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The Theory of HPLC

Column Chemistry

Wherever you see this symbol, it is important to access the on-line course as there is interactive material that cannot be fully shown in this reference manual.

Aims and Objectives

Aims

- To introduce silica as a major stationary phase support for Reverse Phase HPLC separations and for Normal Phase applications
- To explain how bonded phases are produced and to highlight the deactivation process used
- To explain the use of bonded phases in various chromatographic applications and the influence of surface silica silanol groups on chromatographic separations
- To introduce modern bonded phases that give an advantage to today's chromatographer – including water wettable phases, Polar Embedded phase and Phase capable of operating at extremes of pH

Objectives

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

- To highlight new and emerging stationary phase types
- To explain how columns and stationary phases may be characterised

Content

Silica as a Packing Material

Silica is often used as a support material for adsorption (normal phase) chromatography and with chemical modification of the surface for partition (reverse phase), normal phase, ion-exchange, chiral and size exclusion chromatography.

Porous silica has a high surface area that leads to high efficiency columns (through a higher number of possible surface interactions – theoretical plates).

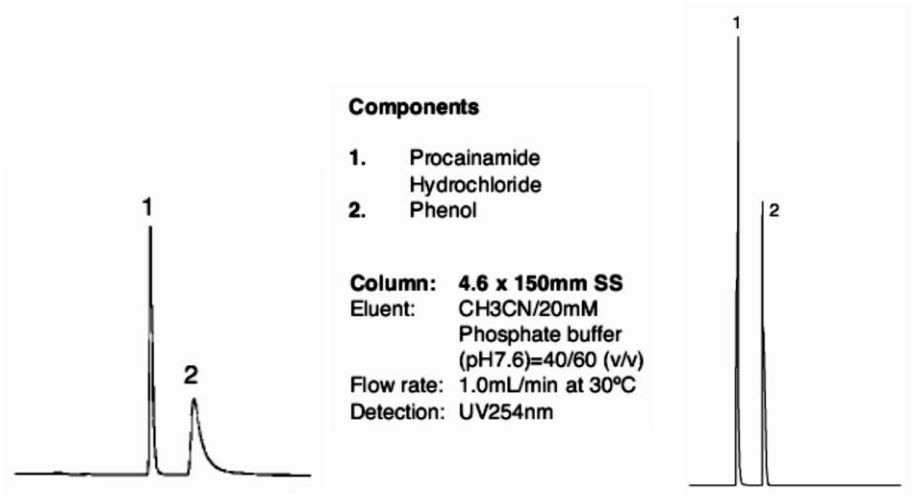
The following factors are important in the manufacture of silica-based columns for HPLC:

- Purity (especially metal ion content)
- Sil-gel or zero gel silica type
- Deactivation / surface treatment
- Hybridisation with alkyl groups
- Shape (spherical or irregular)
- Particle size
- Particle size distribution

Use the information opposite to explore the importance of each of these silica parameters.

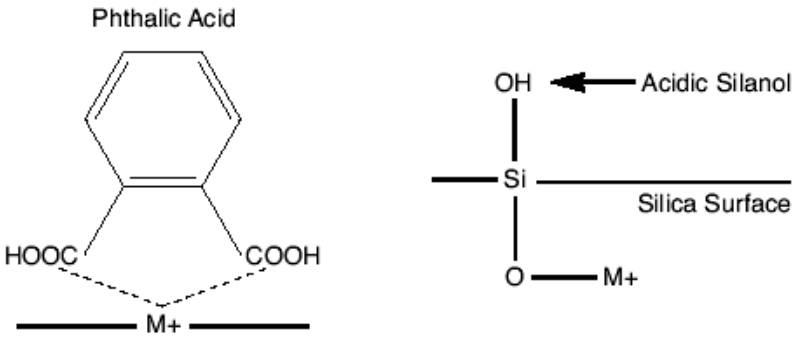
Metal Ion Content

The presence of metal ions within silica can cause peak tailing. Analyte molecules interact with metal or silanol groups on the silica surface and are retained longer than the 'bulk' of the analyte molecules. The molecules undergoing this 'unwanted secondary retention' form the 'tail' of the peak.



Peak tailing caused by the activation of surface silanol species by metal ions in the silica matrix

Surface metal ions act as 'chelating' agents to retain analyte molecules with multiple polar groups. Metal ions near the surface 'activate' silanol groups which become more likely to interact with acidic or basic analytes. Both of these effects can lead to peak tailing or in extreme cases irreversible adsorbtion (retention) of the analyte.



Mechanism of surface silanol activation by silica matrix metal ions

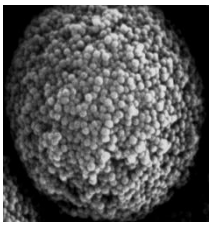
Manufacturers may 'acid wash' the silica in order to remove the metal ion contamination to improve chromatographic performance. Iron and Aluminium are particularly troublesome.

Silica	Na	K	Mg	Al	Fe	Ca	Ti	Zn
Common	37-4220	Not Determined	4-64 ppm	20-150 ppm	20-303 ppm	6-444 ppm	Not Determined	Not Determined

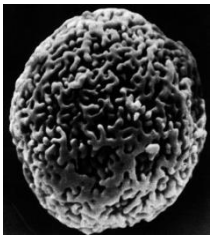
Typical metal ion profile of HPLC grade silica

Particle Type

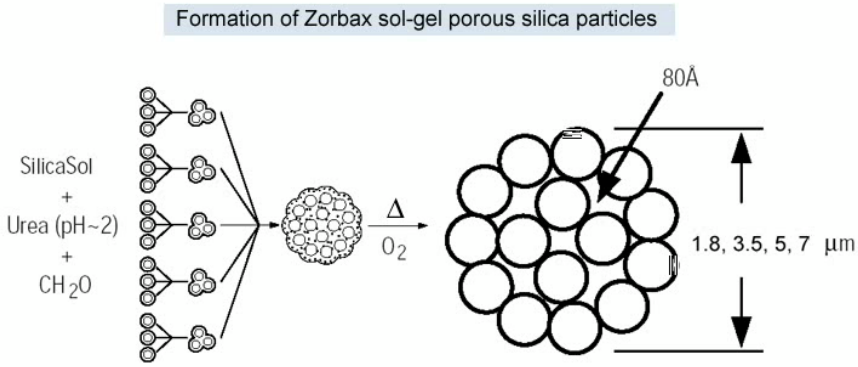
Sol-gel materials are formed via the agglomeration of small silica particles to form 1.8 - 10μm sols. Under the reaction scheme shown involving heating the sol in formaldehyde, the sub-particles fuse into the larger final particle. The process has been likened to melting marbles together to form one larger marble 'particle'. Silica sols generally have lower surface area, porosity, and surface reactivity. Evidence suggests that silica sol gels may be less soluble at pHs above 7 than silica gels.



Silica Sol-gel



Silica Xerogel

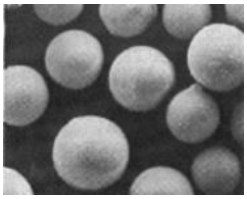


Silica Gel Types and Manufacturing Process for Sol-gel materials

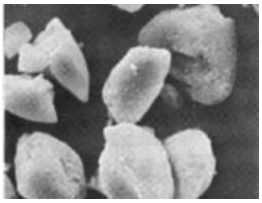
Another common type of particle is a silica gel (Xerogel). The silica sol is subjected to a heat treatment creating a rigid porous silica. This type of particle has higher surface area and porosity. At pH 7 and above, silica gels dissolve readily in the mobile phase.

Manufacture from vendor to vendor will vary producing silica gels with different shapes, surface areas, pore sizes, and surface chemistries.

Particle Shape



Spherical Silica



Irregular Silica

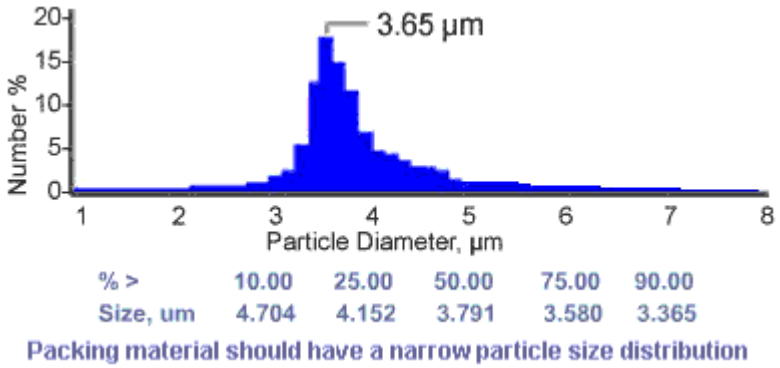
Two basic shapes of silica particle are available, 'irregular' and 'spherical'. Milling silica xerogels followed by sieving to obtain the appropriate particle size and distribution produces irregular particles.

Although irregular particles are somewhat less expensive, they are known to have poorer efficiency than spherical particles. This is due to the way that the particles pack into the HPLC column. With irregular materials, the 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 all cause the irregular particles to sheer forming smaller sub-particles know as 'fines' which may migrate and eventually block the outlet frit of the column.

Spherical particles are generally favoured for the reasons outlined above.

Particle Size Distribution



Typical Silica Particle Size Distribution

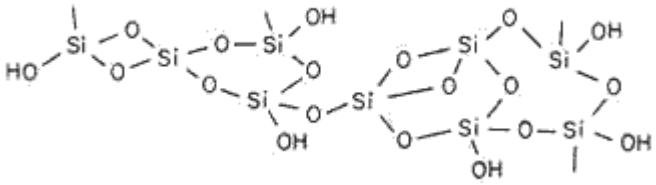
Analytical columns have particle sizes from 3 to 10 µm in diameter. Large particles produce greater band broadening leading to poor resolution. Small particles can lead to high column pressures. The most popular particle sizes are 3.5 – 5 µm.

Besides particle diameter, the particle size distribution is also an important characteristic of the packing material. Most HPLC packings have an approximate Gaussian distribution around the mean particle diameter. Of course, the narrower this distribution, the higher the column efficiency, due to the homogeneity of the resulting packed chromatographic bed.

If the particle size distribution is not tightly controlled, large particles can produce band broadening and small fines can block the 2 μm frits used to retain the packing material inside the column body

Chemical Composition (Hybrid Materials)

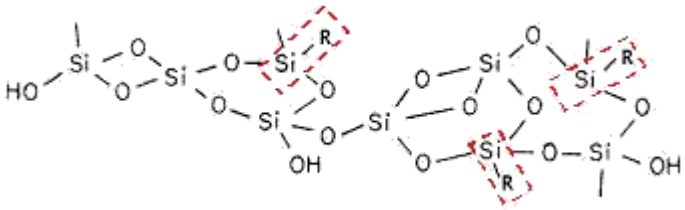
The fundamental chemical composition of the silica used for HPLC supports is SiO2 xH2O



Chemical Structure of the Silica Particle

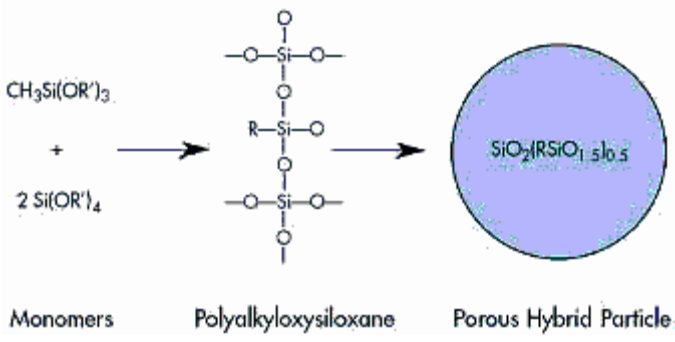
At HIGH pH, the silica substrate used for HPLC columns becomes susceptible to hydrolysis.

When the silica begins to degrade symptoms include rapid loss of efficiency, and an increase in backpressure. Several manufacturers have now resorted to a new generation of support material that are generally referred to as 'hybrid' materials.



Chemical Structure of the Hybridised Silica Particle

Hybrid silica is usually manufactured according to the general reaction scheme below, from a mixture of both organic (alkylsiloxane) and inorganic (silane) monomers to give a polyalkyloxysilane:



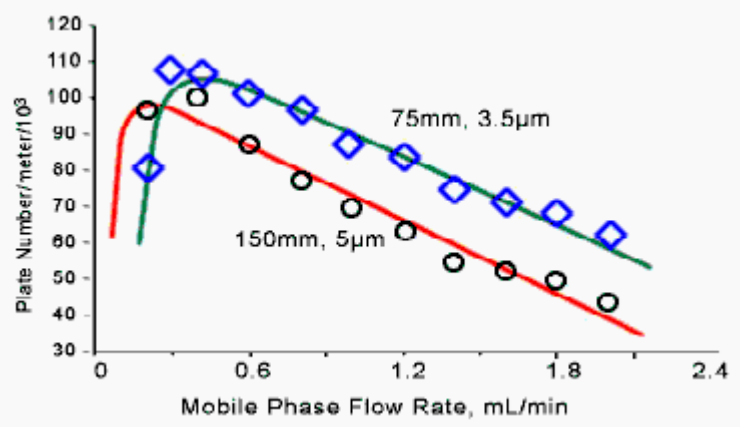
Hybrid Particle Reaction Scheme

These materials show good stability at high pH as the surface is no longer exclusively silanol in nature and therefore not as susceptible to hydrolysis.

The reduction in silanol surface activity also ensures that the activity towards basic analytes is reduced and peak shape improves.

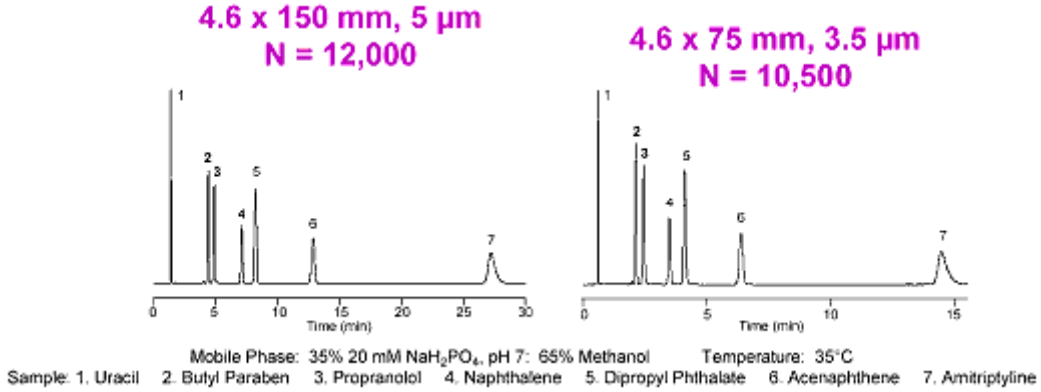
Particle Size

One of the most important column parameters is choice of stationary phase particle size. In the graph above, you can see that at any given flow rate, the smaller the stationary phase particle, the better the efficiency. The improved efficiency will translate into improved resolution.



