3µm Monodisperse IEX Media

3µm monodisperse particles for Ion Exchange Chromatography stationary phases and their impact on the separation of biomolecules

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Abstract

Monodisperse particles are highly uniform in size with a narrow particle distribution. This consistency presents opportunities to design high efficiency chromatography solid phases with high reproducibility. Here, monodisperse particles are investigated as a platform for protein ion exchange phases with uses in a range of biopharmaceutical applications.

Introduction

Fundamentally, liquid chromatography (LC) separations are achieved by taking advantage of differences in analyte affinities for the solid phase and liquid phase in the chromatography column. The equilibration of each analyte with the solid and liquid phase is determined by the surface chemistry of the particle and chemical make up of the liquid phase. Additionally, the fluid mechanics, determined by packed bed structure, and analyte mass transfer, controlled by diffusion and convection, significantly influence the analyte separation quality, particularly for analytes that are closely eluting due to small differences in affinity for the solid phase. As such, chromatographers continually seek to develop new base particles and new surface chemistries to improve analyte separation within complex samples for quantitative and qualitative characterization.

Biological samples typically have many different analytes owing to molecular heterogeneity, low sample purity, and sample matrix complexity. Protein samples are inherently very complex due to their size (ranging from ~1 kDa for peptide fragments to 150 kDa for monoclonal antibodies and MDa for AAV capsids) and general heterogeneity arising from variability in the cellular manufacturing process (e.g., glycosylation, deamidation, and other post-translational modifications). Protein sample peaks are rarely baseline resolved and variants and impurities often appear as shoulders making analysis difficult. As such, precise control over the properties of the solid phase are critical to ensuring high resolution, reproducible separations.

The solid phase is often composed of a base particle with surface chemistry that is subsequently conjugated to the particle surface to control sample interactions. Base particle morphology (e.g., porous vs non-porous, size, and shape) determines column fluid mechanics via packing structure and mass transfer properties (to the particle surface and within pores) of the chromatography column. The base particle chemistry (e.g, silica vs polymeric, hydrophobic vs hydrophilic, etc.) itself may be used for the chromatographic separation or be modified with additional chemical layers to control particle-sample interactions. The particle morphology combined with each layer of chemistry increases the overall complexity of the solid phase and requires precise control to ensure good separations.

Tightly controlling the chemical and phsyical base particle properties provides a robust foundation for surface modification and column packing. Silica and polymeric particles commonly used for LC columns have variation in their particle size (i.e., polydispersity), which can lead to variability in the surface chemistry modification (e.g., through surface area differences) and packing (e.g., column pressure, bed stability, etc.). By contrast, monodisperse particles have a near uniform, single particle size providing consistency in particle properties compared to polydisperse separations. This size control is of particular importance as particle size decreases as the relative quantity of "fines" (particles much smaller than the modal particle size) increases and may cause irreproducibility in particle functionalization and column packing. Figure 1 highlights this difference by comparing monodisperse (left) and polydisperse (right) particle distributions that both measure a 3µm modal pore size (scale bars = 10µm) on a Coulter Counter. Notably monodisperse particles are consistent in size with no fines. By contrast, a wide range of particle sizes are observed for the polydisperse particle with examples of fines indicated by red arrows.

In this poster we evaluate the use of a 3µm monodisperse particle as a platform for Ion Exchange Chromatography with a specific focus on protein chromatography applications. Initial experiments focus on evaluation of the particle itself using a Weak Cation Exchange (WCX) chemistry. Subsequent data looks at applications related to Strong Cation Exchange (SCX) and Strong Anion Exchange (SAX) chromatography. We evaluate the performance of the resulting media using samples of varying complexity and molecular weight.
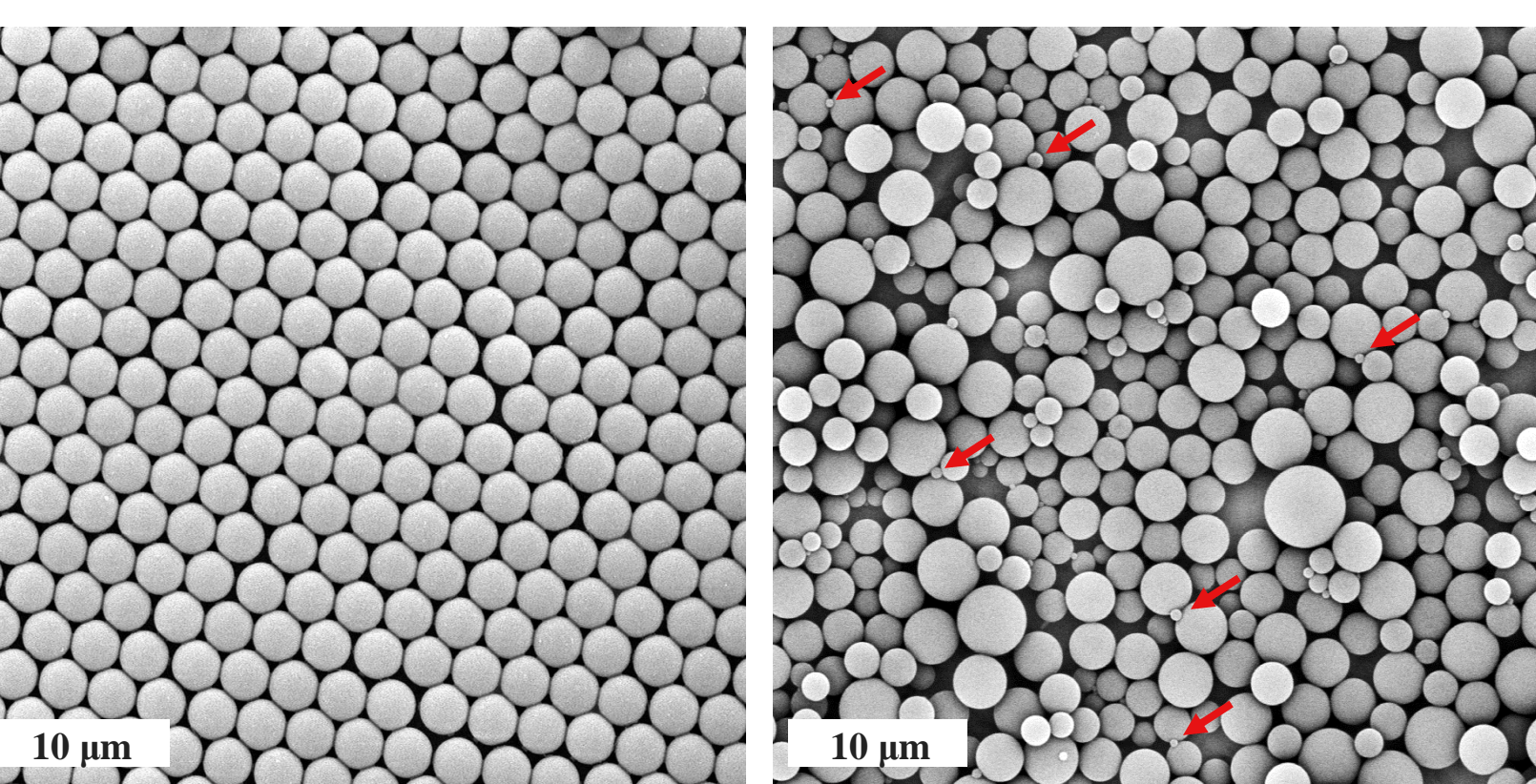

