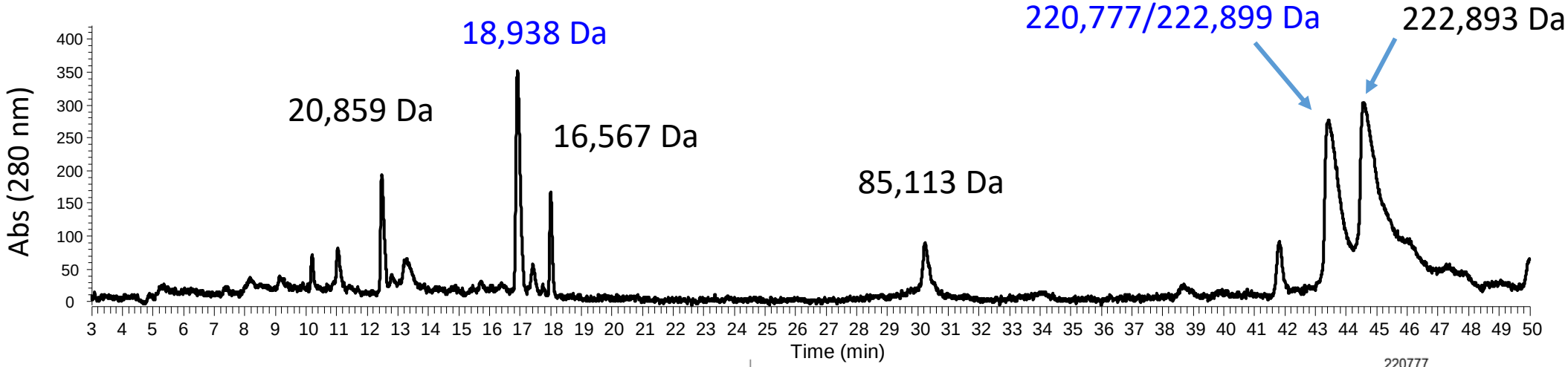
High Resolution Separations for Protein LC/MS. ASMS 556
B Boyes, B Libert, S Schuster, B Wagner, W Miles, J Kirkland

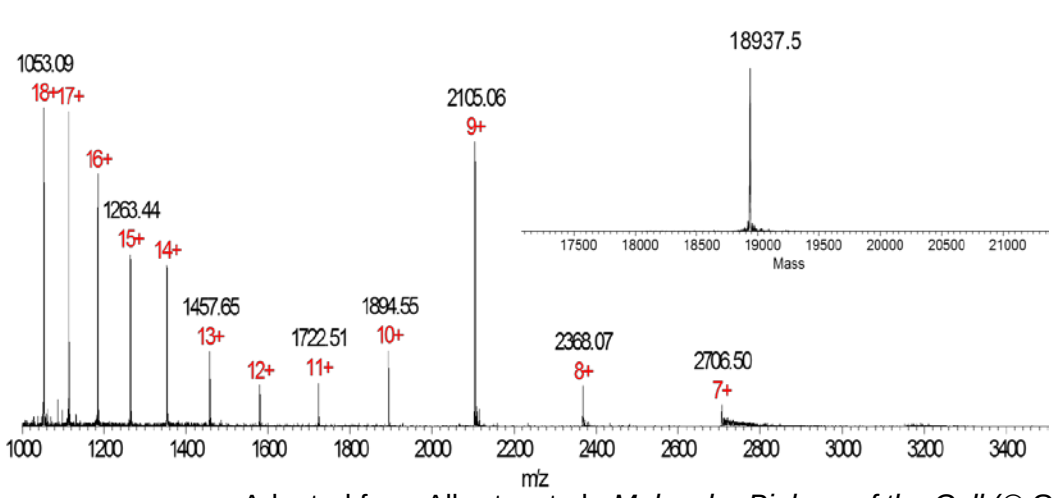
Myosin LC/MS using 1000 Å Fused-Core Particles

