Liquid chromatography

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What is chromatography?

- Derived from the Greek word Chroma meaning colour, chromatography provides a way to identify unknown compounds and separate mixtures.

Introduction to Chromatography

Definition:

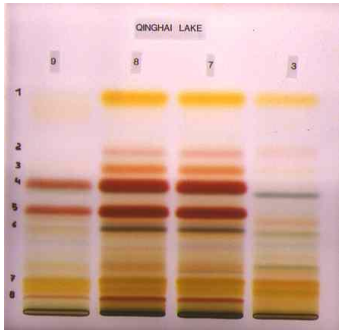
- *Chromatography* is a separation technique based on the different interactions of compounds with two phases, a *mobile phase* and a *stationary phase*, as the compounds travel through a supporting medium.
- **Components:**
  - **Mobile phase:** a solvent that flows through the supporting medium.
  - **Stationary phase:** a layer or coating on the supporting medium that interacts with the analytes.
  - **Supporting medium:** a solid surface on which the stationary phase is bound or coated.

Milestones in Chromatography

- 1903 Tswett - plant pigments separated on chalk columns
- 1931 Lederer & Kuhn - LC of carotenoids
- 1938 TLC and ion exchange
- 1950 reverse phase LC
- 1954 Martin & Synge (Nobel Prize)
- 1959 Gel permeation
- 1965 instrumental LC (Waters) Milestones in Chromatography

Types of Chromatography

Paper (PC)



Thin Layer (TLC)

Gas (GC)

Liquid (LC)

Chromatography

Supercritical Fluid (SFC)

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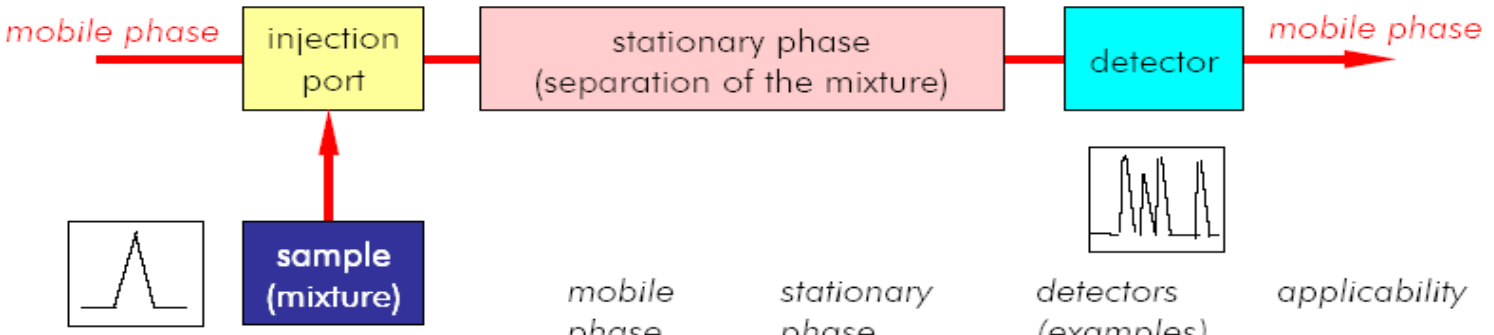
Types of Chromatography

Principle



	mobile phase	*stationary phase*	*detectors (examples)*	*applicability*
Gas chromatography (GC):	gases (H_2, N_2, Ar, He)	capillary or packed columns with	TCD, FID, MSD	volatile organic compounds, permanent gases
High pressure liquid chromatography (HPLC):	H_2O, organic solvents	substituted siloxanes	UV-VIS, RI, MSD	organic compounds and inorganic salts
Thin layer chromatography (TLC):	vapor of organic solvents	impregnated plates	optical detection (UV-VIS, fluorescence)	organic compounds

→ *large number of standardized procedures (norms) for industrial and environmental analysis*

Chromatographic methods classification

Geometry of the system:

- ***Column chromatography:*** the stationary phase (solid particles) is in a tube called the column.
- ***Planar chromatography:*** In this geometry the stationary phase is configured as a thin two-dimensional sheet.
  - In ***paper chromatography*** a sheet or a narrow strip of paper serves as the stationary phase.
  - In ***thin-layer chromatography*** a thin film of a stationary phase of solid particles bound together for mechanical strength with a binder, such as calcium sulfate, is coated on a glass plate or plastic or metal sheet.

Chromatographic methods classification

Mode of operation

Development chromatography

- In terms of operation, in development chromatography the mobile phase flow is stopped before solutes reach the end of the bed of stationary phase. The mobile phase is called the developer, and the movement of the liquid along the bed is referred to as development.

Example of planar development chromatography – TLC or PC

Elution chromatography.

- This method, employed with columns, involves solute migration through the entire system and solute detection as it emerges from the column. The detector continuously monitors the amount of solute in the emerging mobile-phase stream—the eluate—and transduces the signal, most often to a voltage, which is registered as a peak on a strip-chart recorder.

Chromatographic methods classification

Phases involved (mobile phase)

- The primary division of chromatographic techniques is based on the type of mobile phase used in the system:

Type of Chromatography	Type of Mobile Phase
• Gas chromatography (GC)	gas
• Liquid chromatography (LC)	liquid

Chromatographic methods classification

Phases involved (stationary phase)

Further divisions can be made based on the type of stationary phase used in the system:

Gas Chromatography:

Name of GC Method:		***Type of Stationary Phase***
Gas-solid chromatography	→	Solid, underivatized support
Gas-liquid chromatography	→	Liquid-coated support
Bonded-phase gas chromatography	→	Chemically-derivatized support

Chromatographic methods classification

Phases involved (stationary phase)

Further divisions can be made based on the type of stationary phase used in the system:

Liquid Chromatography:

Name of LC Method:	*Type of Stationary Phase*
Adsorption chromatography →	Solid, underivatized support
Partition chromatography →	Liquid-coated or derivatized support
Ion-exchange chromatography →	Support containing fixed charges
Size exclusion chromatography →	Porous support
Affinity chromatography →	Support with immobilized ligand

Chromatographic methods classification

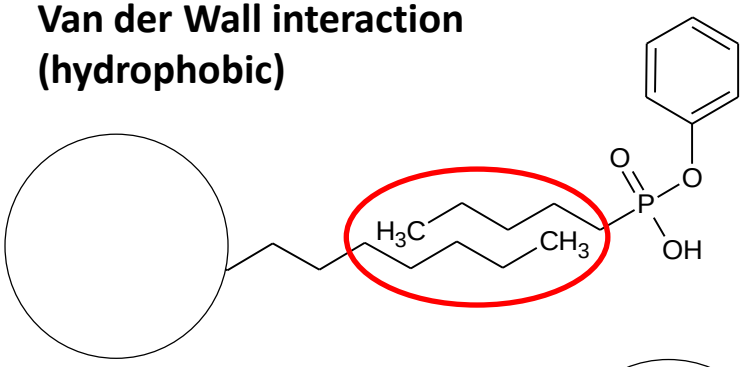
Retention mechanism

- Classification in terms of the ***retention mechanism*** is approximate, because the retention actually is a mixture of mechanisms. The main types of molecular interactive force are:
  - Van der Wall interactions (hydrophobic)
  - Polar (dipole-dipole: hydrogen bonding, π-π interactions)
  - Ionic
  - Special (affinity, chiral)