Effect of mobile phase flow rate on efficiency for different silica particle sizes

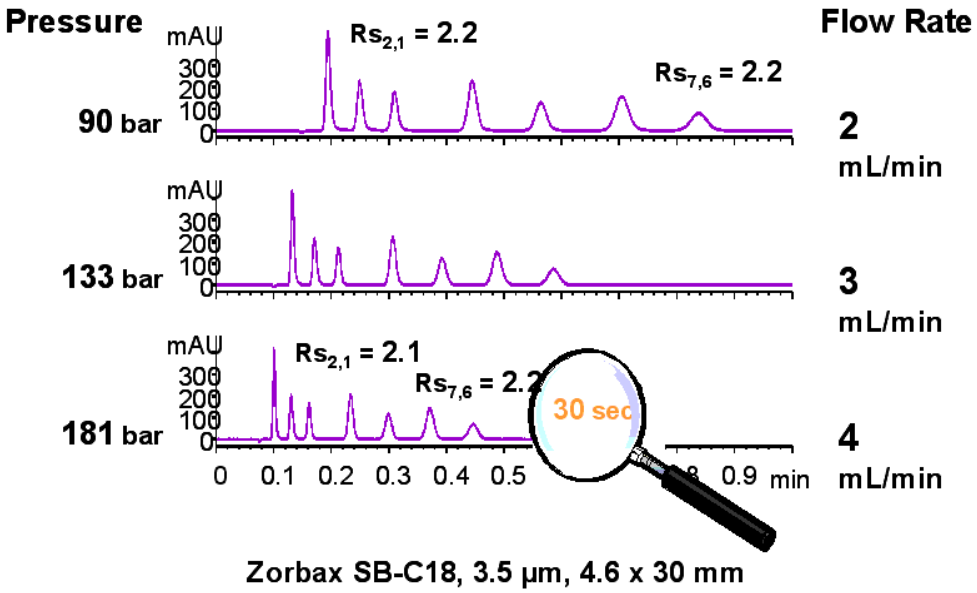
Why not use the smallest particle size possible? The answer is the column back pressure. Smaller particles lead to higher back pressures.
Because of improved efficiencies realized from smaller particles, two types of applications have resulted.



Reduced analysis time with comparable chromatography when using short columns with smaller particle size

First, one can use smaller particles but keep the flow rate the same as the original application (Figure a). This results in increased resolution even with shorter column lengths. Second, use the smaller particles in short columns at high flow rates. This type of

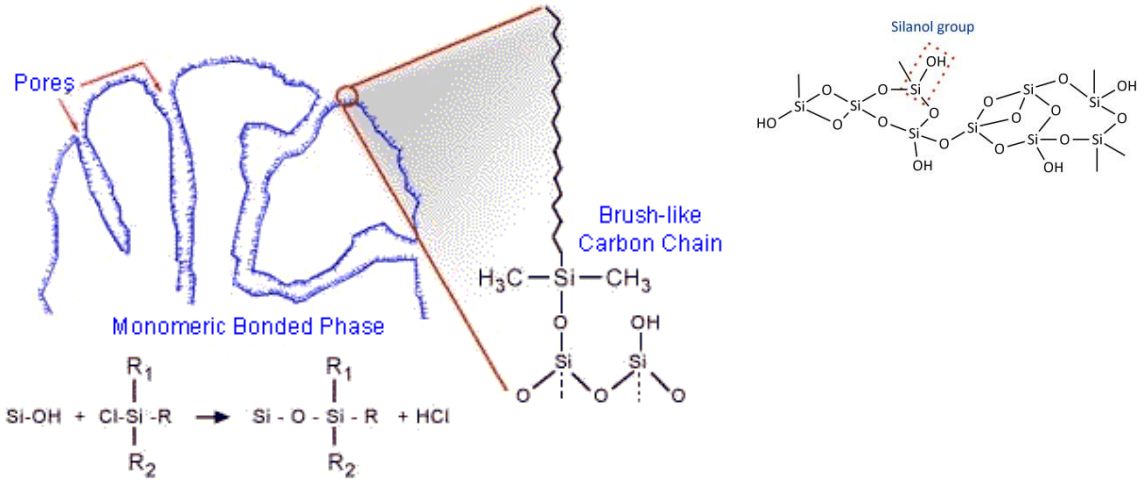
column is known as a fast or high-speed column and is available for high throughput applications needing moderate efficiency.



Use of smaller silica particle size columns at higher mobile phase flow rate to reduce analysis time.

Chemically Bonded Phases

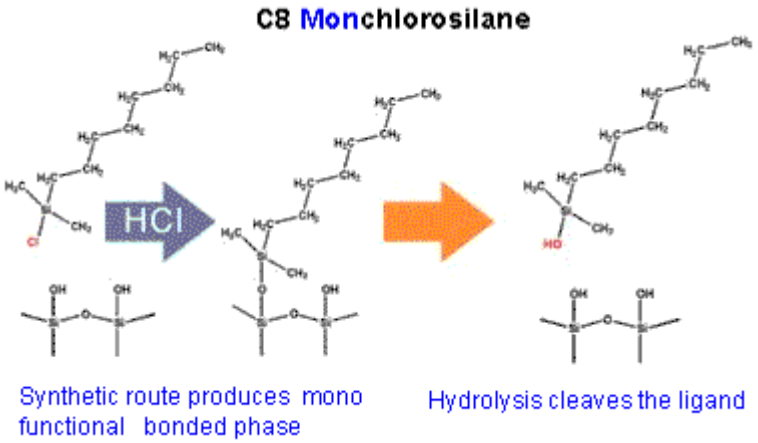
The fundamental composition of the silica used for HPLC is $SiO_2.xH_2O$ – this is shown and the silanol group, through which chemically bonded phases are attached, is highlighted.



Bonded phase application to a silica support in reverse phase HPLC

Reacting a chlorosilane with the surface silanol groups produces many useful stationary phases. The chlorosilane may be a mono-, di-, or tri-chlorosilane.

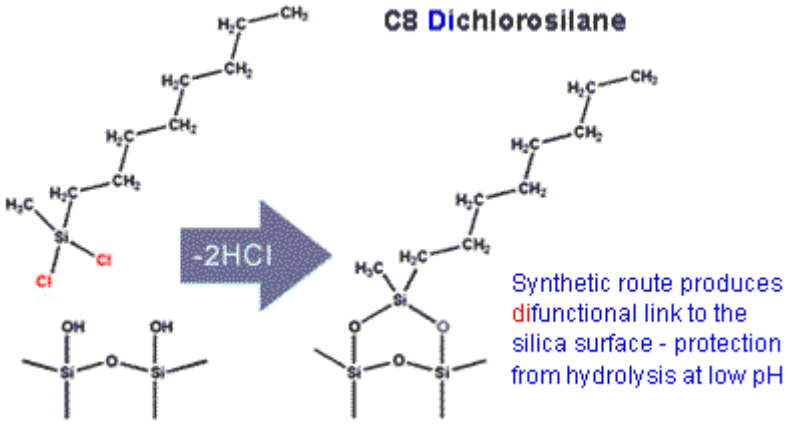
Monochlorosilanes produce monomeric packing materials that attach to the silica surface through a single bond (called a silyl ether linkage).



Monomeric reverse phase ligand application and subsequent hydrolytic degradation

Monomeric stationary phases are highly efficient, but not as chemically robust as those produced from di- or trichlorosilanes. These bonded phases are known as 'polymeric-bonded phases'.

Bonded phases are useful because they change the surface chemistry, thus changing the column selectivity.

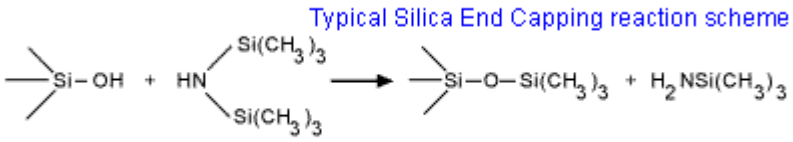


Di-functional reverse phase ligand application used to prevent from hydrolytic degradation

The choice of monomeric or polymeric phases, the type of alkyl species (R1, R2 & R3), extent of the bonding reaction, and other chemical bonding treatments can produce widely differing stationary phase selectivity for any given bonded phase type.

Surface Treatment –End Capping

The surface of non-hybrid silica is covered with silanol groups that are used in adsorption (normal phase) chromatography to interact with polar molecules, or in reversed phase (as well as ion exchange, chiral etc.) chromatography to attach the bonded phase material.



Silanol End Capping Reaction Scheme

Whilst most manufacturers are able to provide good bonded phase surface coverage, even the best manufacturing techniques will still leave a high number of silanol groups unreacted (due to **steric** effects).

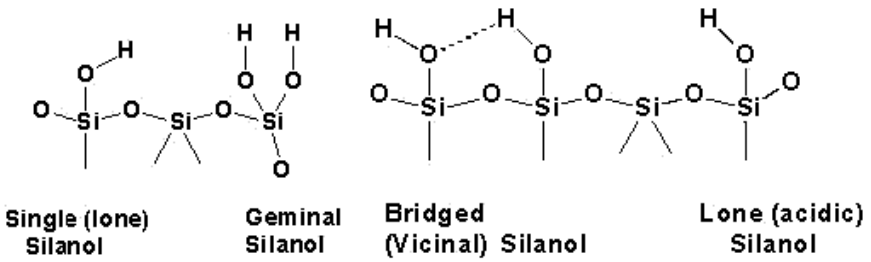
The remaining silanol groups (depending upon their **conformation**) are able to interact with analytes containing ionic or polar functional groups to give rise to peak tailing, through unwanted secondary interactions.

Manufacturers may choose to end-cap the column surface, which involves reacting the silanol groups with a sterically small but highly reactive reagent that 'caps' the polar surface silanol with a non-polar (and much less reactive) trimethylsilyl group.

As we will see later, some manufacturers use the less reactive silanol conformers to give a packing material with an alternative selectivity.

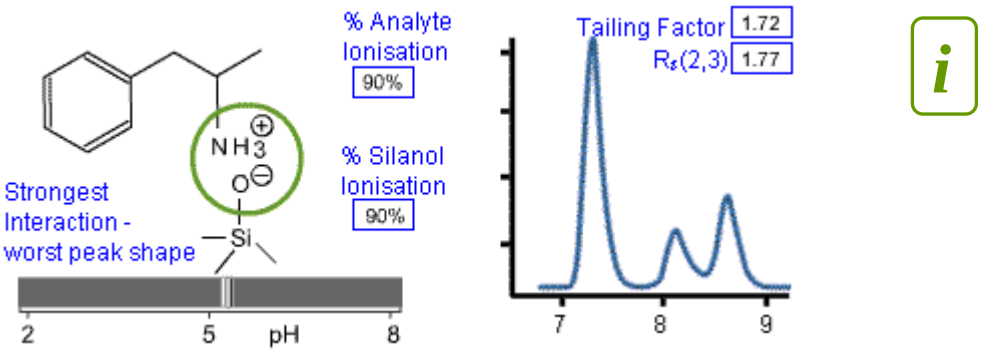
Nature of Silanol Species

All of the various silanol group conformations interact with polar and ionic analytes to different extents. Acidic (or lone) silanol groups give the strongest interaction – and therefore the worst peak tailing. Some of the silanol conformations can be usefully employed to aid selectivity!



Typical Silanol Conformations

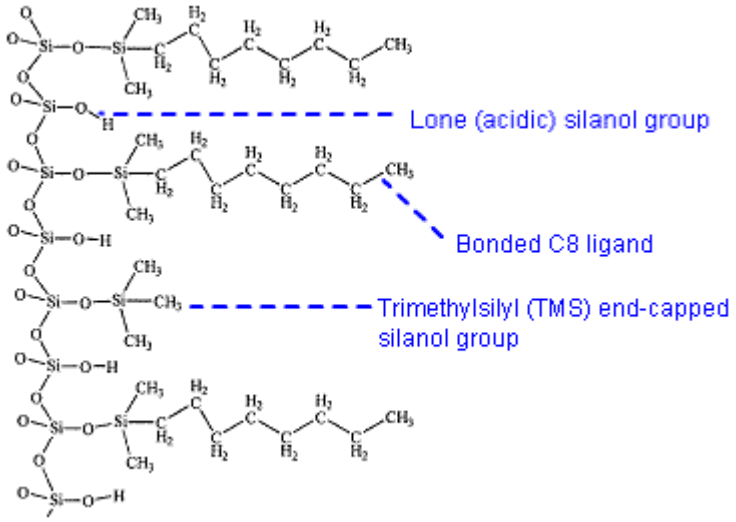
Peak tailing will be at its worst when a fully ionic interaction occurs between the silanol group and analyte. The magnitude of the interaction will depend upon the mobile phase pH, as this effects the degree of ionisation of both species. We will study this concept later, however, you should be aware that a typical lone silanol group will have a pKa value of around 3.8-4.2.



The effect of mobile phase pH on the severity of peak tailing with basic compounds caused by secondary silanol interactions

Use the slider bar to investigate the magnitude of interaction and therefore the extent of peak tailing between this basic analyte and the silica surface.

