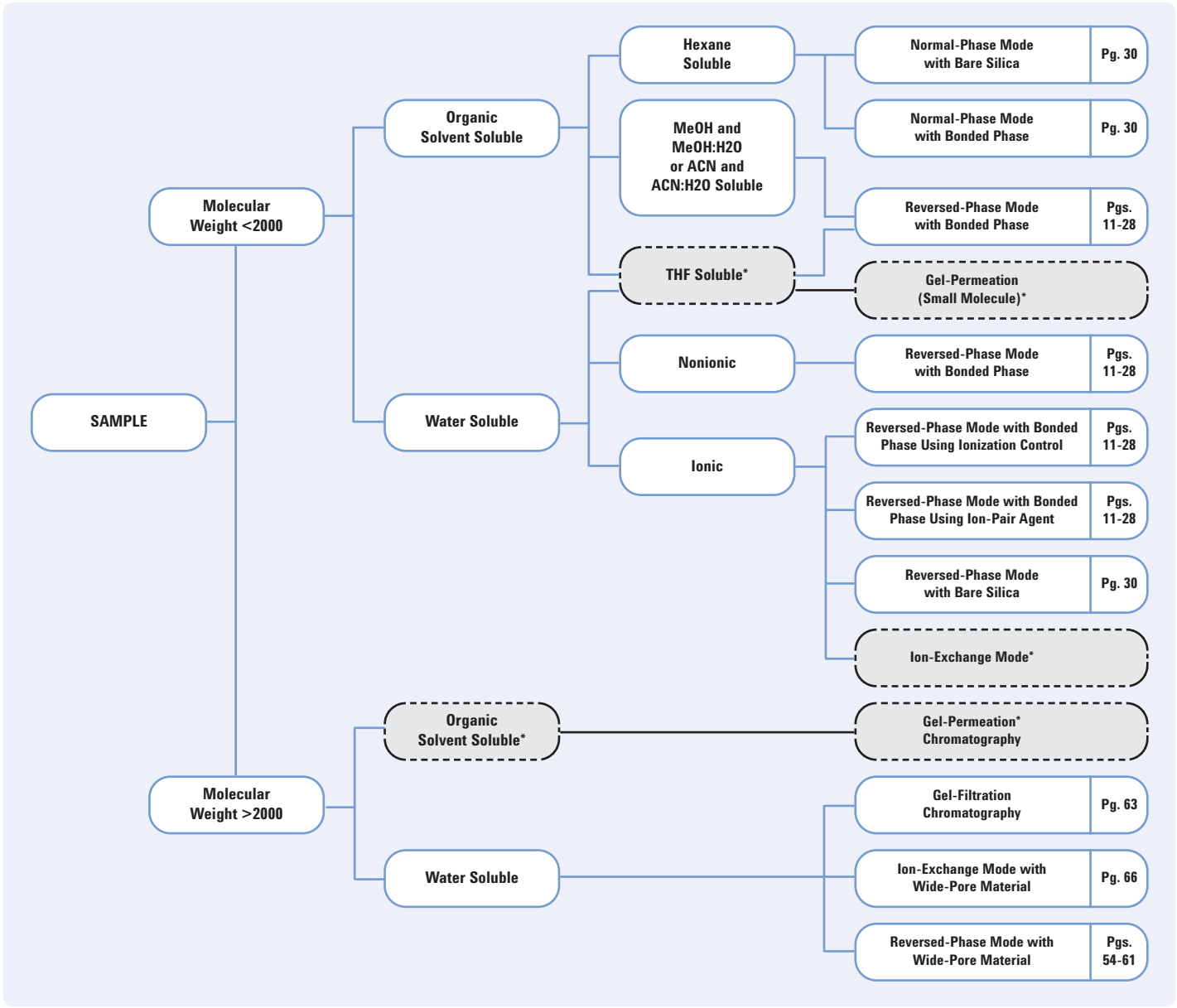
LC and LC/MS Column Selection Flow Chart

To use the column selection diagram below, simply follow the path for your analyte and mobile phase. At the far right, follow your final column selection to the pages indicated. The solid outlined boxes below show which products are located in this Column Selection Guide.



