Waters

THE SCIENCE OF WHAT'S POSSIBLE.™

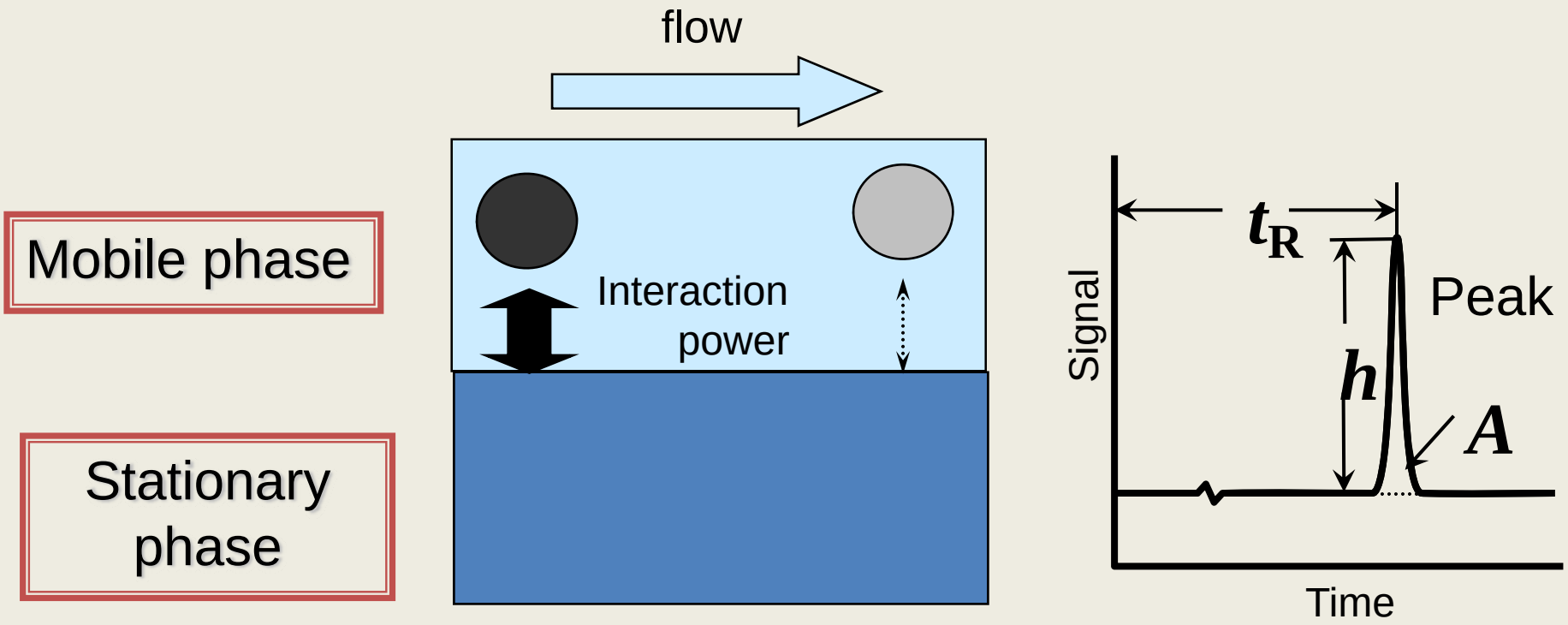
What is HPLC?

Principals and Theory

Liquid chromatography (LC) is a physical separation technique conducted in the liquid phase.

