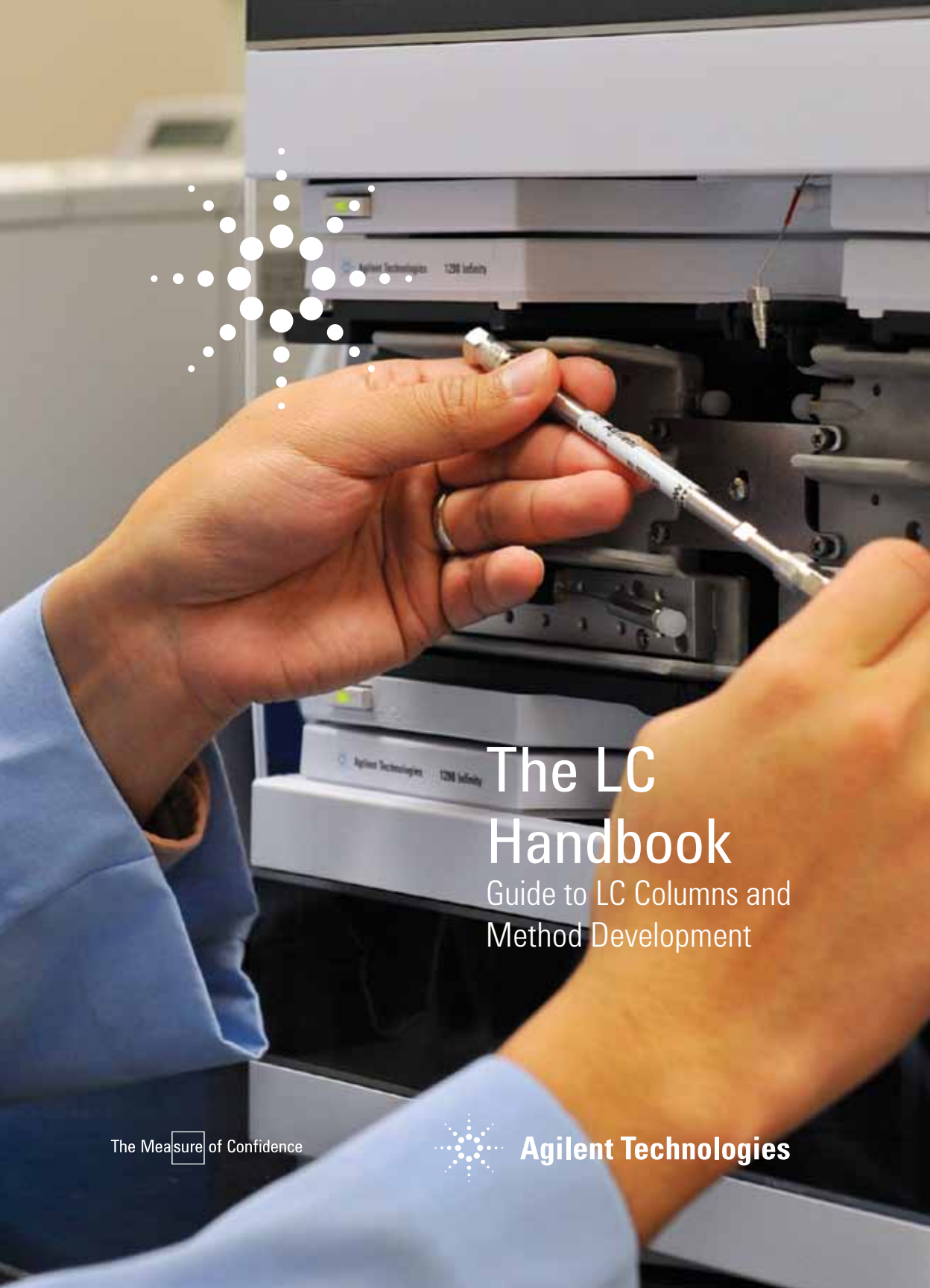

# The LC Handbook

## Guide to LC Columns and Method Development

The Measure of Confidence

Agilent Technologies

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# Contents

# Introduction

## Where to begin? Liquid chromatography is a vast and complex subject, but one for which we never lose our interest.

Chromatographers around the world are using HPLC techniques to ensure the safety of our food and water, develop life-saving pharmaceutical products, protect our environment, guard public health, and that's just the start of it. The more you know about chromatography, the more you can get done with this amazing technology.

Today, you have more choices of columns and packing materials to suit an ever expanding range of uses. Agilent now offers more than 2,000 column choices covering the broadest array of applications and conditions. This increases your opportunities to select the most appropriate column for your needs.

As part of our commitment to helping you get the best results from your liquid chromatography, we have compiled this handy guide to choosing LC columns, with plenty of tips and tricks to make your job easier and more productive. In addition, we've drawn on more than 40 years of experience to provide suggestions for overcoming some of the common problems that can occur with columns and fittings in everyday use. The guide covers the main columns used in LC, with particular emphasis on reversed-phase high performance liquid chromatography.

How to use this guide:

- Sections are color-coded for your easy reference.
- The glossary in the back is fairly comprehensive. It's intended to be a good resource, although we have not touched on every glossary term in the rest of the book, due to space considerations.
- This book primarily focuses on reversed-phase HPLC although we highlight other techniques elsewhere in the book.

# Essential chromatography concepts

We all remember the feeling we had in school as we learned math, wondering how it would actually come into practical use. Scientists have to learn more math than many professionals, and this section reminds us why.

Here, we will briefly review the equations and theory behind many of the concepts that drive chromatography. Understanding these concepts will help you to get the best results, and to troubleshoot if you encounter problems.

We start with fundamentals of performance:

- Efficiency
- Retention
- Selectivity
- Resolution
- Pressure

These are all key to understanding how to optimize results and successfully develop methods.

We also explore a few more complex concepts:

- van Deemter curves
- The gradient equation

These two topics are also important for method development.

## Efficiency (N)

Column efficiency is used to compare the performance of different columns. It is probably the most frequently cited parameter of column performance and is expressed as the theoretical plate number, N.

Efficiency  Retention time  Peak width at base

$$N = 16 \left(t_R / w_t\right)^2$$

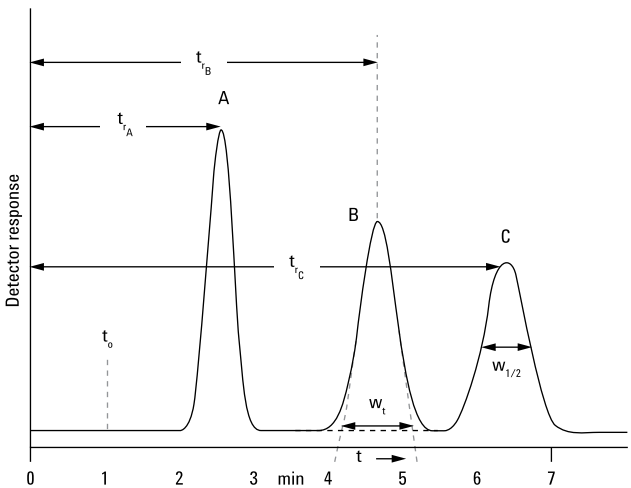
***Equation 1.*** *Efficiency equation*

Retention time  Peak width at half height

$$N = 5.54 \left(t_R / w_{1/2}\right)^2$$

***Equation 2.*** *Alternate equation for calculating efficiency*

Columns with high plate numbers are more efficient. A column with a high N will have a narrower peak at a given retention time than a column with a lower N number.




**Efficiency**
$N = 16 (t_R/w_t)^2$
For Peak B, $16(4.5 \text{ min.}/0.9 \text{ min.})^2 = 400$ plates
$k = (t_R - t_0)/t_0$

**Retention**
$k_A = (2.5 - 1)/1 = 1.5$
$k_B = (4.6 - 1)/1 = 3.6$
$k_C = (6.2 - 1)/1 = 5.2$

**Selectivity (C-B)**
$\alpha = k_2/k_1$
$\alpha = k_c/k_b = 5.2/3.6 = 1.44$
$\alpha = 1.44$

**Selectivity (B-A)**
$\alpha = k_2/k_1$
$\alpha = k_b/k_a = 3.6/1.5 = 2.4$
$\alpha = 2.4$

***Figure 1.*** *Chromatographic illustration of efficiency, Retention Factor and resolution*

If we measure the distance $t_w$ here (Figure 1), by drawing tangent lines to approximate the four-sigma peak width, we can measure the theoretical plates for peak B, using Equation 1, $N = 16 (t_R/t_W)^2$. Sometimes the four-sigma peak width is difficult to measure (e.g., with a noisy baseline), so an alternate equation (Equation 2) involves measuring the peak width at half-height ($w_{1/2}$): $N = 5.54 (t_R/w_{1/2})^2$.

High column efficiency is beneficial since less selectivity is required to completely resolve narrow peaks. Column efficiency is affected by column parameters (diameter, length, particle size), the type of eluent (especially its viscosity), and flow rate or average linear velocity. Efficiency is also affected by the compound and its retention. When comparing columns, the number of theoretical plates per meter (N/m) is often used. However, the same chromatographic temperature conditions and peak retention (k) are required for the comparison to be valid. On stationary phases where $\alpha$ is small, more efficient columns are beneficial.

## Retention Factor (k)

Formerly referred to as capacity factor or k´ (k prime), the Retention Factor measures the period of time that the sample component resides in a stationary phase relative to the time it resides in the mobile phase. It is calculated from the Retention Time divided by the time for an unretained peak ($t_0$).

Retention time for the sample peak

$$k = \frac{(t_R - t_0)}{t_0}$$

Retention Factor

Retention time for unretained peak

***Equation 3.*** *Retention Factor equation*

## Selectivity or separation factor ($\alpha$)

The separation factor is a measure of the time or distance between the maxima of two peaks. If $\alpha = 1$, the two peaks have the same retention time and co-elute.

Selectivity

Retention Factor of first peak

$$\alpha = k_2/k_1$$

Retention Factor of second peak

***Equation 4.*** *Selectivity equation*

Selectivity is defined as the ratio in capacity factors. In Figure 1, you will see that there is better selectivity between peaks A and B than between B and C. Calculations are provided to demonstrate.

Selectivity can be changed by changing the mobile phase constituents or changing the stationary phase. Temperature may also be a factor in adjusting selectivity.

## Resolution ($R_s$)

Resolution describes the ability of a column to separate the peaks of interest, and so the higher the resolution, the easier it is to achieve baseline separation between two peaks. Resolution takes into consideration efficiency, selectivity and retention, as can be seen in Equation 5. One can improve resolution by improving any one of these parameters.

$$R_s = \frac{\sqrt{N}}{4} \frac{(\alpha-1)}{\alpha} \frac{k}{(k+1)}$$

***Equation 5.*** *Resolution equation*

In Figure 2, we see the different effects of each component on the separation process. All of these terms show a diminishing return. This means that the more you try to work on something to improve the separation, the less effective it will become.

If you double the column length, you will obtain more theoretical plates, but your separation will take twice as long; you will only get a square root of 2, or 1.4 improvement in the resolution.

A value of 1 is the minimum for a measurable separation to occur and to allow adequate quantitation. A value of 0.6 is required to discern a valley between two equal-height peaks. Values of 1.7 or greater generally are desirable for rugged methods. A value of 1.6 is considered to be a baseline separation and ensures the most accurate quantitative result.

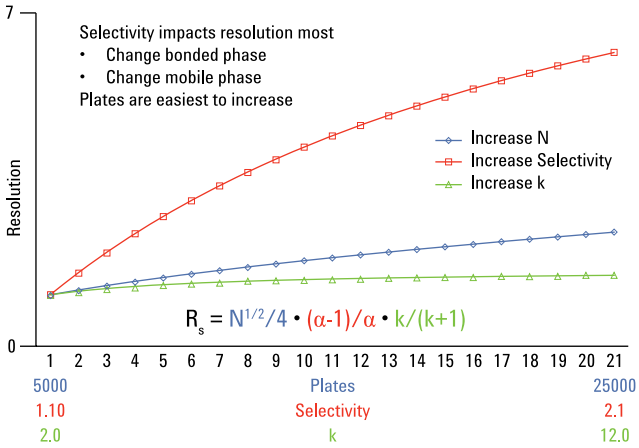
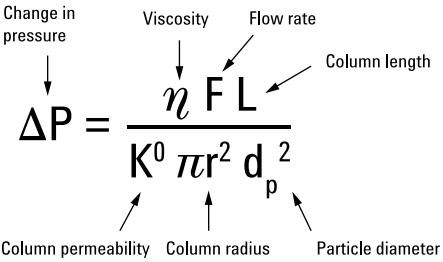



***Figure 2.*** *Resolution as a function of selectivity, column efficiency or retention*

## Pressure

The Pressure equation (Equation 6) identifies five key factors that affect system pressure: solvent viscosity($\eta$), flow rate (F), column length (L), column radius (r) and particle diameter ($d_p$). It is a good idea to familiarize yourself with the pressure equation to understand these key contributors to system pressure.




***Equation 6.*** *Pressure equation*

As noted in the formula, even a small decrease in the particle size ($d_p$) has a significant impact on backpressure.

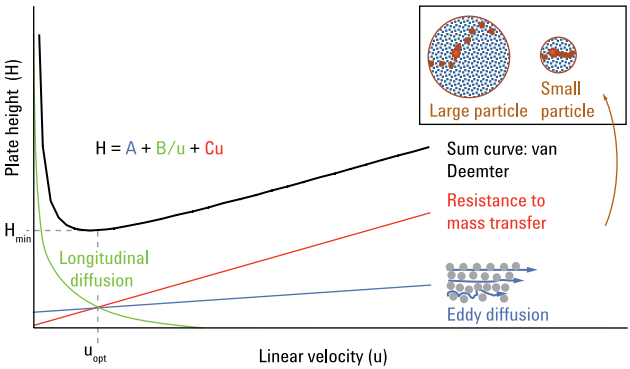
## van Deemter Curves

The van Deemter equation evaluates efficiency (expressed as H, see Equation 7) as a function of linear velocity (u) or flow rate. The H – called plate height, or height of a theoretical plate – is determined by dividing the column length (L) by the calculated number of theoretical plates. The goal is to get a small plate height. We can do this most effectively with smaller particle columns, optimum linear velocities and low viscosity mobile phase. As particle size decreases, the optimum linear velocity increases.

$$H = A+B/u+Cu$$

$$H = L/N$$

***Equation 7.*** *van Deemter equation*




The smaller the plate height, the higher the plate number and the greater the chromatographic resolution

***Figure 3.*** *Illustration of the van Deemter equation*

We often plot van Deemter curves to evaluate the performance of different columns, and to understand the optimum linear velocity ($u_{opt}$) for a method.

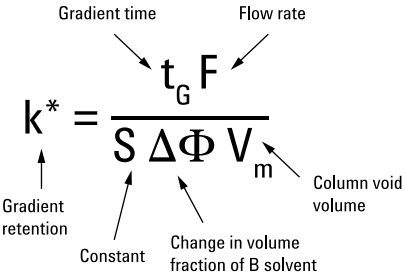
## The gradient equation

Whenever your sample has a wide variety of components present, it can be difficult to separate all of the components in a reasonable time using isocratic elution (e.g. constant mobile phase composition). Gradient elution is a process to increase the mobile phase strength as a function of time, resulting in faster analyses and better peak shape and quantitation. With gradient elution, peak widths are typically more narrow and of constant width (see p. 39 for more about gradients).

The gradient equation (Equation 8) shows key variables that affect your analysis, and may cause issues with your chromatography if you don't account for them. The equation shows how the retention factor is influenced by flow rate (F), gradient time ($t_G$), gradient range ($\Delta\Phi$), and column volume ($V_M$). It's important to

remember that in order to keep the retention factor constant, changes in the denominator need to be offset by proportional changes in the numerator, and vice versa.

Increasing the retention factor k (or k*, in a gradient) is an easy way to increase resolution, but as shown in Figure 2, it is not as effective as increasing efficiency or selectivity. If the retention factor is increased by increasing gradient time, you will have a longer run time, as Equation 8 shows.




***Equation 8.*** *Gradient equation*

In the gradient equation, S is a constant and is dependent on the size of the molecule being separated. For small molecules, the value of S is about 4 to 6. For peptides and proteins, S lies between 10 and 1,000.

These days, it is common to change the dimension of the column, either to something shorter (e.g. for higher throughput) or with a narrower internal diameter (e.g. for mass spectrometric detection). Any decrease in column volume must be offset by a proportional decrease in gradient time ($t_G$) or flow rate (F). Any change in the gradient compositional range ($\Delta\Phi$), using the same column, needs to be adjusted by a proportional change in gradient time ($t_G$) or flow rate (F) if you want to maintain the same gradient slope and k* value.

A great way to get help when transferring a method to a column with different dimensions is to use the Agilent *Method Translation Software*. You can find the method translator by typing in 'LC Method Translator' in the search field at www.chem.agilent.com.

# Selecting your HPLC column

Selecting a column is part science and part art. We say this because there are certainly some selection criteria that are straightforward: column phase for application, appropriate sizes for your system pressure limits, etc.

In many cases, a specific phase or column dimension may be specified for a pre-defined method, so your selection will mostly have to do with meeting these specifications and ensuring a reliable, high quality column provider (we recommend Agilent, but we admit a bias).

Beyond that, your method development may enable you to get more artful, in that you will want to test the performance of various stationary phases and mobile phases to get just the selectivity your application demands.

This section will first review a few basics:

- HPLC modes
- Common HPLC column materials

Then we'll talk specifically about the key types of columns most often used today and what makes them each unique.

- HPLC columns
- UHPLC columns
- Superficially porous particle columns
- Columns for LC/MS
- Columns for Gel Permeation Chromatography (GPC), Size Exclusion Chromatography (SEC) and Gel Filtration Chromatography (GFC)
- Columns for biochromatography

We'll also explain the importance of key column characteristics

- Packing – silicas, bonded phases, polymers
- Pore size
- Particle size
- Column dimensions

At the end of this section, we will provide an overview of cartridge-style columns that are available, including guard, semi-preparative, prep and process columns.

The information in this section should be used in conjunction with the column selection information in the Method Development section (p. 45), which begins to incorporate more information about column stationary phases and selectivities.

## HPLC mode

The HPLC mode is the most important factor to decide before you can begin evaluating columns. There are a number of common HPLC modes or techniques in use today:

- Reversed-phase
- HILIC
- Normal phase
- Ion-exchange
- Size exclusion

Generally, surveys have shown that 95% of all chromatographers use reversed-phase chromatography sometime during their work, and, for this reason, this booklet will focus mostly on this technique. We'll touch on some of the others, however, to point out key differences and propose good resources for more information.

See pp 46 - 48 for more information about the differences between the various modes.

## Column selection basics: conventional columns

Columns for liquid chromatography are made from cylinders of stainless steel or polymers or, more rarely, glass, containing bonded silica or polymer particles. They are available in many dimensions to suit the different needs of chromatographers and their applications. They range from short, narrow-bore columns for high throughput LC/MS, to 50 mm internal diameter (id) preparative columns for gram-scale purification, all the way up to preparative column packing stations with dimensions up to 600 mm for pilot, scale-up and production facilities. Column dimensions affect sensitivity and efficiency, and determine the amount of analyte that can be loaded onto the column. For example, small id columns improve sensitivity compared to larger id columns, but with reduced loading capacity.

Modern stainless steel analytical columns (1 to 4.6 mm id, 20 to 250 mm long) use very low, or zero, dead-volume fittings. The column packing material is held in place by stainless steel frits at both ends.

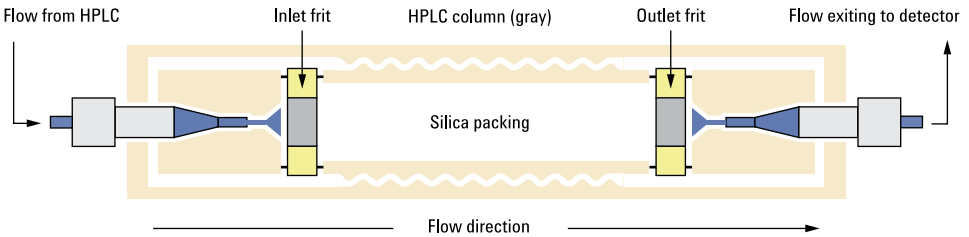



***Figure 4.*** *Diagram of a column*

| Column construction | Column type | Column internal diameter (mm) | Particle type | Particle size (µm) | Use |
|---|---|---|---|---|---|
| HPLC | | | | | |
| Stainless steel | Analytical | 4.0-4.6 | Silica, polymer | 1.8-10 | Traditional quantitative analysis |
| | Analytical solvent saver | 3 | Silica | 1.8-5 | Reduced solvent consumption |
| | Analytical narrow bore | 2.0-2.1 | Silica | 1.8-5 | Reduced solvent consumption |
| | Analytical microbore | 1 | Silica, polymer | 3-5 | Increased sensitivity, sample size ng to µg |
| | Analytical capillary | 0.3-0.5 | Silica, polymer | 3-5 | Sample size pg to ng |
| | Analytical nano | 0.075-0.1 | Silica, polymer | 3 | Sample size < 1 pg |
| | Prep or semi-prep | 9.4-50 | Silica, polymer | 5-50 | Sample purification |

***Table 1.*** *Some characteristics of columns for liquid chromatography*

*Continued on next page*

| Column construction | Column type | Column internal diameter (mm) | Particle type | Particle size (µm) | Use |
|---|---|---|---|---|---|
| LC/MS UHPLC/MS | | | | | |
| Stainless steel | Analytical | 3 | Silica | 1.8-5 | Good choice to match flow rate capability of Agilent MS |
| | Analytical narrow bore and microbore | 1-2.1 | Silica, polymer | 1.8-10 | Where higher sensitivity or specialty detectors are required |
| Fused silica | Analytical capillary | 0.3-0.5 | Silica, polymer | 1.8-10 | With MS detectors |
| UHPLC | | | | | |
| Stainless steel | Analytical | 4.6 | Silica | 1.8-2.7 | For all fast and high resolution applications |
| | Analytical solvent saver | 3 | Silica | 1.8-2.7 | |
| | Analytical narrow bore | 2.1 | Silica | 1.8-2.7 | |
| Prep | | | | | |
| Stainless Steel | prep | 10-100 | polymer | 10-50 | Compound isolation |
| Process | | | | | |
| Stainless Steel | process | 10 cm to 100 cm | polymer | 10-50 | Compound production |

Note: Certain bioHPLC columns are available in PEEK for a metal-free sample path.

*Use ZORBAX Rapid Resolution High Throughput (RRHT) 1.8 µm columns and Poroshell 120 2.7 µm columns , up to 600 bar. Use ZORBAX Rapid Resolution High Definition (RRHD) columns, 1.8 µm, up to 1200 bar.*

**High Performance Liquid Chromatography (HPLC) columns**

Silica gel is commonly used as a stationary phase in normal phase, adsorption HPLC, and is the support for numerous chemically bonded stationary phases. The surface of the silica is covered with strongly polar silanol groups that interact with molecules in a non-polar mobile phase, or serve as reaction sites for chemical bonding. Normal phase HPLC works well with analytes that are insoluble in water, and organic normal phase solvents are more MS 'friendly' than some of the typical buffers used in reversed-phase HPLC. However, the technique sometimes suffers from poor reproducibility of retention times because water or protic organic solvents (which have a hydrogen atom bound to an oxygen or nitrogen atom) change the hydration state of the silica. This is not an issue for reversed-phase HPLC, which has become the main HPLC technique. In reversed-phase chromatographic systems, the silica particles are chemically modified to be non-polar or hydrophobic, and the mobile phase is a polar liquid.

**UHPLC columns**

Ultra High Pressure Liquid Chromatography (UHPLC) generally refers to liquid chromatography performed at pressures in excess of 400 bar (6000 psi), which was the conventional maximum system operating pressure for decades. Generally, UHPLC columns contain small particles (<3 µm) that provide key benefits in terms of speed, resolution and efficiency compared to conventional HPLC columns packed with 3 to 5 µm particles. However, as shown in the pressure equation (Equation 6, p. 9), the smaller the particle, the greater the backpressure needed to force mobile phase through the column, so UHPLC columns are designed to operate at pressures above 400 bar (6,000 psi).

For UHPLC operation, Agilent provides three types of columns: ZORBAX Rapid Resolution High Throughput (RRHT) 1.8 µm particle size columns for operation up to 600 bar, Poroshell 120 columns with 2.7 µm superficially porous particles (also for up to 600 bar) and ZORBAX Rapid Resolution High Definition (RRHD) 1.8 µm columns, stable to 1200 bar. The separation speed achievable in UHPLC can be very fast. In general, UHPLC separations that are less than 10 min are fast, and separations less than 1 min are commonly known as ultrafast.

Another aspect of UHPLC is the increased efficiency and peak capacity when longer UHPLC columns with 1.8 µm packing are used. Analytical column lengths up to 150 mm and ids up to 4.6 mm are available. It is now possible to obtain almost 300% greater peak capacity, which is valuable for improving many complex separations ranging from drug discovery to food safety and environmental applications, such as pesticide screening.

***Figure 5.*** *ZORBAX Rapid Resolution High Definition (RRHD) Columns*

**Superficially porous particle columns**

Superficially porous particle (SPP) columns have enjoyed a recent resurgence in smaller particle sizes than the older 'pellicular' particle columns. As depicted in Figure 6, the Agilent Poroshell 120 particle has a solid core (1.7 µm in diameter) and a porous silica layer (0.5 µm thickness) surrounding it. The current interest in this technology is driven by its re-introduction in smaller particle sizes, such as the sub-3 µm sizes, for use in typical small molecule reversed-phase separations.

The Poroshell 120 offers significant method development advantages to chromatographers using conventional totally porous columns. Because diffusion only occurs in the porous outer shell, not the solid core, efficiency is increased compared to a totally porous particle of the same size. In fact, a 2.7 µm SPP will give efficiency comparable to a 1.8 µm totally porous particle. A big advantage is the fact that the backpressure created by the SPP column is greatly reduced due to its larger particle size, allowing chromatographers to increase flow rate and improve the speed of their analysis, while enjoying exceptional resolution. It is also important to know that Poroshell 120 columns are packed with a standard 2 µm frit, so they are more forgiving for dirty samples and do not clog as readily as columns with smaller frits. Sample filtration, especially when using 1.8 µm columns, will also aid in reducing clogging.

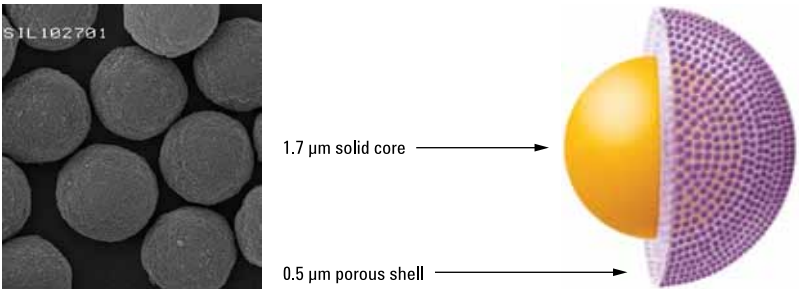



***Figure 6.*** *Poroshell Particles*

The separation of peptides and proteins is challenging because they diffuse slowly, so flow rates must be kept low to prevent peak broadening. Agilent's Poroshell 300 columns use a superficially porous 5 µm particle made with a thin layer of porous silica (0.25 µm thickness) surrounding an impervious solid-silica core. This technology reduces the diffusion distance, permitting rapid HPLC separation of peptides and proteins from 500 Da to 1,000 kDa.

### Columns for LC/MS

There are many columns for LC/MS, depending on the sample. For simple analytical samples, use short columns (with high resolution) to reduce analysis time for high throughput LC/MS. For higher resolution, use longer columns.

Flow rate also affects your choice of a column. LC/MS systems typically operate at flow rates from 1 µL/min to 1 mL/min. This makes smaller id columns such as Agilent Solvent Saver (3.0 mm id), narrow bore (2.1 mm id), and capillary and nanobore columns (see Table 1, p. 15) good options for high sensitivity and fast analyses.

The best bonded phase choice is a high performance end capped C18 bonded phase, stable over a wide pH range, compatible with the typical volatile mobile phase additives used for LC/MS, including formic acid and acetic acid.

**Columns for Gel Permeation Chromatography (GPC), Size Exclusion Chromatography (SEC), and Gel Filtration Chromatography (GFC)**

Separations based on molecular size in solution are generally performed by size exclusion chromatography (SEC). This chromatographic mode is used to separate polymers, including biopolymers. It is used to characterize the molecular weight and molecular weight distribution of polymers. The columns are usually stainless steel and contain gels, cross-linked polymers, or silica particles with tightly controlled pore sizes. The separation mechanism relies solely on the size of molecules in solution, and unlike the other chromatographic modes, there should be no chemical interactions between the particles and the stationary phase. We use the term gel permeation chromatography (GPC) to describe the analysis of organic polymers (e.g. plastics) in organic solvents, and aqueous SEC to describe the analysis of water-soluble organic polymers (e.g. polyvinylalcohol) and water-soluble biopolymers (e.g. proteins, nucleic acids and polysaccharides) using predominantly aqueous mobile phases. Gel filtration chromatography (GFC) has been historically used to describe the low pressure separation of biopolymers such as proteins.

For a comprehensive guide to GPC/SEC we recommended Agilent's *Introduction to Gel Permeation Chromatography and Size Exclusion Chromatography*. (Agilent publication number 5990-6969EN)

**Columns for biocharacterization**

Biochromatography columns, or biocolumns, are columns for the separation of biological compounds such as proteins and peptides, oligonucleotides and polynucleotides, and other biomolecules and complexes, including virus particles. Biocolumns are designed to greatly minimize or eliminate irreversible or non-specific binding of the sample to the packing and to retain biological function (enzymatic activity). Frequently, biocolumns are constructed so that active metals do not contact the sample. They may be made with polymers (e.g. PEEK), fused silica and glass-lined stainless steel, or metallic components that are coated to render the column biocompatible.

## Column characteristics

### Silica

Silica is an ideal material for chromatography, and has been the main base packing material for bonded phase HPLC columns for decades. Silica particles are rigid and resist compaction due to flow, particularly important when particle sizes are 5 µm or less and higher pressures are necessary. Their extremely large surface areas provide the adsorptive capacity for HPLC and UHPLC, and the silanols or Si-OH groups on the particle surface are ideal bonding sites for functional carbon chains.

Not surprisingly, spherical silica particles used for column packing are available in a variety of sizes, purity and acidity. Most modern packings are Type B silicas, which are very low in trace metals and are less acidic than older Type A silicas. Less acidic silica means less potential for interaction between a basic analyte and silanol groups on the silica surface, which contributes to improved peak shape. Columns made with high purity silica are the most common choices for today's chromatographer.

### Bonded phases

For reversed-phase chromatography, the most common bonded phase is a C18 (octyldecylsilane, ODS). This is just one of the many types of alkyl or carbon chains attached to the surface of the silica particle. Some other popular choices for linear alkylsilane phases include C8 and C4. Phenyl, including Phenyl-Hexyl and Diphenyl, and AQ, CN, and PFP phases can offer significant differences in selectivity from the straight-chain alkyl phases and may provide a successful separation. There are an increasing number of bonded phase choices with some specific to high aqueous mobile phases or other applications.

In general, larger solutes, such as proteins, are best separated on short-chain reversed-phase columns (C3, CN, diol) bonded to wide pore silica gels (pore size: 300Å). Peptides and small molecules are separated on longer-chain columns (C8, C18). There are many cases, however, where this convention does not apply. Therefore, it is a good idea initially to select a phase in the middle of the hydrophobic spectrum (for example, C8), then change to a more hydrophobic phase or more hydrophilic phase depending on initial results and the solubility of your sample.

There are other options to consider for ion-exchange, gel permeation/size exclusion or HILIC chromatography. We discuss these further on pp. 76 - 81.

### Polymers

When a column is needed that can operate at very low and high pH, polymeric packings provide an alternative to silica-based materials. Polymeric particles are ideal for small-scale chromatography, particularly LC/MS, as they are chemically stable and do not leach soluble or particulate species. Reversed-phase spherical polymeric packings used in PLRP-S columns, for example, are based on a styrene/divinylbenzene copolymer with an inherently hydrophobic surface. No bonded phase is required for reversed-phase chromatography with polymeric particles. These rigid macroporous particles can be coated and derivatized to give a range of functionalities, including weak and strong cation and anion-exchangers.

**Pore size**

The choice of the pore size is determined by the molecular weight of the component which is analyzed. For reversed-phase separations of small molecules, choose a column packing with small pores (60-120Å). For small molecules and peptides use 100-150Å, for polypeptides and many proteins choose 200-300Å, and 1,000Å and 4,000Å for very high molecular weight proteins and vaccines.

For GPC/SEC separations the molecular weight range for separations is typically given with the pore size information so the right column can be selected. Tables of this information are listed with the column choices.

**Particle size**

The standard particle size for HPLC columns was 5 µm for a long time, until the mid-1990s, when 3.5 µm became popular for method development. More recently, as higher speed and/or higher resolution is required, chromatographers have turned to packings with sub-2-3 µm, including 1.8 µm particles made by Agilent. Shorter columns with these particles can produce faster high-resolution separations. The 3.5 µm particle size operates at a routine operating pressure and may be used on all LCs, including those with a 400 bar operating limit. Short (50 mm and shorter) 1.8 µm columns may be employed on optimized standard LCs, while longer columns may require a higher-pressure LC (e.g. Rapid Resolution LC or UHPLCs), operating at pressures from 600 to 1200 bar. Recently, newer technology (e.g. superficially porous particles) has been developed that enables performance similar to sub-3 µm columns, but generates lower backpressure, so it can be used with conventional HPLC instruments (see Superficially Porous Particles, p.16).

If the particle size of a column is reduced by half, the plate number doubles (assuming column length remains the same). However, if particle size halves, column backpressure increases four times ($\sim 1/d_p^2$). If column length doubles, the plate number and analysis time also double. As column length increases, backpressure increases linearly. For example, a 2.1 x 100 mm column packed with 3.5 µm particles generates about 12,000-14,000 theoretical plates, an efficiency that can provide adequate separation for many samples. By reducing the particle size from 3.5 µm to 1.8 µm, the efficiency of the same 2.1 x 100 mm column is doubled to about 24,000 theoretical plates. However, this column generates a backpressure that is four times greater than the pressure of the same size column filled with 3.5 µm particles.

Very often, an efficiency of 24,000 plates is not required, so the column length can be halved to 50 mm, with an expected efficiency of 12,000 plates. The analysis time will be cut in half with this shorter column and the backpressure is only twice as great as the 100 mm column with 3.5 µm particles.

**Column dimensions**

For many years the column sizes most often recommended for analytical method development were 4.6 x 150 mm or 4.6 x 100 mm with a 5 µm particle size. If more resolution was needed, a 4.6 x 250 mm column was recommended. But with the range of modern choices available analytical method development with 4.6 x 100 mm columns with 3.5 µm or 2.7 µm superficially porous particles are the recommended starting point.

During method development, choose the column id (for example 2.1 or 3.0 mm) to accommodate additional application objectives (such as sensitivity, solvent usage) or compatibility with certain instrument types (capillary, nano, or prep columns).

Nano, capillary or microbore columns are used when increased sensitivity is required or when the sample is extremely limited.

- Nano columns for sample sizes below 1 pg used with nL/min flow rates
- Capillary columns for sample sizes in the range pg to ng with flow rates around 4 µL/min
- Microbore columns for sample sizes from ng to µg typically operate at flow rates around 40 µL/min

| Application objective | Column diameter (mm) |
|---|---|
| Very high sensitivity, LC/MS, peptides and proteins | 0.1, 0.075 |
| Very high sensitivity, limited sample, LC/MS, peptides and proteins | 0.3, 0.5 |
| High sensitivity, limited sample, LC/MS | 1.0 |
| Save solvent; special low-volume instrumentation is available | 2.1 |
| Special detectors such as MS | 2.1 |
| High sensitivity, limited sample | 2.1 |
| Save solvent; standard HPLC equipment available, LC/MS | 3.0 |
| Standard separations | 4.6 |
| Small-scale (mg) preparative separations | 9.4 |
| Medium-scale preparative separations (100 mg to g) or semi-prep | 21.2 |
| Large-lab-scale preparative separations (up to 100 g) | 30, 50 |
| Pilot and process scale | 100 mm to 1 m |

***Table 2.*** *Applications and column diameters*

When you want to establish a routine method, consider reducing the column dimensions to the smallest available size for your analysis and instrument; smaller columns are often less expensive to buy and use less solvent. In some cases, if column diameter is reduced by half, sensitivity increases by four to five times (assuming the injection mass is kept constant). For example, when a sample is injected onto a 2.1 mm id column, the peaks are about three to five times higher than on an optimized LC than when the same amount of sample is injected onto a 4.6 mm id column. If your instrument is optimized for low-volume columns, as long as linear velocity is maintained, column efficiency, theoretical plates, backpressure and analysis time are not significantly affected by reducing the diameter of the column.

It is easy to calculate whether a shorter column will achieve the same results as your longer development column, by using data produced by the development column. Decision making by simple calculation will save you a great deal of time. You can use Agilent's Method Translation Software (search for *LC Method Translation Software* at www.chem.agilent.com) to help you with these calculations.

## Cartridge column systems

Cartridge systems can provide flexibility and economy because the cartridge is fitted to existing column hardware. Cartridges for analytical systems are pre-packed, whereas you can pack your own systems for preparative and semi-preparative applications using media purchased in bulk.

| Type of cartridge | Features | Benefits |
|---|---|---|
| **Analytical Columns and Analytical/Guard combinations** | | |
| Agilent HPLC Cartridge | Can reverse collets in the end fitting to add guard cartridges | Inexpensive |
| | | Extends column lifetime |
| | | Permits rapid column changes |
| | | Can use 2, 3, 4 and 4.6 mm cartridges |
| | Cartridges have a unique filter and sieve at each end | Helps prevent blockage |
| ChromSep: standalone system comprising holder, analytical cartridge, and guard column | Wide combination of column lengths and diameters | Modular flexibility |
| | Cartridges and guards in multi-packs | Economical |
| | No need for special tools | Easy to use |
| ZORBAX Rapid Resolution and Rapid Resolution HT Cartridge Columns: 1.8 and 3.5 µm packings, standalone system | For high throughput LC/MS, LC/MS/MS and combinatorial separations | For all analyte types |
| | Packed with Eclipse XDB for pH 2-9 | For all analyte types |
| | Packed with StableBond for low pH use | Low bleed |
| | Sold individually or as three packs | For all analyte types |
| **Guards** | | |
| ZORBAX Guard Cartridge: standalone system | High efficiency, standalone, low dead volume cartridge | Seals up to 340 bar |
| | Polymeric cartridge designed for leak-tight seals against metal surfaces | No gaskets required |
| | Reusable fittings | Adapt for connections to 1/16 in. LC fittings |

***Table 3.*** *Cartridge Systems*

*Continued on next page*

| Type of cartridge | Features | Benefits |
|---|---|---|
| **Semi-Preparative Guards** | | |
| ZORBAX Semi-Preparative Guard HPLC Hardware Kit: standalone system | Easy low-dead-volume assembly | Seals up to 2,000 psi (135 bar, 13.5 MPa) |
| | Tubing (polyphenylene sulfone) designed for leak-tight seals against metal surfaces | No gaskets required |
| | Reusable fittings | Adapt for connections to 1/16 in. LC fittings |
| ZORBAX and Agilent Prep Cartridge Column and Guard HPLC System: standalone and integral hardware options | Easy low-dead-volume assembly | Extends column lifetime |
| | Reusable fittings | Permits rapid column changes |
| | Hardware options for integral and external guards | Use with 21.2 and 30 mm id columns |
| **Preparative** | | |
| Load & Lock Preparative Columns and Packing Station: standalone system for dynamic and static 'locked' axial compression | For laboratory and process purification, at high quality and high volume with three column sizes, up to 24 in. id | Easy scale-up from g to multi-kg quantities |
| | Mobile packing station | Use anywhere |
| | Runs on compressed air | Safe to use in hazardous environments |
| | Quick release to pack and unpack in minutes | Maximize productivity |
| Dynamax Preparative Cartridge Column and Guard: standalone dynamic axial compression system | Modular design with reusable end fittings | Reduced hardware costs |
| | 10, 21.4 and 41.4 mm id | Easy scale-up |
| | Integral guard column option | Delivers longer column lifetimes with complex samples |
| **Process** | | |
| Polymeric PLRP-S, PL-SAX and PL-SCX | Range of pore sizes and particle sizes to give high sample throughput | Increased productivity |
| | Chemical and thermal stability enables cleaning in place and sanitization | Increased column lifetime |
| | Robust packing methods for process hardware | Improved packed column performance |

| Column type | Guard cartridge holder | ID (mm) | Phases |
|---|---|---|---|
| Cartridge/Guard Cartridge Systems Compatibility Guide* | | | |
| Cartridge column cartridge holder 5021-1845  | Guard cartridge (internal system) cartridge holder 5021-1845  | 2.0<br>3.0<br>4.0<br>4.6 | Asahipak<br>LiChrospher<br>Nucleosil<br>Purospher<br>Superspher<br>ZORBAX |
| Standard fitting  | Column guard cartridge (standalone) cartridge holder 820888-901  | 2.1<br>3.0<br>4.6 | ZORBAX |
| Rapid Resolution cartridge holder 820555-901  | No guard cartridge holder | 4.6 | ZORBAX |
| Semi-preparative column  | Semi-prep guard cartridge (standalone) cartridge holder 840140-901  | 9.4 | ZORBAX |
| PrepHT (no photo available) | Guard cartridge 820444-901  | 21.2 | ZORBAX<br>Agilent Prep |

*Standalone guard cartridges fit all cartridge and standard fitting columns available from Agilent.

***Table 4.*** *Cartridge/Guard Cartridge Systems Compatibility Guide*

# Keys to performance: column configurations and settings

## There have been many books written about chromatography, and there are many more 'keys to performance' than we can possibly cover here.

Our objective here is to highlight a few areas which are often overlooked or tend to cause confusion.

We start with system and 'mechanical' items:

- Reducing extra-column volume
- Making good fittings
- Sample injections
- Understanding and measuring system dwell volume
- Setting the data collection rate for high efficiency columns

Next, we move to topics that have more to do with the process of chromatography, and method development:

- Understanding chelating compounds
- Evaluating pH
- Working with gradients
- Optimizing column re-equilibration

We finish up the section with a few things you'll likely need to deal with over time:

- Column aging
- Column cleaning – for reversed-phase (silica-based vs. polymeric) and normal phase

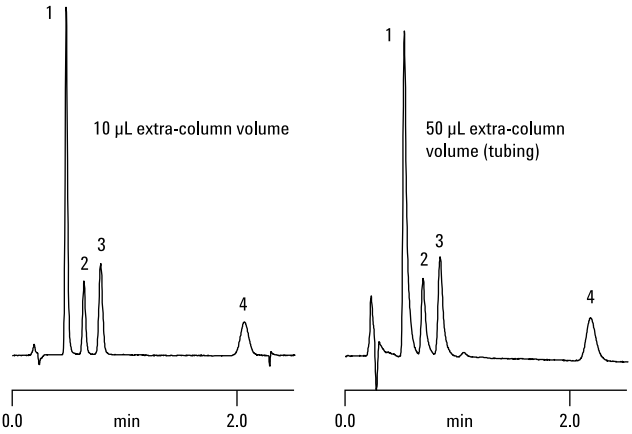
## The importance of reducing extra-column volume

Extra-column volume refers to the 'extra' or 'external' (to the column) volume that is part of your system, most specifically, the connecting tubing carrying the sample aliquot between your LC components and your column, your injection volume and your flow cell volume.

The larger your column, the larger your column volume ($V_m$), and the less important it is to reduce your extra-column volume. However, when you use smaller, high efficiency columns such as Poroshell 120 and sub-2 µm particle columns, you'll want to reduce your extra-column volume as much as possible, to avoid seeing it impact your chromatographic results. Unnecessary extra-column volumes may lead to a loss of efficiency and, in some instances, tailing.

The following examples show the effects of extra-column volume (Figure 9). A small 4.6 x 30 mm column with only 10 µL of extra-column volume produced a nice separation. The same column with added extra tubing, which created an extra-column volume of 50 µL, gave broader peaks and loss of resolution as can be seen by comparing the first three peaks in both chromatograms. This situation is often created when someone introduces tubing to a system without knowing the volume. The addition of a piece of tubing that is too long, or a short tube with a larger id, can lead to poorer chromatography for very efficient columns. It is important to know the internal diameter tubing you are using and what volume it adds. Also, if you are using smaller-volume columns, you may need to use a micro or semi-micro flow cell. Standard flow cells increase extra-column volume.

As a guideline, the maximum extra column volume of the system (tubing volume, injection volume and detector volume) should not exceed 10% of the column volume.




**Peak identification**

1. Phenylalanine
2. 5-Benzyl-3,6-dioxo-2-piperazine acetic acid
3. Asp-phe
4. Aspartame

**Conditions**

| | |
|---|---|
| Column: | StableBond SB-C18, 4.6 x 30 mm, 3.5 µm |
| Mobile phase: | 85% $H_2O$ with 0.1% TFA:15% ACN |
| Flow rate: | 1.0 mL/min |
| Temperature: | 35 °C |

***Figure 7.*** *The influence of tubing volume on chromatographic performance*

Agilent sells capillary kits which contain capillary tubing and Swagelok connectors in various sizes, so you can find just the right length to minimize your connections and your tubing volume. Tubing is color-coded, to identify the diameter of the tubing. As you move to high efficiency columns, you'll want to use the narrow-diameter red tubing (0.12 mm id) for your connections, instead of the green 0.17 mm id tubing that is often used on conventional HPLC instruments.

## Preparing the perfect fitting connection

Problems with improper stainless steel tubing connections are often mistaken for column issues, and are the source of many calls to Agilent's technical support line.

Connection issues can arise because different manufacturers supply different types of fittings (see Figure 10).

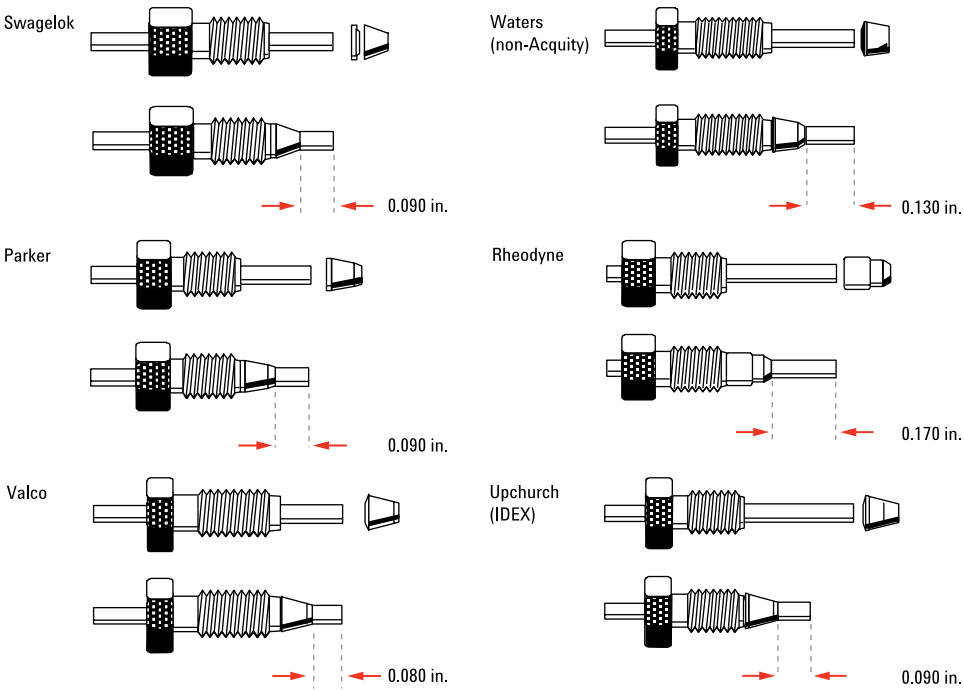



***Figure 8.*** *Column connectors used in HPLC*

You should ideally use the fittings that are recommended by your column manufacturer. Most analytical reversed-phase columns are compatible with Swagelok or Parker type fittings when seated correctly in the column that you will be using.

Stainless steel fittings are the best choice for permanent high pressure sealing. Agilent recommends Swagelok type fittings with front and back ferrules that give the best performance throughout the Agilent LC system. You can use them on all instrument connections such as valves, heaters, column connections, etc. Alternately, for convenience and lower pressure operation to 600 bar, finger-tight polymeric fittings allow for adjustment of the end-fitting to seat the capillary into the column properly, helping to avoid extra-column voids and leaks. These connectors can be tightened without wrenches. There are also new high pressure fittings, for use up to 1200 bar (PN 5067-4733) which are designed to be able to be removed and resealed. See Figure 9 for examples of the various fittings.