End Capping Efficiency



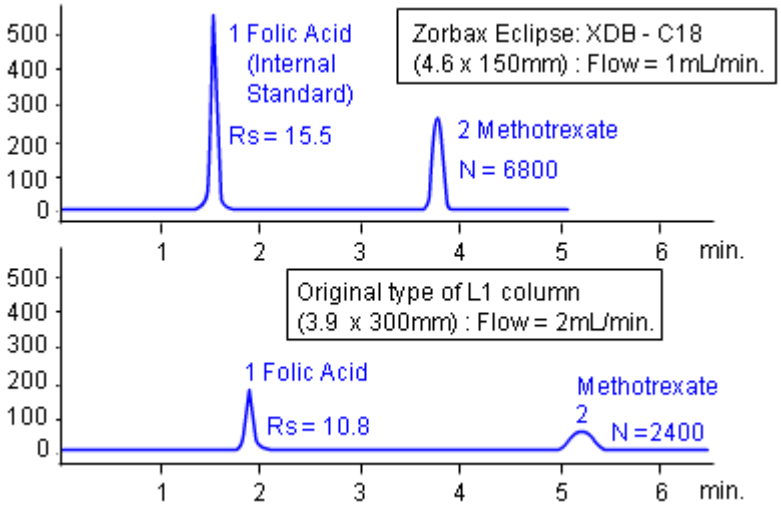
Typical surface chemistry of an end capped reverse phase stationary phase

The table below shows the extent of surface coverage prior to end capping various silica RP bonded phases –the longer chain lengths provide more **steric hindrance** and achieve a lower surface coverage.

Table 1. Typical ligand surface coverage on a silica substrate

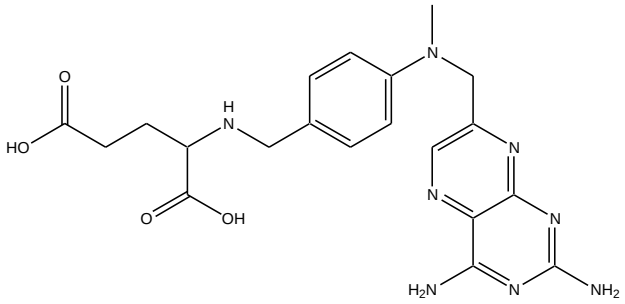
Bonded Phase	Surface Coverage (µmol/m^2)	Reacted Silanols (%)
Trimethyl	4.1	51
Dimethyl-3-cyanopropy	3.6	45
Dimethyl-n-butyl (C4)	3.5	44
Dimethyl-n-octyl (C8)	3.2	40
Dimethyl-n-octadecyl (C18)	2.7	34
Triisopropyl	2.22	8
Diisopropoyl-3-cyanopropyl	2.1	26
Diisopropoyl-n-octyl	2.0	25
Diisobutyl-n-octadecyl	1.9	25

This separation of **lypophilic** bases is carried out using a C18 column. The non-endcapped column shows poor chromatographic performance for the methotrexate analyte molecule (2).

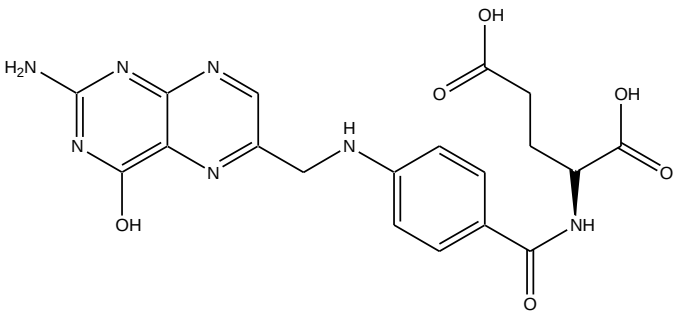


Separation of lipophilic bases using end capped and non-end capped stationary phases

Where:



Methotrexate

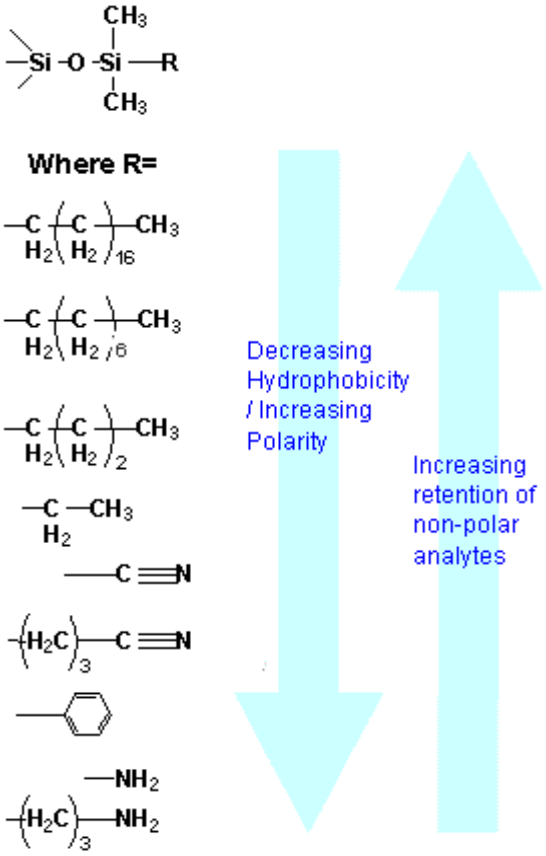


Folic acid

The top chromatogram shows the same separation carried out using an end capped (base deactivated) column with high surface coverage of bonded phase, effectively blocking analyte access to the silica surface. The analyte elutes in a much shorter time and shows much improved peak shape and efficiency.

Reverse Phase Stationary Phases

Reverse phase separations are characterised by having a stationary phase that is less polar than the mobile phase.

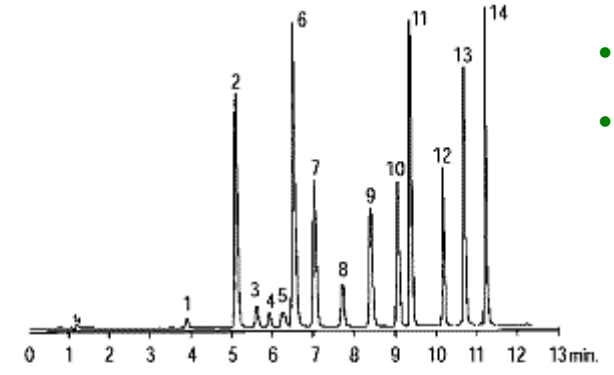


Several popular reverse phase, bonded stationary phases are shown. Octadecylsily (or C18) is commonly used, as it is a highly robust hydrophobic phase, which produces good retention with hydrophobic (non-polar) analyte molecules. This phase can also be used for the separation of polar compounds when used with mobile phase additives, which will be discussed later.

In general, shortening the alkyl chain will shorten the retention time. There are only very slight selectivity differences between, for example, C18 and C8 columns.

The use of more polar phases such as cyano, phenyl or amino phases show altered selectivity compared to the alkyl phases. These phases are able to interact with polar analyte functional groups, via dipole-dipole interactions and the phenyl column can interact with analyte aromatic moieties via π-π electron interactions.

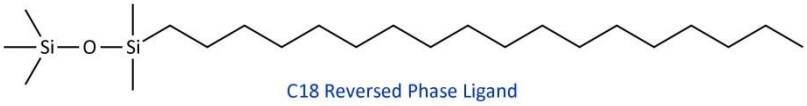
C18 (Octadecysilyl functional silica)



- Separation of substituted phenols using a C18 stationary phase
- This phase is widely used as it is both retentive and rugged for a wide range of application types

1. Phenol
2. 4-Nitrophenol
3. m-Cresol
4. o-Cresol
5. 2-Chlorophenol
6. 2,4-Dinitrophenol
7. 2-Nitrophenol
8. 2,4-Nimethylphenol
9. 4-Chloro-3-methylphenol
10. 2,4-Dichlorophenol
11. 2-Methyl-4,6-dinitrophenol
12. 2,4,6-Trichlorophenol
13. 2,3,4,6-Tetrachlorophenol
14. Pentachlorophenol

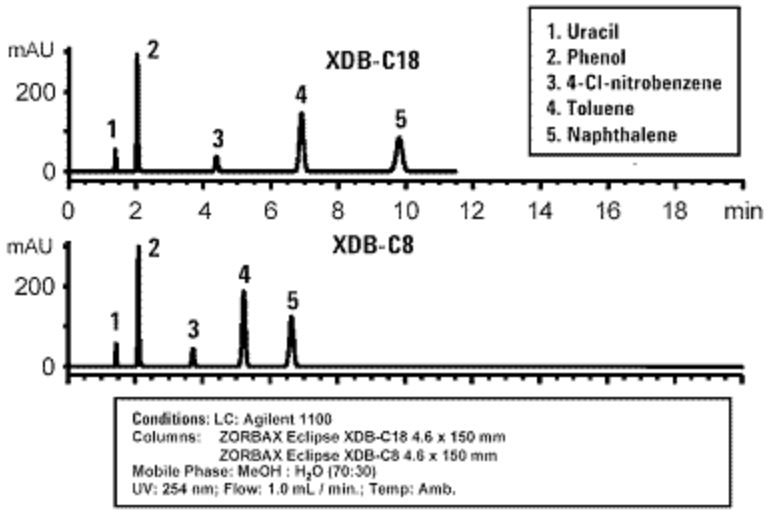
Sample:	Phenols
Column:	ZORBAX SB-C18, 4.6 x 150 mm, 5 µm
Mobile phase:	20% ACN/80% 0.01 M H_3PO_4 to 45% ACN in 7.5 min.
Gradient:	80% ACN in 2.0 min.
Flow rate:	1.5 mL/min
Temperature:	35°C
Detection:	UV 254 nm

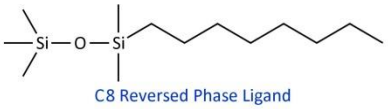


C18 Reversed Phase Ligand

C18 Reverse Phase Stationary Phase ligand and typical application using the phase

C8 (Octyl functional silica)



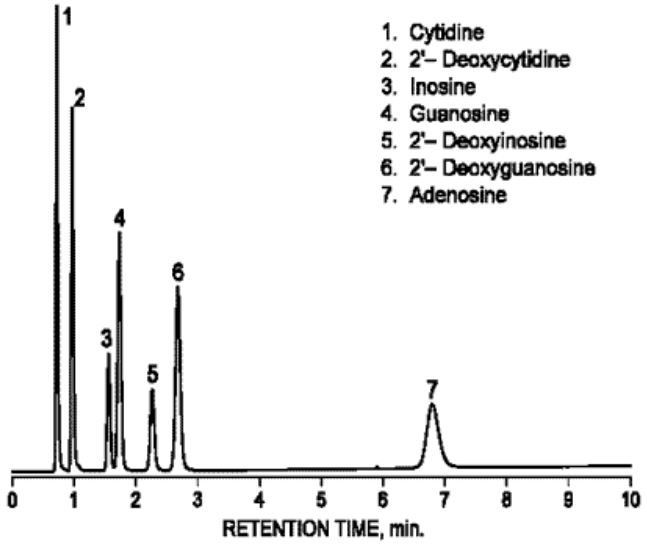


C8 Reversed Phase Ligand

- Separation of test compounds using both C18 and C8 stationary phases
- Retention times on the C8 phase are reduced whilst selectivity remains largely unchanged

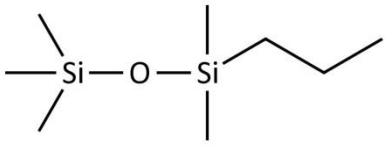
C8 Reverse Phase Stationary Phase ligand and typical application using the phase

C3 (Propyl functional silica)

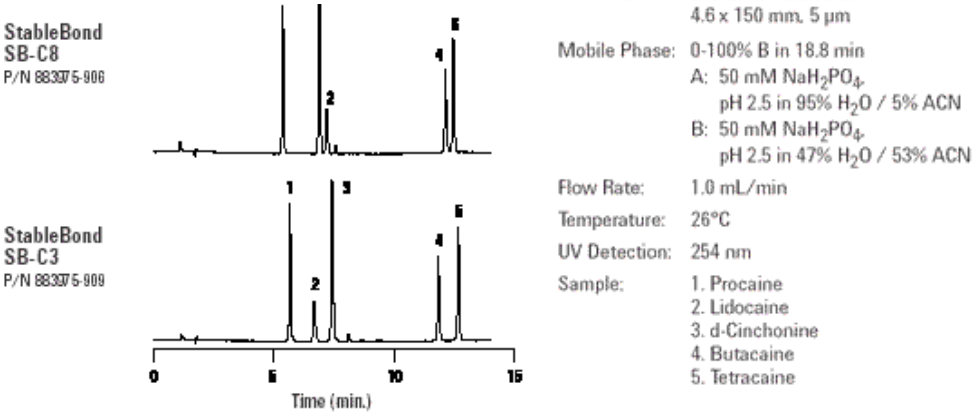


Conditions:
ZORBAX SB-C8 (3.5 µm) (4.6 x 75 mm) (Agilent P/N: 866953-906)
Mobile Phase: 5% methanol, 95% phosphate buffer, pH 4.0
Flow: 2.0 mL/min; Detect. UV(254 nm); 1µl with 1.6 µg each

Separation of nucleosides using short chain bonded phase in reverse phase HPLC



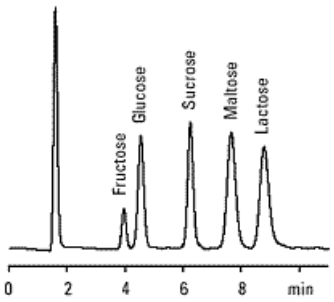
C3 Reversed Phase Ligand



C3 Reverse Phase Stationary Phase ligand and typical application using the phase

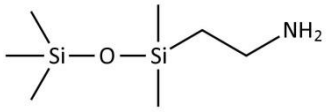
- Separation of local anaesthetics on C8 and C3 columns. The separation is via gradient LC and therefore hydrophobicity differences are not as apparent. It is interesting to note that the columns have notably different selectivity due to the influence of the silanol surface with the short C3 ligand
- C2-4 columns are often used for protein and peptide analysis. It should be noted that these short columns are not as chemically robust as their longer chain counterparts

Aminopropyl Functional Silica



Conditions:

Instrument:	Agilent 1100 RID
Column:	ZORBAX Carbohydrate Analysis Column 4.6 x 150 mm
Mobile Phase:	75% CH_3CN / H_2
Temperature:	30°C
Flow Rate:	1.4 mL/min
Sample:	Carbohydrate standards

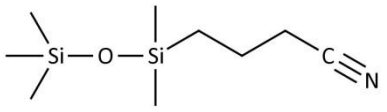


Aminopropyl Reversed Phase Ligand

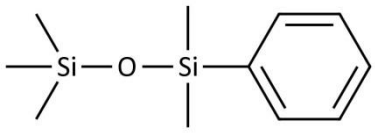
Aminopropyl Reverse Phase Stationary Phase ligand and typical application using the phase

