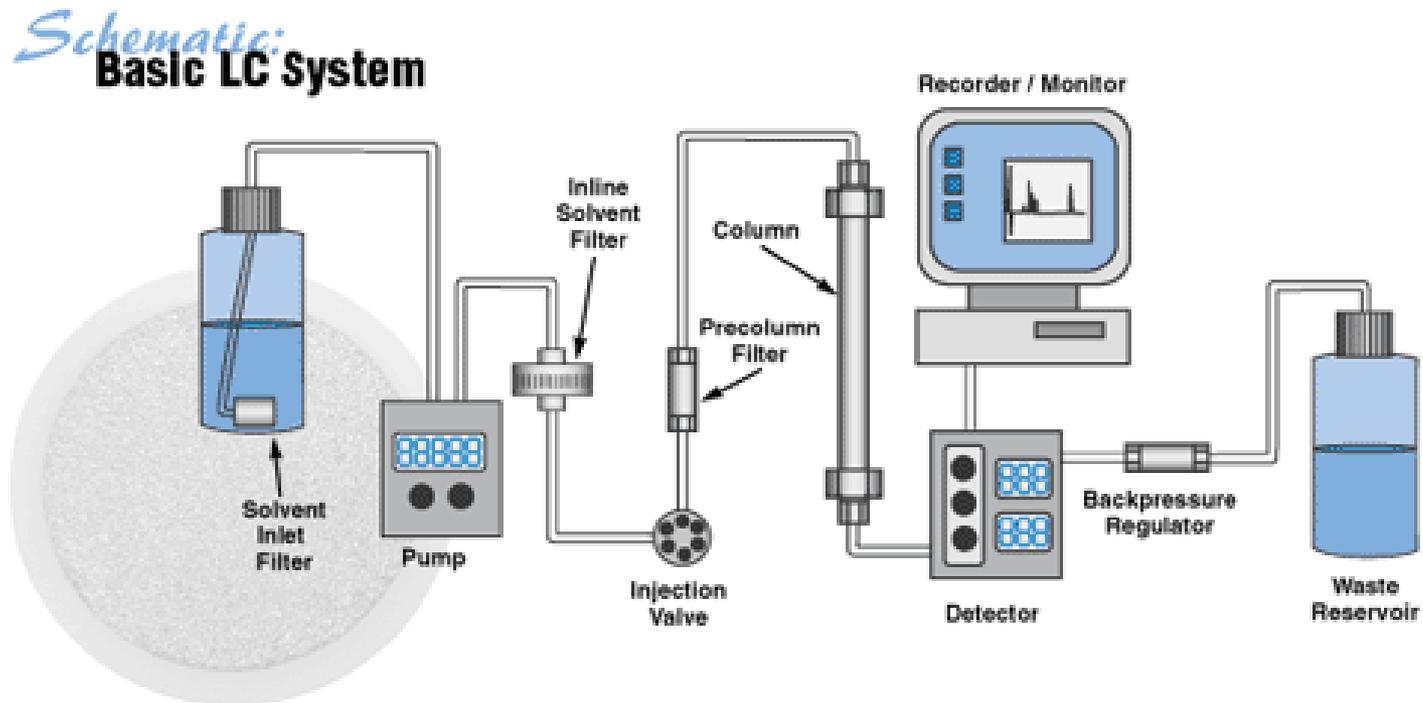


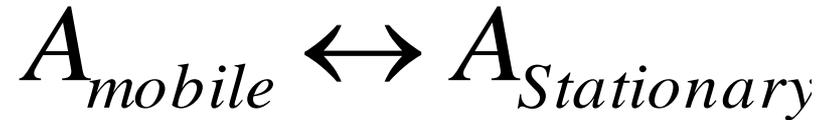
High Performance Liquid Chromatography (HPLC)

Column separation (liquid-liquid, liquid-solid) used for separating and analyzing compounds based on differences in their interaction with a stationary phase.

