1. Aims and Objectives

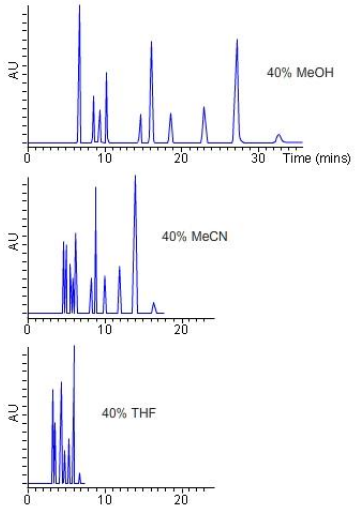
Aims

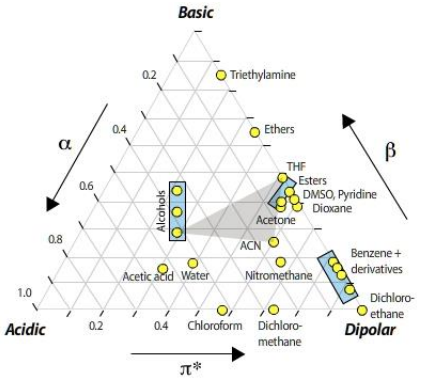
- To give an overview of the mechanism of Reversed Phase High Performance Liquid Chromatography (RP-HPLC) and explain the basis of the retention mechanism.
- To highlight typical RP-HPLC applications.
- To explain retention order in RP-HPLC and demonstrate the influence of mobile phase composition on retention.
- To explain how the mobile phase composition and constituents might be manipulated to optimize chromatographic separations in RP-HPLC.
- To illustrate the principles which are used to select appropriate stationary phases and column geometry in RP-HPLC.
- To explain how mobile phase pH affects the retention of ionizable compounds in RP-HPLC.

Objectives

At the end of this Section you should be able to:

- To interactively demonstrate how to use mobile phase pH to optimize separations.
- To give practical advice on how to choose buffers for RP-HPLC and how to effectively use the principles of ion-suppression to optimize separations.





The IUPAC Definition of Chromatography

"Chromatography is a physical method of separation, in which the components to be separated are distributed between two phases, one of which is stationary whilst the other moves in a definite direction".

In HPLC the stationary phase is either a solid, porous, surface active material in small particle form, or a liquid which is coated onto micro-particulate beads of an inert solid support (usually silica).

The mobile phase is a liquid that moves through the packed bed of stationary phase in the column under pressure.[1]

2. Mechanism of Reversed Phase HPLC

Reversed phase HPLC is characterized by a situation in which the **mobile phase** used is MORE **POLAR** than the **stationary phase**. The name 'Reversed Phase' arises as this was the second, (chronologically), mode of chromatography after Normal (or 'Straight') phase in which a polar stationary phase is used in conjunction with a less polar mobile phase.

Typical reversed phase stationary phases are **hydrophobic** and chemically bonded to the surface of a silica support particle (Figure 1). The most commonly used stationary phases are shown (Figure 2). Other support materials and bonded phases are available.

For more information on bonded phases see the CHROMacademy Column Chemistry module:

http://www.chromacademy.com/frameset-chromacademy.html?fChannel=0&fCourse=0&fSco=4&fPath=sco4/hplc_2_1.asp

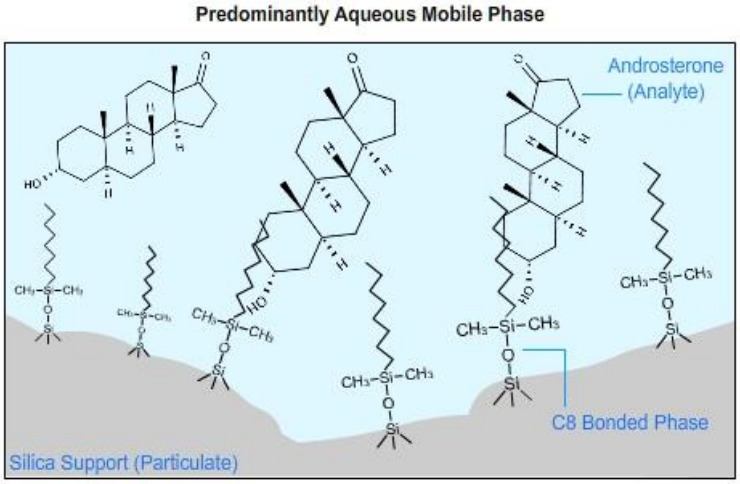


Figure 1: Schematic representation of reversed phase HPLC.

