High Resolution Separations for Protein LC/MS

Barry E Boyes^{1,2}, Benjamin Libert¹, Stephanie Schuster¹, Brian Wagner¹, William Miles¹, Joseph Kirkland¹ ¹Advanced Materials Technology Inc., Wilmington, DE; ²Complex Carbohydrate Research Center, University of Georgia, Athens, GA

Outline

- Improved protein analyses are obtained by selection of appropriate mobile phase conditions, combined with a new generation of superficially porous particles (SPPs).
- Mobile phase acidic modifiers are compared, including several novel acid modifiers which exhibit improved LC/MS performance.
- Formic acid (FA) exhibits relatively poor LC performance and trifluoroacetic acid (TFA) the best. ESI-MS intensities show the opposite trend, with FA the best, TFA the worst, and Difluoroacetic acid (DFA) as a good compromise between MS and LC performance.
- Optimized SPP column packing materials show high resolution, moderate backpressures, and fast separation capabilities, relative to sub-2μm column packing materials, particularly for large proteins (IgG and above).

Introduction

Protein therapeutics and protein reagents continue to find expanded use in research and health care. This contributes to a highly active growth in protein analysis by LC and LC/MS. Many of the proteins of interest are quite large, for example monoclonal antibodies and other multi-subunit proteins, and these present special problems in terms of resolution and separation speed. Present methods for separating and characterizing proteins include various chromatographic separation approaches such as ion-exchange, size-exclusion, hydrophilic interaction, hydrophobic interaction, and reversed-phase. The latter method is especially attractive for many applications because of the capability for efficient and fast separations, using conditions that can be integrated with subsequent analytical tools, most importantly, with MS detection. Improvements in protein separations using conditions that take advantage of ongoing improvements in MS instrumentation are needed. We have previously described the use of superficially porous silica particle materials for small and moderate size molecules, and most recently have extended this approach to much larger molecules, including proteins [Schuster, Wagner, Boyes and Kirkland; J. Chromatogr. A <u>1315</u> (2013) 118; other references]. Examples of high resolution protein separations with novel advanced variant SPP particles are shown herein. In the course of conducting this analysis of stationary phase materials for protein separations, we have identified significant opportunities to improve resolution, while addressing limitations of typical LC conditions (eg., use of TFA), for application to MS analysis. Of the examined mobile phase modifiers, we found that difluoroacetic acid (DFA) has shown the best combination of separation performance and signal generation for online MS detection.

Materials and Methods

Columns of HALO Protein C4 were produced at Advanced Materials Technology Inc. (Wilmington, DE). These materials employ superficially porous Fused-Core® silica particles of 1.5 - 3.4 μm diameter, shell thicknesses of 0.1-0.35 μm, and pore sizes of 400 to 1200 Å.

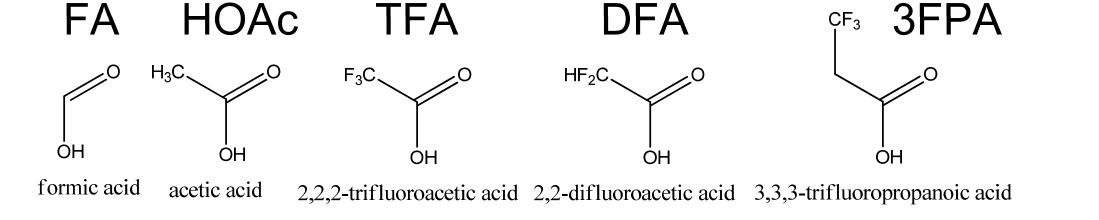
Mobile phase modifiers were obtained from Pierce (TFA, FA), Sigma/Millipore (TFA, FA, DFA, AF), or Synquest Laboratories (DFA, 3FPA). Acetonitrile was MS grade from JT Baker. Synthetic peptides were from AnaSpec, and IdeS protease from Genovis.

Analytical protein separations used the Shimadzu Nexera LC-30 components (40 μL mixer), with the SPD 20A UV detector and MS-2020 quadrupole MS operated in series at +4.5 kV capillary potential. A special low volume flow cell was obtained from Shimadzu Scientific for this effort, to minimize band dispersion effects. Capillary column separations used the Dionex RSLC 3000 with a trap column, connected to the Orbitrap VelosPro MS (ThermoScientific, Inc.), with the low flow IonMax ESI interface operated at 3.8 kV potential. Intact protein MS spectra were recorded in the Orbitrap, using 15,000 resolution, whereas fragments used 30,000 or 60,000 resolution scans.