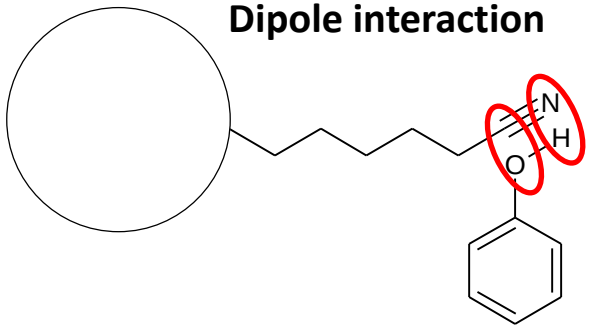
LC Separation

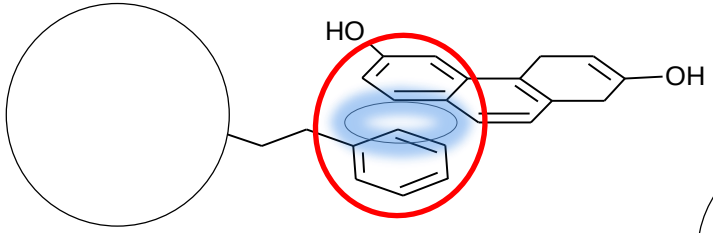
Main type of interactions in liquid chromatography

Van der Wall interaction (hydrophobic)



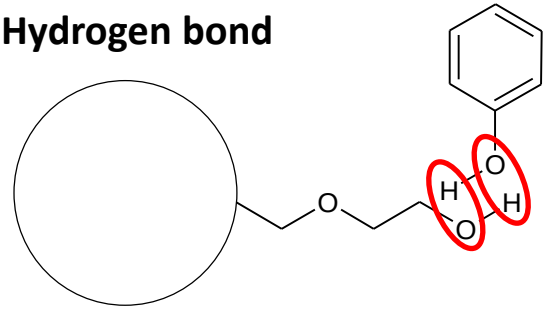
Dipole interaction

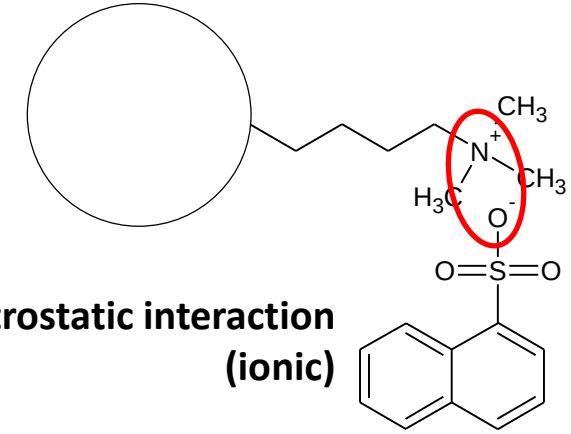




π-π interactions

Hydrogen bond



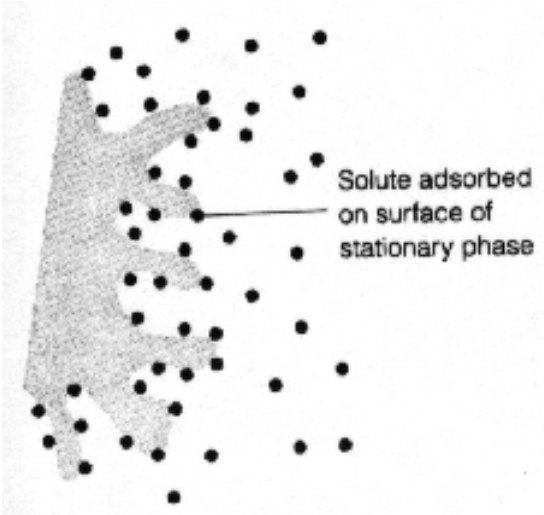


Electrostatic interaction (ionic)

Principle of separation

Adsorption chromatography

- Chromatography in which separation is based mainly on differences between the adsorption affinities of the sample components for the surface of an active solid.
- Usually used anorganic sorbents Al_2O_3, SiO_2 etc.
- Traditional adsorption chromatography is a „normal phase“ chromatography.

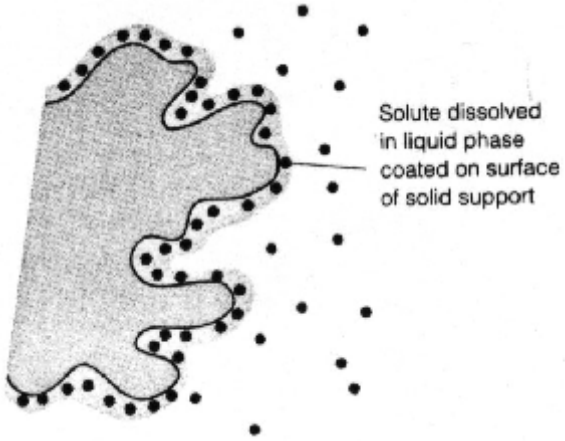


Principle of separation

Partition chromatography

- Chromatography in which separation is based mainly on differences between the solubility of the sample components in the stationary phase (*gas chromatography*), or on different solubilities of the component between mobile and stationary phases (*liquid chromatography*).

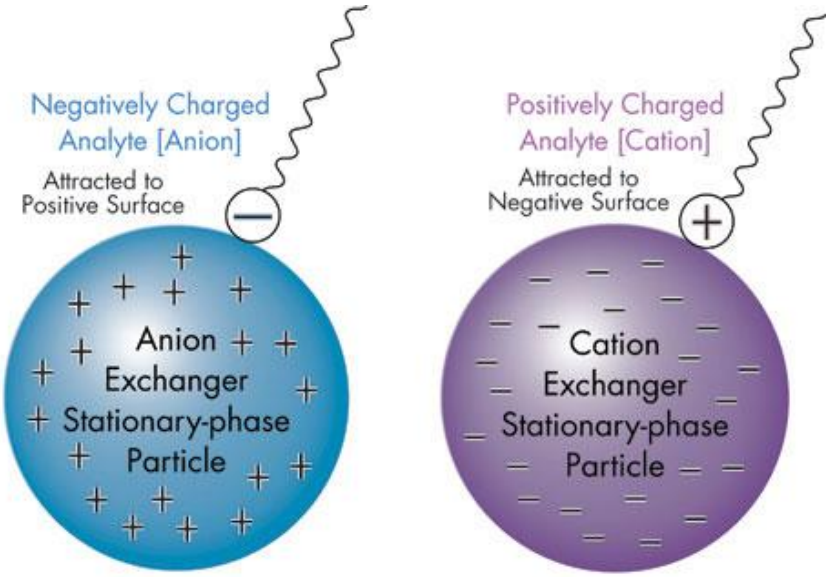
- Modified anorganic support
- In LC could be both normal (polar stationary phase) and reversed (non-polar stationary phase).

