Developing Protein Separation Method On a Reversed-Phase UPLC® Column

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Hillary B. Hewitson, Thomas E. Wheat, and Diane M. Diehl
Waters Corporation, Milford, MA

INTRODUCTION

Complete and accurate characterization of biological macromolecules requires both increasing sensitivity and chromatographic resolution. With the application of Waters® ACQUITY® UPLC® system and the development of a new column packing material for the reversed-phase separation of proteins, users can use optimized technologies for increased resolution, sensitivity, and speed.

The development of this packing material will be described, including the influence of the base particle, pore size, bonded phase on protein separations. The impact of these development decisions was measured based using protein probes representing a range of properties. The test samples include large and small, acidic and basic, hydrophobic and hydrophilic proteins, as well as, monoclonal antibodies and their subunits. The measured properties include peak shape, peak area, resolution, and carryover. The selected packing material is an ethyl bridged hybrid particle with 300Å pores and a C4 bonded phase. Columns packed with this material were used to evaluate variables in developing a separation method. The same set of proteins was used to measure the effect of mobile phase constituents, solvent choice, separation temperature, gradient slope, and flow rate in developing a separation. We will show that simple changes to operating parameters can provide useful alterations in the selectivity that can be appropriate for particular samples. The Waters BEH300 C4 column provides a suitable material for reversed-phase protein separations.

METHODS

Chromatographic Conditions

All separation conditions as indicated, unless otherwise noted in the figure legends.
ACQUITY UPLC® System w/TUV detection @ 220nm
Eluent A: 0.1% Trifluoroacetic acid (CF_3COOH) in water
Eluent B: 0.1% CF_3COOH in acetonitrile
Needle Washes:
Weak: 0.1% CF_3COOH in 5% acetonitrile
Strong: 0.1% CF_3COOH in 75% acetonitrile
Flow rate: 0.2 mL/min
Temperature: 40ºC for Protein Mixture
80ºC for IgG and Reduced/Alkylated IgG
Injection volume: 3.3 µL
Gradient for 2.1mm x 50 mm column:
(time scaled proportionally to column length)

Time (min)	% A	% B	Curve	Column Volumes	% Change / col. vol.
Initial	80	20	*	*	*
25	28.6	71.4	6	29	1.8
27	28.6	71.4	1	2	*
45	80	20	1	21	*

Samples

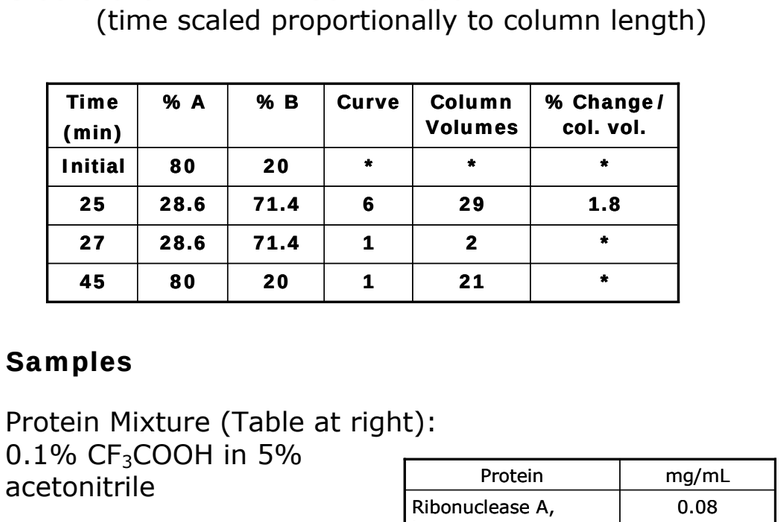
Protein Mixture (Table at right): 0.1% CF_3COOH in 5% acetonitrile

Monoclonal antibodies (murine, chimeric, and fully humanized): ~0.5mg/mL in 0.1% CF_3COOH

Reduced/partially alkylated murine monoclonal antibody:~0.5mg/mL in 0.1% CF_3COOH

Protein	mg/mL
Ribonuclease A, bovine pancreas	0.08
Cytochrome c, horse heart	0.11
Albumin, bovine serum	0.40
Myoglobin, horse heart	0.25
Enolase, baker's yeast	0.43
Phosphorylase b, rabbit muscle	1.18