Figure 1. SEM images of monodisperse (left) and polydisperse (right) 3µm particle distributions as measured by a Coulter Counter. Red arrows indicate the examples of particle "fines" observed in the polydisperse particle distribution. Scale bars are 10µm in length.

Materials and methods

Sample preparation

Reference text and figures for specific sample information. IdeS Protease (FabRICATOR) was purchased from Genovis AB (Lund, Sweden). NISTmAb was purchased from NIST. Protein G was purchased from Thermo Fisher Scientific.

Test methods

Isocratic and salt gradient separations were performed using the indicated buffers and methods as indicated in each figure. All pH gradient separations were performed using Thermo Scientific™ CX-1 pH gradient buffers A (250 mL, PN: 085346) and B (250 mL, PN: 085348).

Instrument: A Thermo Scientific™ Vanquish™ Flex instrument configuration was used and included a System Base Vanquish Flex (P/N: VF-S01-A) Quaternary Pump (P/N: VF-P20-A), Column Compartment H (P/N: VH-C10-A), Split Sampler FT (P/N: VF-A10-A) with 25 µL sample loop, and a Variable Wavelength Detector (P/N: VF-D40-A) with a 2.5 µL Semi-Micro Bio Flow Cell (P/N: 6077.0300)

Columns: Prototype 3 and 5µm monodisperse particle WCX columns Thermo Scientific ProPac™ 3R SCX (4x100mm PN: 43103-104068) Thermo Scientific ProPac™ 3R SAX (4x100mm PN: 43203-104068) Reference text and figures for specific formats.

Data analysis

Thermo Scientific™ Dionex™ Chromeleon™ 7 Chromatography Data System Version 7.2.10 ES was used for data analysis.

Results

Particle design and modification

Particle size is a primary determinant of chromatography column performance with smaller particles giving higher efficiency columns with narrower peaks and thus higher resolution. A target 3µm particle size (see Figure 1) was chosen to maximize column efficiency compared to larger 5-10µm particles commonly used for many protein IEX columns. The 3µm non-porous particles are based on a highly cross-linked divinylbenzene polymer to produce a non-porous particle that is both mechanically and chemically robust. To mask the DVB and reduce hydrophobic secondary interactions, all particles were initially coated with a conformal, cross-linked hydrophilic polymer layer possessing ethoxy-hydroxyl functionality. Initial experiments focused on Weak Cation Exchange chemistry given our familiarity with 5µm and 10µm polydisperse particle medias. PEEK hardware was used for all columns in this work as PEEK has bio-inert properties that minimize secondary interactions with samples compared to metals.[1] As such, observed results can reliably be attributed to the functionalized media itself and not be attributed to hardware secondary interactions.

3µm Monodisperse Particle Performance – WCX Chemistry

Initial experiments sought to determine the benefits derived from particle distribution (i.e., dispersity) versus particle size. Figure 3 compares the isocratic separation of cytochrome C on 3µm and 5µm monodisperse particle columns and a 5µm polydisperse particle column. The 5µm monodisperse particle shows an efficiency gain of 72% over the polydisperse column. Further gains were made by decreasing the particle size to 3µm resulting in an additional 92% efficiency gain. A valley separating the basic variant of cytochrome C was also observed for the 3µm monodisperse media column further illustrating the gains in efficiency. These results show significant performance gains using monodisperse media with expected gains as particle size decreases.
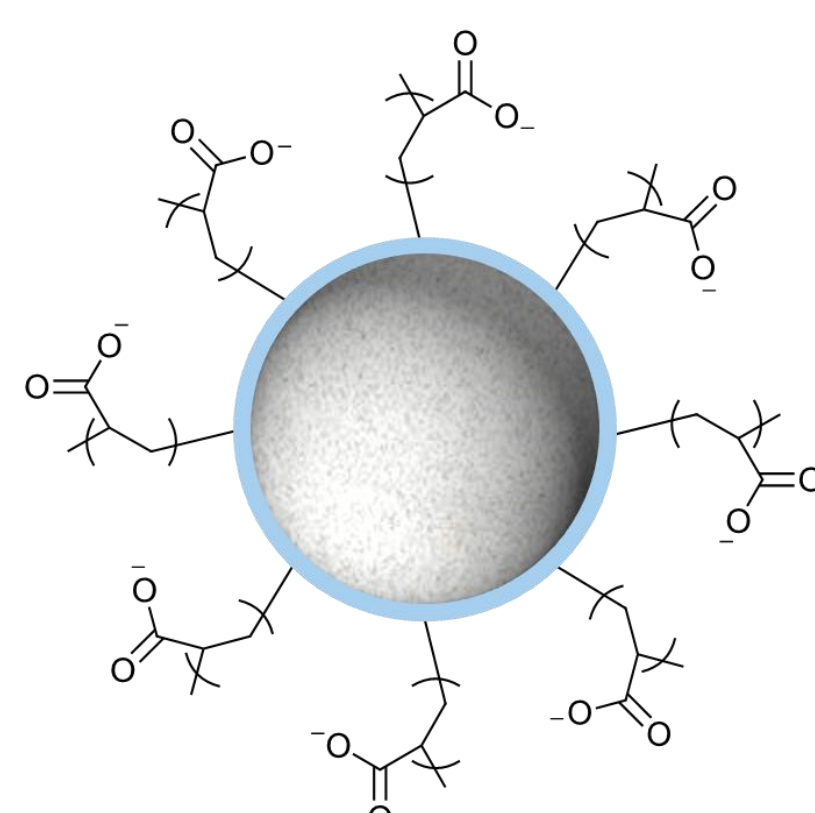