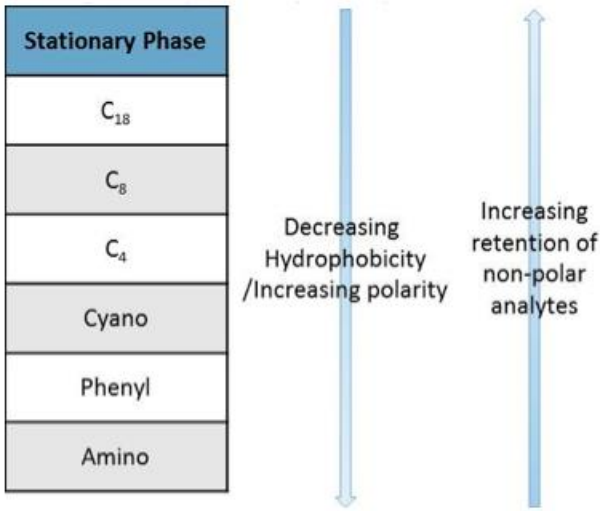


Figure 2: Common reversed phase HPLC stationary phases.

For neutral analytes, the mobile phase consists of water (the more polar component) and an organic modifier that is used to vary the retention of analytes by lowering the polarity of the mobile phase. The most common organic modifiers are shown.

Increasing the water content will repel ('squeeze') hydrophobic (non-polar) analytes out of the mobile phase and onto the non-polar stationary phase where they will reside for a time until 'partitioning' out into the mobile phase again. Each 'on-off' partition is called a '**Theoretical Plate'**.

When **ionizable** (or **ionic**) analytes are present, other additives such as **buffers** or **ion pairing** reagents can be added to the mobile phase to control retention and reproducibility.

The Chromatogram shown (Figure 3) illustrates the general elution order of hydrophilic and hydrophobic analytes. When working with ionizable analytes the hydrophobicity and, hence, retention characteristics of the analyte will be affected depending on its ionization state (ionized or non-ionized), this will be discussed later in the module.

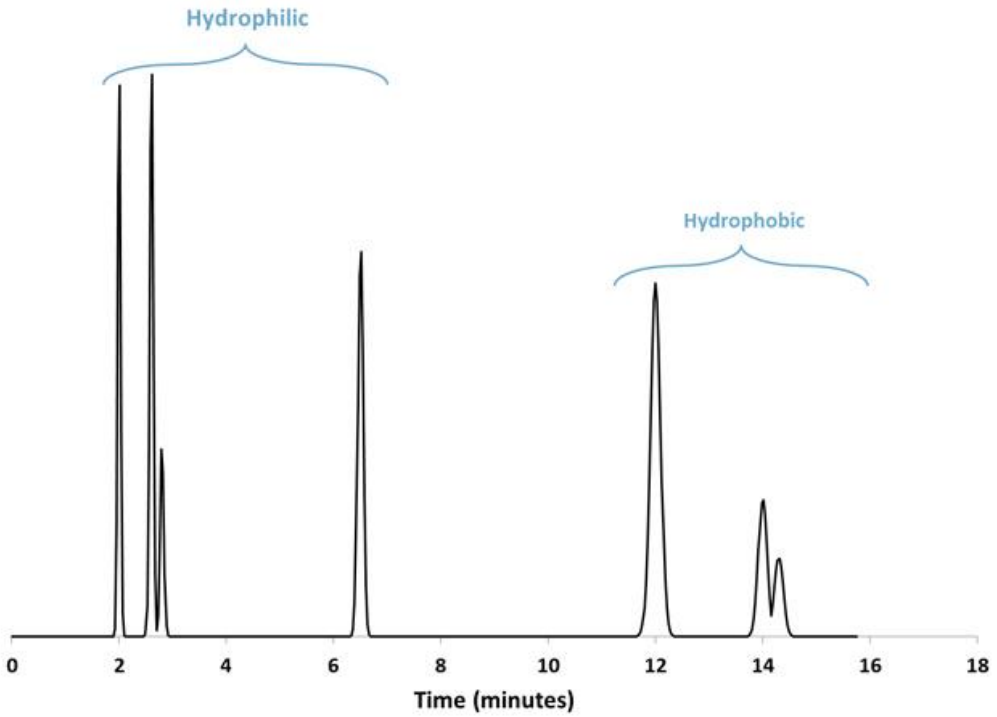


Figure 3: Representative reversed phase chromatogram detailing analyte retention order based on hydrophobicity or Hydrophilicity.

