

UFFLC of Proteins: Optimizing Speed and Separation Simultaneously

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ABSTRACT

Various proteins were resolved on ion-exchange columns; mixed-bed columns (PolyCAT A and PolyWAX LP) in the case of cell lysates and cation-exchange (PolyCAT A) in the case of hemoglobin variants. Pressures of ~ 13,000 psi over the entire column resulted in elution 30% earlier in the gradient. This effect of pressure on retention in ion-exchange chromatography is the opposite of that reported for reversed-phase chromatography. High linear velocities and pressures of ~ 5000-6000 psi resulted in both an increase in the speed of separation by 4x and a simultaneous improvement in resolution in some cases. This method of operation is termed Ultra Fast Flow Liquid Chromatography (UFFLC). The effect on separation involves selectivity; certain proteins were seen to shift in retention time relative to others. These effects may be traced to the increase in energy in the system conferred by high pressure, analogous to an increase in temperature, and possibly to differences in compressibility of different proteins. This technique can be used as a rapid top down separation step to distribute complex mixtures of proteins into fractions for identifications in proteomics. For convenience, proteins can be fractionated with gradients of ammonium acetate, a volatile salt.

INTRODUCTION

Ultra High Pressure Liquid Chromatography (UHPLC) has been applied successfully to separations of many small molecules, including peptides. Few studies have involved proteins and these were narrowly focused on the behavior of several purified standards in reversed-phase chromatography (RPC). We have investigated the separation of proteins in complex mixtures by ion-exchange chromatography (IEX), mixed-bed columns in the case of cell lysates and cation-exchange in the case of hemolyzates. While most UHPLC studies generate high backpressures as a consequence of using small-diameter particles as the stationary phase, we used conventional 3- or 5- μm materials at flow rates 4-6x faster than usual. This combination is termed **Ultra Fast Flow Liquid Chromatography** (UFFLC). These flow rates generated backpressures \sim 5000-7000 psi. This is at the top of the range of a conventional HPLC system, so we have used a new UHPLC system capable of an unusually fast flow rate. In an effort to assess the role of pressure in the chromatography, we have also raised the backpressure to \sim 13,000 psi (896 bar) over the entire column by adding a capillary restrictor to the outlet.

MATERIALS AND METHODS

The UFFLC system was a Model SYS0201 from Scientific Systems Inc. (State College, PA). It consisted of two pumps, a Model 525 UV/Vis detector, and automatic sample injector. Control and data collection was via EZStart® (Scientific Software).

All stationary phases were from PolyLC Inc. (Columbia, MD). The mixed-bed column contained PolyCAT A™ (a weak cation-exchange material) and PolyWAX LP™ (a weak anion-exchange material) in a 1:1 ratio. Column dimensions, pore and particle diameter were as indicated.

Hemolyzates were a gift of Beverly Vispo (Texas Childrens' Hospital, Houston, TX). They were kept frozen until just before analysis. Lysates of yeast cells were a gift of Pierre Havugimana (Dept. of Molecular Genetics, Univ. of Toronto, Toronto, Ont., Canada). Mobile phase salts were HPLC-grade if available; otherwise, *puriss.*-grade from Fluka.