Figure 2. Schematic of particle functionalized with hydrophilic layer (blue) and polymeric WCX (acrylate) layer.
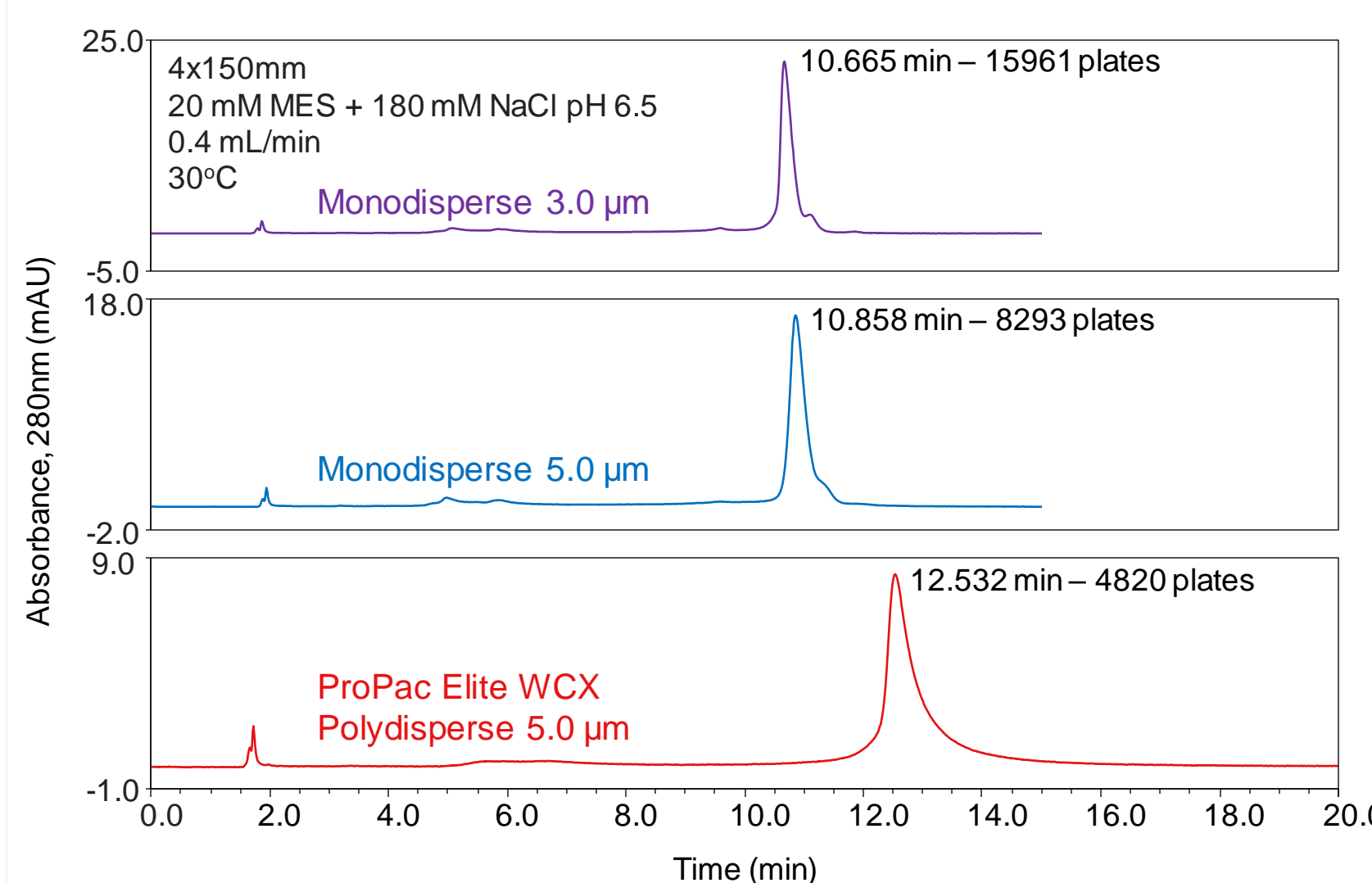

Figure 3. Comparison of isocratic cytochrome C separation using prototype WCX chemistry on 3µm monodisperse (top) and 5µm monodisperse (middle) particles and a polydisperse 5µm (bottom) particle.