Design Considerations



Particle considerations for reversed-phase protein separations
- Base particle—Bridged Ethyl Hybrid (BEH) was chosen for its particle stability and reduced silanol activity
- Pore diameter—Wider pores (300Å) selected for generally sharper protein peaks
- Chain length—Shorter chain length (C_4) selected for narrower peaks and increased recovery of some proteins

RESULTS

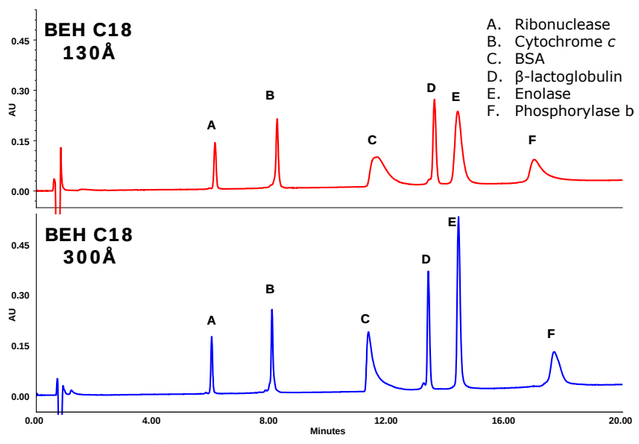


Figure 1A Effect of pore size on the separation.

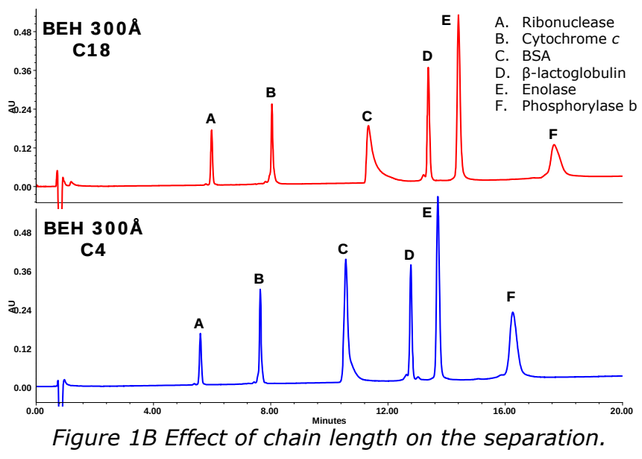


Figure 1B Effect of chain length on the separation.

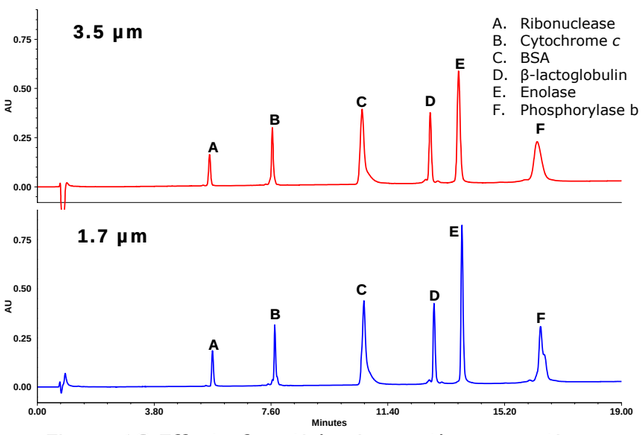


Figure 1C Effect of particle size on the separation.

Figure 1. All protein mixture separations were performed on 2.1mm x 50mm columns. The larger pore diameter produces sharper peaks, especially for the later eluting proteins (1A). Shorter chain length results in sharper peaks and higher recovery of Phosphorylase b (1B). Decreased particle size gives sharper peaks with improved resolution (1C).