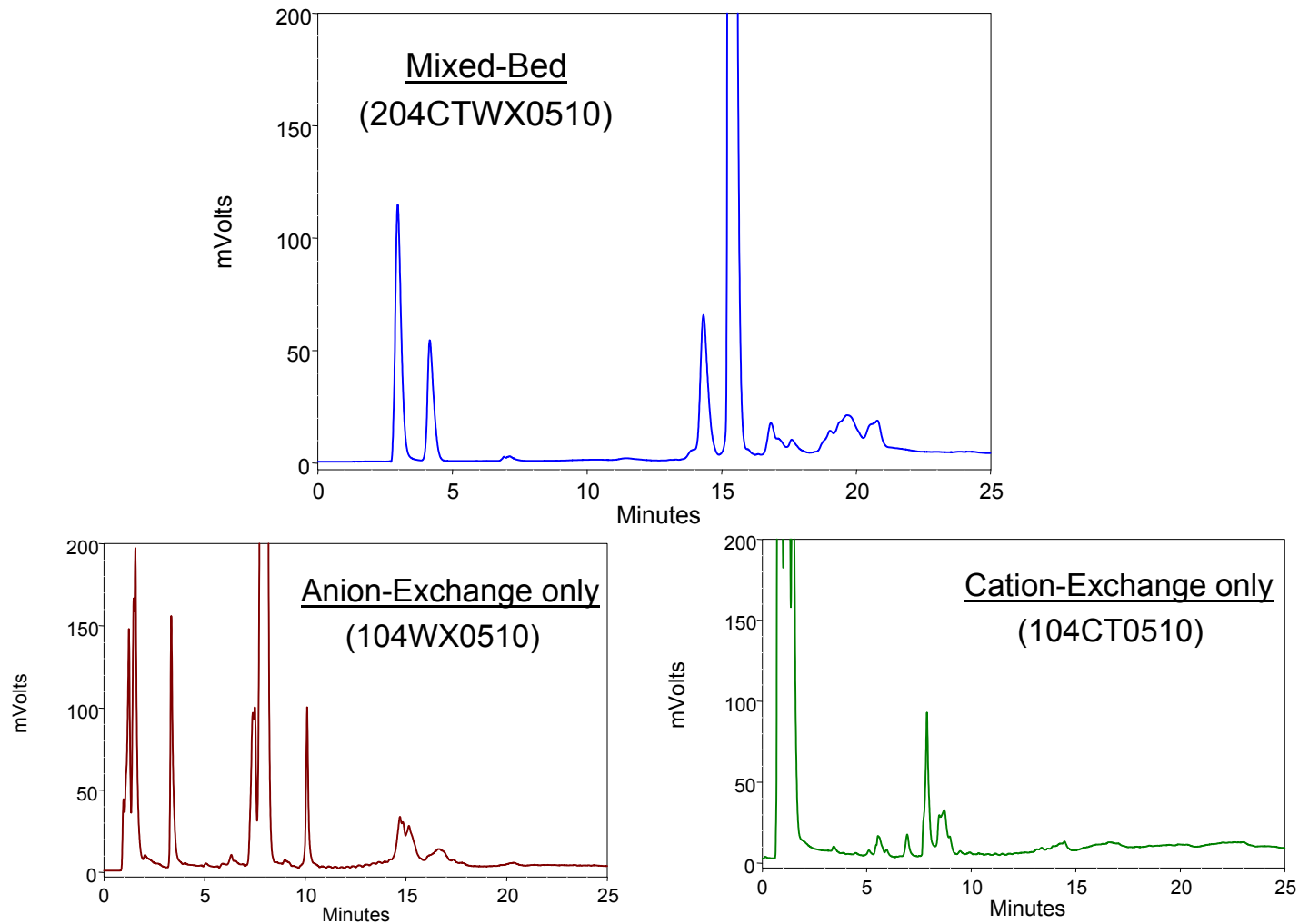


Fig. 1. Comparison of regular IEX columns to the mixed-bed column. Sample: Yeast lysate. All materials were 5- μ m, 1000-Å pore diameter. The mixed-bed column was 200x4.6-mm while the others were 100x4.6-mm. Flow rate: 1 ml/min. Detection: A280. Gradient: 0-300 mM NaCl in 20 mM MES, pH 6.0.

A complex mixture of proteins always contains some that elute in the void volume on a single IEX column. With a mixed-bed column the number that do this is minimal^{1,2}. This helps to insure more uniform distribution of the proteins for proteomics fractionations.

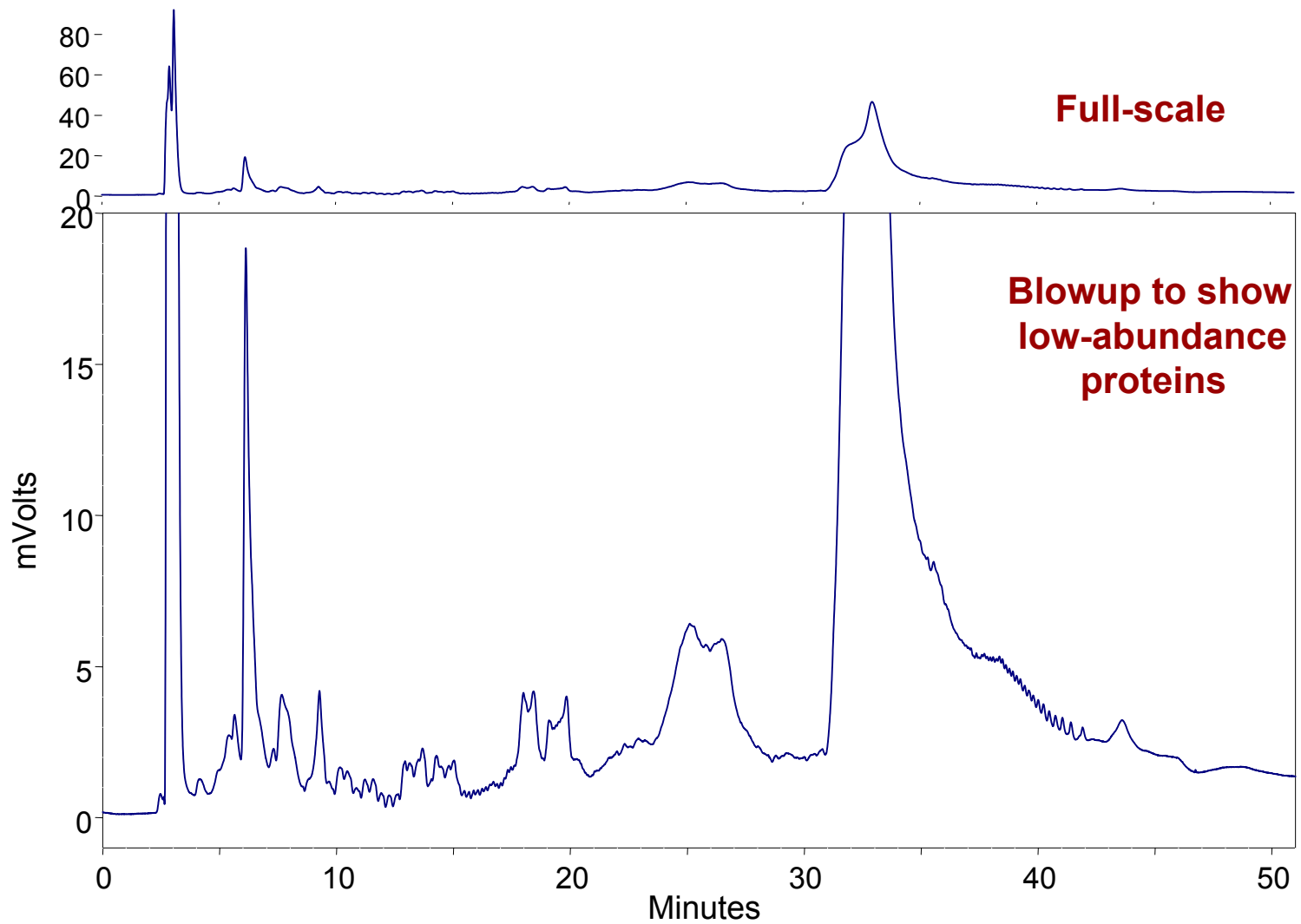


Fig. 2. Mixed-bed IEX of yeast lysate with a volatile mobile phase.

