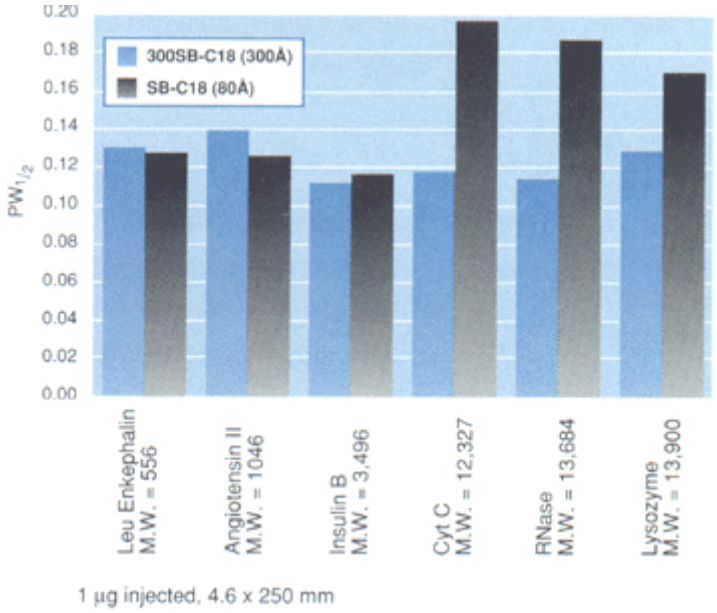
Agilent Technologies

Choosing Right Column for Reverse Phase HPLC Separations

1. **Pore Size**
   Choose a column packing with small pore size (60-80Å) if the solute molecular weight is less than about 2000 - 4000 Daltons. Otherwise, use a column packing with 300Å pore size. Figures 1 and 2 shows the effects of pore size on peak width and sample load. Note that the higher surface area, 80Å packing, tolerates higher sample load.

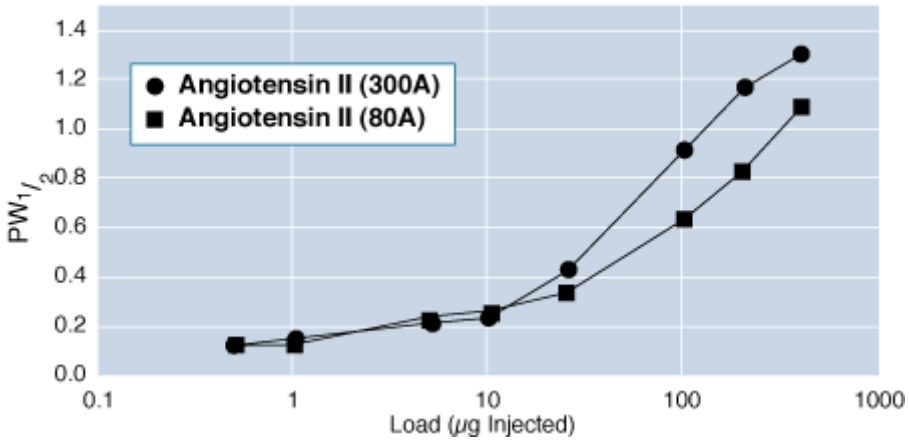
Figure 1: Effect of Pore Size and Molecular Size on Peak Width (PW1/2)



2. Particle Size

The standard particle size for HPLC is 5 µ m. If high-speed (faster than 5 minutes/run) analyses are required, packing with 3.0 or 3.5 um particles in shorter columns can produce high-resolution separations in less time. Many products that use 3 um packing use 0.5 um frits that plug easily. However, using ZORBAX Rapid Resolution columns* (3.5 um particles with a narrow particle-size distribution, no fines, and 2 um frits) offers relief from plugging and permits use of standard equipment while reducing run times.