- Separation of carbohydrate (sugar) standards on an (aminopropyl) carbohydrate column
- Amino columns can be used in normal as well as reversed-phase. They are most commonly used for the analysis of sugars and polysaccharides. They may also be used can be used for the analysis of organic ions. Avoid mobile phases containing aldehydes and ketones

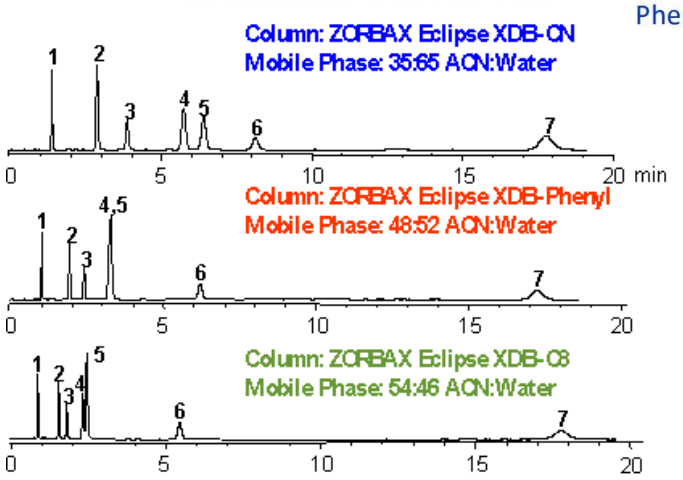
Phenyl and Nitrile Functional Silica



Cyanopropyl Reversed Phase Ligand



Phenyl Reversed Phase Ligand



Column Dimensions: 4.6 x 150 mm, 5 μm
Flow Rate: 2.000 ml/min
Injection Volume: 2.00 μl
Column Temperature: 25 °C
Detector: UV, 210 nm

Sample:
1. Estriol (0.00130 μg/μl),
2. β-Estradiol (0.00130 μg/μl),
3. Ethinyl Estradiol (0.00147 μg/μl),
4. Dienestrol (0.00123 μg/μl),
5. Diethylstilbestrol (0.00128 μg/μl)
6. Ethynylestradiol 3-methyl ether (0.00103 μg/μl)
7. Ethynodiol Diacetate (0.00139 μg/μl)

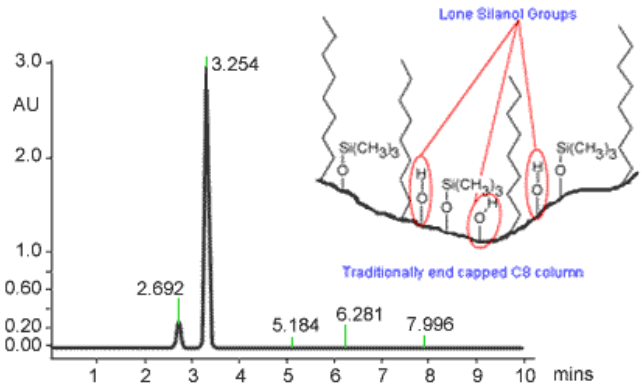
Phenyl and Nitrile Reverse Phase Stationary Phase ligands and typical application using the phase

- Separation of estrogens using C8, Phenyl and Cyano reverse phase bonded phases
- The phenyl column shows different selectivity from the straight chain phases due to the pi-electron cloud. The cyano column interacts with polar functional groups and can separate based on polar functionality. Cyano columns can be used either in reversed or normal phase

Silanol and Separation

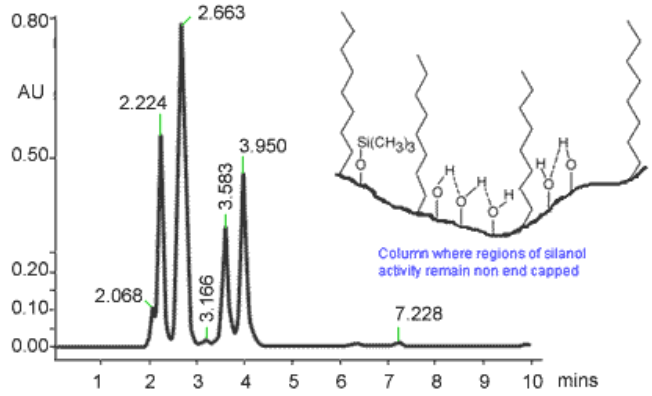
It is important to note the influence that the silica surface may have on the selectivity of a separation, especially in reverse phase chromatography.

Whilst lone (acidic) silanol groups can lead to peak tailing and irreversible retention, it is also possible to use the silanol surface to lend specific selectivity attributes to a separation. In this example, a column in which only the lone silanol groups have been end capped is used. The remaining silanol groups are in the vicinal (low energy) conformation – this adds a polar influence to the separation that is capable of separating closely structurally related compounds, crucially, without giving rise to peak tailing.



Traditionally end capped C18 phase. After the application of the mono-functional bonded phase, the phase is aggressively end capped using highly reactive reagents under high temperature and pressure. This can result in the disruption of homogeneous regions of silanol groups, giving rise to lone silanol groups.

Conditions:
Column: ODS (5µm)
Dimesions: 150 x 4.6mm
Mobile Phase:
Flow Rate: 1mL/min.
Temp: 40°C
Detection: UV @ 254nm



In this phase, the silanol groups on the silica surface remain undisturbed by end capping reagent and the low energy silanols are able to influence the selectivity of the separation without giving rise to peak tailing.

Importantly, by controlling the spacing of the bonded phase ligands, the degree of influence that the silanol species exert on a separation may be controlled.

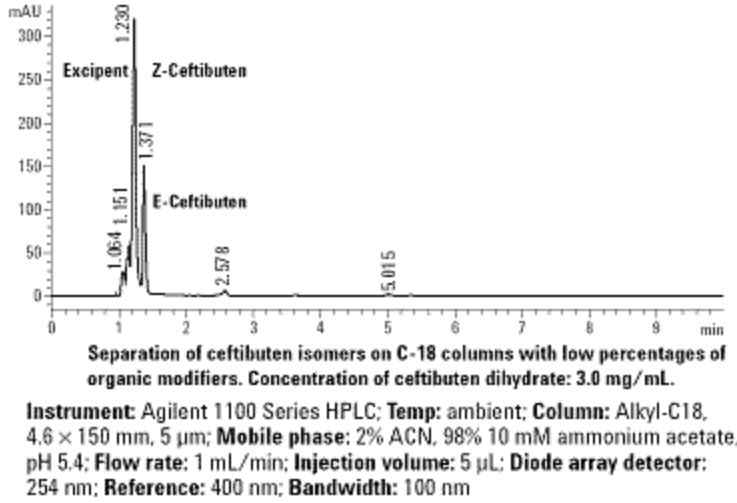
Conditions:
Column: Optimal L (5µm)
Dimesions: 150 x 4.6mm
Mobile Phase: MeCN / H_2O (0.5% Surfactant)
Flow Rate: 1mL/min.
Temp: 40°C
Detection: UV @ 254nm

In the example shown, the single peak due to processing impurities (ditriazinylphenylenediamine compounds), obtained using a traditionally end capped C18 column is shown to contain four separate components when analysed using a column where the silanol surface is utilised to offer an alternative selectivity to the separation, highlighting minor structural differences between analytes.

Water Wettable Phases

Several modern applications in HPLC require the use of highly aqueous mobile phase compositions.

Electrospray LC-MS techniques dictate that for optimum performance, the analyte species should be in the ionised form. As we shall see in later topics, this dictates that (to operate in reverse phase mode), the mobile phase used must be highly aqueous in order to gain any retention of the, potentially highly polar, (ionised) analyte



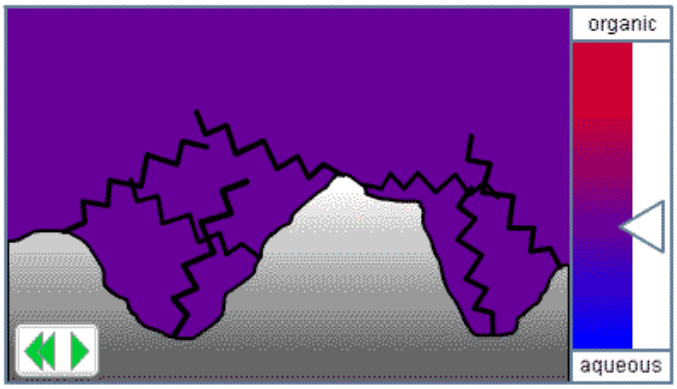
Separation of ceftibuten isomers on C-18 columns with low percentages of organic modifiers. Concentration of ceftibuten dihydrate: 3.0 mg/mL.

Instrument: Agilent 1100 Series HPLC; **Temp:** ambient; **Column:** Alkyl-C18, 4.6 × 150 mm, 5 µm; **Mobile phase:** 2% ACN, 98% 10 mM ammonium acetate, pH 5.4; **Flow rate:** 1 mL/min; **Injection volume:** 5 µL; **Diode array detector:** 254 nm; **Reference:** 400 nm; **Bandwidth:** 100 nm

Separation of Ceftibuten Isomers on a C18 column with increased access to the silica surface

Separation of anti-biotic analyte isomers using a water wettable column (Zorbax StableBond Aq). These compounds are highly polar at pH 5.4 and the analysis is traditionally carried out using ion-pairing reagent. Use of such reagents is not possible with LC-MS systems because they are involatile and foul the source.

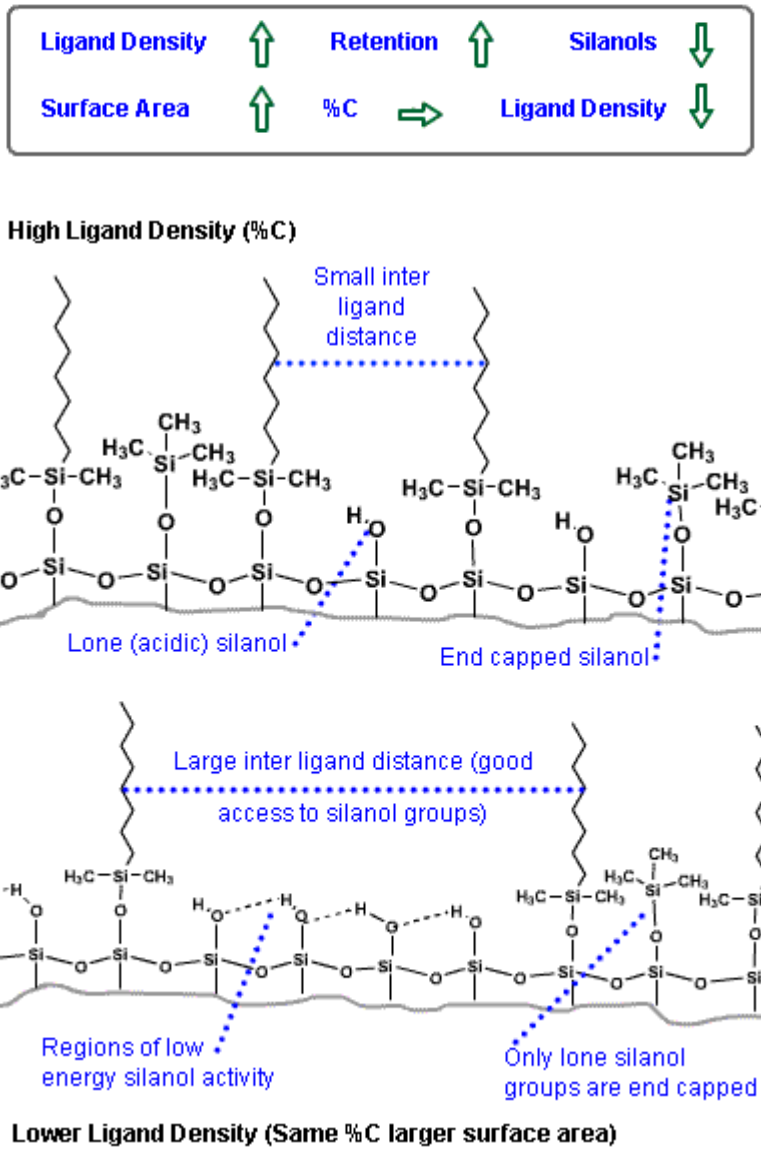
Traditional reverse phase columns are not suitable for use with highly aqueous mobile phases (>95% H_2O), due to a phenomenon known as 'phase collapse' or 'self-association'. Hydrocarbon bonded phases will tend fold into themselves to escape the highly polar mobile phase, and gross efficiency losses occur. The columns may be restored but only after long and complex column washing procedures.



Mechanism of phase collapse in reverse phase HPLC columns exposed to high aqueous content mobile phases

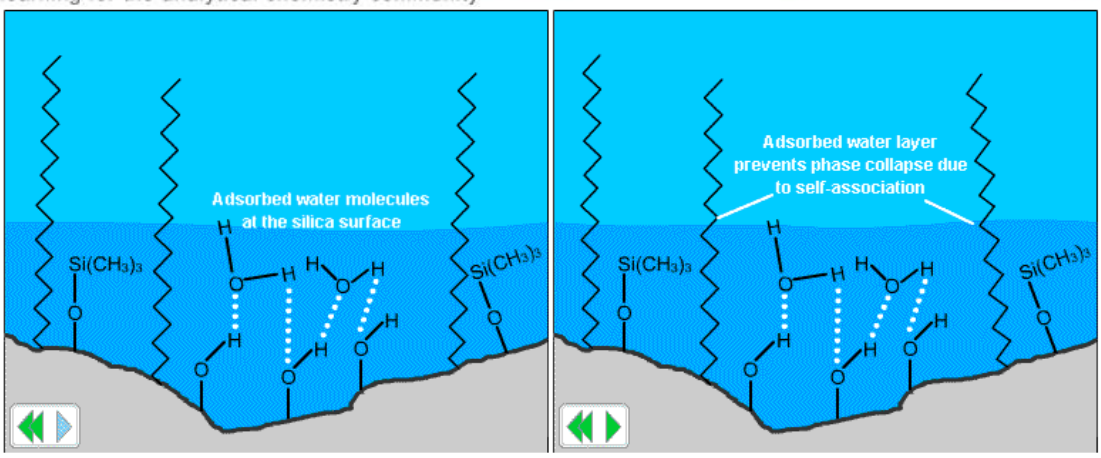
There are two ways of manufacturing water wettable phases for use in reverse phase HPLC – we will study both of them on the next page.