COLUMN: Same mixed-bed as in Fig. 1. DETECTION: 280 nm

MOBILE PHASE: 20-800 mM ammonium acetate, pH 6.0 FLOW: 1 ml/min

GRADIENT: 0-12': 0-10%B; 12-30': 10-60%B; 30-40': 60-100%B 40-50': 100%B

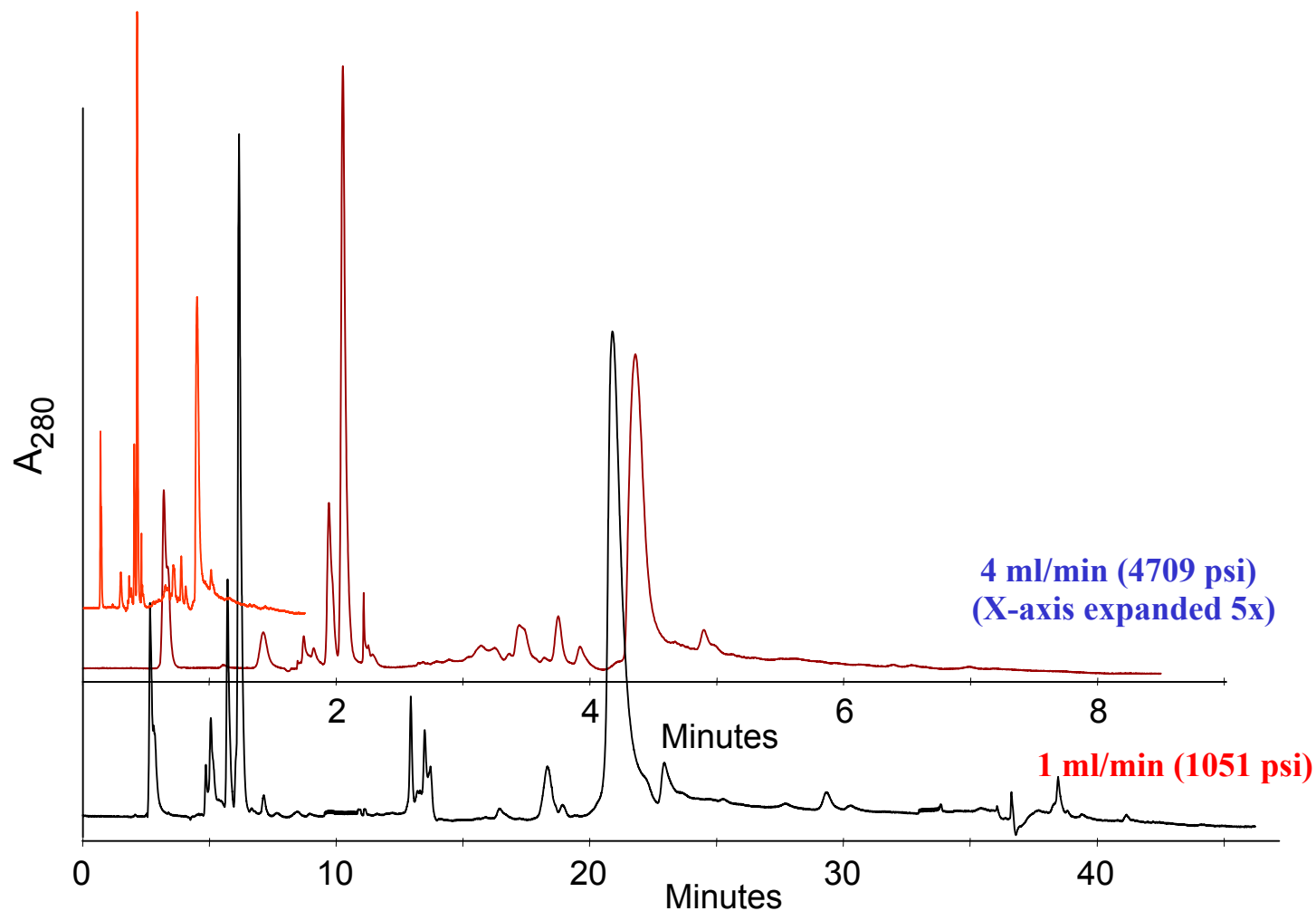


Fig. 3. Effect of flow rate on yeast lysate proteins. Sample: Yeast lysate. Column: Mixed-bed IEX column from Fig. 1. Mobile phases: a) 20 mM HEPES, pH 7.0.; B) same + 0.8 M NaCl.

Black trace: 1 ml/min. Gradient: 0-50% B in 30', then 50-100% B in 10'.

Red trace: 4 ml/min. Gradient: 0-8% B in 2', then 8-100% B in 5'.

The fast-flow trace (red) was elongated along the X-axis (dark red) to permit comparison of the peak shapes and separations. These appear not to have changed significantly even though the running time was 4-5x faster.