Typical Examples

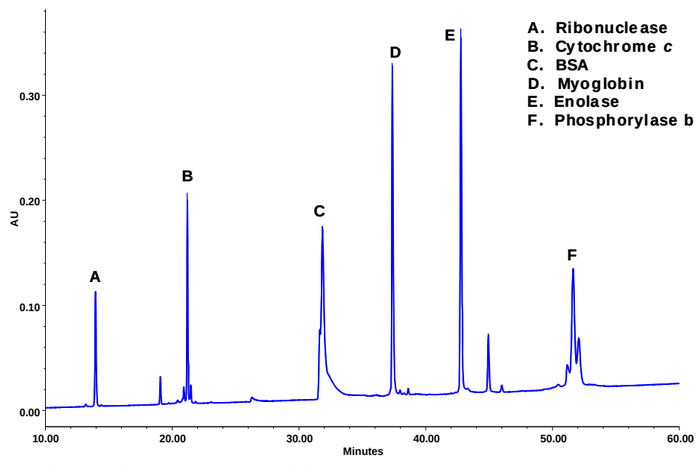
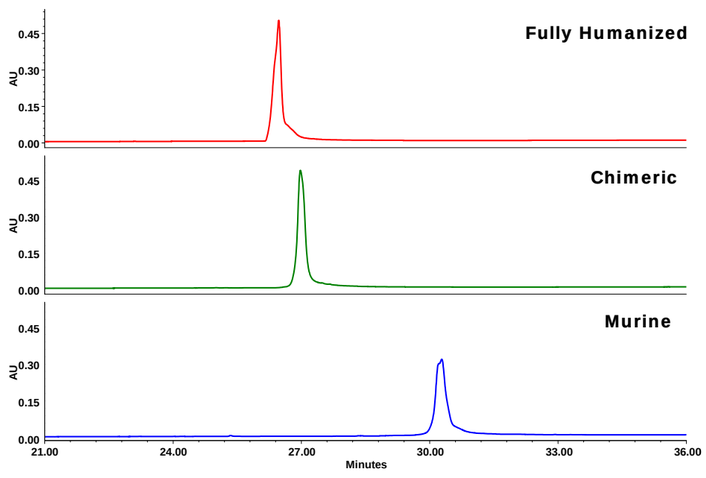


Figure 2A Separation of Protein Mixture



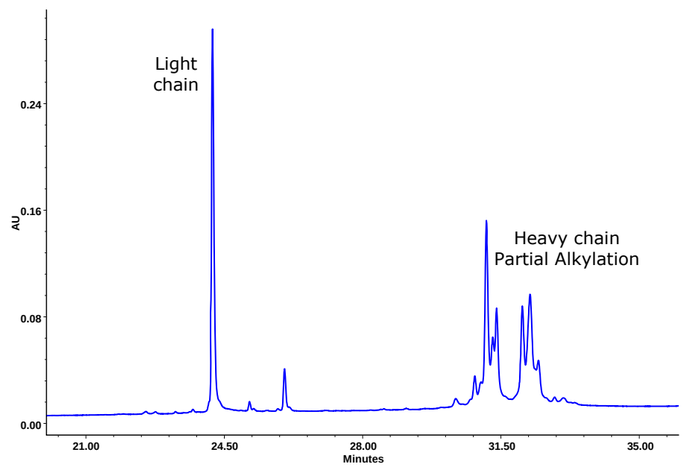


Figure 2C Separation of a reduced and partially alkylated murine monoclonal IgG

Figure 2. All separations were conducted on a 2.1 x 150 mm, 1.7 µm column. (2A) The proteins in the mixture represent a wide range of physical and chemical properties. The useful chromatographic peaks for all of these proteins suggest this column chemistry's general utility. (2B)Good chromatographic peaks were observed for the separation of different monoclonal antibodies. (2C) IgG was reduced and partially alkylated to illustrate a heterogeneous sample of very similar proteins that are partially separated.

