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

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Hillary B. Hewitson, Thomas E. Wheat, Uwe D. Neue, Paula Hong, Kenneth J. Fountain
Waters Corporation, Milford, MA

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 use of this new column packing material is described, focusing on factors that control the separation. The impact of operating 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 set of proteins was used to evaluate the influence of mobile phase constituents, solvent choice, separation temperature, column length 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. We also show the use of separation measurements for estimating plate count in reversed-phase gradient protein separations. 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

Factors Affecting Protein Separations

Physical Factors

- Particle size—Smaller particles give narrower peaks
- Column length—Longer column gives higher plate count
- Flow rate—Best resolution is dependent on protein size

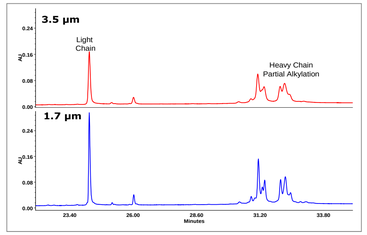

Figure 1. The use of smaller particle size packing, as implemented in UPLC® technology, provides the best resolution while also increasing sensitivity. These characteristics are most apparent in the partially alkylated heavy chain.

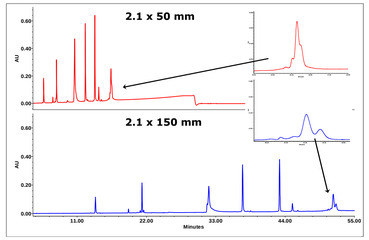

Figure 2. With increased column length, resolution increases. However, sensitivity is decreased and run time is increased.

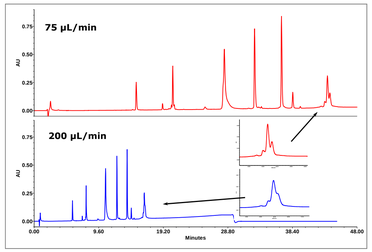

Figure 3. Best sensitivity and resolution are observed at relatively low flow rates, as shown in the inset. Run time is, however, increased proportionally.

Chemical Factors

Chemical factors affect the separation by more complex mechanisms than the physical parameters. These effects are dependent on the structure of the proteins being separated. Modifier concentration, organic solvent, temperature, and gradient slope were examined.

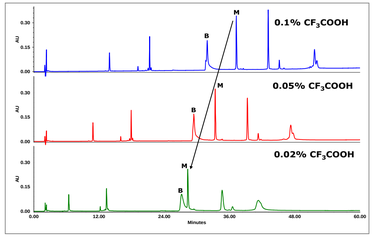

Figure 4. Retention is lower at reduced TFA concentrations, as expected for ion-pairing effects. In this example, Myoglobin is most sensitive, reflecting its structural characteristics. Peaks, especially the larger proteins, are wider with lower acid concentration.

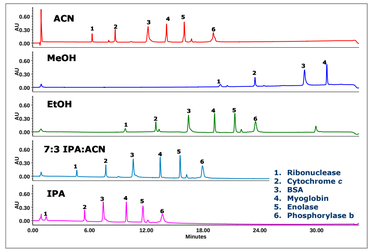

Figure 5. Effect of organic solvent on the separation of the Protein Mixture at 40°C. Although there is reduced retention, little change in selectivity is seen when part or all of acetonitrile is replaced with another organic solvent.

Mobile Phase B	Temp (ºC)	Initial PSI	Highest PSI	% B at Highest PSI
100% ACN	40	4800	4800	20
	80	3900	3900	20
100% MeOH	40	5800	6700	45
	80	4900	5500	45
100% EtOH	40	7000	10300	60
	80	5700	8000	60
7:3 IPA: ACN	40	6300	8200	55
	80	5100	6400	55
100% IPA	40	7600	13600	70
	80	6100	11200	70

Table 1. Observed system pressures for alternative organic solvents with the gradient separation. These guidelines facilitate successful protein separations with high viscosity mobile phases.

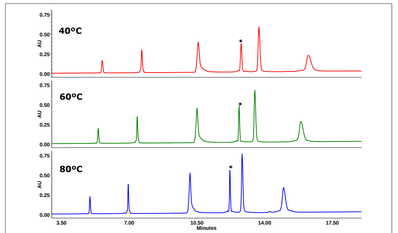

*Figure 6. Separation of Protein Mixture at increasing temperature. For this sample, only small decreases in retention and peak volume are observed. *β-lactoglobulin in place of Myoglobin in this sample.*

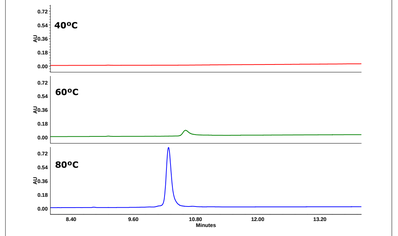

Figure 7. In contrast to Figure 6, the chromatographic behavior of IgG is dramatically improved at elevated temperature.

Separation of Similar Proteins

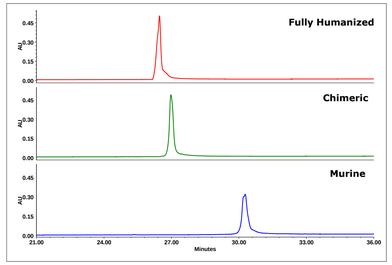

Figure 8. Most of the prior examples show mixtures of protein with very different structure. Reversed-phase can provide separation of similar proteins, as shown for these three monoclonal antibodies.