## Which type is used when?

Stainless steel (SS) fittings are the best choice for reliable high pressure sealing

- Agilent uses Swagelok pressure-type fittings with front and back ferrules - which gives best sealing performance - throughout all our LC systems

PEEK (<400 bar system pressure) fittings are ideal where:

- Connections are changed frequently, i.e. connecting columns
- Pressure is less critical

Polyketone

- Easy, hand tightened column connections
- 600 bar Pressure rating PN 5042-8957 (10/pk)
- Fits to SS Tubing

High pressure fitting for 1290 Infinity LC

- 1200 bar, PN 5067-4733 (also available in long and extra-long sizes, for compatibility with columns with long and extra-long nuts)
- Removable

***Figure 9.*** *Different types of HPLC fittings*

PEEK and polyketone fittings are ideal for connections that need to be changed frequently, or when biocompatibility is required. They are also useful where pressure is less critical, such as the exit fitting on a column. PEEK fittings can be used up to 200 bar and polyketone fittings up to 600 bar.

### Making a good connection

Relative to the distance from the end of the tubing to the bottom of the ferrule, tubing can be too long or too short – these situations can lead to leaks or peak tailing/splitting, as shown in the illustration on p 29. If the tubing is too long, the ferrule will not seat properly and leaks will occur (see Figure 10). If the tubing is not pushed in far enough, a void space is created that acts as a mixing chamber and introduces extra-column volume, resulting in poor peak shape. If you use columns from different manufacturers make sure you use the correct fittings and that the fitting is correctly seated in the column end fitting.

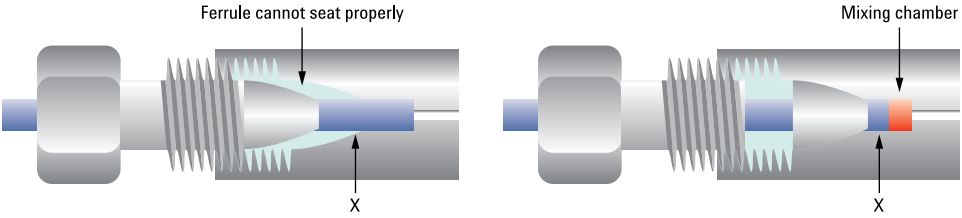
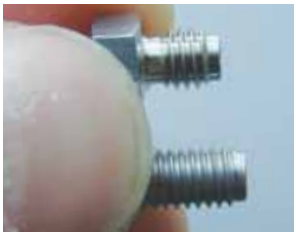
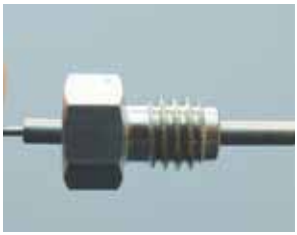



***Figure 10.*** *Examples of incorrect fittings*

## Steps for making good connections



Step 1



Step 2

Step 3

Step 4

Step 5 - a perfect fitting

**Step 1:**
Select a nut that is long enough for the fitting you'll be using

**Step 2:**
Slide the nut over the end of the tubing

**Step 3:**
Carefully slide the ferrule components on after the nut and then finger-tighten the assembly while ensuring that the tubing is completely seated in the bottom of the end fitting.

**Step 4:**
Use a wrench to gently tighten the fitting, which forces the ferrule to seat onto the tubing. Don't over-tighten it, though, because that will shorten the useful life of the fitting.

**Step 5:**
Once you believe you have the fitting complete, loosen the nut, and inspect the ferrule for the correct position on the tubing

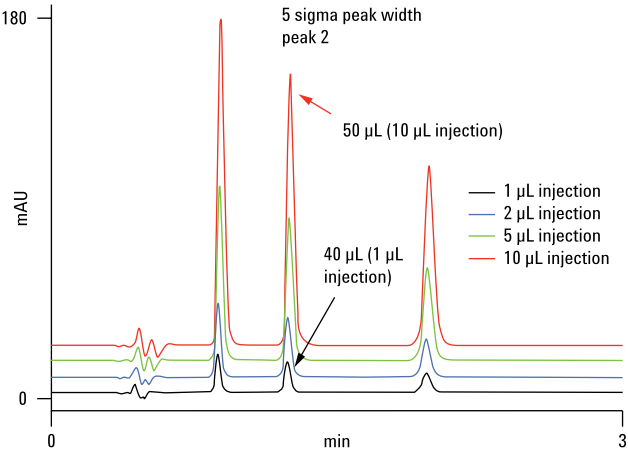
***Figure 11.*** *Steps to make a proper fitting*

To see more tips for making connections in action, check out the LC Troubleshooting series for more information: www.agilent.com/chem/lctroubleshooting, and look for the Peak Broadening Video.

## Sample injection

The injection volume of your sample is important to your results. If you have your injection volume too large the column can be overloaded, which will lead to peak broadening, most often peak fronting or in some cases, peak tailing.

In Figure 12, we see an injection using 1, 2, 5 and 10 µL injections, and you can see how the peaks broaden as the injection volume is increased.

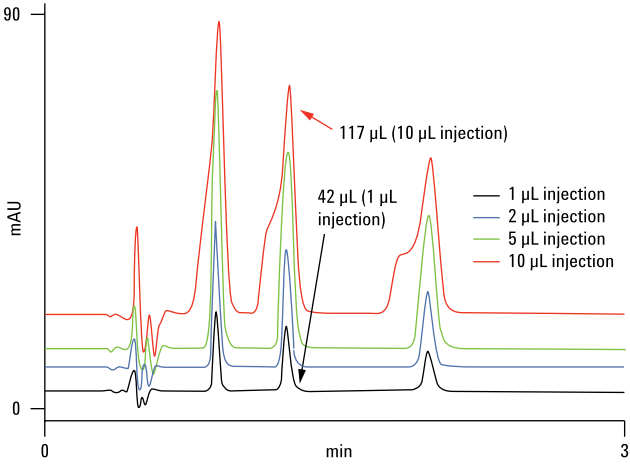



| Conditions | |
|---|---|
| Column: | ZORBAX SB-C18, 3.0 x 50 mm, 1.8 µm |
| Flow rate: | 1 mL/min. |
| Temperature: | 45°C |
| Mobile phase: | 30 - 65% ACN at 2.5 min. |
| Detection: | 250 nm UV |
| Diluent: | methanol |
| Sample: | methyl, propyl and butyl paraben |

***Figure 12.*** *Sample loading volumes compared*

After you have decided your injection volume, you need to make sure your injection solvent is similar enough to the mobile phase to reduce band broadening or splitting.

The sample injection solvent and volume can have an impact on peak shape. In the example (Figure 13), even a 5 µL injection of a paraben sample in a methanol solvent shows evidence of the beginning of peak band broadening. The reversed-phase column had the dimensions of 3 x 50 mm and the instrument was operated in a low dispersion configuration. With a 10 µL injection, the loss of peak symmetry is clearly a problem. In reversed-phase LC, 100% organic, or 100% of the strong solvent (in this case methanol), for larger injection volumes, will cause the peaks to be prematurely swept down the column, resulting in peak distortion. The problem can be overcome by incorporating an evaporation step to concentrate the analytes so that a smaller injection volume can be used. Alternatively, the injection solvent can be diluted with water to make it more compatible with the mobile phase, allowing for larger injection volumes without peak distortion.




**Conditions**

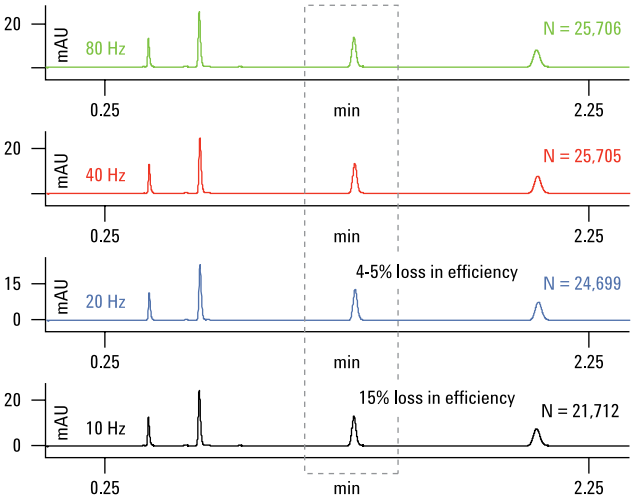
| | |
|---|---|
| Column: | ZORBAX SB-C18, 3.0 x 50 mm, 1.8 µm |
| Flow rate: | 1 mL/min. |
| Temperature: | 45°C |
| Mobile phase: | 30 - 65% ACN at 2.5 min. |
| Detection: | 250 nm UV |
| Sample: | methyl, propyl and butyl paraben |
| Diluent: | methanol |

***Figure 13.*** *Solvent effects: strong diluents with 1-10 µL of injection volume*

## Setting the data collection rate

When using small volume columns, the data collection rate is a common source of 'artificial' peak broadening. For rapidly eluting peaks, you'll want to make sure that you sample enough points across the peak so that the algorithms in the data system can accurately determine peak widths, peak area and retention time. If you take too few points by sampling slowly (e.g. low data collection rate), your peaks appear wider than they actually are as they elute from the column. You'll want to look at your data collection rate and ensure it is properly set to optimize your results for the specific column you are using. Figure 14 illustrates the impact of the data collection rate.

If you are using the Agilent Method Translator with your current column and gradient conditions, the translator will estimate the expected peak width (5 sigma) under your conditions. That information may be helpful when setting up the initial method.




**Conditions**

| | |
|---|---|
| Column: | Poroshell 120 EC-C18, 4.6 x 100mm |
| Instrument: | 1200 SL 2 µL flow cell |
| Flow rate: | 2.00 mL/min |
| Sample: | 2 µL injection. |
| Mobile phase: | 60:40 MeCN:Water |

***Figure 14.*** *Comparison of peak efficiency on Poroshell 120 EC-C18 4.6 x 100 mm with different data collection rates*

Here, we're measuring the efficiency, and as you can see, efficiency increases as the data collection rate used went higher.

You can optimize your data collection rate by adjusting the detector setting and/or the time constant to the fastest possible value that does not compromise signal-to-noise. The peak width control in ChemStation enables you to select the peak width, or response time, for your analysis. The peak width, as defined in the ChemStation software, is the width of a peak at half height. Set the peak width to the narrowest expected peak in your sample. You should not use a faster response time than you need since this may lead to greater noise at the baseline.

## Dwell volume and its impact on chromatography

For low pressure mixing systems dwell volume equals all of the volume from the proportioning valve, through the pump and other system components and on to the head of the column (see Figure 15). For high pressure mixing systems, dwell volume equals all of the volume from where the solvents first meet, after the two metering pumps, to the head of the column (see Figure 16).

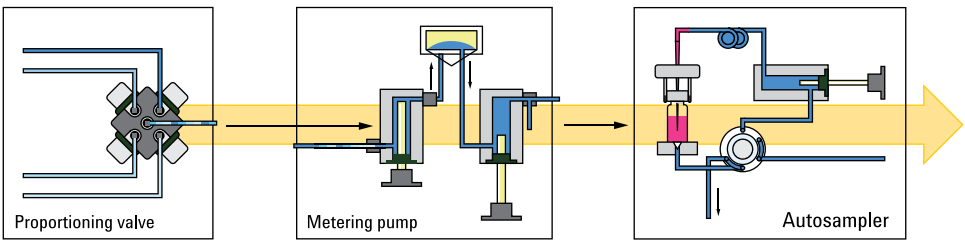



***Figure 15.*** *Dwell volume: low pressure mixing quaternary pump*

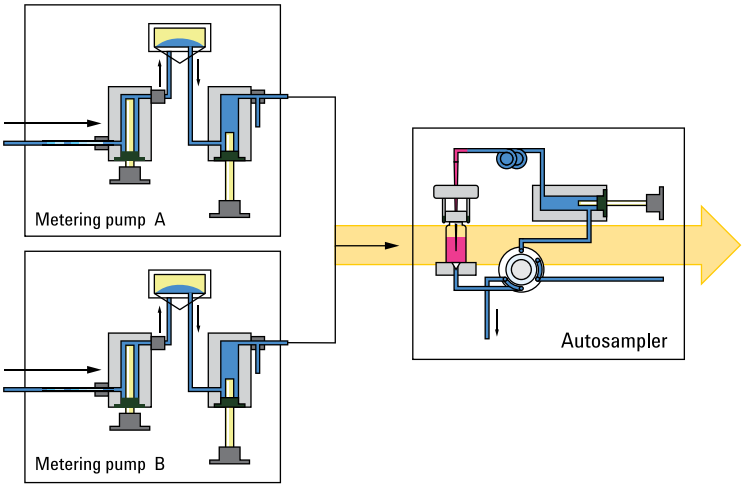
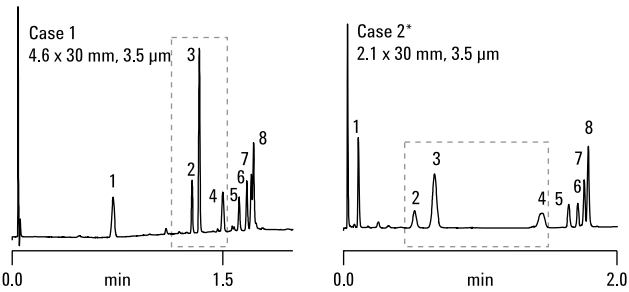



***Figure 16.*** *Dwell volume: high pressure mixing binary pump*

For gradient separations, dwell volume imposes a de facto isocratic hold time at the beginning of the gradient, equal to the dwell volume divided by the flow rate. Too large a dwell volume makes some instruments impractical or unusable for narrow-bore gradient separations.

When using narrow-bore columns, instrument configuration is crucial. Both dwell volume and extra-column volume must be minimized for optimal use of narrow-bore (2.1 mm id) and microbore (1 mm, and < 1 mm id) columns.

Figure 17 shows a chromatographic example that demonstrates the effect of dwell volume on analytical results. Note that the early eluting peaks are broader. The issue here is the early peaks are eluting quite late due to the dwell volume - essentially, they are eluting isocratically. If this interferes with separation or detection, we will need to reduce the dwell volume or move the application to another system.




* Broadening more likely with low flow rates, and narrow-bore (low-volume) column.

***Figure 17.*** *Impact of dwell volume on gradient chromatographic results*

**Peak identification**

1. phenacetin
2. tolmetin
3. ketoprofen
4. fenoprofen
5. ibuprofen
6. phenylbutazone
7. mefenamic acid
8. flufenamic acid

**Conditions**

| | |
|---|---|
| Column: | ZORBAX Rapid Resolution Eclipse XDB-C8 |
| Mobile phase: | A: 0.1% TFA in $H_2O$<br>B: 0.1% TFA in ACN |
| Temperature: | 35°C |
| Gradient: | Case 1: 15-60% B/ 6 min, 1.0 mL/min<br>Case 2: 30-75% B/6 min, 0.2 mL/min |

**Measuring your system's dwell volume**

Start by replacing the column with a short piece of HPLC stainless steel tubing. Prepare mobile phase components as follows: A—Water (UV-transparent), B – Water with 0.2% acetone (UV-absorbing). Monitor at 265 nm. Run the gradient profile at 0 - 100% B/10 min. at 1.0 mL/min. Record, then print out your gradient trace (Figure 18).

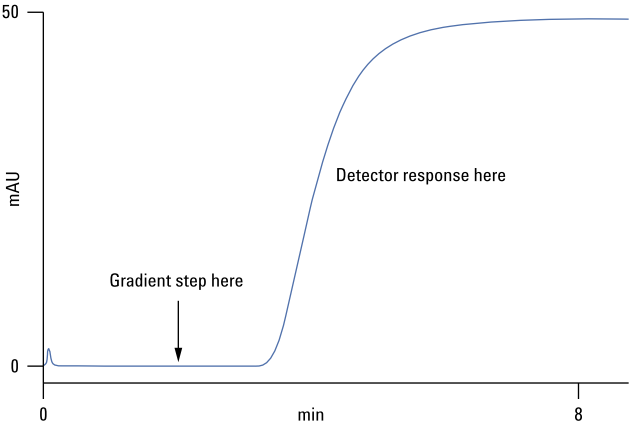



***Figure 18.*** *Calculating dwell (delay) volumes, step one*

Using a ruler on paper or by inserting lines in PowerPoint, draw lines parallel to the x or y axis at the following points:

1. At the zero signal defined by the retention of 90% B
2. At the maximum stable signal defined by the region 100% B
3. Vertical line at 2.0 minutes. Your image should look like Figure 19.

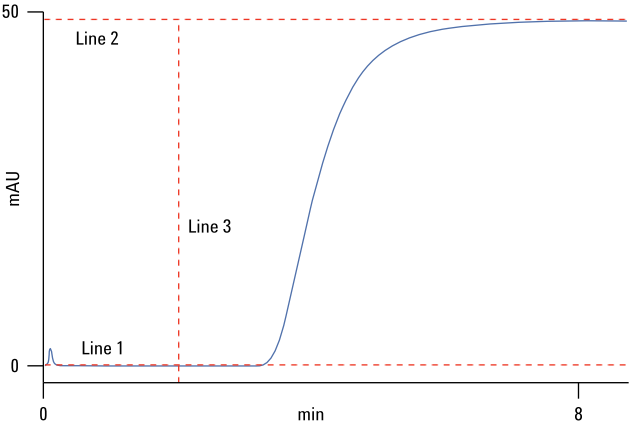



***Figure 19.*** *Calculating dwell (delay) volumes, step two*

After the three lines have been added, add two additional lines. Calculate the 50% response of the step, in this case, 24.5 mAU, and draw a horizontal line across the image. Next, draw a vertical line that intersects the junction of the 50% response line and the observed detector signal. Your new image should look like Figure 20.

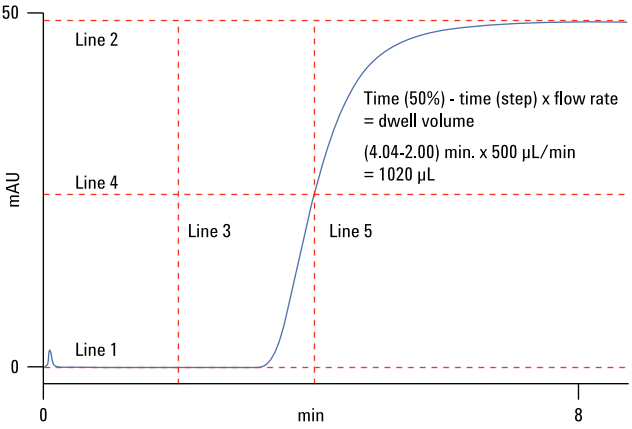



***Figure 20.*** *Calculating dwell (delay) volumes, step three*

Follow the x axis to the 50% B vertical line and determine the time, as closely as possible, at which the 50% response is observed. An error of 0.1 minute will be a 50 µL error in your delay volume estimate, assuming your test was run as we did at 500 µL/min flow rate. In this example, we estimated the 50% time at 4.04 minutes.

To calculate the dwell volume the simple formula is Time (50%) - Time (step) x Flow Rate = Dwell Volume. In this example, (4.04 - 2.00) minutes x 500 µL/min = 1020 µL.

This measurement was made on an Agilent 1200 RRLC system (1200 SL) with nominal 340 bar backpressure via a nominal 100 cm PEEK restrictor with 0.062 mm (0.0025 inch) internal diameter using water as the specified solvent for compressibility compensation in flow delivery. The system included the standard mixer and pulse damper and the autosampler was used in the normal flow path (vs. bypass mode) to a restrictor as described and fitted with a 3 mm 2 µL flow cell.

**Evaluating the impact of dwell volume**

Running a method on instruments with different dwell volumes can produce erratic results, as demonstrated in Figure 21.

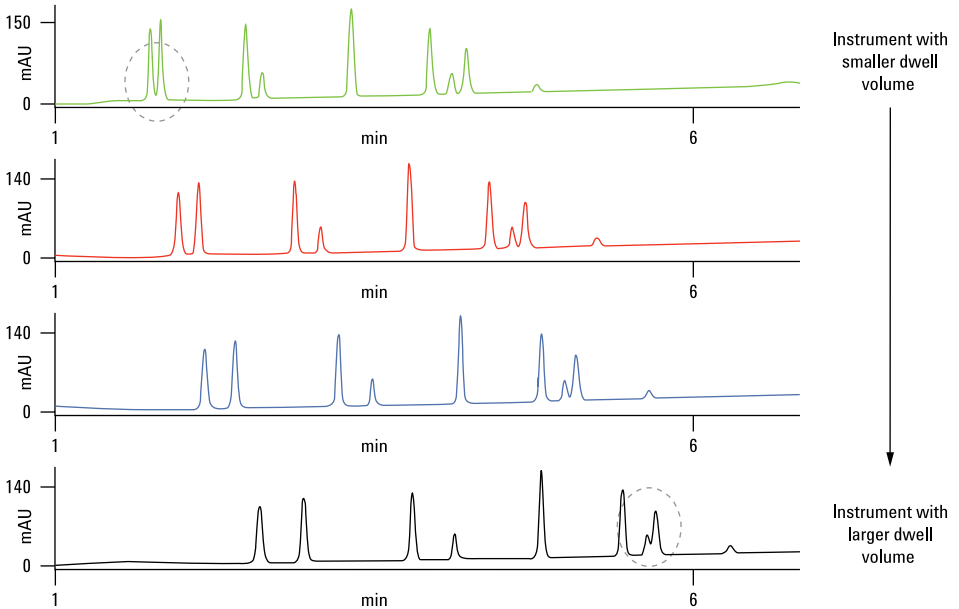



***Figure 21.*** *Dwell volume and resolution*

Here, the systems with the smallest and largest dwell volumes did not produce good results. It is likely that this method was originally developed on an instrument with a dwell volume in the medium range.

To investigate the effect of dwell volume on your gradient separation, you can either increase or decrease the isocratic hold at the beginning of the gradient run.

To simulate larger dwell volume on a system with lower dwell volume, set a hold time at the beginning of the gradient program, equal to the difference between the two dwell volumes, in mL, divided by the flow rate in mL/min.

To simulate smaller dwell volumes than that of your instrument, you must modify the injector program so you impose an injection delay time equal to the same dwell time. Some instruments have this capability; others do not.

If the method is to be transferred to another laboratory with a different instrument, it is extremely important to document, in your written method, the dwell volume of the instrument(s) used in method development, and any effect of dwell volume on the separation.

**Dwell volume and analysis time**

Dwell volume also includes injector volume, so it is important to minimize the internal volume and connecting tubing around the injector. Most Agilent autosamplers offer the option of running in the bypass mode or with Automatic Delay Volume Reduction (ADVR), where the injector is switched back to the load position after the sample is flushed from the sample loop. Depending on which injector you are using, this reduces volume in the system by up to 300 µL. It is possible to start with a delay volume of about 1100 µL on a standard Agilent 1200SL or 1260 Infinity binary gradient instrument and get it down to about 280 µL, by changing to smaller diameter tubing, using the autosampler bypass function, and removing the mixer and damper.

You can reduce analysis time in rapid gradient separations by overlapping injections. In an overlapped injection, the autosampler draws up the sample during the previous run and injects when the system is ready. This can reduce run time by about 30 seconds or more per run. If you are doing fast, gradient combinatorial chemistry runs, this is up to 1/3 of your analysis time.

If you are going to do a lot of gradient work with very small columns, like the capillary and microbore columns, a delay volume of 280 µL will be too high. It is important to choose the Agilent Capillary LC instrument for those columns.

Don't forget that it is also important to minimize extra column volume and inject in an appropriate sample diluent. This helps minimize band broadening that degrades the resolution.

In addition, as discussed earlier, it is necessary to make sure the detector response time is set correctly to capture enough points on the quickly eluting peaks. If it is not, then distorted, artificially broad peaks may appear.

## Chelating compounds

Some analytes have structures that are suitable for chelating metals. Metal on the frit or on the column walls may interact with a chelating compound. A phosphoric acid wash may help to eliminate this problem.

Here is a good way to tell if metals pose a problem. With a compound that has a lone pair of electrons, the addition of a metal may form a ring structure; this may cause irreproducible retention or a peak shape issues. (Figure 22).

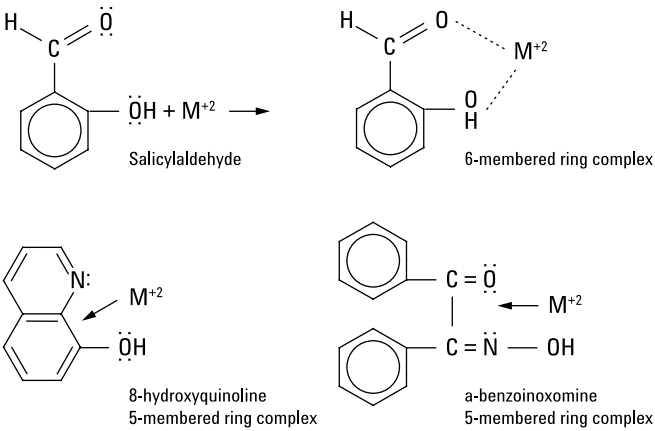



***Figure 22.*** *Evaluating compounds to identify chelating issues*

In a case such as this, a phosphoric acid wash may be beneficial. Notice in the 'before' chromatogram, compound 2 has a high peak-tailing factor (Figure 23). After the acid wash, we see a sharper peak and an improved tailing factor on compound 2. A 1% phosphoric acid wash is acceptable for ZORBAX StableBond columns because they are designed to be rugged in acidic conditions. If you use an Eclipse-XDB, Eclipse Plus, or any end-capped column that is designed for the mid pH range, reduce the acid concentration to 0.5%.

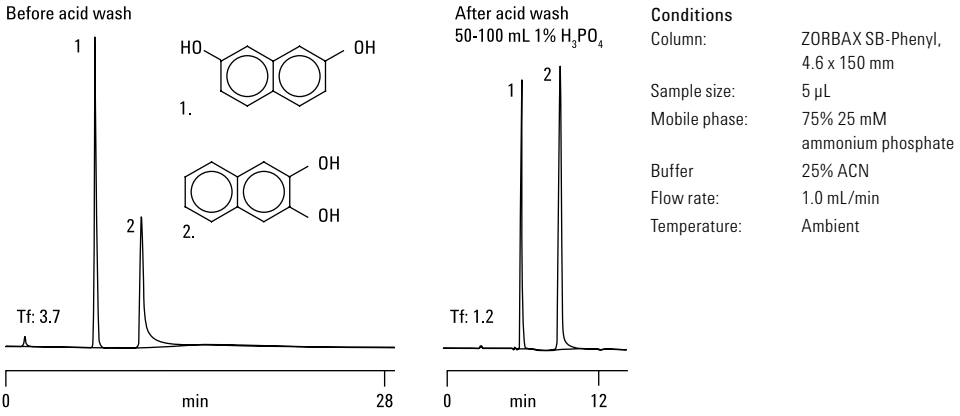



***Figure 23.*** *Washing with phosphoric acid restores column performance for chelating compounds*

## pH and mobile phase modifiers

The pH of your mobile phase and your sample can have a big impact on your method development (see 'Working with Mobile Phases' and 'Method Development' sections for more information). If possible, it is good to avoid extremes – either high or low – in pH, as working at these extremes may shorten the lifetime of your column.

Review the recommended pH range of the column you are using for the optimal pH range. Most separations will take place between pH 2 and 8. The retention of acidic and basic – ionizable – analytes will be sensitive to pH and can often change dramatically with pH. Evaluating the optimum pH for your separation is a key part of method development and more detail can be found in the Method Development section.

## Working with gradients

The more complex your sample, the more likely you are to use a gradient method. Gradient separations are useful for compounds that differ widely in polarity, or are of high molecular weight, such as peptides and proteins.

Although isocratic methods are easy to use, peak broadening of late-eluting peaks can occur because peak width increases with retention time. Gradient elution can overcome this problem by decreasing the retention of late-eluting peaks. Benefits of gradients include sharper peaks because of gradient compression effects and reduced build-up of contamination, because the gradient exposes the column to continuously increasing solvent strength.

Figure 24 shows a comparison between an isocratic and gradient method. When separated isocratically, this eight-component herbicide mixture is not fully resolved in 70 minutes, and components 1 and 2 co-elute. Reducing the percent organic to resolve peaks 1 and 2 will only lead to an unacceptable retention time and possibly unacceptable limits of detection for peak 8. When using a gradient of 20-60%, all eight components are well-resolved in less than 30 minutes with essentially equivalent limits of detection. The analysis time could be further reduced by starting the gradient at a higher percent organic. One way to do this would be to run a 25-65% organic.

Gradient separation for reversed-phase chromatography is accomplished using two to four mobile phase components. In a binary gradient they are usually referred to as A and B. The A solvent is weaker (often water or buffered water), and allows the analyte to slowly elute from the column. The B solvent is stronger and causes the analyte to elute more rapidly. B is an organic solvent miscible with water, such as acetonitrile (ACN), methanol (MeOH), tetrahydrofuran (THF), or isopropanol (IPA). Start by running a test during gradient development.

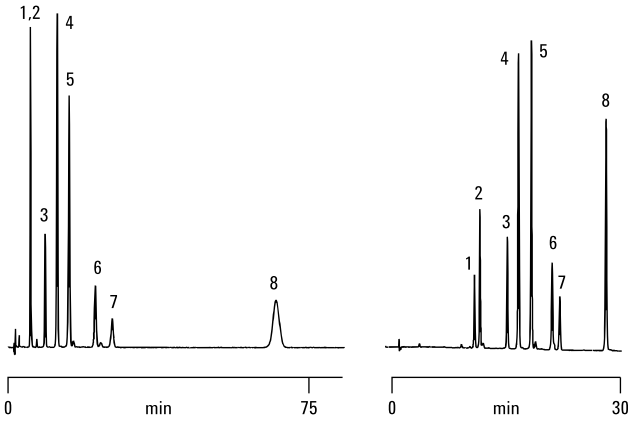
Here are the key steps to creating a gradient:

1. Start by running a test using a linear gradient from 5 - 10% to 100% organic over a set time period. Limit your gradient to ~70% organic if your buffer is insoluble in the B solvent.
2. Hold the final composition for some additional time to ensure all sample components have eluted.
3. Examine the chromatogram to determine the appropriate initial gradient composition and gradient profile.

Most gradient separations are achieved using a linear gradient with a constant change in organic composition over time. However, other types of gradient are possible. For example, some gradient programs involve different slopes at different times, or sudden step changes in the relative concentrations of A and B, depending on the separation and retention time needed.

See more about gradients, and some examples, in the Method Development section.

Keep your gradient simple (using shorter gradient times) and use a shorter column (50-75 mm) to reduce run times and improve peak detection.




**Peak Identification**

1. Tebuthiuron
2. Prometon
3. Prometryne
4. Atrazine
5. Bentazon
6. Propazine
7. Propanil
8. Metolachlor

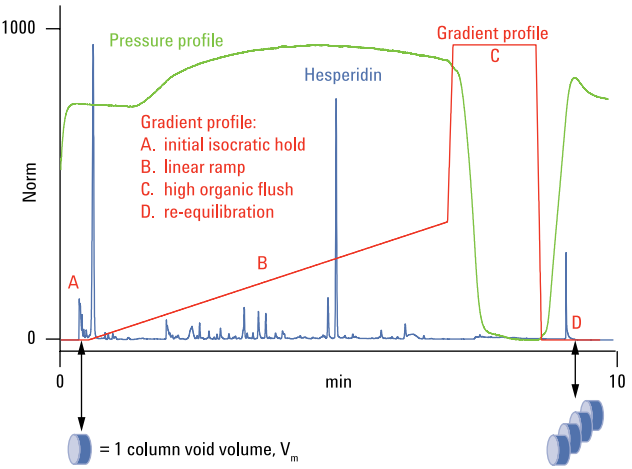
**Conditions**

| | |
|---|---|
| Column: | ZORBAX SB-C8<br>4.6 x 150 mm, 5 µm |
| Mobile phase: | A: $H_2O$ with 0.1% TFA, pH 2<br>B: Acetonitrile |
| Flow rate: | 1.0 mL/min |
| Temperature: | 35 °C |

***Figure 24.*** *Comparison of isocratic and gradient analyses.*

## Optimizing column re-equilibration

Sufficient column equilibration is necessary so your retention times during method development or method transfer are reproducible. Looking at the gradient separation in this grapefruit juice analysis (Figure 25), we can see the column void volume and amount of time dedicated to column re-equilibration.




| Time | %B | mL/min |
|---|---|---|
| 0 | 0 | 0.8 |
| 0.5 | 0 | 0.8 |
| 7 | 40 | 0.8 |
| 7.1 | 100 | 0.8 |
| 8.6 | 100 | 0.8 |
| 8.7 | 0 | 0.8 |
| 10 | 0 | 0.8 |

**Conditions**

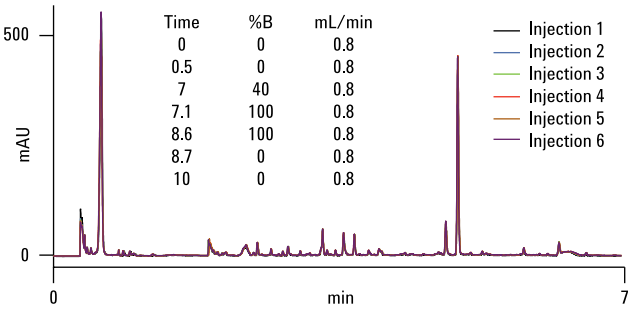
| | |
|---|---|
| LC: | Agilent 1290 Infinity with DAD |
| Column: | Agilent RRHD SB-C18 2.1 x 150 mm, 1.8 µm PN 859700-902 |
| Injection : | 1 µL of 0.2 µm filtered grapefruit juice |
| Temperature: | 40 °C |
| Detection: | UV, 276 nm |
| Solvent A: | Water |
| Solvent B: | Acetonitrile |

***Figure 25.*** *Gradient example: grapefruit juice analysis*

This is the time it takes to return the column to starting mobile phase conditions after a previous gradient has run. In this case, re-equilibration time was 1.4 min, equivalent to 4 column volumes, for this column size.

You can estimate your column void volume by looking at a chromatogram and measuring the time from the start of the chromatogram to the first disturbance on the baseline. Take this time in minutes and divide by the flow rate in mL/min to find the mL of void volume. Alternately, use the Agilent Method Translator which will calculate the void volume for you.

The rule of thumb is to use ten column volumes of equilibration during method development, but for established methods, the equilibration time can be evaluated and shortened, for instance, like in this case to 4 column volumes. The minimum equilibration time has to be determined by trial and error, by running the gradient repeatedly and looking for any changes in retention. If several repeated injections are identical, as in Figure 26, equilibration time is sufficient.




| Conditions | |
|---|---|
| LC: | Agilent 1290 Infinity with DAD |
| Column: | Agilent RRHD SB-C18 2.1 x 150 mm, 1.8 µm PN 959759-902 |
| Injection: | 1 µL of 0.2 µm filtered grapefruit juice |
| Temperature: | 40 °C |
| Detection: | UV, 276 nm |
| Solvent A: | Water |
| Solvent B: | Acetonitrile |
| Sample: | Grapefruit juice |

***Figure 26.*** *Overlay of chromatograms confirms equilibration*

Why does this have to be done by trial and error?

- The rate of stationary phase re-equilibration back to the starting conditions varies. How slow or fast you go depends on the mobile phase solvents, the buffers and gradient range used. This is why ten column void volumes are recommended for method development, to be sure the stationary phase is re-equilibrated for the next experimental run. Once you have the separation you want, you can then look at shortening the re-equilibration time.
- Because the equilibration volume includes the dwell volume of the instrument, this additional volume also can vary from instrument to instrument. Dwell volume has no effect on retention in isocratic methods, but in gradient methods it has a significant effect on retention. If you don't know your system's dwell volume, it could have serious implications, because it's possible that your dwell volume is much larger than your column volume. If you move a gradient method from one instrument to another and the retention times shift, compare the dwell volumes of the two systems. The closer the flow path volumes match, the closer the retention times will match.

Tip: View the pressure trace in the online signal window. The pressure changes as the mobile phase composition changes. When the pressure trace returns to its starting value, you know you are near re-equilibration.

Tip: Know your system's dwell volume! Check out p. 34 for a method to do this. Check your system's documentation, or contact Agilent tech support for help. See Agilent resources section in this book.

## Column aging

As you use your column and expose it to various mobile phases, analytes, and sample matrices, it will be subtly affected by these interactions and over time, there may be changes in its resolution. It's important to keep a test chromatogram, from when your column was new, to compare and understand these changes in performance over time. If resolution degrades beyond an acceptable value for good quantitation, the column should be discarded and replaced with a new one.

**Loss of bonded phase**

While column contamination is one of the most common reasons for decline in silica-based column performance, loss of bonded phase will change retention over time. If certain non-compatible solvents are used in the mobile phase, the bonded phase can be stripped away from the base silica, leading to changes in resolution and retention times, and an increase in peak tailing. Loss of bonded phase is most severe below pH 2. You can limit its effects by using a stabilized bonded phase column, or by operating between pH 2 and pH 7, and below 60 °C. On the high pH side, the dissolution of the underlying silica gel can lead to a loss of packing, formation of a void and poor peak shapes. If you must work at pH values greater than 9, make sure you use a specialty bonded phase column suited for this operation or a polymer-based column which can easily withstand high pH mobile phases.

To determine when a column is wearing out, you should monitor and look for changes in its capacity factor, selectivity and tailing factor. This will also help you predict when your column needs to be replaced.

## Cleaning a reversed-phase silica column

Sometimes you can alleviate a high pressure or peak tailing issue by cleaning your column. Before cleaning your column, we recommend that you disconnect it from your detector and run your wash solvents into a beaker or other container. In some cases, it is advisable to backflush the column while cleaning it. This keeps the column contamination from flowing through the column. Check with your column manufacturer, or review the instructions that came with your column before doing this.

Cleaning the column requires solvents that are stronger than your mobile phase. You should use at least ten column volumes (see Figure 27) of each solvent for cleaning analytical columns. See the following procedure for cleaning reversed-phase columns. When finished cleaning your column, return your column to the flow direction recommended by the manufacturer.

A pressure problem may not be due to column blockage, but to seals breaking down in your system and depositing particulates on the frit in your inline filter; try changing your inline filter frit before cleaning your column.

Do not attempt to change the inlet frit on your column. Due to the high efficiency processes used today to pack columns, this may cause irreversible damage to your column

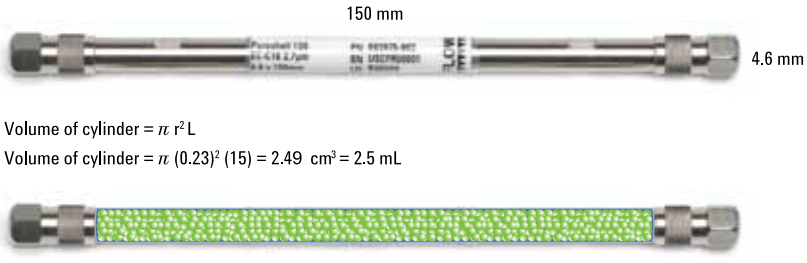



***Figure 27.*** *Calculating your column volume*

Tip: To avoid precipitation if there are non-volatile buffers in your system, first flush with non-buffered aqueous mobile phase before introducing pure organic solvent.

Steps to backflush or clean your column:

1. Disconnect the column from the detector, attach tubing to the end of the column and place it in a beaker to capture the liquid.
2. Start with your mobile phase without buffer salts (water/organic)
3. Next, use 100% organic (methanol and acetonitrile)
4. Check pressure to see if it has returned to normal; if not, then
5. Discard column or consider stronger conditions: 75% acetonitrile/25% isopropanol
6. 100% isopropanol
7. 100% methylene chloride*
8. 100% hexane*

*When using either hexane or methylene chloride, the column must be flushed with isopropanol prior to use and before returning to your reversed-phase mobile phase.

Buffer salts can precipitate out and cause backpressure build-up inside the column. If this occurs, slowly pump warm water through the column to remove them.

Wash solutions containing propanol will generate higher operating pressures due to increased viscosity. You may need to reduce the flow rate during that stage of cleaning to maintain safe pressure operation.

## Cleaning a normal phase silica column

For normal phase, you can only use organic solvents. Use at least 50 mL of each solvent, assuming your column is the traditional analytical 4.6 x 250 mm column (20 column volumes). Try these solvents, in order of increasing strength:

1. 50% methanol:50% chloroform
2. 100% ethyl acetate

Cleaning a column used in normal phase mode may depend on the sample type. If these solvents do not work, contact Agilent so we can recommend a solvent that may be more effective with your application and sample matrix.

## Cleaning a reversed-phase polymeric column

We strongly recommend that you maintain a minimum of 1% organic modifier in the mobile phase when using polymeric reversed-phase columns. Column performance can be degraded when reintroducing organic mobile phases after prolonged use in 100% aqueous eluents; nor should columns be 'washed' with 100% aqueous buffer. You can reverse the direction of flow, and if starting pressures are high, reduce the flow rate. Here are the steps to clean a reversed-phase polymeric column:

1. Run a clean-up gradient using the current mobile phase, moving to a high-percent organic (95%) and hold for several column volumes. Repeat this step two or three times. Buffers may precipitate at high organic. If your mobile phase contains a buffer, you may want to limit the organic to ~70% or flush out the buffer with the mobile phase minus the buffer prior to cleaning.
2. Try a higher-strength organic modifier such as TFA to remove hydrophobically-bound contaminants
3. Peptide or protein contamination may sometimes be removed by aqueous/ACN containing 1.0% v/v TFA
4. Strong acids and bases, including 1 M sodium hydroxide, can be used for cleaning in place and depyrogenation

Tip: Filtering your sample will increase the lifetime of your column and will help reduce instrument wear.

# Method development

If you don't have an established HPLC method, you'll need to do some method development in order to have a robust and reproducible analysis. Having the skill to develop a successful method will help you when encountering difficult assays in the future.

In this section, we start off broadly with an overview that encompasses all HPLC modes:

- Key steps of method development
- Mode selection
- Column packing and dimensions
- Stationary phase selection

Then, we focus on reversed-phase method development:

- Selection of stationary phase for reversed-phase chromatography
- Selection of mobile phase for reversed-phase chromatography
- Tips for working with mobile phases
- Troubleshooting examples involving mobile phase and mobile phase modifiers
- Managing pH with mobile phase modifiers
- Troubleshooting examples involving pH
- Reversed-phase method development with polymeric columns
- Tips for transferring existing methods on conventional columns to superficially porous particle columns
- A step-by-step guide to 'hands-on' method development
- Automated method development

At the end of this section, we also discuss method development for other HPLC modes:

- HILIC
- Normal Phase Chromatography
- Ion-exchange Chromatography
- Gel Permeation/Size Exclusion Chromatography

## Method development: where to start

The overall goal in method development is to optimize the resolution for the desired analyte(s) in the shortest possible time. When you start out thinking about method development, it's a good idea to review the key drivers of resolution. So, as a refresher, you may want to review the Essential Chromatography Concepts section (pages 5 - 11)

A typical method development scheme has the following steps, which will guide our content in this section:

1. Choose the mode
2. Choose the column and column packing dimensions
3. Choose the stationary phase chemistry
4. Choose the mobile phase solvents
5. If the mode requires it, adjust the mobile phase pH
6. Run some initial isocratic or gradient experiments to define boundary conditions
7. Optimize the experimental conditions

**Mode Selection**

The first thing you should do is to choose the HPLC mode. The mode is generally decided by the type and solubility of the analyte(s) of interest, its molecular weight (MW), the sample matrix and the availability of the appropriate stationary phase and column. Figures 28 and 29 outline the steps for evaluating the best choice of mode, based on the molecular weight of your compound and the solvents you'll be using.

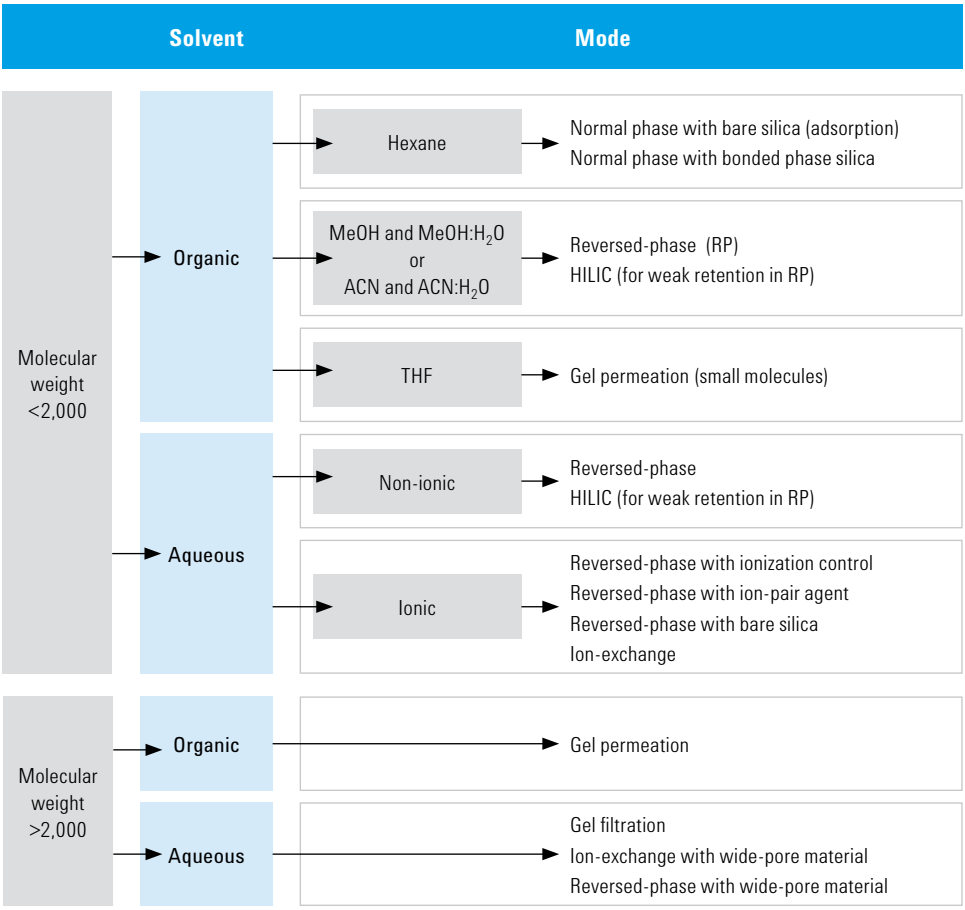



***Figure 28.*** *LC and LC/MS column selection by solvent and mode*

| Molecular size | Solvent | Compound class | Separation mechanism |
|---|---|---|---|
| Small | Aqueous/ Organic | Lipids | Silver ion complexation |
| | | Polycyclic aromatic hydrocarbons | Reversed-phase C18 |
| | | Organic acids | Ligand interaction reversed-phase ion pair |
| | | Monosaccharides and disaccharides | Ligand interaction |
| | | | Normal phase amino |
| | | | Ion-exchange |
| | | Oligosaccharides | Ligand interaction and ion-exchange |
| | | Sugar alcohols | Ligand interaction |
| | | Normal phase | Normal phase amino/cyano/diol |
| | | Basic, polar | Polar reversed-phase C8 or C18 or HILIC |
| | | | Reversed-phase C18 ion suppression |
| | | H-bonding | Reversed-phase C8 or C18 |
| | | Positional isomers | Reversed-phase C8 |
| | | Aromatic or structurally similar | Reversed-phase phenyl/phenyl-hexyl/ diphenyl |
| | | Very polar | Reversed-phase other or HILIC |
| | | Extreme conditions | Reversed-phase polymeric |
| | Organic | Non-polar | Normal phase Si |
| | | Polar | Normal phase amino/cyano/diol |

***Figure 29.*** *LC and LC/MS column selection by analyte and separation mechanism*

Sometimes, more than one mode may work for a particular set of analytes. For example, ionic compounds can be separated by ion-exchange chromatography on a resin or silica-based column or on a reversed-phase column using ion pair partition chromatography.

Many chromatographers start with reversed-phase HPLC since there are many published applications. Reversed-phase chromatography can be used for non-polar, nonionic, ionic, and polar compounds and with a judicious choice of mobile phase and operating conditions, sometimes the entire analysis can be accomplished by this mode alone. We'll discuss the other modes at the end of this section, after we cover reversed-phase.

**Choosing the column and packing dimensions**

Figure 30 shows some of the parameters to consider when evaluating a column stationary phase and column dimensions. To perform high throughput analysis, a short column with small particles (e.g., sub-2 µm) may be the best choice. If you have a complex separation involving many sample components, then a long column packed with small particles could be chosen, keeping in mind that the operating pressure of such a column may increase dramatically. If you are performing mass spectrometry, a small internal diameter column (e.g. 2.1 mm id) may be the best choice, due to the lower flow rates used with an MS detector. For preparative chromatography, larger particles (5 or 10 µm) packed into larger diameter columns are often used. For such columns, it is preferable to have a higher flow rate pump to match the flow requirements of a preparative column.

The pore size of the packing is important since the molecules must 'fit' into the porous structure in order to interact with the stationary phase. Smaller pore size packings (pore size 80 to 120Å) are best for small molecules with molecular weights up to a molecular weight of 2000. For larger molecules with MW over 2000, wider pore packings are required; for example, a popular pore size for proteins is 300Å.

For most separations, stainless steel column hardware is sufficient. However, if you are analyzing fragile molecules that may interact with the metal surface such as certain types of biomolecules, then column materials such as PEEK or glass-lined stainless steel might be used. For the separation of trace cations, sometimes PEEK columns are the most inert. Note, though, that PEEK columns are limited to 400 bar.