Factors Influencing Separations

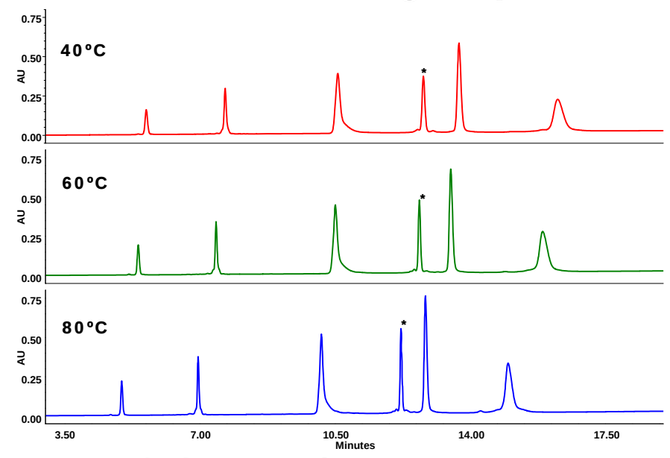


Figure 3A Separation of Protein Mixture

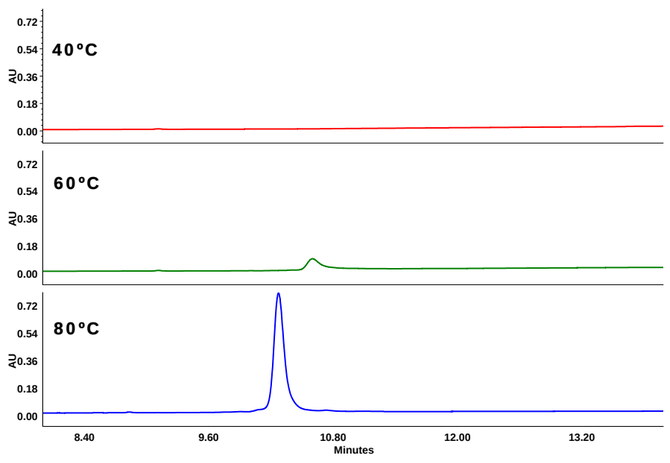


Figure 3B Separation of murine monoclonal IgG

*Figure 3. There is little effect of increasing temperature on the chromatography of some proteins (3A). In contrast, IgG is dramatically improved at elevated temperature (3B). *Protein mixture contains β-lactoglobulin in place of Myoglobin.*

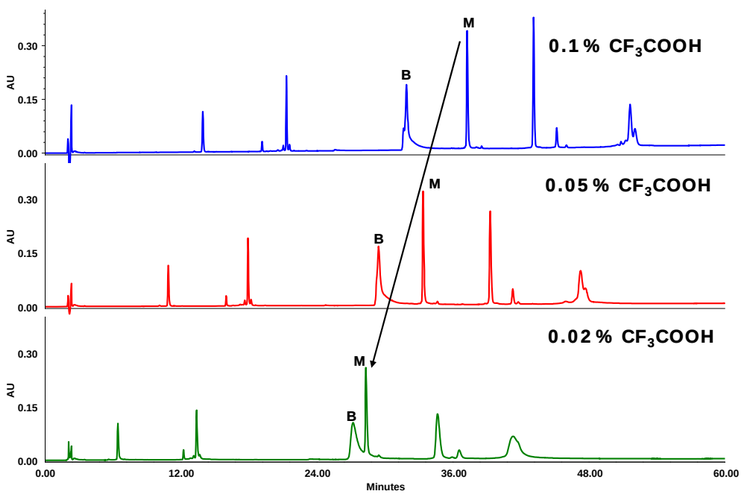


Figure 4. Selectivity is altered with a change in acid concentration. This is most apparent in the relative retention of BSA (B) and Myoglobin (M). All proteins are less retained, and there is less resolution around Phosphorylase b with lower acid concentration.

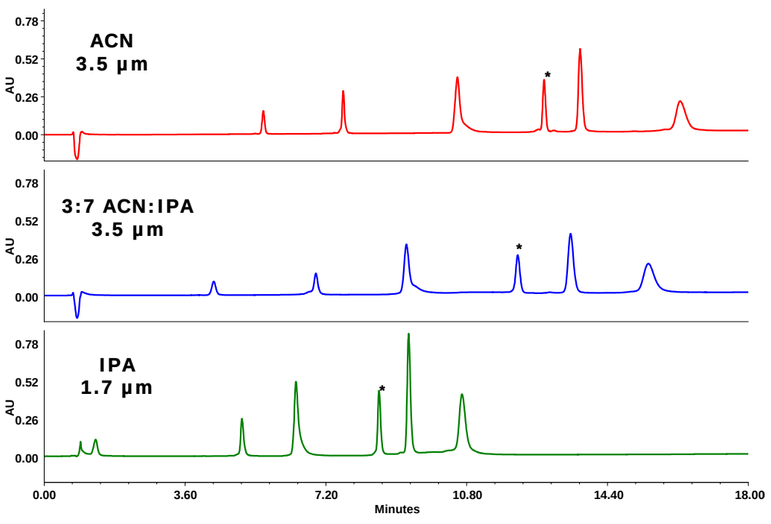


Figure 5. Effect of organic solvent on the separation of the Protein Mixture. Although there is reduced retention, little change in selectivity is seen when part or all of acetonitrile is replaced with IPA.
**Protein mixture contains β-lactoglobulin in place of Myoglobin.*

CONCLUSION AND SUMMARY

- **BEH300 C4 is a good general use column packing material for reversed-phase protein separations**
- **The 1.7µm UPLC® technology particles give improved resolution**
- **Some proteins are dramatically affected by the temperature of the separation**
- **Separation selectivity can be modified**
  - **Temperature**
  - **Modifier concentration**
  - **Organic solvent**
- **Method parameters can be varied concurrently, as required by a specific sample and application**

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