Gradient separation using a 3-protein mix for monodisperse media was also evaluated with gradients ranging from 2.8-3.2µm shown in Figure 4. Retention time and variant separation was highly reproducible across all particle sizes indicating monodisperse particles provide a consistent surface for grafting both hydrophilic and functional chemistries. Minor differences in Ribonuclease A variants were observed for the 3.0µm particle which is attributable to a differences in sample age. The low MW proteins Cytochrome C (12.4 kDa) and Ribonuclease A (13.7 kDa) specifically showed significant decreases in PWHH (cytochrome C decreased from 0.097 to 0.072 min for polydisperse to monodisperse, data not shown). We note that the WCX media was created using a free radical polymerization approach that may also contribute to variability in media capacity. For SCX and SAX media, ATRP polymerization was used in combination with the hydrophilic layer for improved control of chain length and thus column capacity.
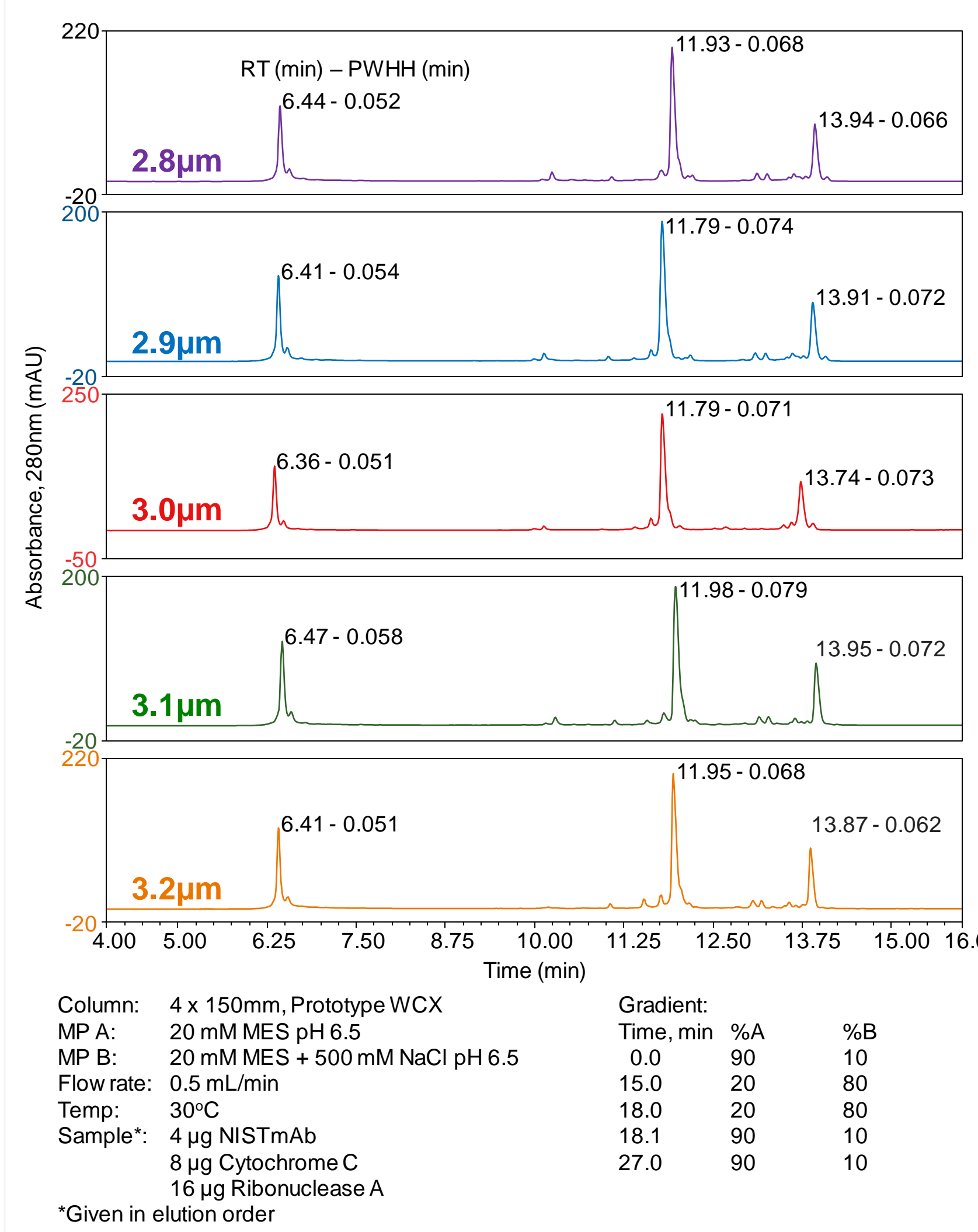

Figure 4. Comparison of gradient separation of a 3-protein mixture using prototype WCX chemistry on monodisperse particles sized 2.8, 2.9, 3.0, 3.1, and 3.2µm.

Column:	4 x 150mm, Prototype WCX	Gradient:		
MP A:	20 mM MES pH 6.5		Time, min	%A	%B
MP B:	20 mM MES + 500 mM NaCl pH 6.5		0.0	90	10
Flow rate:	0.5 mL/min		15.0	20	80
Temp:	30ºC		18.0	20	80
Sample*:	4 µg NISTmAb		18.1	90	10
	8 µg Cytochrome C		27.0	90	10
	16 µg Ribonuclease A				

*Given in elution order

Strong Cation Exchange 3µm Particle

The SCX particle is based on a sulfonate chemistry to provide cation exchange capability across the entire pH range. Initial experiments compared the 3µm monodisperse particle with commercially available 10µm and 3µm polydisperse particles using NISTmAb as a representative monoclonal antibody analyzed by SCX chromatography (Figure 5). Improved acidic peak separation is observed for the 3µm monodisperse column (blue trace) specifically for the proximal peak. Basic variant separation is also greater though each column achieves baseline resolution for each peak save the proximal basic peak.
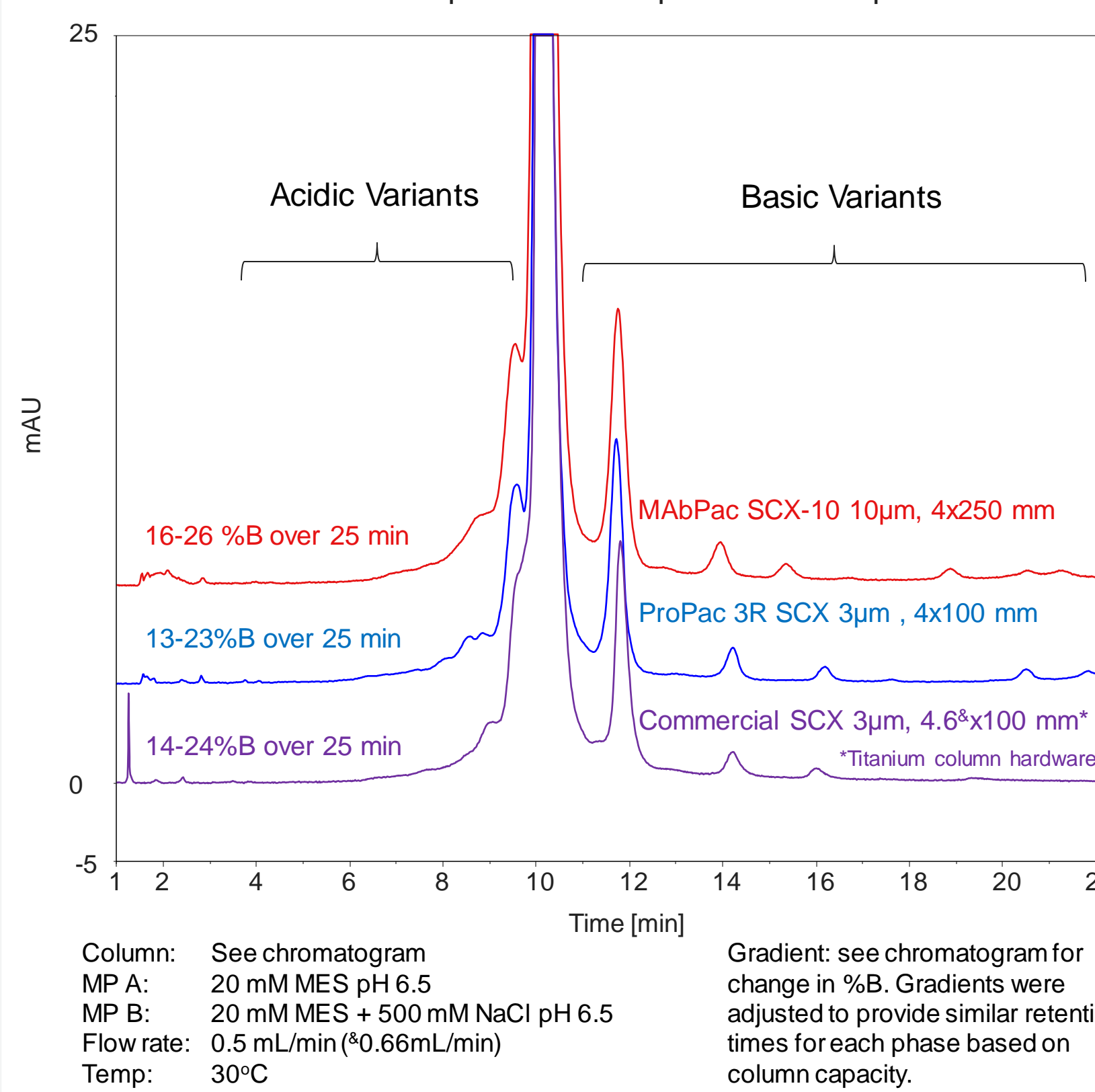