Deconvolution of MS spectra used MagTran v1.02 (based on ZScore [Zhang and Marshall; JASMS <u>9</u> (1998) 225]), or Thermo Scientific Protein Deconvolution v 4.0. Chromatographic peak widths are reported as half height (PW_{1/2}).

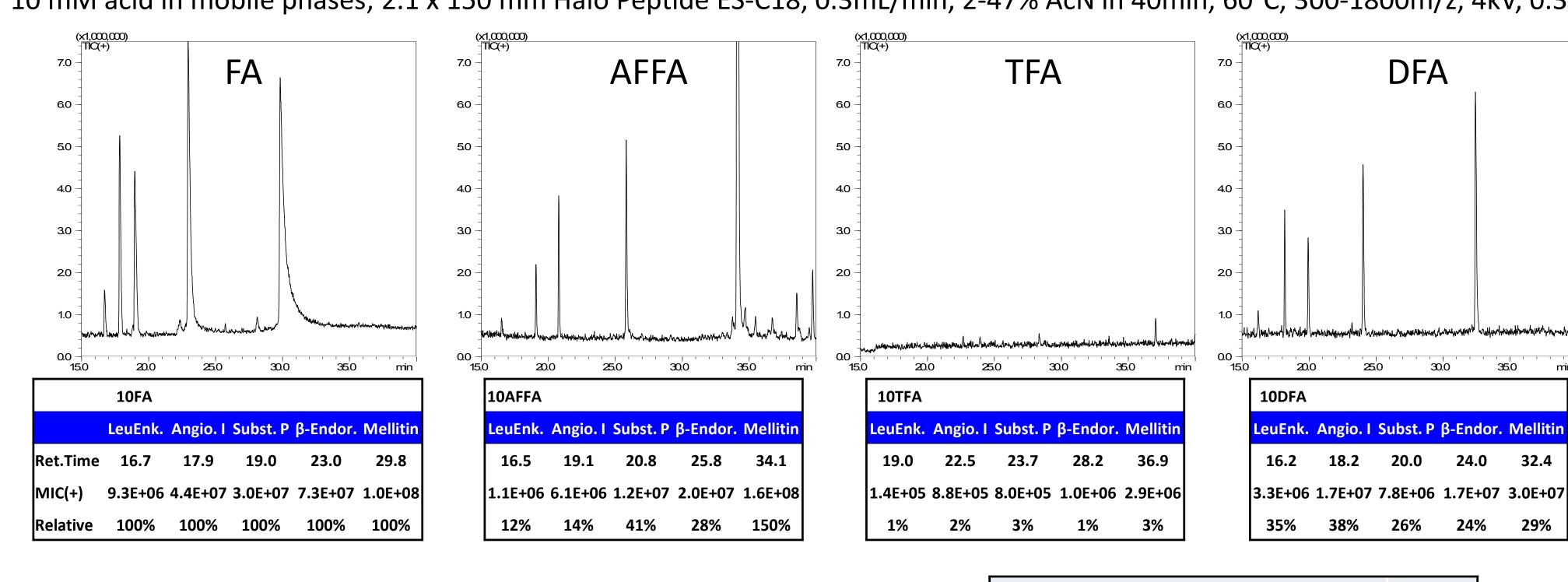
Acidic Mobile Phase Modifiers for LC/MS Analysis