Figure 2: Effect of Sample Load on Peak Width (PW1/2): Pore Size Influence



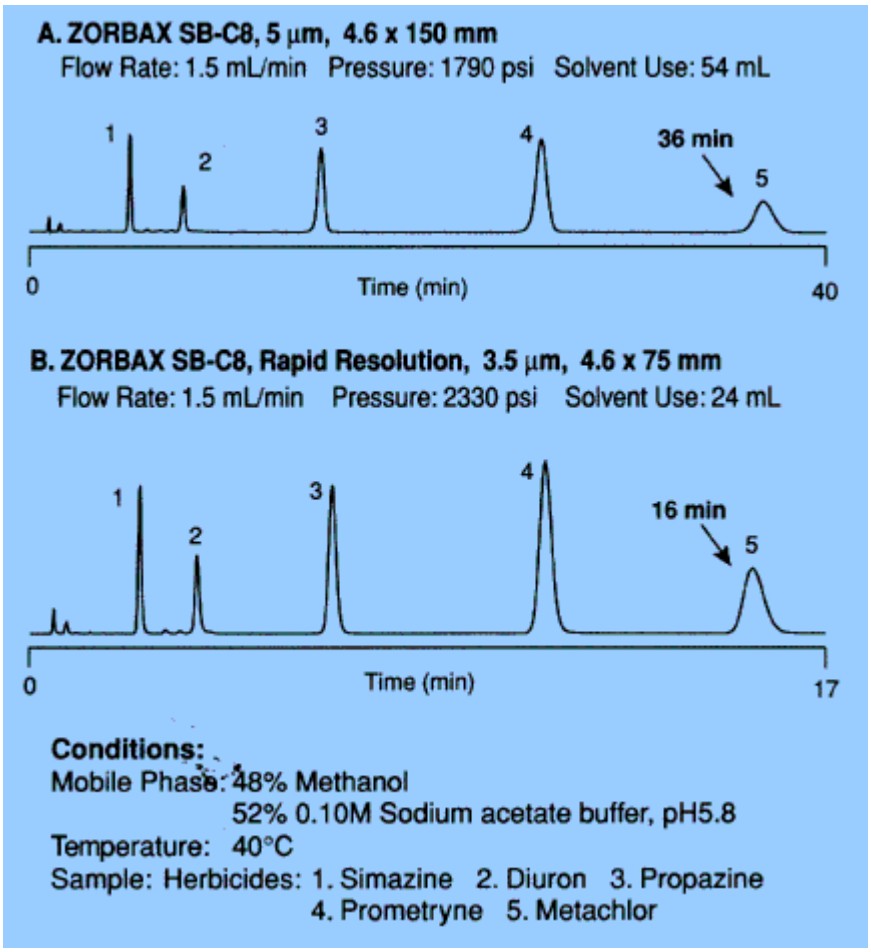
3. Column Configuration

The column configuration most often recommended for analytical method development is 4.6 x 150 mm. If more resolution is needed, use a longer column, 4.6 x 250 mm. After method development, choose the column internal diameter (e.g., 2.1, 3.0 mm) to accommodate additional application objectives (e.g., sensitivity, solvent usage).

Table 1: Column Choice Relative to Application Objective

APPLICATION OBJECTIVE	COLUMN DIAMETER(mm)
Save solvent; special low-volume instrumentation is available	2.1
Save solvent; standard HPLC equipment available	3.0
Special detectors, e.g., mass spec	2.1
High sensitivity, limited sample	2.1
Standard Separations	4.6
Small-scale (mg) preparative separations	9.4
Large-scale preparative separations(hundreds of mg-gm)	21.2