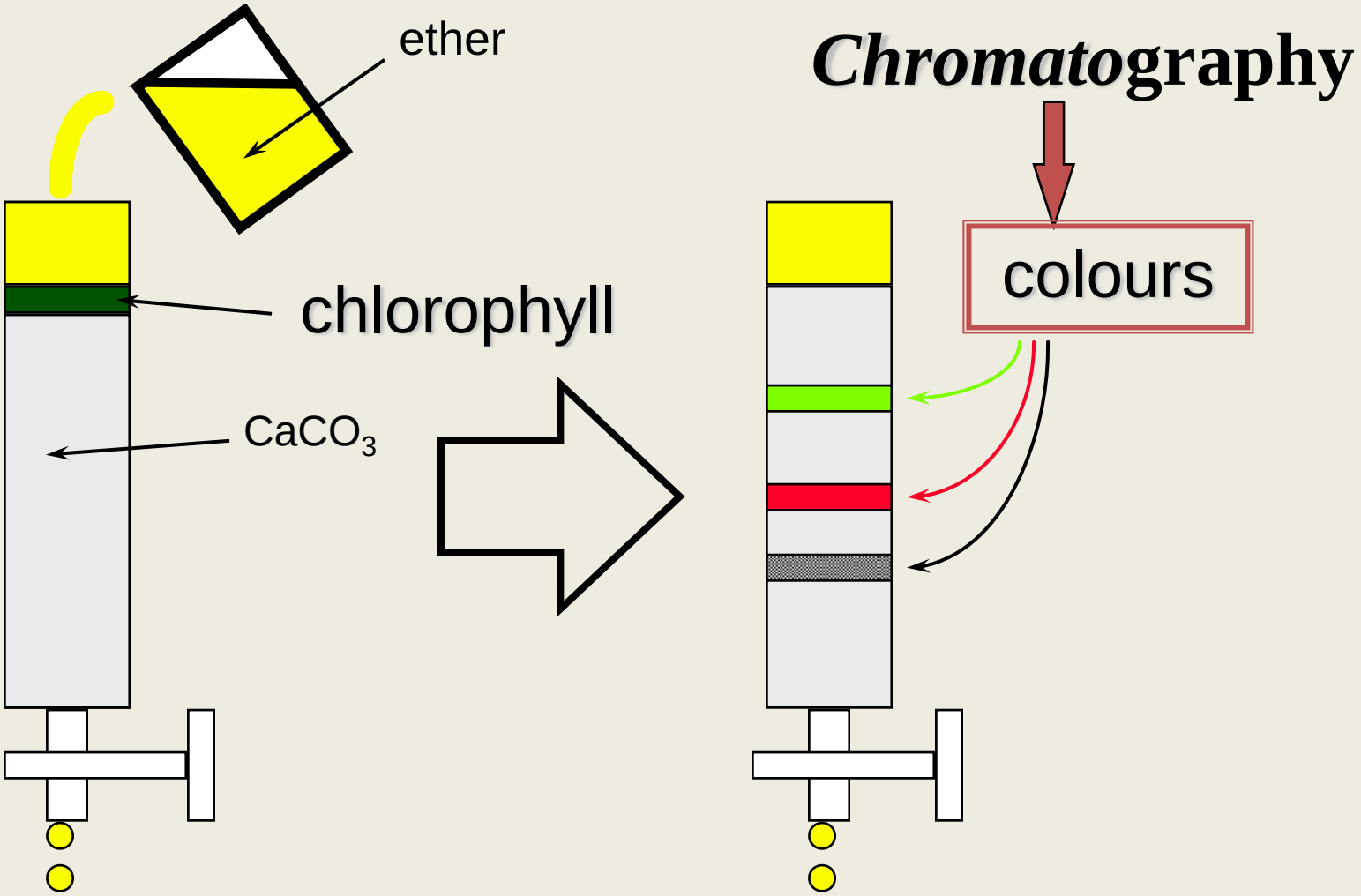
A sample is separated into its constituent components (or analytes) by distributing between the mobile phase (a flowing liquid) and a stationary phase (sorbents packed inside a column).

Mobile phase / Stationary phase



A Brief History

Classical LC, the term *chromatography meaning "color writing," was first discovered by* **Mikhail Tswett**, a Russian botanist who separated plant pigments on chalk (CaCO3) packed in glass columns in 1903.