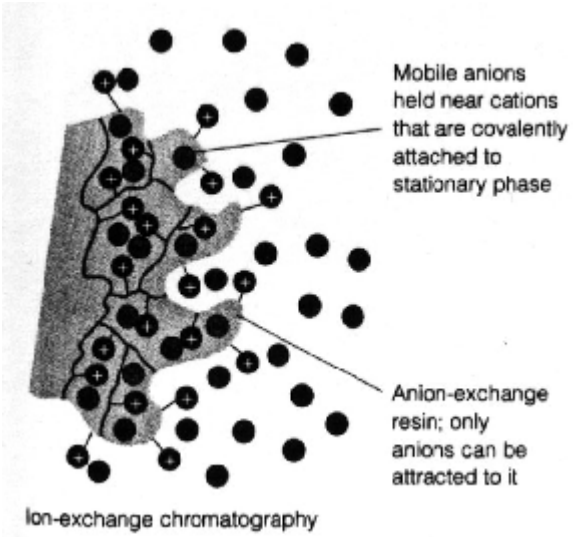


Principle of separation

Ion-exchange chromatography

- Chromatography in which separation is based mainly on differences in the ion-exchange affinities of the sample components. Anions like SO_3^- or cations like $N(CH_3)_3^+$ are covalently attached to stationary phase, usually a resin,

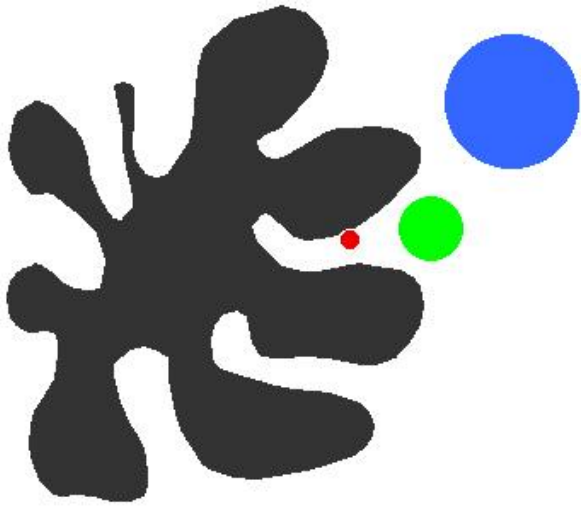




Principle of separation

Molecular exclusion chromatography

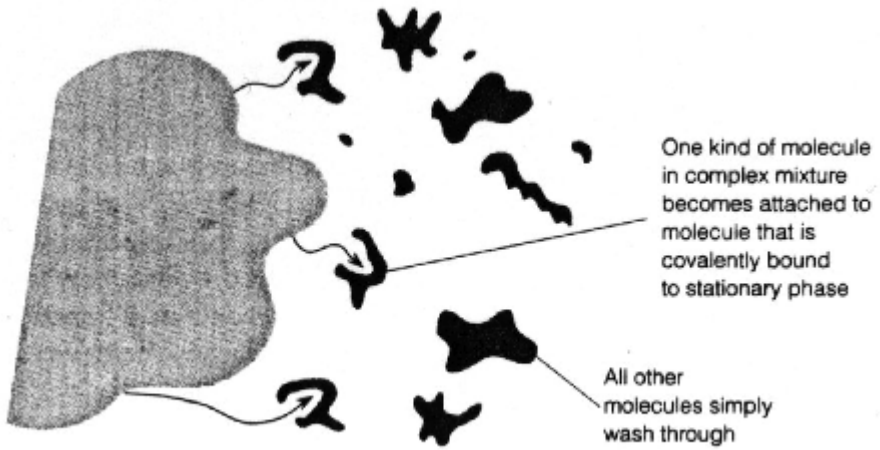
- A separation technique in which separation mainly according to the *hydrodynamic volume* of the molecules or particles takes place in a porous non-adsorbing material with pores of approximately the same size as the effective dimensions in solution of the molecules to be separated.



Principle of separation

Affinity chromatography

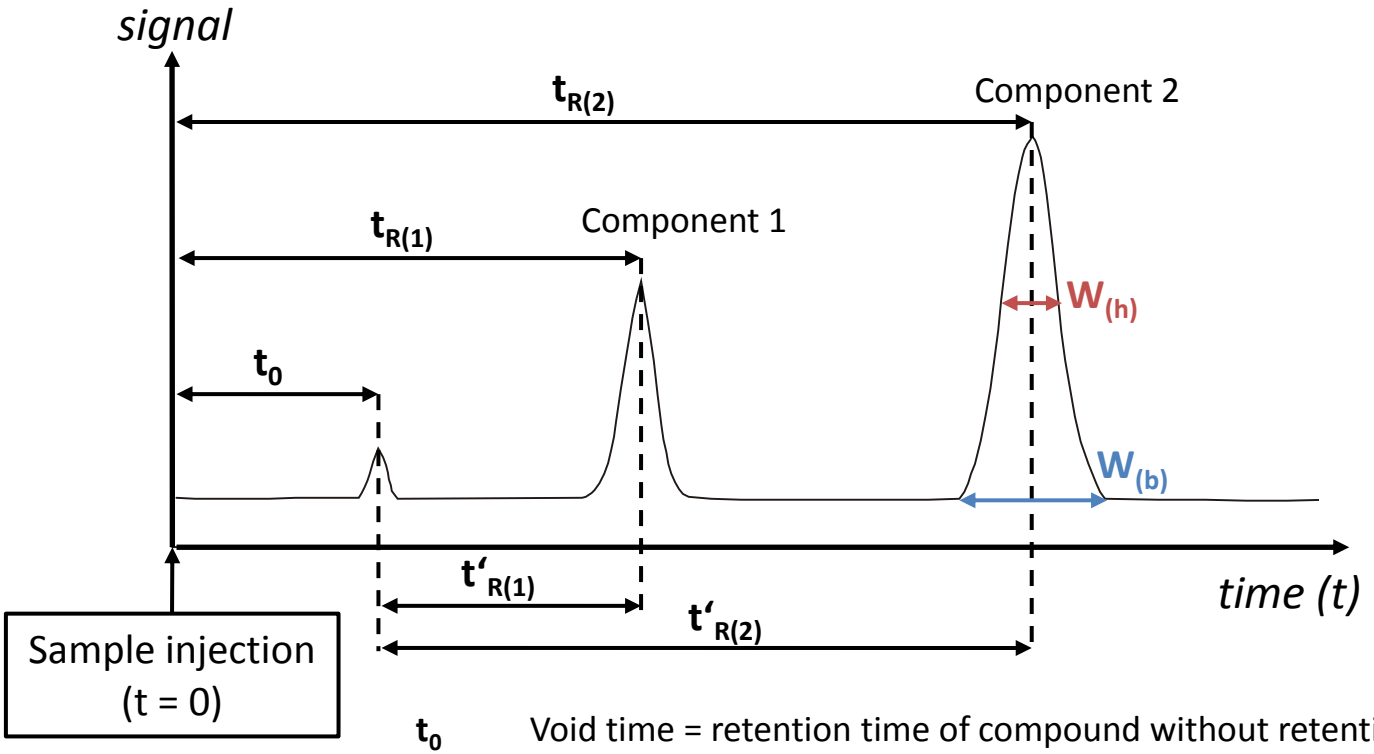
- The particular variant of chromatography in which the unique biological specificity of the analyte and ligand interaction is utilized for the separation.