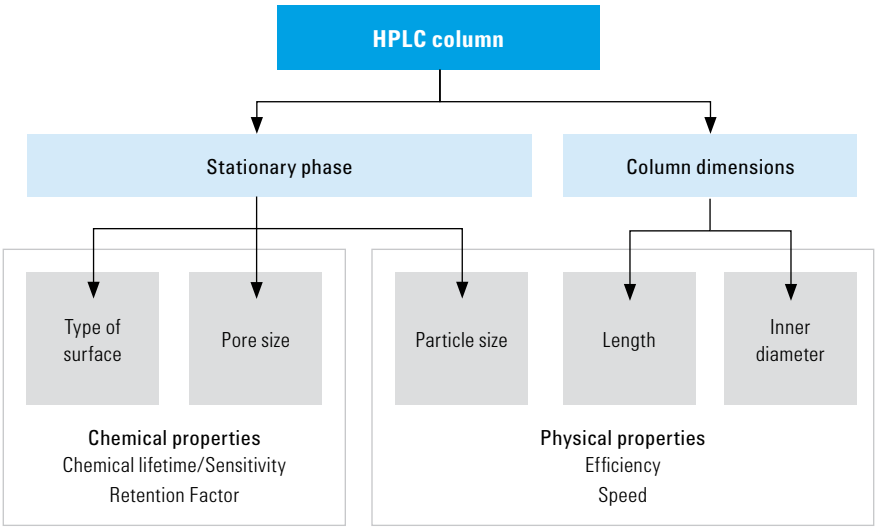



***Figure 30.*** *Some column and chemistry effects*

**Choosing the stationary phase**

There are a wide variety of stationary phases that are available for each of the modes. Many chromatographers practicing reversed-phase chromatography start with the most popular phase, octadecylsilane (C18), especially for small molecule separations. We will focus on reversed-phase separations in the next discussion but will cover other modes later in the chapter.

## Method development for reversed-phase chromatography

Reversed-phase chromatography is by far the most common type of method used in HPLC - it probably accounts for 60% of all methods, and is used by nearly 95% of all chromatographers.

In reversed-phase chromatography, we partition analytes between the polar mobile phase and the non-polar stationary phase – the opposite of normal phase chromatography. Typically, we get non-polar, non-specific interaction of analytes with hydrophobic stationary phase, meaning the sample partitions into the stationary phase. We use stationary phases like C18, C8, phenyl, or C3, which give polarity discrimination and/or discrimination based on the aromatic structure of a molecule.

More polar analytes are less retained than non-polar analytes in reversed-phase chromatography. Retention is roughly proportional to the hydrophobicity of the analytes. Those analytes that have large hydrophobic groups and with longer alkyl chains will be more retained than molecules that have polar groups (e.g., amine, hydroxyl) in their structure. If you have a series of fatty acids, such as C12, C14, C16 and C18, the C12 would be the least retained and the C18 would be the most retained.

The mobile phase is comprised of two main parts:

1. Water with an optional buffer, or perhaps an acid or base to adjust pH
2. Water-miscible organic solvent.

Reversed-phase chromatography is quite versatile and it can be used to separate non-polar, polar, ionizable and ionic molecules, sometimes in the same chromatogram. Typically, with ionizable compounds, to improve retention and peak shape, we will add a modifier to the mobile phase to control pH and retention.

## Selection of stationary phase for reversed-phase chromatography

Let's consider an approach to developing a reversed-phase chromatography method. Figure 31 gives a general flow chart on how to select an appropriate stationary phase based on the molecular weight of a particular analyte. First, the pore size must be chosen to ensure that the molecules of interest will penetrate the packing material and interact with the hydrophobic stationary phase within the pores. Next, we choose the stationary phase; most start with a C18 phase initially. Depending on the ultimate goal of your method, you may choose a conventional analytical column, or if you are interested in high throughput, you might choose a 'fast analysis' reversed-phase chromatography column.

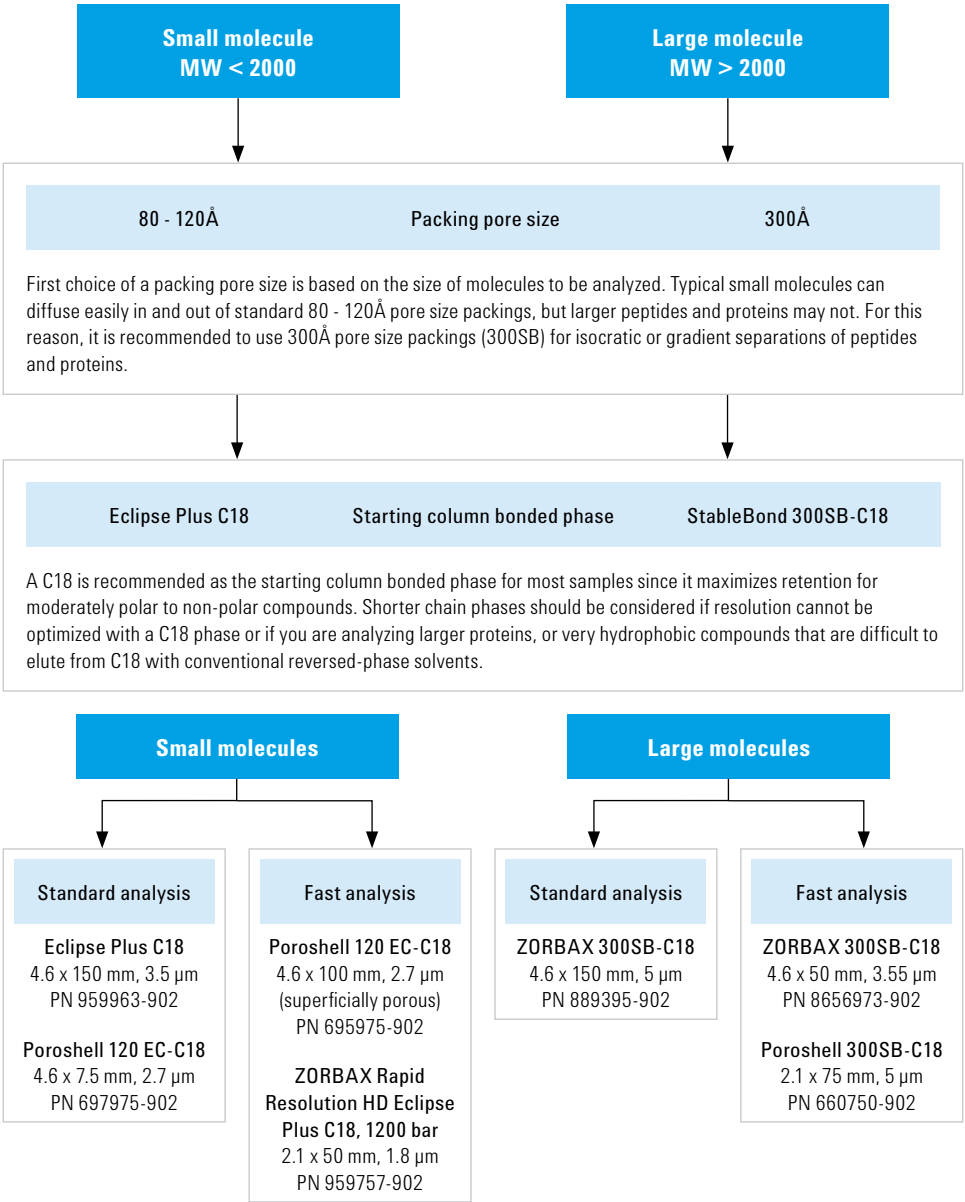
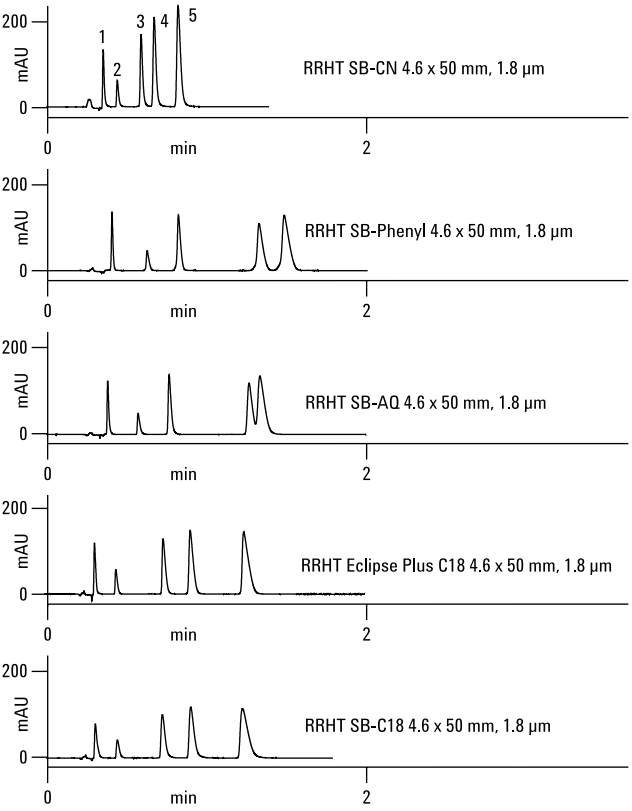



***Figure 31.*** *Reversed-phase chromatography: Overview for selecting stationary phase*

Most chromatographers begin with a C18 stationary phase but as Figure 32 demonstrates, other phases may show different selectivity that can help if C18 doesn't do the job. In this example, cardiac drugs were separated on short Rapid Resolution HT columns containing different sub-2 µm packing materials using an isocratic buffered mobile phase consisting of 70% phosphate buffer adjusted to pH 3.0 and 30% acetonitrile.

Although all of the columns gave a complete or partial separation of each of the five drugs in the sample, the SB-CN column gave the fastest separation with more than adequate resolution.




**Peak identification**

1. Pindolol
2. Diisopyridamide
3. Propranolol
4. Dipyridamole
5. Diltiazem

**Conditions**

| | |
|---|---|
| Columns: | ZORBAX RRHT, 4.6 x 50 mm, 1.8 µm |
| Mobile phases: | A: 25 mM $NaH_2PO_4$,pH 3.0 B: ACN |
| Mobile phase composition: | 30% B |
| Flow rate: | 2.0 mL/min |
| Temperature: | 30 °C |
| Detection: | UV 240 nm |
| Sample: | Cardiac Drugs |

***Figure 32.*** *Bonded phase selectivity differences in reversed-phase chromatography*

Essentially, you should choose a phase that matches the requirements of the sample. When working with hydrophobic small molecules, a longer chain alkyl phase such as C18 should be the first choice. If there is too strong of a retention on the C18 phase, then choose a shorter chain C8 or C3 phase. The C8 phases normally have similar selectivity to a C18 phase but show slightly lower retention. For very hydrophobic molecules, it is preferable to use a very short chain phase like C3. If the analyte molecules have aromatic character and can't be sufficiently separated on an alkyl phase, an aromatic bonded phase such as phenyl or diphenyl could be used. In the case where the desired analytes are strongly polar and unretained or slightly retained on a typical reversed-phase chromatography packing, consider hydrophilic interaction chromatography (HILIC) as an alternative HPLC mode (see Section on HILIC, p. 76). If in the course of method development, you discover that high pH must be used, you'll want to select a stationary phase designed for high pH work such as Extend-C18 or a polymeric phase such as PLRP-S. Silica gel columns with short chain bonded phases may be unstable at high pH values due to dissolution of the silica.

## Selection of mobile phase solvents for reversed-phase chromatography

Typical mobile phases for reversed-phase LC include water with either acetonitrile or methanol as the organic modifier. Less common modifiers are tetrahydrofuran (THF) and isopropanol. We recommend that you always work with HPLC grade or better solvents and modifiers. For UHPLC, Agilent recommends that you only use LC/MS grade solvents or better. Selectivity differences and sample retention will vary significantly between mobile phases. Sample solubility is also likely to differ and dictate the use of a specific solvent or solvents.

In reversed-phase chromatography, both pH and ionic strength of the aqueous portion of mobile phases are important in developing rugged methods not sensitive to small variations in conditions. With ionic compounds, retention of typical species shows significant changes with pH. It is very important to control pH in such reversed-phase systems to stabilize retention and selectivity. A pH between 2 and 4 generally provides the most stable condition for retention versus small changes in pH, and this pH range is recommended for starting method development with most samples, including basic compounds and typical weak acids. For reproducibility, the pH used should be ± one pH unit above or below the $pK_a$ or $pK_b$ of the solutes being separated.

You may not know the $pK_a$s of your analytes, so testing more than one mobile phase pH may provide the best results. Most reversed-phase columns can be used between pH 2-8 or more, allowing a wide range to find the optimum mobile phase pH for your separation. Note that when you are determining the mobile phase pH, measure and adjust it on the aqueous component, before mixing with organic modifiers for the most accurate and reproducible results.

### Working with mobile phases

When you begin using a new column right out of the box, you should only use solvents that are compatible with the shipping solvent. To prevent the buffer precipitating in the column, the buffer should not be pumped through a column shipped or stored in 100% organic for reversed-phase operation. Instead, we recommend equilibrating the column first, without the buffer, then equilibrating with buffered mobile phase. Both the CN and $NH_2$ columns can be used with normal and reversed-phase solvents, so you need to check that your solvents are miscible with the shipping solvents before equilibration. If you want to convert a normal phase column to a reversed-phase column you may have to flush it with a mutually miscible solvent, such as isopropanol. Then you may equilibrate with your desired mobile phase. Check the Useful References section in back for a solvent miscibility chart.

Look to your mobile phase as a potential source of problems that may develop in your HPLC column. To avoid potential problems, check out the tables with common solvent properties and miscibility information in the reference section, see p. 107.

### Troubleshooting mobile phases and mobile phase modifiers

In Figure 33, see an example of an analysis that was initially done on an older column (column 1) that gave acceptable performance. However, when the chromatographer put on a new column (column 2) the resolution of key components was quite different and the new column was 'blamed' for the discrepancy. However, the chromatograher made up fresh mobile phase buffer and the resolution returned to normal, as can be seen in the right chromatogram. In this case, the problem was narrowed down to a ‘bad’ bottle of TEA or phosphoric acid. These solvents had been used for a while and changes or contamination had occurred. See more about using buffers, or mobile phase modifiers, in the next section.

It is important to try to prepare your sample in the same solvent as the mobile phase. See the example on page 30 for the band broadening and splitting that can occur when the injection solvent is much stronger than the mobile phase.

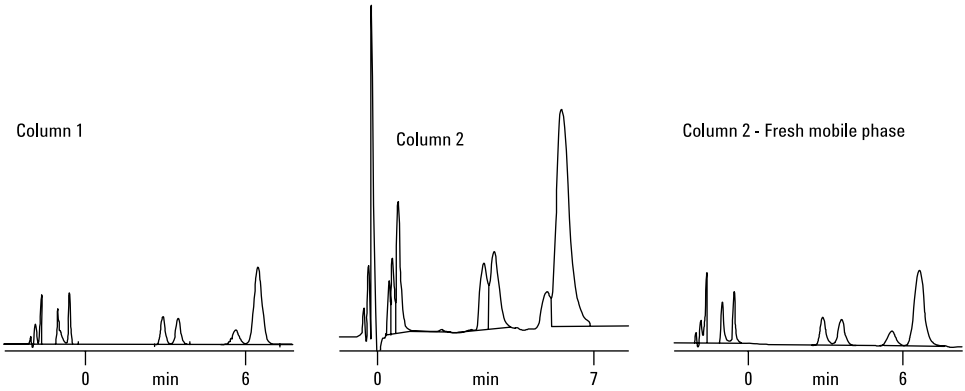



***Figure 33.*** *Variations in mobile phase can have a marked effect on results*

**Mixing mobile phases**

Sometimes, a mobile phase differs because of something as simple as the way it is mixed in your lab. If you are making a 50/50 methanol/water mixture offline, for example, it's important to measure each volume separately, in clean glassware, before mixing them together, because the volume of the MeOH:$H_2O$ mixture is more than the sum of the individual components. If you mix them in the same container, the mixture will differ in total volume. Therefore these two mobile phases, prepared in a different manner, are not the same composition.

**Degassing mobile phases**

Degassing your mobile phase is important, too. Dissolved gas in the solvents can come out of solution, forming an air bubble in the flow path, and possibly interfere with the pump or detector's performance.

Fortunately, nowadays, most LC systems have degassers built in, but if the degasser is bypassed, absent, or not working correctly, be sure to sparge with helium or use some other means to degas.

## Managing your pH with mobile phase modifiers

The pH of the mobile phase can affect your chromatography in a number of ways. Depending on the compound you are analyzing, pH can impact selectivity, peak shape and retention. If you have a fairly non-polar or neutral compound, the effect of pH will typically be insignificant for the resolution and retention.

See Figure 34 for a simple example of how pH can affect resolution. On the left hand side, we have examples of non-polar samples being run at pH values of 3 and 7. Notice that there is not much difference between the two chromatograms.

The polar compounds, which can be seen in the middle panel, tend to be less retentive on C18 columns. Notice that pH has little or no effect on the retention time or peak shapes of the compounds.

If you have ionizable compounds, such as acids or bases, you will see significant changes in retention factor and selectivity with changes in pH. Look at the example of benzoate and benzanilide in the right hand panel and notice the change in retention factor time with pH. Benzanilide, a neutral compound, shows almost no change in retention while benzoic acid has a very noticeable change in retention as the pH is shifted from 3 to 7. At pH 7, well above the $pK_a$ of benzoic acid, it exists as the ionized benzoate anion. This form is more ionic, prefers the aqueous mobile phase, and elutes from the column much faster than at pH 3, where it exists as the predominantly non-ionized* form.

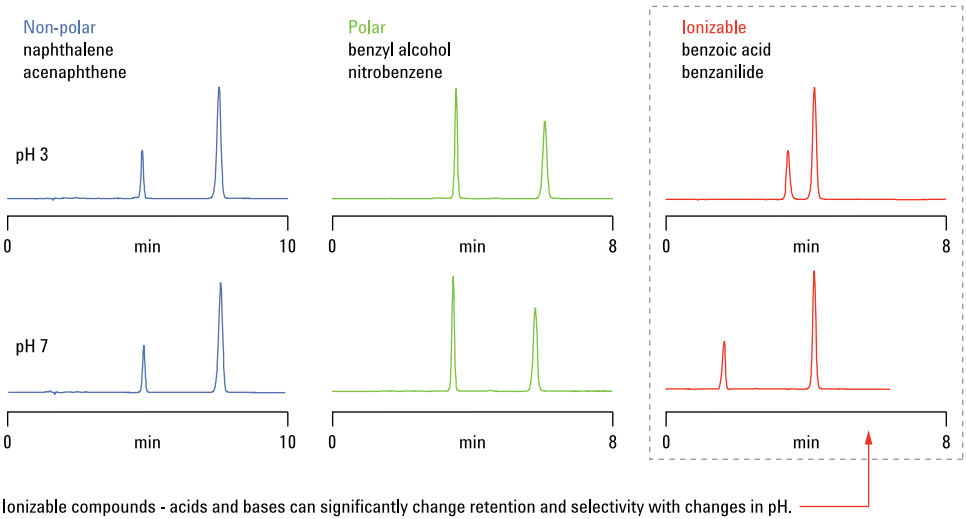



***Figure 34.*** *pH and resolution*

When considering method development with ionizable analytes, it is important to know that non-ionized analytes have better retention than ionized analytes.

If you have acidic analytes, choose a low mobile phase buffered pH to keep the analytes from being ionized. Knowing the pka of the analytes allows you to effectivity choose a mobile phase pH. A buffer is effective at +/- 1 pH units from the pK of the buffering ion, giving you some flexibility in optimizing your mobile phase. Acetate, for example has a $pK_a$ of 4.8 and buffers from pH 3.8-5.8. Formate is more acidic and buffers from pH 2.8-4.8. There are additional buffer choices if your acidic analytes would be not be ionizable at lower pH. For more details on buffers, refer to the chart on p. 110.

If you have basic compounds, the non-ionized form may be at a high pH that is not suitable for the column. But many basic compounds are adequately retained at low pH. While greater retention can be achieved in an non-ionized form, this may not be practical or necessary for all basic compounds.

**Generally, "non-ionized" can be used interchangeably with the term "ion-suppressed".*

An additional issue impacting retention of acidic and basic compounds is the potential ionization of silanols on the silica surface at mid pH. Typically, these silanols will get de-protonated and become negatively charged. This may result in more retention for positively-charged compounds, such as amines. This can result in ion-exchange interactions, a type of secondary interaction. The end result is often peak broadening or peak tailing due to an interaction other than the partitioning that is expected with a reversed-phase column. This does not happen at low pH and is another reason why acidic mobile phases are preferred for the separation of ionizable compounds by reversed-phase chromatography.

Tips for choosing a buffer

- It is not necessary to fully suppress ionization for success with HPLC – 90% suppression is generally considered adequate when sufficient buffer capacity is employed in the mobile phase.
- The buffering capacity of any mobile phase is related to the prepared molarity and how close the desired eluent pH is to the pK of the buffering ion.
- Buffering is typically effective at up to 1 pH unit above or below the pK of the buffering ion. See the reference section, p 110 for a chart with pK and pH ranges for common buffers.
- Chromatographers may also choose a non-buffered mobile phase for pH modification. It is not unusual for acidic analytes to be chromatographed with simple acid solutions, where the concentration of acid is sufficient to create a much lower pH than needed.
- On the alkaline side, choices are limited. TEA (triethylamine) is not freely water soluble and has a high pK (11) and ammonia itself dissolves freely but also has a pK too high for most columns.

**Common buffers for UV detectors**

The choice of buffers strongly influences the means of detection. For chromatographers working with UV detectors, the buffer needs to be effectively transparent at the wavelength of interest. Buffers with UV cut-offs – below 220 nm – work best. Many popular buffers have the needed UV transparency, especially when labeled HPLC grade or better. For example, the excellent low UV transparency of phosphoric acid and its salts make it, along with ACN (acetonitrile) the favored starting point of many method development chemists. With bases, TEA-phosphate is a ready alternative. Phosphate salts have limited high organic solubility, to their disadvantage, and it is recommended not to exceed 70% organic with phosphate buffer in the mobile phase. Fortunately, those compounds requiring ionic control are normally polar enough that very high organic is not required to elute them, even in their non-ionized state, from most reversed-phase columns. Acetate, like formate and TFA, contributes to UV background at wavelengths below about 240 nm and becomes very difficult to use, in significant concentrations, anywhere below 210 nm. Because method development chemists relying on UV detection often start at low UV wavelengths to acquire 2D channels and 3D spectra, ACN/phosphate combinations certainly meet many of the development requirements.

Tip: In the reference section, p. 110, we have a chart that shows UV cutoffs for common mobile phase modifiers. As wavelengths decrease and approach the UV cutoff of the modifier, you will start to see problems with your detection.

**Considerations for LC/MS**

However, when working with MS detectors, with readily usable LC/MS ionization sources, it is absolutely important to exclude non-volatile materials from the mobile phase, and even the sample in some cases, to prevent fouling of the ionization source as the mobile phase is nebulized and partially dried in the source. Significant amounts of inorganic salts in the sample matrix that emerge in the solvent front, for example, can enter the source. Due to the possibility that a developed method on LC/UV might later need to be used in LC/MS as well, many chemists are moving away from phosphoric acid, phosphate salts, non-volatile counter-ions and also any volatile modifiers with a history of causing ion suppression in the source (TFA and TEA being the most common examples).

As a result, today we see universal trends toward formate and acetate, and their volatile ammonium salts, as the dominant ionic modifiers. While they are freely soluble in organic solvents, volatile and available in high HPLC or LC/MS purity, they present significant difficulties when optical detectors (UV or Diode Array UV) are used at lower wavelengths. The modifiers must be added to the aqueous and organic phases and often a slightly lower concentration of modifier (usually 85% of what is in the aqueous phase) is added to the organic due to different absorbance properties in the organic solvent. Anecdotally, ELSDs (evaporative light scattering detectors) also require a wholly volatile mobile phase and have also driven many applications once done with UV and/or RID (Refractive Index Detection) away from the familiar pre-MS modifiers.

| Concentrated reagent | Formula weight[1] | Density | Approx. strength[2] | Molarity (M) | Normality (N) | Volume (mL) required to make 1000 mL solution[3] | |
|---|---|---|---|---|---|---|---|
| | | | | | | 1 M | 1 N |
| Acetic Acid ($CH_3COOH$) | 60.062 | 1.05 | 99.8% | 17.4 | 17.4 | 57.5 | 57.5 |
| Formic Acid (HCOOH) | 46.026 | 1.13 | 90% | 23.6 | 23.6 | 42.5 | 42.5 |
| Ammonium Hydroxide ($NH_4OH$) | 35.046 | 0.90 | 56.5%[4] | 14.5 | 14.5 | 69 | 69 |

1. Based on Atomic Weight Table (32 °C = 12)
2. Representative value, w/w %.
3. Rounded to nearest 0.5 mL
4. Equivalent to 28.0% w/w $NH_3$

***Table 5.*** *Mobile phase modifiers for LC/MS*


Use Agilent Captiva Premium Syringe Filters with low extractables for sample filtration; certified for use with LC/MS

***Figure 35.*** *Syringe filters*

A few notes about pH:

- Non-ionized analytes have better retention (i.e. acids at low pH and bases at high pH if feasible)
- Silanols on silica ionize at mid pH, increasing retention of basic analytes (i.e. possible ion-exchange interactions)
- Choose mobile phase pH to optimize retention and selectivity during method development
- Avoid extreme pH values – very high or very low – in order to lengthen the life of your column. You can use buffers to help modify your pH value. Buffers will maintain a consistent pH and improve reproducibility.
- Remember to consider your detector when choosing a mobile phase modifier; modifiers that work well with UV detectors may not be compatible with MS detectors.
- Eclipse Plus can be used over a wide pH range (pH 2-9)
- Other choices exist for high pH (Extend-C18, polymeric phases) and low pH (StableBond and polymeric phases).

When mixing triethylamine acetate or triethylamine phosphate, put in the phosphate or acetate first, then add the triethylamine, because you won't have water solubility with pure triethylamine.

Good practices for mixing buffers:

1. Use a good pH meter. Calibrate your pH meter, bracketing your target pH. This is key for reliably measuring pH with a pH meter.
2. Make sure your reagents are as fresh as possible.
3. Start by dissolving the solid in the liquid, very close to the final volume desired. After you have adjusted the mixture to the pH you need, then add additional liquid to bring your solution to the right volume.
4. pH adjustments should be made to the aqueous solution before addition of the organic. There is no reliable way to measure pH after adding the organic.
5. When your buffer solution is complete, filter it before using it in your HPLC, to remove any particulates that may have been in the water or in the solid buffer. Use a 0.45 µm filter for most HPLC applications; use a 0.22 µm filter for UHPLC applications.

Tip: Some salts such as ammonium acetate and ammonium formate are particularly hydroscopic, so they will collect water if they are sitting long on the shelf, and as they are opened and used over time. It is a good idea in your method development practice to try variations of the molarities of these salts in your method, to ensure your method is sufficiently rugged to account for these inevitable variances.

**Troubleshooting issues with mobile phase modifiers**
Try altering the mobile phase pH to determine if peak shape or retention problems can be attributed to secondary interactions. Adding trifluroacetic acid (TFA) can be beneficial, but remember that adding more components to your mixture may create more opportunities for error. Try using a low pH first before working with additives. 'Keep it simple' is a key to your starting point with pH-modified mobile phases.

One of the reasons people like to use acetonitrile is its low UV cutoff – 190 nm – whereas methanol is 205 nm and THF is 212 nm. Even THF at 215 nm can have such a high absorbance that it would be difficult to use in a gradient. (Note that fresh THF protected from oxygen is very good but difficult to maintain. Bottles that have been previously opened are vulnerable to higher UV absorbance). Depending on the range of your gradient, the absorbance from the THF could be as high as 2 AU. At a higher wavelength such as 254 nm you would not have this problem.

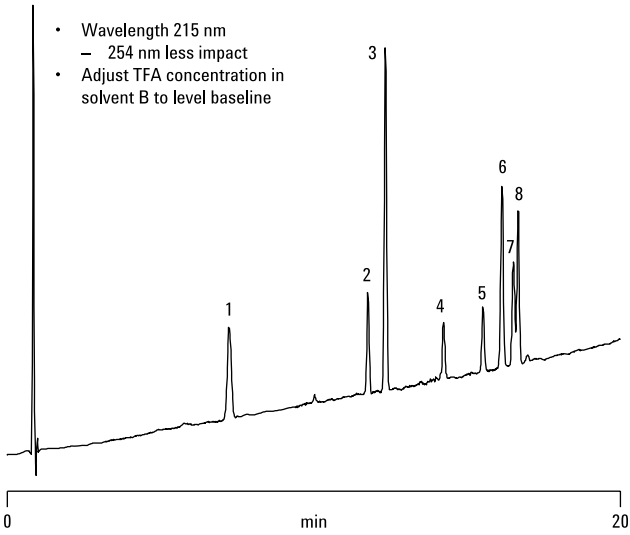
It's important to think about solvent choice and modifiers. The UV cutoff for 1% acetic acid is 230 nm, for 0.1% TFA it is 205 nm. See more on mobile phase modifiers in the Essential Chromatographic Concepts section, p. 5.

Historically, many chromatographers have used phosphate buffers or have diluted phosphoric acid. Whether at acidic or neutral pH, it has excellent UV transparency. However, phosphate can have solubility problems and is non-volatile, and it is not appropriate for use with mass spectrometry. You should avoid using phosphate buffers at concentrations greater than ~25-50 mM, especially at high organic mobile phase concentrations where precipitation may occur.

Ideally, you should have the same level of buffer or modifier in both mobile phase A and B. In other words, your best chromatographic results will be obtained when only the organic concentration varies during the gradient. Of course, there are always exceptions. Sometimes, you won't need to use a buffer; $H_3PO_4$ may be sufficient.

**Troubleshooting example: drifting baseline**
For some mobile phase modifiers absorbance is different in water versus acetonitrile, which can cause drift as a gradient is formed (see Figure 36). TFA is one such example. When using TFA, try using 0.1% TFA in Solvent A and about ~0.09% in Solvent B.

In Figure 36 we have 0.1% TFA in both mobile phases A and B and, at 215 nm, the baseline in our gradient is drifting up. If we adjust the TFA concentration in B to a lower amount, such as 0.09%, we can level the baseline and fix the problem. Using a higher wavelength such as 254 nm could also help to address the problem, but this may not always be possible for detectability reasons.



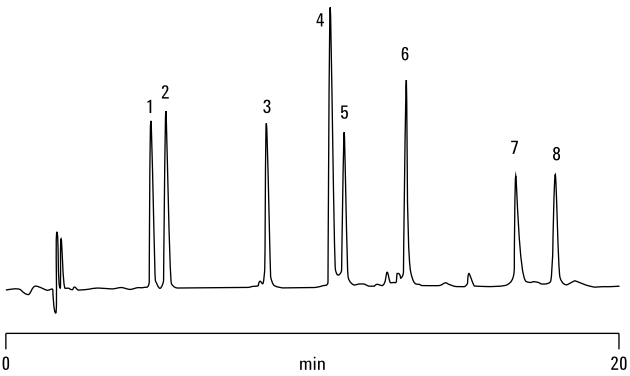

Peak identification
1. phenacetin
2. tolmetin
3. ketoprofen
4. fenoprofen
5 ibuprofen
6. phenylbutazone
7. mefenamic acid
8. flufenamic acid

**Conditions**

| | |
|---|---|
| Column: | Eclipse XDB-C8 4.6 x 150 mm, 5 µm |
| Solvent A: | 0.1% TFA in $H_2O$ |
| Solvent B: | 0.1% TFA in ACN |
| Temperature: | 35°C |
| Gradient: | 5 - 100% B in 30 min |
| Flow rate: | 2.0 mL/min |

***Figure 36.*** *Effect of TFA on baseline*

See here (Figure 37) a chromatogram that illustrates the desired state – with a nice flat baseline. Note that this is a different analysis than the example used in Figure 36.




**Peak identification**
1. leucine enkephalin
2. angiotensin
3. rnase A
4. insulin
5. cytochrome C
6. lysozyme
7. myoglobin
8. carbonic anhydrase

**Conditions**

| | |
|---|---|
| Column: | ZORBAX 300SB-C3 4.6 x 150 mm, 5 µm |
| Gradient: | 15-35% B in 19 min |
| Mobile phase: | A: 95:5 $H_2O$:ACN with 0.1% TFA |
| | B: 5:95 $H_2O$:ACN with 0.085% TFA |

***Figure 37.*** *Gradient separation with a desirable flat baseline*

This shows a typical gradient elution of peptides/proteins of varying molecular weights. We have 0.1% TFA in mobile phase A and 0.085% in B. The result is a level baseline which not only looks better, but helps with peak integration.

There are going to be times when the baseline drift is small enough that it may not matter, especially if your peaks stay on scale and you're able to integrate accurately.

**Troubleshooting example: broadening or splitting caused by high pH**

In Figure 38, see a dramatic example on a ZORBAX StableBond column, which is not designed to be used at high pH. The chromatographer made up his mobile phase which contained 0.2% TEA but forgot to reduce the pH, so the pH of his mobile phase was actually 11. He began his overnight series of chromatographic injections, came back the next day, and the chromatogram on the right is what he obtained after 30 injections. Basically, pH 11 dissolved the silica and formed a void, thereby causing catastrophic band broadening. A new column was the only option.

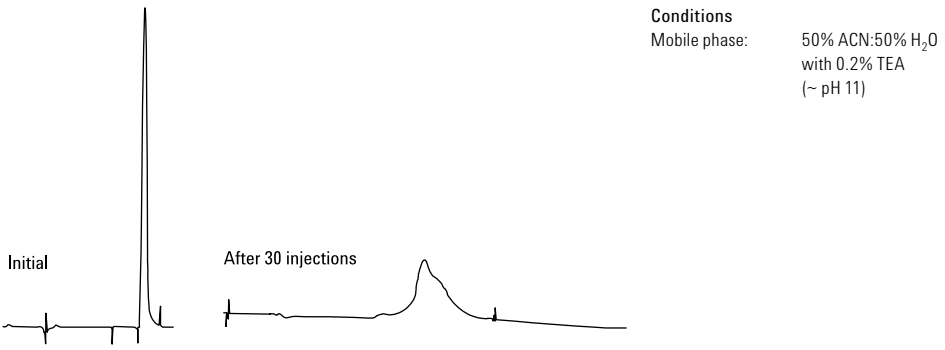



***Figure 38.*** *The effect of operating a silica column at high pH*

Thus, pH should be considered a key method development parameter simply because it can have such an impact on retention and column integrity. We recommend that you use a buffer so you can control your pH and maintain it constantly.

**Troubleshooting example: mobile phase modifiers and selectivity**

When you are choosing a mobile phase modifier, or buffer, make sure it is well within your buffering range. Look up the range in which the buffer solution is effective, and then use that range – generally, 1 pH unit above or below the pK of the buffer. See the reference section in back for a chart with typical buffer ranges.

Let's take a look at some method development schemes for a low pH application (Figure 39). This scheme uses the ZORBAX Eclipse Plus C18 and is being run at low pH. We can do this by using a buffer or weak acid solution and we can adjust the percentage of organic to control peak retention.

A mid pH level (~4-7) can provide better selectivity, and may be more compatible with your sample. The process for investigating mid pH is the same as for low pH. Eclipse Plus delivers outstanding performance at mid pH. Alternate bonded phases should also be considered if improved selectivity is desired.

The Eclipse Plus column is ideal for method development. It has a very wide pH range from 2-9, and is ideal for method development in the low and mid pH ranges. It provides excellent peak shape and efficiency.

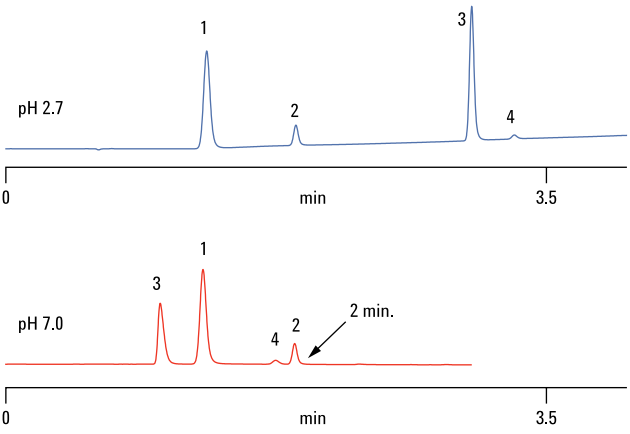



***Figure 39.*** *Selectivity differences at pH 2 and pH 7 can be dramatic*

**Peak identification**

1. Acetaminophen
2. Caffeine
3. Acetylsalicylic acid
4. unknown

**Conditions**

| | |
|---|---|
| Column: | Eclipse Plus C8<br>4.6 x 50 mm, 5 µm,<br>PN 959946-906 |
| Gradient: | 10-60% B/3 min. |
| pH 2.7 – | A: 0.1% formic acid<br>B: 0.1% FA in ACN |
| pH 7.0 – | A 20 mM Na phosphate adjusted to pH 7.0 with phosphoric acid<br>B: ACN |
| Sample: | 'generic Excedrin tablet' |

If you are still having resolution problems in the mid pH range, you may also want to consider trying a higher pH. Sometimes, low pH level or mid pH level applications do not work and they will not give you the retention you desire. With high pH applications, you may increase the retention of basic compounds by analyzing them in non-ionized form and improve selectivity.

The ZORBAX Extend-C18 is a bidentate column that can withstand the rigors of high mobile phase pH. It can be used up to pH 11.5 using organic buffers such as TEA. Just like method development at low pH, the organic modifier concentration can be adjusted for optimal resolution. We will now get into a more detailed discussion of our suggested method development scheme for reversed-phase chromatography.

**Easy, Reliable pH Testing**

Agilent offers a full line of pH meters and electrodes. Designed for chromatographers, these pH meters offer intuitive user design and exceptional ruggedness for your lab.
Learn more at **www.agilent.com/chem/AgilentpH**

***Figure 40.*** *ph meter and electrodes*

| Low pH < 3 | Mid pH 7 | High pH > 9 |
|---|---|---|
| Region A | Region B | Region C |
| Start method development at low pH, where silanols on a reversed-phase-HPLC column are protonated, to minimize peak tailing by eliminating silanol/base interactions | Develop methods at pHs at least 1 pH unit above or below the $pK_a$ to minimize changes in retention with small changes in pH | Basic compounds may be in their free base form |
| At low pH, basic compounds are positively charged and their retention may be reduced | Some silica surface SiOH groups become $SiO^-$ above pH 4 to 5; tailing interactions may occur | Increased retention and resolution of basic compounds is likely |
| Acidic compounds may be protonated and have increased retention | Minimize interactions by selecting a well-designed and endcapped column,using additives such as TEA (triethylamine) or using 'polar-linked' bonded phases | Retention changes little in this region, thus robust methods can be developed |
| Retention times are usually stable with small changes in pH, producing a robust method | Silica breakdown is prevented by innovative bonding chemistry, heavy endcapping, and use of very high purity silica with lower silanol activity due to low metal content (Rx-SIL) | Silica breakdown is prevented by innovative bidentate column chemistry, heavy endcapping, use of Rx-SIL, and optimum mobile phase |
| Volatile mobile phase additives, such as formic acid or TFA, are often used at low pH with LC/MS | | Ammonium hydroxide is an excellent volatile mobile phase modifier at high pH |

***Table 6.*** *Method development at different pH for silica columns*

## Optimizing your chromatographic conditions for reversed-phase chromatography

Once you have chosen your column dimensions, column packing with appropriate stationary phase, mobile phase solvents and modifiers, you should begin to optimize your method. Optimization is dependent on your ultimate goal. If you are developing a method for quality control, you may want to try to develop an isocratic separation so that there are fewer variables in the method. If the goal is to get the best resolution, and saving time is not a priority, you might opt for longer columns for maximum resolution between all compounds. If speed is important, use a shorter column and a fast flow rate. For a complex sample with a number of compounds of interest that have different degrees of retention, an isocratic separation may not be practical and a gradient method must be developed and optimized.

Let's consider the two approaches for condition optimization:

1. Isocratic (constant mobile phase composition)
2. Gradient (changing mobile phase strength as a function of time)

In our discussions, we will mostly deal with binary solvent systems but the same approach can be used with ternary or quaternary mobile phases. We will refer to the common designations as mobile phase 'A', the aqueous-based solvent, and the weaker solvent in reversed-phase chromatography, and the 'B' solvent, higher in organic composition, and considered to be the stronger solvent. As we increase the % of B, retention will generally decrease in the reversed-phase chromatography mode. Note that, in the absence of an actual method for their sample, most people developing HPLC methods use the 'trial and error' approach, where different mobile phase conditions are tried in order to find the optimum conditions.

**Isocratic optimization**

The general approach for isocratic optimization is to vary mobile phase strength (% B) until the right retention range is achieved. This approach is sometimes referred to as 'solvent scouting'. For simple separations, the k value should be between 1 and 10. If k is too low (i.e. <1), then early eluting peaks may run into the unretained peak or matrix components and their quantitation may be very difficult and irreproducible. In addition, low k peaks are greatly influenced by extra column effects and may be broader than one would desire. If the k value is too large (i.e. > 10), the separation time may become excessive and detection limits may be higher due to broader peaks.

For isocratic method development in reversed-phase chromatography, start with the highest percentage of organic modifier in the mobile phase and work downward. The idea here is to make sure that all components are eluted from the column before starting to decrease the mobile phase strength. If components remain on the column, they may elute at the lower % B values, resulting in unexplained 'ghost' peaks. Normally, changes of mobile phase % are made in steps of +/-10% (e.g. 90% B, 80% B, 70% B, etc.) or +/-20%. With some of the modern software (e.g. ChromSword by ChromSword, AutoChrom by ACD), as the retention profile unfolds, the system makes automatic adjustments to get to the optimized conditions faster. Manually, as you approach the optimum isocratic conditions, the increments should be decreased to 5% or even 3%.

Note that if the 'A' component of the mobile phase contains a high concentration of buffer (e.g. greater than 25 mM), you may not want to use 100% 'B' due to the possibility of precipitation of salt when the two solvent systems begin to mix with each other as the % B decreases.

Once the optimum retention is established, if peaks of interest are not completely resolved, work on improving selectivity ($\alpha$). Tweaking a separation to resolve closely spaced peaks can involve a number of experimental parameter adjustments. Temperature can be used as a variable. Most modern instruments have some type of column temperature control; most reversed-phase columns can withstand temperatures up to 60 °C and some even higher. In general, an increase in temperature will shorten the retention time of all the peaks but some may be affected differently than others, resulting in a change in selectivity.

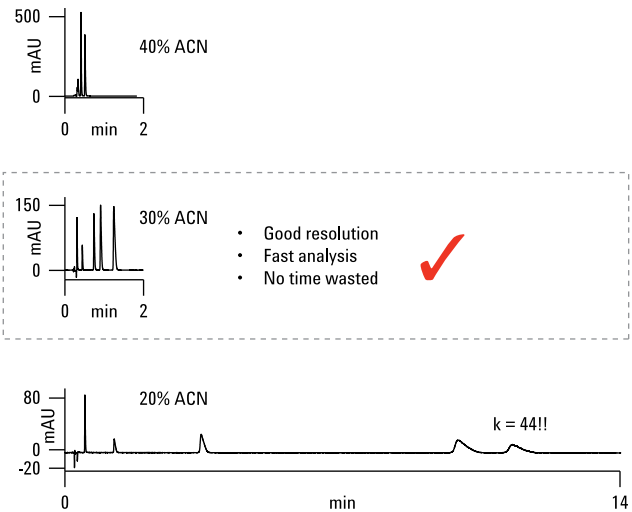
Other variables that can be used to change selectivity would be:

1. pH (for ionizable compounds)
2. Buffer (ionic) strength
3. Buffer type (e.g. phosphate to acetate or formate, depending on pH range desired)
4. Mobile phase organic modifier (e.g. change from acetonitrile to methanol or mixtures of the two; can use ternary and quaternary mobile phase solvent mixtures)
5. Flow rate (generally lower flow rates will give slightly better separations due to improved efficiency, but this is not always the case – see the tip below)
6. Ion pair reagent concentration (if using ion pair RPC)

If adjusting these parameters does not give an improved separation, then changing the column or stationary phase may be the best solution. A longer column will give more plates and therefore aid separation, but remember that resolution only improves with $L^{1/2}$. Doubling the column length doubles the analysis time and solvent consumption, and reduces sensitivity, but only improves resolution by about 40%. Going to a smaller particle size will also provide more theoretical plates but will also increase the pressure by $1/d_p^2$. Changing to a new stationary phase (e.g. C18 to phenyl) may require a new set of solvent scouting experiments and cost more time, but may give the best separation. It does pay to have some additional reversed-phase columns with different stationary phases available for substitution.

As the particle size gets smaller, optimal flow rates get higher, so for a sub-2 µm column, you may need to use a higher flow rate than you're accustomed to using with conventional columns to optimize your separation.

Let's look at one subset of scouting experiments to find the best isocratic conditions for a simple multi-component sample with 5 components. Figure 41 shows 3 chromatograms at 10% increments – 40%, 30% and 20% organic. Higher % organic eluted everything too quickly, in the void volume (not shown), while lower than 20% organic was too time consuming (not shown). At 30% organic all 5 components were well resolved and the analysis was quick. Small 1-2% organic changes can be made around 30% to further optimize this separation if desired. Those steps are not shown here. Note that all other conditions stayed the same for these experiments and it is only the % organic changing for this isocratic method optimization. The column used was an Eclipse Plus C18 4.6 x 50mm, 1.8 µm column allowing these experiments to all be done quickly and the method development scouting process to be time efficient.




**Peak identification**

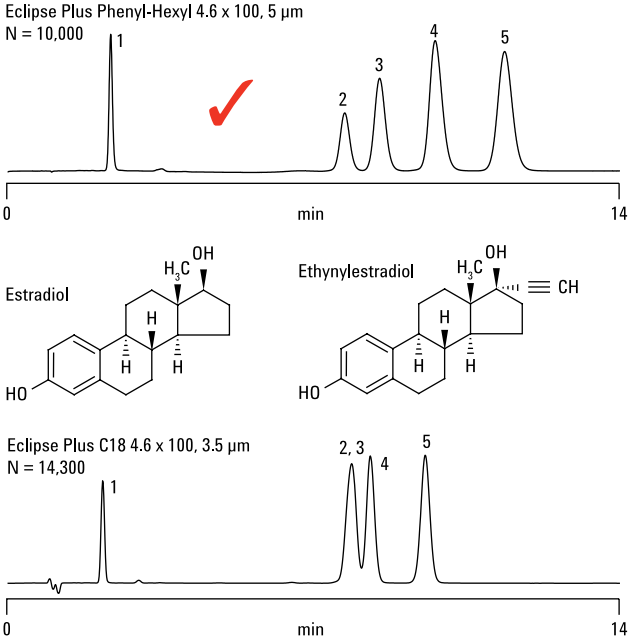
1. Pindolol
2. Diisopyridamide
3. Propranolol
4. Dipyridamole
5. Diltiazem

**Conditions**

| | |
|---|---|
| Column: | ZORBAX RRHT Eclipse Plus C18, 4.6 x 50 mm, 1.8 µm |
| Mobile phase: | A: 25 mM $NaH_2PO_4$, pH 3.0, B: ACN |
| Flow rate: | 2.0 mL/min |
| Temperature: | 30°C |
| Detection: | UV 240 nm |

***Figure 41.*** *Example of optimization of an isocratic method by adjusting the organic modifier*

To further illustrate an approach for getting better resolution, Figure 42 shows a comparison of different stationary phases. The same optimum mobile phase is used on each column; only the bonded phase changes. In this case the traditional C18 did not provide optimum resolution, but an alternate phase did. This approach is sometimes referred to as 'Stationary Phase Scouting' and can be done with one or more different stationary phases. Many laboratories have 'walk up' LC and LC/MS systems using this approach where the mobile phase is fixed and, through column selection valving, users can employ isocratic or gradient elution to develop and optimize their separation with different stationary phases.




**Peak identification**

1. Estriol
2. Estradiol
3. Ethynylestradiol
4. Diethylstilbestrol (DES)
5. Dienestrol

**Conditions**

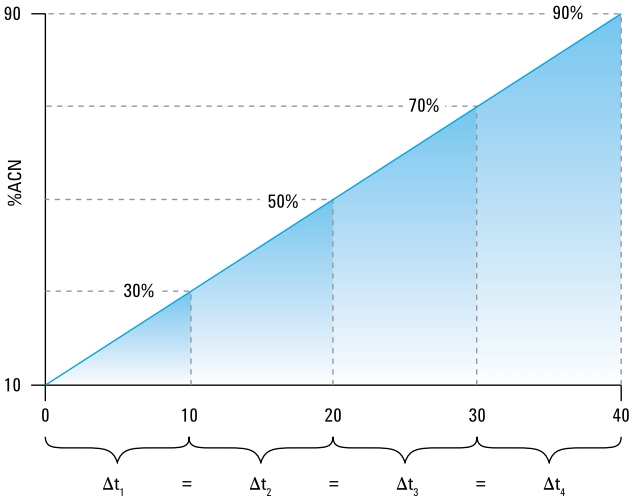
| | |
|---|---|
| Mobile phase: | 60% MeOH, 40% water |
| Flow rate: | 1 mL/min, |
| Detection: | DAD=220 nm |

The 3.5 µm column has 30% more efficiency, but selectivity has greater role in resolution

***Figure 42.*** *Selectivity scouting for isocratic methods*

**Gradient optimization**

For sample mixtures containing a wide variety of components, choosing a single mobile phase composition will not result in a satisfactory solution (i.e., the general elution problem). For example, some sample components such as very polar analytes might elute very quickly from a reversed-phase column, while hydrophobic components may stick to the hydrophobic C8 or C18 phase very strongly and may never elute. The solution to this problem is to change the mobile phase composition with time (gradient elution). In most cases, the initial mobile phase is very weak (e.g. highly aqueous) and with time, the % of organic solvent is increased, usually in a linear manner (Figure 43).