One method of producing a water wettable phase involves close control of the spacing between the bonded phase ligands on the silica surface. Knowing the surface area of the silica and adjusting the carbon loading allows the 'inter ligand distance' to be controlled. This gives more (or less) access to the silica (silanol) surface, allowing the polar influence of low energy surface silanol groups to alter the selectivity of the separation.



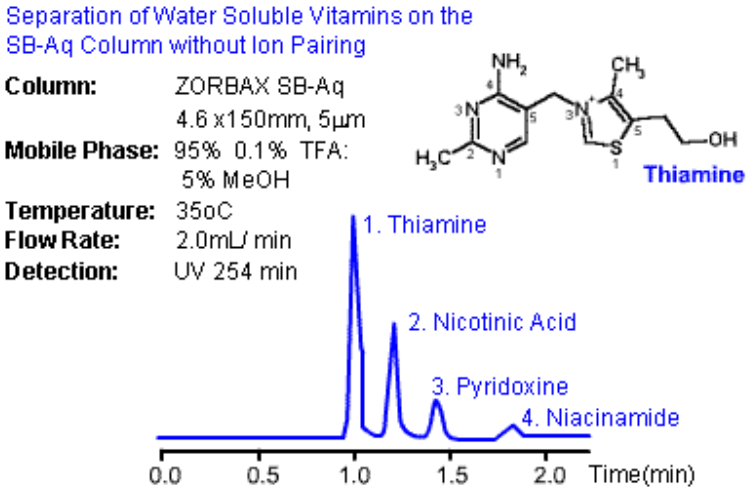
Production of water wettable phases via manipulation of ligand spacing on the silica surface

Stability in highly aqueous mobile phases is achieved via the adsorption of a layer of water at the silica surface. The vicinal (low energy) silanol groups become hydrated and so the driving force towards ligand self-association is lost as the layer of adsorbed water at the silica surface effectively repels the ligands and they remain 'activated'



Mechanism of avoiding phase collapse in water wettable phases

The alternative method of introducing stability in 100% aqueous mobile phases is to use polar end-capping reagents. These reagents are chosen to retain good peak shape with almost all applications whilst again introducing a polar characteristic to the separation and hence altering the column selectivity. The mechanism of high aqueous stability is similar to the previous case where an adsorbed layer of water at the surface prevents phase collapse.



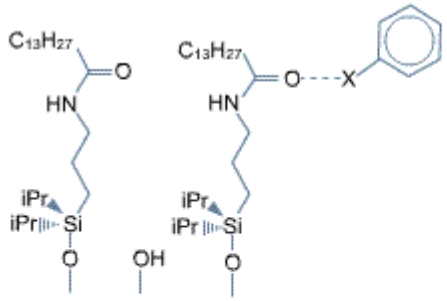
Use of water wettable phases for the separation of highly polar vitamin molecules without the use of ion-pairing reagents in Reverse Phase HPLC

Polar embedded ligands contain a modification to the alkyl chain which is usually an amide, carbamate or other suitable polar functional group.

The phase allows several distinct advantages including:

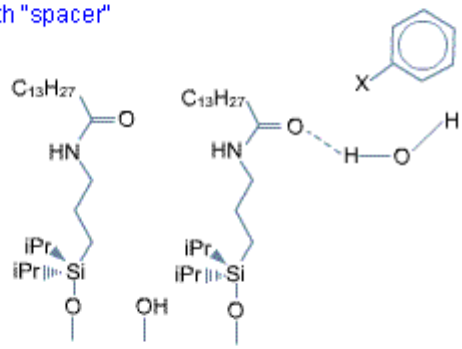
- Use with 100% aqueous mobile phases.
- Polar group that gives an alternative selectivity.
- The ability to separate polar, ionisable and especially highly basic compounds with excellent efficiency and peak shape
- Enhanced robustness over shorter chain and polar bonded phases.

The proposed mechanisms of interaction are shown along with some useful applications



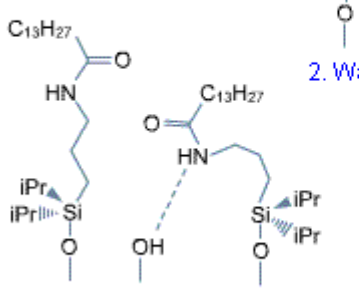
1. Analyte interaction with "spacer"

1. Here the analyte polar functional group is shown interacting with the amide 'spacer' – this is analogous to the interaction with the silanol surface to produce alternative selectivity.



2. Water interaction with modification layer

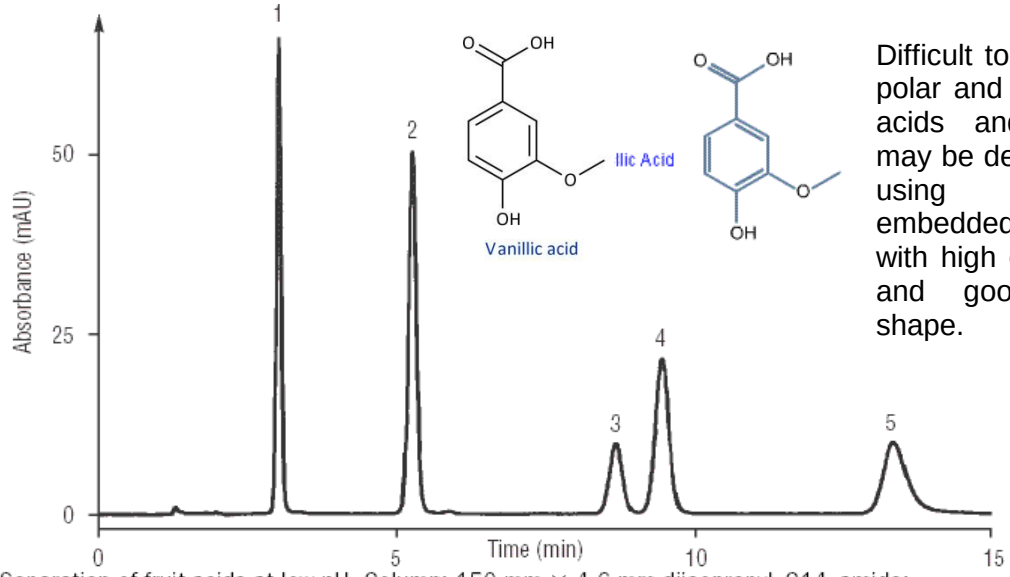
2. A layer of water attracted to the polar embedded moiety acts to stop phase collapse.



3. "Spacer" interaction with silanol

3. The polar moiety interacts with a lone silanol group to shield the surface and reduce secondary interactions (peak tailing).

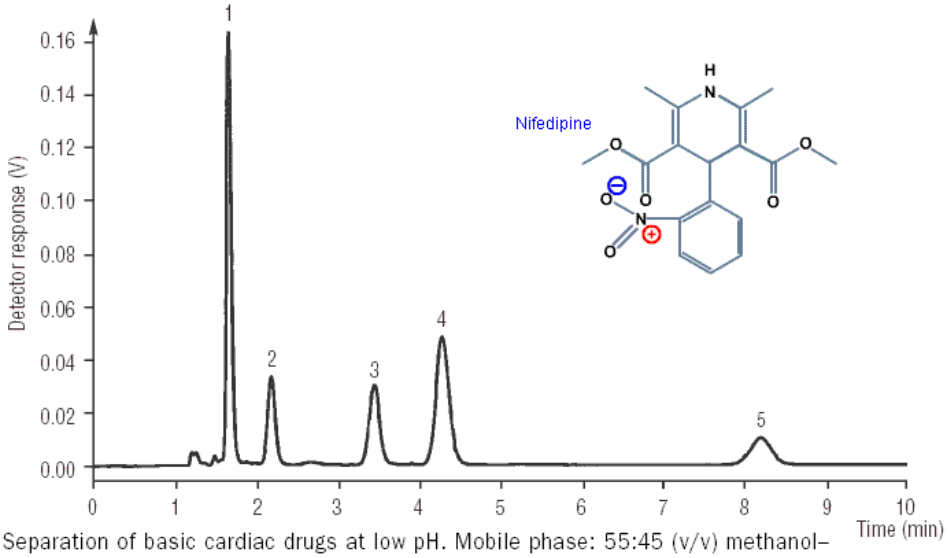
Polar Embedded Phases



Separation of fruit acids at low pH. Column: 150 mm × 4.6 mm diisopropyl–C14–amide; mobile phase: 70:30 (v/v) aqueous (0.1%) trifluoroacetic acid–methanol; flow rate: 1.0 mL/min; temperature: 24 °C; detection: UV absorbance at 254 nm; sample volume: 5 µL. Peaks: 1 = gallic acid, 2 = protocatechuic acid, 3 = syringic acid, 4 = vanillic acid 5 = gentisic acid.

Difficult to analyse, polar and ionisable acids and bases may be determined using polar embedded phases with high efficiency and good peak shape.

Separation of polar fruit acids at low pH using an amide embedded stationary phase

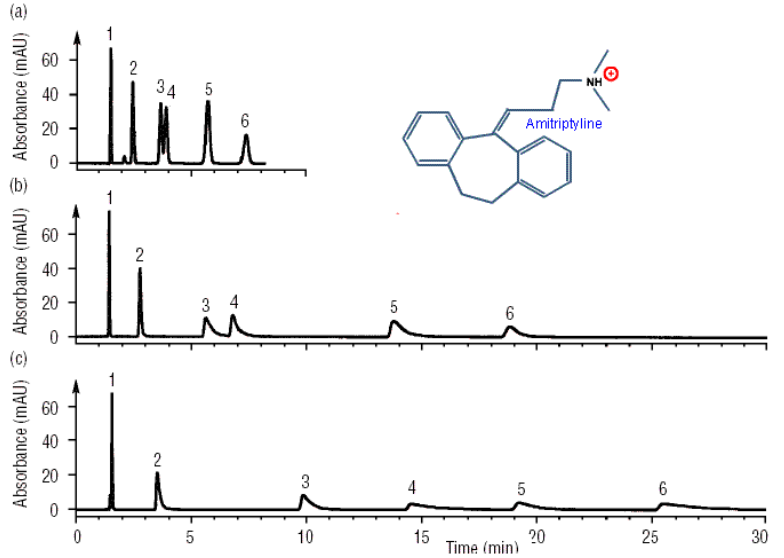


Separation of basic cardiac drugs at low pH. Mobile phase: 55:45 (v/v) methanol–0.025 M sodium phosphate buffer (pH 3.0); sample volume: 3 µL.
Peaks: 1 = diltiazem, 2 = dipyrimidole, 3 = nifedipine, 4 = lidoflazine, 5 = flunarizine

Separation of polar cardiac drugs at low pH using an amide embedded stationary phase

The separation of highly basic drug molecules using various bonded phases. It is interesting to note that the polar embedded column gives much less retention – perhaps due to the reduced hydrophobicity of the phase in comparison to the C8 or C18 column – and also due to reduced interactions between the analyte molecules and the silanol groups of the tradition alkyl phases.

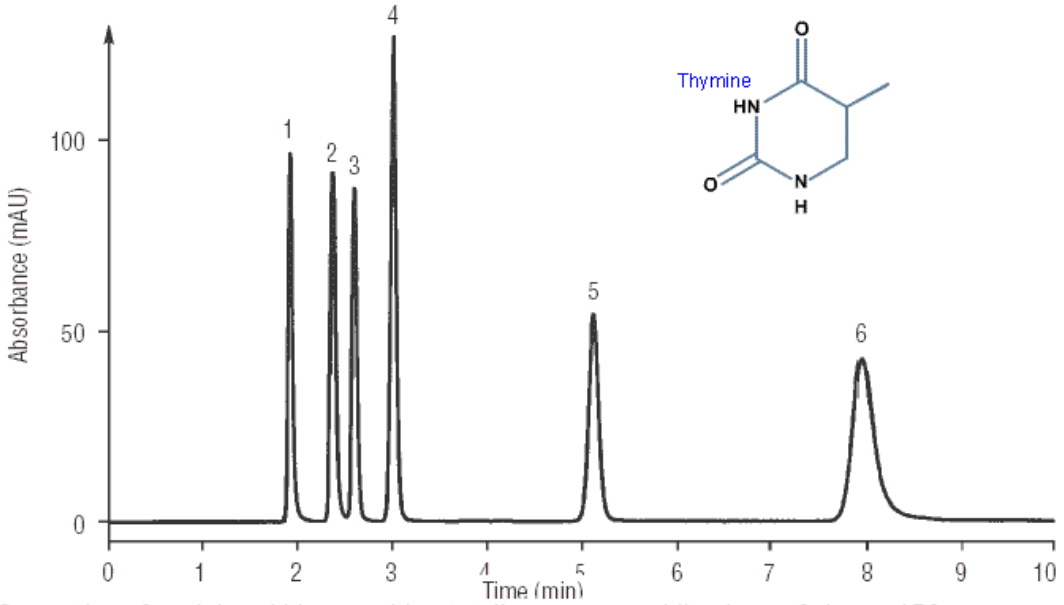
Of course – the polar embedded phase show superior performance, even over the C18 end capped column. This is due to the shielding of the silanol groups on the silica surface by the bulky side groups on the ligand (diisopropyl) and the interaction of the polar embedded group which 'shields' the analyte molecule from lone silanol groups on the silica surface.



Separation of basic drugs at pH 6 for (a) endcapped diisopropyl–C14–amide, (b) end-capped C18, and (c) nonendcapped C8 columns. Column dimensions: 150 mm × 4.6 mm; mobile phase: 62:38 (v/v) methanol–0.01 M citrate buffer (pH 6.0); flow rate: 1.0 mL/min; temperature: 30 °C; detection: UV absorbance at 254 nm. Peaks: 1 = uracil, 2 = propranolol, 3 = nortriptyline, 4 = doxepin, 5 = amitriptyline, 6 = trimipramine.