Adapted with permission from Brian A. Bidlingmeyer *Practical HPLC Methodology and Applications*, NY: John Wiley & Sons, Inc., New York, (1992) 109.

* Additional column choices can be found in the Agilent Technologies Chromatography and Spectroscopy Suppiles Catalog.

ZORBAX Reversed-Phase HPLC Column Selection Flow Chart

For small and large molecules

Most chromatographers use reversed-phase HPLC as one of their key analysis techniques. Reversed-phase HPLC can be used to analyze ionic and nonionic analytes. Therefore this ZORBAX Column Selection Flow Chart will focus on reversed-phase columns. To more easily select a reversed-phase column for method development of small and large molecules, follow the outline on these pages.

This flow chart provides information on choosing an initial column for method development of small molecule and protein and peptide samples, and includes decisions on bonded phase and column configuration.

Small Molecules
MW < 2000

Large Molecules
MW > 2000

80-120Å — **Packing Pore Size** — **300Å**

First choice of a packing pore size is based on the size of molecules to be analyzed. Typical small molecules can diffuse easily in and out of standard 80-120Å pore packings, but peptides and proteins may not. For this reason, it is recommend to use 300Å pore-size packings (300SB) for isocratic or gradient separations of peptides and proteins.

Eclipse XDB-C18 (see page 11) — **Starting Column Bonded Phase** — **StableBond 300SB-C18** (see page 54)

A C18 is recommended as the starting column bonded phase for most samples since it maximizes retention for moderately polar to non-polar compounds. Shorter chain phases should be considered if resolution cannot be optimized with a C18 phase or if you are analyzing larger proteins.

4.6 mm — **Column Diameter** — **4.6 mm**

Column diameter defines how much material (mass) you can inject on a column – the smaller the diameter, the less material that can be injected while maintaining good peak shape. Smaller diameter columns provide increased sensitivity and therefore are commonly used for LC/MS and sample limited applications.

Column Type	Inner Diameter (mm)	Sample Load (approx)	Typical Flow Rate	Increased Sensitivity	Applications
Analytical	4.6	0.1-1.5 mg	0.5-3 mL/min		Standard separations
Solvent Saver	3.0	150-500 ug	0.3-1.5 mL/min	+	Save solvent, uses standard HPLC instrument
NarrowBore	2.1	50-120 ug	0.1-0.5 mL/min	++	High sensitivity, limited sample, LC/MS, save solvent
Microbore	1.0	10-50 ug	10-100 µL/min	++++	High sensitivity, limited sample, LC/MS
Capillary	0.5, 0.3	1-10 ug	1-15 µL/min	+++++	Very high sensitivity, LC/MS, peptides and proteins
Nano	0.1, 0.075	100-200 ng	200-500 nL/min	++++++	Very high sensitivity, LC/MS, peptides and proteins
Semi-Prep	9.4	1-10 mg	5-10 mL/min		mg preparative separations
Preparative	21.2	20-250 mg	20-60 mL/min		Hundreds of milligrams up to 1 gram

Small Molecules
MW < 2000

Large Molecules
MW > 2000

5 µm — **Standard Porous Particle Size** — **5 µm**

For conventional analyses of small and large molecules, the standard particle size is 5 µm. However, smaller particle sizes are available and provide higher efficiency and higher resolution. They are also available in short column lengths.

Fast Analysis Particle Sizes

Rapid Resolution 3.5 µm
Rapid Resolution HT 1.8 µm

Rapid Resolution, 3.5 µm particles provide 60% greater efficiency than 5 µm particles while Rapid Resolution HT columns, 1.8 µm particles provide 200% greater efficiency. Shorter columns can be used to significantly reduce analysis time of small molecules.

Rapid Resolution 3.5 µm
Poroshell 5 µm

For peptides, Rapid Resolution, 3.5 µm particles can be used with shorter columns for faster, high-resolution gradient analyses. But for proteins, Poroshell columns, with a solid core and porous shell, provide the fastest analysis.