3. Applications of Reversed Phase HPLC

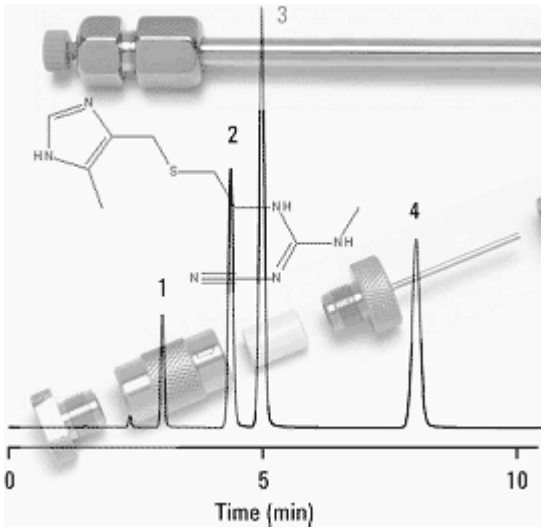
Reversed-phase HPLC is very widely applicable for small molecule analyses (M.Wt. < 2000 **Da**). A C_{18} or Octadecylsilane (**ODS**) column is usually the first choice for method development, although this may differ in certain industries where analytes are chemically very similar (little difference in hydrophobicity).

Reversed phase HPLC successfully separates both polar and nonpolar neutral molecules with molecular weights below 2000 **Daltons**. Even **homologs** and **benzologs** can be baseline separated. Homologs may differ from their counterparts by only one carbon unit (methylene, CH_2).

Reversed-phase HPLC can be extended to the separation of weak acids and bases with the addition of mobile phase modifiers and buffers to control ionization of the sample molecules. Stronger acids and bases can be separated using an analog of reversed-phase HPLC - Ion Pair HPLC. Here, an additive is used to bind with the sample ion producing a neutral compound complex for separation. Alternatively stronger acids and bases have been analyzed alongside hydrophobic analytes using **mixed-mode chromatography** in recent times.

Reversed-phase HPLC is a good choice for peptide and protein separations when short chain alkyl stationary phases are used. Here, analysis of compounds with a molecular weight above 2000 Daltons is possible.

The separation of amines requires more attention, but can be easily accomplished by the use of additives, pH control, or the use of specially treated columns.



Reversed phase HPLC is the first choice for:

- Neutral and non-polar compounds with a molecular weight less than 2000 Da
- Homologs and Benzologs
- Weak acids and bases
- Strong acids and bases (ion pair HPLC)
- Proteins and peptides

More difficult analyses:

- Amines
- Water insoluble compounds

Samples that cannot be easily separated by reversed phase HPLC and the alternative methods that can be used:

- Very hydrophilic compounds – may not have sufficient retention in RP. Use normal phase or Hydrophilic Interaction Liquid Chromatography (HILIC).
- Very hydrophobic compounds – strongly retained under reversed phase conditions and may require the use of non-aqueous conditions. Use non-aqueous reversed phase chromatography (NARP).
- **Achiral** isomers (**stereoisomers**, **diastereomers**, **positional isomers** etc.) – there are some cases where these have been separated by reversed phase, however, often reversed phase conditions will require the use of a **cyclodextrin** bonded phase. Normal phase chromatography will work well.
- **Chiral** isomers (**enantiomers**) – reversed or normal phase HPLC.
- Inorganic ions – use mixed mode or ion exchange chromatography.