Figure 5. Comparison of NISTmAb salt gradient separation on a 3µm monodisperse SCX particle (ProPac 3R), 10µm SCX particle, and a commercial, polydisperse 3µm SCX particle. Retention time normalized for ease of comparison.

Column:	See chromatogram	Gradient: see chromatogram for
MP A:	20 mM MES pH 6.5	change in %B. Gradients were
MP B:	20 mM MES + 500 mM NaCl pH 6.5	adjusted to provide similar retention
Flow rate:	0.5 mL/min (*0.66mL/min)	times for each phase based on
Temp:	30ºC	column capacity.
Sample:	NISTmAb 10 mg/mL	

The 3µm monodisperse particle was used to analyze IdeS digested NISTmAb, which yields an ~50 kDa Fc/2 and ~100 kDa F(ab')₂ domain by both salt and pH gradients (Figures 6 and 7, respectively). Both showed higher retention of the F(ab')₂ domain which may be expected since glycosylations which may impart negative charge are commonly located on the Fc region. This is supported by the presence of clear acidic variants for the Fc/2 as well as basic peaks attributable to C-terminal +1K and +2K. Interestingly, the pH gradient showed more acidic variants for the F(ab')₂ domain suggesting salt and pH gradients could be used as complementary techniques to investigate mAb structure.
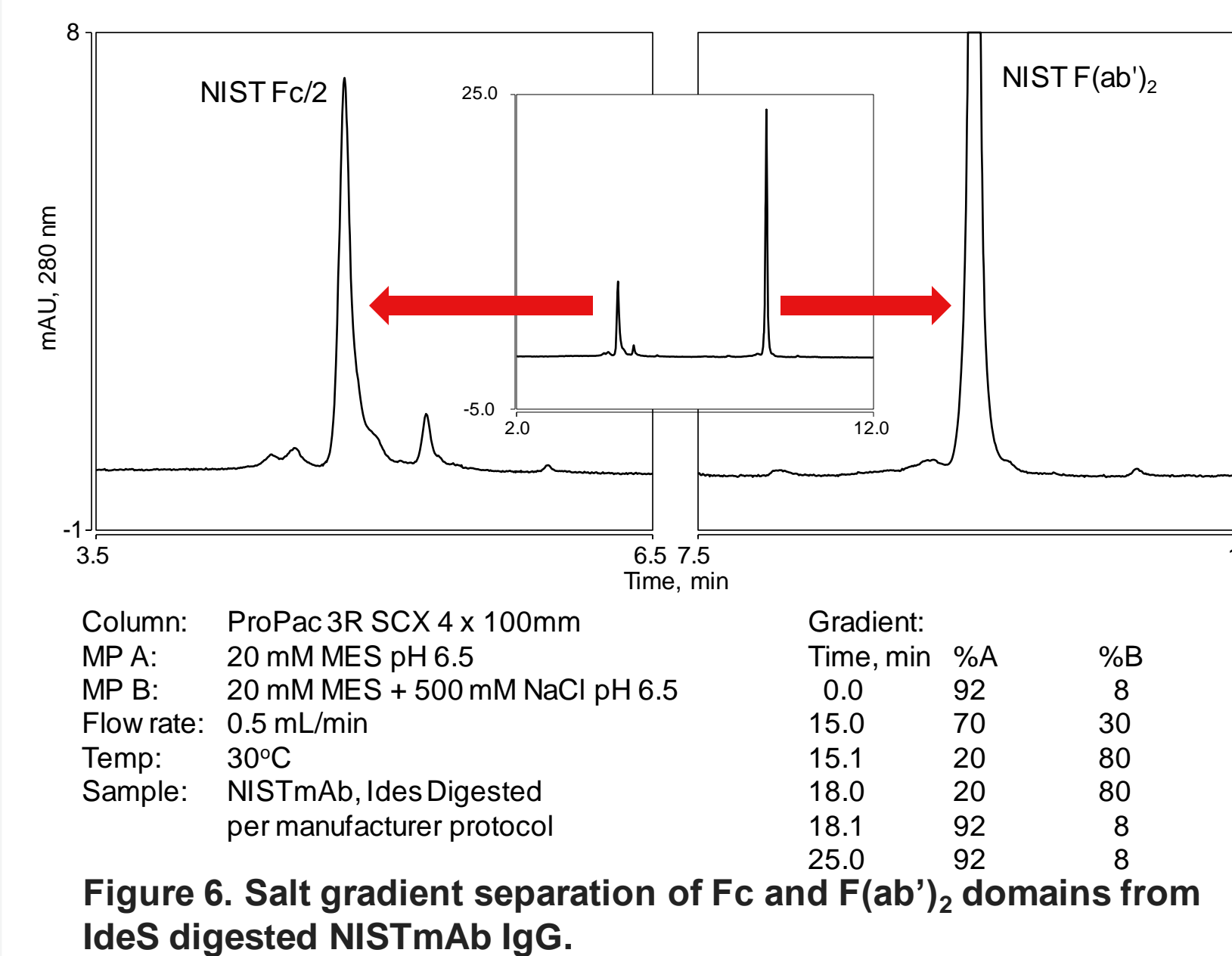