Increasing the solvent strength = Increasing the % organic in the mobile phase

Linear solvent strength gradient = % per min is a constant

$\Delta\Phi = 80\%$

$t_G = 40$ min

$\frac{\Delta\Phi}{\Delta t_G} = 2\%/\text{min}$

***Figure 43.*** *Gradient elutions: In this example, for every 20% change in acetonitrile, Δt is 10 minutes*

There are two cases where gradient elution can be used for method development. One approach is to use a gradient to predict the best starting isocratic conditions for a reversed-phase separation. Most method development software (e.g. DryLab, ChromSword, AutoChrom) have the capability to make a minimum of two gradient runs, then predict what the optimum isocratic conditions might be. One can then use those conditions to further optimize the isocratic separation.

The second case is for the development of a gradient method and uses a wide range and rapid gradient (e.g. 5% B to 95% B in 10 min) to zero in on the best range for one's compounds of interest. In fact, some of the software systems will actually optimize the separation by interacting with the chromatography data system/controller to set up a nearly optimum gradient. However, manually one can accomplish the same by noting the composition at which the peaks of interest are eluting and then fine tune the next gradient to adjust the k* (the gradient equivalent to k) range so that the gradient separation can occur in a reasonable time.

Then, just as in isocratic optimization, once the separation time is reasonable, selectivity should next be addressed.

To illustrate a simple optimization of a relatively complex mixture of 10 sulfa drugs, see Figures 44 - 46. First, run a relatively rapid, wide range gradient as depicted in Figure 44. Here we have run the gradient from 8% B to 90% B in 20 minutes using a 250 mm column. With this initial chromatogram, we can make some observations. First, no peaks eluted after 12.5 minutes, which indicates that 90% is too extreme in terms of the % B required. Also note that the pairs of peaks 5 and 6 and peaks 7 and 8 are unresolved.

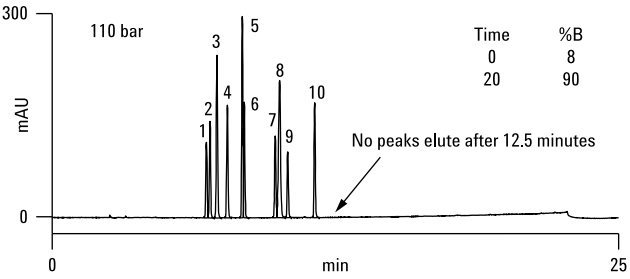



***Figure 44.*** *Initial gradient of sulfa drug analysis*

**Peak Identification**

1. Sulfadiozine
2. Sulfathiozole
3. Sulfapyridine
4. Sulfamerazine
5. Sulfamethazine
6. Sulfamethazole
7. Sulfamethoxypyrifadazine
8. Sulfachloropyridazine
9. Sulfamethoxazole
10. Sulfadimethoxine

**Conditions**

| | |
|---|---|
| Column: | 4.6 x 250 mm Eclipse Plus C18, 5 µm |
| Flow rate: | 1 mL/min |
| Detection: | 254 nm |
| Injection: | 5 µL |
| Mobile phase: | A: 0.1 % Formic Acid in Water, B:0.1 % Formic Acid in MeCN |

The next step we took was to decrease the organic range and increase the gradient time in order to increase the gradient retention, k*, to get our optimal separation in Figure 45. There were intermediate steps taken (not shown) to optimize this gradient. It's all a matter of adjusting gradient time and %B until we have the optimum separation.

At this point, we have gotten baseline resolution of our 10 compounds, but our analysis time is 30 minutes. By looking at column dimensions and flow rates, we can optimize our gradient method further. Keep in mind that as we adjust our column dimensions and flow rate, we need to adjust the gradient accordingly, as discussed in the first section, on p. 10.

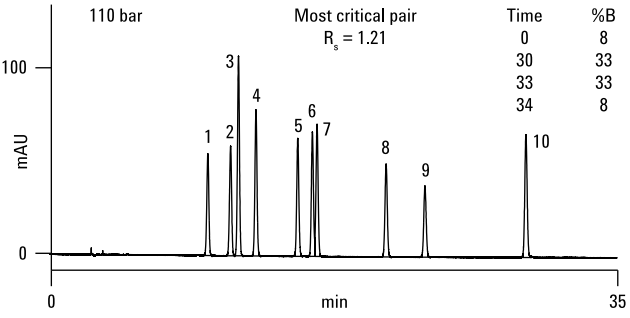


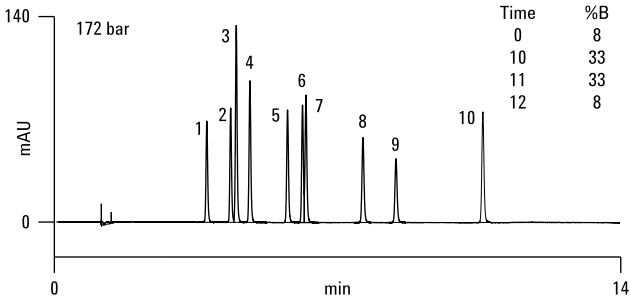

***Figure 45.*** *Gradient optimized for 250 mm column*

**Peak Identification**

For peak identification, see Figure 44

**Conditions**

| | |
|---|---|
| Column: | 4.6 x 250 mm Eclipse Plus C18, 5 µm |
| Flow rate: | 1 mL/min |
| Detection: | 254 nm |
| Injection: | 5 µL |
| Mobile phase: | A: 0.1 % Formic Acid in Water, B:0.1 % Formic Acid in MeCN |




**Peak Identification**
For peak identification, see Figure 44

**Conditions**

| | |
|---|---|
| Column: | 4.6 x 100 mm Poroshell 120 EC-C18, 2.7 µm |
| Flow rate: | 1 mL/min |
| Detection: | 254 nm |
| Injection: | 2 µL |
| Mobile phase: | A: 0.1 % formic acid in water, B: 0.1% formic acid in MeCN |

***Figure 46.*** *Injection volume and gradient scaled from a 250 mm column to a 100 mm Poroshell 120 column*

Here, we have used a Poroshell 120 column, 100 mm length, and reduced our analysis time to a little over 10 minutes, with good resolution. By further optimizing flow rate, we can get our analysis time down even further.

## Polymeric columns for reversed-phase chromatography

Polymeric columns offer significant advantages for analyses of 'difficult' samples. They provide chemical and extreme pH stability. These columns have no reactive sites and, due to their polymeric nature, the stationary phase will not dissolve in extreme pH environments.

A good starting point for reversed-phase gradient separations with polymeric columns is to use ACN/water + 0.1% TFA mobile phase screening gradient, i.e. 5% to 95% ACN. Again, the gradient can be modified to improve resolution of all components depending on where your analytes elute.

You can use polymeric media with acidic, neutral and basic eluents. For example, a synthetic peptide can be screened using ACN eluents at four different pH levels; 0.1% TFA, 20 mM ammonium acetate at pH 5.5, 20 mM ammonium carbonate at pH 9.5 and 20 mM ammonium hydroxide at pH 10.5. For more complex samples you may have difficulty obtaining the desired purity or recovery, or both, due to limited solubility or co-eluting species. The net charge on the peptide depends on the pH of the buffer, and it will have zero net charge at its isoelectric point (pI). Therefore, in reversed-phase HPLC, changing the pH alters the net charge of the sample and any closely related components, and hence changes the retention and selectivity of the separation.

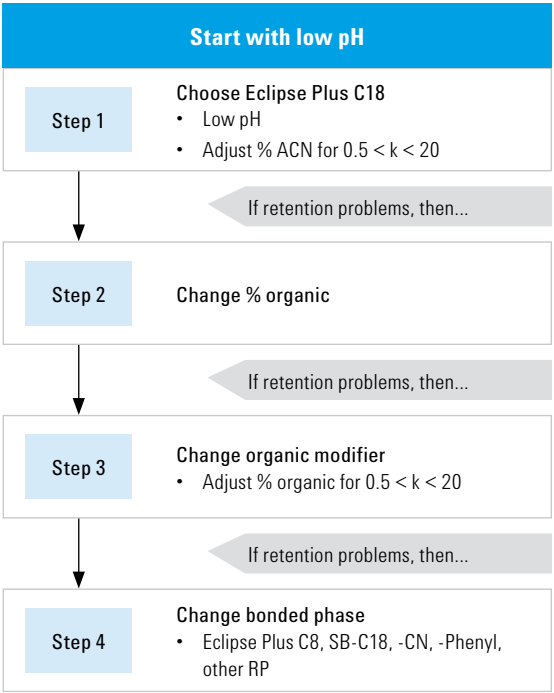
## A step-by-step guide for 'hands-on' isocratic method development in reversed-phase chromatography

The method development practice most often used is the 'hands-on' approach. Figure 47 presents a flow process for developing methods using this approach. In this practice, you follow a preferred method development scheme by selecting a specific column or bonded phase. You will vary the mobile phase by adjusting the pH or trying different organic modifiers.

You could also try utilizing method development software. Run a few experimental runs and obtain a prediction for the best method.

You may choose the practice of evaluating multiple columns or multiple mobile phases in a manual or automated fashion. In this practice, you will connect several different columns with different mobile phases and try different combinations.

Tip: The ZORBAX Eclipse Plus Column is a particularly robust and flexible column for method development, over a wide pH range.




- Select a high quality C18 or C8 bonded phase first for good peak shape, retention and resolution with typical acidic, basic and neutral samples. Note that a short column minimizes development time, and newer technologies such as Poroshell 120 EC-C18 can improve results based on efficiency at reduced operating pressure and improved peak shapes.
- Start with a mobile phase modifier that is reliable and works with many samples, such as a phosphate buffer at pH 3, TFA or formic acid in aqueous portion, and acetonitrile or methanol as the organic modifier.
- Look for adequate resolution of all peaks, Rs>2.0. The retention of the first peak should be at least k = 2 (see p. 7-8 for more info)
- Optimize the organic component of the mobile phase to change selectivity.
- Choose alternate bonded phases to completely optimize method, if needed
- Choose ZORBAX StableBond SB-C18 for pH 1-2

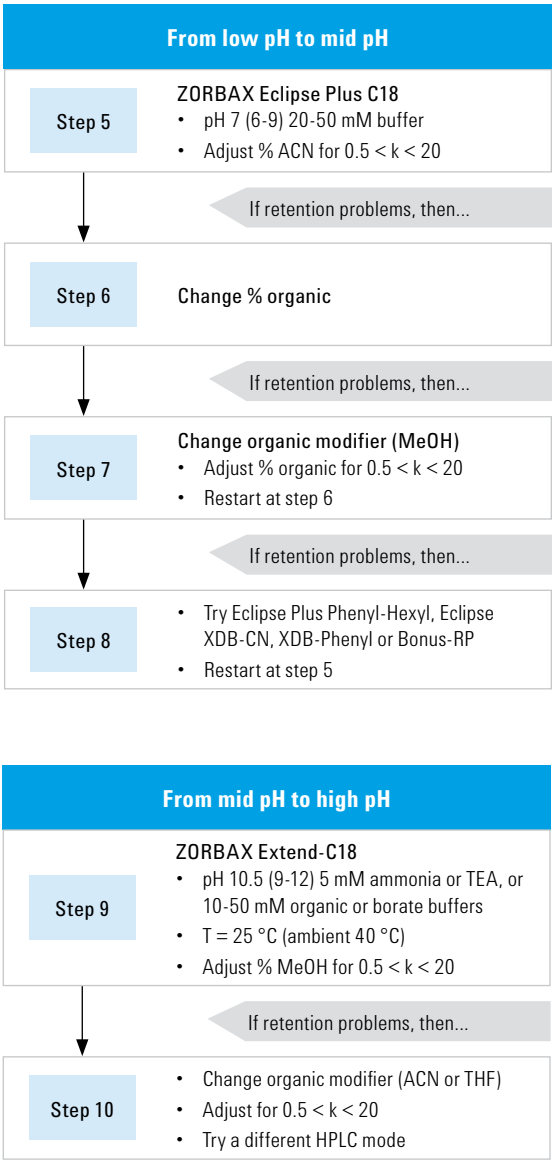



***Figure 47.*** *Method development flowchart*

Mid pH can provide better selectivity:

- It may be more compatible with your sample
- The process for investigating mid pH is the same as for low pH
- ZORBAX Eclipse Plus and Poroshell 120 columns deliver outstanding performance at mid pH
- Alternate bonded phases should also be considered if improved selectivity is desired, so long as appropriate buffers are used (ammonium acetate or formate at mid pH vs. phosphate buffer, phosphoric acid, formic acid or TFA at low pH)

Reasons to Consider High pH:

- Increase retention of basic compounds by analyzing them in non-charged form
- Improve selectivity

A word of caution regarding using buffers at high pH: Depending on buffering capacity, these are very liable to absorb $CO_2$ and the pH will change as carbonate is added inadvertently to the buffer. The only protection against this is blanketing the mobile phase bottle to exclude air except through an Ascarite trap.

## Tips for transferring methods from conventional columns to high efficiency columns

High efficiency columns for UHPLC/Fast LC will help you increase your analytical speed and resolution. Depending on the instrument configuration you are using, you may need to make a few adjustments to get the most from these columns.

Poroshell 120 columns have particles that are 2.7 µm outer diameter, but these are superficially porous particles which have a solid core and porous shell. Because of faster diffusion into and out of the porous shell and a very homogeneous packed bed, these columns provide analytical performance that's comparable to a sub-2 micron particle, but at the pressure of a 2.7 µm particle. They are able to be used up to 600 bar, so you can optimize UHPLC performance with Poroshell 120 columns. Sub-2 µm columns are able to be used up to 1200 bar.

Because of their high efficiency, very narrow peaks elute from higher efficiency columns rather quickly. While modern HPLC instrumentation and data systems are able to capture the benefits of these particles, attention to the instrumental configuration is important to get the best results.

The selectivity of these columns is very similar to other like phases, so it is easy to transfer your method and get great results. Some of the things you'll want to check are just part of routine LC optimization.

Steps to transfer your method:

- **Check the specifications and instructions that came with your instrument** – As your instrument may already be configured appropriately for high efficiency columns. If not, then continue.
- **Optimize your data collection rate (at least 40 Hz detector with fast response time)** – With Poroshell 120 columns at higher velocities, expect narrow peaks similar to those generated with sub-2 µm columns. Set the detector to the fastest setting, then to the second fastest setting and evaluate if the resolution is different. See p. 31 for more information.
- **Use a semi-micro or micro-flow cell** – The standard flow cell on the Agilent 1200 has a path length/volume of 10 mm/13 µL (note that not all detectors have the same flow cells). This may diminish the performance achievable using Poroshell 120 columns. Smaller volume flow cells such as the semi micro (6 mm/5 µL) or micro (3 mm/2 µL) are recommended for best performance. Generally, the smaller the volume of the flow cell, the shorter the path length, which may decrease sensitivity for a UV method. Note that some low-volume flow cells accomplish this by reducing path length which will decrease absolute peak height.
- **Minimize tubing volume in the instrument** – Use Red (0.12 mm id) tubing instead of Green (0.17 mm id) as it has only half of the volume that the sample has to travel through. This cuts down the extra column band broadening. Ensure that your connections are as short as possible (see p. 26 for more information). You'll see there are three or four places where you might have to change tubing, so you'll want to make a note of the connection lengths you need:
  - The autosampler needle seat
  - The autosampler to the Thermal Column Compartment – or 'TCC'
  - The TCC to the column
  - The column to the flow cell, including the internal diameter of the integral flow cell inlet capillary

  If you're not using elevated temperatures in your method, you can take a shortcut and connect your autosampler directly to your column, and then from the column to your flow cell, which reduces

extra-column volume. This operation can cause problems if the temperature is not controlled, depending on the compounds you're analyzing. All these specific capillaries can be ordered individually from Agilent, in the lengths you need.

- **Take care to make proper connections** – Agilent recommends Swagelok fittings with front and back ferrules, which give best sealing performance throughout our LC system (use this on the instrument connections, i.e. valves, heaters, etc). Polyketone fittings are highly recommended for up to 600 bar. Use this fitting (PN 5042-8957) on column connections with Poroshell 120. See more about fittings on p. 27.
- **Optimize your flow rate** – For Poroshell 120, if you're using a 2.1 mm id, the suggested starting flow rate is 0.42 mL/min; for 3.0 mm id Poroshell 120 columns, we suggest starting at 0.85 mL/min, and for 4.6 mm id, we suggest starting at 2 mL/min.

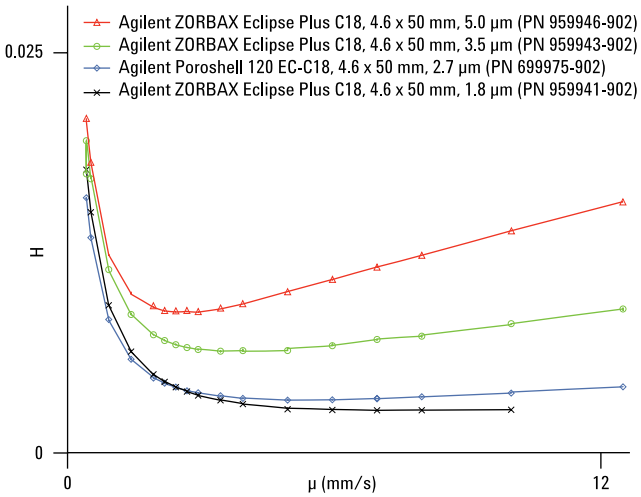



***Figure 48.*** *Overlay of van Deemter plots: the optimal flow rate for Poroshell 120 is faster than for 5 or 3.5 µm columns*

- **Scale your gradient profile and injection volume** – If using gradient elution to optimize your chromatographic results, you'll want to be sure to properly scale the gradient profile and injection volume to the new smaller column to quickly transfer the method and avoid overloading. Use our free method translation software, available at the Agilent website, to help select the proper conditions (see 'Other Agilent Resources' on p. 113). For isocratic and gradient elution, make sure that you scale the injection volume to match the overall column volume.
- **Minimize injection sample dispersion in the column** – You need to use an injection solvent whose solvent strength is equivalent to or weaker than the mobile phase, especially when using an isocratic method. This is good practice in general for any column, and a little more important with very high efficiency columns.

See a video that takes you through these steps at www.agilent.com/chem/poroshell120video

## Automated method development tools

The manual, or 'hands on' method development process is still in use in many labs. However, there are advances in instrumentation that help to make LC method development easier. New method development software, such as the Agilent 1200 Infinity Series Multi-method Solutions, provides special hardware and dedicated software solutions to automate many aspects of the method development process.

***Figure 49.*** *The Agilent 1200 Infinity Series Multi-method Solution*

The 1290 Infinity LC has wide flow and pressure ranges. This feature, combined with a minute gradient delay volume, facilitate the development of methods for other HPLC or UHPLC systems. Depending on the complexity of the separation problem, different requirements are placed on the software to be able to support the experimental setup. New software such as the Agilent ChemStation Method Scouting Wizard provides a tool to define a sequence and all methods to screen a multidimensional matrix of columns, solvents, gradients and temperatures.

With the Agilent 1290 Infinity, up to three TCCs can be clustered together – regardless of whether the system is based on 1260 Infinity or 1290 Infinity modules. Quick-Change valves give the user easy access to capillary fittings for straightforward installation and maintenance.

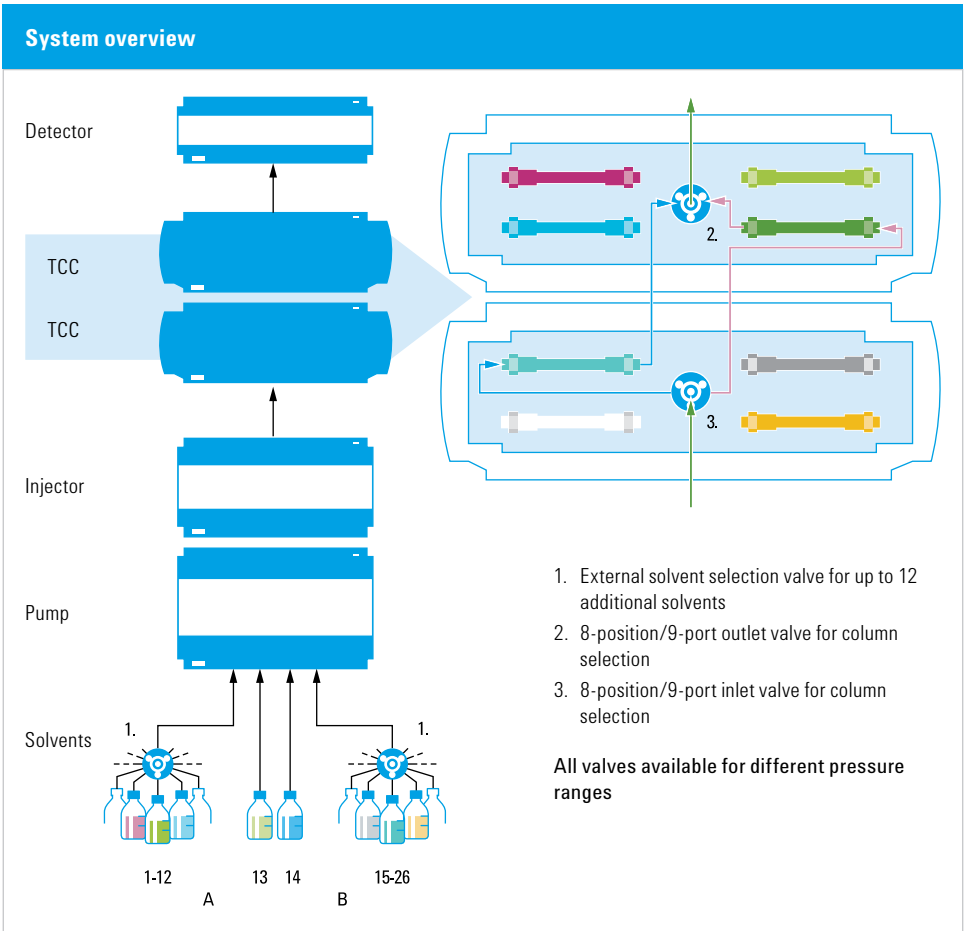



***Figure 50.*** *Flexible automated method development*

The number of solvents available can be extended, using one or two external 12-channel selection valves. With the automated process, the user can select their solvents from a list on the computer screen. Valve mounting aids and tubing kits are available for tidy and optimized setup. With a binary pump in the system, up to 169 binary solvent combinations are possible. A quaternary pump allows up to 193 combinations. When eight columns are installed, there are more than 1000 unique separation conditions available, all automated for easy implementation. The Agilent system facilitates the use of all typical analytical column dimensions.

Tip: For more information about automated method development solutions, look for Agilent publication 5990-6226EN at the Agilent website.

## Method development for other HPLC modes

### HILIC

Hydrophilic Interaction Liquid Chromatography (HILIC) – sometimes called 'aqueous normal phase' (ANP) – is a technique that has been around for several decades. The technique has received renewed attention in recent years for the analysis of polar compounds that are unretained or poorly retained on reversed-phase columns. And, the technique is readily adaptable to MS and MS/MS detection. When HILIC separations are performed with high organic mobile phases, the result is enhanced MS sensitivity due to lower ion suppression than with high aqueous buffered systems.

HILIC uses a polar stationary phase, such as silica, amino, mixed mode, zwitterionic, etc. with a water-miscible, non-polar mobile phase containing a small amount of water (at least 2.5% by volume) and high organic content.

In HILIC methods, the hydrophilic, polar and charged compounds are retained preferentially, compared to hydrophobic, neutral compounds. This directly contrasts reversed-phase liquid chromatography.

The addition of water to the mobile phase reduces the retention. HILIC provides good peak shapes for strongly polar solutes, compared to normal phase. It is a complementary method for reversed-phase chromatography in that it retains hydrophilic compounds and often reverses elution order.

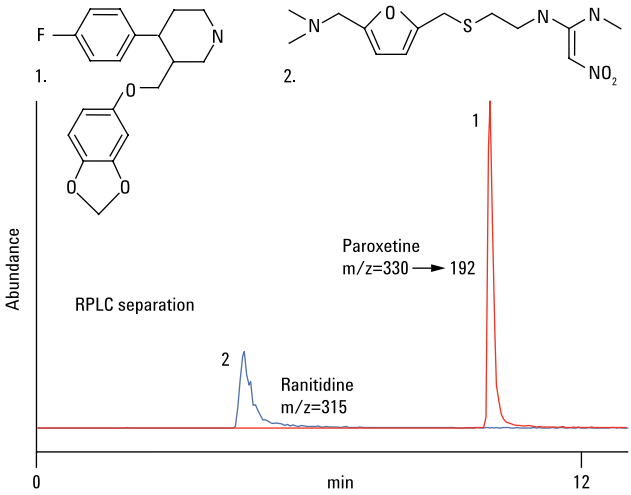
When developing a HILIC method, you may need to take care to optimize the following parameters:

- Stationary phase
- Organic solvent concentration
- Type of buffer
- Buffer (salt) concentration
- pH
- Temperature

The typical columns used in HILIC methods:

- Silica
- Amino
- Mixed-mode (Alkyl-diol, Alkyl-carboxyl, C18-amide, Aromatic-cyano, Alkyl hydroxyl, C18- SCX and Polyhydroxyethylaspartamide)
- Hydride and modified hydride
- Zwitterionic (e.g. tetraalkylamine-sulfonic)
- Proprietary phases

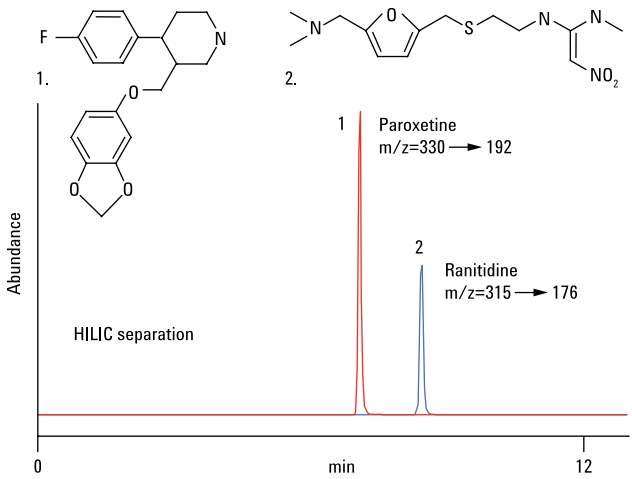
In Figures 51-53, we show a pharmaceutical separation of two compounds – ranitidine and paroxetine – using reversed-phase chromatography and followed by a HILIC method and MS data. The results show the key advantages of the HILIC mode. The ranitidine is more retained in HILIC and has much greater sensitivity in the MS.




**Conditions**

| | |
|---|---|
| Instrument | Agilent Series 1100 LC |
| Column | ZORBAX Eclipse XDB C18, 2.1 x 150 mm, 5 µm) |
| Mobile phase: | A: 8 mM $HCOONH_4$ in water<br>B: 8 mM $HCOONH_4$ in 95% acetonitrile (ACN)/5% water |
| Gradient: | 5% B to 90% B in 10 min |
| Column temperature: | 40 °C |
| Sample volume: | 5 µL |
| Flow rate: | 0.3 mL/min |

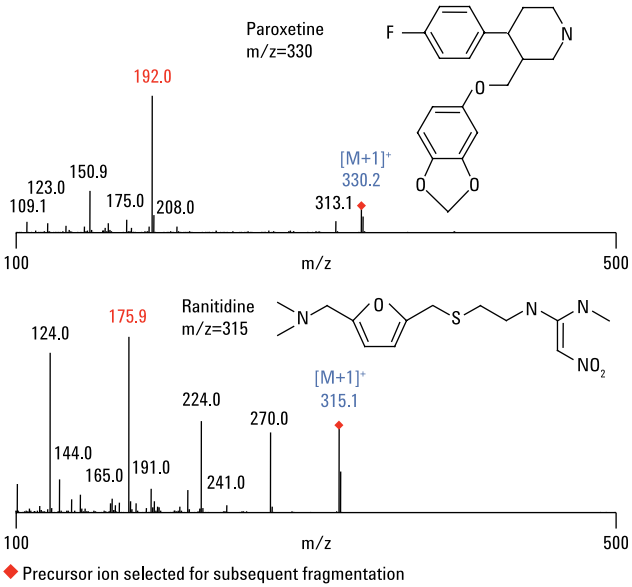
***Figure 51.*** *LC/MS/MS separation of paroxetine and ranitidine on ZORBAX Eclipse XDB-C18 column (reversed-phase HPLC mode)*




**Conditions**

| | |
|---|---|
| Column: | ZORBAX Rx-SIL, 2.1 x 150 mm, 5 µm |
| Gradient: | 100% B to 50% B in 10 min |

***Figure 52.*** *LC/MS/MS separation of paroxetine and ranitidine on ZORBAX Rx-Sil column (HILIC mode) – 100 ppb level*




**MS Conditions**

| | |
|---|---|
| Instrument: | Series 1100 LC/MSD Trap |
| Ionization: | Positive ESI |
| Scan range: | 100-500 m/z |
| SIM ions: | m/z = 315, 330 |
| Drying gas: | 10 L/min at 350 °C |
| Nebulizer gas: | 45 psi |
| Fragmentor voltage: | 0.25 V |

***Figure 53.*** *MS/MS spectra of drug standards*

## Normal phase chromatography

Normal phase or adsorption chromatography predates reversed-phase chromatography. The term 'normal' comes from the original idea that it was normal for the stationary phase to be polar, the mobile phase to be non-polar, and for the polar components to be more retained.

In normal phase chromatography, we use silica or another polar stationary phase, such as short-chained amines or diols. The mobile phase is non-polar – usually hydrocarbons, dichloromethane, ethyl acetate, or another water-immiscible solvent. In normal phase chromatography, the polar components are more retained. Retention decreases as polarity of mobile phase increases. If you have a more polar mobile phase, the analytes will come off much faster. For normal phase gradient separations with silica columns, you can start with a non-polar solvent such as hexane and then introduce a polar solvent such as ethyl acetate, i.e. 5% to 95% ethyl acetate. Depending on where the analyte(s) of interest elute, the gradient can be modified to improve resolution of all components. Sometimes a controlled amount of water or a small amount of isopropanol is added to moderate the surface activity of the silica gel packing. Bonded phase columns may not need water present, and often an alcohol is used as a modifier. Consult solvent tables for UV cutoffs if your detector is a UV or fluorescence type. UV cutoffs for some of the most common solvents can be found in reference section of this book, p. 110.

One of the reasons we use normal phase chromatography is to get more polar components to be retained. This mode can also be used to elute hydrophobic compounds which would be highly retained in reversed-phase chromatography.

Normal phase chromatography has a number of other uses. It is good for separating geometric and positional isomers. It allows for more discrimination than we find with reversed-phase chromatography. We can use normal phase chromatography when we do not have a water-miscible solvent. It's also used in preparative separations where the mobile phase doesn't contain water and is easy to evaporate.

Summary: Normal phase

- Column packing is polar: silica (strongest)>amino>diol>cyano (weakest)
- Mobile phase is non-polar: hexane, iso-octane, methylene chloride, ethyl acetate, etc.
- Polar compounds are more retained
- Retention decreases as polarity of mobile phase increases

Choose normal phase for:

- Resolution of strongly-retained hydrophobic samples
- Isomer separations
- Sample injection solvents that are non-polar and/or not water miscible.
- Recovery in non-polar solvents

**Ion-exchange chromatography**

In ion-exchange chromatography, ionic and ionizable compounds can be separated. In this mode, we use packing materials containing ionic functional groups with a charge opposite of the analytes. In strong cation-exchange (SCX) chromatography we would be analyzing positively charged molecules or cations, so we would use an anionic, or negatively charged stationary phase. If we were analyzing negatively charged molecules or anions, we would use a cationic or positively charged stationary phase.

For this technique, the mobile phase is typically highly aqueous with some buffer or salts. Elution takes place by increasing the ionic strength (salt concentration) either in a continuous or step-wise gradient. It is commonly used for large biomolecule separations but is also useful for small molecule separations such as amino acids, inorganic cations and anions and ionizable compounds like amines or carboxylic acids.

In the example in Figure 54, we are separating proteins, which due to their dual ionic functionality (both positive and negative charges are present), functionality, certainly can take on a charge. Depending on the net charge, proteins and peptides may be separated on cation- or anion-exchange columns.

Summary for ion-exchange:

- Column packing contains ionic groups (e.g. sulfonate, tetraalkylammonium)
- Mobile phase is an aqueous buffer (e.g. phosphate, formate, TRIS, etc.)
- Used less frequently than reversed-phase chromatography
- Similarities to ion-pair chromatography (see glossary for more information)

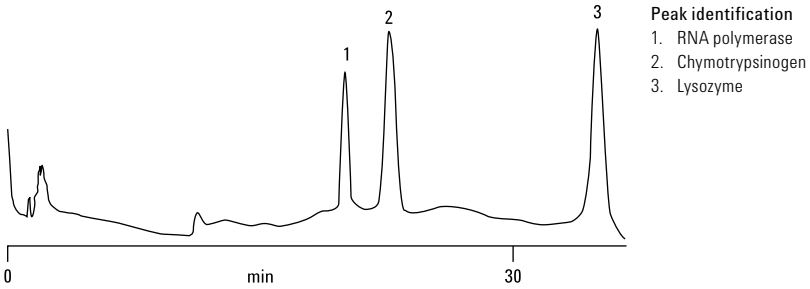


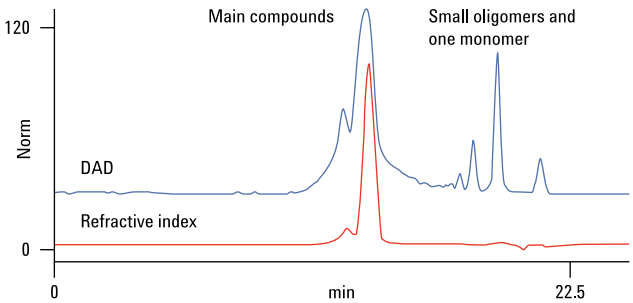

***Figure 54.*** *Basic proteins on strong cation-exchanger (-$SO_3$)*

**Gel permeation chromatography/size exclusion chromatography**

In GPC/SEC, there should be no interaction between the sample compounds and packing material. Molecules diffuse into the pores of a porous polymeric or silica medium. They are separated depending on their size relative to the pore size. Molecules larger than the pore opening do not diffuse into the particles while molecules smaller than the pore opening enter the particle and may be separated. Different from reversed-phase chromatography, large molecules elute first, smaller molecules elute later.

In general, the larger molecules are excluded from the pores, so they elute from the column quickly, in the total exclusion volume and the smallest molecules can penetrate all pores in the column and elute last, in the 'total permeation volume'. All other molecules elute in between and are therefore separated by size. If we want to estimate a molecular weight (MW) value for the individual components or the overall distribution, a calibration of log MW versus elution volume is constructed using standards of a known MW. Then, the MW and MW distribution of a polymer can be estimated by running the polymer sample under the same conditions as the standards.

The mobile phase is chosen mainly to dissolve the analyte.




Conditions

| | |
|---|---|
| Column: | PLgel MIXED-D |
| Mobile phase: | Tetrahydrofuran (THF) |

The monomer elutes after the polymer.

***Figure 55.*** *Gel permeation chromatogram of polybutadiene polymer on non-aqueous GPC/SEC column*

Summary for GPC/SEC:

- Two modes: non-aqueous GPC and aqueous SEC (also Gel Filtration Chromatography, or GFC)
- In size exclusion chromatography, there should be no interaction between the sample compounds and packing material. Molecules diffuse into pores of a porous medium. They are then separated depending on their size in solution relative to the pore size. Different solvents or mobile phases may result in different hydrodynamic radii.
- The mobile phase is chosen mainly to dissolve the analyte
- Used mainly for polymer characterizing, polymer molecular weight determination and for separating proteins

***Figure 56.*** *GPC columns and standards*

# Protecting your chromatographic results

## Reproducibility is one of the most highly prized qualities of chromatography. Getting good reproducibility starts with high quality columns and robust HPLC methods.

There are practices that can help you increase your reproducibility, and lengthen the life of your columns.

In this section, we'll talk about these practices in the order of the workflow:

- Sample preparation
- Using high quality solvents
- Special solvent considerations for UHPLC
- Inline filters
- Inlet frits
- Guard columns
- Solvent saturation columns

Next, we'll discuss how to care for columns:

- Maximizing column lifetime
- Unblocking columns
- Warning signs that your column is deteriorating

We'll wrap up the section with a few words about how to protect your method when it passes to other labs

- Ensuring method reproducibility around the world
- Changes in retention or selectivity from lot to lot

## Sample Preparation

Sample preparation is an essential part of HPLC analysis, intended to provide a representative, reproducible and homogenous solution that is suitable for injection into the column. The aim of sample preparation is to provide a sample aliquot that (a) is free of interferences, (b) will not damage the column and (c) is compatible with the intended HPLC separation and detection methods. It may be further desirable to concentrate the analytes and/or derivatize them for improved detection or better separation.

Sample preparation begins at the point of collection, extends to sample injection onto the HPLC column, and includes sample collection, transport, storage, preliminary processing, laboratory sampling and subsequent weighing/dilution, all form an important part of sample preparation. All of these steps in the HPLC assay can have a critical effect on the accuracy, precision, and convenience of the final method. This section will be devoted mainly to sample pre-treatment prior to injection into the column.

While there are some samples that require no special preparation prior to LC analysis, there are a variety of easy-to-use sample preparation products available. The table below provides a quick guide to some of these and the types of samples that may require these tools.

***Table 7.*** *Bond Elut sample preparation products overview*

| Technique | | Supported Liquid Extraction (SLE) | Precipitation/ Filtration | 'Smart' Filtration | Solid Phase Extraction |
|---|---|---|---|---|---|
| Interference | Dilute and shoot | Chem Elut | Captiva | Captiva ND Lipids | Bond Elut SPE |
| Particulates | No | No | Yes | Yes | Yes |
| Proteins | No | Partial | Yes | Yes | Yes |
| Lipids | No | No | No | Yes | Yes |
| Oligomeric Surfactants | No | No | No | Yes | Yes |
| Salts | No | Yes | No | No | Yes |

### Sample filtration

Why filter the sample? Because better performance requires better sample hygiene. Filtering your sample prevents the blocking of capillaries, frits, and the column inlet. If you take special care when preparing your samples beforehand, it will result in less wear and tear on your instruments. Taking careful action before analysis will result in less downtime for repair, and will also reduce the risks of contamination.

1. Sample filtering can extend column lifetime. Column frits can get clogged on smaller columns. As column particle size goes down, frit size decreases as well. A 5 µm or 3.5 µm column should have a 2 µm frit, a 2-3 µm column should have a 0.5 µm frit and a 1.8 µm column should have a 0.3-0.5 µm frit. The smaller the particle size, the more important sample filtration becomes.

2. Filtering the mobile phase will reduce wear on the instrument parts, such as check valves, piston seals, and autosampler plungers and needles. It also helps avoid plugging of capillary tubing.

3. Sample filtering helps to reduce contamination of the detector. For example, in MS detectors, solvent is evaporated away and non-volatiles and particulates are deposited.

**Solid Phase Extraction**

Solid Phase Extraction offers the highest degree of analyte selectivity and sample clean-up of any sample preparation technology. In its simplest form, SPE employs a small plastic disposable column or cartridge (see Figure 57), often the barrel of a medical syringe packed with an aliquot of functional sorbent. The sorbent is commonly a reversed-phase material, e.g., C18-silica, and will have very similar chemical properties to the associated HPLC phase. Although bonded-silica-based sorbents are the most popular, polymeric packings have become very popular in recent years, due to some nice end user benefits. Compared to silica-based SPE packings, polymeric packings have the advantage of higher surface area (thus higher capacity), chemical balance of hydrophilic-hydrophobic properties (better wetability and can dry out somewhat after the conditioning step without affecting recovery and reproducibility), absence of silanols (less chance of irreversible adsorption of highly basic compounds), and wide pH range (more flexibility in adjusting chemistries).

***Figure 57.*** *SPE cartridges and 96-well plate*

The large array of selectivities and formats available in SPE affords the analytical chemist a 'toolbox' approach to isolating an analyte or compound of interest. The SPE workflow is simple, with four key steps: precondition, load, wash and elute. Robust methods can be easily achieved with minimal method development and time.

SPE is also amenable to high sample throughput environments. Automation-friendly formats such as 96-well plates and functionalized pipette tips offer speed, flexibility and reproducible results.

SPE is used for five main purposes in sample preparation:

- Removal of matrix interferences and 'column killers'
- Concentration or trace enrichment of the analyte
- Desalting
- Solvent exchange (or solvent switching)
- In-situ derivatization

Because SPE is based around a bonded silica platform, many phases used in HPLC are also available in SPE versions. See the reference section for a table with SPE phases and conditions. In addition to the generic phases referenced there, specialty phases are available for a wide variety of specific applications, including: the isolation of drugs of abuse in urine, aldehydes and ketones from air, catecholamines from plasma and many other popular assays. SPE can also employ polar phases: florisil (activated magnesium silicate) and alumina are popular examples; many published methods exist for the isolation of pesticides using florisil. The use of graphitized carbon black has increased, especially for the removal of chlorophyll-containing plant extracts, a matrix interference that can cause significant decrease in column performance.

**Liquid-Liquid Extraction (LLE)**

Liquid-liquid extraction (LLE) is useful for separating analytes from interferences by partitioning the sample between two immiscible liquids or phases. One phase in LLE will usually be aqueous and the second phase an organic solvent. More hydrophilic compounds prefer the polar aqueous phase, while more hydrophobic compounds will be found mainly in the organic solvent. Analytes extracted into the organic phase are easily recovered by evaporation of the solvent, while analytes extracted into the aqueous phase often can be injected directly onto a reversed-phase HPLC column. The following discussion assumes that an analyte is preferentially concentrated into the organic phase, but similar approaches are used when the analyte is extracted into an aqueous phase.

Since extraction is an equilibrium process with limited efficiency, significant amounts of the analyte can remain in both phases. Chemical equilibria involving changes in pH, ion-pairing, complexation, etc. can be used to enhance analyte recovery and/or the elimination of interferences.

The LLE organic solvent is chosen for the following characteristics:

- A low solubility in water (<10%).
- Volatility for easy removal and concentration after extraction.
- Compatibility with the HPLC detection technique to be used for analysis (avoid solvents that are strongly UV-absorbing).
- Polarity and hydrogen-bonding properties that enhance recovery of the analytes in the organic phase
- High purity to minimize sample contamination

One of the main problems that can occur when one is using two dissimilar phases is the formation of emulsions. So instead of settling out into two layers, the two immiscible phases stay in a suspended state. In order to avoid emulsions, Supported Liquid Extraction, or SLE, can be used.

**Supported Liquid Extraction (SLE)**

Instead of using a separatory funnel to perform LLE, one can immobilize one liquid phase in an inert medium packed into a polypropylene tube and percolate the other immiscible liquid phase through the immobilized liquid in a manner similar to chromatography. The most frequently used inert material is high-purity diatomaceous earth with a high surface area and high capacity for aqueous adsorption. The process is termed solid-supported liquid-liquid extraction or supported liquid extraction (SLE), and is a popular alternative to the classical LLE experiment. In practice, the aqueous phase, which could be diluted plasma, urine or even milk, is coated onto the diatomaceous earth and allowed to disperse for a period of time, usually a few minutes. The aqueous sample spreads over the hydrophilic surface of the diatomaceous earth in a very thin layer. Next, the immiscible organic solvent is added to the top of the tube and comes in contact with the aqueous layer finely dispersed over the high surface area packing. Rapid extraction of analyte occurs during this intimate contact between the two immiscible phases. The solvent moves through the packing by gravity flow or by use of a gentle vacuum.

The tubes used in SLE resemble SPE cartridges and their volumes can range from 0.3- to 300 mL. Some SLE tubes are pre-buffered for extracting acidic and basic substances, respectively. For example, at low pH, acids will be in their unionized form and thus will be extractable from the immobilized aqueous phase. At high pH, amines will be in their neutral form and thereby be extracted into the organic phase. It is possible to add salt to the aqueous sample so that a 'salting out' effect occurs thereby leading to better extraction efficiency of certain analytes. The SLE tubes can also be used to remove small amounts of water from organic samples.

There is no vigorous shaking as in conventional LLE, so there is no possibility of emulsion formation. Since the packed tubes are considered disposable, there is no glassware to be cleaned after use. The entire process is amenable to automation and packed 96-well plates with several hundred milligrams of packing in each well are readily available to perform this task. The 96-well plates are suitable for extraction of 150- to 200 µL of aqueous sample and thus miniaturize the conventional LLE experiment as well. An example of a commercial product that performs SLE is Agilent's Chem Elut (Santa Clara, CA),

**QuEChERS**

QuEChERS (pronounced 'Catchers') stands for Quick Easy, Cheap, Effective, Rugged and Safe – all qualities that describe this sample prep method for food. In a few simple steps, you can prepare your food samples for multi-class, multi-residue pesticide analysis. With QuEChERS, samples are prepared by mixing them with extraction salts that separate out the matrices which need to be analyzed from the interferences. Kits are available with pre-measured, pre-weighed salts for standard sample sizes.

The original QuEChERS method is non-buffered, and was developed by M. Anastassiades, S.J. Lehotay, D. Stanjnbaher and F. J. Schenck in 2003 and published in the Journal of AOAC.

Later, refinements were made to ensure efficient extraction of pH dependent compounds, to minimize degradation of susceptible compounds (e.g. base and acid labile pesticides) and to expand the spectrum of matrices covered.

Today there are two commonly used buffered methods: A European standard (EN 15662) available from individual country members of the CEN http://www.cen.eu/research and a standard recognized by the Association of Analytical Communities (AOAC 2007.01), used in the US and other countries. Members have access to the method http://www.aoac.org.

The versatility of QuEChERS has been demonstrated by its acceptance outside of its traditional application areas.

***Figure 58.*** *Agilent QuEChERS kits, pre-weighed for the sample size and method*

Some emerging applications include:

- The extraction of veterinary drugs in animal tissue (such as kidney and chicken muscle)
- The extraction of environmental compounds in soil
- Non-pesticide extraction of analytes such as antibiotics, acrylamide, perfluorinated compounds, mycotoxins, PAHs, and alkaloids
- Matrices such as grains (barley and rice), nuts, dough, seeds, oils (soybean, peanut, olive, and cooking), chocolates, coffee, baby food, tobacco, and beverages (milk, wine, and water)

## The importance of using high-quality-grade solvents

In general, for HPLC applications, use only HPLC grade or better solvents. Filter all buffers. A 0.22 µm filter is preferred, especially for UHPLC applications, and a 0.45 µm filter can be used for standard HPLC applications. It is usually not necessary to filter HPLC grade or better organic solvents, and this practice can actually add contaminants if the filters and glassware used for filtration are not chromatographically clean.

Always remember to flush your column appropriately. At the end of the day, flush the buffers from the system and leave it in water/organic mobile phase. Use mobile phases that are miscible with your sample solvents (see the reference charts in the back of this guide for help). If you are only using water or up to 5% ethanol in your mobile phase A and you leave it sitting for a long period of time, bacteria will start to grow.

This can cause pressure problems and is difficult to eliminate, so be sure to put in fresh buffer every day, or at least every couple of days.

## Special considerations for UHPLC

Because the particles in high-efficiency columns are so small, the column needs to have a smaller frit on each end to contain them. And that frit is a filter that will trap particulates that enter your system, which can cause pressure increases. So keeping your system free of contaminants becomes even more important at the higher pressures of UHPLC applications.

For UHPLC, Agilent recommends Certified HPLC/MS grade solvents only. Be sure to check with your solvent provider for certification on the following:

- Low solvent and metal impurities, to reduce interference with minute or unknown samples
- Trace metal specifications should be very low – 5 ppb max is a good guide
- Positive mode and negative mode specifications
- Testing on LC-MS, and other QC test (the more QC testing, the better the solvent!)

Tips for solvent and buffer usage:

- Filter aqueous mobile phases (including buffers). Use 0.45 µm for standard HPLC and 0.2 µm for UHPLC.
- Buffers can increase your chance of clogging. Make sure the concentration used will not precipitate out in your solvent.
- When preparing mobile phase, it is important that the procedure be consistent and carefully followed, e.g., order of addition of solvents.
- Algae and bacteria can grow in aqueous mobile phases, especially in the pH 4-8 range. Replace buffers on a regular basis (every 2 days is good practice), always using a clean bottle.
- Use an amber bottle for aqueous mobile phases.

## Inline filters

Install an inline filter between your autosampler and column. This can catch particulates and keep them from traveling to the top of the column and plugging the frit. If you have a 3.5 µm column, a 2 µm frit is a good fit. For a 1.8 µm column use a 0.5 µm frit.