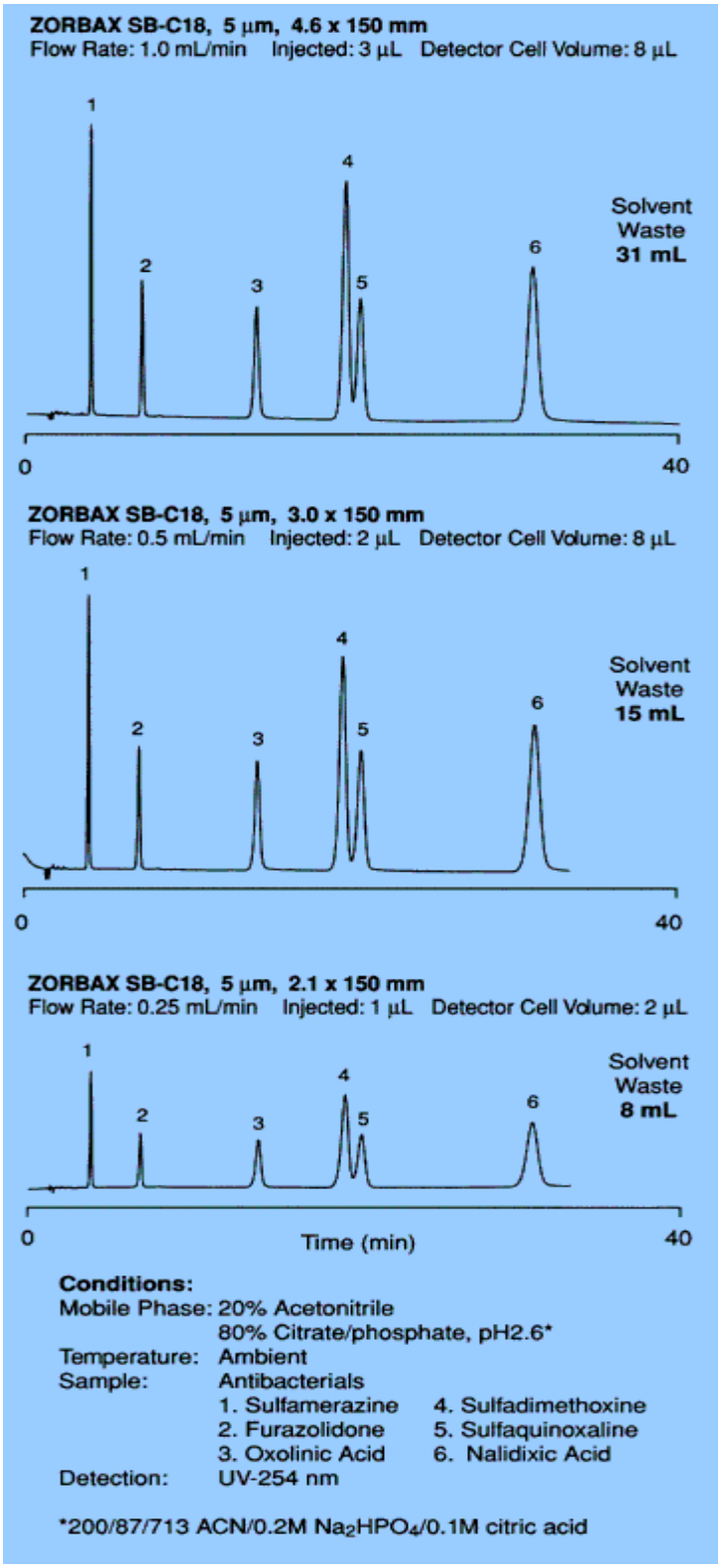
Figure3: Increase in Speed and Reduction in Solvent Usage in Herbicide Separation Using 3.5 um Rapid Resolution ZORBAX SB-C8



4. Silica Type and Bonded Phase

A) SILICA TYPE. ZORBAX reversed-phase columns use two different types of porous silica microspheres, the original ZORBAX SIL and ZORBAX Rx-SIL. The original ZORBAX SIL is a less-purified, more-acidic silica than ZORBAX Rx-SIL. Increased acidity means a larger potential for interaction between the analyte and silanol groups on the silica surface, especially if the solutes are basic. If doing research or method development on a new procedure, we strongly recommend using reversed-phase products based on ZORBAX Rx-SIL. However, many excellent methods have been developed on ZORBAX SIL and we continue to manufacture these high quality reliable products.

Figure 4: Saving Solvent Using Different Column Configurations



B) BONDED PHASE. A good first choice for bonded phase is C8 or C18. If the sample solutes of interest are not adequately separated on these columns, the CN and Phenyl columns may offer sufficient differences in selectivity from the straight-chain alkane phases to effect the separation. Refer to the method development section to choose the phase appropriate to anticipated mobile phase PH.