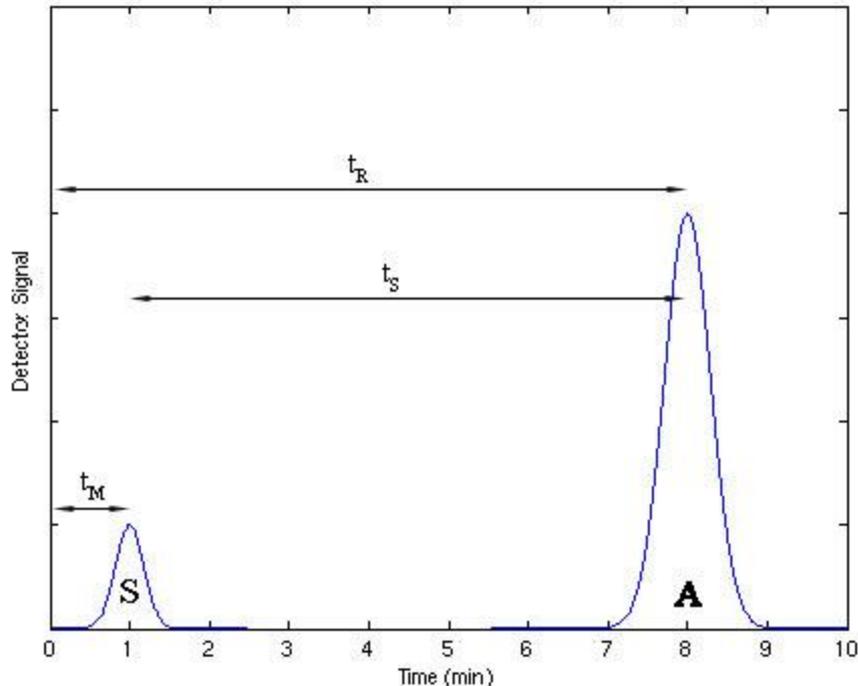


Adsorption, partition, ion exchange, molecular exclusion and affinity

The Chromatogram



Retention of Compound A



$$k' = \frac{K_D V_s}{V_m} = \frac{t_r - t_m}{t_m}$$

$$N = \frac{L}{H} = 16 \left(\frac{t_r}{w} \right)^2$$

Remember, relating the peak height or area to an injected analyte concentration requires a response curve obtained under the same separation conditions.

When analytes are not volatile...then?

There are many different ways to use liquid chromatography to separate compounds of interest and the choice of technique usually depends on the physicochemical characteristics of the molecule of interest. The solid phases used for each type of chromatography are highly engineered porous, chemically inert supports functionalized with various chemical groups that determine the interactions with the molecules to be separated.

Commonly used modes of separation are based on:

- Specific binding interactions ([affinity chromatography](#))
- Charge ([ion exchange chromatography](#))
- Size ([size exclusion chromatography/gel filtration chromatography](#))
- Hydrophobic surface area ([hydrophobic interaction chromatography](#) and reverse phase chromatography)
- Multiple properties ([multimodal or mixed-mode chromatography](#))

Affinity Chromatography

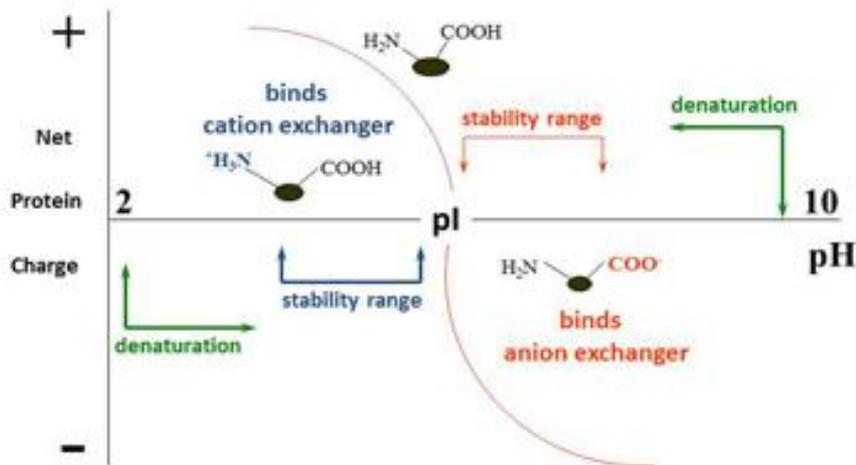
Affinity chromatography is a separation method based on a specific binding interaction between an immobilized ligand and its binding partner. Examples include antibody/antigen, enzyme/substrate, and enzyme/inhibitor interactions. The degree of purification can be quite high depending on the specificity of the interaction and, consequently, it is generally the first step, if not the only step, in a purification strategy.



The molecule of interest can then be released from the resin using a buffer with a high salt concentration, a pH shift, or a competing ligand.

Ion Exchange Chromatography

Ion exchange chromatography involves the separation of ionizable molecules based on their total charge. This technique enables the separation of similar types of molecules that would be difficult to separate by other techniques because the charge carried by the molecule of interest can be readily manipulated by changing buffer pH.