***Figure 59.*** *The 1290 Inline Filter installed in a 1290 Infinity LC*

### Low-volume inline filters

Filters are available for every column and provide column protection from particulate materials. An inline filter will increase analytical column lifetime by preventing particulates (from unfiltered samples and eluents, or both) from plugging the analytical column frit. Using guard columns can compromise the efficiency of very low-volume columns and columns with very small particle sizes. For these columns, low volume inline filters are strongly recommended.

The inline filter for the 1290 Infinity LC (PN 5067-4638) contains a 0.3 µm filter designed for low carryover. It can be used at up to 1200 bar pressure and has 1.3 µL dead volume. This same filter can be used on the 1260 and 1220 Infinity LC as well as the 1200 RRLC.

## Guard columns

Injecting dirty samples without a guard column can reduce the lifetime of the analytical column depending on the number of injections. Choosing to use guard columns is an economic decision the chromatographer makes, based on the number of injections typically run and the sample type.

Fit a guard column in the solvent line right before the analytical column.

The guard column prevents damage caused by particulate matter and strongly adsorbed material. To maintain an adequate capacity for sample impurities, choose a guard column with an internal diameter similar to the column internal diameter. Ideally, the packing of the guard column should be the same as the analytical column so that the chromatography of the analytical column is not altered. Guard columns contribute to the separation, so you should include a guard column inline during method development.

Judging when to replace a guard column can be difficult and best comes from experience. As a rough guide, if plate number, pressure or resolution change by more than 10%, the guard column probably needs replacing. You will need to make a judgment call on how often to replace your guard columns based on your application type. It is always preferable to change the guard column sooner rather than later.

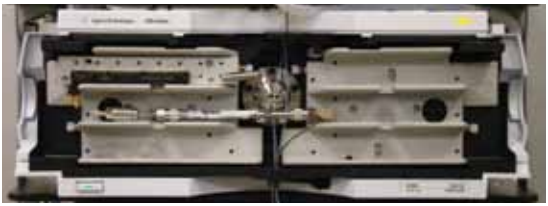


***Figure 60.*** *Fast Guard columns for UHPLC protect analytical columns*

## Solvent-saturation columns

A solvent- or silica-saturation column can be useful for protecting the analytical column if you use very harsh mobile phase conditions, such as pH above 7 and 40 °C, and buffer salt concentration over 50 mM. The solvent-saturator column is placed between the pump and injector, and releases silica as the mobile phase passes through it, saturating the mobile phase in the process. This prevents the dissolution of silica in the analytical column, prolonging its lifetime. Solvent-saturation columns can add to the dwell volume and delay gradient effects, which is a disadvantage when using gradient techniques. This additional dwell volume should be taken into account during method transfer to ensure reproducibility.

## Column inlet frits

If HPLC columns are used without a guard column or inline pre-column filter, the analytical column may become blocked. Due to the high efficiency packing processes used today replacing the column inlet frit is discouraged and may not be possible in many columns. Column efficiency may be compromised if the frit is replaced.

## Column care and storage

### Maximizing column lifetime

Modern columns are robust and are designed to operate for long periods under normal chromatographic conditions. You can maximize column lifetimes by running them within specifications. Always review the specifications before putting in place a final method.

| Tip | Additional info |
|---|---|
| Use a guard column and/or an inline 0.5 µm filter | See section on Guard columns, p. 90. |
| Frequently flush columns with a strong solvent | Use 100% B solvent. If you suspect a pressure build-up, use stronger solvents. See guidelines for cleaning columns on p. 42. |
| Pre-treat 'dirty' samples to minimize strongly retained components and particulates | Use solid phase extraction, liquid-liquid extraction, filtering sample through 0.45 µm filters or 0.22 µm filters for UHPLC, or high speed centrifugation. |
| Follow manufacturer specifications for maximum temperature limits for your column | Many newer methods call for higher temperatures, and some columns can withstand them; ideally, you should operate your column below the maximum temperature specified. |
| Use below the maximum pressure limit | Choose a flow rate that keeps your pressure below the maximum, ideally 10% below. |
| Operate the column in the direction marked on it. Check the column documentation or check with the manufacturer to determine if a column can be backflushed | Be sure to check with the manufacturer if you have questions. |
| Use a mobile phase between pH 2 and pH 7 for maximum column lifetime. | If you work outside of this pH range, use a StableBond column (for low pH) or a phase that is designed for high pH (e.g. Eclipse Extend-C18) or polymeric column. |
| Use fresh solvents, and avoid bacterial growth | If bacterial growth is a concern, make a stock solution of mobile phase and store it in the refrigerator, using only what you need daily. Sodium azide has been used to prevent bacterial growth, but due to carcinogenic properties, caution should be used. |
| When storing the column, purge out salts and buffers. Leave the column in pure acetonitrile, or a 50/50 blend with pure water and acetonitrile | This prevents precipitation of buffer salts in the column. Acetonitrile is a good solvent for storage because aqueous and alcohol mobile phases can increase the rate of stationary phase hydrolysis. |
| When using elevated temperatures always increase the column temperature gradually, and only with mobile phase running | After the analysis, leave the mobile phase running through the column until it reaches room temperature. |
| Take care not to over-tighten the end fittings of the column when attaching them to the instrument. Use short-handled wrenches/spanners to avoid excessive tightening of the end fittings | Since columns have 3/8 in. end nuts, a short 3/8 in. spanner or wrench should be used to attach the columns to the instrument to avoid any additional tightening of the end fittings. |

***Table 8.*** *Tips for extending column lifetime*

### Care in storage

To avoid potential metal corrosion, long-term storage of any column in halogenated solvents (for example, butyl chloride, methylene chloride, etc.) should be avoided. If the column has been used with a buffered mobile phase, the column should be purged with 20 to 30 column volumes of ACN and water followed by the same volume of the pure organic solvent. Leaving buffer in a column encourages growth of bacteria, which can block columns or frits, or lead to ghost peaks. Storage of unbonded silica columns in most other liquids is acceptable. Avoid storing in solvents that degrade easily, such as THF, TEA or TFA.

For overnight storage, rather than column shutdown, you can maintain mobile phase flow through the column at 0.1 to 0.2 mL/min. This will also reduce equilibration time the following day. For longer-term storage use the solvent that the column manufacturer recommends, often the solvent used to ship the new column.

### Unblocking a column

If backpressure increases and you suspect a blocked column, then it may be able to be backflushed. Disconnect the column from the detector and pump mobile phase through it in the reverse direction, if the manufacturer indicates it is safe. See p. 42, for more details about cleaning columns.

## Quickly determining when a column is going bad

In addition to keeping a sample chromatogram of your column's performance right out of the box (see p. 95), you can go back to the original essential chromatography concepts to evaluate when your column needs to be replaced:

| Parameter | Warning signs |
|---|---|
| Theoretical plates, N (efficiency) | Column voiding and column contamination over time will lead to reduced efficiency. Peak broadening is a sign of decreasing efficiency. By monitoring N, you can detect these problems. See p. 6 for more info. |
| Retention Factor, k | Retention Factor measures retention independent of flow rate and column dimension. Changes in k may indicate problems with loss of bonded phase or problems with column contamination due to non-eluting compounds. It could also be related to mobile phase changes that give a false impression of a column problem. See p. 7 for more info. |
| Selectivity, α | Shifts in selectivity are an additional indication, along with k, of problems with loss of bonded phase, column contamination or changes in mobile phase conditions. See p. 7 for more info. |
| Tailing factor, Tf | Tailing factor is a measurement of peak symmetry. An increase in tailing factor may indicate a problem with column voiding but may also result from an interaction between polar solutes and silanol sites, permitted by the loss of bonded phase. |
| Column backpressure, P | Increasing backpressure is almost always due to particulates clogging the column inlet frit. However, column voiding induced by column packing collapse can cause a large surge in pressure, too. See p. 9 for more info. |

***Table 9.*** *Parameters that help monitor column performance*

## Ensuring method reproducibility around the world

Different column histories often affect retention if a column is used to develop a method, and then replaced with another column that produces different results. The second column may not have been through similar conditions as the first column.

Insufficient or inconsistent equilibration may occur when a chromtographer develops a method and gives it to other chemists to reproduce without clear instructions about the equilibration. Each chemist may equilibrate their columns differently, which is likely to create some varation in results. Once they equilibrate their columns in the same manner as the development chemist, they will obtain the same results.

Other causes of retention change include poor column/mobile phase combinations, because the method may not be robust; changes in the mobile phase, flow rate, and other instrument issues; and slight changes in column-bed volume from one packed column to another.

### Increasing method robustness

There can be variations that are very compound-specific from lot to lot among different columns. There can also be changes in reagents from lot to lot, which will affect chromatographic results. When developing a method, good practice includes testing multiple lots and assessing that the conditions can accommodate slight changes from lot to lot. Manufacturers attempt to ensure reproducibility from lot to lot, but there are always going to be differences. Careful method development will help eliminate problems later on.

When evaluating lot-to-lot changes, first make sure that you have eliminated all column-to-column issues. Then check the robustness of your method. If you are working with ionizable compounds, make sure you have buffers and you are not working near the $pK_a$ of your analytes. In addition, check the pH sensitivity of sample and column, and secondary interactions. If you have determined that pH sensitivity is the problem, you may need to reevaluate your method.

In the example (Figure 61), lot-to-lot changes involve pH. A method was developed at pH 4.5 using lot 1. Recall that silanols become active for basic compounds around pH 4.5 (see more about pH and your method on p. 54). You can see two basic compounds with good peak shape. In lot 2, base 2 was shifted dramatically and the peak shape is not as sharp. Solutions to this problem could involve adding TEA or dropping the pH. In this case, the pH was lowered. Lot 1 at pH 3 changed the selectivity, but still produced good baseline separation. In addition, reproducibility between lot 1 and lot 2 at pH 3 was good. In this particular case, peak 4 reduced, but this was due to sample degradation.

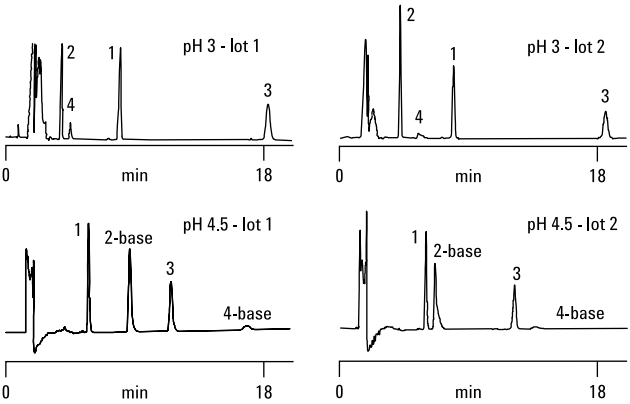



***Figure 61.*** *Lot-to-lot changes in retention due to pH levels*

In summary, for lot-to-lot retention changes.

- Eliminate all causes of column-to-column selectivity change
- Reevaluate method ruggedness and modify your method
- Determine pH sensitivity and, again, modify your method
- Classify different selectivity changes and contact your manufacturer

**A reminder about dwell volume implications**

Understanding dwell volume is very pertinent to the transfer of methods from laboratory-to-laboratory (see p. 32 for more informaton about dwell volumes). If the HPLC instrument used in one lab has a different dwell volume to an instrument in another lab and the same method is used in both labs, there is a strong possibility that the method will not perform exactly the same. The reason for this mismatch is that the gradient formed at the point of mixing of the two (or more) mobile phases will take a different amount of time in the flow path of the instruments to reach the head of the column. Thus, after injection the analytes will see a different mobile phase composition as a function of time and their retention and resolution may be affected. Therefore, the dwell volumes should be adjusted for by adding additional volume to the instrument whose dwell volume is smaller. Alternatively, the instrument whose dwell volume is larger can attempt to match the dwell volume of the smaller instrument by reducing connecting tubing id and lengths. Another 'trick' is to build a gradient delay into the method to compensate for the different residence time in the flow system. However, some validated methods may not allow this change to be used for analysis.

# Quick troubleshooting reference

## We know how it is. You'd like to read the entire book in one sitting, but who has the time?

Odds are, you're turning to this book because you have a question or issue. Therefore, we have a quick guide table with the most common issues our technical support team hears from chromatographers in the field, to help you quickly identify the potential cause of your trouble, and provide time-saving tips to help you address them. We refer you to other parts of this guide for more detail on specific issues.

### Tips for effective troubleshooting

One principle to remember is that you can only know if something is wrong if you first know what it looks like when it's right. So here are two practices to put into place in your lab to help you as a troubleshooter:

- **Run a test chromatogram with every new column** – Start by looking at the test chromatogram that is supplied with the column. Most of the time, the test components are easy-to-find chemicals that are common around the lab or can be purchased from chemical suppliers. Prepare the test sample (0.1 mg/mL of each is a good starting concentration) and run it on your instrument with your new column to compare. This initial test injection will help you identify if you have any system issues that prevent you from getting optimum results. Some people prefer to use their own sample, or standard, because the test mix may not be relevant to their application. It is best to employ isocratic conditions because sometimes a gradient may disguise poor column performance by 'compressing' peaks so that they look artificially sharper. Over time, comparing your own test chromatogram to this original chromatogram can help you evaluate whether your column has lost efficiency, or if there are other changes that affect performance. It's a good idea to quantitatively analyze parameters such as efficiency, selectivity, resolution and pressure, using the equations discussed on pp. 5 - 11. By understanding your column's comparative performance, you can begin to isolate a potential source of problems. Manufacturer's instruments for testing columns are optimized for column performance. You should expect a value lower than the plate number reported on the manufacturer's test chromatogram.
- **Keep a system map of your optimized instrument** – When you install your instrument and optimize your method, make a note of exactly how your instrument is stacked, part numbers and/or lengths of all connecting tubing and accessories and all electrical connections. This map is a handy reference if you have trouble, to ensure that no one has changed the configuration, thereby changing your results or instrument performance.

| Issue | Potential causes | Try this |
|---|---|---|
| **Pressure** | | |
| High backpressure (see pressure equation for more information, p. 9) | Plugged inlet frit on column | Backflush column (see p. 42) |
| | Column blockage (chemical contaminaiton) | Clean column with solvent or replace column if irreversible |
| | Column particle size too small | Review column selection (see p. 12) |
| | Plugged frit in inline filter or guard column | Inspect frits in filters and replace as necessary |
| | Blocked tubing | Remove tubing to confirm it is the culprit; replace as necessary |
| | Polymeric columns: solvent change causes swelling | Consult manufacturer's solvent compatibility info |
| | Mobile phase viscosity too high | Use lower viscosity solvent or higher temperature |
| | Salt/buffer precipitation | Ensure mobile phase compatibility with buffer |
| Fluctuating pressure | Bubble in pump | Degas the solvent (see p. 54); sparge solvent with helium or use inline degasser |
| | Leaking check valve or seals | Replace or clean check valve; replace pump seals |
| Decreasing pressure or low pressure | Insufficient flow from pump | Vent mobile phase reservoir, replace inlet line frit in reservoir; check for pinched tubing; check flow rate setting; look for leaks throughout system |
| | Leaking pump check valve or seals | Replace or clean check valves; replace pump seals; check for salt residues |
| | Pump cavitation | Degas solvent; check for obstruction in line from solvent reservoir to pump; replace inlet line frit |
| **Peak shapes** | | |
| No peaks | Instrument problem | Make sure all HPLC components are turned on and working; check to see if there is flow from detector exit tube; inject unretained compound to ensure system suitability; increase solvent strength or run gradient. |
| | Wrong mobile phase or stationary phase combination | |
| Extra peaks, or 'ghost' peaks | Analytes retained from a previous injection | Use a fast gradient, such as 10% ACN to 90% ACN in 10 - 15 min. to get a feel for the number of components in a sample and their relative retention. Start with a strong mobile phase, such as 75% MeOH and/or higher flow rate to get the components to come off the column more quickly. |
| | Mobile phase contamination | Use high purity HPLC grade or better (LC/MS or gradient grade) solvents only. Use high purity water from an in-house water purification system. Use TFA in the aqueous mobile phase solvent and TFA at a lower concentration in the organic mobile phase solvent (e.g. 0.1% TFA in water/0.086% in ACN); use a longer wavelength where TFA has poorer absorbance (see p. 59) |
| | Sample preparation/sample prep contamination | Use sample prep to reduce contamination in general - filtration, SPE, liquid-liquid extraction, centrifugation,etc |

***Table 10.*** *Quick Troubleshooting Tips*

*Continued on next page*

| Issue | Potential causes | Try this |
|---|---|---|
| Peak shapes, *continued* | | |
| Extra peaks, or 'ghost' peaks, *continued* | System contamination | Inject the sample solvent to ensure that there is nothing in the sample solvent that contributes to the issue; make a blank injection during the course of multiple runs to demonstrate that there are not ghost peaks present due to carryover. |
| | | Remove the autosampler from the flowpath and make a blank run to see if ghost peaks disappear. If so, clean the autosampler. If not, then work back through the flowpath to other system components to isolate the source. |
| | Column contamination (note: a less common cause of ghost peaks) | Backflush your column (if it is OK to do so; check manufacturer's information); clean your column (see p. 42) |
| Peak fronting | Channelling in column | Replace column; use guard columns |
| | Column overload | Use higher capacity column (increase length or diameter); decrease sample amount. |
| Peak tailing | Silanol interactions (silica-based columns) | Use endcapped or specialty columns; increase buffer concentraion; decrease mobile phase pH to suppress silanol interactions; use a competing base; derivatize solution to change polar interactions; if remedies fail, run in reverse direction; if better results are seen, column contamination is the likely cause; clean column or replace column. |
| | Extra-column effects | Check system for long tubing lengths between components and replace with shorter connection lengths; if using a high efficiency column, replace green 0.18 mm id tubing with red 0.12 mm id tubing. |
| | Degradation of column at high temperature (silica-based columns) | Reduce temperature to less than 40 °C, especially for high pH mobile phases; use high temperature compatible column such as sterically-protected silica, hybrid, polymeric, zirconia-based, etc. |
| | Degradation of column at high pH (silica columns) | Use column with high-coverage or bidentate phase specified for higher pH work (e.g. ZORBAX Extend-C18), or use polymeric, hybrid or zirconia-based reversed-phase column. |
| | Column void | Run in reverse direction; if poor peak shapes or peak doublets are seen for all peaks, a column void may be present; discard column. |
| | Interfering co-eluting peak | Improve selectivity by adjusting the mobile phase (see p. 53) or choosing a new stationary phase, improve sample clean-up. |
| Peak splitting/ doubling | Interfering component | Use sample prep to clean up sample; change mobile phase or stationary phase to adjust selectivity. |
| | | If component is suspected to be from previous injection, flush column with strong solvent at end of run; add gradient at higher solvent concentration; extend run time. |

*Continued on next page*

| Issue | Potential causes | Try this |
|---|---|---|
| **Peak shapes, *continued*** | | |
| Peak splitting/ doubling, *continued* | Partially plugged column frit | Backflush column (if it is OK to do so) (see p. 42; use 0.2 µm or 0.5 µm (UHPLC) inline filter between injector and column; filter samples, use guard columns (p. 90) |
| | Column void | Replace column; in future, use guard columns to protect analytical column (see p. 90); use less aggressive mobile phase conditions |
| | Injection solvent effects | Use mobile phase or weaker injection solvent (see p. 30-31) |
| | Sample volume overload | Use smaller sample injection volume (see p. 30) |
| | Sample solvent incompatible with mobile phase | Use mobile phase or weaker miscible solvent as injection solvent. |
| | Worn injector rotor | Replace injector rotor. |
| Peak broadening/wide peaks | Improper fittings/connections | Ensure your fittings are made correctly (see p. 29) |
| | Extra tubing volume on system | Ensure that the tubing is narrow and as short as possible, to avoid extra-column volume (see p. 26) |
| | Injection volume too large | Reduce injection volume (see p.30) |
| | System settings (e.g. data sampling rate too low for conditions) | Check data collection rate. Adjust the detector setting and/ or time constant to the fastest possible value that does not compromise signal-to-noise (see p. 31) |
| | Sample diluent strength too high | Reduce diluent strength (see p. 31) |
| | For gradients: dwell volume | Reduce initial gradient concentration, to focus peaks, or use injector programming to start the gradient before the sample injection is made (see p. 33) Check to see that the peak is eluting during the gradient, and not isocratically. |
| **Retention** | | |
| Retention time shifts | The column is getting old | Check chromatogram against test chromatogram (see p. 95) to compare and understand column changes; use guard columns to extend column life. |
| | Change in column dimensions or flow rate | Ensure that your method parameters are adjusted to reflect any change in flow rate or column dimension (see p. 21-22). This is especially important for gradients. |
| | Poor column/mobile phase combination for your analytes (poor retention for bonded phase, pH too close to $pK_a$, pH range of column not compatible with mobile phase etc.) | It's possible that your mobile phase is not consistent. Ensure that you prepare your mobile phase the same way every time – buffers and solvents should be measured separately in clean glassware, then mixed; degas mobile phase; replace aged mobile phase (see p. 63 - 66) |
| | For gradients: insufficient column re-equilibration | Measure your column void volume and system dwell volume and determine the optimal equilibration time for your method (see p. 40 and p. 67) |

*Continued on next page*

| Issue | Potential causes | Try this |
|---|---|---|
| Retention, *continued* | | |
| Decreasing retention | Active sites on column packing | Use mobile phase modifier, competing base (basic compound such as triethylamine) or increase buffer strength, use high coverage column packing |
| | Sample overload | Decrease sample amount or use larger diameter or longer column |
| | Loss of bonded stationary phase or base silica | Use mobile phase pH between 2 and 8; use specialty high pH or low-pH silica-based columns, polymers or other high/low pH column |
| | Column aging | Use guard column or high stability bonded phase polymeric, hybrid or high-stability column (e.g. zirconia, titania, graphitized carbon) |
| Increasing retention | Decreasing flow rate | Check and reset pump flow rate; check for pump cavitation; check for leaking pump seals, and faulty check valves and other system leaks. |
| | Changing mobile phase composition | Cover solvent reservoirs; ensure that gradient system is delivering correct composition; premix mobile phase for isocratic runs (see p. 53) |
| | Loss of bonded stationary phase | For regular silica-based columns, keep mobile phase pH between pH 2 and 8; use high-stability bonded phase, polymeric or high stability stationary phase columns for very high pH (>10) or very low pH (<2) work |
| Baselines | | |
| Drifting baseline | For gradients: absorbance of mobile phase A or B | For negative drift: use non-UV-absorbing mobile phase solvents; use HPLC-grade mobile phase solvents; add UV-absorbing additive in mobile phase A to mobile phase B to balance/compensate for drift. |
| | | For positive drift: use higher UV-absorbance detector wavelength where analytes can still be present; use non-UV-absorbing mobile phase solvents. Reduce amount of UV-absorbing compound added to mobile phase B to balance/compensate for drift. (p. 59) |
| | Wavy or undulating - temperature changes in room | Insulate column or use column oven; cover refractive index detector and keep it out of the air currents. |
| | Positive direction - LC/MS, stationary-phase bleed | Use low-bleed, MS-compatible or high-stability stationary phase column |
| | Positive direction - MS contamination | Clean MS interface, avoid THF and chlorinated solvents with PEEK tubing columns. |
| | Contaminated column (bleed from column) | Flush column with strong solvent; improve sample clean-up; use/replace guard column; replace analytical column. |
| | For continuous issues: look to detector lamp or flow cell. | Replace UV lamp; clean and flush flow cell. |

*Continued on next page*

| Issue | Potential causes | Try this |
|---|---|---|
| Baselines, *continued* | | |
| Drifting baseline, *continued* | For gradients or isocratic: lack of solvent mixing | Use proper mixing device; check proportioning precision by spiking one solvent with UV-absorbing compound and monitor UV-absorbance detector output. |
| | Gradient or isocratic proportioning - malfunctioning proportioning valves | Clean or replace proportioning values; partially premix solvents (e.g. 5% B in A and/or vice versa). |
| | Occasional sharp spikes - external electrical interference | Use voltage stabilizer/constant voltage power supply for LC system; check for local interfering sources such as cycling oven; use independent electrical current. |
| | Periodic - pump pulses | Service or replace pulse damper; purge air from pump; clean or replace check valves; degas mobile phase. |
| | Random - contamination build-up | Flush or backflush column (if it is OK to do so) with strong solvent (see p. 42); clean up sample; use HPLC-grade solvents. |
| | Spikes - bubbles in detector | Degas mobile phase (p. 54); use backpressure restrictor at detector outlet; ensure that all fittings are tight and not leaking (see p. 29.) |

# Useful references

## Here's another good reason you'll want to keep this book on hand.

We've included a few tables which are useful to reference for method development:

- USP designations
- Solvent properties table, including polarity index value and UV cutoffs
- Solvent miscibility table
- UV cutoffs for commonly used mobile phase modifiers
- Solid Phase Extraction (SPE) phase overview
- SPE sorbent usage conditions

### USP designations

The USP specifies columns by packing material rather than by manufacturer. In most categories several different types of columns are listed which differ in particle size, particle shape, and perhaps carbon load or surface area. In these cases, the USP column specifications are quite broad and several column types meet the basic specifications. For example, an L1 specification calls for octadecyl silane chemically bonded to porous silica or ceramic micro-particles, 1.5 to 10 µm in diameter, or a monolithic rod.

Many commercially available columns meet the basic specifications and may be different particle types, including totally porous, superficially porous and monoliths. However, not all C18 columns are alike, and only a limited number of the available C18 columns from any vendor will actually perform the desired separation. For increased efficiency and resolution, we recommend spherical particles, totally porous or superficially porous from 1.5-3.5 µm in diameter. The USP specifically gives you the flexibility to determine the best column for your needs (Table 11).

## USP designations (12/2010)

| USP Method | USP Packing Materials | Column | Particle Size (µm) | Pore Size (Å) |
|---|---|---|---|---|
| L1 | Octadecyl silane chemically bonded to porous silica or ceramic micro-particles, 1.5 to 10 µm in diameter, or a monolithic rod | Poroshell 120 EC-C18 | 2.7 | 120 |
| | | Poroshell 120 SB-C18 | 2.7 | 120 |
| | | ZORBAX Eclipse Plus C18 | 1.8, 3.5, 5 | 95 |
| | | ZORBAX Eclipse XDB-C18 | 1.8, 3.5, 5, 7 | 80 |
| | | ZORBAX SB-C18 | 1.8, 3.5, 5, 7 | 80, 300 |
| | | ZORBAX Rx-C18 | 3.5, 5 | 80 |
| | | ZORBAX Extend-C18 | 1.8, 3.5, 5, 7 | 80, 300 |
| | | ZORBAX ODS | 3.5, 5, 7 | 70 |
| | | ZORBAX ODS Classic | 5 | 70 |
| | | Pursuit XRs C18 | 3, 5, 10 | 100 |
| | | Pursuit C18 | 3, 5, 10 | 200 |
| | | Polaris C18-A | 3, 5, 10 | 180 |
| | | Polaris C18-Ether | 3, 5 | 200 |
| | | SepTech ST60 C18 | 10 | 60 |
| | | SepTech ST150 C18 | 10 | 150 |
| L3 | Porous silica particles, 5 to 10 µm in diameter, or a monolithic silica rod | ZORBAX SIL | 5 | 70 |
| | | ZORBAX Rx-Sil | 3.5, 5 | 80, 300 |
| | | Pursuit XRs Si | 3, 5, 10 | 100 |
| | | Polaris Si-A | 5, 10 | 180 |
| L7 | Octylsilane chemically bonded to totally porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod | Poroshell 120 EC-C8 | 2.7 | 120 |
| | | Poroshell 120 SB-C8 | 2.7 | 120 |
| | | ZORBAX Eclipse Plus C8 | 1.8, 3.5, 5 | 95 |
| | | ZORBAX Eclipse XDB-C8 | 1.8, 3.5, 5, 7 | 80 |
| | | ZORBAX SB-C8 | 1.8, 3.5, 5, 7 | 80, 300 |
| | | ZORBAX Rx-C8 | 1.8, 3.5, 5, 7 | 80 |
| | | ZORBAX C8 | 5 | 70 |
| | | Pursuit XRs C8 | 3, 5, 10 | 100 |
| | | Pursuit C8 | 3, 5, 10 | 200 |
| | | Polaris C8-A | 3, 5 | 180 |
| | | Polaris C8-Ether | 3, 5 | 200 |

***Table 11.*** *USP designations and Agilent columns*

*Continued on next page*

## USP designations (12/2010)

| USP Method | USP Packing Materials | Column | Particle Size (µm) | Pore Size (Å) |
|---|---|---|---|---|
| L8 | An essentially monomolecular layer of aminopropylsilane chemically bonded to totally porous silica gel support, 3 to 10 µm in diameter | ZORBAX NH2 | 5 | 70 |
| | | Polaris NH2 | 5 | 180 |
| L9 | Irregular or spherical, totally porous silica gel having a chemically bonded, strongly acidic cation-exchange coating, 3 to 10 µm in diameter | ZORBAX SCX | 5 spherical | 300 |
| L10 | Nitrile groups chemically bonded to porous silica particles, 3 to 10 µm in diameter | ZORBAX CN | 5 | 70 |
| | | ZORBAX SB-CN | 3.5, 5, 7 | 80, 300 |
| | | ZORBAX Eclipse XDB-CN | 3.5, 5 | 80 |
| L11 | Phenyl groups chemically bonded to porous silica particles, 1.5 to 10 µm in diameter | ZORBAX Eclipse XDB Phenyl | 5 | 70 |
| | | ZORBAX Eclipse Plus Phenyl-Hexyl | 1.8, 3.5, 5 | 95 |
| | | ZORBAX SB-Phenyl | 3.5 | 80 |
| | | ZORBAX 300-Diphenyl | 1.8 | 300 |
| | | Poroshell 120 Phenyl-Hexyl | 2.7 | 120 |
| | | Pursuit XRs Diphenyl | 3, 5, 10 | 100 |
| | | Pursuit Diphenyl | 3, 5, 10 | 200 |
| L13 | Trimethylsilane chemically bonded to porous silica particles, 3 to 10 µm in diameter | ZORBAX TMS | 5 | 70 |
| L14 | Silica gel having a chemically bonded, strongly basic quaternary ammonium anion-exchange coating, 5 to 10 µm in diameter | ZORBAX SAX | 5 | 70 |
| | | IonoSpher A | 5 | 120 |
| L17 | Strong cation-exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the hydrogen form, 7 to 11 µm in diameter | Hi-Plex H | 8 | N/A |
| L19 | Strong cation-exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the calcium form, 9 µm in diameter | Hi-Plex Ca | 8 | N/A |
| | | Hi-Plex Ca (Duo) | 8 | N/A |
| L20 | Dihydroxypropane groups chemically bonded to porous silica particles, 3 to 10 µm in diameter | LiChrospher Diol | 5 | N/A |

*Continued on next page*

## USP designations (12/2010)

| USP Method | USP Packing Materials | Column | Particle Size (µm) | Pore Size (Å) |
|---|---|---|---|---|
| L21 | A rigid spherical styrene-divinylbenzene copolymer, 5 to 10 µm in diameter | PLRP-S | 3, 5, 8, 10, 10-15, 15-20, 50 | 100 |
| | | PLRP-S | 3, 5, 8, 10, 10-15, 15-20, 50 | 300 |
| | | PLRP-S | 5, 8, 10, 30, 50 | 1000 |
| | | PLRP-S | 5, 8, 10, 30, 50 | 4000 |
| | | PLgel | 3, 5, 10, 20 | 50, 100, 500, 103, 104, 105, 106, MIXED |
| L22 | A cation-exchange resin made of porous polystyrene gel with sulfonic acid groups, about 10 µm in size | Hi-Plex H | 8 | N/A |
| L25 | Packing having the capacity to separate compounds with a MW range from 1,000 to 5,000 da (as determined by the polyethylene oxide), applied to neutral, anionic and cationic water-soluble polymers. A polymethacrylate resin base, cross-linked with polyhydroxylated ether (surface contained some residual carboxyl functional gruops) was found suitable | PL aquagel-OH | 5, 8 | 30 |
| L33 | Packing having the capacity to separate dextrons by molecular size over a range of 4,000 to 500,000 da. It is spherical, silica-based, and processed to provide pH stability | ZORBAX GF-250 | 4 | 150 |
| | | Bio SEC-3 | 3 | 100, 150, 300 |
| | | Bio SEC-5 | 5 | 100, 150, 300, 500, 1000, 2000 |
| | | ProSEC | 5 | 300 |

*Continued on next page*

## USP designations (12/2010)

| USP Method | USP Packing Materials | Column | Particle Size (µm) | Pore Size (Å) |
|---|---|---|---|---|
| L34 | Strong cation-exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the lead form, about 9 µm in diameter | Hi-Plex Pb | 8 | N/A |
| L35 | A zirconium-stabilized spherical silica packing with a hydrophilic (diol-type) molecular monolayer bonded phase having a pore size of 150 Å | ZORBAX GF-250 | 4 | 150 |
| | | ZORBAX GF-450 | 6 | 300 |
| L43 | Pentafluorophenyl groups chemically bonded to silica particles by a propyl spacer, 5 to 10 µm in diameter | Pursuit PFP | 3, 5 | 200 |
| L45 | Beta cyclodextrin bonded to porous silica particles, 5 to 10 µm in diameter | ChiraDex Chiral | 5 | 100 |
| L50 | Multifunction resin with reversed-phase retention and strong anion exhange functionalities. The resin consists of ethylvinylbenzene, 55% cross-linked with divinylbenzene copolymer, 3 to 15 µm in diameter, and a surface area of not less than 350 $m^2$ per g. Substrate is coated with quarternary ammonium functionalized latex particles consisting of styrene cross-linked with divinylbenzene. | ZORBAX 300SCX | 5 | 300 |
| L52 | Weak cation-exchange resin made of porous silica with sulfopropyl groups, 5 to 10 µm in diameter | IonSpher C | 5 | 120 |
| L53 | Weak cation-exchange resin consisting of ethylvinylbenzene, 55% cross-linked with divinylbenzene copolymer, 3 to 15 µm diameter. Substrate is surface grafted with carboxylic acid and/or phosphoric acid functionalized monomers. Capacity not less than 400 µEq/column | Bio SAX | 3, 5, 10 | 300 |
| L56 | Propyl silane chemically bonded to totally porous silica particles, 3 to 10 µm in diameter | ZORBAX SB-C3 | 1.8, 3.5, 7 | 80, 300 |
| L57 | A chiral-recognition protein, ovomucoid, chemically bonded to silica particles, about 5 µm in diameter, with a pore size of 120Å | Ultron ES-OVM | 5 | 120 |

*Continued on next page*

## USP designations (12/2010)

| USP Method | USP Packing Materials | Column | Particle Size (µm) | Pore Size (Å) |
|---|---|---|---|---|
| L58 | Strong cation-exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the sodium form, about 6 to 30 µm in diameter | Hi-Plex Na | 10 | N/A |
| | | Hi-Plex Na (Octo) | 8 | N/A |
| L60 | Spherical, porous silica gel, 10 µm in diameter, the surface of which has been covalently modified with alkyl amide groups and endcapped | Bonus-RP | 1.8, 2.1, 3.0, 3.5, 4.6, 5 | 80 |
| | | Polaris Amide-C18 | 3, 5 | 180 |

## Solvent miscibility

For liquid chromatography, it is important to understand the miscibility of various solvents, because incompatible solvents in your system can cause erratic chromatographic results. Miscibility refers to the ability of a solution to mix with another in all proportions and become a new homogenous solution.

For organic compounds, the polarity of a solvent determines what type of compounds are able to be dissolved in it and with what other solvents or liquid compounds it is miscible. Generally, polar solvents dissolve polar compounds best and non-polar solvents dissolve non-polar compounds best: 'like dissolves like'. Water (strongly polar) and hexane (strongly non-polar), therefore are not miscible with each other and will quickly separate into two layers even after being shaken well.

For inorganic compounds, the length of the carbon chain often determines miscibility relative to members of the homologous series. For example, in the alcohols, ethanol has two carbon atoms and is miscible with water, whereas butanol has four carbon atoms and is not miscible with water.

## Properties for common HPLC solvents

| Solvent | Viscosity (cP at 20 °C) | Boiling Point (°C) | UV Cutoff (nm) | Polarity Index (P′) | Miscibility Number (M) |
|---|---|---|---|---|---|
| Acetone | 0.36 | 56.29 | 330 | 5.1 | 15. 17 |
| Acetonitrile | 0.38* | 81.60 | 190 | 5.8 | 11, 17 |
| *n*-Butyl Acetate | 0.74 | 126.11 | 254 | 4.0 | 22 |
| *n*-Butyl Alcohol | 2.98 | 117.50 | 215 | 3.9 | 15 |
| *n*-Butyl Chloride | 0.45 | 78.44 | 220 | 1.0 | |
| Chlorobenzene | 0.80 | 131.69 | 287 | 2.7 | 21 |
| Chloroform | 0.57 | 61.15 | 245 | 4.1 | 19 |
| Cyclohexane | 1.00 | 80.72 | 200 | 0.2 | 28 |
| Cyclopentane | 0.44 | 49.26 | 198 | 0.1 | |
| *o*-Dichlorobenzene | 1.32** | 180.48 | 295 | 2.7 | |
| Dichloromethane | 0.44 | 39.75 | 233 | 3.1 | 20 |
| Dimethyl Acetamide | 2.14 | 166.10 | 268 | 6.5 | |
| N,N-Dimethylformamide | 0.92 | 153.00 | 268 | 6.4 | 12 |
| Dimethyl Sulfoxide | 2.24 | 189.00 | 268 | 7.2 | 9 |
| 1,4-Dioxane | 1.37 | 101.32 | 215 | 4.8 | 17 |
| Ethyl Acetate | 0.45 | 77.11 | 256 | 4.4 | 19 |
| Ethyl Alcohol | 1.10 | 78.32 | 210 | | |
| Ethyl Ether | 0.24 | 34.55 | 215 | 2.8 | 23 |
| Ethylene Dichloride | 0.79 | 83.48 | 228 | 3.5 | |
| Heptane | 0.42 | 98.43 | 200 | 0.1 | 29 |

***Table 12.*** *Solvent Properties*

*Continued on next page*

## Properties for common HPLC solvents

| Solvent | Viscosity (cP at 20 °C) | Boiling Point (°C) | UV Cutoff (nm) | Polarity Index (P') | Miscibility Number (M) |
|---|---|---|---|---|---|
| Hexane | 0.31 | 68.70 | 195 | 0.1 | 29 |
| Iso-Octane | 0.50 | 99.24 | 215 | 0.1 | 29 |
| Isobutyl Alcohol | | 107.70 | 220 | 4.0 | 15 |
| Isopropyl Alcohol | 2.40 | 82.26 | 205 | 3.9 | 15 |
| Isopropyl Myristate | | 192.60 | | | |
| Methanol | 0.59 | 64.70 | 205 | 5.1 | 12 |
| Methyl *t*-Butyl Ether | 0.27 | 55.20 | 210 | 2.5 | |
| Methyl Ethyl Ketone | 0.43 | 79.64 | 329 | 4.7 | 17 |
| Methyl Isobutyl Ketone | 0.58 | 117.40 | 334 | 4.2 | |
| N-Methylpyrrolidone | 1.67** | 202.00 | 285 | 6.7 | |
| Pentane | 0.23 | 36.07 | 190 | 0.0 | |
| Petroleum Ether | | | | 0.1 | |
| *n*-Propyl Alcohol | 2.30 | 97.20 | 210 | 4.0 | |
| Propylene Carbonate | | 241.70 | 220 | 6.1 | |
| Pyridine | 0.95 | 115.25 | | 5.3 | 16 |
| Tetrahydrofuran | 0.55 | 66.00 | 212 | 4.0 | 17 |
| Toluene | 0.59 | 110.62 | 284 | 2.4 | 23 |
| 1,2,4-Trichlorobenzene | | 213.50 | 308 | | |
| Triethylamine | 0.36** | 89.50 | | | |
| Trifluoroacetic Acid | 0.93 | 71.80 | 210 | | |
| Water | 1.00 | 100.00 | 190 | 10.2 | |
| *o*-Xylene | 0.81 | 144.41 | 288 | 2.5 | |

* cP at 15 °C
** cP at 25 °C
Missing values indicate data was not available

Miscibility (M) number:

1. All pairs whose M number differs by 15 units or less are miscible in all proportions at 15 °C
2. Each pair whose M number difference is 16 has a critical solution temperature between 25 and 75 °C, generally about 50 °C
3. A difference of 17 or more corresponds to immiscibility or to a critical solution temperature above 75 °C

*Information in this chart supplied by Honeywell Burdick & Jackson, www.honeywell.com/contactbandj*

## Solvent miscibility table

| | Acetone | Acetonitrile (ACN) | *n*-Butyl Alcohol | Chloroform | Cyclohexane | Dichloromethane (DCM) | *N,N*- Dimethylformamide | Dimethyl Sulfoxide (DMSO) | 1,4 -Dioxane | Ethyl Acetate | Ethyl Alcohol | Ethyl Ether | Ethylene Dichloride | Heptane | Hexane | Iso-Octane | Isopropanol (IPA) | Methanol | Methyl *t*-Butyl Ether | Methyl Ethyl Ketone | Pentane | Tetrahydrofuran (THF) | Toluene | Water | *o*-Xylene |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Acetone | | | | | | | | | | | | | | | | | | | | | | | | | |
| Acetontrile (ACN) | | | | | ■ | | | | | | | | | ■ | ■ | ■ | | | | | ■ | | | | |
| *n*-Butyl Alcohol | | | | | | | | | | | | | | | | | | | | | | | | ■ | |
| Chloroform | | | | | | | | | | | | | | | | | | | | | | | | ■ | |
| Cyclohexane | | ■ | | | | | ■ | ■ | | | | | | | | | | ■ | | | | | | ■ | |
| Dichloromethane (DCM) | | | | | | | | | | | | | | | | | | | | | | | | ■ | |
| *N,N*- Dimethylformamide | | | | | ■ | | | | | | | | | ■ | ■ | ■ | | | | | ■ | | | | ■ |
| Dimethyl Sulfoxide (DMSO) | | | | | ■ | | | | | | | ■ | | ■ | ■ | ■ | | | | | ■ | | | | ■ |
| 1,4-Dioxane | | | | | | | | | | | | | | | | | | | | | | | | | |
| Ethyl Acetate | | | | | | | | | | | | | | | | | | | | | | | | ■ | |
| Ethyl Alcohol | | | | | | | | | | | | | | | | | | | | | | | | | |
| Ethyl Ether | | | | | | | | ■ | | | | | | | | | | | | | | | | ■ | |
| Ethylene Dichloride | | | | | | | | | | | | | | | | | | | | | | | | ■ | |
| Heptane | | ■ | | | | | ■ | ■ | | | | | | | | | | ■ | | | | | | ■ | |
| Hexane | | ■ | | | | | ■ | ■ | | | | | | | | | | ■ | | | | | | ■ | |
| Iso-Octane | | ■ | | | | | ■ | ■ | | | | | | | | | | ■ | | | | | | ■ | |
| Isopropanol (IPA) | | | | | | | | | | | | | | | | | | | | | | | | | |
| Methanol | | | | | ■ | | | | | | | | | ■ | ■ | ■ | | | | | ■ | | | | |
| Methyl *t*-butyl Ether | | | | | | | | | | | | | | | | | | | | | | | | ■ | |
| Methyl Ethyl Ketone | | | | | | | | | | | | | | | | | | | | | | | | ■ | |
| Pentane | | ■ | | | | | ■ | ■ | | | | | | | | | | ■ | | | | | | ■ | |
| Tetrahydrofuran (THF) | | | | | | | | | | | | | | | | | | | | | | | | | |
| Toluene | | | | | | | | | | | | | | | | | | | | | | | | ■ | |
| Water | | | ■ | ■ | ■ | ■ | | | | ■ | | ■ | ■ | ■ | ■ | ■ | | | ■ | ■ | ■ | | ■ | | ■ |
| *o*-Xylene | | | | | | | ■ | ■ | | | | | | | | | | | | | | | | ■ | |

■ Immiscible □ Miscible

***Table 13.*** *Solvent miscibility*

This chart classifies two solvents as miscible if they can be mixed together in all proportions without forming two separate phases. Information for this chart supplied by Honeywell Burdick & Jackson. Contact B&J with questions at www.honeywell.com/contactbandj.

## UV cutoffs for mobile phase modifiers

The selection of a mobile phase modifier is a balance among various requirements. Does it produce a stable pH solution within the safe range of the column, and appropriate to control the ionization (either by ion suppression, ion pairing or a combination) of the analytes? Does it not interfere significantly with the detector performance, including the disparate properties of volatility, MS ion suppression and UV transparency? The challenge today is to work with a smaller range of acceptable modifiers, often requiring the analyst to investigate a more diverse range of stationary phases (embedded polar, alkyl phenyl, fluorinated RP in addition to typical alkyl phases like C8 and C18) in order to enhance selectivity for specific analytes.

The chart below provides UV cutoffs for common HPLC mobile phase modifiers.

| | v/v | $pK_a$ at 25 °C | Max. pH Range | UV Cutoff (nm) |
|---|---|---|---|---|
| Trifluoroacetic acid (TFA) | 0.1% | 0.3 | | 210 |
| | 0.05% | 0.3 | | 210 |
| | 0.01% | 0.3 | | 210 |
| Phosphate, $pK_1$ | | 2.1 | 1.1. – 3.1 | <200 |
| Phosphate, $pK_2$ | | 7.2 | 5.2 – 8.2 | <200 |
| Phosphate, $pK_3$ | | 12.3 | 11.3 – 13.3 | <200 |
| Citrate, $pK_1$ | | 3.1 | 2.1 – 4.1 | 230 |
| Citrate, $pK_2$ | | 4.7 | 3.7 – 5.7 | 230 |
| Citrate, $pK_3$ | | 6.4 | 5.4 – 7.4 | 230 |
| Carbonate, $pK_1$ | | 6.1 | 5.1 - 7.1 | <200 |
| Carbonate, $pK_2$ | | 10.3 | 9.3 – 11.3 | <200 |
| Formate | | 3.8 | 2.8 – 4.8 | 210 (10 mM) |
| Acetic Acid (HAC) | 1.0% | 4.8 | 3.8 – 5.8 | 210 |
| Acetate | | 4.8 | 3.8 – 5.8 | 210 (10 mM) |
| Ammonia | | 9.2 | 8.2 – 10.2 | 200 (10 mM) |
| Borate | | 9.2 | 8.2 – 10.2 | n/a |
| Triethylamine (TEA) | | 10.8 | 9.8 – 11.8 | <200 |
| TRIS-HCl | | 8.3 | 7.3 - 9.3 | 205 (120 mM) |

Shaded area indicates buffers that are more commonly used in LC/MS applications.

***Table 14.*** *UV Cutoffs for Common Mobile Phase Modifiers*

## Solid Phase Extraction sorbents

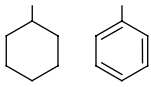
| Mechanism of Separation | Typical Phases | Structure(s) |
|---|---|---|
| Normal Phase (Adsorption) | Silica | Si-OH |
| | Alumina | AlOH |
| | Florisil | $Mg_2SiO_3$ |
| Normal Phase (polar bonded phase) | Cyano | -CN |
| | Amino | $-NH_2$ |
| | Diol | -CH(OH)-CH(OH)- |
| Reversed-phase (nonpolar bonded phase – strongly hydrophobic) | Octadecylsiloxane (C18) | $(-CH_2-)^{17}CH_3$, |
| | Octylsiloxane (C8) | $(-CH2-)_7CH_3$ |
| | PS-DVB | PS-DVB |
| | DVB (Polymeric) | DVB |
| Reversed-phase (nonpolar bonded phase – intermediate hydrophobicity) | Cyclohexyl | |
| | Phenyl |  |
| Reversed-phase (nonpolar bonded phase – low hydrophobicity) | Ethyl (C2) | $-C2H_5$ |
| | Methyl (C1) | $-CH_3$ |
| Polymeric Reversed-phase (Hydrophilically Modified) | Polyamide, | Various polymers |
| | hydroxylated DVB | Various polymers |
| | Methacrylate-DVB | Various polymers |
| WAX (Weak Anion-exchange) | Amino | $(-CH_2-)_3NH_2$ |
| | 1°, 2°-amino | $(-CH_2-)_3\ NHCH_2CH_2NH_2$ |
| SAX (Strong Anion-exchange) | Quaternary Amine | $(-CH_2-)_3N+(CH_3)_3$ |
| WCX (Weak Cation-exchange) | Carboxylic Acid | $(-CH2-)_3COOH$ |
| SCX (Strong Cation-exchange) | Alkyl Sulfonic acid | $(-CH2-)_3SO_3H$ |
| | Aromatic Sulfonic Acid | $SO_3H$ |

***Table 15.*** *Overview of Solid Phase Extraction (SPE) Phases*

## SPE sorbent conditions

| Mechanism of Separation | Use for analytes that are: | Loading Solvent | Eluting Solvent |
|---|---|---|---|
| Normal Phase (Adsorption) | Slightly to moderately polar | Low polarity (P'), e.g. hexane, $CHCl_3$ | High polarity (P'), e.g. methanol, ethanol |
| Normal Phase (polar bonded phase) | Moderately to strongly polar | Low P', e.g. hexane, $CHCl_3$ | High P', e.g. methanol, ethanol |
| Reversed-phase (nonpolar bonded phase-strongly hydrophobic) | Hydrophobic (strongly nonpolar) | High P', e.g. $H_2O$, $CH_3OH/H_2O$, $CH_3CN/H_2O$ | Low P', e.g. hexane, $CHCl_3$ |
| Reversed-phase (nonpolar bonded phase-intermediate hydrophobicity) | Moderately nonpolar | High P', e.g. $H_2O$, $CH_3OH/H_2O$, $CH_3CN/H_2O$ | Intermediate, e.g. Methylene chloride, ethyl acetate |
| Reversed-phase (nonpolar bonded phase-low hydrophobicity) | Slightly polar to moderately nonpolar | High P', e.g. $H_2O$ to moderate P', e.g ethyl acetate | High P', e.g acetonitrile, methanol |
| Polymeric Reversed-phase (Hydrophilically Modified) | Acidic, basic, neutral | Water or buffer | High P', e.g. acetonitrile, methanol |
| WAX (Weak Anion-exchange) | Ionic (ionizable), acidic | Water or buffer (pH = $pK_a + 2$) | A. Buffer (pH = $pK_a - 2$)<br>B. pH value where sorbent or analyte is neutral<br>C. Buffer with high ionic strength |
| SAX (Strong Anion-exchange) | Ionic (ionizable), acidic | Water or buffer (pH = $pK_a + 2$) | A. Buffer (pH = $pK_a - 2$)<br>B. pH value where analyte is neutral<br>C. Buffer with high ionic strength |
| WCX (Weak Cation-exchange) | Ionic (ionizable), basic | Water or buffer (pH = $pK_a - 2$) | A. Buffer (pH = $pK_a + 2$)<br>B. pH where sorbent or analyte is neutral<br>C. Buffer with high ionic strength |
| SCX (Strong Cation-exchange) | Ionic (ionizable), basic | Water or buffer (pH = $pK_a - 2$) | A. Buffer (pH = pKa + 2)<br>B. pH value where analyte is neutral<br>C. Buffer with high ionic strength |

***Table 16.*** *Guidelines for SPE Sorbent Conditions*

## Other suggested reading

Snyder, Lloyd R, Kirkland, Joseph J. and Dolan, John W., *Introduction to Modern Liquid Chromatography.* Third Edition, John Wiley & Sons Inc., 2010.

