CONSIDERATIONS FOR REVERSED-PHASE METHOD DEVELOPMENT OF PROTEIN MOLECULES

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

INTRODUCTION

Increased emphasis on the development of protein biopharmaceuticals has brought along with it the need to accurately and reliably characterize these macromolecules. There are many techniques available to the users towards this end, such as biological binding assays, electrophoresis, chromatography, and mass spectrometry. These large molecules require the use of multiple techniques for complete characterization. Each technique can reveal unique information about the protein being analyzed. Reversed-phase chromatography is a powerful probe technique that provides a great deal of sensitivity and resolution, and hence, information about identity, variants, and quantity of protein present.

The combination of instrumentation, chemistry, and methodology optimized for protein separations will be discussed. The physical and chemical factors that affect the separations will be systematically evaluated, including particle size, column length, flow rate, modifier type and concentration, organic solvent, temperature, and gradient slope. These factors will be quantitatively investigated with the analysis of proteins representing a wide range of properties. The comparisons encompass large and small, acidic and basic, hydrophobic and hydrophilic proteins, as well as monoclonal antibodies and their subunits. The measurement probes include peak retention, shape, area, resolution, and carryover. We will show how simple modifications to these method parameters can alter the separation to achieve the desired selectivity and sensitivity for particular protein samples. In addition to providing basic guidelines for the development of reversed-phase protein separations, we will show that the Waters BEH300 C4 column provides a suitable material for these separations, both qualitatively and quantitatively.

METHODS

Chromatographic Conditions

All separation conditions as indicated, unless otherwise noted in the figure legends.
ACQUITY UPLC® System w/TUV detection @ 220nm

Column:	BEH300 C_4, 2.1 x 50 mm, 1.7 µm
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:	40°C for Protein Mixture 80°C for IgG and Reduced/Alkylated IgG
Injection volume:	3.3 µL

Gradient for 2.1 mm x 50 mm column:(otherwise scaled proportionally to specified conditions)

Time (min)	% A	%B	% B / CV
Initial	80	20	-
25	28.6	71.4	1.8
27	28.6	71.4	-
45	80	20	-

Samples

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

Monoclonal antibodies mixture (murine, chimeric, and fully humanized):
0.3—1 mg/mL in 0.1% CF_3COOH

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

Fully humanized and murine monoclonal antibody (quantitative linearity set)
Samples prepared in 0.1% CF_3COOH for mass load targets:
0.1, 0.5, 1, 5, 7.5, 10, 20, 30, 40, 50 µg

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

RESULTS

Factors Affecting Quality of Protein Separations

Physical Factors

- Particle size—Smaller particles give narrower peaks
- Column length—Longer column gives higher plate count
- Flow rate—Lower optimum flow rate due to larger size of protein molecules

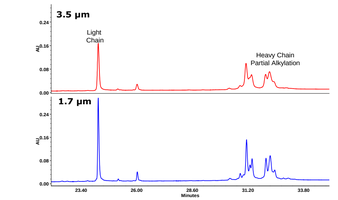


Figure 1. The use of a smaller particle size packing, as implemented in UPLC® technology, provides the best resolution, as can be seen in the heavy chain partial alkylation species.

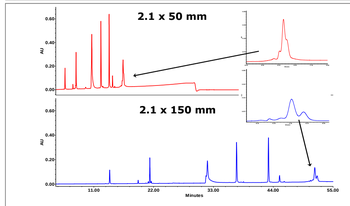


Figure 2. With the use of longer columns, resolution can be increased, as shown in the inset. However, run time is proportionally increased and sensitivity is sacrificed.

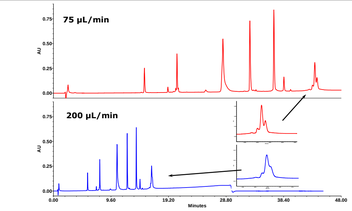


Figure 3. The best sensitivity and resolution are observed at relatively low flow rates, as shown in the inset. Run time is, however, increased proportionally.

Chemical Factors

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

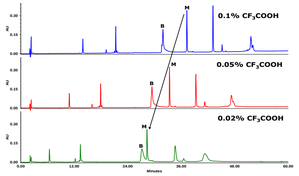


Figure 4. A decrease in retention at reduced TFA concentrations is observed for all proteins, due to ion-pairing effects. In this example, Myoglobin is most sensitive, reflecting its structural characteristics. With lower acid concentration, the peaks are wider, especially the larger proteins such as BSA and Phosphorylase b.

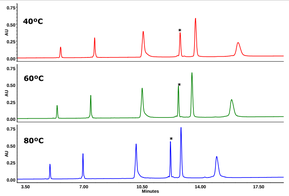


*Figure 5. Separation of Protein Mixture at increasing temperature. For this sample, only small decreases in retention and peak volume are observed. *β-lactoglobulin replaces Myoglobin in this sample.*

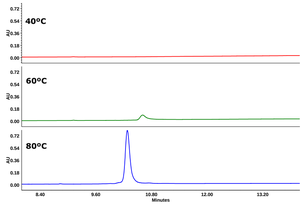
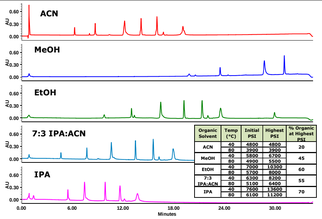


Figure 6. While only minor changes are observed with the protein mixture in Figure 5, the chromatographic behavior of IgG is dramatically improved at elevated temperature.