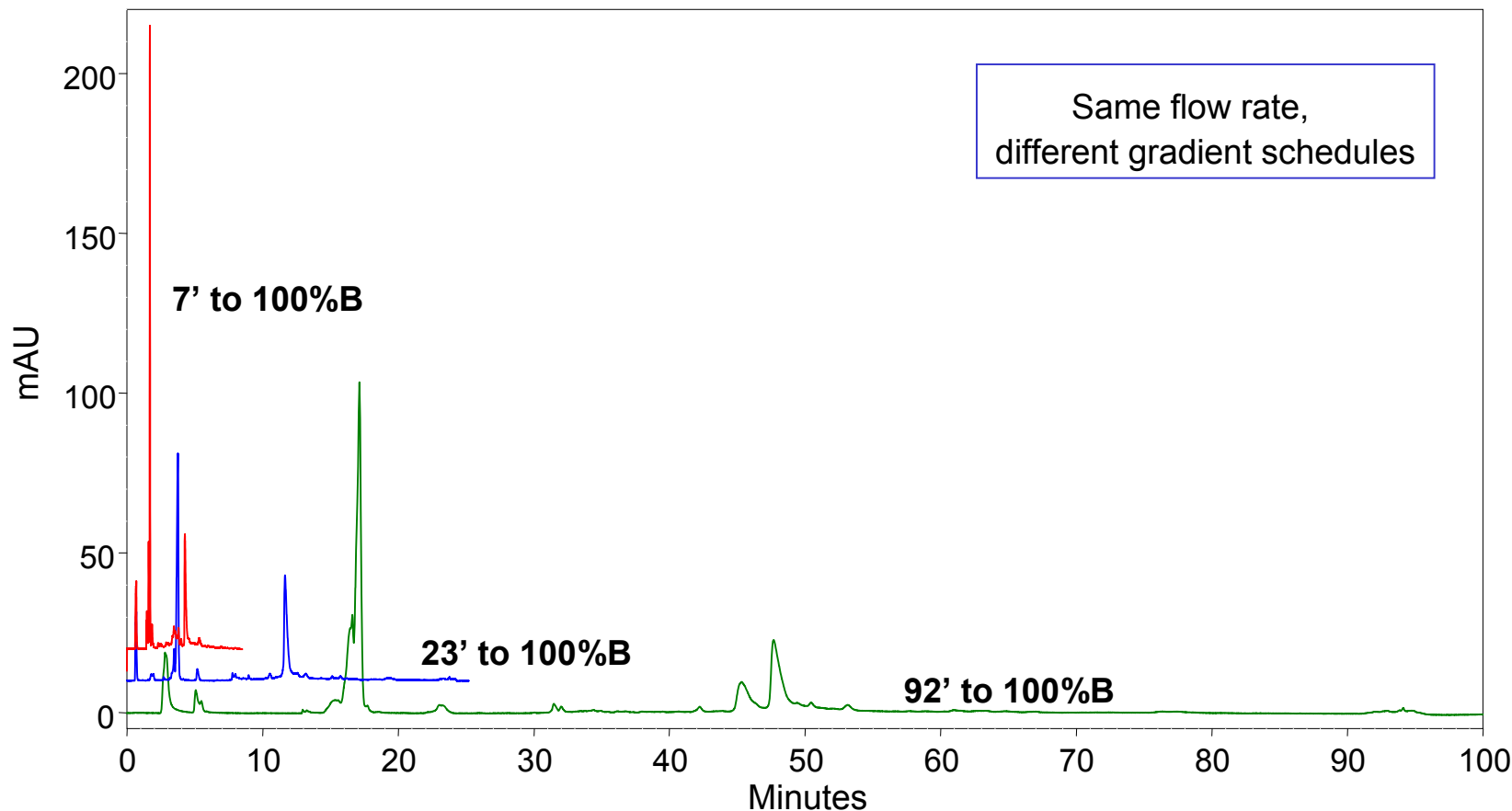


Fig. 4a. Effect of gradient slope. Sample: Yeast lysate. Column: Mixed-bed IEX column from Fig. 1. Mobile phases: a) 20 mM HEPES, pH 6.6.; B) same + 0.8 M NaCl. Flow rate: 4 ml/min.

TOP: Gradient time to 100% B as noted.

MIDDLE: Same chromatograms elongated to facilitate comparison.

BOTTOM: Same as middle, elongated along the Y-axis to facilitate comparison of minor peaks.

It is generally accepted in chromatography that shallower gradients afford better separations. That was not obviously the case here, with either the major or minor protein peaks. The results from the combination of fast flow plus fast gradient are more than acceptable.

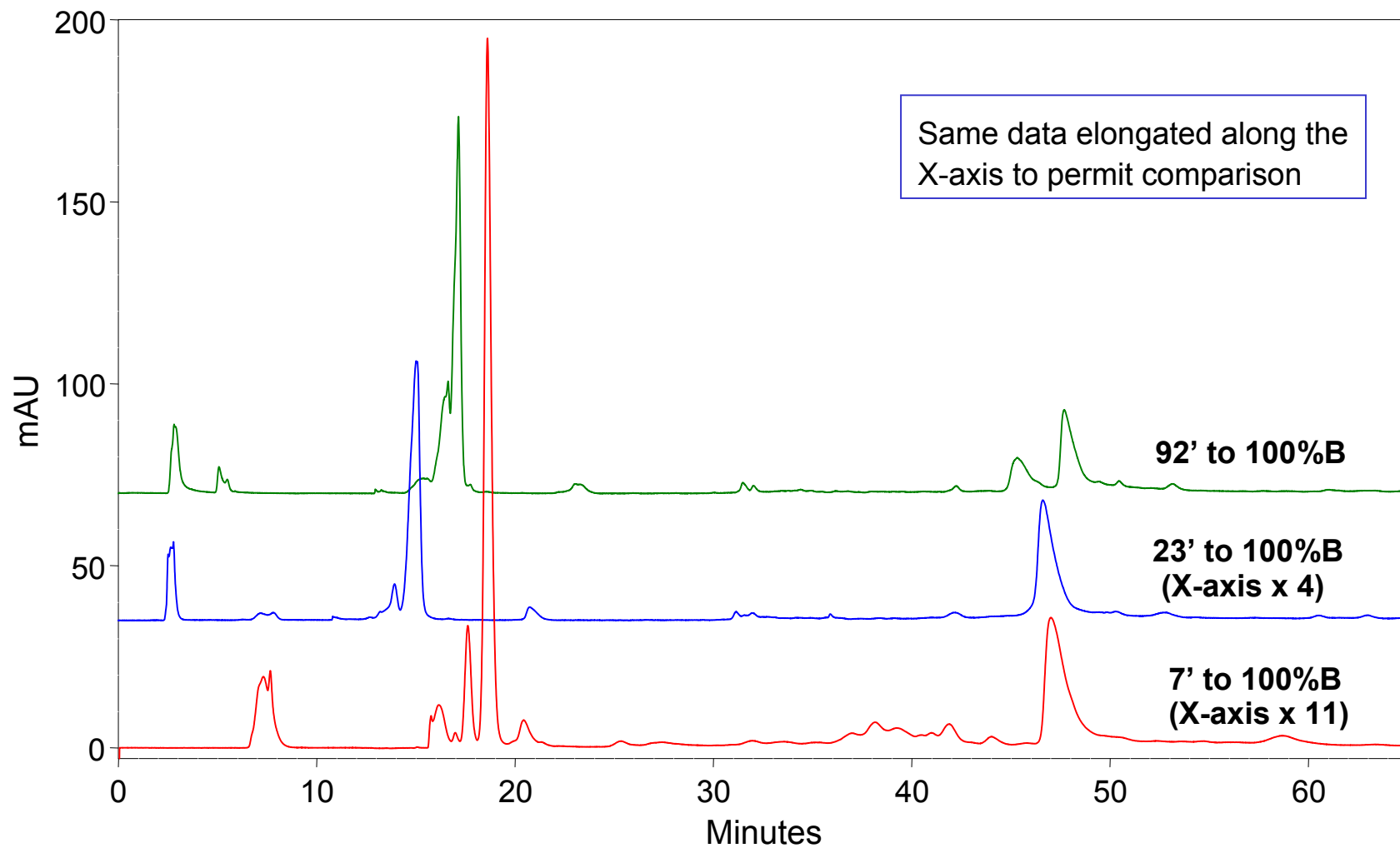


Fig. 4b.