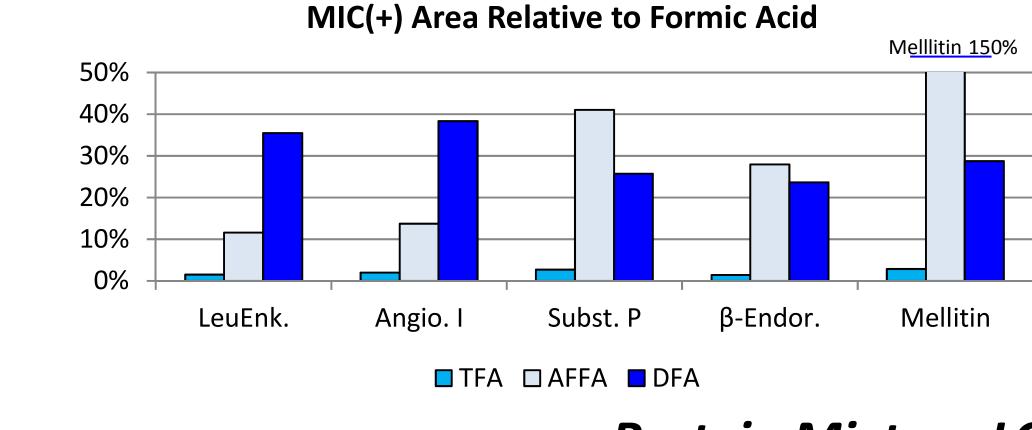
- TFA is an acidic mobile phase modifier of choice for protein and peptide separations, with good peak shape and high column efficiency.
- TFA can be a bad choice for LC/MS, due to ESI suppression (low signal), background problems (chemical noise), and system persistence (requiring extensive cleanup of an LC/MS for eliminating carryover).
- Alternative acidic modifiers that allow good peak shape, recovery, selectivity, and detection capabilities (absorbance and ESI signal intensities) are needed; many have been examined, preferred acids are shown below.
- Acceptable HPLC separation performance was obtained for FA. TFA, DFA and the mixture of FA with ammonium formate (FAAF), as assessed with UV and MS detection retention, peak widths and resolution. Comparisons of peptide separations (mixtures of synthetic and tryptic digests) and protein mixtures with inline MS detection shows TFA very strongly suppressed ionization, DFA was moderate, and FA or FAAF supported robust detection.