Improved peak shape of basic drugs using an amide functional polar embedded phase

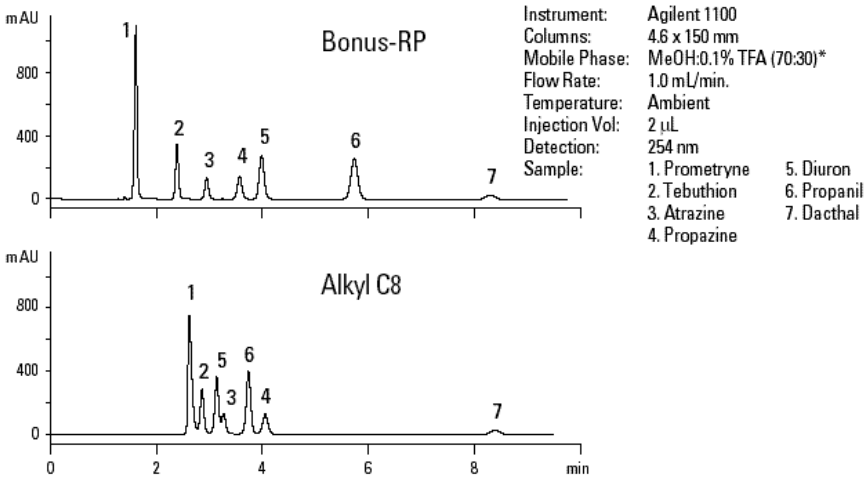
The next analysis shows the separation of nucleic acids using a totally aqueous mobile phase – demonstrating the ability of polar embedded phases to resist phase collapse.



Separation of nucleic acid bases with a totally aqueous mobile phase. Column: 150 mm × 4.6 mm alkyl–amide; mobile phase: 0.050 M sodium acetate (pH 4.6); flow rate: 1.0 mL/min; temperature: 24 °C; detection: UV absorbance at 254 nm. Peaks: 1 = cytosine, 2 = 5-fluorocytosine, 3 = uracil, 4 = 5-fluorouracil, 5 = thymine, 6 = adenine.

Separation of nucleic acids using a totally aqueous mobile phase

The next application presents the separation of triazine pesticides, showing the alternative selectivity between a conventional alkyl C8 column and a polar embedded amide alkyl (C14) column.



Alternative selectivity demonstrated by an amide functional C14 polar embedded phase for the separation of triazine pesticides

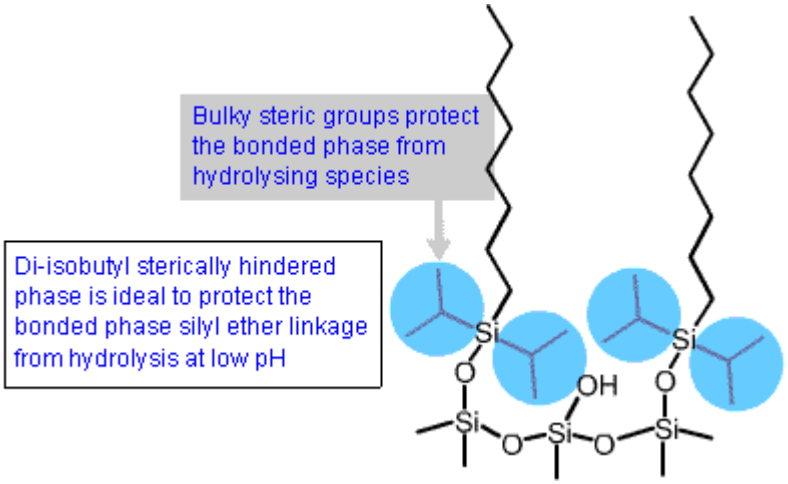
Working at High and Low pH

The traditional 'working' mobile phase pH range for silica based columns was 2.5 – 7.5. Outside this range, serious column damage would be inflicted with long-term use. This is unfortunate as there are many advantages to working at high and low pH.

At low pH (1-2.5), the risk is due to hydrolysis of the silyl ether linkage between the bonded phase and the silica surface. Symptoms of phase 'bleed' at low pH include deterioration of peak shape and loss of efficiency.

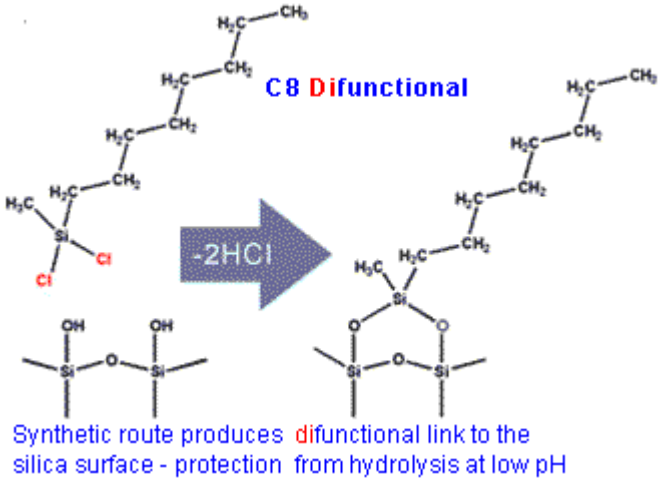
Most manufacturers offer bonded phase choices to operate at low pH. Most are based on one of two mechanisms:

- Groups on the ligand to offer steric protection of the silyl ether linkage



Sterically Protected Ligands to avoid hydrolysis at Low pH

- Difunctional binding to the silica surface



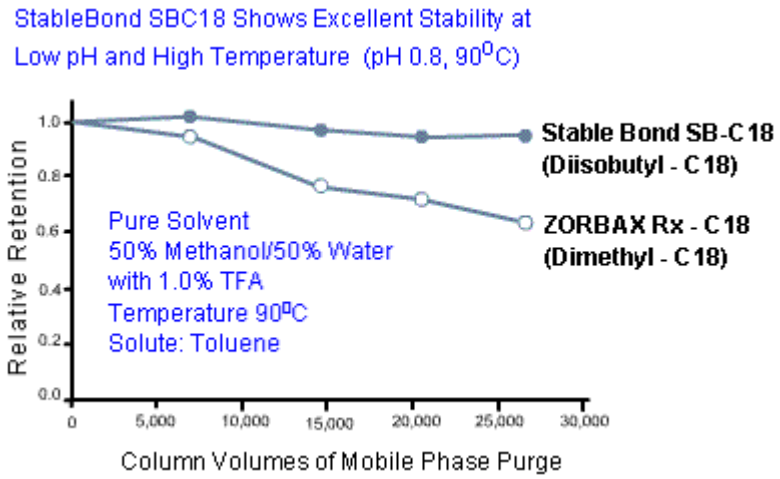
Difunctional ligands to protect silica from hydrolysis at

It should be noted that tri-functional bonding is sterically impossible – however, some column manufacturers will use trifuctional ligands in order to increase the extent of difunctional bonding.

Working at low pH allows strong(er) acidic analytes to be analyzed without the need for ion pairing reagents.

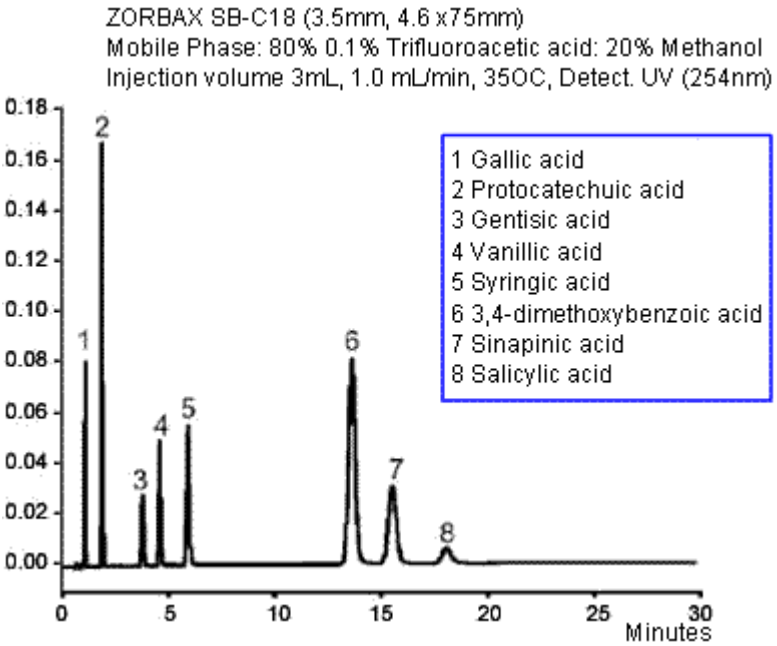
Extreme pH applications

The sterically hindered phase (SB C18) is very stable as measured by the retention time of toluene at pH 0.8 and 90°C over three working months of continuous column usage.



Stability of protected and non-protected C18 phases at low pH

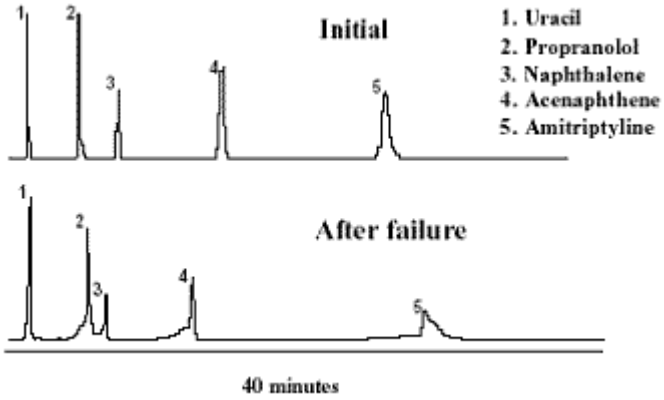
The next graph presents \ separation of organic acids that is carried out with 0.1% TFA which adjusts the mobile phase to pH 2.1. At this pH all of the organic acids are in the non-ionic form resulting in improved peak shape.



Separation of acidic analytes using a sterically protected C18 phase at low pH

Column Failure due to Hydrolysis

Loss of efficiency and peak shape (including fronting as well as tailing) are symptoms of loss of bonded phase when working at low (<2.5) mobile phase pH.



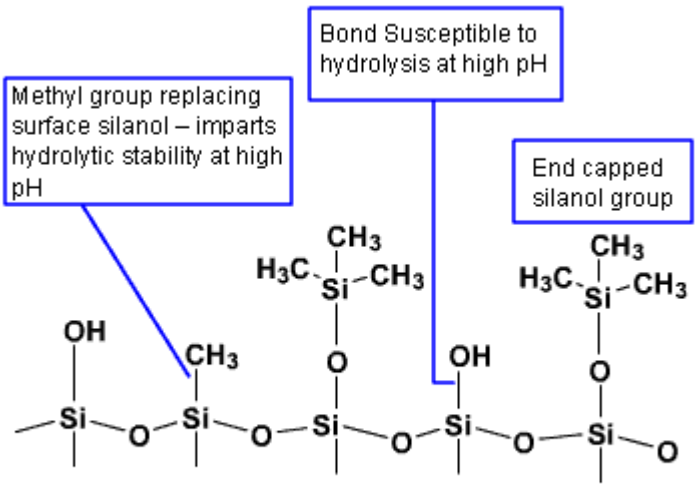
Typical Symptoms of column failure using Low pH mobile Phase

At higher pH it is the silica surface itself that is most at risk from hydrolysis, primarily via the surface silanol species.

At mobile phase pH above 7.0 traditional silica based packing materials may begin to hydrolyse, leading eventually to the formation of small 'fines' which migrate to, and block, the outlet frit – leading to increased system back pressure. Other, earlier symptoms again include loss of peak shape and efficiency.

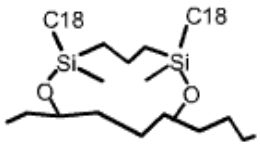
Many approaches have been taken to protect HPLC stationary phases at high pH including:

- Using 'hybrid' silica that contains fewer surface silanol groups

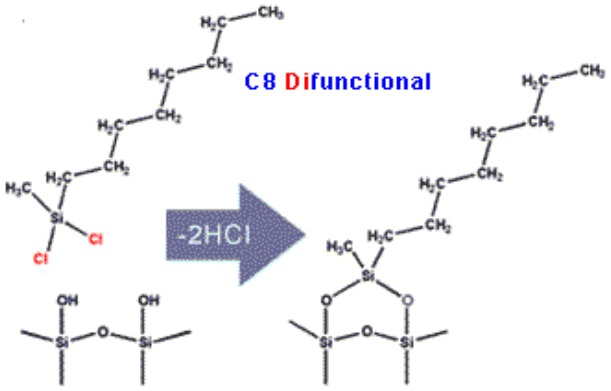


Typical Hybrid Silica Structure

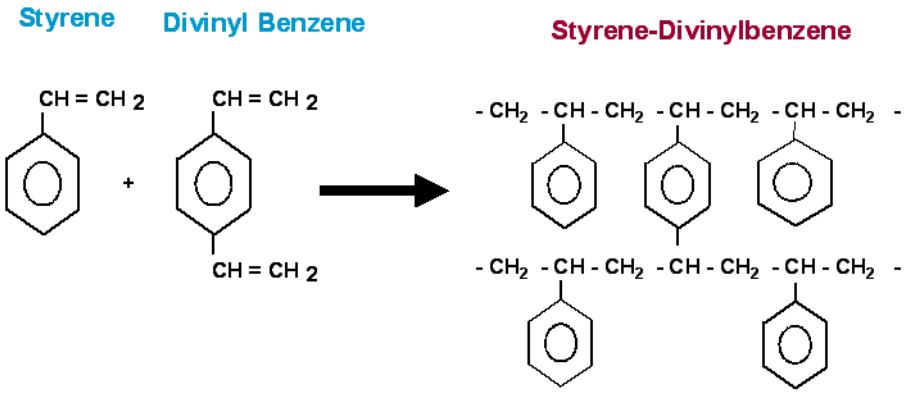
- Using multiply bonded silica to reduce the instance of surface silanol species



Difunctional and Multi-dentate Ligands



- Using polymeric and non-silica based material



Polymeric Ligands

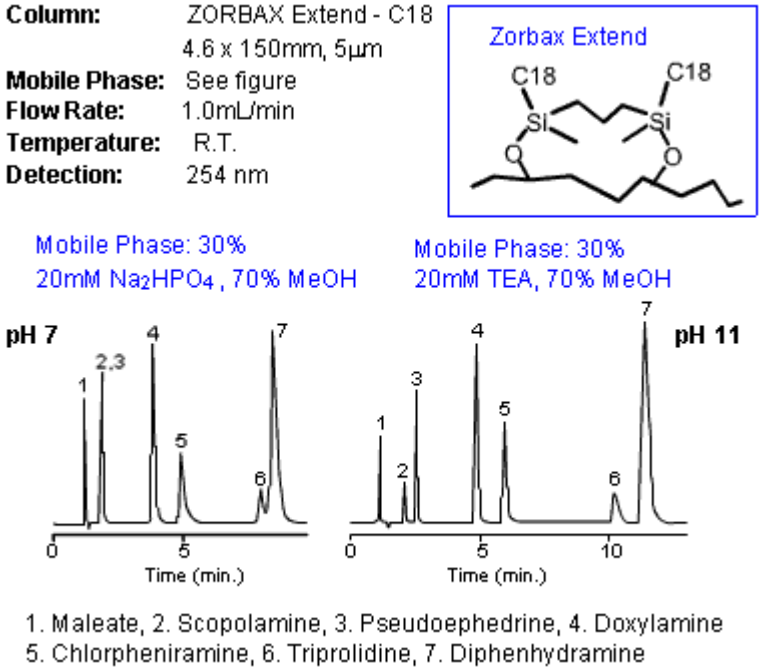
Polymeric supports such as polystyrene-divinylbenzene (PSDVB) are often used in ion exchange and size exclusion applications. PSDVB phases can be very rigid and resist pressure up to 5000 psi (345 bar). This stationary phase is very hydrophobic and can be used without derivatization as a pH resistant alternative (pH 1-13). Unfortunately, due to the lack of porosity, PSDVB does not have the efficiency of silica gels, so its applications have been limited. Peak shape can be improved with THF/acetonitrile mobile phases. This mobile phase combination is said to deactivate the aromatic rings of the PSDVB.