For more information on:

HILIC

http://www.chromacademy.com/frameset-chromacademy.html?fChannel=0&fCourse=0&fSco=105&fPath=sco105/hilic_1_1_1.asp

Normal Phase Chromatography

http://www.chromacademy.com/frameset-chromacademy.html?fChannel=0&fCourse=0&fSco=7&fPath=sco7/hplc_2_4.asp

Ion Chromatography

http://www.chromacademy.com/frameset-chromacademy.html?fChannel=0&fCourse=0&fSco=111&fPath=sco111/ic_1_1_1.asp

4. Analyte Retention in Reversed Phase HPLC

The hydrophobicity of an analyte molecule will be the primary indicator as to the retentivity in reversed phase HPLC. Hydrophobicity is often expressed as Log P which is a measure of the way an analyte (in its neutral form) partitions between two immiscible solvents (usually octanol and water) under standard conditions (Equation 1). The higher the value of Log P (between –1 and +1) the more hydrophobic the molecule.

$$logP_{oct/wat} = log\left(\frac{[solute]_{octanol}}{[solute]_{water}^{un-ionised}}\right) \quad (1)$$

Polar analytes interacting with silica surface silanol groups undergo an adsorption type interaction as well as their partitioning behavior – this can lead to detrimental peak shape effects along with an increase in retention time.

The structure of the sample molecules will also give clues as to their elution order. The elution order is governed by the water solubility of the molecule and the carbon content for an analogous series. Some observations governing sample elution order include (again, these assume compounds are of analogous series):

- The less water soluble a sample is, the more retention
- Retention time increases as the number of carbon atoms increase
- Branched-chain compounds elute more rapidly than normal isomers
- Unsaturation decreases retention
- Neutral polar and charged species typically show the least retention followed by acid, then basic compounds all eluting early

The general order of elution is:

Aliphatics > induced dipoles (e.g. CCl_4) > permanent dipoles (e.g. $CHCl_3$) > weak Lewis bases (**esters**, **aldehydes**, **ketones**) > strong **Lewis bases** (amines) > weak **Lewis acids** (**alcohols, phenols**) > strong Lewis acids (**carboxylic acids**)

Ionic compounds generally elute with the void volume of the column.

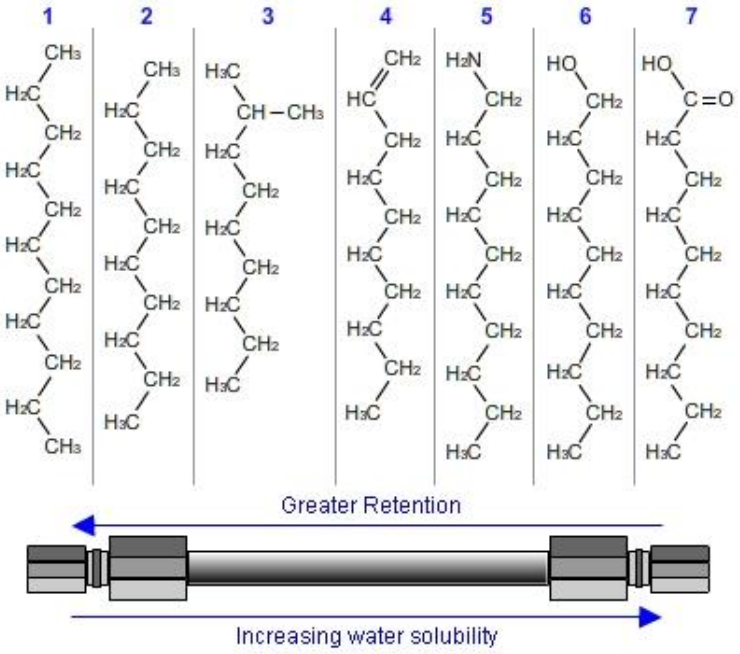


Figure 4: Retention order in reversed phase HPLC

Retention order:

1. Straight chain hydrocarbon – most hydrophobic (C11)
2. Straight chain hydrocarbon – less hydrophobic (C10)
3. Branched n-hydrocarbon (C10)
4. Unsaturated C10 - (more polar due to π electron dipole in the double bond)

5-7. Analytes with functional groups. Ionized analytes will elute fastest of all

6. Reversed Phase Mobile Phase Solvents

The mobile phase in reversed phase HPLC usually consists of water/aqueous solution (commonly an aqueous buffer) and an organic modifier. When ionizable compounds are analyzed, buffers and other additives may be present in the aqueous phase to control retention and peak shape.