150 mm — **Column Length** — **150 mm**

A conventional column length is 150 mm or 250 mm for high resolution with 5 µm particles. This is a good place to start a typical analysis because it will provide high resolution in a 20-30 minute analysis. For fast analysis, shorter columns (75 mm and 50 mm) with smaller particle sizes (3.5 µm) should be used.

For proteins and peptide digests, 150 mm is also a good starting column length. The typical gradient time on a column of this length will be 30-60 minutes for a high-resolution analysis. For fast analysis, shorter columns (50 mm) with smaller particles (3.5 µm) should be used.

Starting Column Choices

Small Molecules

Standard Analysis
Eclipse XDB-C18
4.6 x 150 mm, 5 µm
P/N 993967-902
(see page 14)

Fast Analysis
Rapid Resolution
Eclipse XDB-C18
4.6 x 75 mm, 3.5 µm
P/N 966967-902
(see page 14)

Rapid Resolution HT
Eclipse XDB-C18
4.6 x 50 mm, 1.8 µm
P/N 922975-902
(see page 15)

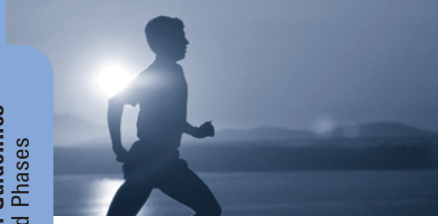
For additional method development guidelines, review the section on method development from low to high pH on page 8.

Large Molecules

Standard Analysis
300SB-C18
4.6 x 150 mm, 5 µm
P/N 883995-902
(see page 57)

Fast Analysis
Rapid Resolution
300SB-C18
4.6 x 50 mm, 3.5 µm
P/N 865973-902
(see page 57)

Poroshell
300SB-C18
2.1 x 75 mm, 5 µm
P/N 660750-902
(see page 59)



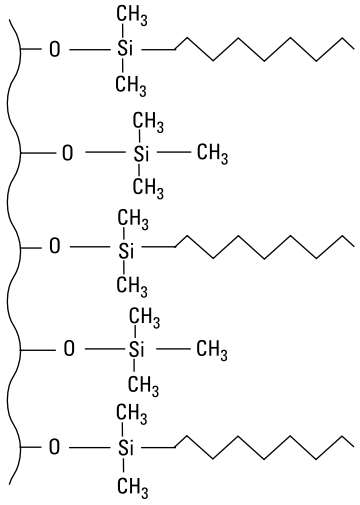
Quick Guide to ZORBAX Reversed-Phase Bonded Phases

Excellent peak shape and easy method development

Below is a brief description of each ZORBAX bonded phase. For more detail on these bonded phases and for column selection, use the tabs to turn to the appropriate section in this guide. It will provide a complete list of all the columns available.

Agilent ZORBAX reversed-phase bonded phases based on ultra-pure silica (Type B, Rx-Sil) — Eclipse XDB, StableBond, Bonus-RP, and Extend-C18 — provide the best peak shape and sample resolution, as well as long column lifetimes. These four choices provide extraordinary method development flexibility from pH 1-12 and provide options for both small and large molecules.

Eclipse XDB, pH Range 2-9
First Choice for Method Development of Small Molecules at Low and Mid pH

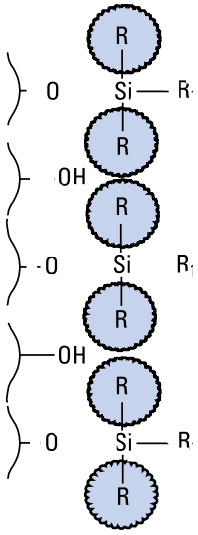


Eclipse XDB Columns:

see page 11

- First choice for method development of small molecules
- Excellent peak shape for acids, bases, neutrals
- High performance over a wide pH range (pH 2-9)
- Extra densely bonded and double endcapped for superior peak shape
- Three bonded phases – C18, C8 and Phenyl for selectivity optimization
- Available in dimensions from capillary to prep for all sample sizes