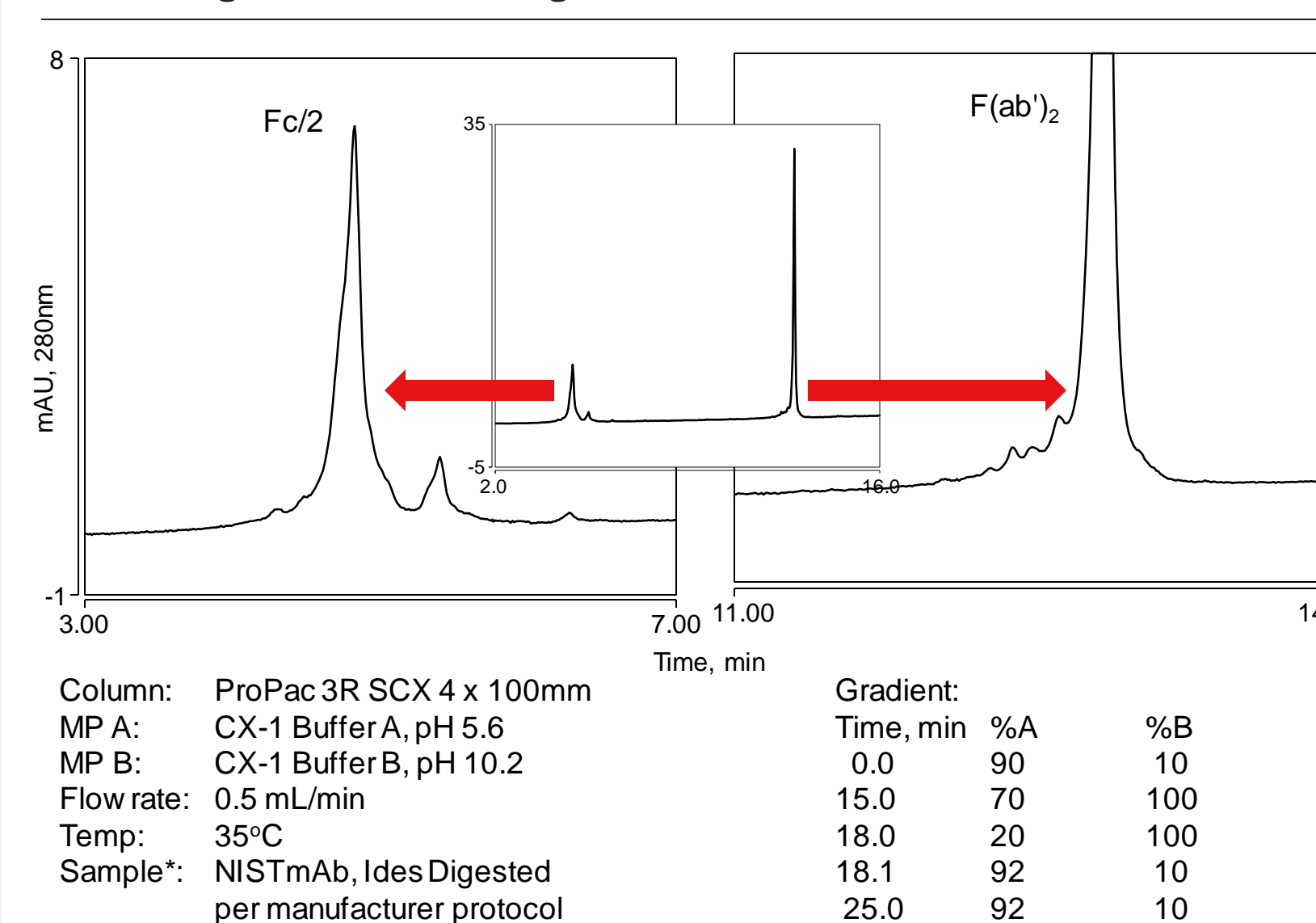**Figure 6. Salt gradient separation of Fc and F(ab')₂ domains from IdeS digested NISTmAb IgG.**

Column:	ProPac 3R SCX 4 x 100mm	Gradient:		
MP A:	20 mM MES pH 6.5		Time, min	%A	%B
MP B:	20 mM MES + 500 mM NaCl pH 6.5		0.0	92	8
Flow rate:	0.5 mL/min		15.0	70	30
Temp:	30ºC		15.1	20	80
Sample:	NISTmAb, Ides Digested		18.0	20	80
	per manufacturer protocol		18.1	92	8
			25.0	92	8

Figure 7. pH gradient separation of Fc/2 and F(ab')₂ domains from IdeS digested NISTmAb IgG.

Column:	ProPac 3R SCX 4 x 100mm	Gradient:		
MP A:	CX-1 Buffer A, pH 5.6		Time, min	%A	%B
MP B:	CX-1 Buffer B, pH 10.2		0.0	90	10
Flow rate:	0.5 mL/min		15.0	70	100
Temp:	35ºC		18.0	20	100
Sample*:	NISTmAb, Ides Digested		18.1	92	10
	per manufacturer protocol		25.0	92	10