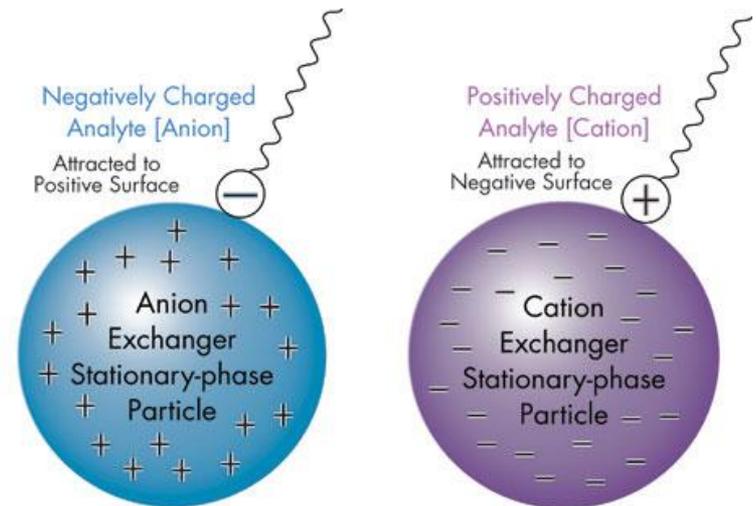
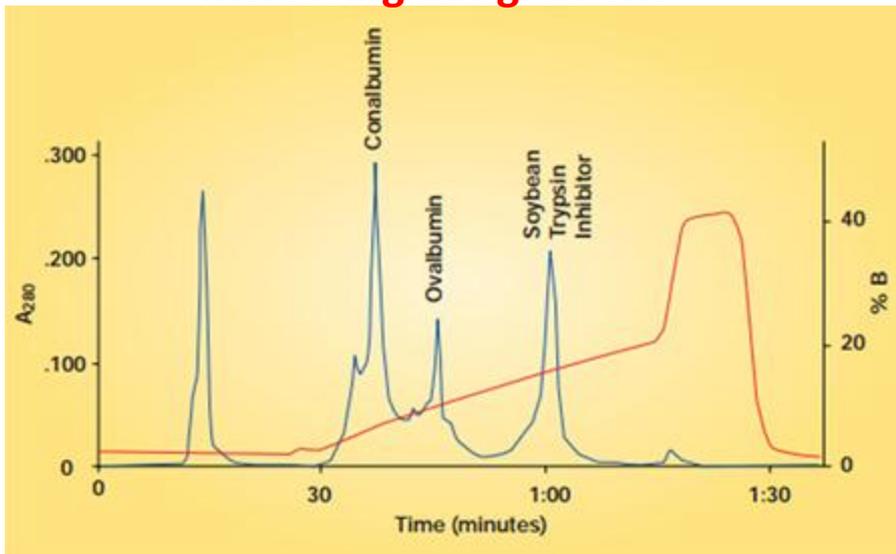
Resin Type	Cation Exchanger	Anion Exchanger
Net charge of molecule of interest	+	-
Charge of resin	-	+
Running conditions	0.5–1.5 pH units below the pI of the molecule of interest	0.5–1.5 pH units above the pI of the molecule of interest

Depending on the pH of their environment, proteins may carry a net *positive* charge, a net *negative* charge, or *no* charge. The pH at which a molecule has no net charge is called its isoelectric point, or pI.

Ion Exchange Chromatography

Ion exchange chromatography is a technique that is used analytically unlike some other separation methods that are used more for preparative purposes. Example shows a series of proteins separated and eluted off the column with increasing salt content in the mobile phase. **Site competition!**

Increasing salt gradient



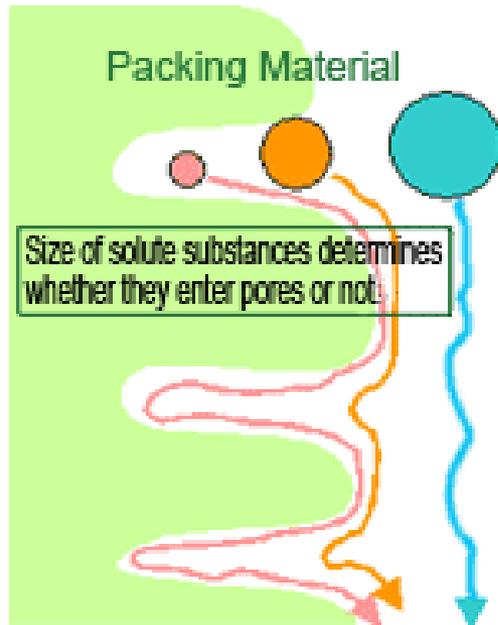
IEC Stationary Phase Types

Strong ion exchangers are often preferred resins for many applications because their performance is unaffected by pH. However, weak ion exchangers can be powerful separation tools in cases where strong ion exchangers fail because the selectivities of weak and strong ion exchangers often differ.