Affinity chromatography

Theory of Chromatography

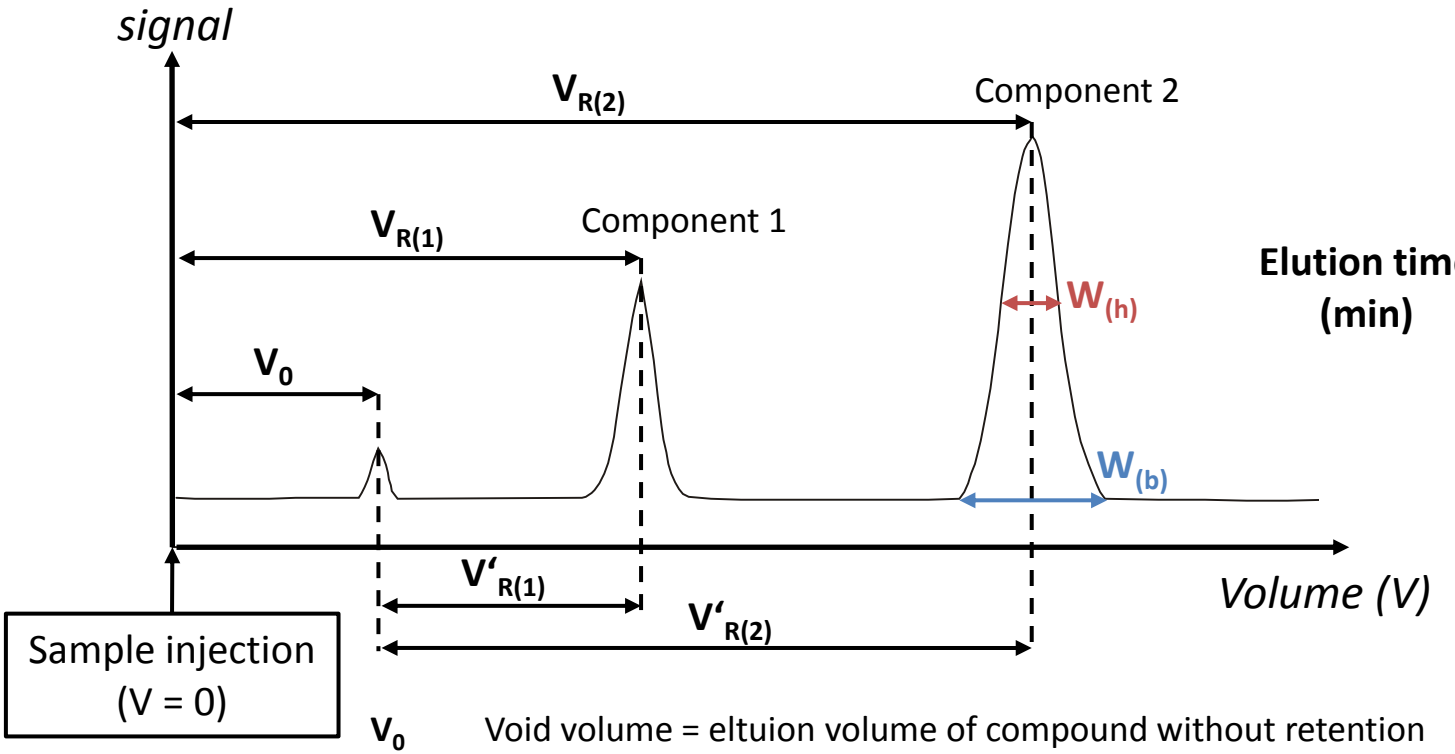
- Typical response obtained by chromatography (i.e., a chromatogram): chromatogram - concentration versus elution time.



t_0	Void time = retention time of compound without retention
t_R	Retention time
t'_R	Net retention time($t'_R - t_0$)
$W_{(b)}$	Base peak width
$W_{(h)}$	Half-of-height peak width

Theory of Chromatography

- A similar plot can be made in terms of elution volume instead of elution time.



Elution volume (ml)

Elution time (min) → $t_R = \frac{V_R}{F}$

Flow rate of mobile phase (ml/min)

V_0	Void volume = eltuion volume of compound without retention
V_R	Retention volume
V'_R	Net retention volume ($V'_R - V_0$)
$W_{(b)}$	Base peak width
$W_{(h)}$	Half-of-height peak width

Theory of Chromatography

Capacity factor (*k'*):

- More universal measure of retention, determined from t_R or V_R.

$$k' = \frac{t_R - t_M}{t_M} = \frac{V_R - V_M}{V_M} = \frac{t'_R}{t_M} = \frac{V'_R}{V_M}$$

- **Capacity factor** is useful for comparing results obtained on different systems since it is independent on column length and flow-rate.

Theory of Chromatography

Capacity factor (*k'*):

- The value of the capacity factor is useful in understanding the retention mechanisms for a solute, since the fundamental definition of k' is:

$$k' = \frac{moles\ A_{stationary}}{moles\ A_{mobile}} = \frac{(n_A)_s}{(n_A)_m}$$

k' is directly related to the strength of the interaction between a solute with the stationary and mobile phases

- Moles $A_{stationary}$ and moles A_{mobile} phase represents the amount of solute present in each phase at equilibrium. Equilibrium is achieved or approached at the center of a chromatographic peak.

k' ≈ 1.0, separation is poor

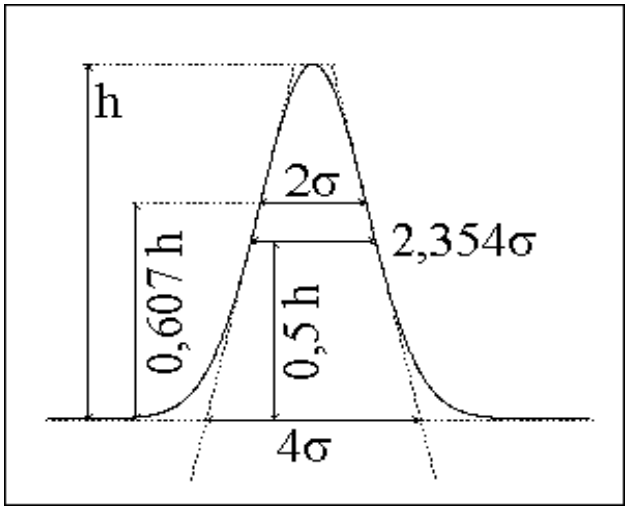
k' > 30, separation is slow

k' = 2-10, separation is optimum

Theory of Chromatography

Efficiency of chromatography

- Efficiency is related experimentally to a solute's peak width.
  - An efficient system will produce narrow peaks
  - Narrow peaks ≈ smaller difference in interactions in order to separate two solutes
- Efficiency is related theoretically to the various kinetic processes that are involved in solute retention and transport in the column
  - Determine the width or standard deviation (**σ**) of peaks



Estimate (**σ**) from peak widths, assuming Gaussian shaped peak:

$W_b = 4\sigma$

$W_h = 2.354\sigma$

Theory of Chromatography

Number of theoretical plates (N):

- Compare efficiencies of a system for solutes that have different retention times:

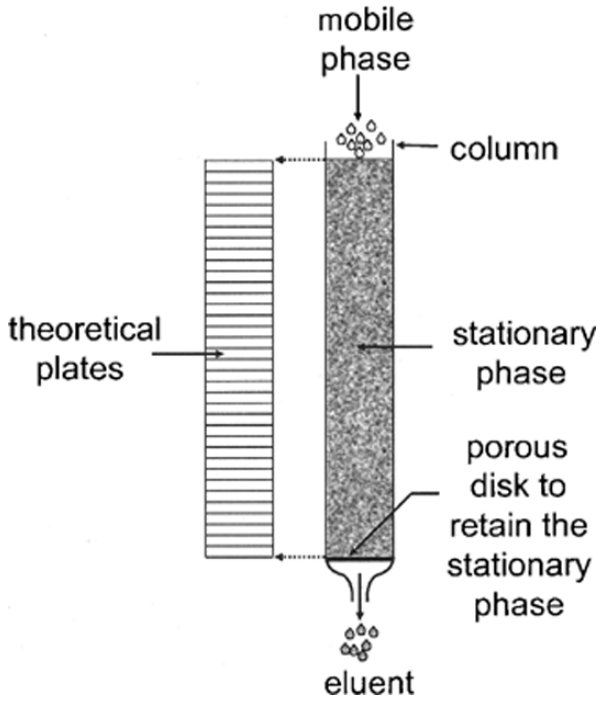
$$N = \left(\frac{t_R}{\sigma}\right)^2$$

- Gaussian shaped peak:

$$N = 16\left(\frac{t_R}{W_b}\right)^2 = 5.54\left(\frac{t_R}{W_h}\right)^2$$

The larger the value of N is for a column, the better the column will be able to separate two compounds:

- The better the ability to resolve solutes that have small differences in retention.
- N is independent of solute retention
- N is dependent on the length of the column



Theory of Chromatography

Plate height or height equivalent of a theoretical plate (H or HETP):

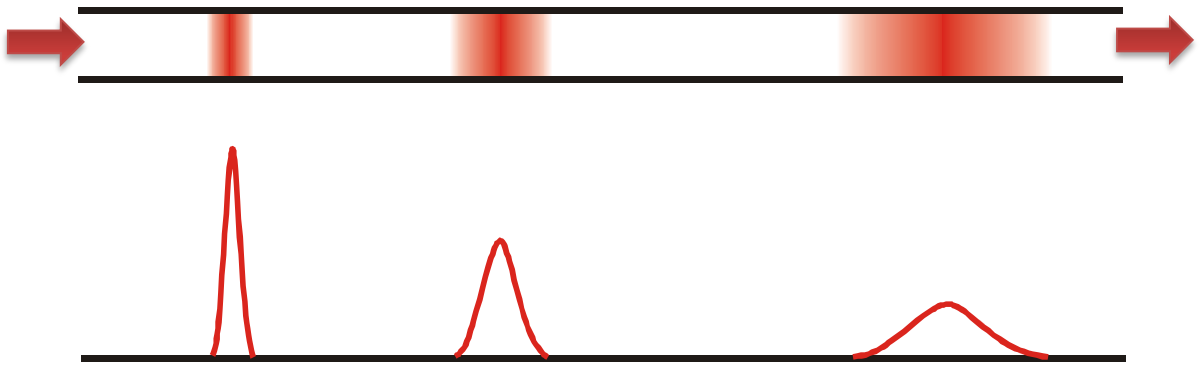
- Compare efficiencies of columns with different lengths:

$$H = \frac{N}{L}$$

L = column length
N = number of theoretical plates for the column

Note: H simply gives the length of the column that corresponds to one theoretical plate.

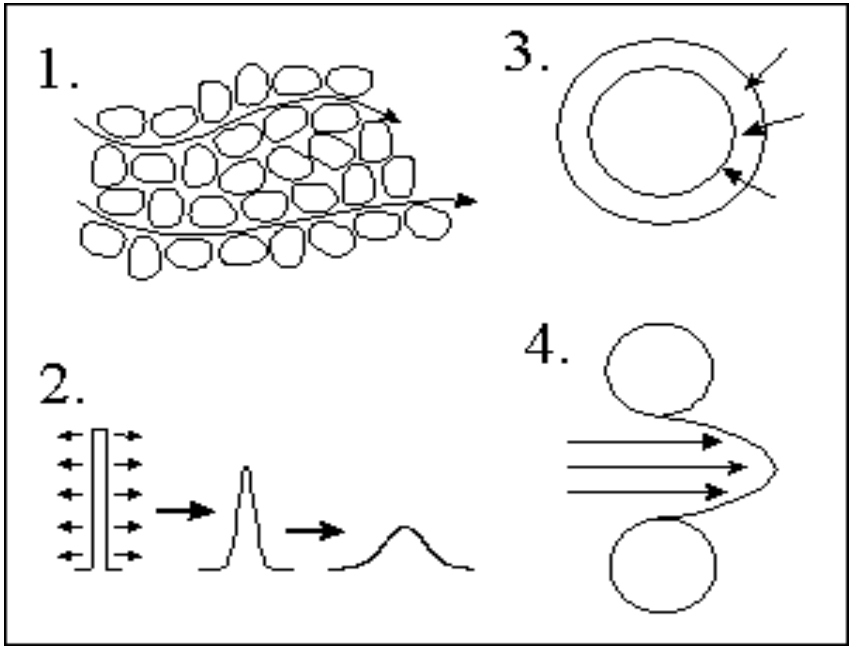
- H can be also used to relate various chromatographic parameters (e.g., flow rate, particle size, etc.) to the kinetic processes that give rise to peak broadening.



Theory of Chromatography

Why Do Bands Spread?

1. **Eddy diffusion** – different molecules have to travel different distance
2. **Longitudinal diffusion** – molecules move from the place with higher concentration to place with lower concentration
3. **Stationary phase mass transfer** – different depth of molecules diffusion into the stationary phase particle.
4. **Mobile phase mass transfer** – diffusion asociated with different mobile phase velocity



Theory of Chromatography

Rate Theory of Chromatography

- The HEPT height depends on mobile phase velocity ($\boldsymbol{u}$):

$$\mathbf{H = H_L + H_S + H_M + H_{SM}}$$

$$H_L = \frac{B}{u} \qquad H_{SM} = A \qquad H_S = C_S\, u \qquad H_M = C_M\, u$$

H	Plate equivalent to the theoretical plate (as in Plate Theory)
H_L	Contribution to the longitudinal diffusion
H_S	Stationary phase mass transfer contribution
H_M	Diffusion associated with mobile phase effect
H_{SM}	Diffusion into or mass transfer across a stagnant layer of mobile phase

$$H = A + \frac{B}{u} + (C_S + C_M)\, u = A + \frac{B}{u} + C\, u$$

Van Deemter equation

Theory of Chromatography

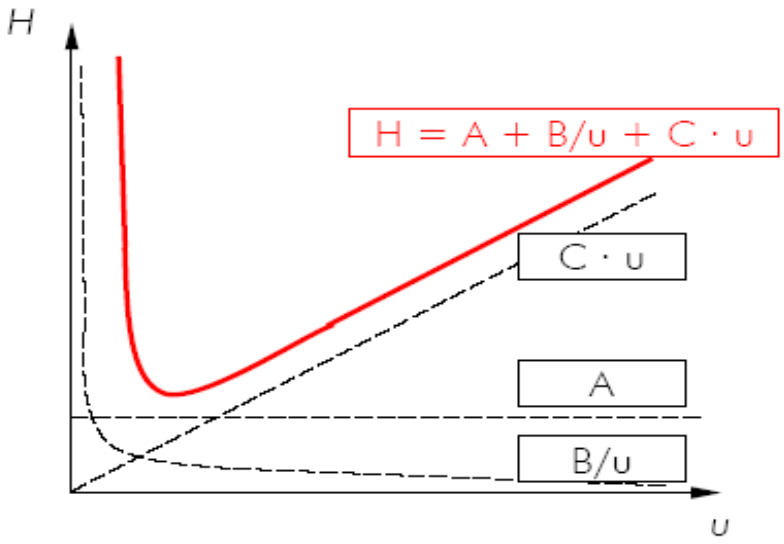
Van Deemter equation

$$H = A + \frac{B}{u} + C \cdot u$$

Eddy diffusion term (peak broadening by different ways of sample molecules in a column)

axial diffusion term (statistic axial distribution of sample molecules)

term for mass exchange between stationary and mobile phases (adsorption/desorption, diffusion/back diffusion)