Chromatographically, in reversed phase HPLC water is the 'weakest' solvent. As water is most polar, it repels the hydrophobic analytes into the stationary phase more than any other solvent, and hence retention times are long – this makes it chromatographically 'weak'. The organic modifier is added (usually only one modifier type at a time for modern chromatography), and as these are less polar, the (hydrophobic) analyte is no longer as strongly repelled into the stationary phase, will spend less time in the stationary phase, and therefore elute earlier. This makes the modifier chromatographically 'strong' as it speeds up elution/reduces retention.

As progressively more organic modifier is added to the mobile phase, the analyte retention time will continue to decrease.

The common organic modifiers are detailed below (Figure 1). The Snyder polarity index value is shown which gives a measure of the polarity of the solvents. The ε° values are also shown which give a measure of relative elution strength (the values quoted are for elution on C_{18}).[2]

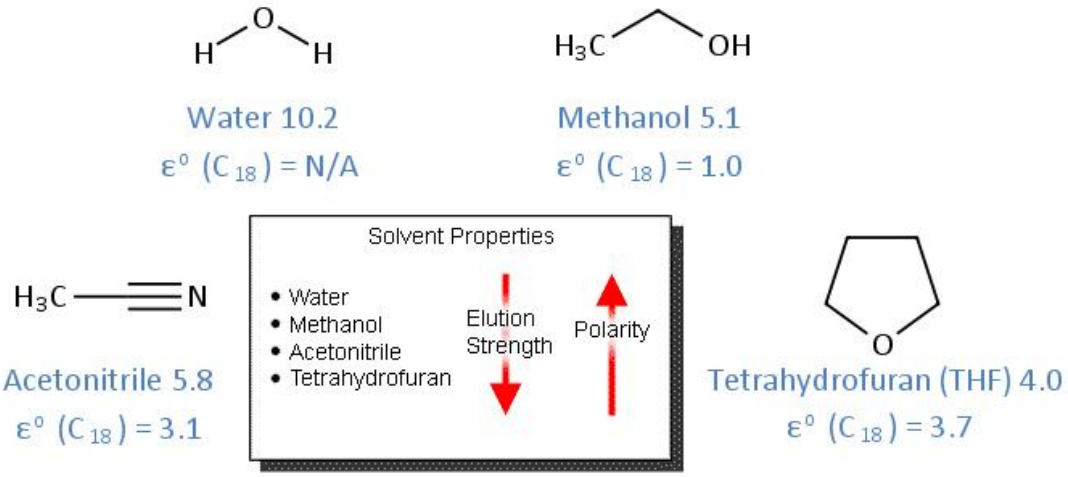


Figure 5: Typical solvents used in reversed phase HPLC.

Solvent selection may be one of the most important parameters in an HPLC separation due to the effect it can have on the selectivity. In fact selectivity may be the most effective tool for optimizing resolution (Figure 6).

7. Mobile Phase Strength and Retention

Retention can be altered by changing the mobile phase strength. Retention values in the range 2 < ***k*** < 10 are desirable. This operating range for *k* holds well for conventional HPLC (150 x 4.6 mm, 5 µm and 400 bar), however, for high efficiency HPLC, typically with core-shell and sub-2µm columns and UHPLC we can operate over the range 0.5 < *k* < 5.

Selectivity (***α***) can be changed by changing mobile phase composition (swap MeOH for MeCN), column stationary phase, and temperature.

Increasing the percentage organic modifier in the mobile phase has a profound effect on analyte retention due to the change in polarity of the mobile phase. An increase in the organic content of the mobile phase of 10% will decrease *k* for each analyte by a factor of 2 to 3.

The interactive experiment below can be used to investigate the influence of the organic modifier on and HPLC experiment. It is possible to start at a high % organic modifier (to save time as all components will elute very quickly), and then reduce the amount of modifier to obtain the optimum chromatogram. It should be noted that the target is to obtain a suitable resolution of all peaks (R_S > 1.7 for all peaks in this instance) in the minimum analysis time.

Make sure ALL modifier concentrations have been investigated before responding to the question below.