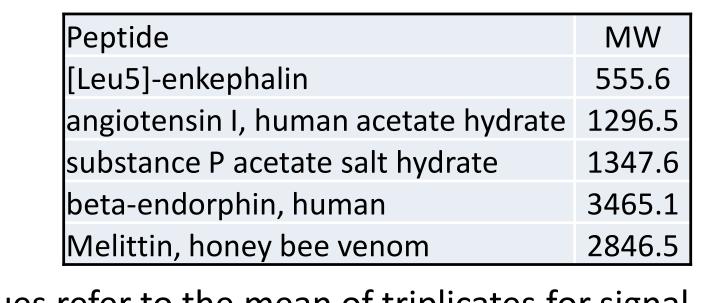


Synthetic Peptide Mixture LC/MS in Several Acidic Modifiers

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

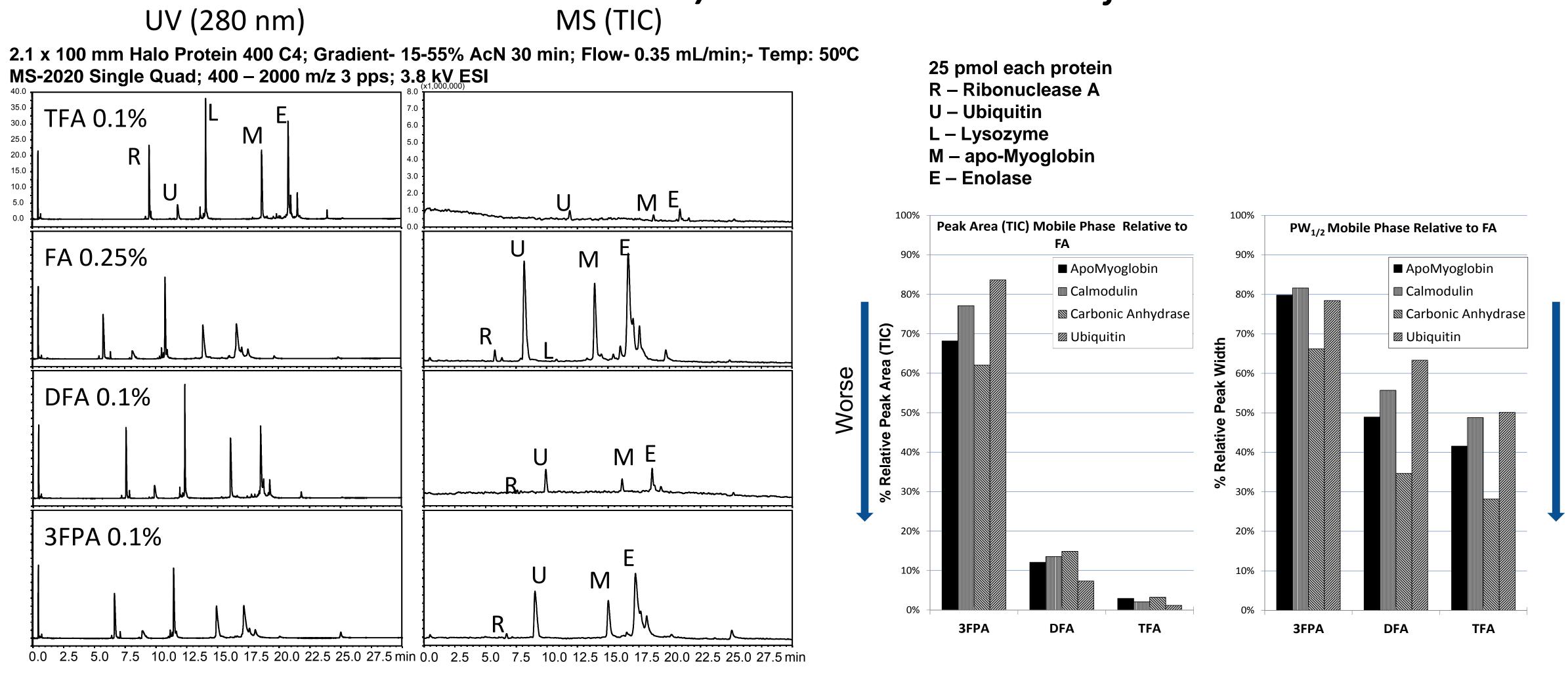






alues refer to the mean of triplicates for signal intensities at 50 pmol of each peptide. Relative Standard Deviations were less than 10%.

Protein Mixture LC/MS in Several Acidic Modifiers

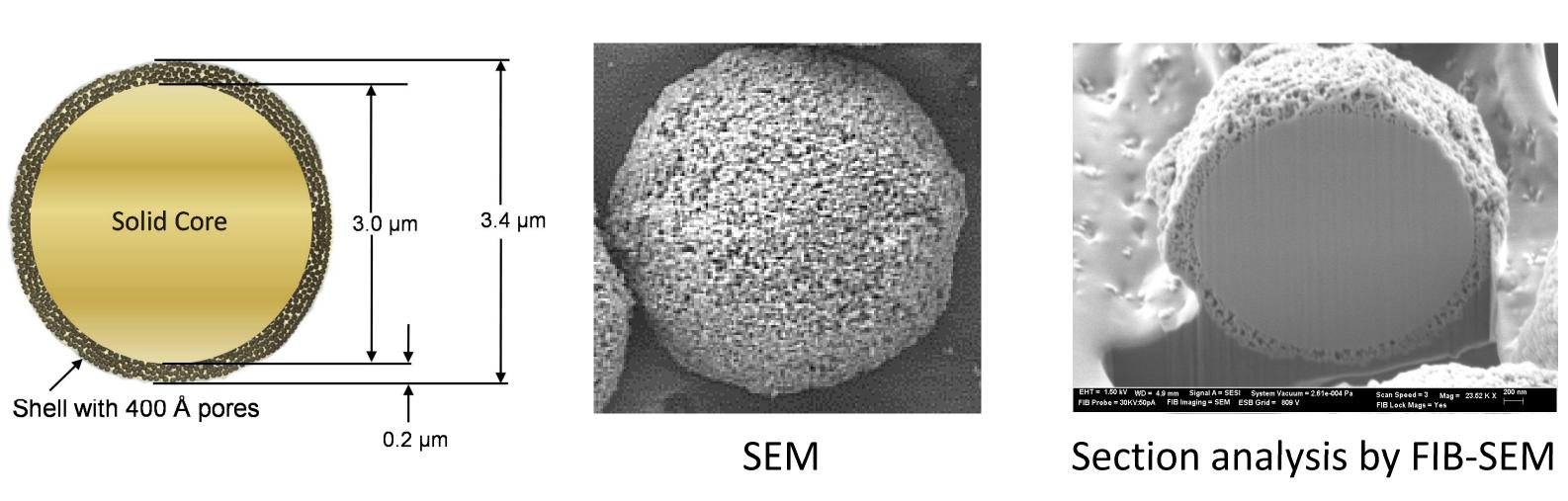


- Chromatograms compare LC/MS and LC performance for either 0.1% or 10 mM of each acid modifier to 0.25% or 10 mM FA. The higher FA content was required for reasonable protein peak shape and recovery.
- In addition, each acid was examined at varying concentrations (2-50 mM for fluorinated acids; 20-500 mM for FA), exhibiting progressive suppression of ESI signal with concentration: plateau at 50 mM for FA, 10-20 mM the other acids
- Improved peak widths and tailing for proteins over the range of 5-20 mM, except FA, which required 100 mM to maximize performance