Advantages and Limitations

Table 1.1 highlights the advantages and limitations of HPLC. HPLC is a premier separation technique capable of multi component analysis of real-life samples and complex mixtures. Few techniques can match its versatility and precision of <0.5% relative standard deviation (RSD)

Table 1.1. Advantages and Limitations of HPLC

Advantages

- Rapid and precise quantitative analysis
- Automated operation
- High-sensitivity detection
- Quantitative sample recovery
- Amenable to diverse samples

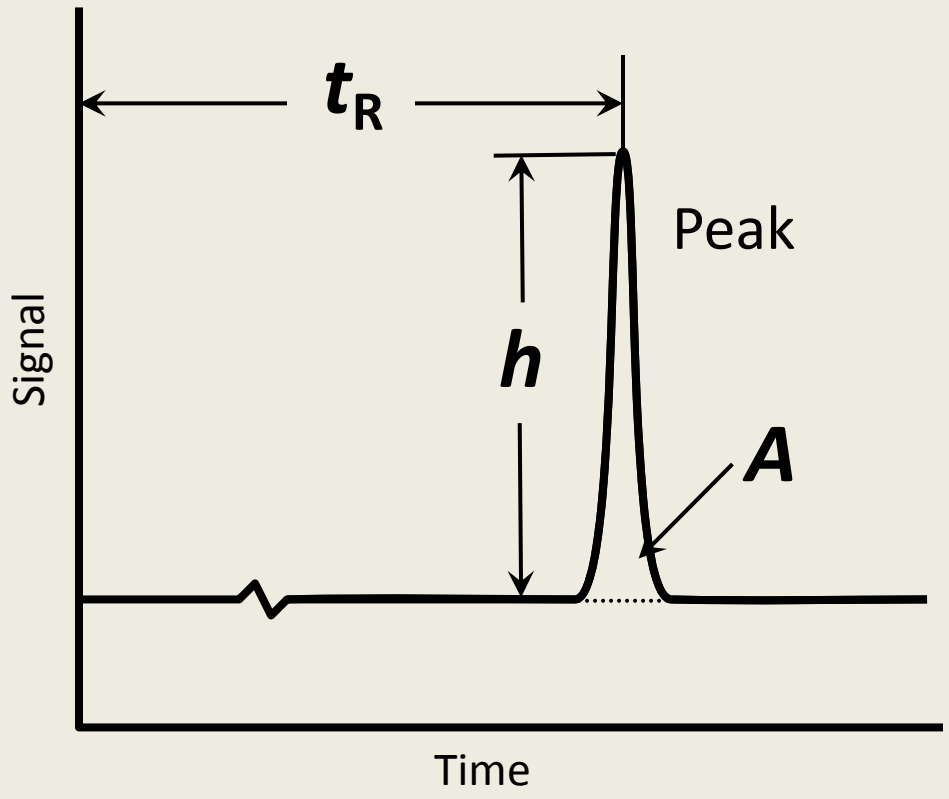
Limitations

- No universal detector
- Less separation efficiency than capillary GC
- More difficult for novices

Chromato-graphy / -graph / -gram / -grapher

- Chromato (-graphy) : Method
- Chromato (-graph) : Instrument
- Chromato (-gram) : Picture
- Chromato (-grapher) : Person