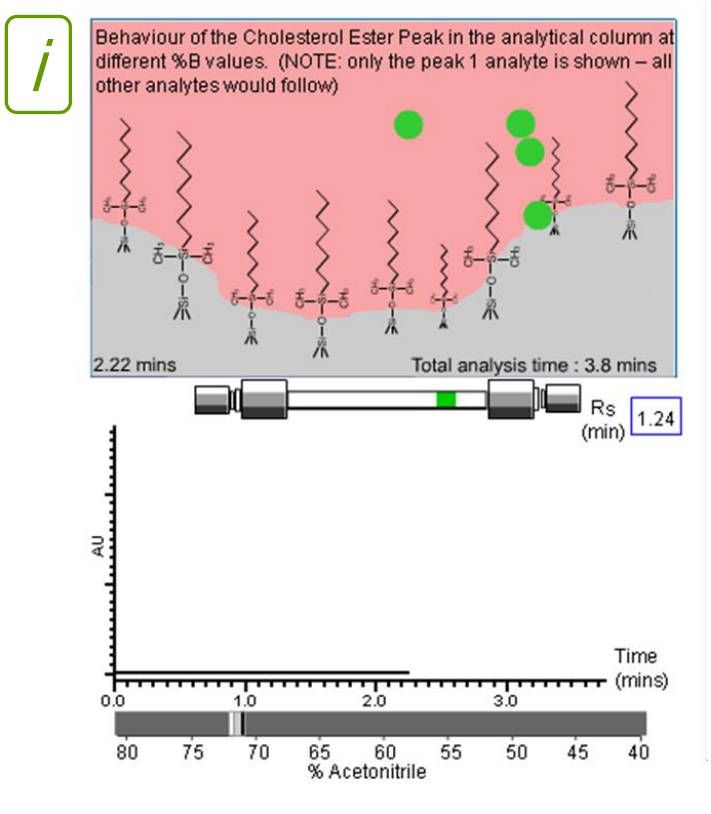


Figure 11: Relationship between retention and % organic modifier in reversed phase HPLC.

Solvent strength is the most powerful tool available to alter the resolution between analytes.

10. Selecting Reversed Phase Columns

The "Column Chemistry" module gives comprehensive information on HPLC column stationary phases. A reminder of the stationary phase properties that are often reported by column manufacturers is shown (Table 5).

http://www.chromacademy.com/frameset-chromacademy.html?fChannel=0&fCourse=0&fSco=4&fPath=sco4/hplc_2_1.asp

Column	Particle Size (µm)	Pore Size	Surface Area (m^2g^{-1})	Temp. Limits (°C)	pH Range	Endcapping	Carbon Load (%)
Symmetry C_{18}	3.5, 5	100 Å	-	45	2-8	Yes	19
Luna C_{18}	3, 5, 10	100 Å	440	-	1.5-10	-	19
Zorbax Eclipse Plus C_{18}	1.8, 3.5, 5.0	95 Å	160	60	2-9	Double	9
Hypersil BDS C_{18}	3, 5	120 Å	170	-	2-9	Yes	10
YMC-Pack ODS-A	3, 5	12 nm	330	-	2-7.5	Yes	17

Table 5: C_{18} column parameters from various manufacturers. 1 nm = 10 Å.

Particle Size

The particle size or particle diameter (d_p) is the average diameter of the column packing particles.

Pore Size

The pore diameter chosen is directly related to the **hydrodynamic volume** of the molecules in the sample. Larger pores allow large molecules to access the bonded phase found within pores for maximum separating ability. Select pore sizes of 150 Å or less for small molecules. Large pores, 300 Å or greater, are used for samples having a molecular weight greater than 2000 Da. As a rule of thumb the pore size should be at least three times the hydrodynamic diameter of the molecule.

Surface Area

The surface area refers to the total area of the solid surface. This is often determined using an accepted measurement technique such as the Brunauer-Emmet-Teller (BET) method, which uses the physical adsorption of nitrogen to measure the surface area. The surface area can have an effect on several chromatographic parameters. High surface area columns may exhibit greater retention, capacity, and resolution. Low surface area packings will generally equilibrate more quickly which can be advantageous in gradient elution.

pH Range

The pH range gives the pH values at which the HPLC column can be used without degradation of the solid support and stationary phase which will ultimately result in deleterious chromatographic results.