Support	DEAE	High Q	CM	High S
Type of exchange	Weak anion	Strong anion	Weak cation	Strong cation
Functional group	$-\text{N}+(\text{C}_2\text{H}_5)_2$	$-\text{N}+(\text{CH}_3)_3$	$-\text{SO}_3^-$	$-\text{COO}^-$
pH Range*	5–9	0–14	5–9	0–14

Size Exclusion Chromatography

Size exclusion chromatography (SEC), also called gel filtration chromatography, separates molecules based on their sizes. SEC resins are gels that contain beads with a known pore size. When a complex protein mixture is passed over SEC resin, small molecules move through the bead pores, whereas molecules too large to fit into the pores move around the beads and through the void space between beads.

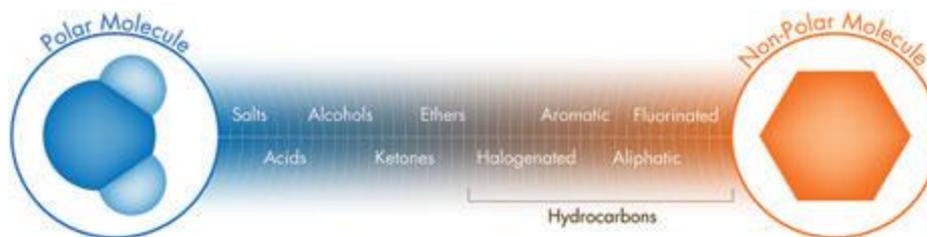


When dissolved molecules of various sizes flow into the column, smaller dissolved molecules flow more slowly through the column because they penetrate deep into the pores, whereas large dissolved molecules flow quickly through the column because they do not enter the pores.

Purification method

Normal- and Reversed-Phase HPLC

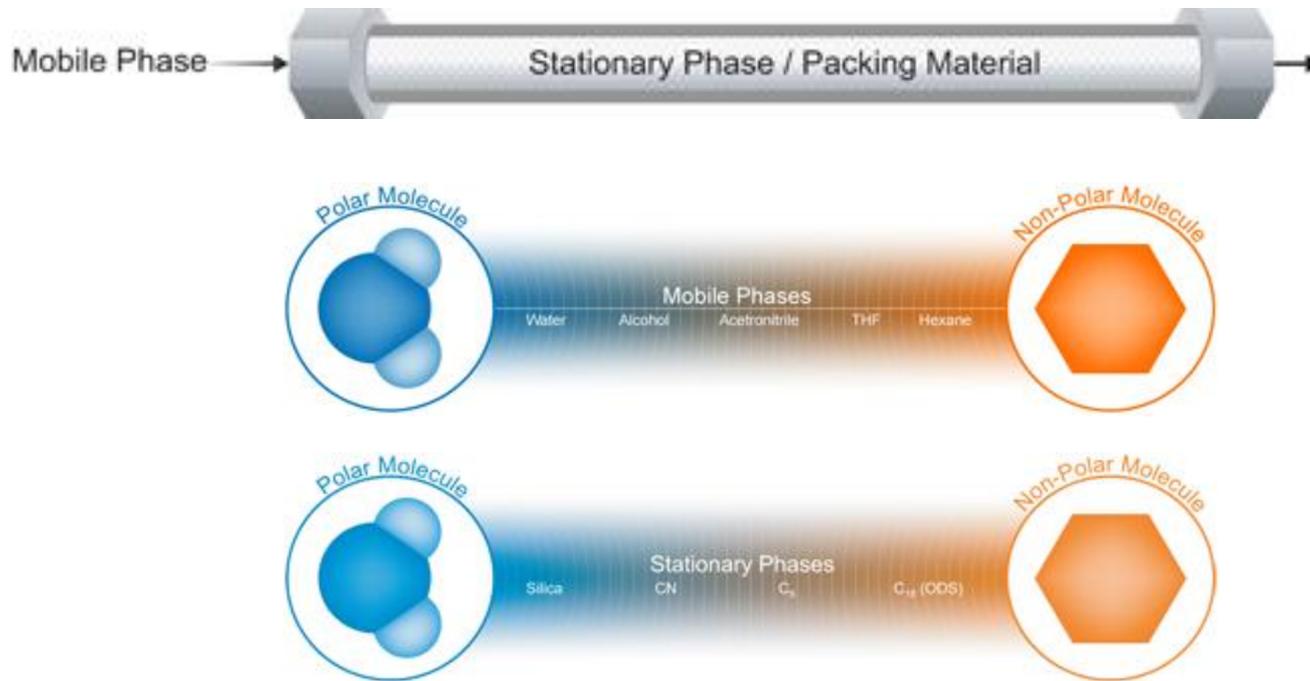
A molecule's structure, activity, and physicochemical characteristics are determined by the arrangement of its constituent atoms and the bonds between them. Within a molecule, a specific arrangement of certain atoms that is responsible for special properties and predictable chemical reactions is called a functional group. This structure often determines whether the molecule is *polar* or *non-polar*.