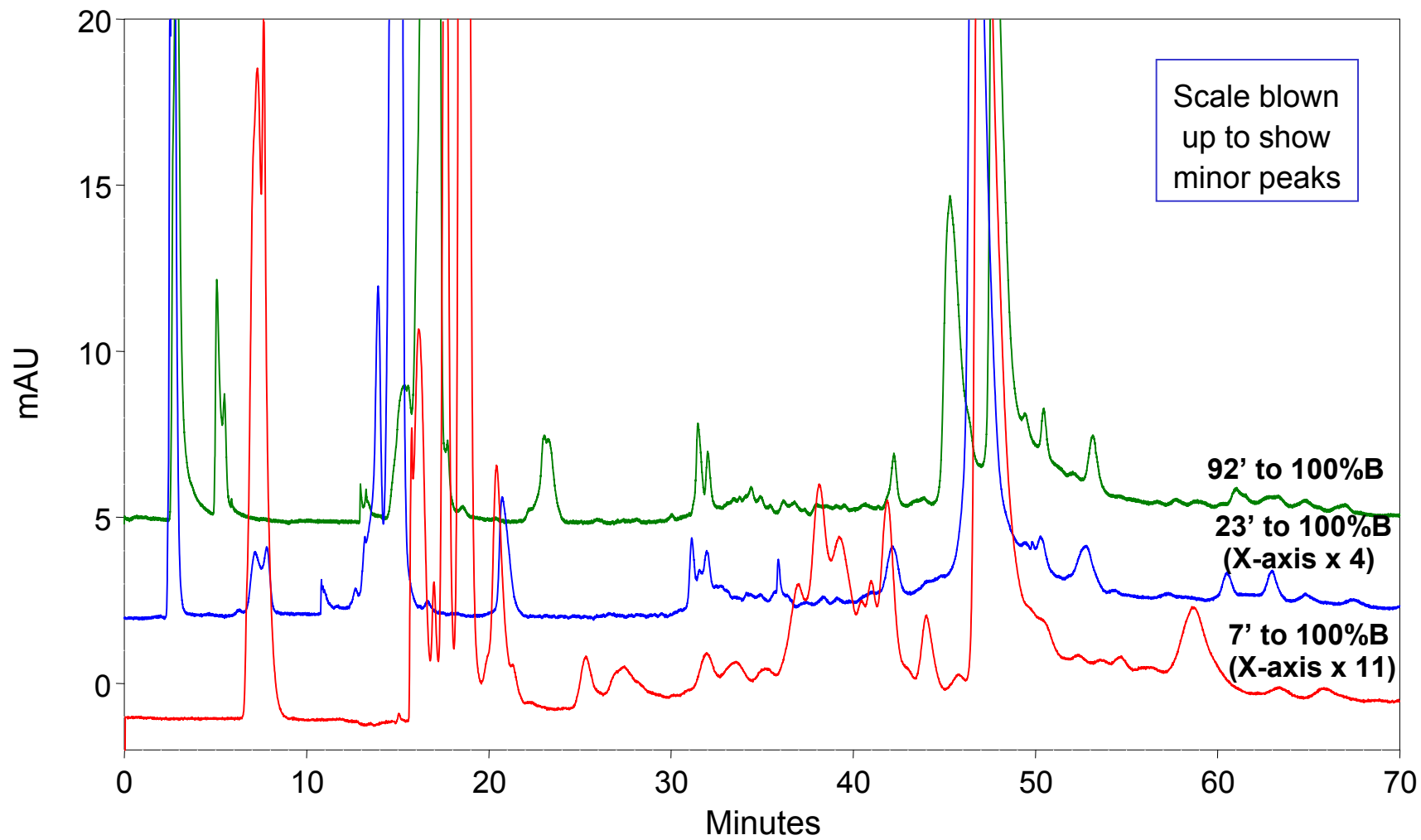


Fig. 4c.