Traditional silica has a working range of pH 2.5-7.5. At low pH acidic hydrolysis of the silyl ether linkage between the bonded phase and silica surface will occur, resulting in column bleed (loss of stationary phase), poor peak shape, and loss of efficiency. At high pH the silica surface itself is at risk of basic hydrolysis, sometimes referred to as silica dissolution, whereby, the solid silica support is cleaved apart and fines are created which block the support material pores, interstitial gaps between the particles, and the column outlet frit resulting in the system over pressurizing and shutting down.

Temperature Limit

Some column manufacturers will give an operating range or upper temperature limit at which the columns can be used without damaging the stationary phase. Elevated temperatures will also promote basic hydrolysis of the silica support and acidic hydrolysis of the stationary phase.

Endcapping

Endcapping is used to remove surface silanol groups on the solid support which can cause unwanted analyte secondary interactions resulting in poor peak shapes; this is particularly evident when analyzing basic, ionizable, and ionic compounds. A small silylating reagent (i.e. trimethylchlorosilane or dichlorodimethylsilane) is reacted with the surface silanol groups to produce, for example, a trimethylsilyl (TMS) capped group (Figure 18).

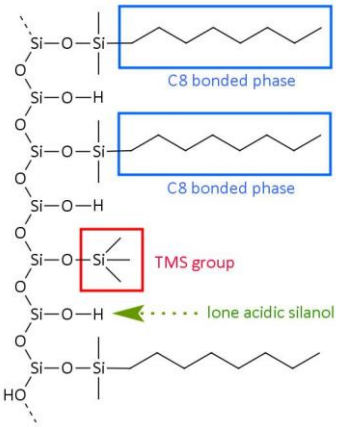


Figure 18: Endcapped silica surface.

Fully hydroxylated silica will have a silanol surface concentration of ≈8 µmol/m^2. Following chemical modification > 4 µmol/m^2 of these silanols may remain; even with optimum bonding conditions due to steric limitations of the modifying ligands. This indicates that on a molar basis there are more residual silanols remaining than actual modified ligand. This is only a partial solution; however, as not all of the surface silanol groups will be reacted even when using sterically very small ligands and optimized bonding conditions, also the endcapping ligand is prone to hydrolysis especially at low pH.

Carbon Load

Carbon load is simply the elemental load of carbon on the support material (which is carbon free in its native form for traditional silica). Hybrid particles such as BEH, XBridge, TriArt and Gemini contain a mixture of inorganic silica and organic groups and will always have a larger background

carbon content present, even in its native, unmodified state. The carbon load is normally expressed as % carbon. A higher carbon loading will result in greater analyte retention. It should be noted that C18 columns will always have a higher % carbon and columns with different endcapping cannot be subjected to a like to like comparison as the % carbon for the different endcapping groups will differ.

Particle Size Distribution

The particle size distribution will give a measure of the distribution of the size of particles used to pack the column. It is desirable to having a narrow particle size distribution as this gives a more homogeneous packing and ultimately more reproducible chromatography. A particle size distribution of d_p ± 10% would indicate that the 90% of the particles are between 9-11 µm for a 10 µm average d_p packing.

D10/D90 ratios are often quoted for packing materials as a relative measure of the particle diameter distribution. D10 = particle diameter at 10% of the total size distribution and D90 = particle diameter at 90% of the total size distribution. The closer this value is to unity, the more homogeneous the particle diameter distribution.

Particle Shape

Two basic silica particle shapes are available, spherical and irregular (Figure 19). Milling of silica particles followed by sieving to obtain the appropriate particle size and distribution produces irregular particles. Although irregular particles are somewhat less expensive they are known to have poorer efficiency than spherical particles. This is due to the way in which the particles pack into the HPLC column; with irregular particles packing homogeneity is much poorer leading to an exaggeration of eddy diffusion and mass transfer effects.

The use of columns for high flow (pressure) applications and mechanical shock can cause the irregular particles to shear, forming smaller sub-particles known as fines which may migrate and eventually block the outlet frit of the column.

Therefore, spherical particles are generally favored for the reasons outlined above.

Figure 19: Silica particle shapes.

Porosity

The porosity is related to both the pore size and number of pores. The greater the porosity, the less stable the stationary phase. Size exclusion columns have high porosity; thus they often have lower pressure limits than other columns.