Theory of Chromatography

Separation factor

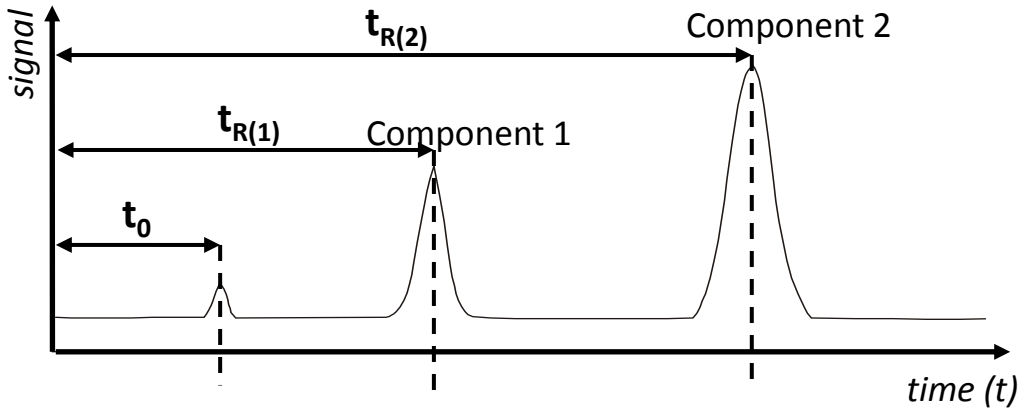
- Universal measure of retention, determined from t_R or V_R:

$$\alpha = \frac{k'_{(2)}}{k'_{(1)}}$$

$k'_{(1)}$ – capacity factor of solute 1

$k'_{(2)}$ – capacity factor of solute 2,
when $k'_{(2)} > k'_{(1)}$

- A value of α > 1.1 is usually indicative of a good separation Does not consider the effect of column efficiency or peak widths, only retention.



Theory of Chromatography

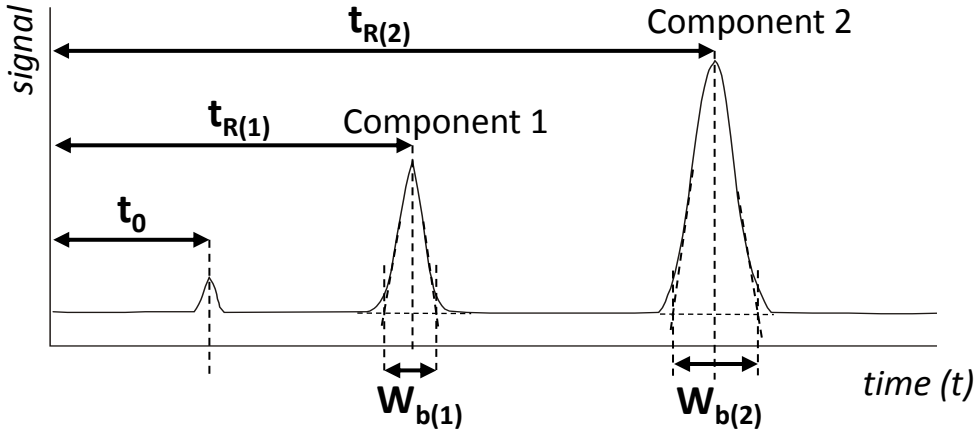
Resolution (R_S):

- Resolution between two peaks is a second measure of how well two peaks are separated:

$$R_S = \frac{t_{R(2)} - t_{R(1)}}{\left(W_{b(2)} + W_{b(1)}\right)/2}$$

t_{r1}, W_{b1} retention time and baseline width for the first eluting peak

t_{r2}, W_{b2} retention time and baseline width for the second eluting peak



R_S is preferred over α since both retention (t_r) and column efficiency (W_b) are considered in defining peak separation.

Theory of Chromatography

Factors affecting resolution

- Selectivity expressed as *Separation factor* (α)
- Retention of later eluted compound expressed as *Capacity factor* (*k'*)
- Efficiency expressed as number of effective plates (N)

$$R = \frac{(\alpha - 1)}{\alpha} \cdot \frac{k'_2}{k'_2 + 1} \cdot \frac{\sqrt{N}}{4}$$

Selectivity term

- Type of stationary phase
- Used mobile phase
- Solubility of analysed compounds in mobile phase