Chromatogram



t_R : Retention time

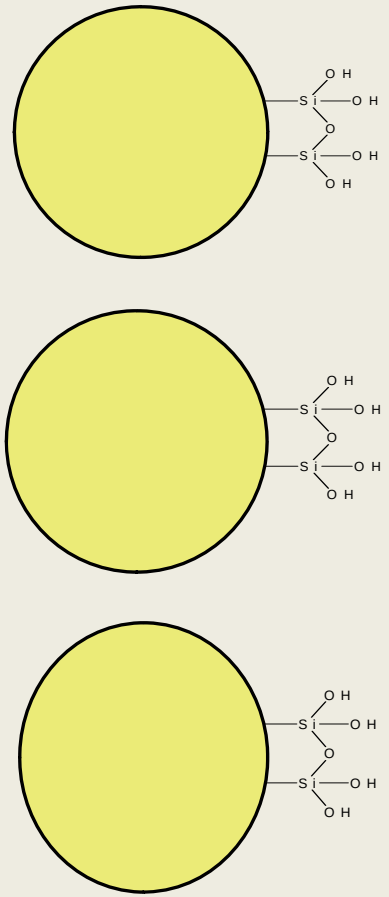
A : Area

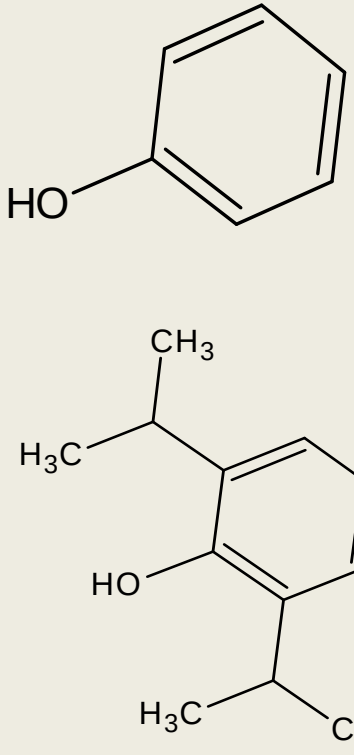
h : Height

MODES OF HPLC

- **Normal-Phase Chromatography (NPC):**

  Also known as liquid-solid chromatography or adsorption chromatography, NPC is the traditional separation mode based on adsorption/desorption of the analyte onto a polar stationary phase (typically Silica or Alumina).





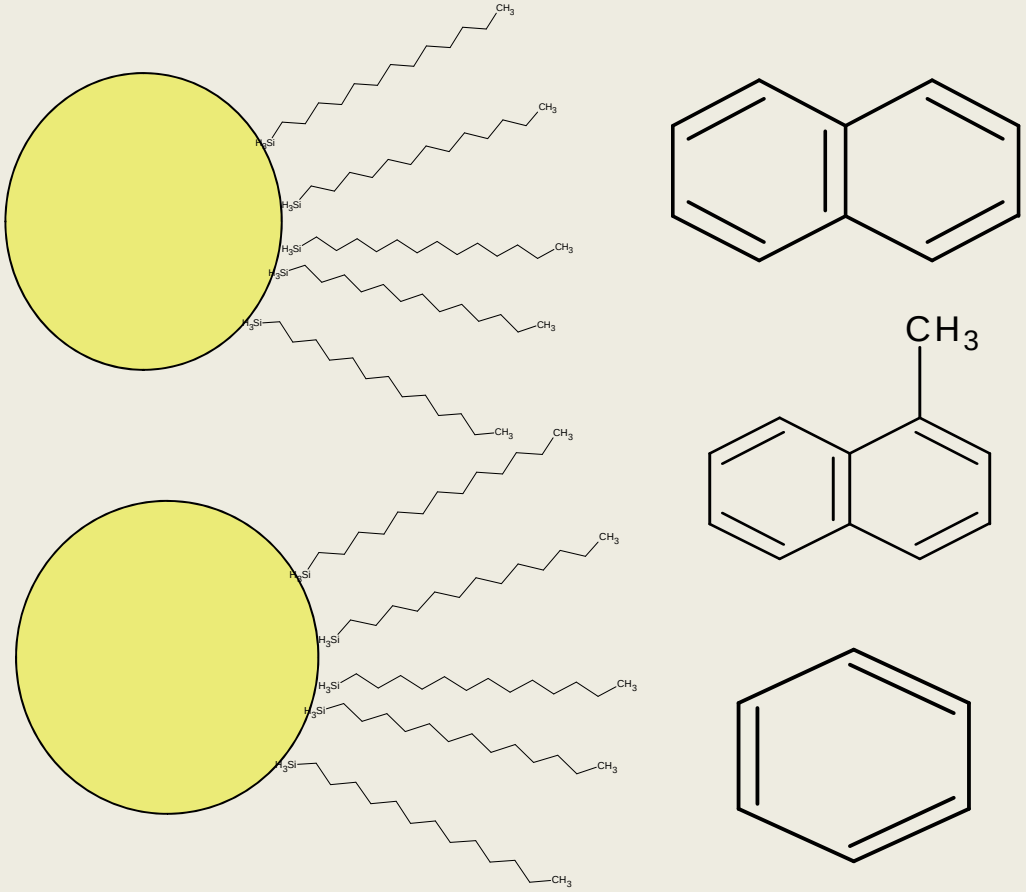
NPC is particularly useful for the separation of non polar compounds and isomers.