Polystyrene-divinylbenzene is most often applied to size exclusion and ion exchange HPLC applications and chemical modification may be performed to produce bonded phases where required.

Working at high pH, especially with basic analytes, has the advantage of removing an experimental variable (the degree of analyte ionisation, and the necessity to very accurately adjust mobile phase pH), and increases method robustness and retention for high basic analytes.

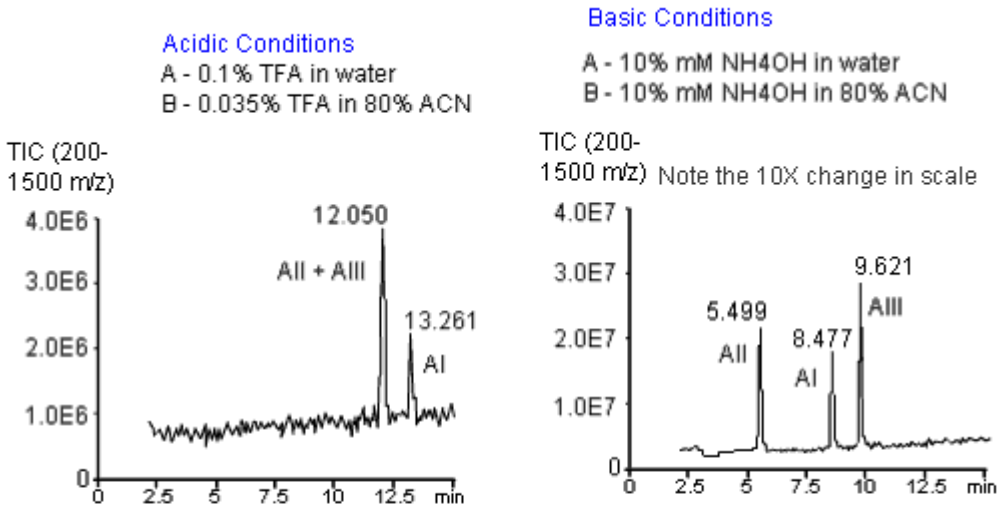
High pH Applications

The next application presents the separation of antihistamines shows improved selectivity, resolution and peak shape when the basic analytes are in the free base form (non-ionised) at pH 11. The column uses bidentate ligand chemistry - two 'chemically bridged' ligands are applied to the silica surface as an alternative to using multi-functional ligands, which can re-hydrolyse to form acidic silanol groups.



High pH separation of antihistamines using a bidentate stationary phase

The next application presents the LC-MS analysis of peptides can be improved at high pH using ammonium hydroxide as the buffering reagent rather than TFA for low pH that can suppress analyte signal. In this application, signal to noise ratio is improved and an alternative selectivity is seen at high pH.



Column:	ZORBAX Extend-C18, 2.1x150mm
Flow Rate:	0.2mL/min
Temperature:	35ºC
Mobile Phase:	As indicated
Gradient:	15-50% B in 15 min.
LC/MS:	Pos. Ion ESI-Vf70V, Vcap 4.5kV, N_2-35 psi, 12 L/min., 3250C
Sample:	2.5ml sample (50pmol each)

Peptide Separation at High pH using a Difunctional Stationary Phase

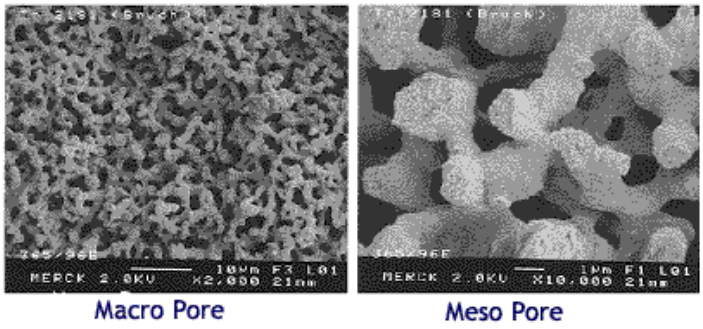
Other Stationary Phase Types

There are many other stationary phase types available and these will be dealt with in subsequent modules. However, a broad overview is given here for reference. Other stationary phase types include:

- Chiral Phases
- Ion Exchange Stationary Phases
- Normal Phase Stationary Phases
- Gel-permeation and Size Exclusion Phases
- Fluorous Phases
- Monolithic and Cast Carbon Phases

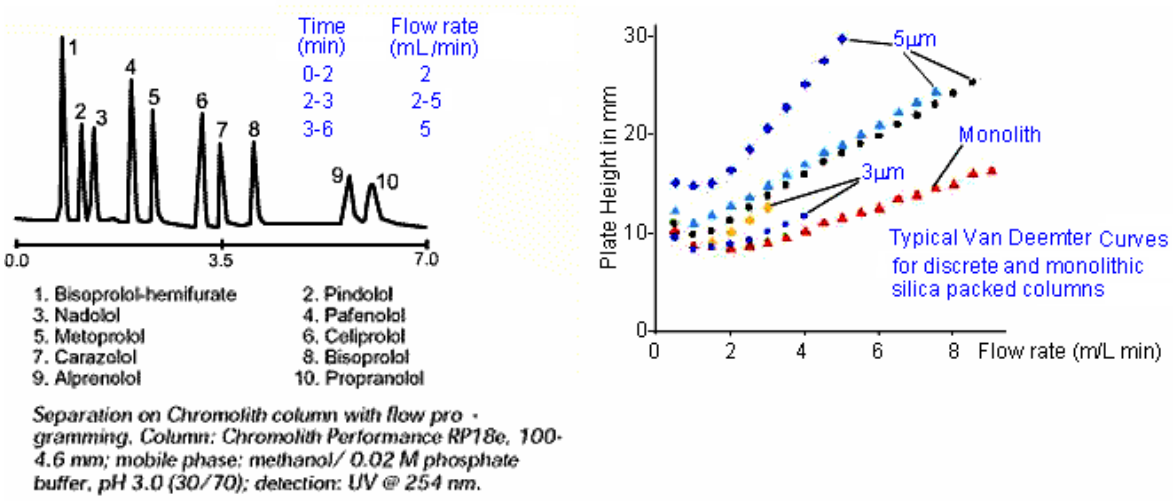
Monolithic Phases

Monolithic columns are formed from dried rods of polymeric silica gel. The columns are highly porous with a bi-modal pore structure containing mesopores with diameters of approximately 13 nm and macropores with diameters of approximately 2 µm. The surface area created by the mesopores is approximately 300 m^2/g.



Scanning Electromicrograph ~ (SEM) of the typical bimodal pore structure of monolithic silica columns

Because the total porosity of the monolithic silica matrix is greater than 80%, users can perform chromatography using a much lower back pressure. Because of the better mass transfer properties of a monolithic skeleton over distinct particles (i.e., flatter Van Deemter profiles), high-speed separation is possible without a noticeable effect on resolution.

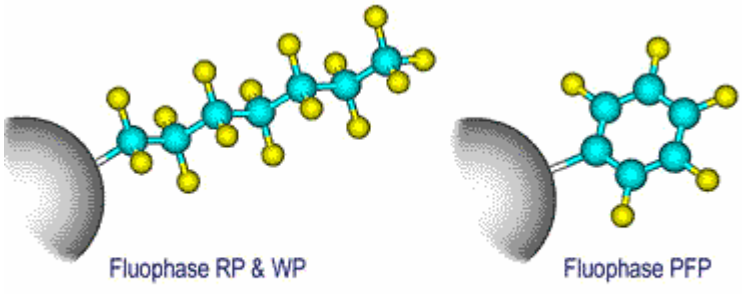


Typical Flow Programming application using Monolithic C18 column and the Van Deemter curve for various stationary phase particle sizes versus the monolith material

The low back pressures created also introduce the possible of Flow-Programming, where the mobile phase flow rate is increased during the analysis to shorten analysis time.

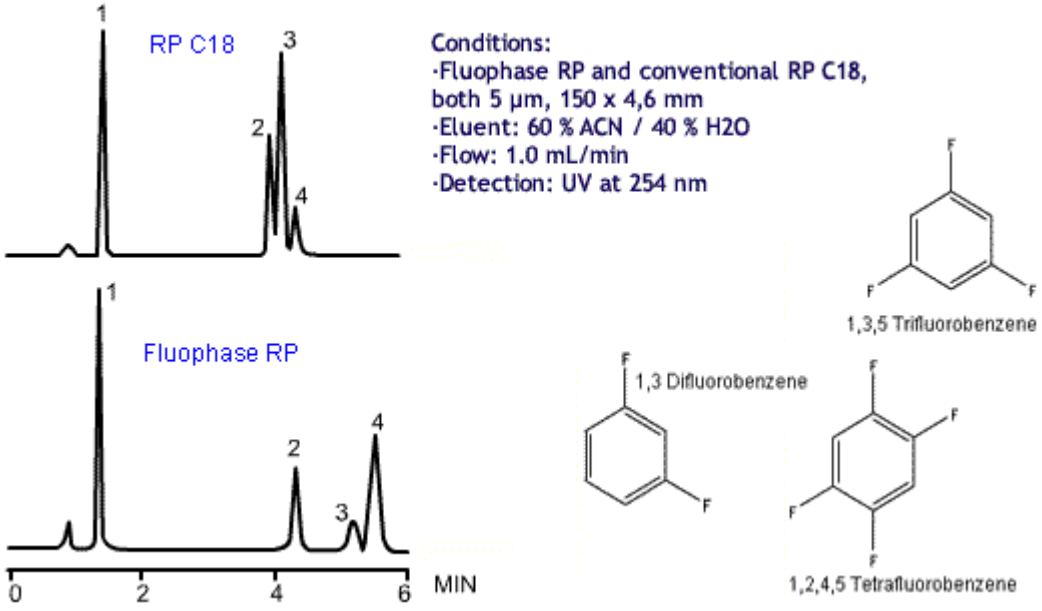
Monolithic columns, which carry a C18 surface-modification and endcapping are comparable in selectivity to conventional reversed-phase columns. A comparison shows that although the selectivity of monolithic and particle based columns are the same, the retention times achieved by the monolithic column are much shorter, therefore resulting in a faster separation.

Fluorine Based (Fluorous) Phases



Typical Structure of Fluorinated Stationary Phase

Fluorinated phases exhibit extra retention and selectivity for halogenated compounds and shape selectivity for positional isomers involving aromatic rings and other rigid systems.



Altered selectivity of Fluorine based stationary phases versus conventional C18 for halogenated analytes

Introducing Fluorine groups into the stationary phase gives the fluorinated columns unique capabilities in solute-stationary phase interactions. The carbon-fluorine bond has greater dipole character than a carbon-hydrogen bond, giving greater interactions with polar and halogenated compounds.

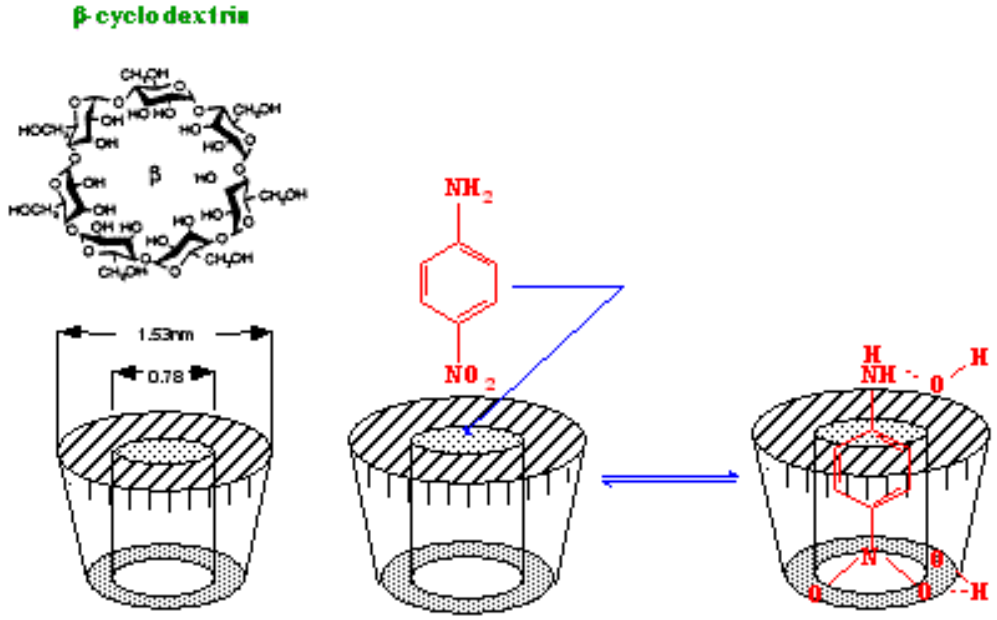
Chiral Phases

There are several distinct types of chiral stationary phase (CSP) and these will be covered in more depth in a subsequent topic.

The basis for many chiral separations, especially in the reversed phase mode is a phenomenon called inclusion complexing. First described for the polyglucose structures, cyclodextrins (CD), it has been identified as a mechanism for the macrocyclic glycopeptides as well as the cellulose and amylose CSPs.