Considering Non-Ideal Behavior in SEC of Proteins:

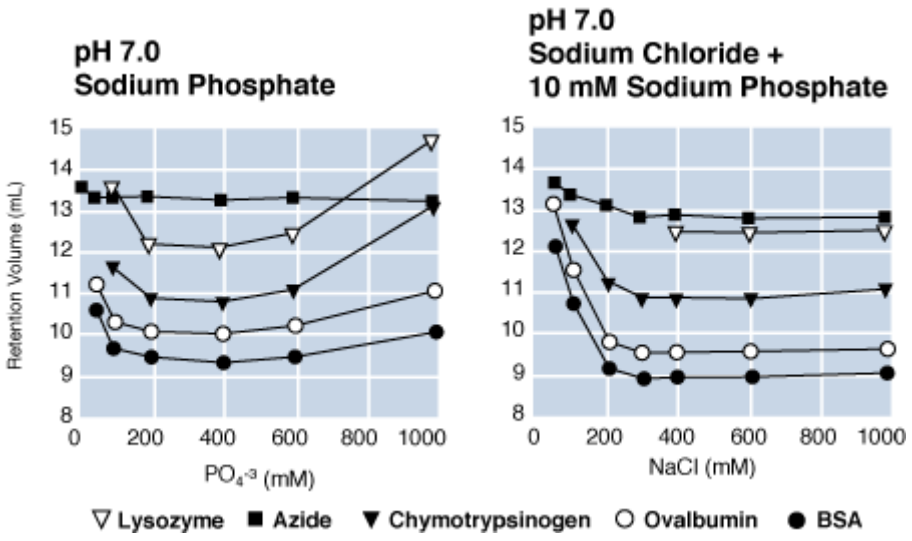
Size-exclusion columns separate proteins based on molecular weight, or more accurately, hydrodynamic volume. Ideally, the surface of the chromatography media should not interact with the protein, a requirement difficult to meet for a wide variety of proteins. ZORBAX GF-250 and ZORBAX GF-450 columns are manufactured to minimize protein interactions with the bonded silica surface and to insure the highest reproducibility.

Before choosing the mobile phase for separating protein mixtures, consider whether proteins in the sample may exhibit non-ideal behavior. Most proteins are well behaved when separated on GF-250 and GF-450 (Figure 6). Some proteins, however, display non-ideal behavior at either low ionic strength or high ionic strength, resulting in a mixed-mode separation instead of a purely size exclusion separation.

Figure 7A shows that at low ionic strength, cytochrome C displays electrostatic-type interactions with the silica surface, as shown by increased retention volume and peak broadening1. In Figure 7B, insulin shows hydrophobic interaction at high ionic strengths, again, leading to retention and peak broadening. In Figure 7C, lysozyme shows both electrostatic and hydrophobic interaction at both extremes of ionic strength.

Table 1 summarizes data for several proteins separated on the GF-250 column. To avoid non-ideal behavior, we recommend using 200 mM sodium phosphate at pH 7.0. Alternatives are 50 mM sodium phosphate pH 7.0 or 50 mM Tris-HCl, pH 7.0-8.0, both containing 200-400 mM NaCl, or 50 mM Tris-HCl, pH 7.0-8.0, containing 200 mM ammonium sulfate. If your protein is similar to a protein that displays non-ideal behavior, adjust the ionic strength to the middle of a region in the chart where the retention remains constant for changes in ionic strength.
For more information about GF-250, view our technical note.

Figure 4: Effect of Mobile Phase Ionic Strength on Protein-Stationary Phase Interactions Using GF-250



At low ionic strength, electrostatic interactions can cause a change in retention, whereas at high ionic strength, hydrophobic interactions can cause a change in retention. At lower (e.g., pH 5.5) or higher (e.g., pH 8.0) pH, these effects can become even more pronounced. (Ref. 2)

With phosphate buffer, a mobile phase of 200 mM sodium phosphate, pH 7.0 is recommended. Use of high concentrations of phosphate at higher temperatures or pH may cause slow dissolution of silica. Alternatives are 50 mM sodium phosphate pH 7.0 or 50 mM Tris-HCl, pH 7.0-8.0, both containing 200-400 mM NaCl, or 50 mM Tris-HCl, pH 7.0-8.0, containing 200 mM ammonium sulfate.