MODES OF HPLC

- **Reversed-Phase Chromatography (RPC) :**

The separation is based on analytes partition coefficients between a polar mobile phase and a hydrophobic (non polar) stationary phase.

The earliest stationary phases were solid particles coated with non polar liquids. These were quickly replaced by more permanently bonding hydrophobic groups, such as octadecyl (C18) bonded groups, on silica support.

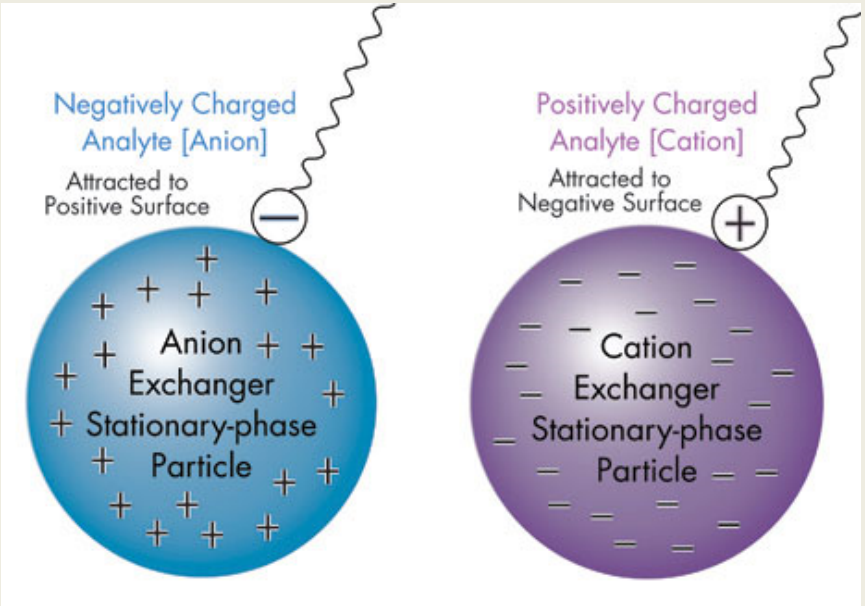


RPC is the most popular HPLC mode and is used in more than 70% of all HPLC analyses.3,4 It is suitable for the analysis of polar (water-soluble), medium-polarity, and some non polar analytes.

MODES OF HPLC

- **Ion-Exchange Chromatography (IEC) :**

In ion-exchange chromatography,3–5 the separation mode is based on the exchange of ionic analytes with the counter-ions of the ionic groups attached to the solid support.
Typical stationary phases are cationic exchange (sulfonate) or anionic exchange (quaternary ammonium) groups bonded to polymeric or silica materials.
Mobile phases consist of buffers, often with increasing ionic strength, to force the migration of the analytes.
Common applications are the analysis of ions and biological components such as amino acids, proteins/peptides, and poly nucleotides.



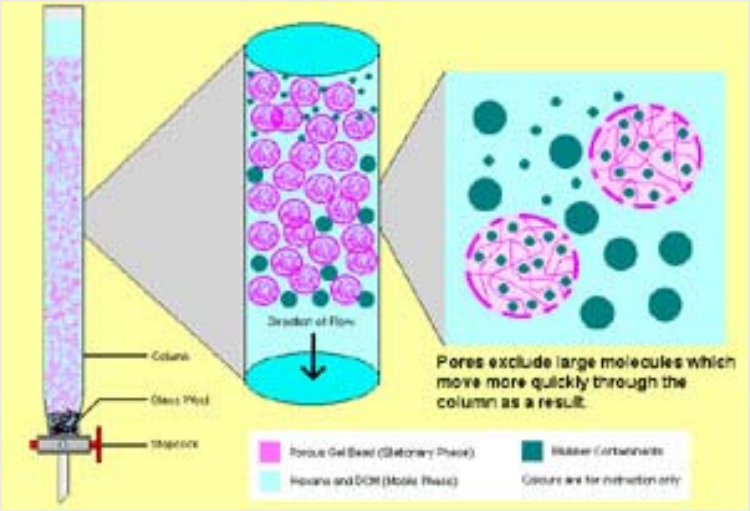
MODES OF HPLC

- **Size-Exclusion Chromatography (SEC) :**

Size-exclusion chromatography15 is a separation mode based solely on the analyte's molecular size.