Gradient Effects

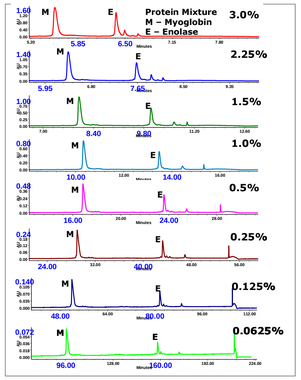

Figure 9. The most common tool for improving resolution is adjustment of gradient slope, expressed as % increase in acetonitrile per column volume of gradient duration. Shallower gradients improve resolution while reducing sensitivity and increasing run time.

Gradient Slope (Δ %B/Col. Vol.)	Myoglobin / Enolase Resolution	Enolase Peak Height (AU)	Enolase Peak Volume (µL)
3.00	10.9	1.35	18
2.25	12.8	0.88	25
1.50	15.7	0.67	33
1.00	18.9	0.51	46
0.50	24.8	0.30	52
0.25	29.9	0.16	88
0.125	35.4	0.08	158
0.0625	41.0	0.04	276

Table 2. The quantitative relationship among gradient slope, resolution, and peak sensitivity and volume. Particularly with shallow gradients, sensitivity is lost more rapidly than resolution is gained.

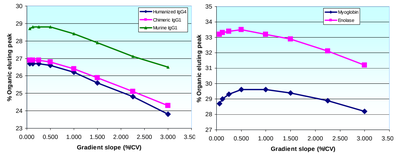

Figure 10. In measuring the effect of gradient slope, it is possible to estimate the concentration of acetonitrile at the point of elution. This eluting solvent strength varies with gradient slope. For each protein, a maximum is observed, and that maximum occurs at a gradient slope that appears to be approximately related to the molecular weight of the protein. This effect probably reflects adsorption-desorption kinetics.

True Plate Counts
Proteins in Reversed-phase Gradients

Theory

For a linear reversed-phase gradient, the (true) retention factor at the point of elution k_e is shown by equation (1), where k_0 is the retention factor of the analyte at the beginning of the gradient, and G is the generalized gradient slope, defined by equation (2):

$$k_e = \frac{k_0}{G \cdot k_0 + 1} \quad (1) \qquad G = B \cdot \Delta c \frac{t_0}{t_g} \quad (2)$$

Δc is the difference in the solvent composition over the gradient, t_0 is the retention time of an unretained peak, t_g is the gradient run time, and B is the slope of the relationship between the natural logarithm of the retention factor of the analyte and the solvent composition. We program the gradient parameters, but we do not know the factor B, which is compound specific.
For large molecules such as proteins (and a few other conditions) k_0 is typically very large, and the equation for k_e simplifies to:

$$k_e = \frac{1}{G} \quad (3)$$

If we can determine k_e, we can then calculate the true plate count N of a protein from the measured peak width w (in volume units), shown by equation (4). V_0 is the retention volume of an unretained peak, and f is a factor that depends how we measure the peak width w. We can rearrange this equation to obtain an expression for the peak width, as shown by equation (5):

$$N = f^2 \cdot \frac{V_0^2 \cdot (k_e + 1)^2}{w^2} \quad (4) \qquad w = f \cdot \frac{V_0}{\sqrt{N}} \cdot (k_e + 1) \quad (5)$$

Inserting equations 3 and 2 into equation 5, we obtain a simple relationship between the measured peak width and the gradient slope, where S is the programmed gradient slope:

$$w = f \cdot \frac{V_0}{\sqrt{N}} \cdot \left(\frac{1}{B \cdot S} + 1\right) \quad (6a) \qquad S = \Delta c \cdot \frac{t_0}{t_g} \quad (6b)$$

Therefore, if we plot the peak width w versus the inverse of the gradient slope, we should get a straight line with a slope $f \cdot \frac{V_0}{\sqrt{N}} \cdot \frac{1}{B}$ and the intercept $f \cdot \frac{V_0}{\sqrt{N}}$. Dividing the second value by the first gives us the protein-specific value of B, which in turn permits a calculation of the true plate count.

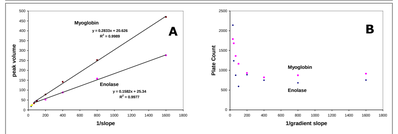

Figure 11. (A) The values for slope B were about 90 for Myoglobin and 160 for Enolase, in agreement with the difference in molecular weight (small molecules typically have a slope near 10). (B) From these slope values, we can calculate the plate counts for proteins as approximately 1000.

CONCLUSION AND SUMMARY

- **BEH300 C4 is a good general use wide-pore column packing material for reversed-phase protein separations**
- **The 1.7µm UPLC® particles give improved resolution as compared to traditional HPLC packing materials**
- **Protein separations are affected by temperature**
- **Modifier concentration affects selectivity**
- **Organic solvent choice affects retention more than selectivity**
- **Flow rate can alter resolution**
- **Column length can alter resolution and sensitivity**
- **Gradient slope affects both resolution and sensitivity**
- **Plate counts can be measured for proteins in reversed-phase gradients**

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