DEVELOPING METHODS FOR PROTEIN SEPARATIONS ON A NEW REVERSED-PHASE COLUMN

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INTRODUCTION

The growing need for fast and accurate characterization of large biological molecules has required ever increasing sensitivity and resolution in the separation of these molecules. With the application of Waters® ACQUITY UPLC® technology and the development of a new column chemistry for the reversed-phase separation of proteins, users can leverage these technologies for increased confidence and efficiency of their processes.

The development of this column packing material is described, including the influence of the base particle, pore size, and bonded phase on the separation. The impact of design choices was measured based on the behavior of protein probes representing a range of properties as well as some representative applications. The comparisons encompass large and small, acidic and basic, hydrophilic and hydrophobic proteins, as well as monoclonal antibodies and their subunits. The measurement criteria include peak shape, peak area, and resolution. The same set of proteins was also used to evaluate the influence of mobile phase constituents, solvent choice, separation temperature, and flow rate in developing a method for separation. We show that simple modifications can provide useful alterations in the selectivity that can be adapted to particular samples. Whether it is for identification and quantitation of target proteins and their possible modification, or monitoring trace impurities, we show that the Waters BEH300 C4 column provides a suitable material for reversed-phase protein separation.

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 Selection

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

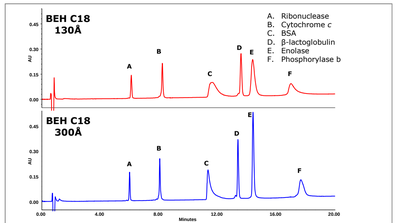

Figure 1A Effect of pore size on the separation.

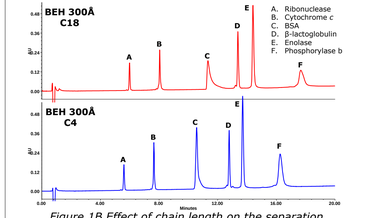

Figure 1B Effect of chain length on the separation.

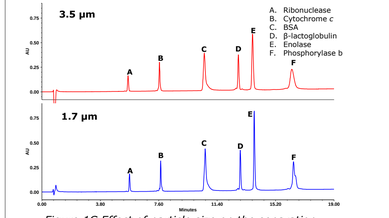

Figure 1C Effect of particle size on the separation.

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

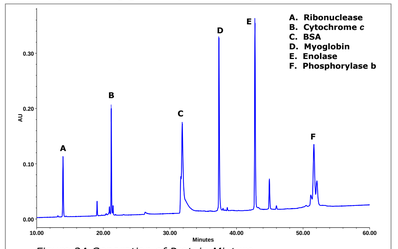

Figure 2A Separation of Protein Mixture

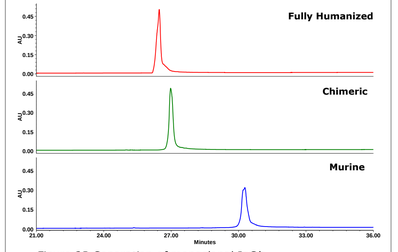

Figure 2B Separation of monoclonal IgG's

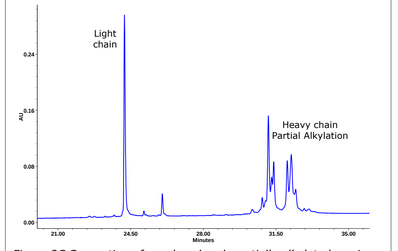

Figure 2C Separation of a reduced and partially alkylated murine monoclonal IgG

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

Some method options for optimizing protein separations
- Temperature
- Acid concentration
- Organic modifier
- Particle size
- Flow rate