A large molecule is excluded from the pores and migrates quickly, whereas a small molecule can penetrate the pores and migrates more slowly down the column. It is often called gel permeation chromatography (GPC) when used for the determination of molecular weights of organic polymers.

It is often called gel-filtration chromatography (GFC) when used in the separation of water-soluble biological materials. In GPC, the column is packed with cross-linked polystyrene beads of controlled pore sizes and eluted with common mobile phases such as toluene and tetrahydro furan.



MODES OF HPLC

Other Separation Modes:

• *Affinity chromatography: Based on a receptor/ligand interaction in which* immobilized ligands (enzymes, antigens, or hormones) on solid supports are used to isolate selected components from a mixture. The retained components can later be released in a purified state.

• *Chiral chromatography: For the separation of enantiomers using a* chiral-specific stationary phase. Both NPC and RPC chiral columns are available.

• *Hydrophilic interaction chromatography (HILIC): This is somewhat* similar to normal phase chromatography using a polar stationary phase such as silica or ion-exchange materials but eluted with polar mobile phases of organic solvents and aqueous buffers. It is most commonly used to separate polar analytes and hydrophilic peptides.

• *Hydrophobic interaction chromatography: Analogous to RPC except* that mobile phases of low organic solvent content and high salt concentrations are used for the separation of proteins that are easily denatured by mobile phases with high concentrations of organic solvents used in RPC.

• *Supercritical fluid chromatography (SFC): Uses HPLC packed columns* and a mobile phase of pressurized supercritical fluids (i.e., carbon dioxide modified with a polar organic solvent). Useful for non polar analytes and preparative applications where purified materials can be recovered easily by evaporating the carbon dioxide.

SOME COMMON-SENSE COROLLARIES

The goal of most HPLC analysis is to separate analyte(s) from other components in the sample for accurate quantitation. Several corollaries are often overlooked by practitioners:

- *Sample must be soluble:* *"If it's not in solution, it cannot be analyzed by* HPLC." Solubility issues often complicate assays of low-solubility analytes or component difficult to extract from sample matrices.
- *For separation to occur, analytes must be retained and have differential migration in the column:* *Separation cannot occur without retention and* sufficient interaction with the stationary phase.
- *The mobile phase controls the separation:* *Whereas the stationary phase* provides a media for analyte interaction, the mobile phase controls the overall separation. In HPLC method development, efforts focus on finding a set of mobile phase conditions for separating the analyte(s) from other components. Exceptions to this rule are size exclusion, chiral, and affinity chromatography.
- *The final analyte solution should be prepared in the mobile phase:* *The* final analyte solution, if possible, should be dissolved in the mobile phase or a solvent of "weaker" strength than the starting mobile phase.
- *Every analytical method has its own limitations, or pitfalls:* *An* experienced method development scientist should identify these potential pitfalls and focus on finding conditions to minimize these problems areas for more reliable analysis.

Prior to Analysis

Mobile Phase

The mobile phase is the solvent that moves the solute (analyte) through the column.

In HPLC, the mobile phase interacts with both the solute and the stationary phase and has a powerful influence on solute retention and separation

- Water
  - Ultra pure water
  - HPLC grade
- Organic solvent
  - HPLC grade
  - Super-high grade may be used in some application.
  - Some solvents such as THF and chloroform include Stabiliser, which cause a problem.

Mobile Phase

General Requirements

Ideally, solvents used as HPLC mobile phases should have these characteristics:

- High solubility for the sample components
- Noncorrosive to HPLC system components
- High purity, low cost, UV transparency
- Other desirable characteristics include low viscosity, low toxicity, and no flammability.