Separations based on an analytes relative affinity for the mobile phase versus the stationary phase. Polar vs. non-polar.

Normal- and Reversed-Phase HPLC

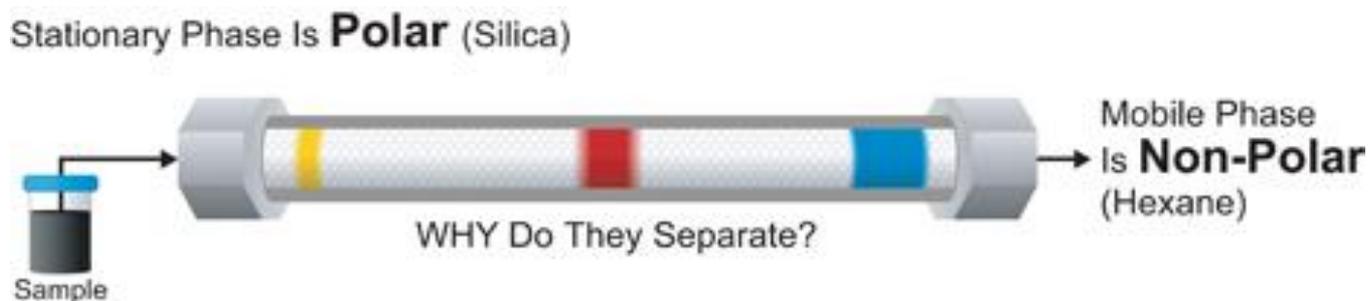
Stationary phases are commonly bonded to a high surface area, inert support (silica).



To summarize, the chromatographer will choose the best combination of a mobile phase and particle stationary phase with appropriately opposite polarities. Then, as the sample analytes move through the column, the rule *like attracts like* will determine which analytes slow down and which proceed at a faster speed.

Normal-Phase HPLC

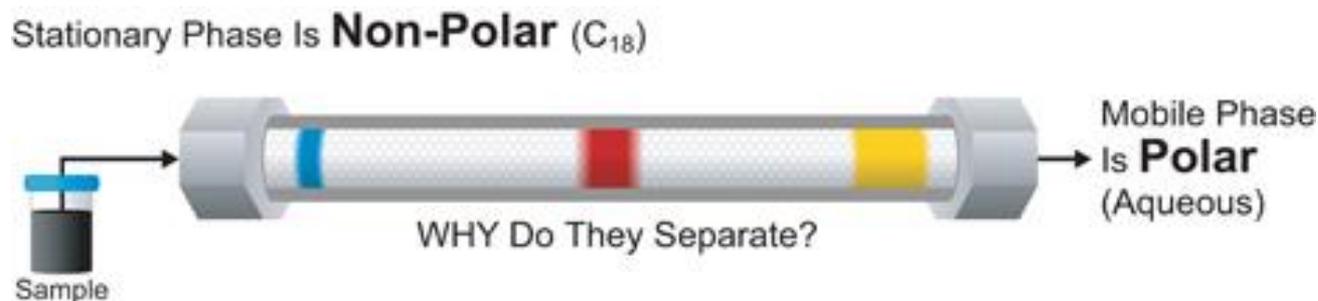
In the separations of plant extracts, Tswett (original developer of separation science) was successful using a *polar stationary phase* with a much *less polar [non-polar] mobile phase*. This classical mode of chromatography became known as normal phase.



The figure represents a **normal-phase chromatographic separation** of a three-dye test mixture. The stationary phase is polar and retains the polar yellow dye most strongly. The relatively non-polar blue dye is won in the retention competition by the mobile phase, a non-polar solvent, and elutes quickly. Since the blue dye is most like the mobile phase [both are non-polar], it moves faster. It is typical for normal-phase chromatography on silica that the mobile phase is 100% organic; no water is used.

Reversed-Phase HPLC (most common method)

The term reversed-phase describes the chromatography mode that is just the opposite of normal phase, namely the use of a *polar mobile phase* and a *non-polar [hydrophobic] stationary phase*. The figure illustrates the black three-dye mixture being separated using such a protocol.