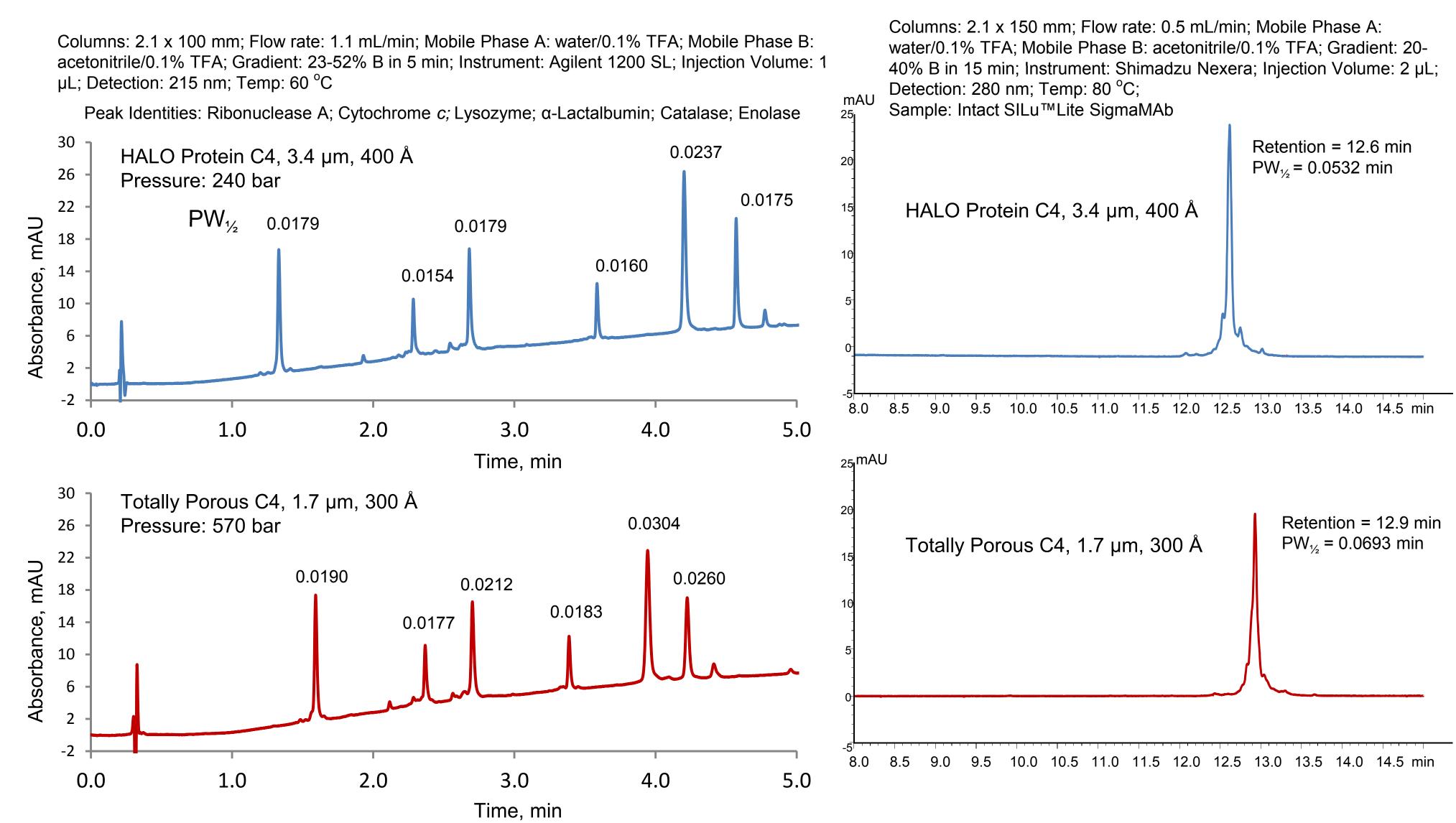
Improved SPP Particles for Separations of Proteins

- New large pore (400 Å and above) SPP silica materials have been created for protein separations. The schematic below shows the characteristics for the 3.4 µm diameter SPP particle with 400 Å pores. These materials allow highly efficient packed columns, exhibiting fast protein separations, even for very high molecular weight proteins and polypeptides (for example, IgG and myosin).
- Comparisons of protein separations show improvements in band widths and resolution, even when compared to much smaller diameter (sub- 2μ m) particles, without the disadvantage of high column backpressures.