Mobile Phase

U.V. Cut-offs for some Common Solvents

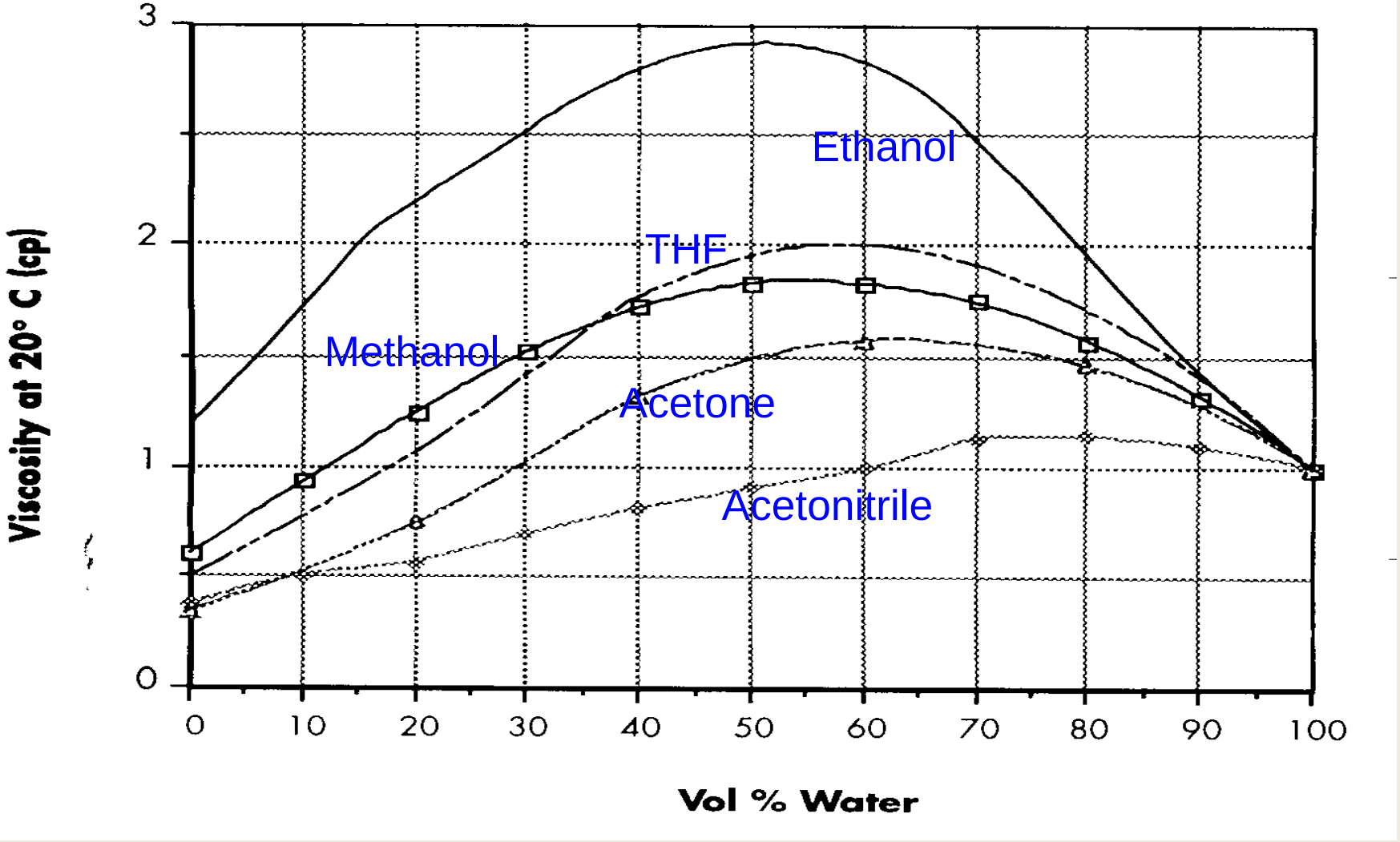
Remember Solvents chosen can affect detection!!

Solvent	UV Cutoff	Solvent	UV Cutoff
Water	180	N-Heptane	197
Methanol	205	Cyclohexane	200
N-Propanol	205	Carbon tetrachloride	265
Acetonitrile	190	Chloroform	245
THF	225	Benzene	280
Acetone	330	Toluene	285
Methyl acetate	260	Methylene chloride	232
Ethyl Acetate	260	Tetrachloroethylene	280
Nitromethane	380	1,2-Dichloroethane	225

All wavelengths reported in nm.