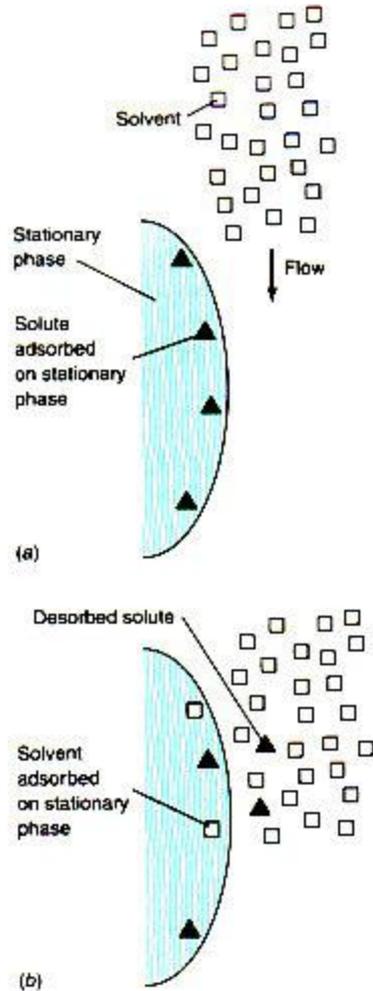
Now the most strongly retained compound is the more non-polar blue dye, as its attraction to the non-polar stationary phase is greatest. The polar yellow dye, being weakly retained, is won in competition by the polar, aqueous mobile phase, moves the fastest through the bed, and elutes earliest *like attracts like*.

Reversed-Phase HPLC (most common method)

Because it is more reproducible and has broad applicability, reversed-phase chromatography is used for approximately 75% of all HPLC methods. Most of these protocols use as the *mobile phase* an **aqueous blend of water with a miscible, polar organic solvent**, such as acetonitrile or methanol. This typically ensures the proper interaction of analytes with the non-polar, hydrophobic particle surface. A C18-bonded silica [sometimes called ODS] is the most popular type of reversed-phase HPLC packing.

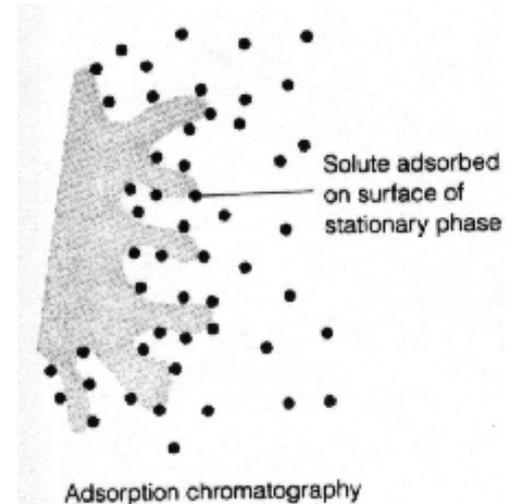
Separation Mode	Stationary Phase [particle]	Mobile Phase [solvent]
Normal phase	Polar	Non-polar
Reversed phase	Non-polar	Polar

Types of NP and RP-HPLC: Adsorption Mechanism



Silica, $\text{SiO}_2 \cdot x\text{H}_2\text{O}$

Alumina, $\text{Al}_2\text{O}_3 \cdot x\text{H}_2\text{O}$



Solvent molecules compete with solute molecules for sites. More strongly the solvent binds to the stationary phase, the greater the *eluent strength*.

Adsorption Chromatography

Table 22-2 Eluotropic series and ultraviolet cutoff wavelengths of solvents for adsorption chromatography on silica^a