Figure 6: Non-Ideal Behavior of Cytochrome C, Insulin, and Lysozyme at Low and High Ionic Strength1

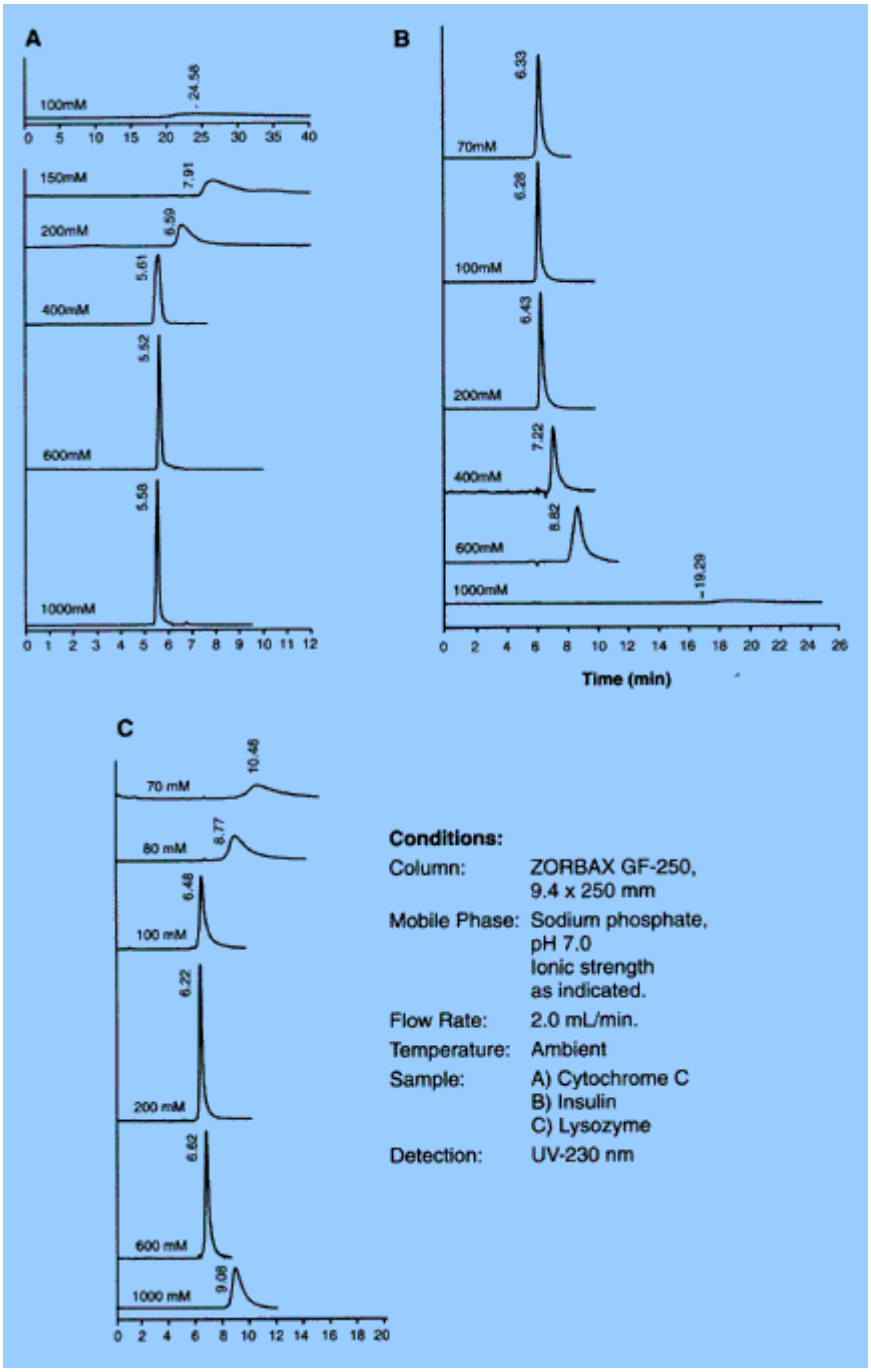


Table 2: K* versus Ionic Strength for Various Proteins Separated on ZORBAX GF-250 (1, 2) {κ* of Sample at Listed Concentration of Sodium Phosphate pH 7.0(3)}