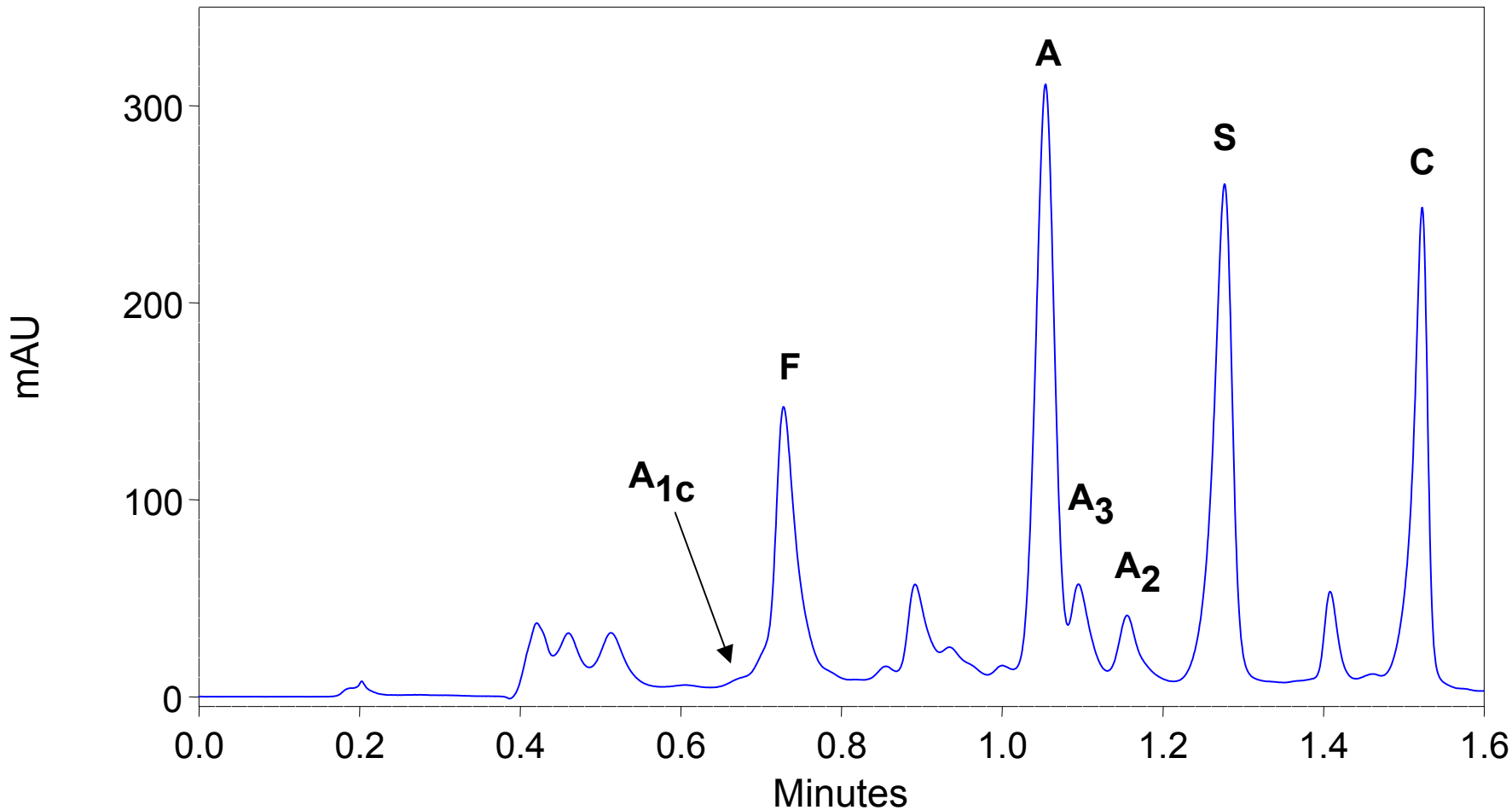


Fig. 5. Rapid Separation of Hemoglobin Variants. Column: PolyCAT A, 100x2.1-mm; 3- μ m, 1500- \AA . Flow: 1.2 ml/min. Backpressure: 6938 psi (478 bar). Detection: 415 nm. Mobile Phases: A) 40 mM Bis-Tris + 2 mM KCN, pH 6.5; B) 40 mM Bis-Tris + 2 mM KCN + 200 mM NaCl, pH 6.8. Gradient: 0-0.8': 18-55% B; 0.8-1.1': 55-90% B; 1.1-1.2': 90-100% B. 1.2-1.3': 100% B.

This separation is 3x faster than existing methods³ but still suffices for quantitation of all these variants. While the flow rate is 6x faster than is customary for a column this size, the consumption of mobile phase is still modest because a narrow-bore column was used.

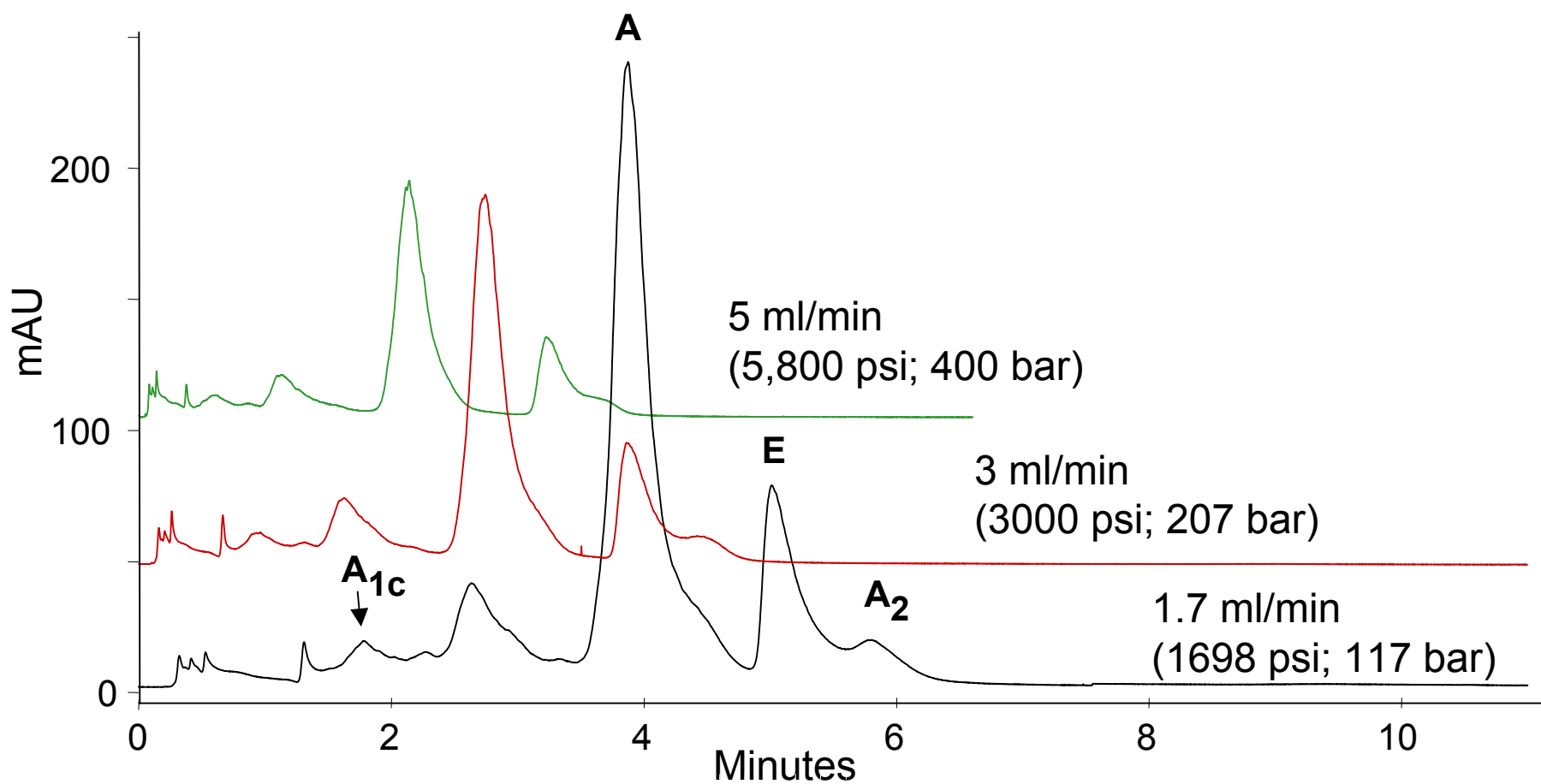


Fig. 6. Effect of Flow Rate with Hemoglobin AE Hemolyzate. Column: PolyCAT A, 100x3.0-mm; 5- μ m, 1000- \AA . Flow and backpressure: As noted. Detection: 415 nm. Mobile Phases: A) 20 mM Bis-Tris + 2 mM KCN, pH 6.96; B) 20 mM Bis-Tris + 2 mM KCN + 200 mM NaCl, pH 6.55. Gradient: 0-8': 10-40% B; 8-10': 40-100% B.