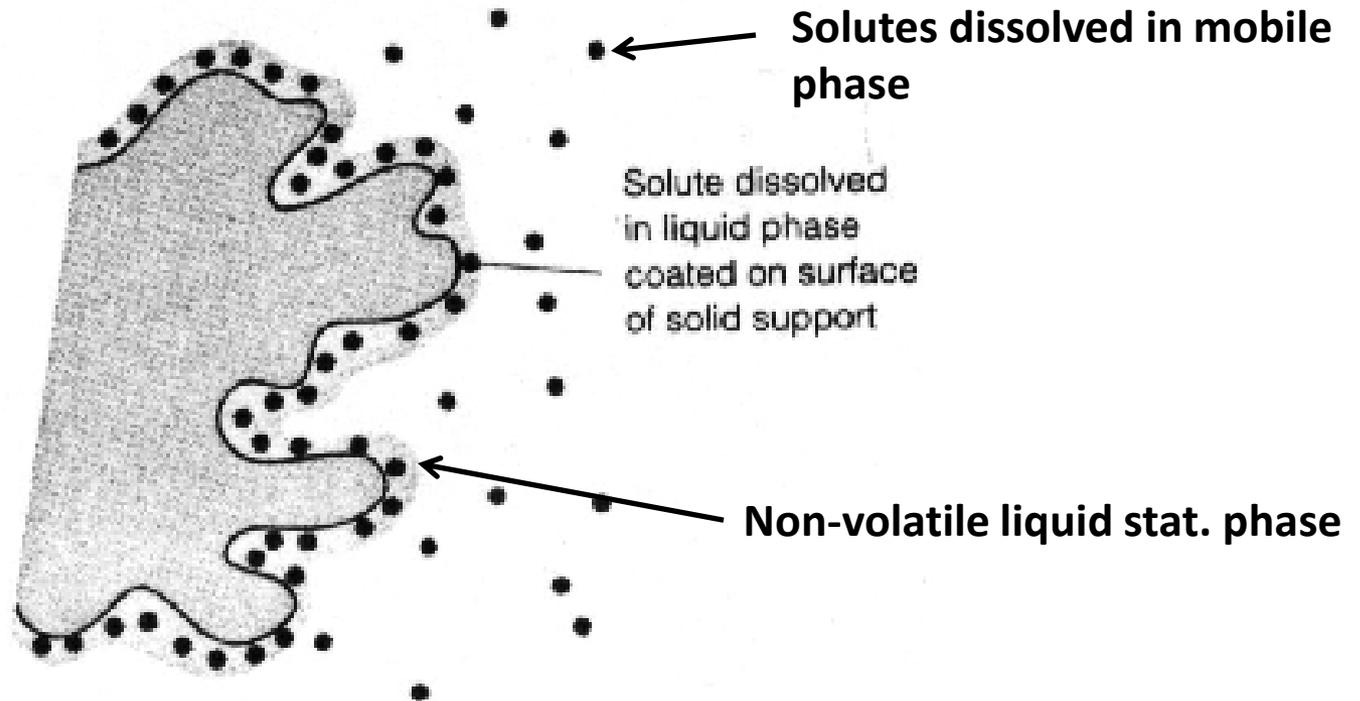
Solvent	Eluent strength (ϵ°)	Ultraviolet cutoff (nm)
Pentane	0.00	190
Hexane	0.01	195
Heptane	0.01	200
Trichlorotrifluoroethane	0.02	231
Toluene	0.22	284
Chloroform	0.26	245
Dichloromethane	0.30	233
Diethyl ether	0.43	215
Ethyl acetate	0.48	256
Methyl <i>t</i> -butyl ether	0.48	210
Dioxane	0.51	215
Acetonitrile	0.52	190
Acetone	0.53	330
Tetrahydrofuran	0.53	212
2-Propanol	0.60	205
Methanol	0.70	205

a. From L. R. Snyder in *High-Performance Liquid Chromatography* (C. Horváth, ed.), vol. 3 (New York: Academic Press, 1983); *Burdick & Jackson Solvent Guide*, 3rd ed. (Muskegon, MI: Burdick & Jackson Laboratories, 1990).

b. Ultraviolet cutoff is the approximate minimum wavelength at which solutes can be detected above the strong ultraviolet absorbance of solvent. The ultraviolet cutoff for water is 190 nm.

Types of NP and RP-HPLC: Partition Mechanism

This form of chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase. Solute equilibrates between the mobile phase and the stationary liquid.

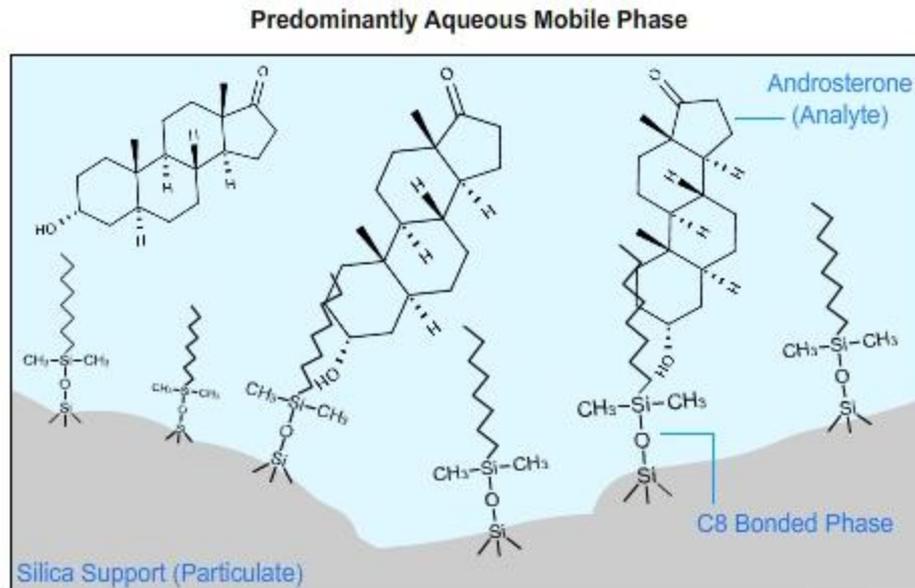


Partition chromatography

Partition or transfer of an analyte in and out of a liquid stationary phase.