Organic Solvent	Temp (°C)	Initial PSI	Highest PSI	% Organic at Highest PSI
ACN	40	4800	6800	20
MeOH	40	2800	4700	45
	80	4800	5800	
EtOH	40	4200	10000	40
	80	4200	11000	
7:3 IPA:ACN	40	6100	8300	55
	80	3100	6400	
IPA	40	7600	13000	70
	80	4100	11000	

Figure 7. 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 another organic solvent. The inset table can facilitate the successful migration of methods to the use of more viscous mobile phases.

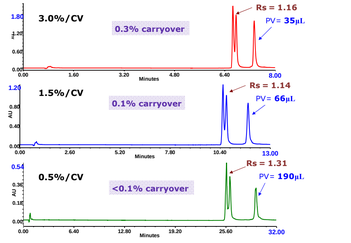
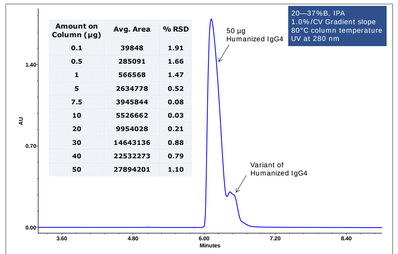


Figure 8. Effect of gradient slope on the separation of a mixture of monoclonal antibodies. Resolution is gained with decreased gradient slope, while run time and sensitivity are lost. There is an improvement in recovery with the shallower gradient.

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 1. Similar to the comparison shown in Figure 8, a larger data set was obtained using a mixture containing Myoglobin and Enolase. The quantitative relationship among gradient slope, resolution, peak sensitivity and volume is shown. Particularly with shallow gradients, sensitivity is lost more rapidly than resolution is gained.

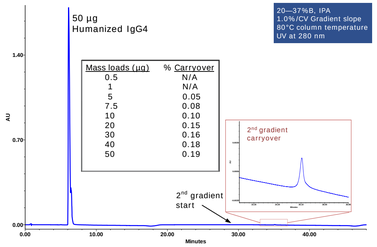
Quantitation



Amount on Column (µg)	Avg. Area	% RSD
0.1	39848	1.91
0.5	285091	1.66
1	566568	1.47
5	2634778	0.52
7.5	3945844	0.08
10	5526662	0.03
20	9954028	0.21
30	14643136	0.88
40	22532273	0.79
50	27894201	1.10

Figure 9. Triplicate injections of a constant volume of humanized IgG were made from 0.1 to 50 µg on-column. A dynamic range of 3 orders of magnitude can be achieved from 0.5 to 50 µg with an R^2 of 0.994. The lower end of linearity is limited by the reproducibility of the integration, while the upper end is constrained by the limit of detector linear response, as well as peak shape distortion affecting integration reproducibility. % RSD values of < 2% was observed for all mass loads. At higher mass loads, variant forms of the protein can also be easily seen and measured.

Memory effect carryover is protein that did not elute in the first gradient, but is still associated with the column. Since there is no movement of valves for injection in the second internal gradient, peaks eluting at the same relative point in the gradient as the protein in the first gradient are determined to be memory effect carryover.



Mass loads (µg)	% Carryover
0.5	N/A
1	N/A
5	0.05
7.5	0.08
10	0.10
20	0.15
30	0.16
40	0.18
50	0.19

Figure 10. The humanized IgG sample used in the determination of linear range, as seen in Figure 9, was also used in the estimation of the carryover of protein in the column. A second internal gradient was performed for each sample injection. The inset table shows that carryover on BEH300 C_4 did not exceed 0.2% for any amount loaded.

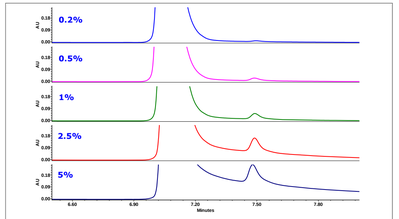


Figure 11. Protein impurities were quantitated in the presence of a large amount of another protein (50 µg IgG on column). The same conditions indicated in Figure 9 were used, but with a 3% gradient slope. An apparent tail on the main API peak can be seen at the higher impurity loads, which is accompanied by a decrease in peak height. This tail is either attributed to a sample degradation throughout the analysis or to interaction of the proteins, which can be seen more readily at the higher impurity mass loads.

Impurity				API	
Nominal % of API	Measured % of API	Retention Time % RSD	Area % RSD	Retention Time % RSD	Area % RSD
0.10	0.05	0.18	3.89	0.24	0.26
0.20	0.13	0.08	0.86	0.08	0.05
0.50	0.42	0.05	0.58	0.08	0.52
1.00	0.94	0.09	1.61	0.06	0.83
2.50	2.48	0.06	1.05	0.07	0.36
5.00	4.88	0.05	2.43	0.08	2.78

Table 2. The quantitative reliability of five replicate injections of the spiked impurity at six different levels was evaluated. The measured impurity amount matched the expected values at levels above 0.1%. While the retention time reproducibility was good at all levels tested, the reproducibility of area of both impurity and API deteriorated at the 5% load. This irreproducibility can be attributed to the apparent tailing, seen in Figure 11. The lowest impurity level tested showed higher %RSD for area as well.

CONCLUSION

- **BEH300 C4 is a good general use column packing material for reversed-phase protein separations.**
- **Resolution can be gained by use of smaller particles, longer columns, and changing the column temperature and gradient slope.**
- **Selectivity can be affected by modifier concentration and column temperature.**
- **Organic solvent can be used to affect retention and recovery.**
- **Sensitivity is influenced by flow rate, column length, modifier concentration, and gradient slope.**
- **Protein recovery is influenced by gradient slope as well as mass load of protein on column.**
- **Three orders of quantitative dynamic range is reliably achieved with < 0.2% carryover.**
- **Reliable quantitation of trace impurities down to 0.2% was successfully demonstrated.**

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