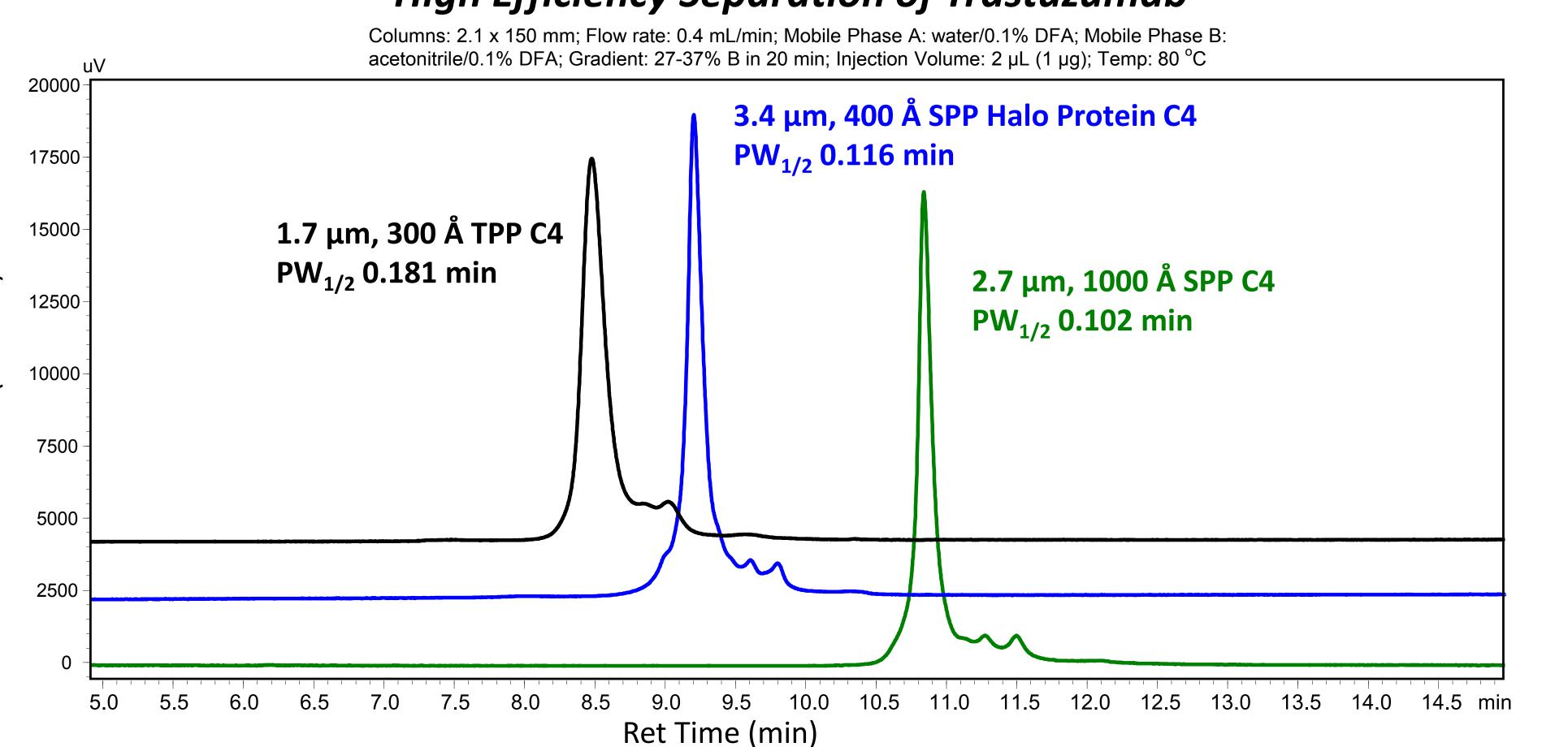
HALO® Fused-Core 400 Å Protein Particle



Large Pore SPP Improve Protein Separation Efficiency



High Efficiency Separation of Trastuzumab

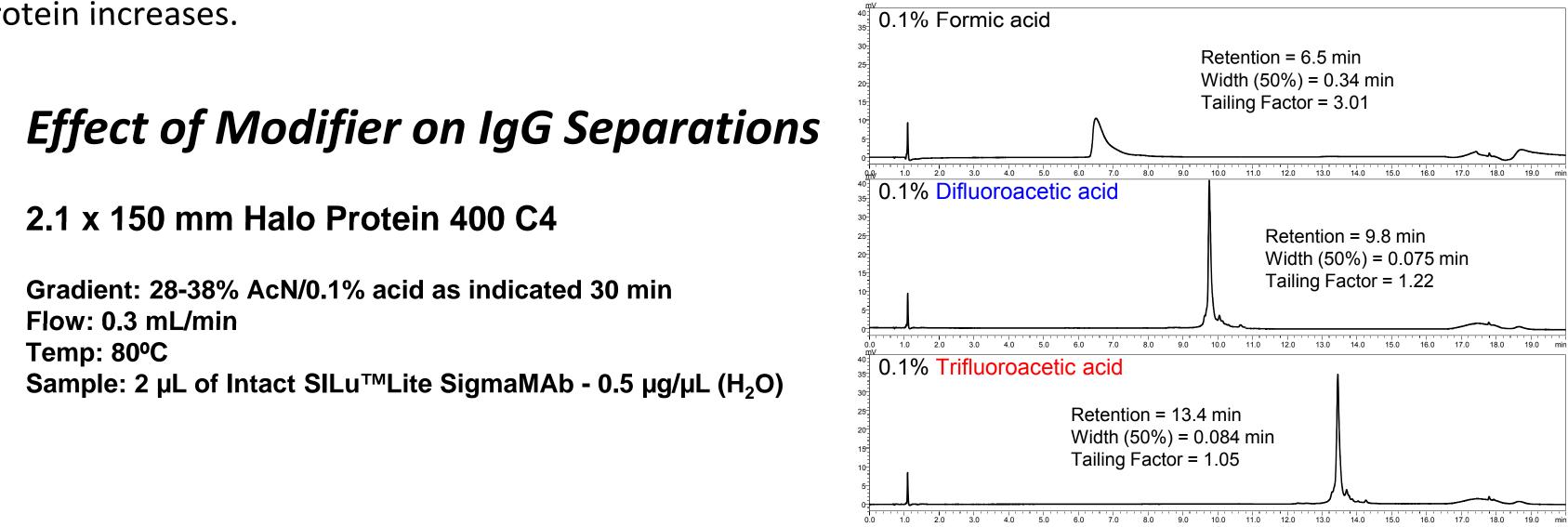


• High resolution and column efficiency is shown for the large pore 400 Å material, as well as an example of a new prototype 2.7 μm particle with 1000 Å pores with a 0.5 μm shell.

Application of Improved Materials and Conditions

Presented at ASMS 2016 Poster 556

- DFA as an alternative to TFA and FA as the acidic modifier for RP-HPLC with and without MS detection has been employed during the past 2 years, with no unwanted issues with LC or MS hardware (normal bore and capillary LC, single quad and Orbitrap MS)
- Separations of several intact IgGs (and other proteins) confirm that LC performance in FA is much poorer than in either TFA or DFA
- LC/MS comparisons of separations using DFA and FA, or mixtures thereof, indicate that DFA has useful LC properties, while allowing high resolution MS analysis, with moderate reduction in ionization. We note that fluorinated ion pairing acids decreased protein average charge state for all proteins examined
- SPP wide pore materials exhibited increased efficiency for protein separations, particularly as the size of the protein increases. 0.1% Formic acid



Analysis of IdeS Fragments of IgG mAb (Trastuzumab)