Nivolumab is an IgG4 mAb that functions as a checkpoint inhibitor by blocking PD-1. Figure 8 compares Nivolumab separation with a non-volatile pH gradient (left) and an MS-compatible volatile pH gradient. The variants detected by UV may be less for the volatile buffer system compared to the linear CX-1 gradient; however, the volatile buffer method enables native mAb detection by MS for direction characterization of variants not attainable with UV.
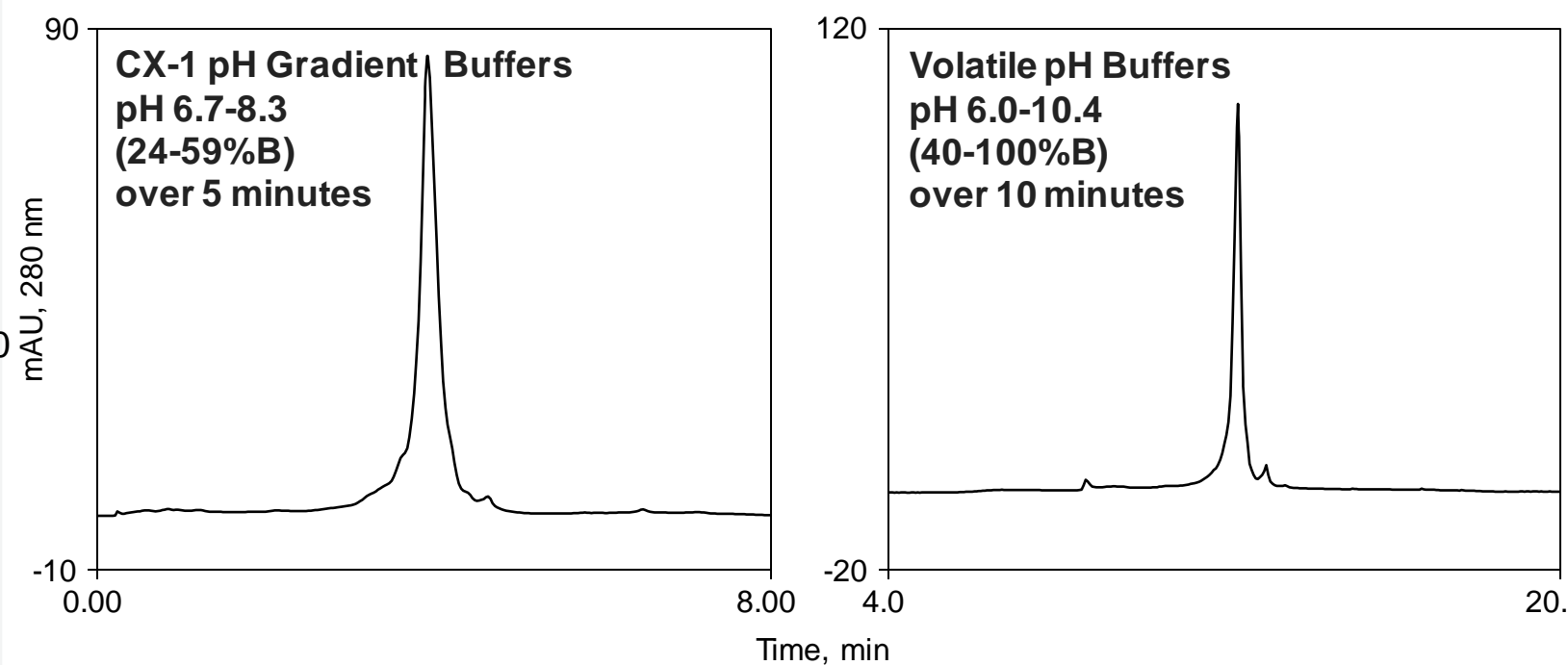

Figure 8. pH gradient separation of Nivolumab using (left) CX-1 pH gradient buffers and (right) MS-compatible volatile pH gradient buffers. Separations were performed on a 2x50mm ProPac 3R SCX column. Volatile Buffers were composed of A: 25 mM Ammonium Bicarbonate, 30mM Acetic Acid pH 5.3 and B: 10 mM Ammonium Hydroxide pH 10.9.

Strong Anion Exchange 3µm Particle

The SAX particle is based on a quaternary amine to provide anion exchange capability across the entire pH range. The performance was compared against a 10µm polydisperse particle column in a 4x100mm and 4x250mm length. The 3µm monodisperse particle has superior separation to the longer 250mm, 10µm column, for all variants despite using a reduced flow rate. This highlights the significant performance improvements observed for low MW proteins (Protein G 21.6 kDa).
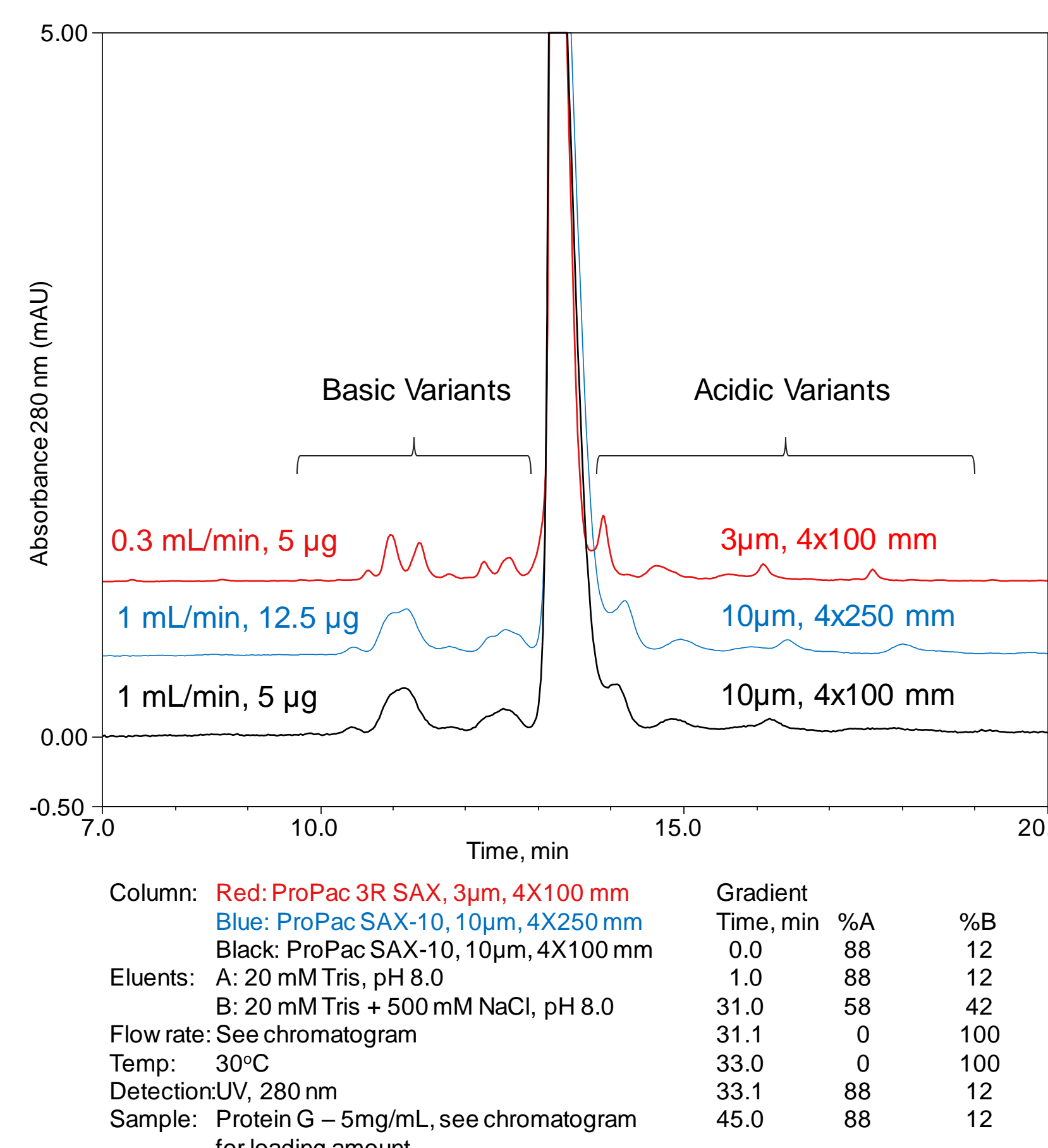

Figure 9. Comparison of salt gradient protein G separation using 4x100mm 3µm ProPac 3R SAX, 4x250mm 10µm ProPac SAX-10, and prototype 4x100mm 10µm ProPac SAX-10. Retention time and peak height normalized for ease of comparison.