StableBond, pH Range 1-6
Use at Low pH

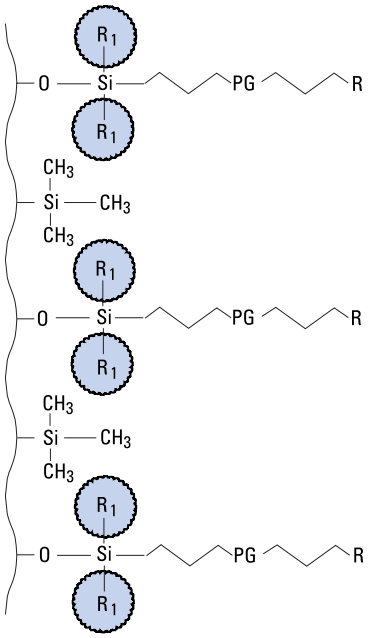


StableBond Columns:

see page 16

- Available in 80Å for small molecules and 300Å for large molecules (proteins and peptides)
- Very stable at low pH — down to pH 1 — and high temperature (80-90°C)
- Excellent stability with TFA (trifluoracetic acid) containing mobile phases
- Available in six different bonded phases for small molecules: SB-C18, SB-C8, SB-Phenyl, SB-CN, SB-C3, and SB-Aq
- SB-Aq can be used with 100% aqueous mobile phases
- Alternate selectivity to Eclipse XDB columns for low pH method development
- Available in four different bonded phases for large molecules: 300SB-C18, 300SB-C8, 300SB-CN and 300SB-C3
- Available in dimensions from nano to prep for all sample sizes

Bonus-RP, pH Range 2-8
Use at Low and Mid pH

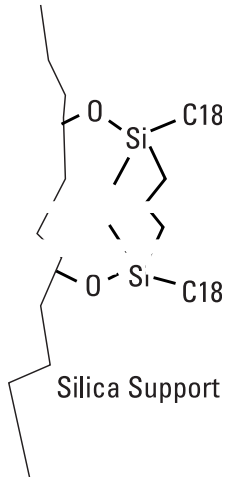


Bonus-RP Columns:

see page 24

- Excellent peak shape for difficult basic compounds
- Triple endcapped for good peak shape
- Polar column with an embedded amide group in long alkyl chain
- Can be used with 100% aqueous mobile phases
- Provides very different selectivity at low and mid pH
- Sterically protected bonding for use at low pH
- Available in dimensions from MicroBore to prep

Extend-C18, pH 2-11.5
Use at High pH



Extend-C18 Columns:

see page 26

- Patented bidentate C18 bonded phase can be used up to pH 11.5
- High pH improves retention and peak shape of difficult basic compounds
- Silica based for high efficiency
- Can be used from pH 2-11.5
- Double endcapped for excellent peak shape of basic compounds
- Available in dimensions from capillary to prep

Use genuine Agilent PerfectFit HPLC consumables for optimal results

Agilent PerfectFit HPLC supplies give you the best results and keep your system running longer. These supplies are specifically engineered and tested for use with Agilent's industry-standard 1100 Series LC systems. For example, Agilent deuterium detector lamps are manufactured to give up to 50% longer lifetimes and noticeably better signal-to-noise.

For more information on PerfectFit HPLC consumables, go to: www.agilent.com/chem/lccolumntips

ZORBAX Reversed-Phase HPLC Column Method Development

Chromatographic resolution between two or more peaks depends upon three factors — column efficiency, selectivity, and retention. ZORBAX columns have a wide variety of bonded phases for optimizing selectivity and retention — the key to the best resolution. These columns are available in a variety of particle sizes — from 1.8-7 µm to provide the efficiency needed for any separation. A simple method development scheme, as shown on page 10, provides the path to achieving high chromatographic resolution.

Start method development at low pH (pH 2-4)
With so many column choices available, how do you know where to start your method development? The recommended starting point for method development is using a buffered low pH mobile phase — around pH 2-3. Using a low pH mobile phase results in the best peak shape for basic compounds on silica-based columns. At low pH, the silanols on the silica are fully protonated so positively charged basic compounds do not interact strongly. The result is good peak shape. Many acidic compounds are non-charged, maximizing their retention at low pH. These observations are key advantages to method development at low pH.

For standard analytical work, start method development with acetonitrile as the mobile phase organic modifier and 20-50 mM phosphate buffer (pH 2-3) as the aqueous component. These conditions provide good pH control, necessary for the most reproducible analyses of ionizable compounds.