RP-HPLC: Retention Order

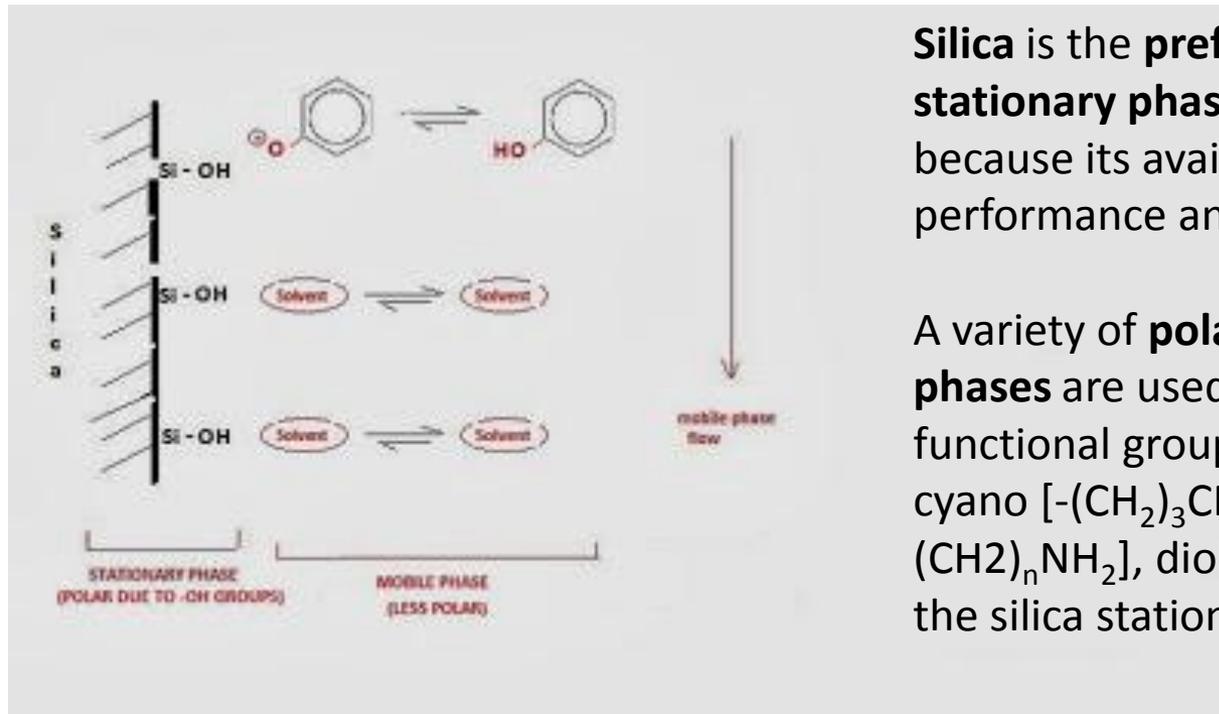
Reversed phase HPLC is characterized by a situation in which the *mobile phase* used is MORE **POLAR** than the *stationary phase*.



More polar analytes elute first, less polar analytes are retained longer. Water and miscible organics (acetonitrile, methanol, ethanol) make for a polar mobile phase. Analytes must be soluble in mobile phase!!!

NP-HPLC: Retention Order

In normal-phase chromatography, the *stationary phase* is **polar** and the *mobile phase* is **nonpolar**.



Silica is the **preferred stationary phase** mainly because its availability, known performance and low cost.

A variety of **polar bonded phases** are used with functional groups such as cyano $[-(\text{CH}_2)_3\text{CN}]$, amino $[-(\text{CH}_2)_n\text{NH}_2]$, diol, bonded to the silica stationary phase.

Least polar analytes elute first, more polar analytes are retained longer. Low to medium polarity solvents are used (hexane, ethyl acetate, methanol). Must eliminate water. Analytes must be soluble in mobile phase!!!

Summary of NP and RP-HPLC

Type	Mobile Phase	Stationary Phase	Elution Order
Normal - Phase	Non-polar (hexane, toluene, methanol)	Polar (silica or chemically-modified Si such as $-\text{O}-(\text{CH}_2)_3-\text{CN}$)	Least polar first, most polar last
Reversed-Phase	Polar (water + miscible organic solvent (acetonitrile, ethanol, methanol))	Non-polar (chemically-modified silica, C8, C12, C18 groups) 5 μm diam. particle size	Most polar first, least polar last

Remember: The analytes must be soluble in the mobile phase in order to use the separation method and the longer the analytes spend interacting with the stationary phase, the broader the peaks will be.