The separation of E and A₂ is a difficult one; the separation obtained here is not quite good enough for clinical quantitation. A longer separation time is required.

As flow rate and backpressure increase, Hb A and A₂ appear to shift as a set to earlier elution times relative to the elution time of Hb E. Similar behavior has been noted with the elution of glycosylated hemoglobins (e.g., A_{1c}) relative to nonglycosylated hemoglobins as a function of pH.

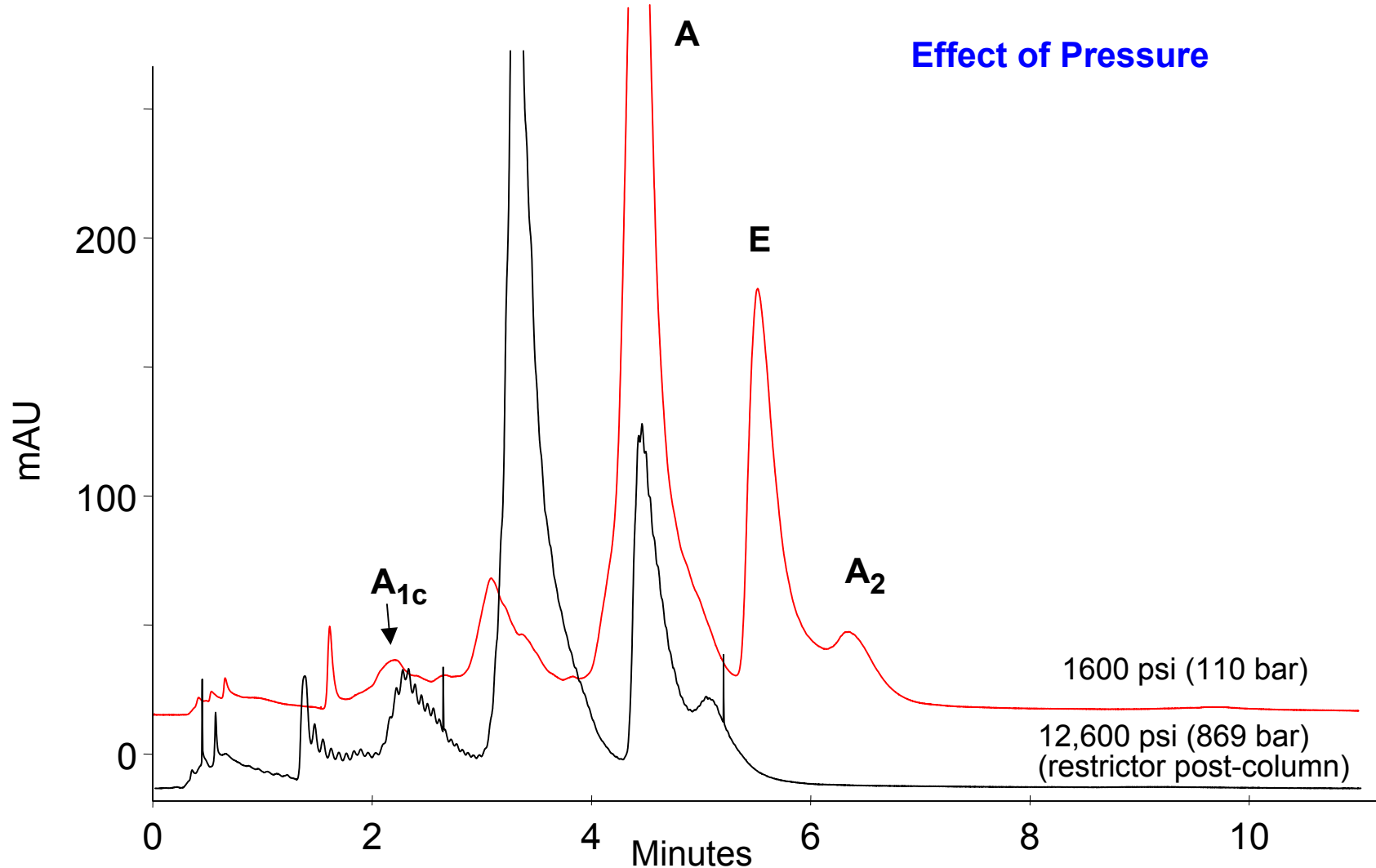
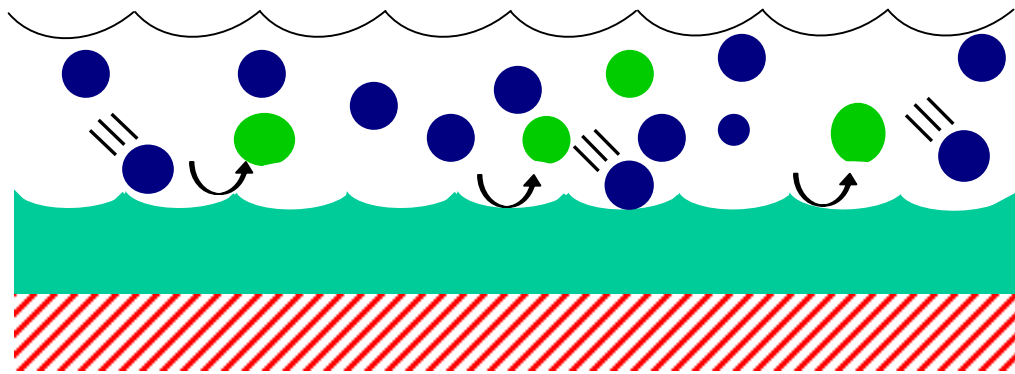


Fig. 7. Effect of Pressure with Hemoglobin AE Hemolyzate. Flow: 1.5 ml/min. Other conditions as noted in Fig. 4.