Choose ZORBAX Eclipse XDB first for best peak shape
Select ZORBAX Eclipse XDB-C18 or C8 columns first for method development at low pH. Eclipse XDB columns are e**X**tra **D**ensely **B**onded and double endcapped to provide good peak shape for basic compounds. Eclipse XDB columns can be used from pH 2-9 providing method development flexibility. They are also very stable down to pH 2. This makes them an ideal choice for initial method development.

Optimize solvents and bonded phases at low pH
The initial method development steps may lead very quickly to a satisfactory separation. But if more optimization is needed, the acetonitrile can be replaced by methanol or tetrahydrofuran and the separation re-optimized. This step may lead to a satisfactory solution, but if still more optimization is needed, the column bonded phase can be changed.

At low pH, there are many bonded phase choices available for optimization. These include the Eclipse XDB-Phenyl and six different StableBond bonded phases: SB-C18, SB-C8, SB-Phenyl, SB-CN, SB-C3, and SB-Aq. The method development guidelines on page 10 show us that we can try several of these phases at low pH with Eclipse XDB-Phenyl and StableBond SB-CN providing the most differences in selectivity.

It may be necessary at low pH to improve the retention of acidic compounds. For these situations, lower the pH even further, down to pH 1-2, and use StableBond columns. These columns provide the greatest stability at very low pH and provide many selectivity options for achieving the highest resolution separations.

Method development at mid pH (4-9)
ZORBAX Eclipse XDB

There are some samples that may not be resolved at low pH or they may have better solubility and stability at mid pH. While still using the Eclipse XDB-C18 column, the mid pH range can be used for method development. The Eclipse XDB column is stable up to pH 9 so it is equally reliable at mid pH. The double endcapped columns have two key advantages — good peak shape at low and mid pH as well as sufficient bonding density to protect the column from degradation from pH 6-9.

At mid pH, basic compounds (e.g. amines) may still have a positive charge and the silanols on the silica surface may have a negative charge. Therefore covering as many silanols as possible leads to the best peak shape at mid pH. This makes the Eclipse XDB-C18 the best starting choice for a column at mid-pH. Phosphate buffer is usually the first choice for mobile phase modifier at pH 7 because its buffer range is pH 6.1-8.1. A second choice for mid pH is acetate buffer since it buffers from pH 3.8-5.8 and its volatility makes it a good choice for LC/MS compatibility.

Alternate selectivities — Eclipse XDB-Phenyl and Bonus-RP

The method development process at mid pH then mimics the process at low pH with optimization of the organic modifier and selecting an alternate bonded phase if resolution is not achieved after that step. The alternate bonded phases at mid pH are the Eclipse XDB-Phenyl and Bonus-RP. They provide very different selectivities for many samples and the method development process is followed again. The Bonus-RP column has a polar embedded amide that provides different selectivity for many samples, provides good peak shape for basic compounds, and allows the column to be used with up to 100% aqueous mobile phases.

Method development at high pH (pH 9-12)
choose ZORBAX Extend-C18 columns

At low or mid pH, some separations of basic compounds may still not have enough retention or the desired selectivity. For these samples, high pH separations may be appropriate. Until recently, high pH separations on silica were avoided because of short column lifetimes, due to dissolution of the underlying silica gel. Newer column technologies, i.e. the ZORBAX Extend-C18, can protect the silica from dissolution, so a reasonable column lifetime can be achieved and the selectivity advantages of high pH can be explored.

The mobile phase buffer choices at high pH with the Extend-C18 column are organic buffers like triethylamine and ammonium hydroxide. These buffers are best used with methanol as the organic modifier to extend the column lifetime at high pH.

Gain insight into LC method development

The *Lab Insights LC Column Applications* CD includes chromatograms, columns (with part numbers), and conditions from 178 applications. Use the insights from Agilent experts as the first step in your method development. Application areas include food, environmental, pharmaceutical, polymers, agriculture, and more. To request this CD:

Go to www.agilent.com/lccolumntips
CD publication number **5988-5500EN**

Method Development Guidelines from Low to High pH