Mobile Phase

Viscosity for mixtures of water and organic solvents



Mobile Phase

Solvent Strength and Selectivity

- Solvent strength refers to the ability of a solvent to elute solutes from a column.

- Solvent strength is related to its polarity. Non polar hexane is a weak solvent in normal phase chromatography whereas water is a strong solvent.

- The opposite is true in RPLC since the stationary phase is hydrophobic. Here water is a weak solvent and organic solvents are strong.

- THF > ACN > MeOH >> water.

- Water is a weak solvent because it is a poor solvent for no strongly H-bonding organics.

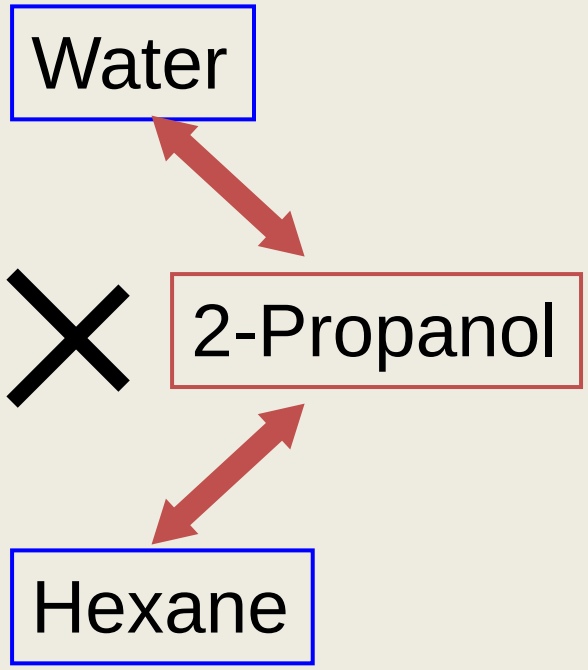
Mobile Phase

Classification of Solvents Polarity Index - Selectivity Group

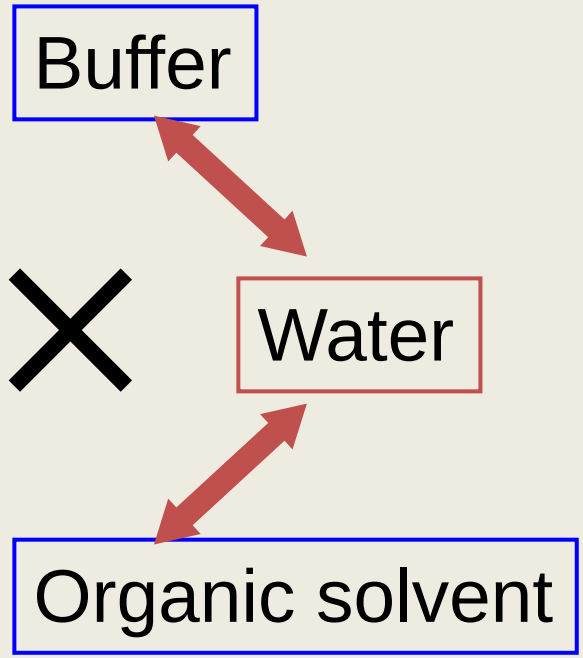
Polarity Index	Solvent	Selectivity group
9.0	Water	9
6.6	Methanol	2
6.2	Acetonitrile	6
5.2	Ethanol	2
4.8	Dioxane	6
4.3	Chloroform	9
4.3	2-propanol	2
4.3	Ethyl Acetate	6
4.2	THF	3
3.9	Butanol	2
3.4	Methylene Chloride	5
2.9	Ethyl Ether	1
2.3	Toluene	7
1.8	Triethyl Amine	1
0.0	Hexane	0

Replacement of mobile phase

- Un-dissolved solvents must not be used in replacement.



- Buffer must not be replaced directly with organic solvent.



Off-line degassing & Filtration