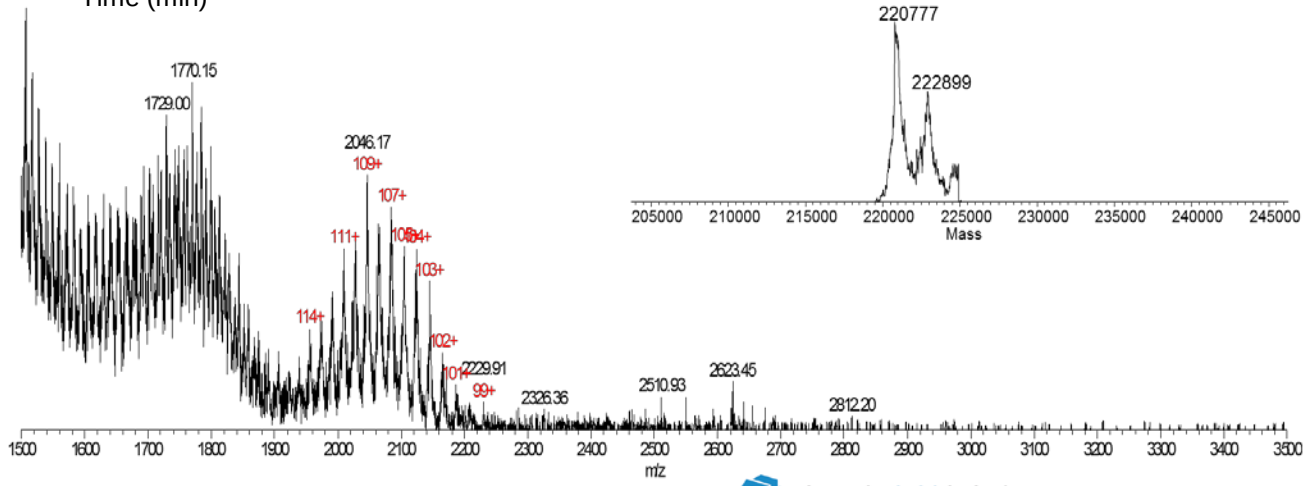
Rabbit Skeletal Myosin Structure
2 HC subunits @ 220 kDa
4 LC subunits (2 x RLC @ 20 kDa + 2 ELC (17, 25 kDa))



2.1 x 100 mm, 2.7 µm Fused-Core, C4, 1000 Å; 0.1% DFA 33 – 48% AcN in 50 min.;
Flow rate: 0.2 mL/min; Temp: 75°C; 2.5 µg myosin (7 M Urea/1% HOAc).







Adapted from Alberts, et al., *Molecular Biology of the Cell* (© Garland Science 2008)

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Summary and Future Work

- Improving protein LC/MS is both materials and chemistry.
- DFA is current best practice in our labs, with more than 2 years of practical experience indicated no detrimental effects on MS or LC hardware. Exploring potential benefits of mixtures of DFA/FA to manipulate selectivity and possibly sensitivity.
- Fused-Core with enlarged pore sizes (400 and 1000 Å) have particular utility for protein analyses, are highly robust, and allow *faster* protein separations with *higher* efficiency.
- Continuing focus on the use of new materials (Mobile Phase and Stationary Phases) to enable larger biomolecule LC and LC/MS analysis.

Acknowledgements

Thank you for your Attention!

- AMT – Drs. Jack Kirkland, Joe DeStefano, Mark Schure, Mr. Mark Haynes, Ben Libert, Will Miles and Bob Moran.
- Proteomics and Glycoproteomics
  - Prof. Ron Orlando, U.Ga.
    - D.J. Johnson, Shujuan Tao, Yining Huang, Emily Betchy and Majors Badgett, CCRC, University of GA.
- AMT and NIH/SBIR Program for Financial Support