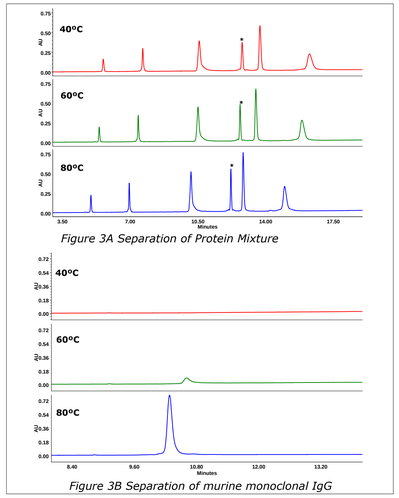

Figure 3A Separation of Protein Mixture

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.*

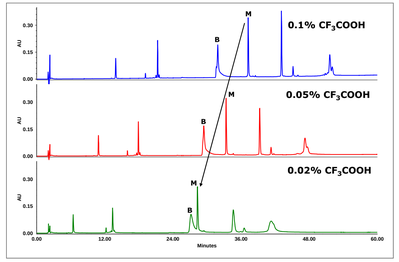

Figure 4. Selectivity is altered with a change in acid concentration. This is most apparent in the relative retention of BSA (B) and Myoglobin (M). All proteins are less retained, and there is less resolution around Phosphorylase b with lower acid concentration.

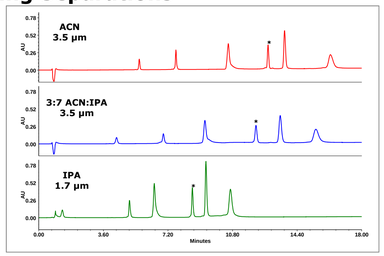

Figure 5. Effect of organic solvent on the separation of the Protein Mixture. Although there is reduced retention, little change in selectivity is seen when part or all of acetonitrile is replaced with IPA.
**Protein mixture contains β-lactoglobulin in place of Myoglobin.*

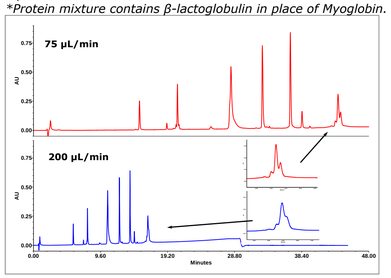

Figure 6. Decreased flow rate can increase resolution, as shown most clearly with the inset chromatogram of Phosphorylase b. Run time is, however, increased proportionally.

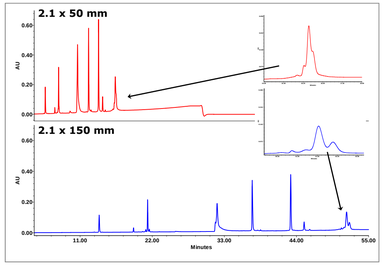

Figure 7. With longer columns, resolution can be increased, as shown in the inset. The small peaks surrounding the main Phosphorylase b peak are more resolved on the longer column. The peaks are sharper in the shorter column, as expected. Run time is proportional to column length.

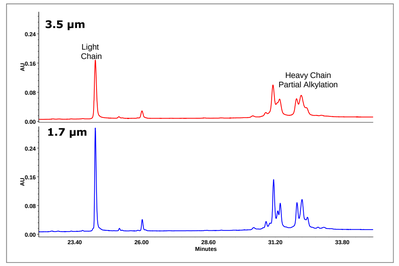

Figure 8. The use of smaller particle size packing, as implemented in UPLC® technology, provides the best resolution, as can be seen in the heavy chain partial alkylation species.

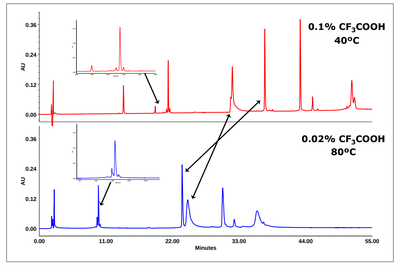

Figure 9. Variables such as temperature and acid concentration can be combined to give the desired separation. The arrows show an elution order changed between BSA and Myoglobin, while the inset shows better separation of trace level impurities from the Cytochrome c peak at lower temperature and higher acid concentration.

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**
- **Flow rate can alter resolution and sensitivity**
- **Column length can alter resolution and sensitivity**
- **Method parameters can be varied concurrently, as required by a specific sample and application**

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