*An Introduction to Gel Permeation Chromatography and Size Exclusion Chromatography,* Agilent Technologies 2011, pub no. 5990-6969EN.

Meyer, Veronika R., *Practical High-Performance Liquid Chromatography.* Fourth Edition, John Wiley & Sons, Inc., 1999.

Snyder, Lloyd R. and Dolan, John W., *High-Performance Gradient Elution*, John Wiley & Sons, Inc., 2007.

Snyder, Lloyd R. Kirkland, Joseph J. and Glajch, Joseph L., *Practical HPLC Method Development,* John Wiley & Sons, Second Edition, 1997.

Cunico, Robert L.; Gooding, Karen M. and Wehr, Tim, *Basic HPLC and CE of Biomolecules*, Bay Bioanalytical Laboratory, Inc., 1998.

Neue, Uwe D., *HPLC Columns: Theory, Technology and Practice*, John Wiley & Sons, Inc., 1997.

## Other Agilent resources

Here are a few good places to go for more information:

| Resource | Details |
|---|---|
| LC Column & Sample Prep Navigator | An interactive online tool which recommends the best HPLC column and sample prep product for your application. **www.agilent.com/chem/navigator** |
| The LC Method Translator | This online tool helps you quickly factor for changes in column length, diameter, system flow rate, etc., and calculate adjustments to your methods. This can be especially helpful for adjusting gradient methods accurately. Go to **www.chem.agilent.com** and search for 'LC Method Translator' |
| The LC Flow Rate Calculator App | If you have a smart phone, you can get a free app for your phone that lets you quickly adjust your flow rate to accommodate other method changes. Find the app at **www.agilent.com/chem/lcapp** |
| Agilent CrossLab Selection Tool | Just by answering a few simple questions on this online tool we can recommend which supplies are best for your non-Agilent instruments. **www.agilent.com/chem/SelectCrosslab** |
| Vial Selector | With so many options to choose from, selecting the right vials, caps and septa for your application can be difficult and time consuming. Simplify the process by using this interactive tool. **www.agilent.com/chem/SelectVials** |
| Syringe Filter Online Selection Guide | This tool makes it fast and easy to choose the best syringe filter for your application. **www.agilent.com/chem/SelectFilters** |
| www.agilent.com/chem/lctroubleshooting | We have created a series of videos to illustrate some of the points in this guide. Over time, we'll be adding other videos. |

***Table 17.*** *Other Agilent Resources*

# Glossary

## A

***a:*** See *separation factor*.

***A solvent:*** Usually the weaker solvent in a binary eluent or gradient elution separation. In reversed-phase liquid chromatography (LC), the A solvent typically is water or a water-rich mixture.

***A term:*** The first term in the van Deemter equation. See *eddy dispersion term* and *van Deemter equation*.

***Adapter:*** A union with different threads on each end; generally used to connect two different types of tubing together.

***Absorption:*** The process of retention in which the solute partitions into a liquid-like coating.

***Activity:*** The relative strength of the surface of the packing in adsorption chromatography. For silica gel, the more available the silanol groups, the more active the surface. Activity can be controlled by adding water or other polar modifier that hydrogen bonds to the active sites, thereby reducing the surface activity.

***Additive:*** A substance added to the mobile phase to improve the separation or detection characteristics; for example, a competing base to negate the effects of silanols, a chelating agent to block metal sites, or a UV-absorbing compound to perform indirect photometric detection.

***Adjusted retention time ($t_R'$):*** A measure of the retention time adjusted for the holdup time; $t_R' = t_R - t_M$, where $t_R$ is the retention time and $t_M$ is the holdup time (the time it takes for a small, unretained compound that completely permeates the pores to be eluted from the chromatographic column).

***Adjusted retention volume ($V_R'$):*** Adjusts the retention volume for the holdup volume; $V_R' = V_R - V_M$, where $V_R$ is the retention volume of the peak of interest and $V_M$ is the holdup volume (the volume corresponding to the total volume of mobile phase in the column). See also ***dead volume*** and ***holdup volume***.

***Adsorbent:*** Packing used in adsorption chromatography. Silica gel and alumina are the most frequently used adsorbents in high performance liquid chromatography (HPLC).

***Adsorption:*** The process of retention in which the interactions between the solute and the surface of an adsorbent dominate. The forces can be strong forces (hydrogen bonds) or weak (van der Waals forces). For silica gel, the silanol group is the driving force for adsorption, and any solute functional group that can interact with this group can be retained on silica. The term adsorption places emphasis on the surface versus penetration or embedding in the stationary phase coated or bonded to a surface.

***Adsorption chromatography:*** One of the basic LC modes that relies upon adsorption to the surface of an active solid to effect the separation. Silica gel and alumina are the most frequently used normal phase adsorbents, and molecules are retained by the interaction of their polar function groups with the surface functional groups; for example, silanols of silica. Carbon is also used as an adsorbent in a reversed-phase mode.

***Adsorption isotherm:*** A plot of the equilibrium concentration of sample in the mobile phase per unit volume versus the concentration in the stationary phase per unit weight in adsorption chromatography. The shape of the adsorption isotherm can determine the chromatographic behavior of the solute; for example, peak tailing, peak fronting, and column overload.

***Aerogel:*** A packing prepared when the dispersing agent is removed from a gel system without collapsing the gel structure. Silica gels and glass beads used for size exclusion chromatography (SEC) are examples of aerogels that can retain their structures even at the high pressures used in HPLC. See also ***xerogels***.

***Affinity chromatography:*** A technique in which a biospecific adsorbent is prepared by coupling a specific ligand – such as an enzyme, antigen, or hormone – for the macromolecule of interest to a solid support (or carrier). This immobilized ligand will interact only with molecules that can selectively bind to it. Molecules that will not bind will be eluted unretained. The retained compound later can be released in a purified state. Affinity chromatography is normally practiced as an on-off separation technique.

***Agarose:*** High molecular weight polysaccharide used as a separation medium in biochromatography. It is used in bead form, often in gel filtration chromatography, with aqueous mobile phases.

***Alkoxysilane:*** A reactant used for the preparation of chemically bonded phases. It will react with silica gel as follows: $R_3SiOR + \equiv SiOH \rightarrow \equiv Si\text{-}OSiR_3 + ROH$, where $R$ is an alkyl group.

***Alumina:*** A normal phase adsorbent used in adsorption chromatography. Aluminum oxide is a porous adsorbent

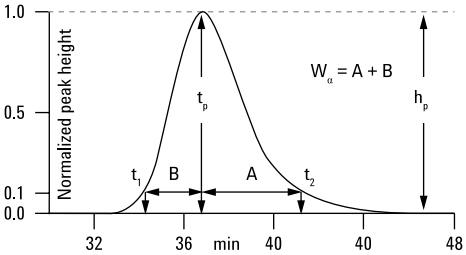



***Figure 62.*** *Example of a tailing peak (modified with permission from reference 3)*

that is available with a slightly basic surface; neutral and acidic modifications also can be made. Basic alumina can have advantages over silica, which is considered to have an acidic surface.

***Amino phase:*** A propylamino phase used in normal bonded-phase chromatography. It is somewhat reactive for solute molecules such as aldehydes or mobile-phase additives that can react with amines. The amino phase has found some applications as a weak anion-exchanger, and it also is used for the separation of carbohydrates with a water-acetonitrile mobile phase. It is a relatively unstable phase.

***Amphoteric ion-exchange resin:*** Ion-exchange resins that have both positive and negative ionic groups. These resins are most useful for ion retardation in which all ionic materials can be removed from solution because the anionic and cationic functionalities coexist on the same material.

***Analyte:*** The compound of interest to be analyzed by injection into and elution from an HPLC column.

***Analytical Column:*** An HPLC column used for qualitative and quantitative analysis; a typical analytical column will be 4.6 mm id x 50-250 cm in length but columns with smaller diameters (down to 0.05 mm id) can also be considered as analytical columns; can be constructed of stainless steel, glass, glass-lined SS, PEEK and other metallic and non-metallic materials.

***Anion-exchange:*** The ion-exchange procedure used for the separation of anions. Synthetic resins, bonded-phase silicas, and other metal oxides can be analyzed in this mode. A typical anion-exchange functional group is the tetraalkylammonium, which makes a strong anion-exchanger. An amino group on a bonded stationary phase is an example of a weak anion-exchanger.

***Asymmetry:*** Factor describing the shape of a chromatographic peak. Chromatographic theory assumes a Gaussian shape and that peaks are symmetrical. A quantitative measure is the peak asymmetry factor, which is the ratio of the distance from the peak apex to the back side of the chromatography curve over the distance from the peak apex to the front side of the chromatography curve at 10% of the peak height. Other measures of asymmetry are commonly used, especially the U.S. Pharmacopeia (USP) method. See Figure 62. See also *Foley-Dorsey equation*.

***Asymmetry factor:*** A factor that denotes band shape. The asymmetry factor is calculated from the chromatographic peak by dropping a perpendicular at the peak apex and a horizontal line at 10% of the peak height; at the intersection, the distance to the tail of the peak along the horizontal line (distance B) divided by the distance along the horizontal line to the front of the peak (distance A) produces a ratio called the peak asymmetry factor (see Figure 62). The ratio is 1 for a symmetrical peak, less than 1 for a fronting peak, and greater than 1 for a tailing peak. The higher the value, the less symmetrical the peak; values greater than 2 are unacceptable.

***Atmosphere (atm):*** A measure of the pressure drop across an HPLC column; *1 atm = 14.7 lb/in.$^2$ (psi)*. See also *bar* and *pascals*.

## B

***β:*** See *phase ratio*.

***$B_o$:*** See *permeability*.

***B solvent:*** Usually the stronger solvent in a binary eluent or gradient separation; typically the organic modifier or modifier-rich binary mixture with water in reversed-phase LC.

***B term:*** The second term of the van Deemter equation. See also *longitudinal diffusion* and *molecular diffusion term*.

***Backflushing:*** A column-switching technique in which a four-way valve placed between the injector and the column allows mobile phase flow in either direction. Backflushing is used to elute strongly held compounds at the head of a column. It can be used for analyzing these compounds or merely removing them from the column.

***Backpressure:*** Same as *head pressure*, *column pressure*.

***Backpressure regulator:*** A device placed online after the detector to maintain a positive pressure on the flow cell minimizing solvent outgassing problems in the detector.

***Band:*** Refers to the chromatographic peak as it moves down and is eluted from the column.

***Band broadening:*** The process of increasing width and concomitant diluting of the chromatographic band as it moves down the column. The peak is injected as a narrow slug and, ideally, each separated component

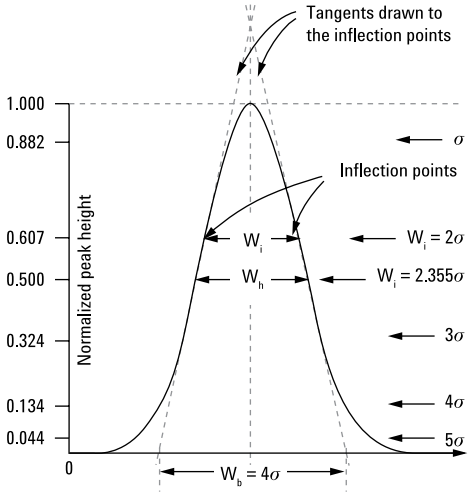



***Figure 63.*** *Widths of a Gaussian peak at various heights as a function of the standard deviation (σ) of the peak. (Modified with permission from reference 2).*

would be eluted as a narrow slug of pure compound if not for the process of band broadening. The measure of band broadening is bandwidth ($t_w$) or, more correctly, the number of theoretical plates ($N$) in the column. Sometimes called band dispersion or band spreading. See Figure 63.

***Bandwidth ($t_w$):*** The width of the chromatographic band during elution from the column. It usually is measured at the baseline by drawing tangents to the inflection points on the sides of the Gaussian curve that represents the peak. Small bandwidths usually represent efficient separations; also called peak width. See Figure 63.

***Bar:*** A unit of pressure measurement in HPLC equal to *1 atm, ~ 15 lb/in.*$^2$, or *0.1 MPa*.

***Baseline:*** The baseline is the line drawn by the recording device representing the signal from the detector when only mobile phase is passing through. It also represents the point from which calculations are often made on peaks to determine peak area or peak height.

***Baseline noise:*** Irregular variations (short term) in the chromatographic baseline due to electrical noise or temperature fluctuations, outgassing in the flow cell, or poorly mixed mobile phase solvents.

***BET method:*** Developed by Bruner, Emmett, and Teller (BET), a method for measuring surface area that uses nitrogen adsorption-condensation in pores at liquid nitrogen temperature. Pore volume and pore size distribution also can be obtained from BET method calculations.

***Bidentate silane:*** A specific type of bonded phase in which a short hydrocarbon bridge connects two silicon atoms in a silane that is bound to the surface through two siloxane groups.

***Binary mobile phase:*** Mobile phase comprising two solvents or buffers.

***Biocompatible:*** A term to indicate that the column or instrument component will not irreversibly or strongly adsorb or deactivate biomolecules such as proteins. Frequently means metal-free or ceramic surfaces and components.

***Bonded-phase chromatography:*** The most popular mode in LC in which a phase chemically bonded to a support is used for separation. The most popular support for bonded-phase chromatography is microparticulate silica gel, and the most popular type of bonded phase is organosilane such as octadecyl for reversed-phase chromatography. Approximately 70% of all HPLC applications are performed using chemically bonded phases.

***Bonded-phase concentration:*** See *coverage*.

***Boxcar chromatography:*** See *column switching*.

***Breakthrough volume:*** The volume at which a particular solute pumped continuously through a column will begin to be eluted. It is related to the column volume and the retention factor of the solute. It is useful to determine the total sample capacity of the column for a particular solute.

***Buffer:*** A solution that maintains constant pH by resisting changes in pH from dilution or addition of small amounts of acids and bases.

***Buffer capacity:*** A quantitative measure of the potential of a buffer solution (defined as the number of equivalents of strong acid or base to cause a one pH unit change in 1 L of a buffer solution) or simply the ability of a buffer to withstand injections of a buffered sample solution without changing mobile phase pH; capacity determined by pH, buffer $pK_a$, and buffer concentration.

***Buffer strength:*** See *ionic strength*.

## C

***C term:*** The interphase mass transfer term of the van Deemter equation. See also ***mass transfer*** and ***van Deemter equation***.

***C8:*** See *octylsilane*.

***C18:*** See *octadecylsilane*.

***C4, C8, C18, etc.:*** Refer to the alkyl chain length of a reversed bonded phase.

***$C_S$:*** See *Langmuir isotherm*.

***Capacity:*** See *sample capacity*.

***Capacity factor (k′):*** Old term for a chromatographic

parameter that measures the degree of retention. Now defined as the retention factor *(k)* by the *International Union of Pure and Applied Chemistry (IUPAC)*. See also *retention factor* for method of calculation.

***Capillary column:*** Refers to columns with inner diameters less than 0.5 mm.

***Capillary electrochromatography (CEC):*** A hybrid technique in which capillary columns are packed with chromatographic sorbents and electroosmotic flow rather than pressure moves mobile phase through the column; technique has the surface-mediated selectivity potential of HPLC and the high efficiency of capillary electrophoresis (CE).

***Capillary LC:*** Generally refers to HPLC performed in a fused-silica or other type of capillary column; the inner diameters typically are less than 0.5 mm; has also been called micro-LC.

***Capillary micellar electrochromatography:*** The CEC version of micellar electrokinetic capillary chromatography (MEKC).

***Capillary tubing:*** Tubing to connect various parts of a chromatograph and direct flow to the proper places. Most capillary tubing used in HPLC is less than 0.020 in. in inner diameter. The smallest useful inner diameter is approximately 0.004 in.

***Capping:*** Same as *endcapping*.

***Carbon load:*** For a bonded phase silica, term usually used to describe the surface coverage or the degree to which the available silanols on the column packing's surface have reacted and been replaced with the bonded phase; the higher the carbon load, the lower the number of residual silanols. The carbon load is normally expressed as a % carbon (e.g. 12% carbon). In reversed-phase LC, the higher the carbon load, the greater the analyte retention.

***Carrier:*** A term most often used in affinity chromatography; refers to the support that binds the active ligand, usually by a covalent bond; can also refer to the support in other chromatography modes such as liquid-liquid chromatography.

***Carrier gas:*** The mobile phase in gas chromatography (GC).

***Cartridge column:*** A column type that has no endfittings and is held in a cartridge holder. The column comprises a tube and packing contained by frits in each end of the tube. Cartridges are easy to change and are less expensive and more convenient than conventional columns with endfittings.

***Cation-exchange chromatography:*** The form of ion-exchange chromatography that uses resins or packings with functional groups that can separate cations. An example of a strong cation functional group would be a sulfonic acid; a weak cation-exchange functional group would be a carboxylic acid.

***CE:*** Capillary electrophoresis.

***CEC:*** See *capillary electrochromatography*.

***CGE:*** See *capillary gel electrophoresis*.

***CZE:*** See *capillary zone electrophoresis*.

***Chain length:*** The length of carbon chain in the hydrocarbon portion of a reversed-phase packing. It is expressed as the number of carbon atoms (C8, C18, etc.). It specifically excludes the short chains - typically methyl, isopropyl, and sec-butyl groups - that also are attached to the silane.

***Channeling:*** Occurs when voids created in the packing material cause mobile phase and accompanying solutes to move more rapidly than the average flow velocity, which in turn allows band broadening to occur. The voids are created by poor packing or erosion of the packed bed.

***Check valve:*** A device inserted into a moving liquid stream that allows flow of the stream in only one direction; most often used on the inlet and outlet sides of an HPLC pump.

***Chemisorption:*** Sorption caused by a chemical reaction with the packing. Most of these interactions are irreversible and usually occur on packings with reactive functional groups such as silanol or bonded amino phases. Chemisorption is common with metal oxide phases that have strong Lewis acid sites.

***Chiral recognition:*** The ability of a chiral stationary phase to interact differently with two enantiomers leading to their HPLC separation.

***Chiral stationary phases:*** Stationary phases that are designed to separate enantiomeric mixtures. The phases can be coated or bonded to solid supports, created in situ on the surface of the solid support, or exist as surface cavities that allow specific interactions with one enantiomeric form.

***Chlorosilane:*** A chemical reagent used to prepare siloxane bonded phases; reactivity changes from a monochlorosilane < dichlorosilane < trichlorosilane; the alkyl portion (octadecyl, octyl, etc.) will dictate the hydrophobicity of the resulting bonded phase; alkoxysilanes can be used but are less reactive.

***Chromatogram:*** A plot of detector signal output or sample concentration versus time or elution volume during the chromatographic process.

***Chromatograph:*** As a noun: a device used to implement a chromatographic separation. As a verb (IUPAC): the act of separating by elution through a chromatographic bed.

***Chromatographic conditions:*** Those chromatographic method experimental parameters that describe how an

analysis was performed. Sufficient information must be presented so that the analysis can be duplicated for verification purposes.

***Classification:*** The process of sizing column packing particles; generally in HPLC, small particle size distribution provides better efficiency and a greater permeability because of the absence of fines. Classification can be performed by sedimentation, elutriation, and centrifugalair techniques.

***Column backpressure:*** See *head pressure*.

***Column chromatography:*** Any form of chromatography that uses a column or tube to hold the stationary phase. Open-column chromatography, HPLC, and open-tubular capillary chromatography are all forms of column chromatography. Most often refers to open-column chromatography used for preparative scale work.

***Column dead time:*** The time associated with the dead volume; determined by the dead volume divided by the flow rate; in reversed-phase LC, uracil is often used to measure dead volume and dead times.

***Column length (L):*** The length of chromatography column in HPLC or capillary in CE used to perform the liquid phase separation.

***Column packing:*** The solid material, usually a porous solid with or without a chemically interactive surface, placed inside of the column used to differentially retain analytes; referred to as the stationary phase; common packings are unbonded and bonded silica, resins, inorganic-organic hybrids, graphtized carbon

***Column performance (N):*** Refers to the efficiency of a column; the number of theoretical plates for a given test compound.

***Column plate number (N):*** Denotes the column efficiency; the larger the plate number, the more theoretical plates the column possesses; a typical well-packed column with a *5-/m* $d_p$ porous packing in a 150 x 4.6 mm column should provide 10,000-12,000 plates.

***Column switching:*** Using multiple columns connected by switching valves for better chromatographic separations or sample clean-up. Fractions from a primary column can be switched to two or more secondary columns, which in turn can be further diverted to additional columns or to detectors; sometimes called multidimensional chromatography.

***Column volume ($V_c$):*** The volume of the unpacked column; $V_c = A_c L$, where $A_c$ and $L$ are the cross-sectional area of the tube and the tube length, respectively.

***Competing base:*** Adding a small basic compound such as triethylamine or dimethyloctylamine at 10-50 mM concentration to the mobile phase in reversed-phase chromatography to inhibit basic analytes from interacting with residual silanols; works by the law of mass action because concentration of competing base is much greater than analyte. See also ***additive***.

***Comprehensive two-dimensional chromatography:*** Two-dimensional chromatography applied to every fraction. See also *two-dimensional chromatography*.

***Controlled surface porosity support:*** Same as *porous layer bead* and *pellicular packing*.

***Counterion:*** The ion in solution used to displace the ion of interest from the ionic site in an ion-exchange process. In ion pairing, it is the ion of opposite charge added to the mobile phase to form a neutral ion pair in solution.

***Coupled columns:*** A form of column switching that uses a primary column connected to two secondary columns by a selector valve. Fractions from the first column can be selectively transferred to the second and third columns for additional separations. This term is also used to describe two or more columns connected in series to provide an increased number of plates.

***Coverage:*** Refers to the amount of bonded phase on a silica support in bonded-phase chromatography. Coverage usually is described in micromoles per square meter or in terms of percentage carbon *(w/w)*.

***Critical micelle concentration:*** The concentration of an ionic surfactant above which a micelle is formed by aggregation; micelles added to a mobile phase improve the separation of nonionic substances in HPLC and CE (MEKC) by a partitioning mechanism.

***Cross-linking:*** During the process of copolymerization of resins to form a three-dimensional matrix, a difunctional monomer is added to form cross-linkages between adjacent polymer chains. The degree of cross-linking is determined by the amount of the monomer added to the reaction. For example, divinylbenzene is a typical cross-linking agent for the production of polystyrene ion-exchange resins. The swelling and diffusion characteristics of a resin are governed by its degree of cross-linking.

***Cyano phase:*** A chemically bonded phase that terminates with the -CN functional group; it can be used in normal phase as a moderate polarity sorbent and in reversed-phase as a short chain bonded phase.

***Cyclodextrins:*** Cyclic oligomers of several D-(+)-glucopyranose units used in chiral HPLC and CE separations; popular ones are named $\alpha$, $\beta$, and $\gamma$*-cyclodextrins*; they have a truncated cone shape, a relatively hydrophobic cavity, and primary and secondary hydroxyl groups at their ends; they separate on the basis of differential inclusion of enantiomers; modified cyclodextrins with derivatized hydroxyl groups also are used for selectivity modification.

## D

***Data acquisition rate:*** A term referring to the rate of sampling of a detector output. To characterize a chromatographic peak at least 20-30 data points must be collected. The data acquisition rate, usually measured in Hertz, defines how many data points per second are collected while the peak is moving through the detector. For fast chromatography, the data acquisition rate must be sufficiently rapid to characterize a narrow peak. Modern detectors have data rates up to 80 Hz; also known as data rate and sampling rate.

***Dead volume ($V_M$):*** The column dead volume comprises the entire space accessible to a small molecule that can fully permeate all pores of a packing material. It includes the interstitial volume and the unoccupied pore volume. It is de-noted as $V_M$. The system dead volume includes the additional volume in the tubing that connects the injector and detector to the column. The system dead volume usually is approximated by injecting a small, essentially unretained species. Uracil, acetone and thiourea are most commonly used species in reversed-phase chromatography. See also ***adjusted retention volume, holdup volume***, and ***void volume***.

***DEAE:*** See *diethylaminoethyl*.

***Degassing:*** The process of removing dissolved gas from the mobile phase before or during use. Dissolved gas may come out of solution in the detector cell and cause baseline spikes and noise. Dissolved air can affect detectors such as electrochemical (by reaction) or fluorescence (by quenching) detectors. Dissolved gases also can cause pumps to lose their prime. Degassing is performed by heating the solvent, helium sparging, or using vacuum (in a vacuum flask) or online evacuation from a tube made of a gas-permeable substance such as polytetrafluoroethylene (PTFE).

***Denaturing HPLC:*** Using reversed-phase HPLC to investigate genetic mutations by the investigation of DNA base pairs.

***Desalting:*** Technique in which low molecular weight salts and other compounds can be removed from nonionic and high molecular weight compounds. An example is using a reversed-phase packing to retain sample compounds by hydrophobic effects yet allowing salts to pass through unretained. Using an SEC column to exclude large molecules and retain lower molecular weight salts is another example.

***Dextran:*** Polydextran-based packing material primarily used for low pressure biochromatography; an example would be Sephadex (GE Healthcare, Piscataway, New Jersey).

***Diethylaminoethyl (DEAE):*** A popular weak anion-exchange functionality (typically attached to cellulose or Sepharose *[GE Healthcare]*) used for separating biomolecules.

***Diffusion coefficient ($D_M$ or $D_S$):*** A fundamental parameter of a molecule in gas, solution ($D_M$), or the stationary phase ($D_S$). Expressed in square centimeters per second. $D_M$ is dependent on the molecular weight of the solute, temperature, solvent viscosity, and molar volume of the solute. A typical value for a 100-Da molecule in reversed-phase chromatography at room temperature is $10^{-5}$ $cm^2/s$.

***Diol phase:*** A hydrophilic phase that is useful in normal and reversed-phase. It is a diol structure (two -OH groups on adjacent carbon atoms in an aliphatic chain). In normal phase work, it is less polar than silica. It has been used to separate proteins and polypeptides in reversed-phase chromatography.

***Displacement chromatography:*** A chromatographic process in which the sample is placed onto the column head and then is displaced by a compound that is more strongly sorbed than the compounds of the original mixture. Sample molecules then are displaced by each other and by the more strongly sorbed compound. The result is that the eluted sample solute zones may be sharpened; displacement techniques have been used mainly in preparative-scale HPLC applications.

***Distribution constant (coefficient) ($K_c$):*** The total equilibrium concentration of a component in all forms or on the stationary phase divided by the total equilibrium concentration of the component in the mobile phase; also called the distribution coefficient or the partition coefficient in partition chromatography. In partition chromatography, $K_c$ is used when the concentration in the stationary phase is expressed per unit volume of the phase ($V_R = V_M + K_cV_S$). In a solid stationary phase, $KV_g$ is used and is expressed per mass (weight) of the dry solid phase. In adsorption chromatography with a well characterized adsorbent of known surface area, the concentration in the stationary phase is expressed per unit surface area.

***$D_M$:*** See *diffusion coefficient*.

***$d_p$:*** See *particle size*.

***$D_S$:*** See *diffusion coefficient*.

***Dwell time:*** The time equivalent to dwell volume; determined by the product of flow rate and the dwell volume.

***Dwell volume:*** The volume between the point of mixing of solvents (usually in the mixing chamber or at the proportioning valves in the liquid chromatograph) and the head of an LC column. Important in gradient elution or in isocratic elution situations when changes in solvent composition are made so that the column experiences

the composition change in the shortest possible time. Low-pressure mixing systems generally have larger dwell volumes than high pressure mixing systems.

***Dynamic coating:*** The formation of in-situ coatings on the packing in HPLC or on capillary walls in CE by adding a substance to the mobile phase that adsorbs onto (or absorbs into) the packing or at the wall surface. The purpose of a dynamic coating is to generate a new stationary phase or to deactivate the packing material or capillary wall to prevent unwanted interactions. One simple example is the adjustment of the mobile phase or running buffer to less than pH 3 to protonate silanols and negate their effect. Another example is coating the phase with a hydrophilic polymeric material to prevent adsorption of proteins.

## E

***E:*** See *separation impedance*.

$\varepsilon$***:*** See *interparticle porosity*.

***Eddy dispersion (diffusion) term ($\lambda$):*** The A term in the van Deemter equation. It is the contribution to plate height from the heterogeneity in axial velocities as a result of the particle size and geometry of the packing, as well as wall effects; $A = 2\lambda d_p$, where I is an empirical column constant. Typical values of I for well-packed columns are 0.8-1.0. Some theories of chromatography indicate a velocity-dependent contribution to the height equivalent to a theoretical plate (HETP) from this process. Also known as eddy diffusion, flow-heterogeneity induced broadening, and the multipath term. See also ***van Deemter equation***.

$\varepsilon_e$***:*** See *interstitial porosity*.

***Effective plate height ($H_{eff}$):*** The column length divided by the effective plate number.

***Effective theoretical plates ($N_{eff}$):*** Also called the effective plate number by IUPAC. The true number of plates in a column, because it corrects theoretical plates for dead volume. $N_{eff} = 16[(t_R'/w_b)^2]$, where $t_R'$ is the adjusted $R_b$ retention time and $w_b$ is the bandwidth of the peak (see Figure 63, p. 116). It is a better figure of merit than simple plate number for comparing devices of very different geometries and phase ratios.

***Efficiency (N or H ):*** A measure typically determined by the number of theoretical plates *(N)* calculated from the equation $N = 16(V_R/w_b)^2 = 16\ (t_R/w_b)^2$, where $w_b$ is the peak width measured at the base (see Figure 63, p. 116). If the peak width is measured at half height, the following equation is used: $N = 5.545\ (V_R/w_h)^2$. The plate height *(H)* or HETP is deter mined by H = L/N. The efficiency of asymmetric peaks is better determined from the peak centroid and variance by mathematical analysis of the peak shape. See also ***Foley-Dorsey equation***.

***Effluent:*** The mobile phase leaving the column; same as *eluate*.

$\varepsilon_i$***:*** See *intraparticle porosity*.

***Eluate:*** Combination of mobile phase and solute exiting the column; also called effluent.

***Eluent:*** The mobile phase used to perform a separation.

***Eluite:*** The species being eluted, the analyte, or the sample.

***Eluotropic series:*** A series of solvents (eluents) with an increasing degree of solvent strength generally used in liquid- solid or adsorption chromatography. In normal phase chromatography, a non-polar solvent such as pentane would be at the low end of the scale, an intermediate solvent such as methylene chloride would be in the middle of the scale, and a strongly polar solvent such as methanol would be near the upper end of the scale. In reversed-phase chromatography, the reverse order of strength would be observed; water would be weak and acetonitrile strong. Thus, when developing a method or running a gradient, an eluotropic series is useful for selecting solvents. See also ***Snyder $\varepsilon^o$***.

***Elute:*** To chromatograph by elution chromatography. The term elute is preferred over develop, which was used in older nomenclature.

***Elution:*** The process of passing mobile phase through the column to transport solutes down a column.

***Elution chromatography:*** The most commonly used chromatographic method in which a sample is applied to the head of the column as a narrow zone and individual analytes are separated and eluted from the end of the column. Compare with displacement chromatography and frontal analysis.

***Elution volume ($V_R$):*** Refers to the volume of mobile phase necessary to elute a solute from a column. It is the volume from the point of injection to the volume at maximum concentration (apex) for a symmetrical peak; $V_R = Ft_R$, where $F$ is the flow rate and $t_R$ is the retention time of the peak of interest.

***Elutriation:*** A technique used to fractionate packing particles by size based on the difference in their Stokes terminal velocities. It most often is used for the separation of ion-exchange resins that require a particularly narrow size range, such as amino acid resins. The technique involves the upward flow of water into a large tube. The unsized beads are added to the moving water, and the particles seek their own level, depending upon their density and particle size. They are removed at certain levels in the tube. High-purity spherical silica gels sometimes are sized by elutriation.

***Enantiomeric compound:*** Chemical compounds that display chiral activity; such compounds will require a

separation mechanism that can differentiate between the R- or S-enantiomer and specialty columns are available for this purpose.

***Endcapping:*** A technique used to remove silica gel silanol groups that may remain after reaction with a large silylatin agent such as octadecyltrichlorosilane. The column is said to be endcapped when a small silylating reagent (such as trimethylchlorosilane or dichlorodimethylsilane) is used to bond residual silanol groups on a silica gel-based packing surface. Most often used with reversed-phase packings to minimize undesirable adsorption of basic, ionizable, and ionic compounds. Endcapping reactions also are used to remove terminal silanol groups from polymeric phases.

***Endfitting:*** The fitting at the end of the column that permits connection to the injector or detector. Most HPLC endfittings have frits to contain the packing and low dead volumes for minimum band spreading. They usually are constructed of stainless steel, but poly-etherether ketone (PEEK) and other polymeric materials also are used.

$\varepsilon_T$**:** See *total porosity*.

***Exchange capacity:*** See *ion-exchange capacity*.

***Excluded volume:*** See *interstitial volume*.

***Exclusion chromatography:*** See *ion exclusion chromatography* and *steric exclusion chromatography*.

***Exclusion limit:*** The upper limit of molecular weight (or size) beyond which molecules will be eluted at the same retention volume, called the exclusion volume. Many SEC packings are known by their exclusion limit. For example, a 105 column of porous silica gel will exclude any compounds with a molecular weight greater than 100,000, based on a polystyrene calibration standard.

***Exclusion volume ($V_0$, $V_{ei}$):*** The minimum retention volume of a molecule on an SEC packing in which all molecules larger than the size of the largest pore are totally excluded. These molecules are incapable of penetrating the pores and are eluted at the interstitial (interparticle) volume of the column.

***Exponentially modified Gaussian peak:*** An asymmetric peak resulting from passing a Gaussian peak through a detector that is excessively slow or has an excessive volume. Frequently used to model peak tailing arising from the column per se. The basis for the Foley-Dorsey equations. See also ***Foley-Dorsey equation***.

***Extracolumn effects:*** The total band broadening effects of all parts of the chromatographic system outside of the column itself. Extracolumn effects must be minimized to maintain the efficiency of a column. Sources of band broadening can include the injector design, injection volume, connecting tubing, endfittings, frits, detector cell volume, and internal detector tubing. The variances of all of these contributions are additive.

***Extracolumn volume:*** The volume between the effective injection point and the effective detection point, excluding the part of the column containing the stationary phase. It comprises the volumes of the injector, connecting lines and frits, and the detector. It determines the extracolumn effects.

## F

***F:*** See *flow rate*.

***F:*** See *flow resistance parameter*.

***Fast LC:*** Use of HPLC of short columns (1.5-7 cm) with conventional inner diameters (2-6 mm) packed with small particles *(3- or 5-µm $d_p$)*. Separation times in the range of minutes, or even seconds, are common.

***Fast protein LC (FPLC):*** A termed coined to cover the specific use of HPLC for separating proteins. Generally, glass columns, moderate pressure, and spherical microbeads are used for FPLC.

***Flow rate (F):*** The volumetric rate of flow of a mobile phase through an LC column. Typical flow rates are 1-2 mL/min for a conventional 4.6-mm id HPLC column.

***Flow resistance parameter (Φ):*** $\Phi = d_p^2/B_o$, where $B_o$ is permeability. See also ***permeability***.

***Fluoro phase:*** One of a family of aliphatic and aromatic reversed-phase materials in which a substantial fraction of the bonded phase is fluorinated. Sometimes called fluorous phases or per-fluoro phases. Typically these phases have different selectivities than hydrocarbon phases.

***Foley-Dorsey equation:*** A correction of the plate count and retention time for peak tailing from extracolumn sources of broadening. See reference 3.

***FPLC:*** See *fast protein LC*.

***Fractionation range:*** Refers to the operating range of a gel or packing in SEC. This range is where a packing can separate molecules based on their size. At one end of the range, molecules that are too large to diffuse into the pores are excluded. At the other end of the range, molecules that can diffuse into all of the pores totally permeate the packing and are eluted (unseparated) at the permeation volume.

***Frit:*** The porous element at either end of a column that contains the column packing. It is placed at the very ends of the column tube or, more commonly, in the endfitting. Frits can be stainless steel or other inert metal or plastic such as porous PTFE or polypropylene. The frit porosity must be less than the smallest particle in the HPLC column; otherwise particles will pass through the frit, and the packing will be lost.

***Frontal analysis:*** A chromatographic technique that involves continuous addition of sample to the column with the result that only the least sorbed compound, which moves at the fastest rate, is obtained in a pure state. The second-least sorbed compound is eluted with the first-eluted compound, the third-least sorbed compound with the first and second compound and so on until the original sample is eluted at the column exit. Frontal analysis is seldom used and is mainly a preparative technique.

***Frontal chromatography:*** Same as *frontal analysis*.

***Fronting:*** Peak shape in which the front part of the peak (before the apex) in a chromatogram tapers in advance of the remainder of the peak; that is, the front is less steep than the rear. The peak has an asymmetric distribution with a leading edge. The asymmetry factor for a fronting peak has a value of less than one. Tailing is the opposite effect. Fronting can result at high sample loads because of positive curvature in the isotherm and from using poorly packed columns.

## G

***g:*** The obstruction or tortuosity factor. Molecular diffusing term. See also *tortuosity*.

***Gaussian curve:*** A standard error curve, based on a mathematical function, that is a symmetrical, bell-shaped band or peak. Most chromatographic theory assumes a Gaussian peak. Using the peak maximum position as a measure of retention and the efficiency equations mentioned above assume Gaussian peak shape. See Figure 63, p. 116.

***Gaussian peak:*** A peak whose shape conforms closely to the equation: $C = C_{max}\ exp[-(t - t_R)^2/2\sigma^2]$.

***Gel:*** The solid packing used in gel chromatography or gel permeation chromatography (GPC). An actual gel consists of two parts: the dispersed medium (solid portion) and the dispersing medium (the solvent). Also defined as a colloidal dispersion of a solid and liquid in which the solid is the continuous phase.

***Gel filtration chromatography (GFC):*** Also called aqueous size exclusion chromatography. Performed with aqueous mobile phases. Generally refers to molecular size separation performed on soft gels such as polydextrans, but analysts also can use highly cross-linked polymers, silica gels, and other porous media. Most gel filtration separations involve biopolymers and water-soluble polymers such as polyacrylic acid.

***Gel permeation chromatography (GPC):*** SEC performed with organic mobile phases used for the separation and characterization of polymers. SEC with aqueous mobile phases is called aqueous GPC, GFC, or aqueous SEC.

***GFC:*** See *gel filtration chromatography*.

***Gigapores:*** See *perfusion chromatography*.

***GPC:*** See *gel permeation chromatography*.

***Gradient:*** A process to change solvent strength as a function of time (normally solvent strength increases) thereby eluting progressively more highly retained analytes. Typically gradients can be binary, ternary, and quaternary solvent mixtures in which solvents are blended to achieve the proper strength.

***Gradient delay volume:*** See *dwell volume*.

***Gradient elution:*** Technique for decreasing separation time by increasing the mobile phase strength over time during the chromatographic separation. Also known as solvent programming. Gradients can be continuous or stepwise. Binary, ternary, and quaternary solvent gradients have been used routinely in HPLC.

***Graphitized carbon:*** Graphitized carbon is a graphitic carbon with more or less perfect three-dimensional hexagonal crystalline order prepared from non-graphitic carbon by graphitization heat treatment; this packing material has a strong affinity for polar compounds in aqueous samples and water miscible organic extracts. Commonly used in pesticide analysis of food samples.

***Graphitized carbon packing:*** A reversed-phase packing material consisting of pure graphitic carbon. Possesses interesting sorbent properties such as preferential separation of geometric isomers such as o-, m- and p-aromatics and cis-trans isomers.

***Guard column:*** A small column placed between the injector and the analytical column. It protects the analytical column from contamination by sample particulates and strongly retained species. The guard column is usually packed with the same material as that in the analytical column and is often of the same inner diameter. It is much shorter, costs less, and usually is discarded when it becomes contaminated. Integrated guard-analytical column systems are often preferred to minimize extracolumn effects caused by connecting tubing with separate guard and analytical columns.

## H

***h:*** Reduced plate height. Defined as $HETP/d_p$, where *HETP* is the height equivalent to a theoretical plate and $d_p$ is the particle diameter. See also ***reduced plate height***.

***H:*** Same as *HETP*. See also ***efficiency***.

$\eta$***:*** See *viscosity*.

***Head pressure (Δp):*** The difference in pressure between the inlet and outlet of a column measured in pounds per square inch. Governed by the following approximate equation for a column packed with spherical particles of

typical internal porosity *(0.5)*:$\Delta p = 3000L\eta/t_M d_p^2$, where $L$ is the column length in centimeters, $\eta$ is the mobile phase viscosity in centipoise, $t_M$ the column holdup time in minutes, and $d_p$ is the particle diameter in micrometers. Pressure can be expressed in pounds per square inch, bars, atmospheres, or pascals.

***Heart cutting:*** Refers to collection of the center of the peak at which purity should be maximum in preparative LC. The term also is used in column switching.

***$H_{eff}$:*** See *effective plate height*.

***Helium sparging:*** See *degassing*. Helium has a very low solubility in most common liquids.

***HETP:*** Height equivalent to a theoretical plate. A carryover from distillation theory; a measure of column efficiency; $HETP = L/N$, where $L$ is column length and $N$ is the number of theoretical plates. HETP should be approximately 2-3 $d_p$ for 5 µm particles with a typical well-packed HPLC column, HETP (or $H$) values usually are in the range of 0.01-0.03 mm. See *also efficiency* and *h*.

***High performance CE:*** A technique in which small-diameter capillaries, buffered conducting solutions, and high voltages (as much as 30,000 V) separate ionic molecules based on their differential electrophoretic mobilities. Nonionic (neutral) molecules can be separated by MEKC.

***High performance liquid chromatography (HPLC):*** The modern, fully instrumental form of liquid-phase chromatography technique that uses small particles and high pressures. Sometimes called high-pressure LC.

***High pressure mixing:*** A configuration of a gradient HPLC system where the solvents are mixed on the high pressure side of multiple pumps (usually 2, binary); such a system offers a lower gradient delay volume than low pressure mixing systems where the solvents are mixed by proportioning valves prior to a single pump.

***Holdup volume ($V_M$):*** The total volume of mobile phase in the column regardless of where it exists; $V_M = V_e + V_i$, where $V_e$ is the interstitial volume and Vi is the intraparticle volume. Also called the column void volume. IUPAC indicates that use of the term dead volume should be eliminated for this concept. The use of dead volume is limited to regions not swept by the flowing mobile phase system. Holdup volume is measured by injecting an unretained species that fits in all the pores. See also *interstitial porosity* and *intraparticle porosity*.

***HPLC:*** See *high performance liquid chromatography*.

***Hybrid silica:*** Silica gel comprising both organic and inorganic moieties with hybrid properties of polymeric packings and silica packings. Synthesized from silanes containing organic functionality. Different selectivity but better high pH stability than bare or uncoated silica gel.

***Hydrodynamic volume:*** The molecular volume defined by the effective diameter of a molecule in free solution at which the hydrodynamic sphere would be a sphere defined by the molecule as it revolves around its central axis in solution. Term used in SEC to define molecular shape and to explain why molecules with the same molecular weight often have different elution volumes. Measured by determining the Stokes radius.

***Hydrophilic:*** Greek word for water loving. Refers to stationary phases that are fully compatible with water and to water-soluble molecules in general. Many columns used to separate proteins - such as ion-exchange, SEC, and affinity columns - are hydrophilic in nature and should not irreversibly sorb or denature protein in an aqueous environment.

***Hydrophilic interaction chromatography:*** An alternative technique to reversed-phase HPLC (RPC) for the separation of highly polar analytes that may be only slightly retained by RPC, HILIC requires a high percentage of a non-polar mobile phase and a polar stationary phase, similar to the requirements in normal phase chromatography (NPC). However, unlike NPC which uses non-polar solvents such as hexane and methylene chloride and tries to exclude water from the mobile phase, HILIC requires some water in the mobile phase to maintain a stagnant enriched water layer on the surface into which analytes may selectively partition. In addition, water-miscible organic solvents are used instead of the water-immiscible solvents used in NPC. With HILIC, sorbents such as bare silica, bonded diol, and polyhydroxyethylaspartamide are used. Polar analytes are well retained and elute in order of increasing hydrophilicity, just the inverse of RPLC.

***Hydrophobic:*** Greek word for water fearing. Refers to stationary phases that are incompatible with water or to molecules that in general have little affinity for water. Hydrophobic molecules have few polar functional groups. Most have a high content of hydrocarbon (aliphatic and aromatic) functionality.

***Hydrophobic interaction chromatography:*** A technique in which weakly polar (non-hydrocarboneous) packings are used to separate molecules by the interactions of their hydrophobic moieties and the hydrophobic sites on their packing surface. High concentrations of salt solutions are used in the mobile phases, and separations are generated by changing the salt concentration. The technique is analogous to salting out molecules from solution. Gradients are run by decreasing the salt concentration. The technique often is used to separate proteins that are sensitive to denaturization by the organic solvents used in regular reversed-phase chromatography. Usually little or no organic solvent is used in the mobile phase in hydrophobic

interaction chromatography.

***Hydroxyapatite:*** A porous calcium hydroxy phosphate solid that chemically resembles bone and tooth. Used as a packing material in biochromatography for nucleic acid constituents, monoclonal antibodies, and proteins.

***Hyphenated techniques:*** Refers to the family of techniques best known by their acronyms, including LC-mass spectrometry (MS), LC-Fourier transform IR spectroscopy (FTIR), and LC-MS-MS. See also *multidimensional chromatography*.

## I

***IC:*** See *ion chromatography*.

***Immobilized metal-affinity chromatography:*** See *metal-affinity chromatography*.

***Immunoaffinity chromatography:*** A specific form of separation where an antibody is bonded or immobilized onto the surface of an HPLC support material. Based on a molecular recognition mechanism, analytes that are specifically targeted by the antibody can be selectively retained via antibody-antigen interactions from a complex mixture. After interferences are washed away, retained analytes can be released by changing the mobile phase conditions such that the strong binding is disrupted.

***Imprinted phases:*** Polymer and silica phases generated in the presence of a template or printing molecule. These phases have enhanced selectivity for the templating molecule.

***Included volume:*** Also known as totally included volume. The volume at which a small molecule that explores the entire pore space of a column is eluted. See also ***size exclusion chromatography***.

***Indirect detection:*** Used for non-UV-absorbing or non-fluorescing analytes. A UV-absorbing or fluorescent compound added to the mobile-phase maintains a high background signal; when a non-absorbing or non-fluorescing analyte is eluted, the background is diluted and a negative peak is observed for that analyte. When an analyte acts to increase the concentration of the indicating species, it produces a positive peak. When a negative signal is detected, the detector signals are reversed to the output device.

***Infinite diameter column effect:*** At a certain column length, a sample injected into the center of a packed bed spreads by radial diffusion but never reaches the column wall, where wall effects can cause band broadening. Phenomenon observed by John Knox, who showed that a sample peak collected in the exact center of the column exit displayed a higher efficiency than a sample peak collected near the wall. The infinite diameter effect depends on column length, internal diameter, particle size, and mobile phase properties. Very seldom applied in HPLC.

***Injection solvent:*** Solvent used to inject sample into an HPLC column; solvent should be of equal or lower strength than the mobile phase to prevent premature movement down the column due to the presence of a stronger solvent.

***Inlet:*** The initial part of the column where the solvent and sample enter. An inlet frit usually holds the packing in place and, in some cases, protects the packed bed.