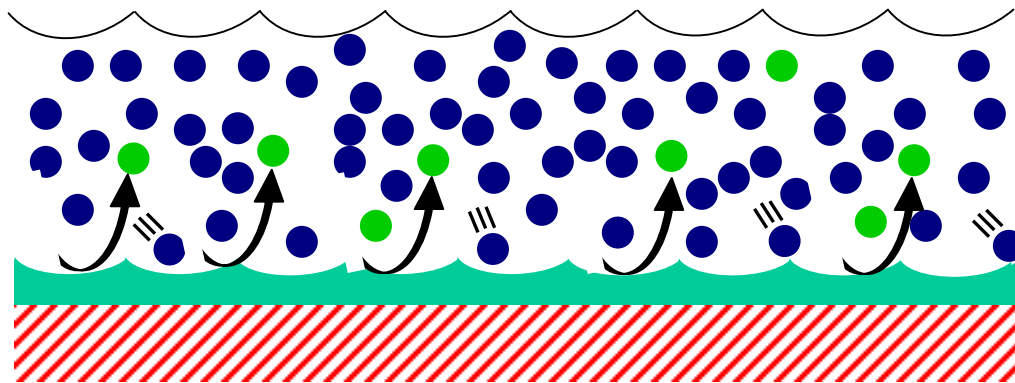
A capillary restrictor was added to the outlet of the cation-exchange column from Fig. 4. The Hb AE hemolyzate was analyzed with and without the restrictor. The only significant effect of the extraordinary increase in column pressure was a decrease of about 30% in the retention time of the hemoglobin proteins.

Effect of Pressure on Kinetics in HPLC/UPLC



- Mobile Phase (● = proteins; ● = water & salt molecules)
- Stagnant Layer of Hydration
- Stationary Phase

Conventional Pressure



Ultra-High Pressure

Compression of solvent ~ 10%

- More molecular collisions
- Higher energy system (like ↑ temperature)
- Thinner surface layer of hydration and earlier elution of solutes in IEX
- Molecules and spheres and layers of hydration compressed
- Faster equilibration times

Fig. 8.

DISCUSSION

The most interesting result of this study is in Fig. 5. All research in the literature on chromatography of proteins at elevated pressures has involved reversed-phase chromatography (RPC). Pressure increases retention of proteins in RPC^{4,5}. It also increases retention of a series of homologous oligophenylalanines⁵, so the effect cannot be due primarily to denaturation of the protein with exposure of the hydrophobic core residues. Protein denaturation is minimal at these pressures in any case^{6,7}. Explanations for these effects have focused on compression of the solute sphere of hydration, compression of the stationary phase coating, an increase in viscosity of the mobile phase, and other phenomena^{5,8}. However, since such elevated pressures caused retention times in IEX to decrease, not increase, it seems that all explanations derived from RPC data are reduced to a special case. The increase in free energy of the mobile phase seems to promote the partitioning of hydrophobic solutes into a hydrophobic stationary phase. We speculate that the same increase in free energy of both water molecules and ions in the mobile phase is responsible for more facile displacement of charged solutes in IEX, as portrayed in the schematic below. Only the most general statements are possible about the effects of elevated pressure on chromatography without additional data from IEX, HILIC, and perhaps other modes; an inductive approach.

The separations obtained here, while preliminary in nature, do indicate that it is feasible to accelerate the speed of protein separation markedly via faster flow with little or no loss of resolution. The work involved stationary phases and column dimensions that are currently conventional for HPLC. There is no reason why one could not combine this approach with a smaller particle diameter and so gain a simultaneous increase in efficiency. The difficulty is the practical one of manufacturing a smaller particle with pores wide enough for satisfactory work with proteins. Perhaps the ultimate challenge would be the synthesis of a material with a particle diameter of 1 μm and pore diameter of 10,000 \AA ...

DISCUSSION (cont.)

Faster protein separations have some practical application. Clinical analysis of proteins such as hemoglobin variants is performed nonstop at many centralized testing labs. Decreasing the time of analysis would increase their throughput directly. In proteomics research, fast-flow separations make HPLC (or UFFLC) a practical alternative to 2-dimensional gel electrophoresis. One could separate proteins in one dimension through IEX, collecting fractions that are then run in a complementary second dimension such as hydrophobic interaction chromatography (HIC). Normally this would be a tedious process. With the flow rates demonstrated here, however, it may be possible to collect and rerun 30-50 fractions in one day. The protein composition of the resulting 30-50 chromatograms would be sufficiently simplified so that it might be possible to identify differences in protein expression between different samples directly. If not, then fractions could be collected from the second dimension and digested for peptide identification via a bottom-up approach to proteomics. The number of different proteins in each fraction would be so small that it should be possible to identify peptides from proteins of unprecedented low abundance, reflecting the lack of masking from peptides from other proteins.

REFERENCES

- 1) El Rassi Z, Horváth C. Tandem Columns and Mixed-Bed Columns in High-Performance Liquid Chromatography of Proteins. *J. Chromatogr.* 1986;359: 255-264.
- 2) Maa Y-F, Antia FD, El Rassi Z, Horváth C. Mixed-Bed Ion-Exchange Columns for Protein High-Performance Liquid Chromatography. *J. Chromatogr.* 1988;452: 331-345.
- 3) Ou C-N, Rognerud CL. Rapid Analysis of Hemoglobin Variants by Cation-Exchange HPLC. *Clin. Chem.* 1993;39: 820-824.
- 4) Liu X, Szabalski P, Kaczmarek K, Zhou D, Guiochon G. Influence of Pressure on the Chromatographic Behavior of Insulin Variants Under Nonlinear Conditions. *J. Chromatogr. A* 2003;988:205-218.
- 5) Chen S-H, Ho C-T, Hsiao K-Y, Chen J-M. Pressure-Induced Retention of the Lysozyme on Reversed-Phase Liquid Chromatography. *J. Chromatogr. A* 2000;891:207-215.
- 6) Pin S, Royer CA, Gratton E, Alpert B, Weber G. Subunit Interactions in Hemoglobin Probed by Fluorescence and High-Pressure Techniques. *Biochemistry* 1990;29:9194-9202.
- 7) Gross M, Jaenicke R. Proteins under Pressure (*review*). *Eur. J. Biochem.* 1994;221:617-630.
- 8) Martin M, Guiochon G. Effects of High Pressure in Liquid Chromatography. *J. Chromatogr. A* 2005;1090:16-38.