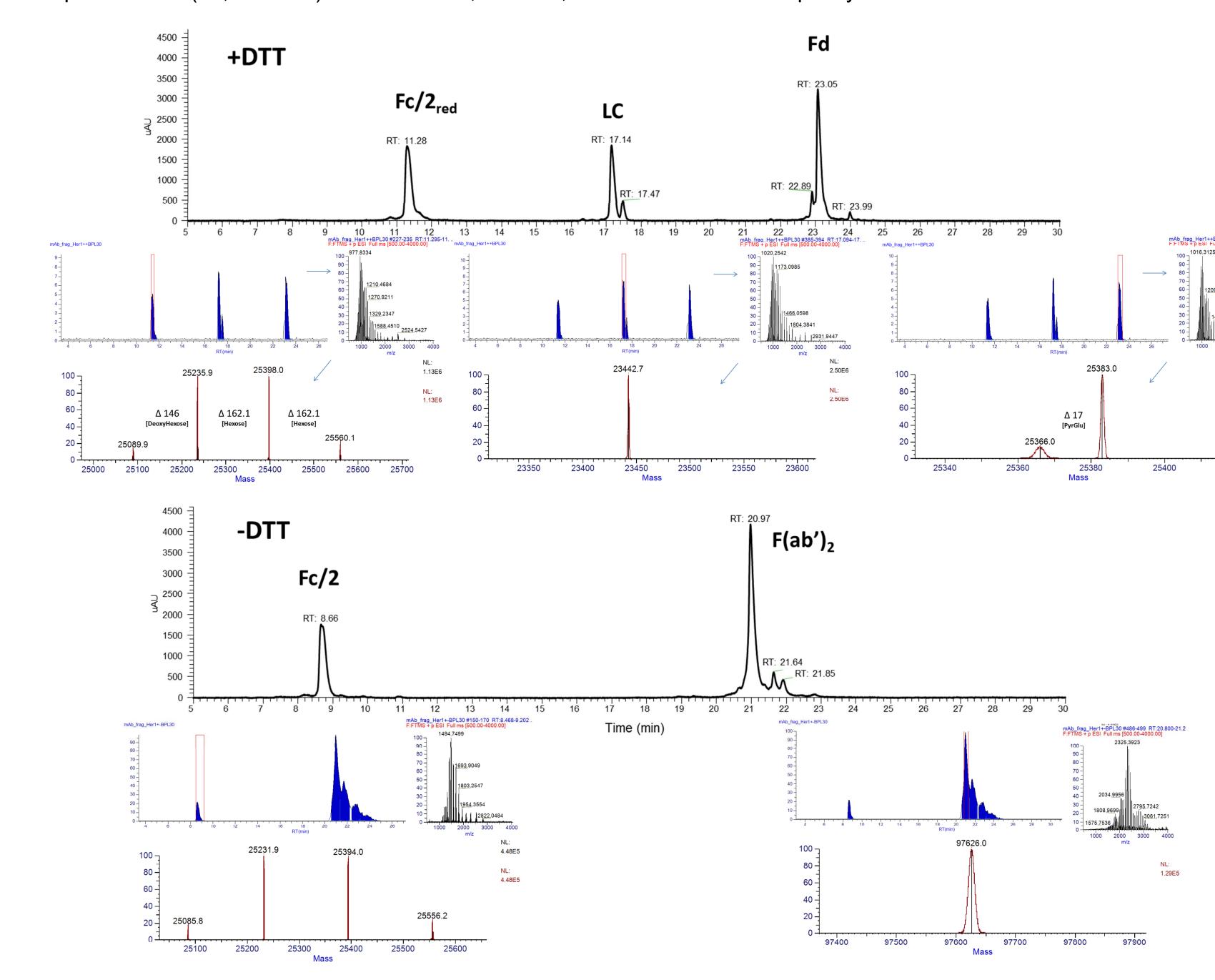
2.1 x 150 mm Halo Protein 400 C4

Flow: 0.3 mL/min

Temp: 80°C

Gradient: 28-38% AcN/0.1% acid as indicated 30 min

Prototype 1000 Å pore size 2.5 µm Halo SPP C4, 2.1 mm ID x 150 mm; 5 mM DFA; 28-38% AcN in 20 min; 0.35 mL/min, 80 °C; Orbitrap Velos Pro (30,000 Res) 500-4000 m/z, +3.8 kV, 275 °C desolvation capillary



• The use of DFA mobile phase with a prototype 2.5 μm Halo 1000 Å pore material allows high resolution separations and accurate high resolution mass analysis of IgG fragments, to confirm glycovariants and disulfide bridges.

Conclusions

- Novel superficially porous particles improve RP HPLC separations of many protein mixtures and fragments, permitting higher efficiency separations than previously available.
- Alternative acidic mobile phase modifiers can be employed for useful protein separations, in some cases exhibiting a better compromise between separations performance and ESI-MS compatibility

Acknowledgements: Tim Langlois, William Miles and Bob Moran for advice and technical assistance. Supported in part by NIH Grant GM116224 (BEB). Opinions expressed are solely those of the author.