***Inlet/outlet check valves:*** The check valve(s) on an LC pump that allow(s) mobile phase to flow in one direction but not in the reverse direction. The inlet check valve allows flow from the reservoir into the pump and the outlet check valve allows mobile phase to flow to the column from the pump.

***Inlet filter:*** Filtration devices attached to the inlet lines of the pump that removes particulate matter from the mobile phase before the solvent reaches the pump; reservoir filters are an inlet filter that resides in the solvent bottle.

***Inline filter:*** A device that prevents particulate matter from damaging the column. Modern low-volume, inline filters can be placed between the injector and the column without major contributions to band broadening. A filter in this position prevents sample particles from entering the packed bed or column inlet frit.

***Interparticle porosity ($\varepsilon_e$):*** The interparticle volume of a packed column per unit column volume; $\varepsilon_e = V_e/V_c$ , where $V_e$ is the interstitial volume and Vc is the total column volume. See also ***interstitial porosity***.

***Interparticle volume ($V_o$):*** The volume of mobile phase located outside the particles.

***Interstitial porosity ($\varepsilon_e$):*** The fraction of the volume in the column located in the interparticle (interstitial) space; $\varepsilon_e = V_e/V_c$.

***Interstitial velocity ($u_e$):*** The actual velocity of the eluent as it moves through the column flowing around the particles; $u_e = F/A_c\varepsilon_e$. The interstitial velocity is the basis for computing the reduced velocity.

***Interstitial volume ($V_e$):*** The volume between the particles. It does not include the volume in the pores of the particles. Also called the excluded volume (see ***SEC***) and interparticle volume. Measured by injecting a molecule that does not permeate any pores and does not interact with the surface of the particles. In SEC, this volume is denoted $V_o$.

***Intraparticle porosity ($\varepsilon_i$):*** The fraction of the particle volume that is the pore volume; $\varepsilon_i = V_{pore}/V_{particle}$.

***Intraparticle volume ($V_i$):*** The volume inside the pores of the particles. Also called the internal and included volume. Can be measured by the BET method or mercury-

intrusion porosimetry.

***Ion chromatography (IC):*** An ion-exchange technique in which low concentrations of organic and inorganic anions or cations are determined using ion-exchangers of low ion-exchange capacity with dilute buffers. Conductivity detectors often are used. IC is practiced in two forms: in suppressed IC, a second column or a membrane separator is used to remove the buffer counter ion from the analyte and simultaneously replace it with a hydrogen or hydroxide ion that concomitantly converts the buffer to an uncharged species thereby suppressing background and enhancing sensitivity. In non-suppressed IC, low concentration, weakly conducting buffers are carefully selected, the entire effluent is passed through the detector, and ions are detected above the background signal.

***Ion-exchange capacity:*** The number of ionic sites on the packing that can participate in the exchange process. The exchange capacity is expressed in milliequivalents (mequiv) per gram. A typical styrene-divinylbenzene strong anion-exchange resin may have 3-5 mequiv/g capacity. Exchangers for IC have very low capacity. Capacity of weak anion and cation-exchangers varies dramatically with pH.

***Ion-exchange chromatography:*** A mode of chromatography in which ionic substances are separated on cationic or anionic sites of the packing. The sample ion, usually with a counter ion, will exchange with ions already on the ionogenic group of the packing. Retention is based on the affinity of different ions for the site and other solution parameters such as pH, ionic strength, and counter ion type. Ion chromatography basically is an ion-exchange technique.

***Ion exclusion:*** The process in which ionized solutes can be separated from un-ionized or partially ionized solutes using ion-exchange resins. Separation results from Donnan potential in which ionic solutes exist at a higher concentration in solution than in the stationary phase, whereas non-ionic solutes are evenly distributed between the mobile phase and resin. Therefore, ionic solutes will move faster down the column than nonionic solutes. Ion exclusion occurs in reversed-phase chromatography when anions are separated at pH values at which the silanol groups are ionized.

***Ionic strength:*** Ionic strength is a characteristic of an electrolyte solution. It is typically expressed as the average electrostatic interactions among an electrolyte's ions. It is related to electrolyte concentration but the main difference between ionic strength and electrolyte concentration is that the former is higher if some of the ions are more highly charged. The higher the ionic strength of a mobile phase the more the mobile phase competes with the analyte for ionic or adsorptive sites.

***Ion-moderated partitioning chromatography:*** A technique used for separating carbohydrates using strong cation-exchange packings that are in specific cationic form (for example, calcium, hydrogen, silver). The separation mechanism is complexation rather than ion-exchange.

***Ion-pair chromatography:*** Form of chromatography in which ions in solution can be paired or neutralized and separated as an ion pair on a reversed-phase column. Ion-pairing agents usually are ionic compounds that contain a hydrocarbon chain, which imparts a certain hydrophobicity so that the ion pair can be retained on a reversed-phase column. Retention is proportional to the length of the hydrophobic chain and the concentration of the ion-pair additive. Ion pairing also can occur in normal phase chromatography when one part of the pair is dynamically loaded onto a sorbent, but this technique is not as popular as reversed-phase chromatography. Also known as ion-interaction chromatography or dynamic ion-exchange chromatography, which stresses that users sometimes do not know the precise mechanistic details of how the additive controls retention.

***Ion retardation:*** Refers to using amphoteric ion-exchange resins, which retard ionic molecules and allow non-ionic molecules or nonelectrolytes to be eluted preferentially.

***Ion suppression:*** Buffering in an aqueous mobile phase at a particular pH to suppress solute ionization. For example, weak carboxylic acids can have their ionization suppressed by the adjustment of the pH below their $pK_a$ value. Useful for improving peak shape of weak acids and bases in reversed-phase chromatography.

***Irregular packing:*** Refers to the shape of a column packing. Irregular packings are available in microparticulate sizes. The packings are obtained from grinding solid materials into small particles and sizing them into narrow fractions using classification machinery. Spherical packings are used more often than irregular packings in analytical HPLC, but the less-expensive, irregular packings are still widely used in preparative-scale LC.

***Irreversible adsorption:*** When a compound with a very strong affinity for an adsorbent is injected onto a column, it can be adsorbed so strongly that it cannot be eluted from the column. A chemical reaction between the sample and the surface of the adsorbent is an example of irreversible adsorption. See also *chemisorption*.

***Isocratic:*** Using a time invariant-eluent composition in LC.

***Isotherm:*** See *adsorption isotherm*.

***Isothermal chromatography:*** Using conditions of constant temperature. The vast preponderance of all LC is performed under isothermal conditions.

## K

***k:*** See *Retention Factor*.

***k':*** An old term that has been replaced by the IUPAC-approved term, retention factor *(k)*.

***K:*** See *partition coefficient*.

***$k_{A/B}$:*** See *selectivity coefficient*.

***$K_c$:*** See *distribution constant (coefficient)*.

***Kieselguhr:*** A diatomaceous earth used in column chromatography and also as a sample clean-up media. Only weakly adsorptive, it can be used as a support in liquid-liquid chromatography. Rarely used in HPLC.

***Kinetic Plot:*** Kinetic plots are methods to characterize the practical limits of column performance, where theoretical plates (H) and separation impedance *(E)* are plotted as a function of the pressure-drop limited plate number *(N)*. The kinetic plot retains the information shown in van Deemter plots but completes it with the information on the bed permeability. See *Poppe Plot*.

***Knox equation:*** A modification of the van Deemter equation developed by John Knox in which the A term that represents eddy dispersion multiplied by *u1/3*, where u is the interstitial eluent velocity. Usually written in terms of the dimensionless or reduced plate height *(h)* and reduced velocity (*v*) as $h = Av\ 1/3 + B/v + Cv$. See also ***van Deemter equation***.

## L

***L:*** See *column length*.

***Laminar flow:*** The smooth time invariant flow that develops when a liquid is moving under conditions in which viscous forces dominate inertial forces. Laminar flow is characterized by a low Reynolds number (see ***Reynolds number***). In a cylindrical tube, fluid streams in the center flow faster than those at the tube wall, which results in a radially parabolic distribution in axial fluid velocity. This non-uniformity of axial velocities in the interstices in a packed bed also causes substantial peak broadening in packed columns.

***Langmuir isotherm:*** A specific form of an isotherm; $C_S = N_0 C_M/(K_d + C_M)$, where $C_S$ and $C_M$ are the equilibrium stationary and mobile-phase concentrations of the solute, $N_0$ the total number of surface sites available for sorption, and Kd the sorption binding constant.

***LC:*** See *liquid chromatography*.

***Ligand:*** In ligand exchange chromatography, it refers to the analyte that undergoes ligand exchange with the stationary phase. In affinity chromatography, it refers to the biospecific material - enzyme, antigen, or hormone - coupled with the support (carrier) to form the affinity column. In bonded-phase chromatography, it refers to the moiety covalently bound to the surface.

***Ligand exchange chromatography:*** A technique in which chelating ligands are added to the mobile phase and undergo sorption onto a packing. These sorbed molecules can act as chelating agents with certain solutes. For example, copper salt can be added to the mobile phase for the chelation and separation of amino acids. Chelating resins function in a similar manner: chelating groups are chemically bonded to the polystyrene backbone.

***Linear elution adsorption chromatography:*** Refers to adsorption chromatography performed in the linear portion of an adsorption isotherm. A term coined by Lloyd Snyder.

***Linear velocity (u):*** The velocity of the mobile phase moving through the column. Expressed in centimeters per second. Related to flow rate by the cross-sectional area of the column. Determined by dividing the column length *(L)* by the retention time of an unretained compound. See also ***void time***.

***Liquid chromatography (LC):*** A separation technique in which the mobile phase is a liquid. Most often performed in a column.

***Liquid-liquid chromatography:*** One of the earliest separation modes of HPLC; it gave way to chemically bonded phases in the early 1970s. Same as ***partition chromatography***.

***Liquid-solid chromatography:*** Same as *adsorption chromatography*.

***Loadability:*** The maximum amount of analyte that can be injected onto a column that no longer permits the isolation of product at the desired level of purity or recovery level; important in preparative chromatography

***Loading (phase loading versus sample loading):*** The amount of stationary phase coated or bonded onto a solid support. In liquid-liquid chromatography, the amount of liquid phase in milligrams of per gram of packing. In bonded-phase chromatography, the loading may be expressed in micromoles per square meter or percentage carbon *(w/w)*. Also called coverage or surface coverage. An alternate and unrelated meaning is the amount of sample mass injected on an analytical or preparative scale column; preparative-scale columns often are operated in an overloaded condition for throughput reasons.

***log $k_w$:*** The extrapolated intercept of a plot of *log k* versus volume fraction of organic modifier in reversed-phase LC. See also ***S***.

***Longitudinal diffusion:*** Same as *molecular diffusion term*. B term in van Deemter equation. See also ***van Deemter equation***.

***Low pressure mixing:*** See *high pressure mixing*.

## M

***μ:*** See *electrophoretic mobility*.

***Macroporous resin (macroreticular):*** Cross-linked ion-exchange resins that have molecular-scale micropores and also macropores of several hundred angstroms. These highly porous resins have large internal surface areas that are accessible to large molecules.

***Mass transfer (interphase):*** The process of solute movement between the moving and stationary zones. The C term of the van Deemter equation is called the interphase mass transfer term. The faster the mass transfer process, the better the column efficiency. In HPLC, slow mass transfer is the most important factor affecting column efficiency. Its rate can be increased by using small particle packings, thin stationary-phase layers, low viscosity mobile phases, and high temperatures.

***Mean pore diameter:*** The average diameter of the pore of a porous packing. It most commonly is determined by the BET method and is reported as fourfold the specific pore volume divided by the specific surface area *(4V/A)* based on the assumption of uniform cylindrical pores. The pore diameter is important in that it must allow free diffusion of solute molecules into and out of the pore so that the solute can interact with the stationary phase. Additionally, the pores must be well-connected, with a minimum of dead ends, so many paths can allow a molecule to access any part of the pore space. In SEC, the packings have different pore diameters; therefore, molecules of different sizes can be separated. For a typical substrate such as silica gel, 60- and 100Å pore diameters are most popular. Pore diameters greater than 300Å are used for the separation of biomolecules. Pores usually are classified as micro (<20Å), meso (20-500Å), and macro (>500Å).

***MECC:*** See *micellar electrokinetic capillary chromatography*.

***Megapores:*** See *perfusion chromatography*.

***MEKC:*** See *micellar electrokinetic capillary chromatography*.

***Metal-affinity chromatography:*** A special form of ligand exchange chromatography used to separate biopolymers with a particular affinity for a specific metal cation, typically copper(II), zinc(II), and iron(II).

***Metalophile:*** A compound that has high affinity for active acidic silanol groups on the surface of silicas. Usually a strongly basic amine or multifunctional carboxylate or phenol.

***Method development:*** A process for optimizing the separation, including the sample pretreatment, to obtain a reproducible and robust separation. Usually, it emphasizes the search for the stationary phase, eluent, and column temperature combination that provides an adequate, if not optimum, separation.

***Method validation:*** A process of testing a method to show that it performs to the desired limits of precision and accuracy in retention, resolution, and quantitation of the sample components of interest.

***Micellar chromatography:*** Adding micelles to the mobile phase to cause separation. The micelles may act as displacing or partitioning agents and provide another parameter to change selectivity. Surfactants at concentrations greater than their critical micelle concentration are used in micellar chromatography and in MEKC.

***Micro-LC:*** Refers collectively to techniques in which a column of smaller than conventional inner diameter is used for separation. The term micro-LC most often is used for HPLC in columns with inner diameters smaller than 0.5 mm; micro-LC is used in high-sensitivity analysis when the sample amount is limited and with certain ionization techniques in LC-MS in which the volume of solvent flowing into the ionization source must be minimized.

***Microbore:*** Refers to the use of smaller-than-usual inner diameter columns in HPLC. Columns of 2 mm and less are considered to be microbore sizes. Inner diameters of 0.5 mm and smaller are considered micro-LC columns.

***Microchip devices:*** Microdevices based on silicon, glass, and other types of microfabricated chips in which experiments can be miniaturized into single or multichannel microfluidic circuits. These devices can be used for CE and CEC. They should be low cost and disposable. Using microdevices for separation currently is in its infancy, and applications should expand with time.

***Microparticulate:*** Refers to the small particles used in HPLC. Generally packings with a particle diameter of less than 10 mm that are totally porous are considered microparticles.

***Microporous resin:*** Same as *microreticular resin*.

***Microreticular resin:*** Cross-linked, synthetic ion-exchange resins that have pores with openings that correspond to molecular sizes. Diffusion into the narrow pores can be impaired, and low exchange rates and poor performance can occur, especially for large molecules.

***Migration rate:*** See *electrophoretic mobility*.

***Migration time ($t_m$):*** The time it takes for a charged molecule to move from the point of injection to the point of detection in a CE capillary. Distinct from holdup time *($t_M$)*.

***Minimum plate height:*** The minimum of the van Deemter curve that results from a plot of H versus v. This value represents the most theoretical plates that can be obtained for a certain column and mobile phase system.

Usually occurs at excessively low flow rates. Also known as the optimum plate height. It typically is two- to threefold the particle diameter of well-packed columns.

***Mixed-bed column:*** Combination of two or more stationary phases in the same column, used most often in IEC (mixed anion and cation resins) and SEC (mixture of different pore size packings). Its advantage in IEC is the total removal of both cationic and anionic compounds. Useful in SEC because a wider molecular weight range can be accommodated by the same column.

***Mixed-mode separation:*** A separation that occurs in a single column caused by the retention and selectivity provided by a dual-retention mechanism. For example, a reversed-phase column with residual silanols at intermediate-to-high pH values can separate by hydrophobic interaction and ionic interaction by the ionized silanols. Sometimes mixed-mode separations can be quite beneficial to the selectivity (band spacing), but they can cause peak asymmetry, and the precise balance of interactions may be difficult to reproduce with subsequent packing batches.

***Mobile phase:*** The solvent that moves the solute through the column. In LC, the mobile phase interacts with both the solute and the stationary phase and, therefore, can have a powerful influence on the separation.

***Mobile phase strength:*** See *solvent strength*.

***Mobile phase velocity ($u_M$):*** The velocity at which the mobile phase percolates through the bed of particles; $u_M = L/t_M$, where $L$ is column length and $t_M$ is holdup time. See also ***adjusted retention volume, holdup volume***, and *dead volume*.

***Mobility:*** See *electrophoretic mobility*.

***Modifier:*** An additive that changes the character of the mobile phase. For example, methanol is the strong solvent in reversed-phase and sometimes is called the modifier (water is the weak solvent); sometimes other additives - competing bases such as triethylamine or ion-pairing reagents - are referred to as modifiers, but they more correctly should be called additives. See also ***additives***.

***Molecular diffusion term (B term):*** Refers to the B term (second term) of the van Deemter equation. Also called longitudinal or axial diffusion term. It dominates band broadening only at very low flow rates below the minimum plate height at which the diffusion of individual solutes can occur in a longitudinal (lengthwise) direction on the column. The contribution to the B term arises from diffusion in the mobile phase and is $2\gamma D_M$, where $\gamma$ is the obstruction factor (typically 0.6-0.8) and $D_M$ is the diffusion coefficient. See also ***van Deemter equation***.

***Molecular weight distribution:*** The distribution of molecular weight of molecules in a polymer sample. Distribution can be defined as weight average and number average. Molecularly imprinted phases: See ***imprinted phases***.

***Monodisperse particles:*** Particles that fall into a narrow range of diameters. See also ***polydisperse particles***.

***Monomeric phase:*** Refers to a bonded phase in which single molecules are bonded to a support. For silica gel, monomeric phases are prepared by the reaction of an alkyl- or aryl- monochloroor or alkoxysilane. Polymeric phases generally are prepared from a di- or trichlorosilane or an alkoxysilane reactant in the presence of water.

***Moving zone:*** To be distinguished from the mobile phase, this zone is the fraction of the mobile phase in the column that occupies the interstitial spaces. See also ***stationary phase***.

***Multidimensional chromatography:*** The use of two or more columns or chromatographic techniques to generate a better separation. It is useful for sample clean-up, increased resolution, increased throughput, and increased peak capacity. It can be used offline by collecting fractions and reinjecting them onto a second column or online by using a switching valve. Also called coupled column chromatography, column switching, multicolumn chromatography, and boxcar chromatography.

## N

***n:*** See *peak capacity*.

***N:*** The number of theoretical plates; $N = 16(t_R/w_b)^2$, where $t_R$ is retention time and $w_b$ is the base width of the peak. A measure of the efficiency of a column. Sometimes measured as $N = 5.54(t_R/w_h)^2$, where $w_h$ (or $w_{1/2}$) is the peak width at half height. See also ***efficiency*** and *theoretical plate*.

***n:*** See *reduced velocity*.

***NanoLC:*** LC practiced with columns less than 100 µm in internal diameter; usually requires specialized instrumentation; often used in proteomic studies where sample is limited and sensitivity is required.

***Narrow-bore column:*** Columns of less than 2 mm id used in HPLC. Also called *microbore*.

***$N_{eff}$:*** See *effective theoretical plates*.

***Noise:*** See *baseline noise*.

***Non-aqueous reversed-phase chromatography:*** Refers to reversed-phase chromatography performed without water as a component of the eluent on a reversed-phase packing. Used for very non-polar compounds that cannot be eluted or are difficult to elute from a reversed-phase column with 100% methanol or acetonitrile. In these cases, solvent A should be acetonitrile, and solvent

B should be a stronger solvent such as tetrahydrofuran. Reversed-phase rules apply to non-aqueous reversed-phase chromatography; that is, the more non-polar the analyte, the greater the retention.

***Non-Polar:*** A non-polar molecule is one that the electrons are distributed more symmetrically and thus does not have an abundance of charges at the opposite sides. The charges all cancel out each other. Non-polar compounds, solvents or bonded phases readily dissolve in organic solvents, such as hexane, or prefer such solvents in place of water. Non-polar substances do not readily dissolve in water.

***Nonporous packing:*** Particles similar to porous-layer bead but with particle diameters in the sub-5 µm range; particles often are in the sub-2 µm $d_p$ range. Used for high-speed separations in short columns. Common column abbreviations include NPS, which refers to nonporous silica; NPR, which refers to nonporous resins; and NPZ, which refers to nonporous zirconia.

***Nonporous particle:*** Refers to a solid particle used as a support for a porous coated or bonded phase; pellicular particles are nonporous particles of large particle diameter (~40 µm). Nonporous silicas and resins with small particle diameters of less than 3 µm usually are microbeads with thin porous outer coatings of silica gel, bonded silica gel, or polymeric phase.

***Normal phase chromatography:*** A mode of chromatography performed when the stationary phase is more polar than the mobile phase. A typical normal phase system would be adsorption chromatography on silica gel or alumina using mixtures of less polar eluents such as hexane-diethethyl ether as a mobile phase. Also refers to the use of polar bonded phases such as cyano and alumina. Sometimes called straight-phase chromatography.

## O

***Octadecylsilane:*** The most popular reversed-phase in HPLC. Octadecylsilane phases are bonded to silica or polymeric packings. Both monomeric and polymeric phases are available. Abbreviated in column names as C18 and ODS.

***Octylsilane:*** A popular stationary phase in reversed-phase chromatography. Usually provides slightly less retention than the more popular C18. Both monomeric and polymeric phases are available. Abbreviated in column names as C8.

***ODS:*** See *octadecylsilane*.

***On-column detection:*** The column itself serves as the flow cell in HPLC or CE-CEC. Generally, the term used with fused-silica capillary applications. Outer polyimide layer is removed, an optical beam is directed through the capillary, and a measuring device such as a photomultiplier tube is located on the opposite side of the capillary.

***Online preconcentration:*** A precolumn is placed in front of the separation column to concentrate analytes before their separation. Different mechanisms - hydrophobic interaction, adsorption, or enzymatic reaction - may be used to retain analyte as a function of time. Then concentrated analytes are transferred to the separation column by a displacement process such as solvent elution or pH change.

***Open tubular columns:*** Small inner diameter columns (less than 100 µm) currently being investigated for use in HPLC, supercritical fluid chromatography (SFC), and CE. Stationary phases can be bonded on the internal walls of these small columns. The most frequently used column material is fused-silica tubing. Used very little in routine HPLC or SFC but frequently in CE.

***Optically active resin:*** Incorporation of optically active groups into an ion-exchange resin to allow separation of optically active isomers. Few commercially available resins for HPLC applications.

***Organic modifier:*** Water-miscible organic solvent added to an aqueous mobile phase to obtain separations in reversed-phase HPLC. Common organic modifiers are acetonitrile, methanol, isopropanol, and tetrahydrofuran.

***Orthogonality:*** Of two separation dimensions, such that the elution times in the two dimensions can be treated as statistically independent. Ideally, the two dimensions should have totally different retention mechanisms (e.g. reversed-phase and normal phase; ion-exchange and reversed-phase)

***Outlet Check Valve:*** See *Check Valve*

***Overload:*** In preparative chromatography the overload is defined as the sample mass injected onto the column at which efficiency and resolution begins to be effected if the sample size is increased further. See also ***sample capacity***.

## P

**Δ*p:*** See *head pressure*.

***Pa:*** See *pascal*.

***Packing:*** The adsorbent, gel, or solid support used in an HPLC column. Most modern analytical HPLC packings are less than 10 µm in average diameter, and 5 µm is the current favorite.

***Paired-ion chromatography:*** Same as *ion-pair chromatography*.

***Particle size ($d_p$):*** The average particle size of the packing in the LC column. A 5 µm $d_p$ column would

be packed with particles with a definite particle-size distribution because packings are never monodisperse. See also *monodisperse particles, particle size distribution*, and *polydisperse particles*.

***Particle size distribution:*** A measure of the distribution of the particles used to pack the LC column. In HPLC, a narrow particle size distribution is desirable. A particle size distribution of $d_p \pm 10\%$ would mean that 90% of the particles fall between 9 and 11 µm for an average 10 µm dp packing.

***Particulates:*** Generally refers to a small particle found in the mobile phase that can cause backpressure problems by lodging in frits; it can also refer to the small particles packed into HPLC columns

***Partition chromatography:*** Separation process in which one of two liquid phases is held stationary on a solid sup-port (stationary phase) while the other is allowed to flow freely down the column (mobile phase). Solutes partition them-selves between the two phases based on their individual partition coefficients. Liquid-liquid chromatography is an example; modern bonded-phase chromatography can be considered to be a form of partition chromatography in which one of the liquid phases is actually bonded to the solid support. Mechanistically partition chromatography implies that the solute becomes at least partially embedded within the stationary phase, which is impregnated, coated, or bonded to the substrate. In contrast to an adsorption process in which the solute does not penetrate into the retentive surface or interphase.

***Partition coefficient (K):*** The ratio of the equilibrium concentration of solute in the stationary phase relative to the equilibrium concentration of solute in the mobile phase. Also called distribution coefficient, $K_D$, and distribution constant ($K_c$).

***Pascal (Pa):*** A unit of pressure. 1 MPa is approximately 10 bar (atm) or 150 psi.

***Peak:*** The profile of an analyte compound as it elutes from a column through a detector; usually depicted on a visual output on a recorder or printer based on the detector's electrical response.

***Peak area:*** The area measured under a chromatographic peak; usually measured by an integrator or data system; the peak area is related to the amount of substance eluted in a peak.

***Peak capacity (n):*** The number of equally well-resolved peaks *(n)* that can be fit in a chromatogram between the holdup volume and some upper limit in retention. For $R = 1$, $n$ is given by the approximation $1 + 0.25[(N)1/2 \ln(1 + k_n)]$, where $R$ is the resolution, $N$ is the number of theoretical plates, and $k_n$ is the retention factor for peak $n$.

***Peak dispersion:*** See *band broadening*.

***Peak doublet:*** A split peak generally caused by a column void. Could be closely eluted compounds.

***Peak height:*** The height of a chromatographic peak as measured from the baseline to the peak apex; the peak height is related to the amount of substance eluted in a peak.

***Peak shape:*** Describes the profile of a chromatography peak. Theory assumes a Gaussian peak shape (perfectly symmetrical). Peak asymmetry factor describes shape as a ratio. See Figures 62 and 63, p. 115 and 116. See also *asymmetry*.

***Peak tracking:*** A way of matching peaks that contain the same compound between different experimental runs during method development. Relies upon detection parameters of each pure analyte. Diode array detectors and mass spectrometers are among the best detectors for peak tracking because of their specificity.

***Peak variance ($\sigma^2$):*** The second central moment of the peak about the retention time. For a Gaussian peak, the variance is the fundamental parameter controlling peak width. See Figure 63, p. 116. See also *Gaussian peak*.

***Peak volume:*** The total volume occupied by a chromatographic peak as it passes through the detector; $V_R = F \times W_b$. See Figure 63, p. 116.

***Peak width ($W_b$):*** Same as *bandwidth*. See Figure 63, p. 116.

***Pellicular packing:*** See *porous-layer bead*.

***Percent B solvent (% B solvent):*** Refers to the stronger solvent in a binary solvent mixture. % A solvent would be the weaker solvent analog.

***Perfusion chromatography:*** Refers to chromatography performed using particles with very large pores (4000-8000Å) called throughpores (megapores or gigapores). Eluent flows between the large pores and through the particles' 300-1000Å interconnecting pores, called diffusive pores. Best suited for the preparative separation of macromolecules.

***Permeability ($B_o$):*** Also called column permeability and specific permeability. A term expressing the resistance of the packed column to the flow of mobile phase. For a packed column, $B_o \approx d_p^2 \varepsilon^3 / [180(1-\varepsilon)^2] \; 5 \; d_p^2 / 1000$. A column with high permeability gives a low pressure drop.

***Permeation:*** Refers to the SEC process in which a solute can enter a mobile-phase-filled pore of the packing.

***Phase ratio ($\beta$):*** The relative amount of stationary to mobile phase in the column. In partition chromatography, $\beta = V_S / V_M$, where $V_S$ and $V_M$ are the volume, of stationary and mobile phase in the column, respectively. The retention factor is the product of the phase ratio and the partition coefficient.

***Phenyl phase:*** A popular non-polar bonded phase prepared by the reaction of dimethylphenylchloro- or alkoxysilane with silica gel. Reportedly has affinity for aromatic-containing compounds and does impart a different selectivity compared with alkyl-bonded phases.

***Pirkle column:*** Chiral, brush-type stationary phases based on 3,5-dinitrobenzoylphenylglycine silica used in the separation of a wide variety of enantiomers. Named after its developer, William Pirkle of the University of Illinois.

***Planar chromatography:*** A separation technique in which the stationary phase is present as or on a plane (IUPAC). Typical forms are paper and thin-layer chromatography.

***Plate height (H):*** See *HETP*.

***Plate number:*** See *column plate number*.

***Plate or plate number:*** Refers to theoretical plates in a packed column (IUPAC). See also ***theoretical plate***.

***Polar:*** Chemical polarity refers to the dipole-dipole intermolecular forces between the slightly positively-charged end of one molecule to the negative end of another or the same molecule. Molecular polarity is dependent on the difference in electronegativity between atoms in a compound and the asymmetry of the compound's structure. For example, water is polar because of the unequal sharing of its electrons. However, methane is considered non-polar because the carbon shares the hydrogen molecules uniformally.

***Polyacrylamide gel:*** Neutral hydrophilic polymeric packings used in aqueous SEC. Prepared by the copolymerization of acryl-amide with N,N'-methylenebisacrylamide.

***Polydisperse particles:*** Particles that have a substantial range of diameters (>10%).

***Polyethyleneimine:*** An anionic polymeric phase used to coat or bond onto silica or a polymeric packing. Most often used for separating proteins and peptides.

***Polymeric packings:*** Packings based on polymeric materials, usually in the form of spherical beads. Typical polymers used in LC are polystyrene-divinyl-benzene (PS-DVB), polydivinylbenzene, polyacryl-amide, polymethylacrylate, polyethylene-oxide, polydextran, and polysaccharide.

***Polymeric phase:*** Refers to a chemically bonded phase in which a polymer species is bonded to silica-based particles.

***Polystyrene-divinylbenzene resin (PS-DVB):*** The most common base polymer for ion-exchange chromatography. Ionic groups are incorporated by various chemical reactions. Neutral PS-DVB beads are used in reversed-phase chromatography. Porosity and mechanical stability can be altered by varying the cross-linking through the DVB content.

***Poppe Plot:*** A kinetic plot named after Prof. Hans Poppe [J. Chromatogr. A 778, 3 (1997)], University of Amsterdam, the Netherlands, where the plate time *[log (t0/N)]* is depicted as a function of the number of theoretical plates *(N)* in order to assess the limits of column performances as a function of particle size, column pressure drop, etc.

***Pore diameter:*** Same as *mean pore diameter*.

***Pore size:*** The average size of a pore in a porous packing. Its value is expressed in angstroms or in nanometers. The pore size determines whether a molecule can diffuse into and out of the packing. See also *mean pore diameter*.

***Pore volume:*** The total volume of the pores in a porous packing, usually expressed in milliliters per gram. More appropriately called the specific pore volume. It is measured by the BET method of nitrogen adsorption or by mercury intrusion porosimetry in which mercury is pumped into the pores under high pressure.

***Porosity:*** For a porous substrate, the ratio of the volume of the pores in a particle to volume occupied by the particle. The pore volume is a measure of the porosity and is expressed in milliliters per gram.

***Porous-layer bead:*** A small glass bead coated with a thin layer of stationary phase. The thin layer can be an adsorbent, resin, or a phase chemically bonded onto the adsorbent. These packings were among the first to be used in HPLC. They had 20-40 µm particle sizes, which were larger than the microparticulate packings of today, but were easy to pack and provided adequate efficiency. Also called controlled surface porosity supports and pellicular materials.

***Porous particle:*** Refers to column packing particles that possess interconnecting pores of specified diameter and pore volume. For HPLC applications, analysts generally use porous particles with diameters less than 10 mm. Larger particles are used in preparative-scale chromatography because of lower cost and higher column permeability.

***Porous polymer:*** A packing material, generally spherical, that is based on organic polymers or copolymers. Popular examples include PS-DVB, polyacrylates, polydextrans, polyacrylamides, and polybutadienes.

***Precolumn:*** A small column placed between the pump and the injector. It removes particulate matter that may be present in the mobile phase, presaturates the mobile phase with stationary phase or with dissolved substrate to prevent a loss of stationary phase or dissolution of the analytical column, and chemically absorbs substances that might interfere with the separation. Its volume has little effect on isocratic elution but contributes a delay to the

gradient in gradient elution.

***Precolumn Filter:*** A filter used between the injector and the column (or guard column) to keep unwanted sample components from reaching the column; sometimes called inline filter, occasionally inlet filter.

***Preconcentration:*** See *trace enrichment*.

***Preparative chromatography:*** Refers to the process of using LC as a technique for the isolation of a sufficient amount of material for other experimental or functional purposes. For pharmaceutical or biotechnological purifications, large columns of several feet in diameter can be used for multiple grams of material. For isolating a few micrograms of valuable natural product an analytical column with a 4.6 mm id can be used. Based on the intended need of the chromatographer, both size of columns are preparative chromatographic approaches.

***Pressure (pressure drop) ($\Delta p$):*** See *head pressure*.

***Pressure injection:*** Pressure-induced injection in CE. Using pressure or vacuum to inject nanoliter-level volumes of sample into a capillary column. Best for narrow-bore capillaries that have inner diameters less than 10 µm. A version of hydrostatic injection.

***Process-scale chromatography:*** Refers to the use of LC at the industrial-scale level outside of laboratories. Generally requires specially designed columns (usually with diameters > 5 cm), recoverable solvents, low-cost packings (larger and irregular-shaped particles), and overloaded operating conditions compared with laboratory-scale HPLC.

***Programmed-temperature chromatography:*** Varying temperature during a chromatographic run. Seldom used in LC.

***PS-DVB:*** See *polystyrene-divinylbenzene resin*.

***Pulsating flow:*** Flow originating from a reciprocating pump. Normally, the pulses are dampened by a pulse damper, an electronic pressure feedback circuit, or an active damper pump head. Detectors such as electrochemical and refractive index detectors are greatly affected by flow pulsations.

## Q

***Quaternary methyl amine:*** A strong anion-exchange functionality popular in resin-based packings. Usually supplied in chloride form.

***Quaternary mobile phase:*** A mobile phase comprising four solvents or buffers.

***Quaternary-solvent mobile phase:*** A mobile phase consisting of four separate solvents which allow for fine tuning mobile phase composition; most often this mobile phase is delivered by a low-pressure quaternary pump.

## R

***r:*** See *relative retention*.

***Radial compression:*** Using radial pressure applied to a flexible wall column to reduce wall effects.

***Radial diffusion-dispersion:*** Diffusion-dispersion across the LC column in a radial direction. If the sample is injected into the exact center of a column, it will spread not only in a longitudinal direction as it moves down the column but also radially, which allows the solute to reach the wall region where the eluent velocity is different than in the center of the column.

***Re:*** See *Reynolds number*.

***Recovery:*** The amount of solute or sample that is eluted from a column relative to the amount injected. Excellent recovery is important for good quantitation, preparative separations, especially biomolecules, and good peak shape and resolution. Reasons for inadequate recovery can be solute interaction with active sites on the packing, column frits, and column tubing. Compound decomposition during the separation process also can affect recovery.

***Recycling chromatography:*** A technique in which the column effluent is recirculated onto the head of the column to take advantage of extended column length. Can be performed on a single column by passing the effluent through the pump again. An alternative technique uses two columns connected by a switching valve where the effluent of one column is directed onto the head of the other column. Very seldom used in HPLC and then only in exclusion chromatography.

***Reduced plate height (h):*** Used to compare efficiencies of different columns; $h = H/d_p$, where H is the height equivalent to a theoretical plate and $d_p$ is the particle diameter. An h value of 2 or less at the optimum velocity is considered to be a well-packed HPLC column.

***Reduced velocity (v):*** Used with the reduced plate height to compare different packed chromatographic columns. It relates the solute diffusion coefficient ($D_M$) in the mobile phase to the particle size of the column packing ($d_p$); $v = ud_p/D_M$, where u is the average interstitial mobile-phase linear velocity. See also *Knox equation*.

***Refractive index peak:*** A pseudo-peak normally found near the dead volume that results from the refractive index sensitivity of absorbance and other detectors. See also *vacancy peak*.

***Regeneration:*** Regenerating the packing in the column to its initial state after a gradient elution. Mobile phase is passed through the column stepwise or in a gradient. The stationary phase is restored or solvated to its initial

condition. In ion-exchange, regeneration involves replacing ions taken up in the exchange process with the original ions, which occupied the exchange sites. Regeneration also can refer to bringing any column back to its original state; for example, removing impurities with a strong solvent.

***Relative retention (r):*** Retention relative to a standard; $r = t_R'/t_{R(st)}' = k/k_{st}$, where $t_R'$ is the adjusted retention time of the component of interest, $t_{R(st)}'$ is the adjusted retention time of the standard, $k$ and $k_{st}$ are the corresponding retention factors. For two adjacent peaks, a expresses the relative retention and is called separation factor (formerly called selectivity or selectivity factor); calculated as $\alpha = t_{R2}'/t_{R1}' = k_2/k_1$, where $t_{R2}'$ and $t_{R1}'$ are the adjusted retention times of peaks 2 and 1, respectively, and $k_2$ and $k_1$ are the corresponding retention factors.

***Residual silanols:*** The silanol (-Si-OH) groups that remain on the surface of a packing after chemically bonding a phase onto its surface. These silanol groups, which may be present in very small pores, may be inaccessible to a reacting bulky organosilane (such as octadecydimethylchlorosilane) but may be accessible to small polar compounds. Often they are removed by endcapping with a small organosilane such as trimethylchlorosilane. See also ***endcapping***.

***Resin:*** A solid polymeric packing used in ion-exchange separations. The most popular resins are PS-DVB copolymers with particle sizes less than 10 µm. Ionic functionality is incorporated into the resin.

***Resolution ($R_s$):*** Ability of a column to separate chromatographic peaks; $R_s = (t_{R2} - t_{R1})/[(w_{b1} + w_{b2})/2]$, where $t_{R2}$ and $t_{R1}$ are the retention times of the two peaks and $w_b$ is the baseline width of the peaks. It usually is expressed in terms of the separation of two peaks. A value of 1 is considered to be the minimum for a measurable separation to occur and to allow good quantitation. A value of 0.6 is required to discern a valley between two equal-height peaks. A value of 1.5 is considered sufficient for baseline resolution for two peaks of equal height. Values of 1.7 or greater generally are desirable for rugged methods. See Figure 63, p. 116.

***Resolution equation:*** Also called the general resolution equation and the Purnell equation; $R = 4N^{1/2}[(\alpha-1)/\alpha][k/(1+k)]$, where $N$ is the efficiency, $\alpha$ is the separation factor, and $k$ is the retention factor.

***Retention factor (k):*** The period of time that the sample component resides in the stationary phase relative to the time it resides in the mobile phase. It is calculated from the adjusted retention time divided by the holdup time; $k = (t_R - t_M)/t_M$, where $t_R$ is retention time for the sample peak and tM is the retention time for an unretained peak. (Formerly, $k'$ was used, and it was called the capacity factor or the capacity ratio.)

***Retention time ($t_R$):*** Also called the total retention time. The time between injection and the appearance of the peak maximum. The total retention volume ($V_R$) is determined by multiplying the retention time by the flow rate. The adjusted retention time ($t_R'$) adjusts for the column void volume; $t_R' = t_R - t_M$. It usually is measured from the point of injection to the apex of the peak, but it should be measured to the center of gravity of the peak for asymmetric peaks.

***Retention volume ($V_R$):*** The volume of mobile phase required to elute a substance from the column; $V_R = Ft_R$ *or* $V_R = V_M + K_DV_S$, where $V_M$ is the void volume, $K_D$ is the distribution coefficient, and $V_S$ is the stationary-phase volume. See also ***retention time***.

***Reversed-phase chromatography:*** The most frequently used mode in HPLC. Uses low-polarity packings such as octadecyl- or octylsilane phases bonded to silica or neutral polymeric beads. The mobile phase usually is water or water-miscible organic solvents such as methanol or acetonitrile. Elution usually occurs based on the relative hydrophobicity or lipophilicity of the solutes. The more hydrophobic, the stronger the retention. The greater the water solubility of the analyte, the less it is retained. The technique has many variations in which various mobile phase additives impart a different selectivity. For example, adding a buffer and a tetraalkylammonium salt to an anion analysis would allow ion-pairing to occur and generate separations that rival those of ion-exchange chromatography. More than 90% of HPLC analysts use reversed-phase chromatography.

***Reynolds number (Re):*** The ratio of viscous to inertial energy of the moving fluid. A measurement of flow in a smooth unpacked pipe; $Re = ud/(\eta/\rho)$, where $u$ is the average velocity (in centimenters per second), $d$ is the pipe diameter, $\eta$ is the viscosity (in grams per centimeter seconds), and $\rho$ is the density (in grams per cubic centimeters). At low *Re*, viscous friction dominates and controls fluid motion, making it slow and steady. In an unpacked tube, flow becomes fully turbulent when *Re* exceeds 4200. In a packed bed, $u$ is replaced with the average interstitial velocity and d with the average particle diameter. Flow becomes turbulent in a packed bed at *Re* values greater than approximately 10 but is not fully turbulent until *Re* exceeds 100-200.

***$R_s$:*** See ***resolution***.

## S

***S:*** The solvent-strength parameter in reversed-phase chromatography. The solute-dependent slope of a plot of $log_{10}\ k$ versus volume fraction of organic modifier. $S$ varies

with modifier type, stationary phase, and temperature.

***SAX:*** See *strong anion-exchanger*.

***SCX:*** See *strong cation-exchanger*.

***WAX:*** See *weak anion-exchanger*.

***WCX:*** See *weak cation-exchanger*.

***$\sigma^2$:*** See *peak variance*.

***Salting-out effect:*** Using a high concentration salt buffer in the mobile phase to cause a low-polarity analyte to have a decreased solubility in water and therefore precipitate or come out of solution. Most often used for the hydrophobic interaction chromatography of proteins when proteins are precipitated first at high salt concentrations and then eluted by gradual dilution using reversed gradient elution.

***Sample capacity:*** Refers to the amount of sample that can be injected onto an LC column without overloading. Often expressed as grams of sample per gram of packing. Overloading is defined as the sample mass injected when the column efficiency decreases by 10% from its normal value; sometimes called sample loading.

***Sampling Rate:*** See *Data acquisition rate*.

***Saturator column:*** See *precolumn*.

***Scalability:*** In going from analytical to preparative chromatography, refers to the reproducibility of results on columns of different internal diameters when using the same particle size and bonded phase; normally a larger diameter column is used to increase capacity; a linear scale-up process minimizes time required to optimize preparative separations.

***SEC:*** See *size exclusion chromatography* and *steric exclusion chromatography*.

***Sedimentation:*** A technique used for the sizing of resins for ion-exchange chromatography. A broad distribution of beads are placed in a solvent, often water, in a container that is affixed to a stationary surface. Based on particle size and particle density, the beads will settle at different velocities into a gradient of sizes, and the fraction of interest is removed. Workers can obtain very narrow cuts of particle size by sedimentation.

***Selectivity or selectivity factor ($\alpha$):*** Old term replaced by the separation factor. Sometimes called relative retention.

***Selectivity coefficient ($k_{A/B}$):*** In ion-exchange chromatography, the equilibrium coefficient obtained by applying the law of mass action to an ion-exchanger and characterizing the ability of an ion-exchanger to select two ions present in the same solution using electroosmotic flow. For example, the exchange of $Na^+$ *for* $H^+$ $k_{Na/H} = ([Na]_S[H]_M)/([Na]_M[H]_S)$.

***Semi-preparative chromatography:*** Refers to preparative LC performed on analytical (4-5 mm id) or slightly larger (6-10 mm id) columns. Normal injection size would be milligram- to low-gram-size samples.

***Separation factor ($\alpha$):*** A thermodynamic factor that is a measure of relative retention of two substances. Formerly called selectivity or selectivity factor. The relative retention; $\alpha = t_{R2}'/t_{R1}' = k_2/k_1$, where $t_{R2}'$ and $t_{R1}'$ are the adjusted retention times of peaks 2 and 1, respectively, and $k_2$ and $k_1$ are the corresponding retention factors.

***Separation impedance (E):*** A figure of merit developed by John Knox to compare the efficiency of two chromatographic systems that normalize for both analysis time and pressure drop; $E = t_R\Delta_p/N^2v(1 + k)$, where $t_R$ is the retention time, $D_p$ is the pressure drop, $N$ is the efficiency, $v$ is the reduced velocity, and $k$ the retention factor. The lower the value of $E$, the better the system.

***SFC:*** See *supercritical fluid chromatography*.

***Silanol:*** The Si-OH group found on the surface of silica gel. Silanols vary in strength depending upon their location, relationship to each other, and the metal content of the silica. The strongest silanols are acidic and often lead to undesirable interactions with basic compounds during chromatography.

***Silanophile:*** A compound that has high affinity for active or acidic silanol groups on a silica surface. Usually a strongly basic amine.

***Silica gel:*** The most widely used HPLC packing. It has an amorphous structure, is porous, and is composed of siloxane and silanol groups. It is used in all modes of LC as a bare packing for adsorption, as the support for liquid-liquid chromatography or for chemically-bonded phases, and as an SEC packing with various pore sizes. Microparticulate silicas of 3, 5, and 10 µm average particle diameter are used in HPLC. Compared with irregular silicas, spherical silicas are preferred in modern analytical HPLC columns because of their packing reproducibility and lower pressure drops. Sometimes called silica.

***Siloxane:*** The *Si-O-Si* bond. A principal bond found in silica gel or a silylated compound or bonded phase. Stable, except at high pH values. Has little effect on the HPLC separation.

***Silylation:*** The reaction process of an organochloro- or organoalkoxysilane with a compound that contains an reactive group. In LC, it refers to the process of derivatizing the solute before chromatography to make it detectable or to prevent unwanted stationary-phase interactions. It also can refer to the process of adding a chemically-bonded phase to a solid support or deactivating the packing to reduce surface activity.

***Simulated moving bed:*** A chromatographic system involving a series of columns and valves set up to simulate the countercurrent movement of the mobile and stationary

phases and enable the continuous removal of product and reapplication of sample. A complex form of recycle chromatography used in preparative-scale chromatography.

***Size exclusion chromatography (SEC):*** Same as *steric exclusion chromatography*.

***Slurry packing:*** The technique most often used to pack HPLC columns with microparticles. The packing is suspended in a slurry of approximately 10% *(w/v)* and rapidly pumped into the empty column using special high pressure pumps.

***Snyder $\varepsilon^o$:*** Solvent-strength parameter in adsorption chromatography. The energy of solvent adsorption per unit surface area occupied by the solvent.

***Soap chromatography:*** The earlier name for ion-pair chromatography. Long-chain soaps or detergents were used as the mobile phase additives.

***Sol gel:*** Silica gel formed by the aggregation of silica sol. Generates Type B silica gel with lower surface acidity, lower trace metal, lower surface area and porosity, and greater high pH stability than older Type A silica gels.

***Solid phase extraction (SPE):*** A technique for sample preparation using a 20-40 µm $d_p$ solid phase packing contained in a small plastic cartridge, disk, or in the wells of a 96-well flowthrough plate. The solid stationary phases used are identical to HPLC packings. Although related to chromatography, the principle of SPE is different and is sometimes called digital chromatography. The process as most often practiced requires four steps: conditioning the sorbent, adding the sample, washing away the impurities, and eluting the sample in as small a volume as possible with a strong solvent.

***Solid support:*** Same as *support*.

***Solute:*** See also *analyte*.

***Solvent:*** The liquid used to dissolve a sample for injection into an HPLC column or CE capillary. Sometimes refers to the mobile phase used. See also ***eluent***.

***Solvent demixing:*** Occurs when two solvents with very different strengths – A is the weak solvent, and B is the strong solvent – are used with unmodified silica or alumina. The strong solvent (B) will be adsorbed preferentially by the active surface of the stationary phase until it is saturated; until this occurs, the weak solvent (A) will be enriched or demixed as it travels down the column. Eventually, when the entire column is saturated with solvent B, this solvent will be eluted, mixed with solvent A at the initial strength, and sample components will be eluted with the sudden change in solvent strength.

***Solvent selectivity:*** Ability of a solvent to influence selectivity. For example, a change in solvent strength from 5% to 10% solvent B or a change from methanol to acetonitrile as the reversed-phase organic modifier will affect band spacing.

***Solvent-selectivity triangle:*** A useful guide for choosing among different solvents for changing band spacing. Solvent selectivity is dependent on dipole moment, acidity, and basicity of the solvent molecule.

***Solvent strength:*** Refers to the ability of a solvent to elute a particular solute or compound from a column. Snyder described this quality for linear elution adsorption chromatography (liquid-solid chromatography) on alumina and quantitatively rated solvents in an eluotropic series. Less-extensive data are available for silica and carbon adsorbents. See also ***Snyder $\varepsilon^o$***.

***Sorb:*** The process of being retained by a stationary phase when the retention mechanism – adsorption, absorption, or partitioning – is unclear.

***Sorbent:*** Refers to a packing used in LC. Common sorbents are polymers, silica gel, alumina, titania, zirconia, and chemically modified materials.

***SPE:*** See *solid phase extraction*.

***Specific surface area:*** The surface area of an LC packing based on measurement by an accepted technique such as the BET method using nitrogen adsorption.

***Spherical packing:*** Refers to spherical, solid packing materials. In analytical HPLC, spherical packings generally are preferred over irregular particles, but irregular particles often are used in preparative work because of their lower cost.