Column:	Red: ProPac 3R SAX, 3µm, 4X100 mm	Gradient		
	Blue: ProPac SAX-10, 10µm, 4X250mm		Time, min	%A	%B
	Black: ProPac SAX-10, 10µm, 4X100mm		0.0	88	12
Eluents:	A: 20 mM Tris, pH 8.0		1.0	88	12
	B: 20 mM Tris + 500 mM NaCl, pH 8.0		31.0	58	42
Flow rate	See chromatogram		31.1	0	100
Temp:	30ºC		33.0	0	100
Detection:	UV, 280 nm		33.1	88	12
Sample:	Protein G – 5mg/mL, see chromatogram		45.0	88	12
	for loading amount				

The SAX phase performed well with volatile pH gradient buffers with good resolution of the many variants of Protein G shown in Figure 10. Interestingly, the volatile pH gradient method showed an increased number of basic variants and reduced number of acidic variants relative to the salt gradient separation method. As with the SCX example, the volatile buffers enable the use of MS detection to allow direct characterization of the native protein and its associated variants.
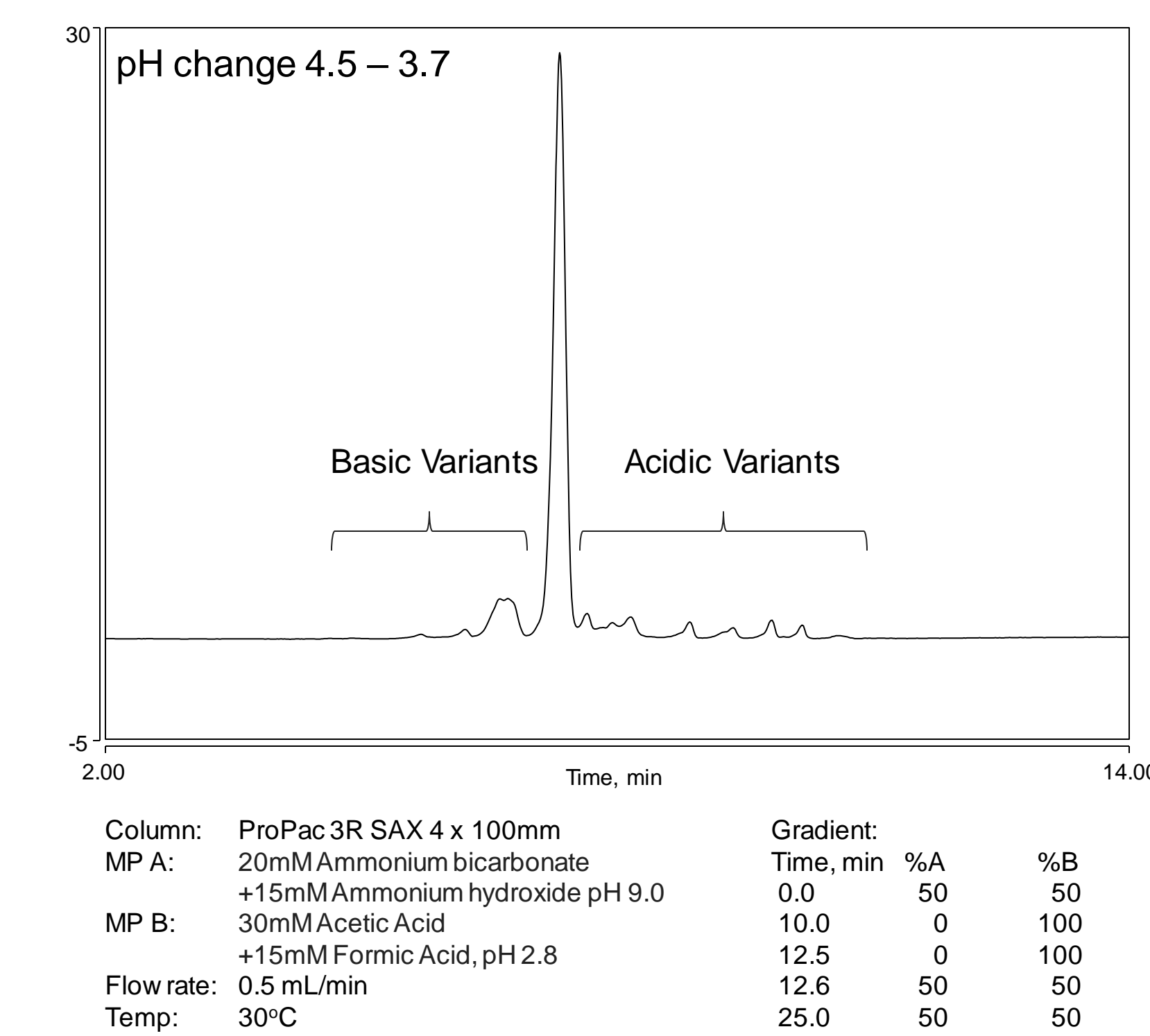

Figure 10. Volatile pH gradient analysis of protein G using 4x100mm 3µm ProPac 3R SAX.

Column:	ProPac 3R SAX 4 x 100mm	Gradient:		
MP A:	20mM Ammonium bicarbonate		Time, min	%A	%B
	+15mM Ammonium hydroxide pH 9.0		0.0	50	50
MP B:	30mM Acetic Acid		0.0	0	100
	+15mM Formic Acid, pH 2.8		12.5	0	100
Flow rate:	0.5 mL/min		12.6	50	50
Temp:	30ºC		25.0	50	50
Sample:	Protein G, 5 µg injection				

Conclusions

- Monodisperse particles provide a high efficiency, reproducible platform for IEX phases capable of analyzing proteins across a wide molecular weight range with separations superior to that of polydisperse particles

- SCX and SAX functionalized particles perform well for both salt gradient and pH gradient applications with limited secondary interactions

- Volatile pH gradient buffers extends the application of columns made with these particles to Mass Spectrometry for native protein analysis

References

1. Rivera, B.; Anspach, J.A.; Rao, S. LCGC Supplements, 2018, 36(6), 24-29.

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