Sample Name	pI	M.W.	50 mM	70 mM	80 mM	100 mM	150 mM	200 mM	400 mM	600 mM	1000 mM
Thyroglobulin	5.1	669,000	0.54	0.54	0.54	0.54	0.54	0.53	0.55	0.55	0.56
Ferritin	4.2	440,000	0.67	0.63	0.64	0.65	0.64	0.64	0.67	0.73	0.88
Catalase	5.4	250,000	0.68	0.68	0.68	0.69	0.68	0.68	0.71	0.75	1.54
Beta Amylase	5.4	200,000	0.64	0.63	0.63	0.64	0.64	0.64	0.65	0.66	0.78
Alcohol Dehydrogenase	6.8	150,000	0.66	0.66	0.66	0.67	0.67	0.67	0.67	0.68	0.72
BSA Dimer	5.4	132,000	0.64	0.63	0.64	0.64	0.64	0.64	0.65	0.66	0.72
BSA	5.1	66,430	0.70	0.70	0.70	0.70	0.70	0.70	0.72	0.73	0.79
Ovalbumin	4.6	44,000	0.75	0.74	0.75	0.75	0.75	0.75	0.78	0.79	0.89
Peroxidase	9.0	44,000	0.73	0.73	0.73	0.73	0.73	0.73	0.73	0.75	0.88
Pepsin	2.0	35,000	0.92	0.82	0.81	0.80	0.81	0.81	0.92	1.16	1.70
Carbonic Anhydrase	5.9	29,000	0.91	0.85	0.85	0.83	0.82	0.82	0.83	0.85	0.97
Chymotrypsinogen A	9.3	25,000	2.51	1.16	1.00	0.88	0.84	0.83	0.83	0.90	1.23
Trypsinogen	9.3	23,970	0.87	0.84	0.83	0.82	0.80	0.82	0.83	0.85	0.89
Trypsin	10.5	23,280	0.93	0.90	0.87	0.85	0.83	0.83	0.85	0.86	0.95
a-Chymotrypsin	8.8	21,600	2.10	1.09	0.96	0.86	0.82	0.82	0.83	0.84	0.92
Myoglobin	7.2	17,600	0.82	0.82	0.82	0.82	0.82	0.82	0.83	0.83	0.87
Lysozyme	10.0	14,300	2.10	1.59	1.33	1.02	0.94	0.95	0.95	1.00	1.38
Ribonuclease A	7.8	13,700	4.25	2.74	1.82	1.78	0.96	0.85	0.86	0.87	0.91
Cytochrome C	9.6	12,400	6.47	4.90	4.26	3.72	1.20	0.92	0.85	0.84	0.85
Aprotinin	10.0	6,500	2.75	2.18	1.72	1.11	0.96	0.94	0.93	0.96	1.10
Insulin	5.7	6,000	0.97	0.97	0.97	0.95	0.96	0.97	1.09	1.34	2.92
Vitamin B12	‹	1,350	0.95	0.96	0.95	0.95	0.96	0.99	1.04	1.19	1.44
Uridine	-	240	0.95	0.99	0.95	0.94	0.95	0.96	0.97	0.97	1.03
Uracil	-	120	0.99	0.99	0.99	0.98	0.99	1.00	1.01	1.01	1.02
Sodium Azide	-	60	0.99	0.99	1.00	1.00	1.00	1.00	1.01	1.02	1.05

1. Ricker, R.D., L.A. Sandoval, J.D. Justice and F.O. Geiser, Multivariate Visualization in the Size-Exclusion Chromatography of Biological Samples (1995) J. Chromatography A, 691, 67-79.
2. K* = Vr/Vm, where Vr = retention volume of protein, Vm = total permeation volume of the column.