***Standards:*** A sample which contains known quantities of the compounds of interest. Standards are used to help identify sample peaks by comparing the time in which they elute to the retention times obtained through the injection of the sample under the same conditions. For quantitation, external standards are compounds that are used to construct calibration curves of detector output (peak area or peak height) vs. concentration; the concentration of unknowns are determined by fitting the detector output to the calibration curve. Internal standards are compounds of known concentration with different retention times that are added to the sample and relative detector responses between the internal standard and the unknown are compared in order to quantitatively measure unknown compounds.

***Stagnant mobile phase:*** The fraction of the mobile phase contained within the pores of the particle.

***Stationary phase:*** The chromatographically retentive immobile phase involved in the chromatographic process. The stationary phase in LC can be a solid, a bonded, an immobilized or a coated phase on a solid support or a wall-coated phase. The stationary phase often characterizes the LC mode. For example, silica gel is used in adsorption chromatography and octadecylsilane bonded phase is used in reversed-phase chromatography.

***Stationary zone:*** To be distinguished from the stationary

phase. The stationary zone includes the stagnant mobile phase and the chromatographically active stationary phase.

***Stepwise elution:*** Using eluents of different compositions during a chromatographic run. These eluents are added in a stepwise manner with a pump or a selector valve. Gradient elution is the continuous version of changing solvent composition.

***Steric exclusion chromatography:*** A major mode of LC in which samples are separated by virtue of their size in solution. Also known as size exclusion chromatography, gel permeation chromatography, gel filtration chromatography, and gel chromatography. Steric exclusion chromatography is used most often for polymer separation and characterization.

***Sterically protected bonded phase:*** Bonded phase that has sterically protecting bulky functional groups such as isopropyl and isobutyl surrounding a siloxane covalent surface bond. Prevents attacks on siloxane bond, catalyzed hydrolysis, and loss of bonded phase at pH levels less than 3.

***Straight phase chromatography:*** Same as *normal phase chromatography*.

***Strong anion-exchanger:*** Anion-exchange packing with strongly basic ionogenic groups such as tetraalkylammonium groups.

***Strong cation-exchanger:*** Cation-exchange packing with strongly acidic ionogenic groups such as sulfonate groups.

***Strong solvent:*** In general, refers to a solvent which is a good solvent for a chemical compound; in chromatography, refers to the mobile phase constituent that provides a higher solvent strength that causes an analyte to elute more quickly from the column; in a water-acetonitrile binary solvent system for reversed-phase LC, acetonitrile would be considered to be the strong solvent.

***Sub-2 µm:*** A term that refers to the use of porous packings below 2 µm average particle diameter; current products vary from 1.5- to 2.0 µm

***Sulfonyl cation-exchanger:*** A strong cation-exchange functionality found in resin-based packings, usually *propyl*$SO_3H$. May come in cationic forms such as sodium, ammonium, silver, and calcium.

***Supercritical fluid chromatography (SFC):*** A technique that uses a supercritical fluid as the mobile phase. The technique has been applied to the separation of substances that cannot be handled effectively by LC (because of detection problems) or GC (because of the lack of volatility). Examples include separations of triglycerides, hydrocarbons, and fatty acids. GC detectors and HPLC pumps have been used together in SFC.

***Superficial velocity (us):*** The hypothetical velocity that a mobile phase would have if the same column were operated unpacked but with the same flow rate; $u_s = F/A_c$, where $F$ is the flow rate and $A_c$ is the cross-sectional area of the tube.

***Superficially porous packing:*** Same as *porous-layer bead*.

***Support:*** Refers to solid particles. A support can be naked, coated, or have a chemically-bonded phase in HPLC. Normally the solid support doesn't contribute to the chromatographic process.

***Suppressor column:*** Refers to the column placed after the ion-exchange column. Its purpose is to remove or suppress the ionization of buffer ions so that sample ions can be observed in a weakly conducting background with a conductivity detector. Sometimes membrane suppressors are used rather than a column.

***Surface area:*** Refers to the total area of the solid surface in an adsorbent as determined by an accepted measurement technique such as the BET method, which uses nitrogen adsorption. The surface area of a typical porous adsorbent such as silica gel can vary from less than 100 to 600 $m^2/g$.

***Surface coverage:*** Usually refers to the mass of stationary phase per unit area bonded to an LC support. Often expressed in micromoles per square meter of surface. Sometimes the percent-age of carbon is given as an indicator of surface coverage.

***Swelling-shrinking:*** Process in which resins and gels increase or decrease their volume because of their solvent environment. Swelling is dependent upon the degree of cross-linking; low-cross-linking resins will swell and shrink more than highly cross-linked resins. If swelling occurs in a packed column blockage, increased backpressure can occur, and column efficiency can be affected.

## T

***Tailing:*** The phenomenon in which a normal Gaussian peak has an asymmetry factor greater than 1. The peak will have an extended trailing edge. Tailing is caused by packing sites that have both a stronger-than-normal retention for the solute and slower desorption kinetics. A typical example of a tailing phenomenon would be the strong adsorption of amines on the residual silanol groups of a low-coverage reversed-phase packing at intermediate pH values. Tailing also can result from injecting an excessive mass or sample, badly packed columns, excessive extracolumn volume, poor fittings, excessive detector volume, and slow detector response. See Figure 62, p. 115.

***Tailing factor:*** U.S. Pharmacopeia measure of peak

asymmetry defined as the ratio of the peak width at 5% of the apex to twofold the distance from the apex to the 5% height on the short time side of the peak. Greater than unity for tailed peaks. See also ***asymmetry factor***.

***Temperature programming:*** Changing column temperature as a function of time during the separation. Rarely used in HPLC; if so, usually in a stepwise manner.

***Ternary mobile phase:*** Mobile phase that is a mixture of three solvents or buffers.

***Theoretical plate (N):*** A concept described by Martin and Synge. Relates chromatographic separation to the theory of distillation. Length of column relating to this concept is called height equivalent to a theoretical plate. See also ***HETP***. Plates are calculated as $N = 16(V_R/w^b)^2 = 16\ (t_R/w^b)^2$, where $V_R$ is the retention volume, $w^b$ is the width at the peak base, and $t_R$ is the retention time. See also ***N***.

***Thermally tuned tandem column chromatography:*** A form of LC in which two columns with distinctly different selectivities are placed in tandem and operated at two temperatures to optimize the resolution or analysis speed. Both columns use a common eluent, and the entire sample passes through both columns and is detected with a single detector. It is not a two-dimensional technique because each sample component provides only one peak.

***Titania:*** An uncommon adsorbent used in adsorption chromatography.

***$t_m$:*** See *migration time*.

***$t_M$:*** Holdup time.

***Tortuosity or tortuosity factor:*** A packed-column property that controls the inhibition of longitudinal diffusion of the solute as it diffuses along the column axis. The B term in the van Deemter equation is proportional to the tortuosity. See also ***B term***, $\gamma$, and *molecular diffusion term*.

***Total mobile-phase volume ($V_t$):*** The total volume of mobile phase in an SEC column. Also known as totally included volume. Same as $V_M$.

***Total permeation volume ($V_p$):*** The retention volume of an SEC packing in which all molecules smaller than the smallest pore will be eluted. In other words, all molecules totally permeate all of the pores at $V_p$ and are eluted as a single peak. Same as $V_M$.

***Total porosity ($\varepsilon_T$):*** Ratio of the total volume of mobile phase in the column to the total column volume; $\varepsilon = V_M/V_c = \varepsilon_e + \varepsilon_i\ (1 - \varepsilon_e)$; where $V_M$ is the mobile phase volume, $V_c$ is the column volume, $\varepsilon_e$ is the interstitial porosity, and $\varepsilon_i$ is the intraparticle porosity.

***Totally porous packing:*** The stationary phase is a porous matrix, and solutes penetrate the porous matrix to interact with the stationary phase.

***$t_R$:*** See *retention time*.

***$t_R'$:*** See *adjusted retention time* and *retention time*.

***Trace enrichment:*** Technique in which trace amounts of compounds are retained on an HPLC or precolumn packing out of a weak mobile phase or solution and then are eluted by adding a stronger mobile phase in a concentrated form. The technique has been applied most successfully in the concentration of trace amounts of hydrophobic compounds such as polynuclear aromatic hydrocarbons from water using a reversed-phase packing. A strong solvent such as acetonitrile will elute the enriched compounds.

***Triethylamine:*** A very common additive used to block silanol groups in reversed-phase chromatography when separating basic analytes.

***Trifluoroacetic acid:*** A very common additive in reversed-phase chromatography for peptides and proteins.

***Tryptic digestion:*** A method for selectively and reproducibly dissecting peptide chains of proteins to yield a characteristic pattern of smaller units that enables analysis of the parent protein by gradient elution reversed-phase LC.

***Turbulence:*** The state in which fluid velocity fluctuates randomly at a point. See also ***Reynolds number*** and *turbulent flow*.

***Turbulent flow:*** A form of fluid motion in which the flow ceases to be smooth and steady and becomes chaotic and fluctuates with time. It is characterized by a pressure drop significantly higher than what would be extrapolated from the laminar region to achieve the same volumetric flow rate.

***Turbulent flow chromatography:*** Chromatography performed at very high linear velocities with large particles under conditions using high Reynolds numbers. At these conditions, the *H* versus *v* curves show a decrease in *H* as *v* increases. See Figure 63, p. 116.

***$t_w$:*** See *bandwidth*.

***Two-dimensional chromatography:*** A procedure in which part or all of the separated sample components are subjected to additional separation steps. It can be performed by conducting a particular fraction eluted from the first column into a second column or system that has a different separation characteristic. It includes techniques such as two dimensional TLC using two eluent systems in which the second eluent is applied after rotating the plate through 90°. It also includes LC followed by GC and one LC mode followed by a different mode such as reversed-phase chromatography followed by SEC. See also *multidimensional chromatography*.

***Type A silica:*** Silica gel formed by gelling soluble

silicates. Generally has higher acidity, higher surface area and porosity, more trace metals, and poorer high-pH stability than Type B silicas.

***Type B silica:*** See *sol gel*.

***$t_0$:*** See *void time*.

## U

***u:*** See *linear velocity* and *velocity*.

***$u_e$:*** See *interstitial velocity*.

***$u_M$:*** See *mobile phase velocity*.

***$u_s$:*** See *superficial velocity*.

***$u_z$:*** See *zone velocity*.

***UHPLC:*** Refers to Ultra High Pressure Liquid Chromatography; often loosely used for any separation performed over the pressures of conventional pumps (400 bar); original meaning was for separations in the 20,000 psi+ range.

## V

***Vacancy chromatography:*** Technique in which a mobile phase additive causes a positive detector signal output. When a solute is eluted from the column, it dilutes the signal and generates a negative peak or vacancy. The technique has been applied primarily to single-column ion chromatography in which mobile phases such as citrate and phthalate buffers absorb in the UV region. When a non-absorbing anion is eluted, it dilutes the UV-absorbing background and causes a negative peak; the detector output leads usually are reversed so that the chromatogram looks normal. It also has been used in CE for detection.

***van Deemter equation:*** An equation used to explain band broadening in chromatography. The equation represents the height of a theoretical plate (HETP) and has three terms. The A term describes eddy dispersion or diffusion that results from axial velocity heterogeneity. The B term is for the contribution of molecular diffusion or longitudinal diffusion of the solute while passing through the column. The C term is the contribution from interphase mass transfer, which allows for the finite rate of transfer of the solute between the stationary phase and mobile phase. In its simplest representation, $h = A + B/v + Cv$. See also ***reduced plate height*** and ***reduced velocity***.

***$V_c$:*** See *column volume*.

***$V_d$:*** See *dead volume*.

***$V_e$:*** See *interstitial volume*.

***Velocity (u):*** Same as *linear velocity*.

***$v_{eo}$:*** See *electroosmotic flow*.

***$V_i$:*** See *intraparticle volume*.

***Viscosity ($\eta$):*** Also called mobile phase viscosity. The viscosity of the mobile phase varies with the temperature of the column. Low-viscosity mobile phases generally provide better efficiency than less-viscous ones because diffusion coefficients are inversely related to solvent viscosity. For example, column efficiency is higher in reversed-phase chromatography with acetonitrile as an organic modifier than with isopropanol, which is more viscous. Column backpressure is directly proportional to solvent viscosity.

***$V_M$:*** See *holdup volume*. Also *mobile phase volume*.

***Void:*** The formation of a space or gap, usually at the head of the column, caused by a settling or dissolution of the column packing. A void in the column leads to decreased efficiency and loss of resolution. Even a small void can be disastrous for small particle microparticulate columns. The void sometimes can be filled with glass beads or the same porous packing used in a column.

***Void time ($t_0$):*** The elution time of an unretained peak; also called the dead time and the holdup time ($t_M$). The void volume is determined by multiplying the void time and the flow rate.

***Void volume ($V_M$):*** The total volume of mobile phase in the column; the remainder of the column is taken up by packing material. This volume can be determined by injecting an unretained substance. Also called dead volume. The symbol $V_0$ is often used to denote the void volume. This is valid only for a column packed with non-porous particles. $V_0$ is valid when used to denote the excluded volume ($V_e$) in SEC.

***$V_p$:*** See *total permeation volume*.

***$V_R$:*** See *retention volume* and *elution volume*.

***$V_R'$:*** See *adjusted retention volume*.

***$V_t$:*** See *total mobile phase volume*.

***$V_o$:*** See *exclusion volume*.

## W

***Wall effect:*** The consequence of a looser packing density near the walls of a rigid HPLC column. The mobile phase has a tendency to flow slightly faster near the wall because of the increased local permeability. The solute molecules near the wall are carried along faster than the average of the solute band, and, consequently, band spreading results and the column loses efficiency.

***$w_b$:*** See *peak width*.

***Weak anion-exchanger:*** Anion-exchange packing with weakly basic ionogenic groups such as amino diethylamino ethyl groups.

***Weak cation-exchanger:*** Cation-exchange packing with weakly acidic ionogenic groups such as carboxyl groups.

***Weak solvent:*** In general, refers to a solvent which is a poor solvent for a chemical compound; in chromatography, refers to the mobile phase constituent that provides a low solvent strength that causes an analyte to elute more slowly from the column in a water-acetonitrile binary solvent system for reversed-phase LC, water would be considered to be the weak solvent.

***Wilke-Chang equation:*** A semi-empirical equation used to estimate diffusion coefficients in liquids as a function of solute molecular size and solvent viscosity.

## X

***Xerogels:*** Gels used in SEC that swell and shrink in different solvents. Also refers to silica-based packings that are prepared from acidification of soluble silicates to generate an amorphous, high surface area, high-porosity, rigid particle.

## Z

***Zero dead volume:*** Any fitting or component that has no volume that is unswept by the eluent.

***Zirconia:*** Porous zirconium oxide. Used as a chromatographic sorbent, usually coated or bonded with polymeric organic phase.

***Zone:*** See *band*.

***Zone velocity ($u_z$):*** The velocity at which the solute zone travels; $u_z = u_M/(1 + k) = L/t_R$, where $u_M$ is the mobile phase velocity, $k$ is the retention factor, $L$ is the column length, and $t_R$ is the retention time.

***Zwitterions:*** Compounds that carry both positive and negative charges in solution.

## Glossary references


***1:*** R.E. Majors and P.W. Carr, *LCGC **19 (2)*** 124-162 (2001).

***2:*** 'Nomenclature for Chromatography', *Pure and Appl. Chem. **65 (4)***, 819-872 (1993).

***3:*** J.P. Foley and J.G. Dorsey, *Anal. Chem. **55***, 730 - 737 (1983)

***4:*** L.R. Snyder, P.W., Carr, and S.C. Rutan, *J. Chromatogr.* A ***656***, 537 (1993).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## Acknowledgements

Special thanks to the Agilent chromatographers who contributed to this book:

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# Index

## Index of factors affecting performance

# Index of products

**Product and Ordering Information**

On the following pages, we've chosen a few products to highlight and provide quick part number reference for you.

# Agilent LC Columns

Poroshell 120 columns help you get more efficiency and analytical speed from any HPLC/UHPLC instrument. See pp. 15 – 17 for more information.

| Poroshell 120 - Particle Size 2.7 µm | | | | | | |
|---|---|---|---|---|---|---|
| | Size (mm) | Eclipse Plus EC-C18 (USP L1) | Eclipse Plus EC-C8 (USP L7) | Eclipse Plus EC-CN | StableBond SB-C18 (USP L1) | StableBond SB-C8 (USP L7) |
| Analytical | 4.6 x 150 | 693975-902 | 693975-906 | 693975-905 | 683975-902 | 683975-906 |
| Analytical | 4.6 x 100 | 695975-902 | 695975-906 | 695975-905 | 685975-902 | 685975-906 |
| Analytical | 4.6 x 75 | 697975-902 | 697975-906 | | 687975-902 | |
| Analytical | 4.6 x 50 | 699975-902 | 699975-906 | 699975-905 | 689975-902 | 689975-906 |
| Analytical | 4.6 x 30 | 691975-902 | 691975-906 | | 681975-902 | |
| Solvent Saver | 3.0 x 150 | 693975-302 | 693975-306 | 693975-305 | 683975-302 | 683975-306 |
| Solvent Saver | 3.0 x 100 | 695975-302 | 695975-306 | 695975-305 | 685975-302 | 685975-306 |
| Solvent Saver | 3.0 x 75 | 697975-302 | 697975-306 | | 687975-302 | |
| Solvent Saver | 3.0 x 50 | 699975-302 | 699975-306 | 699975-305 | 689975-302 | 689975-306 |
| Solvent Saver | 3.0 x 30 | 691975-302 | 691975-306 | | 681975-302 | |
| Narrow Bore | 2.1 x 150 | 693775-902 | 693775-906 | 693775-905 | 683775-902 | 683775-906 |
| Narrow Bore | 2.1 x 100 | 695775-902 | 695775-906 | 695775-905 | 685775-902 | 685775-906 |
| Narrow Bore | 2.1 x 75 | 697775-902 | 697775-906 | | 687775-902 | |
| Narrow Bore | 2.1 x 50 | 699775-902 | 699775-906 | 699775-905 | 689775-902 | 689775-906 |
| Narrow Bore | 2.1 x 30 | 691775-902 | 691775-906 | | 681775-902 | |
| Fast Guard | 4.6 x 5 | 820750-911 | | | 820750-912 | |
| Fast Guard | 3.0 x 5 | 823750-911 | | | 823750-912 | |
| Fast Guard | 2.1 x 5 | 821725-911 | | | 821725-912 | |

*Continued on next page*

| Poroshell 120 - Particle Size 2.7 µm | | | | | |
|---|---|---|---|---|---|
| | Size (mm) | StableBond SB-Aq | Phenyl-Hexyl (USP L11) | Bonus-RP (USP L60) | HILIC |
| Analytical | 4.6 x 150 | 683975-914 | 693975-912 | 693968-901 | 693975-901 |
| Analytical | 4.6 x 100 | 685975-914 | 695975-912 | 695968-901 | 695975-901 |
| Analytical | 4.6 x 50 | 689975-914 | 699975-912 | 699968-901 | 699975-901 |
| Solvent Saver | 3.0 x 150 | 683975-314 | 693975-312 | 693968-301 | 693975-301 |
| Solvent Saver | 3.0 x 100 | 685975-314 | 695975-312 | 695968-301 | 695975-301 |
| Solvent Saver | 3.0 x 50 | 689975-314 | 699975-312 | 699968-301 | 699975-301 |
| Narrow Bore | 2.1 x 150 | 683775-914 | 693775-912 | 693768-901 | 693775-901 |
| Narrow Bore | 2.1 x 100 | 685775-914 | 695775-912 | 695768-901 | 695775-901 |
| Narrow Bore | 2.1 x 50 | 689775-914 | 699775-912 | 699768-901 | 699775-901 |

More Poroshell 120 phases will be developed.
For a complete listing, visit www.agilent.com/chem/poroshell120

For a full list of columns from Agilent, request the current Essential Chromatography and Spectroscopy Catalog from Agilent at www.agilent.com/chem/getguides

## Agilent LC Columns

| ZORBAX Rapid Resolution High Definition (RRHD) Columns, Stable to 1200 Bar | | | | |
|---|---|---|---|---|
| | Eclipse Plus C18 (USP L1) | Eclipse Plus C8 (USP L7) | Eclipse XDB-C18 (USP L1) | Extend-C18 (USP L1) |
| RRHD 2.1 x 150 mm, 1.8 µm | 959759-902 | 959759-906 | 981759-902 | 759700-902 |
| RRHD 2.1 x 100 mm, 1.8 µm | 959758-902 | 959758-906 | 981758-902 | 758700-902 |
| RRHD 2.1 x 50 mm, 1.8 µm | 959757-902 | 959757-906 | 981757-902 | 757700-902 |
| RRHD, 3.0 x 150 mm, 1.8 µm | 959759-302 | 959759-306 | 981759-302 | |
| RRHD 3.0 x 100 mm, 1.8 µm | 959758-302 | 959758-306 | 981758-302 | 758700-302 |
| RRHD 3.0 x 50 mm, 1.8 µm | 959757-302 | 959757-306 | 981757-302 | 757700-302 |
| | StableBond SB-C18 (USP L1) | StableBond SB-C8 (USP L7) | StableBond SB-Phenyl (USP L11) | StableBond SB-CN (USP L10) |
| RRHD 2.1 x 150 mm, 1.8 µm | 859700-902 | 859700-906 | 859700-912 | 859700-905 |
| RRHD 2.1 x 100 mm, 1.8 µm | 858700-902 | 858700-906 | 858700-912 | 858700-905 |
| RRHD 2.1 x 50 mm, 1.8 µm | 857700-902 | 857700-906 | 857700-912 | 857700-905 |
| RRHD 3.0 x 150 mm, 1.8 µm | 859700-302 | 859700-306 | | |
| RRHD 3.0 x 100 mm, 1.8 µm | 858700-302 | 858700-306 | 858700-312 | 858700-305 |
| RRHD 3.0 x 50 mm, 1.8 µm | 857700-302 | 857700-306 | 857700-312 | 857700-305 |
| | Eclipse PAH (USP L1) | StableBond SB-Aq | HILIC Plus | Eclipse Plus Phenyl-Hezyl |
| RRHD 2.1 x 150 mm, 1.8 µm | 959759-918 | 859700-914 | 959759-901 | 959759-912 |
| RRHD 2.1 x 100 mm, 1.8 µm | 959758-918 | 858700-914 | 959758-901 | 959758-912 |
| RRHD 2.1 x 50 mm, 1.8 µm | 959757-918 | 857700-914 | 959757-901 | 959757-912 |
| RRHD 3.0 x 100 mm, 1.8 µm | 959758-318 | 858700-314 | 959758-301 | 959758-312 |
| RRHD 3.0 x 50 mm, 1.8 µm | 959757-318 | 857700-314 | 959757-301 | 959757-312 |
| | Bonus-RP | | | |
| RRHD 2.1 x 50 mm, 1.8 µm | 857768-901 | | | |
| RRHD 2.1 x 100 mm, 1.8 µm | 858768-901 | | | |
| RRHD 2.1 x 150 mm, 1.8 µm | 859768-901 | | | |

More RRHD phases are being developed.
For a full list of available part numbers, visit
www.agilent.com/chem/rrhd

| 300Å for the analysis of proteins and peptides | | | | | |
|---|---|---|---|---|---|
| | StableBond 300SB-C18 | StableBond 300SB-C8 | StableBond 300SB-C3 (USP L56) | 300-Diphenyl (USP L11) | 300-HILIC |
| RRHD 2.1 x 50 mm, 1.8 µm | | | 857750-909 | 857750-944 | 857750-901 |
| RRHD 2.1 x 100 mm, 1.8 µm | 858750-902 | 858750-906 | 858750-909 | 858750-944 | 858750-901 |
| RRHD 2.1 x 150 mm, 1.8 µm | 857750-902 | 857750-906 | | | |

## Agilent LC Columns

| ZORBAX Eclipse Plus | | | | | | |
|---|---|---|---|---|---|---|
| | Size (mm) | Particle size (µm) | Eclipse Plus C18 (USP L1) | Eclipse Plus C8 (USP L7) | Eclipse Plus Phenyl-Hexyl (USP L11) | Eclipse Plus PAH (USP L1) |
| Analytical | 4.6 x 250 | 5 | 959990-902 | 959990-906 | 959990-912 | 959990-918 |
| Analytical | 4.6 x 150 | 5 | 959993-902 | 959993-906 | 959993-912 | 959993-918 |
| Analytical | 4.6 x 100 | 5 | 959996-902 | 959996-906 | 959996-912 | 959996-918 |
| Analytical | 4.6 x 50 | 5 | 959946-902 | 959946-906 | | |
| Rapid Resolution | 4.6 x 150 | 3.5 | 959963-902 | 959963-906 | 959963-912 | 959963-918 |
| Rapid Resolution | 4.6 x 100 | 3.5 | 959961-902 | 959961-906 | 959961-912 | 959961-918 |
| Rapid Resolution | 4.6 x 75 | 3.5 | 959933-902 | 959933-906 | 959933-902 | |
| Rapid Resolution | 4.6 x 50 | 3.5 | 959943-902 | 959943-906 | 959943-912 | 959943-918 |
| Rapid Resolution | 4.6 x 30 | 3.5 | 959936-902 | 959936-906 | 959936-912 | |
| Rapid Resolution HT, 600 bar | 4.6 x 100 | 1.8 | 959964-902 | 959964-906 | 959964-912 | 959964-918 |
| Rapid Resolution HT, 600 bar | 4.6 x 75 | 1.8 | 959951-902 | | | |
| Rapid Resolution HT, 600 bar | 4.6 x 50 | 1.8 | 959941-902 | 959941-906 | 959941-912 | 959941-918 |
| Rapid Resolution HT, 600 bar | 4.6 x 30 | 1.8 | 959931-902 | 959931-906 | 959931-912 | 959931-918 |
| Solvent Saver | 3.0 x 250 | 5 | | | | 959990-318 |
| Solvent Saver | 3.0 x 150 | 5 | 959993-302 | 959993-306 | | |
| Solvent Saver Plus | 3.0 x 150 | 3.5 | 959963-302 | 959963-306 | 959963-312 | |
| Solvent Saver Plus | 3.0 x 100 | 3.5 | 959961-302 | 959961-306 | 959961-312 | |
| Solvent Saver RRHD, 1200 bar | 3.0 x 150 | 1.8 | 959759-302 | 959759-306 | | |
| Solvent Saver RRHD, 1200 bar | 3.0 x 100 | 1.8 | 959758-302 | 959758-306 | | |
| Solvent Saver RRHD, 1200 bar | 3.0 x 50 | 1.8 | 959757-302 | 959757-306 | | |
| Solvent Saver HT, 600 bar | 3.0 x 100 | 1.8 | 959964-302 | 959964-306 | 959964-312 | |
| Solvent Saver HT, 600 bar | 3.0 x 50 | 1.8 | 959941-302 | 959941-306 | 959941-312 | |
| Narrow Bore | 2.1 x 250 | 5 | | | | 959790-918 |
| Narrow Bore | 2.1 x 150 | 5 | 959701-902 | 959701-906 | 959701-912 | 959701-918 |
| Narrow Bore | 2.1 x 50 | 5 | 959746-902 | 959746-906 | | |

*Continued on next page*

## Agilent LC Columns

| ZORBAX Eclipse Plus | | | | | | |
|---|---|---|---|---|---|---|
| | Size (mm) | Particle size (µm) | Eclipse Plus C18 (USP L1) | Eclipse Plus C8 (USP L7) | Eclipse Plus Phenyl-Hexyl (USP L11) | Eclipse Plus PAH (USP L1) |
| Narrow Bore RR | 2.1 x 150 | 3.5 | 959763-902 | 959763-906 | 959763-912 | |
| Narrow Bore RR | 2.1 x 100 | 3.5 | 959793-902 | 959793-906 | 959793-912 | 959793-918 |
| Narrow Bore RR | 2.1 x 50 | 3.5 | 959743-902 | 959743-906 | 959743-912 | |
| Narrow Bore RR | 2.1 x 30 | 3.5 | 959733-902 | 959733-906 | 959733-912 | |
| Narrow Bore RRHD, 1200 Bar | 2.1 x 150 | 1.8 | 959759-902 | 959759-906 | | |
| Narrow Bore RRHD, 1200 Bar | 2.1 x 100 | 1.8 | 959758-902 | 959758-906 | | |
| Narrow Bore RRHD, 1200 Bar | 2.1 x 50 | 1.8 | 959757-902 | 959757-906 | | |
| Narrow Bore RRHT, 600 Bar | 2.1 x 100 | 1.8 | 959764-902 | 959764-906 | 959764-912 | 959764-918 |
| Narrow Bore RRHT, 600 Bar | 2.1 x 50 | 1.8 | 959741-902 | 959741-906 | 959741-912 | 959741-918 |
| Narrow Bore RRHT, 600 Bar | 2.1 x 30 | 1.8 | 959731-902 | 959731-906 | 959731-912 | |

| ZORBAX Eclipse Plus: Guard Cartridges | | | | | | |
|---|---|---|---|---|---|---|
| | Size (mm) | Particle size (µm) | Eclipse Plus C18 (USP L1) | Eclipse Plus C8 (USP L7) | Eclipse Plus Phenyl-Hexyl (USP L11) | Eclipse Plus PAH (USP L1) |
| Guard Cartridges 4 pk. | 4.6 x 12.5 | 5 | 820950-936 | 820950-937 | 820950-938 | 820950-939 |
| Guard Cartridges 4 pk. | 2.1 x 12.5 | 5 | 821125-936 | 821125-937 | 821125-938 | 821125-939 |
| Guard Hardware Kit | | | 820999-901 | 820999-901 | 820999-901 | 820999-901 |

This is only a partial list of available phases and columns. Plus, Agilent offers a range of BioHPLC columns for fast and accurate protein and peptide separations.
For a full list of available part numbers, visit the Agilent website, or request an Agilent Essential Chromatography and Spectroscopy Catalog, or a BioHPLC Column Selection Guide at **www.agilent.com/chem/getguides**.

# Agilent Capillaries and Fittings

## Select the right capillaries for your application

Using the right capillaries is important for best analytical performance. Below are recommended fittings for conventional HPLC and UHPLC systems

**Standard System Capillaries: 1290 Series – 1200 Bar**

| From (A) | From (B) | Material | ID (mm) | Length (mm) | Fitting Type From | Fitting Type To | Notes | |
|---|---|---|---|---|---|---|---|---|
| Pump | Autosampler | SS | 0.17 | 300 | S | S | Pre-swaged on A and B | 5067-4657 |
| Pump | Thermostatted Autosampler | SS | 0.17 | 450 | S | S | Pre-swaged on A and B | 5067-4658 |
| Autosampler | TCC | SS | 0.12 | 340 | S | S | Pre-swaged on A | 5067-4659 |
| Column | DAD | SS | 0.12 | 220 | S | S | Pre-swaged on A | 5067-4660 |
| 1290 System | CTC Autosampler | SS | 0.17 | 600 | S | SH | Pre-swaged on A | 5067-4670 |
| CTC Autosampler | Column | SS | 0.12 | 600 | S | S | | 5067-4669 |
| Detector | Waste | PTFE | 0.8 | 5000* | | | Finger-tight fittings not included (0100-1516, 2/pk) | 5067-2462 |

**Material**

| Key | Description |
|---|---|
| SS | Stainless steel |
| S | Swagelok 1.6 mm port id |
| SH | Swagelok 1.6 mm port id, long head |
| SL | Swagelok 1.6 mm port id, long |
| SLV | Swagelok 1.6 mm port id, long, 1200 bar |
| SX | Swagelok 1.6 mm port id, extra-long |
| M | Metric M4 0.8 mm port id |

## Connections for 1290 valve heads: 600 and 1200 Bar

| From (A) | From (B) | Material | ID (mm) | Length (mm) | Fitting Type From | Fitting Type To | Notes | Valve Information | Part Number |
|---|---|---|---|---|---|---|---|---|---|
| Autosampler | Valve with Swagelok port | SS | 0.12 | 340 | S | SX | Pre-swaged on A | | 5067-4684 |
| Autosampler | Valve with Swagelok port | SS | 0.12 | 340 | S | SX | Pre-swaged on B | G4231A/B 2 Position/6 Port valve head, 600/1200 bar | 5067-4647 |
| Autosampler | Valve with M4 port | SS | 0.12 | 340 | SLV | M | | G4232A 2 Position/10 Port micro valve head, 600 bar | 5067-4744 |
| Autosampler | Valve with M4 port | SS | 0.12 | 500 | SLV | M | | G4234A/B 6 column selector valve, 600/1200 bar | 5067-4745 |
| Valve with 10/32 Swagelok port | Heat exchanger | SS | 0.12 | 90 | SX | S | Pre-swaged on A and B | G4231A/B 2 Position/6 Port valve head, 600/1200 bar | 5067-4649 |
| Valve with M4 port | Heat exchanger | SS | 0.12 | 90 | M | SL | Pre-swaged on B | G4232A 2 Position/10 Port micro valve head, 600 bar | 5067-5106 |
| Short Column | Valve with M4 port | SS | 0.12 | 130 | SV | M | | G4234A/B 6 column selector valve, 600/1200 bar | 5067-4735 |
| Short Column | Valve with M4 port | SS | 0.12 | 150 | SV | M | | G4232A 2 Position/10 Port micro valve head, 600 bar | 5067-5104 |
| Long Column | Valve with M4 port | SS | 0.12 | 280 | SV | M | | G4232A 2 Position/10 Port micro valve head, 600 bar | 5067-5107 |
| Short Column | Valve with Swagelok port | SS | 0.12 | 150 | SL | SX | Pre-swaged on B | G4231A/B 2 Position/6 Port valve head, 600/1200 bar | 5067-4650 |
| Short Column | Valve with 10/32 Swagelok port | SS | 0.12 | 150 | SL | SX | | G4232B 2 Position/10 Port valve head, 1200 bar | 5067-4686 |
| Long Column | Valve with Swagelok port | SS | 0.12 | 280 | SL | SX | Pre-swaged on B | G4231A/B 2 Position/6 Port valve head, 600/1200 bar | 5067-4651 |

*Continued on next page*

## Connections for 1290 valve heads: 600 and 1200 Bar

| From (A) | From (B) | Material | ID (mm) | Length (mm) | Fitting Type From | Fitting Type To | Notes | Valve Information | Part Number |
|---|---|---|---|---|---|---|---|---|---|
| Valve Swagelok port | Detector | SS | 0.12 | 200 | SX | S | Pre-swaged on A and B | G4231A/B 2 Position/6 Port valve head, 600/1200 bar | 5067-4653 |
| Long column | Valve Swagelok port | SS | 0.12 | 280 | SL | SX | | G4232B 2 Position/10 Port valve head, 1200 bar | 5067-4687 |
| Valve Swagelok port | Detector | SS | 0.12 | 200 | SX | S | Pre-swaged on A | G4232B 2 Position/10 Port valve head, 1200 bar | 5067-4689 |
| Valve with M4 port | Detector | SS | 0.12 | 250 | M | SLV | | G4232A 2 Position/10 Port micro valve head, 600 bar | 5067-4746 |
| Heat exchanger | Valve with M4 port | SS | 0.17 | 90 | SL | M | Pre-swaged on A | G4232A 2 Position/10 Port valve head, 1200 bar | 5067-5109 |
| Column | Valve with M4 port | SS | 0.17 | 90 | SV | M | | G4232A 2 Position/10 Port valve head, 1200 bar | 5067-5110 |
| Column | Valve with M4 port | SS | 0.17 | 150 | SV | M | | G4232A 2 Position/10 Port valve head, 1200 bar | 5067-5111 |
| Column | Valve with M4 port | SS | 0.17 | 280 | SV | M | | G4232A 2 Position/10 Port valve head, 1200 bar | 5067-5112 |
| G4232A 2 positions/10 ports valve head, 1200 bar | | SS | 0.17 | 250 | SL | M | Pre-swaged on A | G4232A 2 Position/10 Port valve head, 1200 bar | 5067-5113 |

## Fittings

| Description | Key | Unit | Part No. |
|---|---|---|---|
| Swagelok 1.6 mm stainless steel fitting | S | 10/pk | 5062-2418 |
| Swagelok 1.6 mm stainless steel fitting, long screw | SL | 10/pk | 5065-4454 |
| Swagelok 1.6 mm stainless steel fitting, extra long screw | SX | 10/pk | 5062-9967 |
| Swagelok 1.6 mm 1200 bar removable fitting | SV | 1/ea | 5067-4733 |
| Swagelok 1.6 mm 1200 bar removable fitting, long screw | SLV | 1/ea | 5067-4738 |
| Swagelok 1.6 mm 1200 bar removable fitting, extra long screw | SXV | 1/ea | 5067-4739 |

# Agilent Lamps for LC Instruments

## Don't risk your chromatographic results with an imperfect fit

Only Agilent lamps are designed and built to match Agilent instruments. Don't risk your results due to an ill-fitting or poorly made lamp in your detector. Only Agilent knows the alignment specifications of Agilent instrument detectors. Use the table on the right to easily cross reference your detector to the correct lamp needed for optimal performance.

**To learn more, visit www.agilent.com/chem/lamps**

| Detector | Part No. | Original Lamp Part Number |
|---|---|---|
| DAD | G1315A/B | 5182-1530 |
| | G1315C/D | 2140-0820 |
| | G4212A/B | 5190-0917 |
| MWD | G1365A/B | 2140-0813 |
| | G1365 C/D | 2140-0820 |
| VWD | G1314A/B/C | G1314-60100 |
| | G1314D/E/F | G1314-60101 |
| UV VIS | 8453A | 2140-0605 |

## Improve Acuracy with Agilent Captiva Premium Syringe Filters

Sample filtration prior to HPLC, UHPLC, GC/MS, or LC/MS analysis is critical to achieving optimal system performance. Agilent Captiva Premium Syringe Filters make the process faster than ever with the industry's highest flow rates and loading capacities. Choose from a variety of membrane types and pore sizes to suit your needs.

**For additional products and the learn more about Captiva filtration products, visit www.agilent.com/chem/filtration.**

**Premium Syringe Filters (100/pk)**

| Membrane | Diameter/Pore Size | | | | | |
|---|---|---|---|---|---|---|
| | 4 mm | | 15 mm | | 25 mm | |
| | 0.2 µm | 0.45 µm | 0.2 µm | 0.45 µm | 0.2 µm | 0.45 µm |
| PTFE | 5190-5082 | 5190-5083 | 5190-5084 | 5190-5085 | 5190-5086 | 5190-5087 |
| Nylon | | | 5190-5090 | 5190-5091 | 5190-5092 | 5190-5093 |
| PES | 5190-5094 | 5190-5095 | 5190-5096 | 5190-5097 | 5190-5098 | 5190-5099 |
| Regenerated cellulose | 5190-5106 | 5190-5107 | 5190-5108 | 5190-5109 | 5190-5110 | 5190-5111 |
| Cellulose acetate | | | | | 5190-5116* | 5190-5117* |
| Glass microfiber | | | 5190-5120 | | 5190-5122* | |
| Depth filters: glass/PTFE | | | 5190-5126 | 5190-5127 | 5190-5128 | 5190-5129 |
| Depth filters: glass/nylon | | | 5190-5132 | 5190-5133 | 5190-5134 | 5190-5135 |

Notes: *larger diameter is 28 mm (not 25 mm). PNs in blue are LC/MS certified; all others are LC/UV certified

# Agilent Solvent Safety Caps

## Increase your Lab Safety

Agilent safety caps are freely rotating, PTFE and PFA screw caps for all solvent bottles and waste containers used with HPLC systems in the lab. They reduce solvent emissions by up to 70%, and prevent leakage and evaporation, which can change chromatographic results. They optimize solvent replacement with a freely rotating cap design that helps avoid tube twisting during container exchange.

| **For NS29/32 ground neck bottles** | |
|---|---|
| **Safety Cap II with 2 ports - NS29/32** | **5043-0221** |
| 1 basic cap,PP, blue, NS29/32, with PTFE cone, 2 ports | included |
| 2 one piece fittings, PFA, 3,2mm, (1x blue, 1x red) | included |
| 1 venting valve 1µm,PTFE membrane | included |

| **For GL 45 threaded solvent bottles** | |
|---|---|
| **Safety Cap II with 2 ports - GL 45** | **5043-0222** |
| 1 basic cap,PP, blue, GL45, with PTFE cone, 2 ports | included |
| 2 fittings, PFA 3,2mm, (1x blue, 1x red) | included |
| 1 venting valve, 1µm,PTFE membrane | included |
| **Safety Cap I with 1 port - GL 45** | **5043-0223** |
| 1 basic cap,PP, blue, GL45, with PTFE cone, 1 port | included |
| 1 one piece fitting, PFA, 3,2mm, black | included |
| 1 venting valve, 1µm,PTFE membrane | included |
| **Safety Cap II with 2 stopcocks - GL 45** | **5043-0224** |
| 1 basic cap,PP, blue, GL45, with PTFE cone, 2 ports, 2 Stopcocks | included |
| 2 fittings, PFA 3,2mm, (1x blue, 1x red) | included |
| 2 fittings, PTFE 3,2mm, (white) | included |
| 1 venting valve 1µm,PTFE membrane | included |
| **Safety Cap I with 1 stopcock - GL45** | **5043-0225** |
| 1 basic cap,PP, blue, GL45, with PTFE cone, 1 port , 1 Stopcock | included |
| 1 one piece fitting, PFA, 3,2mm, black | included |
| 1 fittings, PTFE 3,2mm, (white) | included |
| 1 venting valve 1µm,PTFE membrane | included |

# Agilent In-line Filters

## Protect your LC System

Column inlet frit contamination can increase column back pressure and reduce efficiency. Microbore column blockages are a particular problem, due to the small diameter of the inlet frit. To prevent blockages always use the appropriate filters in your LC system. Agilent offers several types of high-pressure in-line filter kits for use with any HPLC system.

Low-dispersion in-line filter: Positioned immediately before the column, removes particles from the sample and injection system. Minimizes external band spreading due to frit diameter of only 2.1 mm and tapered inserts. Can be used with any microbore, high speed or standard analytical column.

Universal in-line filter: Installed between the LC pump and injector to remove particles from the solvent. Uses a high capacity filter. The frit is placed between the tapered edges of the inserts so the solvent is evenly distributed over the filtering frit.

**HPLC In-Line Filters**

| Description | Frit Porosity (µm) | Frit Inlet ID (mm) | Comments | Part No. | Replacement Frits |
|---|---|---|---|---|---|
| RRLC in-line filter, connecting capillary | 0.2 | 4.6 | Max 600 bar | 5067-1553 | 5067-1562, 10/pk. |
| RRLC in-line filter, connecting capillary | 0.2 | 2.1 | Max 600 bar | 5067-1551 | 5067-1555, 10/pk. |
| Low dispersion in-line filter, includes two frits | 2 | 2.1 | <1 mL/min. | 01090-68702 | 280959-904, 10/pk (2 µm). |
| | 0.5 | | | | 280959-907, 10/pk (0.5 µm). |
| Universal in-line filter, two frits, with inserts, 130 x 0.25 mm connecting capillary | 2 | 4.8 | 1 – 5 mL/min. | 01090-69703 | 01090-27609, 2 /pk. |
| Semi-prep filter | 0.5 | 12.7 | 1 – 5 mL/min. | 5064-8273 | 5022-2185 |
| High pressure semi-prep filter | 10 | 19 | 5 – 10 mL/min | 5022-2165 | 5022-2166, 10/pk. |
| Prep filter | 10 | | 10 – 100 mL/min. | 5065-4500 | 5065-9901, replacement glass cartridge |
| In-line filter for G1311A | Recommended when high salt concentrations are used | | | G1311-60006 | |
| 1290 Infinity in-line filter (0.3 µm) | 0.3 | 2.0 | 1200 bar | 5067-4638 | 5023-0271, 5/pk. |

# The Agilent 1200 Infinity Series LC Instruments

## Future Proof Your Lab

Agilent offers unlimited module and system compatibility between the new 1200 Infinity Series and previous 1100 or 1200 Series instruments. This unique capability of Agilent's LC solutions facilitates flexible, stepwise upgrade for any Agilent LC – now or in the future!

**Infinitely more affordable: The Agilent 1220 Infinity LC** is a high quality, integrated system for routine HPLC and advanced UHPLC analysis, for maximum return on investment.

- 600 bar power range up to 5 mL/min and 80 Hz detector speed – prepare your lab to take advantage of latest advances in LC column technology
- Full compatibility with all other detectors within the 1200 Infinity Series and with 6100 Series Quadrupole MS – run any existing HPLC or RRLC method
- Uses same technology and parts as 1260 and 1290 Infinity LC systems

**Infinitely more confident: The Agilent 1260 Infinity LC** raises the standard in HPLC – without raising the price. It offers new levels of productivity, data quality and robustness to give you highest confidence in your investment.

- 600 bar standard pump pressure, 80 Hz standard detector speed and up to 10 times higher UV detection sensitivity – be prepared for today's and tomorrow's challenges
- 100% compatible with all your HPLC methods – ensuring riskless replacement of existing equipment

**Infinitely more powerful: The Agilent 1290 Infinity LC** is the last word in chromatographic performance providing highest speed, resolution and sensitivity.

- Wide power range up to 1200 bar – deploy any particle type, any column dimensions, or any mobile and stationary phases
- Ultimate method flexibility from conventional HPLC to UHPLC – run your existing methods and solve all your LC and LC/MS challenges on one system
- Lower total cost of ownership – get UHPLC productivity at service costs comparable to HPLC equipment

**Keep up to date on the infinitely better technologies coming from Agilent instruments at www.agilent.com/chem/infinity**

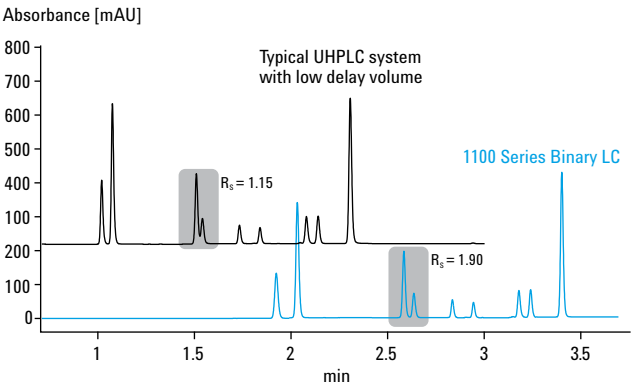
# Agilent 1290 Infinity LC with ISET

## Overcome pitfalls in the transfer of methods between different LC instruments with Intelligent System Emulation Technology (ISET)

Design differences between LC instrumentation – such as power range, delay volume, mixing behavior, temperature control, extra column volume and detector cell design – all affect the ability to transfer a method from one system to another. Therefore identical LC methods used on different LC instrumentation could result in different retention time and chromatographic resolution.

## The impact of delay volume and gradient mixing

The delay (dwell) volume of an LC system determines how fast the gradient reaches the column. Further, the mixing behavior influences the gradient profile. Both these factors – delay (dwell) volume and mixing behavior – are determined by the instrument design and the consequences for method transfer are differences in retention times and in resolution.



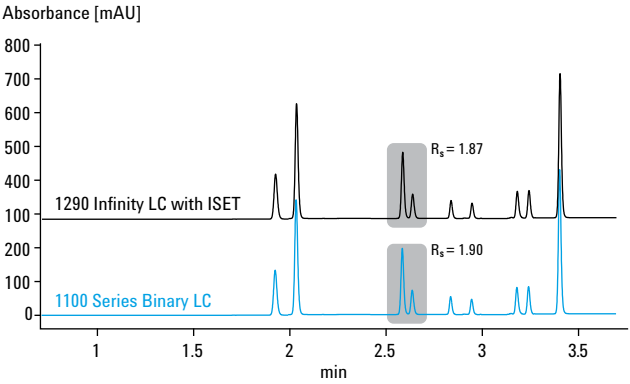

**Conditions**

| | |
|---|---|
| Column: | Poroshell 120, 3 x 50 mm (2.7um) |
| Flow Rate: | 0.85 mL/min. |
| Mobile phase: | Water, Acetonitrile. |
| Gradient: | 0 min (10 % Acetonitrile), 3 min (90 % Acetonitrile) |
| Mobile phase: | Tetrahydrofuran (THF) |

Different solvent compositions at the column due to different delay volumes and gradient mixing behaviours result in different retention times and resolutions.

## The solution for instrument to instrument method transfer: 1290 Infinity LC with ISET

Intelligent System Emulation Technology makes the 1290 Infinity LC the world's first truly universal LC system as it can execute other HPLC and UHPLC methods and deliver the same chromatographic results without any change of the instrument or the original method – all by simple mouse click.




**The result:** same retention times and same resolution, without modifying the instrument or original method.

## Higher productivity and cost savings for both method development and QA/QC

With 1290 Infinity LC and ISET you can speed up your method development with UHPLC performance and then fine-tune your method by emulating the target system – and be confident that the method will run as intended. Simply emulate the LC system on which the original method was developed. You can run your legacy methods with ISET and at the same time take full advantage of the UHPLC speed, resolution and sensitivity of the 1290 Infinity LC. With ISET, there is no need to maintain your old legacy LC systems..

## Learn more about this technology, and keep up to date on the infinitely better technologies coming from Agilent instruments at www.agilent.com/chem/infinity

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Published in USA, October, 2011
Publication Number 5990-7595EN

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