Inclusion complexing is dependent upon the formation of a **diastereomeric** complex based on optimising the chiral interactions between the analyte(s) and stationary phase and reducing the non-chiral interactions.

In the next example using a cyclodextrin phase, the positional isomerism of the substituted aromatic will dictate how far into the CD cavity the analyte will penetrate. Any substituent groups capable of hydrogen bonding may also interact with Hydroxyl groups around the mouth of the CD cavity



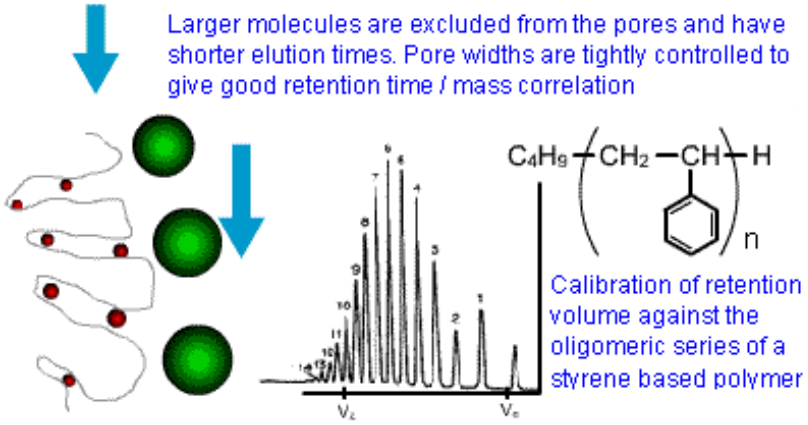
Typical Inclusion Complex Formed between analyte molecules and the CD cavity with Chiral Stationary Phases

Size Exclusion Chromatography

Size exclusion chromatography is a technique for separating molecules based on their effective size and shape in solution. The technique is often called 'gel permeation' chromatography if used with organic solvents or 'gel-filtration' chromatography is used with aqueous solvents.

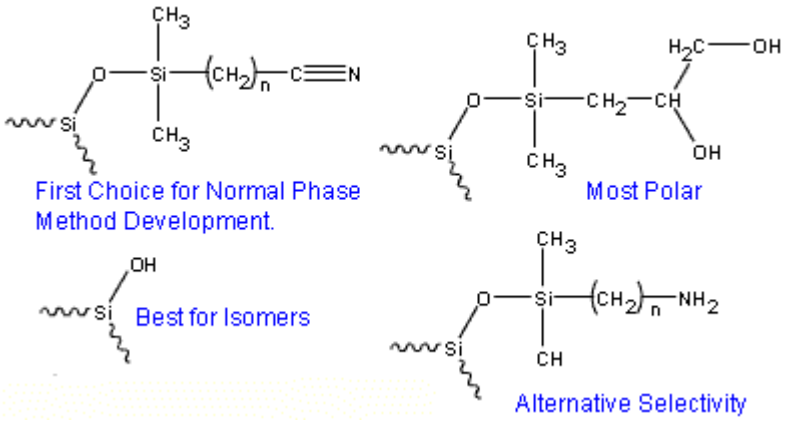
The stationary phases used in exclusion chromatography are porous particles (made from either silica or more typically a polymeric compound) with a closely controlled pore size. Unlike other chromatographic modes, there should be no interaction between the solute and the surface of the stationary phase.

Analytes are separated according to their molecular volume. An oligomeric compound such as styrene, whose oligomeric molecular weights are well known, can be used to calibrate the column for retention time (t_R) (or retention volume V_M) against molecular mass. In this way the molecular weight distribution of unknown analytes such as polymers or proteins may be obtained.



Mechanism of Size Exclusion Chromatography (SEC) and a typical calibration curve for molecular weight against retention volume for a polystyrene standard

Normal Phase Stationary Phases



Typical Normal Phase Chromatography Bonded Phases

Advantages of Bonded Phases:

- Water does not have to be controlled
- Gradient elution possible
- Column equilibrates rapidly
- Wide variety of polarities, selectivity
- High capacity
- Peaks don't tail as with bare silica

The mobile phase water content does not have to be strictly controlled as it does with silica columns (more on this later). As a result of all these advantages, it is recommended that method development in normal-phase mode begin with bonded columns. In particular, with cyano columns which have intermediate polarity and good stability. Diol columns are the most polar and silica like. They however, like amino columns are not as

stable. If the selectivity is not appropriate on the bonded-phase columns, then switch to bare silica.

Bare silica, however, is recommended for the separation of structural isomers.

Ion Exchange Columns

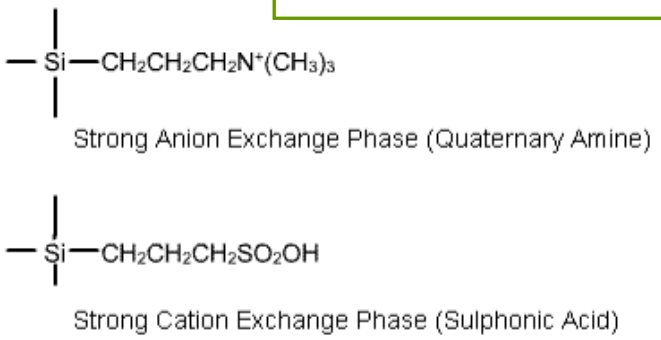
Ion exchange resins can be divided into four categories:

1. Strong Cation Exchangers
2. Weak Cation Exchangers
3. Strong Anion Exchangers
4. Weak Anion Exchangers.

Important:

Strong ion exchangers retain their charge over a wide pH range

- Weak ion exchangers maintain their charge over a limited pH



Range of Strong and Weak Ion Exchange Ligands

Strong anion and cation exchangers maintain their charge and thus their separating ability over a very large pH range. Weak anion and cation exchangers retain their separating capability (charge) over a much narrower pH range. Therefore, strong anion and cation exchangers are generally preferred when the analyte characteristics are not known.

In the case of strong sample anions or cations, a weak exchanger may be more useful as strong acids and bases may be too strongly attracted to the stationary phase – making elution difficult. The pH sensitivity of the weak exchangers helps bring about the sample ion elution, by altering the mobile phase pH to neutralise the charge on the stationary phase.

The charged functional groups may be bonded to either silica or a rigid polymer. Polymers are preferred for durability. They can withstand a wide range of mobile phase pH and higher ionic strengths. The silica backbone is often preferred because of its tolerance of organic modifiers, minimal shrinkage or swelling, high capacity, and availability in a variety of pore sizes.

Test Probes and Column Characterisation

It is useful to define and track the performance characteristics of a column / stationary phase. This can be done for regulatory purposes, as part of your Good Laboratory Practice (GLP) regime or just to save wasting time using a column that is not fit for purpose.

Many methods exist for characterising column performance and many users make a test injection of a standard solution of the analytes of interest for a particular application.

It is also useful to inject a solution of chromatographically 'difficult' or revealing test compounds in order to characterise column performance in terms of:

- Retentivity
- Selectivity
- Efficiency
- Presence of lone (acidic) surface silanol groups
- Metal Chelation

The Engelhardt test mix shown, is one of many standard mixes that can help to characterise column performance. Typically, the results of a standard test will be entered into a database, where changes can be monitored over time and action limits set for column withdrawal from service.

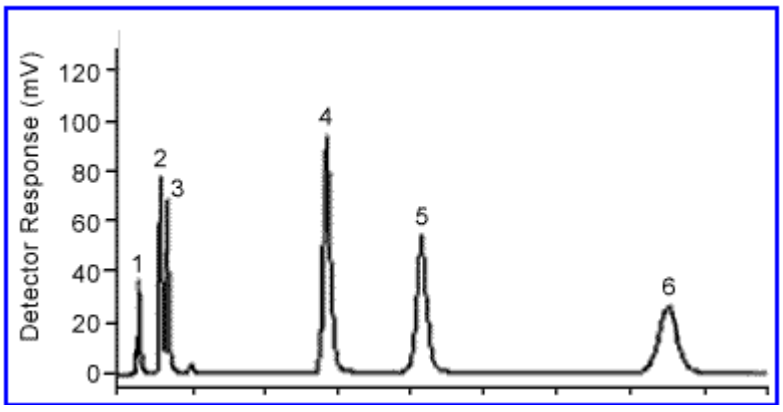
Separation of some components of the Engelhardt test mixture

Mobile phase: 55:45 (v/v) methanol–water
Flow rate: 0.7 mL/min
Detection: UV absorbance at 254 nm
Injection volume: 5 µL.

Peaks: 1 uracil, 2 aniline, 3 phenol,
4 N,N - dimethylaniline,
5 toluene, 6 ethylbenzene.

Test mix characteristics:

Uracil – records the retention time of an unretained component (t_0)
Aniline and N,N dimethylaniline– used to probe trace silanol interactions (using peak asymmetry measurements)
Phenol – characterise any polar interaction with the stationary phase surface (using retention factor k)
Toluene, Ethylbenzene – the selectivity (α) value for the separation between these two peaks can be used to chart the hydrophobicity of a column

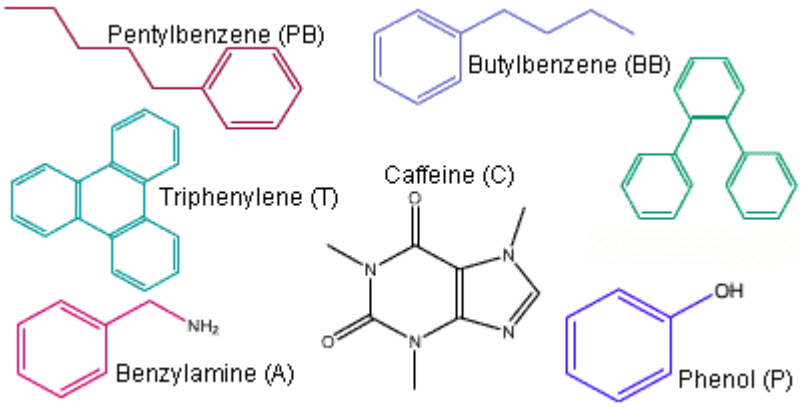


Use of the Englehardt Test Mix as Column Probe

Tanaka (N. Tanaka et al. J. Chrom. Sci. 27 (1989) 721) proposed a system for 'classifying' certain column characteristics in order to make column choices for method development more straightforward. This initiative has since been further developed by several groups of workers and databases exist based on the Tanaka column characteristics that enable direct column comparisons for various application types. These characteristics include:

- Efficiency
- Surface Coverage
- Hydrophobicity
- Steric (shape) selectivity
- Hydrogen Bonding Capacity (degree of endcapping / measure of available silanol groups)
- Ion exchange capacity at pH >7 and <3 (an indication of both total and lone silanol activity)

All of these factors are important in defining the characteristics of reverse phase bonded phase materials. Over 200 phases have now been characterised using these descriptors and databases are useful in comparing and contrasting column characteristics for specific application areas.



Retention factor for pentylbenzene, *k*PB
A measurement of the surface area and surface coverage (ligand density).

Hydrophobicity or hydrophobic selectivity, αCH2
The retention factor ratio between pentylbenzene and butylbenzene,

αCH2 = *k*PB/*k*BB

Shape selectivity, αT/O
The retention factor ratio between triphenylene (planar) and *o*-terphenyl (rotating structure)
αT/O = *k*T/*k*O

Hydrogen bonding capacity, αC/P
The retention factor ratio between caffeine and phenol,

αC/P = *k*C/*k*P

Total ion-exchange capacity, αA/P pH 7.6
The retention factor ratio between benzylamine and phenol,

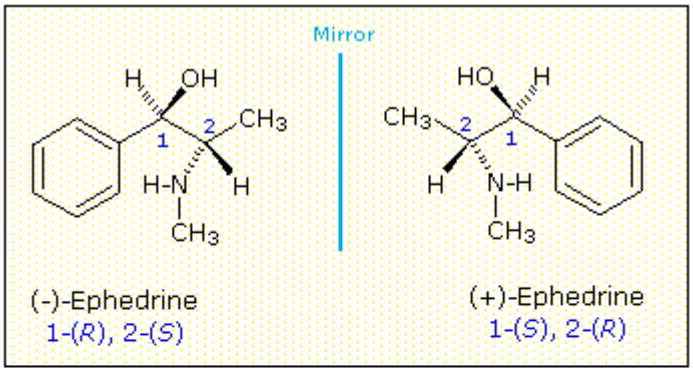
αB/P (pH 7.6) = *k*A/*k*P,

is a measure of the total silanol activity.

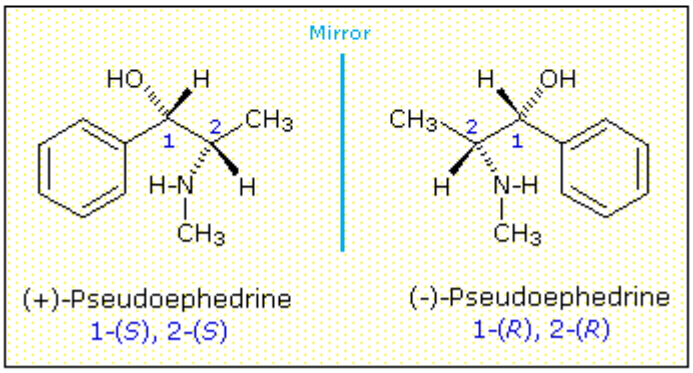
Glossary

Conformation - the various steorochemical arrangements that may be adopted by chemical species.

Diastereomeric -



Ephedrine enantiomers



Pseudoephedrine enantiomers

As a general rule, a structure having 'n' stereogenic centers will have 2n possible combinations of these centers. Depending on the overall symmetry of the molecular structure, some of these combinations may be identical, but in the absence of such identity, we would expect to find 2n stereoisomers. Some of these stereoisomers will have enantiomeric relationships, but enantiomers come in pairs, and non-enantiomeric stereoisomers will therefore be common. We refer to such stereoisomers as diastereomers. In the example above, either of the ephedrine enantiomers has a diastereomeric relationship with either of the pseudoephedrine enantiomers.

Lipophilic - lipophilic literally means "fat-loving." The term is used in chromatography to describe those functional groups or molecules which prefer to be in an environment where there is no water; an oily or (more often) hydrocarbon based non-polar environment.

Steric – relating to the spatial volume of a reacting species

Steric Hindrance – the effects of reactant size and spatial volume on extent and rate of reaction.

Thank You