Retention term

- Elution strenght
- Type and amount of stationary phase
- Temperature

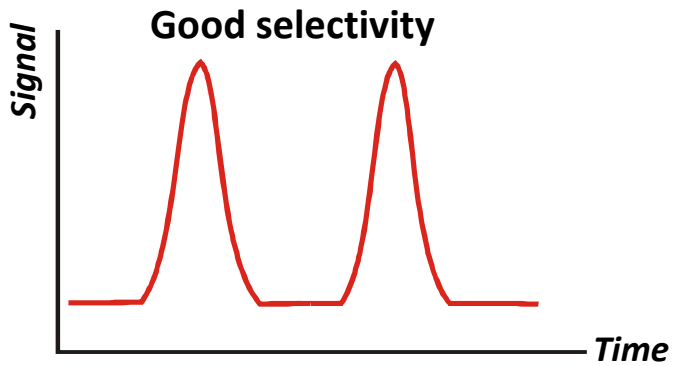
Efficiency term

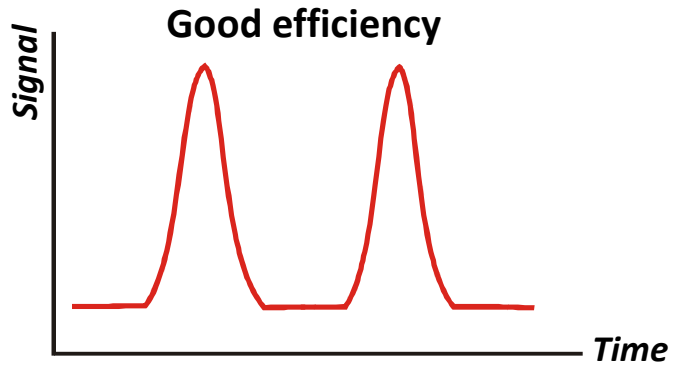
- Column lenght
- Particle size

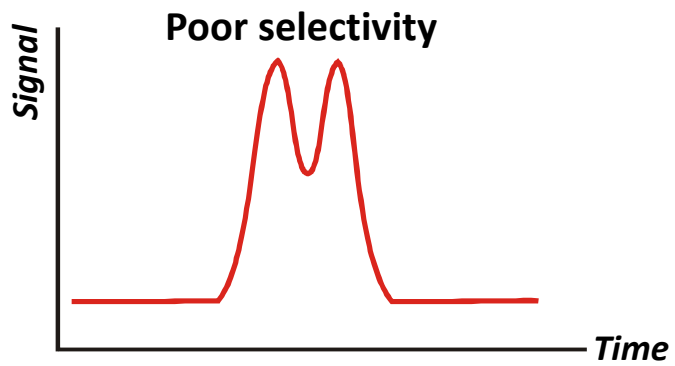
Theory of Chromatography

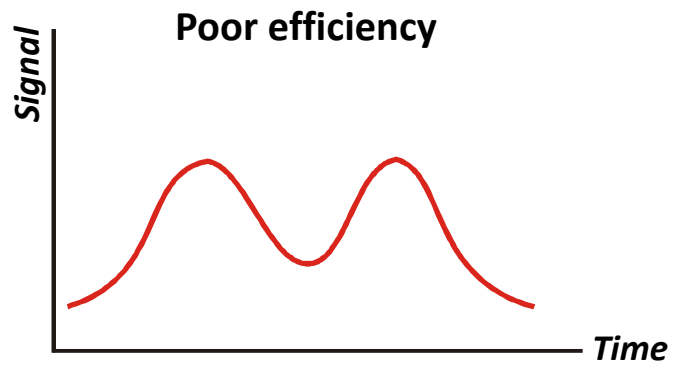
Factors affecting resolution

- Selectivity and column efficiency







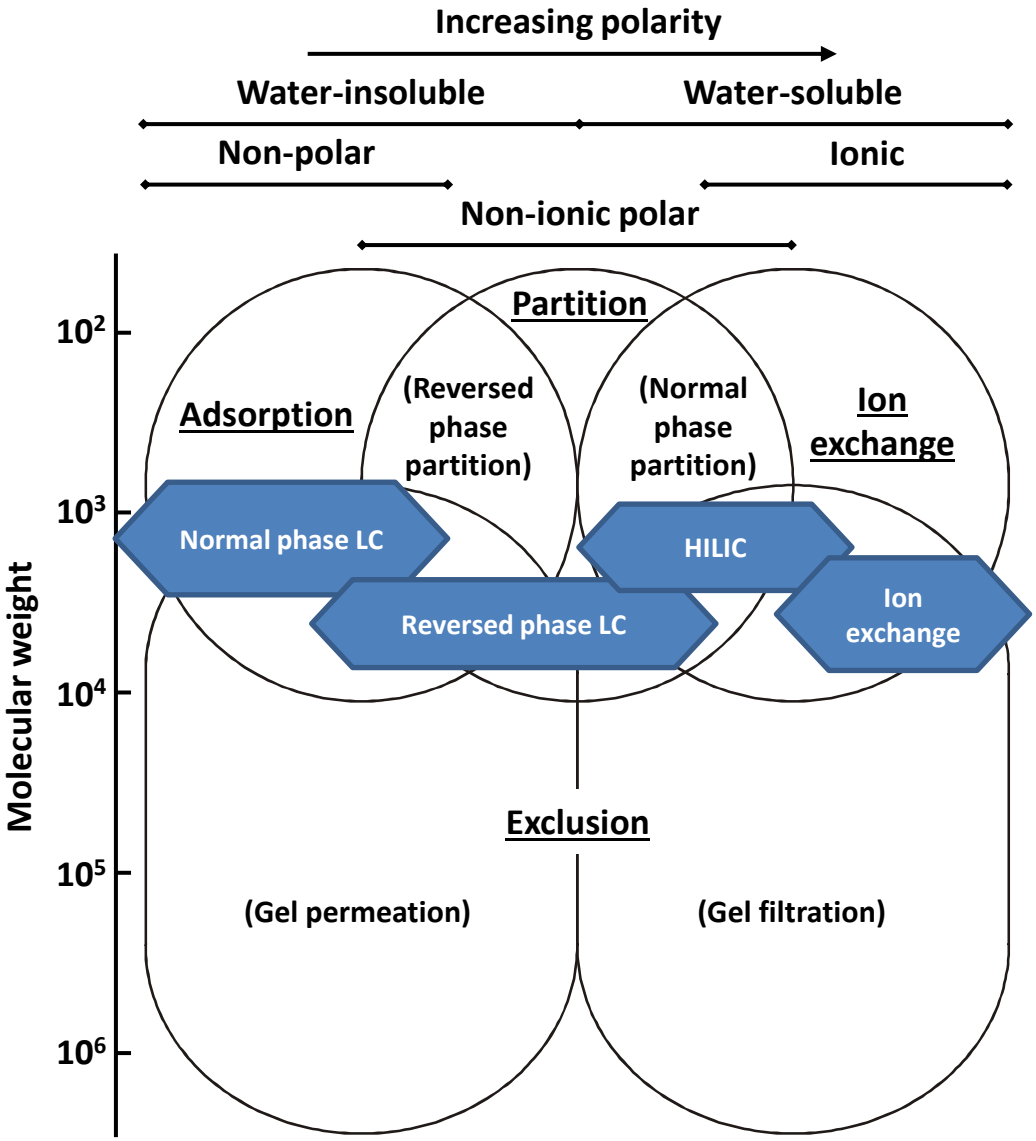


Chromatographic systems

Selection of chromatographic configuration depends on physico-chemical properties of the analyte:

- Analyte solubility
- Analyte polarity
- Analyte weight

Only weak interactions with stationary phase are required
(analytes have to go throught the column)



Chromatographic systems

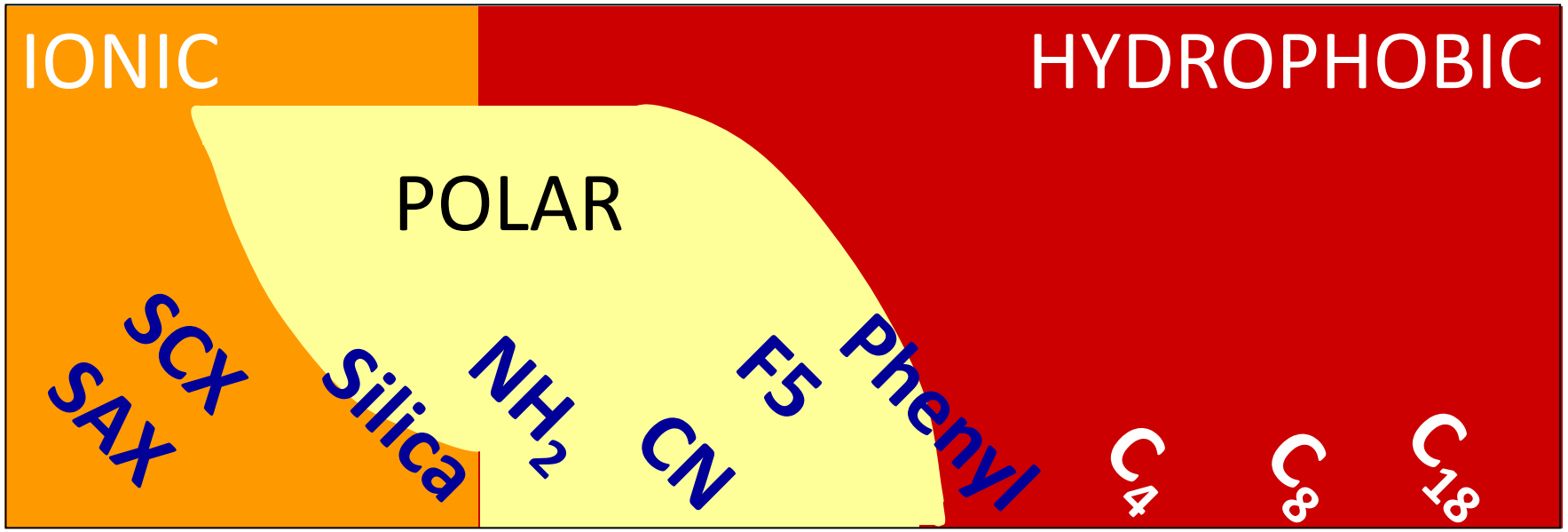
Selection of chromatographic configuration:

	Normal phase Chromatography	Reversed Phase Chromatography	HILIC Chromatography	Ion Exchange Chromatography
Stationary phase	Polar (silica, alumina, florisil, MgO)	Non-polar modified silica (CN, C8, C18, phenyl)	Polar (silica, modified silica (aminopropyl, CN))	Ionic (Resins with bonded ionic groups
Mobile phase	Non-polar (hexan, dichlormethan, tetrahydrofuran, ethylacetate)	Polar (water, methanol, acetonitrile, tetrahydrofuran)	Polar (water, acetonitrile)	Ionic water (up to 50% organic) with buffers (NaHCO3, NaOH…)
Analytes	Non-polar and water insoluble	Non-polar and polar	Ionic and non-ionic polar, water soluble analytes	Ionic, organic and anorganic bases and acids

HPLC System

Columns and Stationary Phases

Stationary phases



SCX…strong cation exchange
SAX…strong anion exchange
Silica …bare silica phase
CN…cyanopropyl phase
NH_2…amino phase

F5…pentafluorophenyl phase
Phenyl…butyl-phenyl phase
C_4…butyl phase
C_8…octyl phase
C_{18}…octadecyl phase

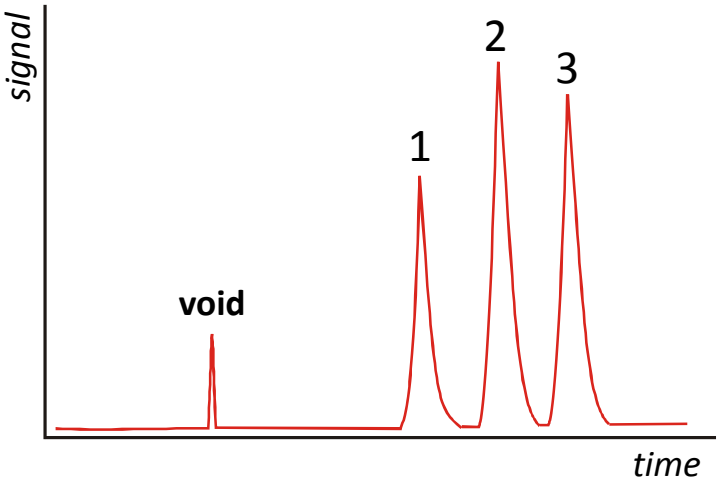
Normal Phase Chromatography

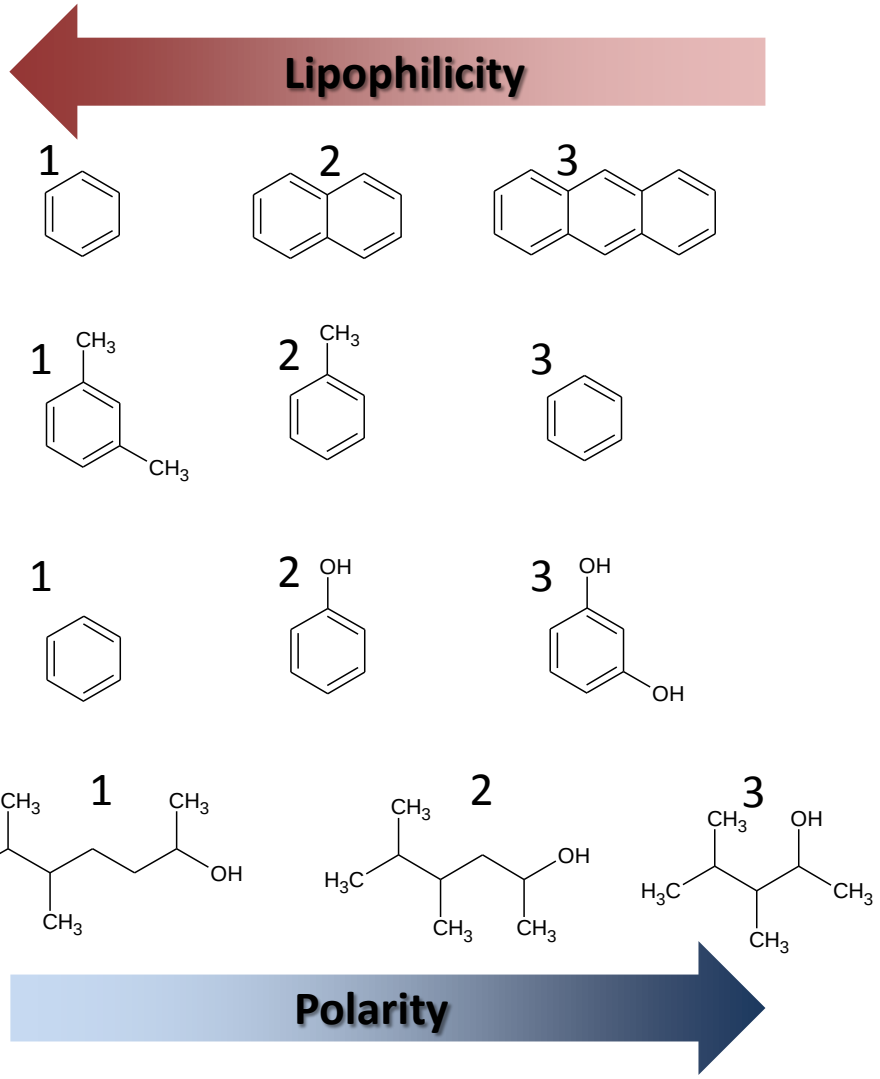
- Adsorption chromatography, -OH on the surface of silica are active sites (or Al^{3+} an O^{2-} in case when alumina is used).
- Types of interactions are *dipole-induced dipole*, *dipole-dipole*, *hydrogen bonding*, *π-complex bonding*.
- Adsorption strengths (*k*) increase in the following order:

  saturated hydrocarbons < olefins < aromatic ≈ halogenated compounds < suphides < ethers < nitro compounds < esters ≈ aldehydes ≈ ketones < alcohols ≈ amines < suphones < suphoxides < amides < carboxylic acids
- Only functional groups or double bond are used for separation, it is not possible to distinguish between molecules that are identical except the aliphatic moiety.
- The most polar functional group in the molecule determines its retention.
- The strength of interaction depends also on steric factors, isomers are suitable for separation by adsorption chromatography.

Normal Phase Chromatography

Polar compounds are eluted **later** than the non-polar.

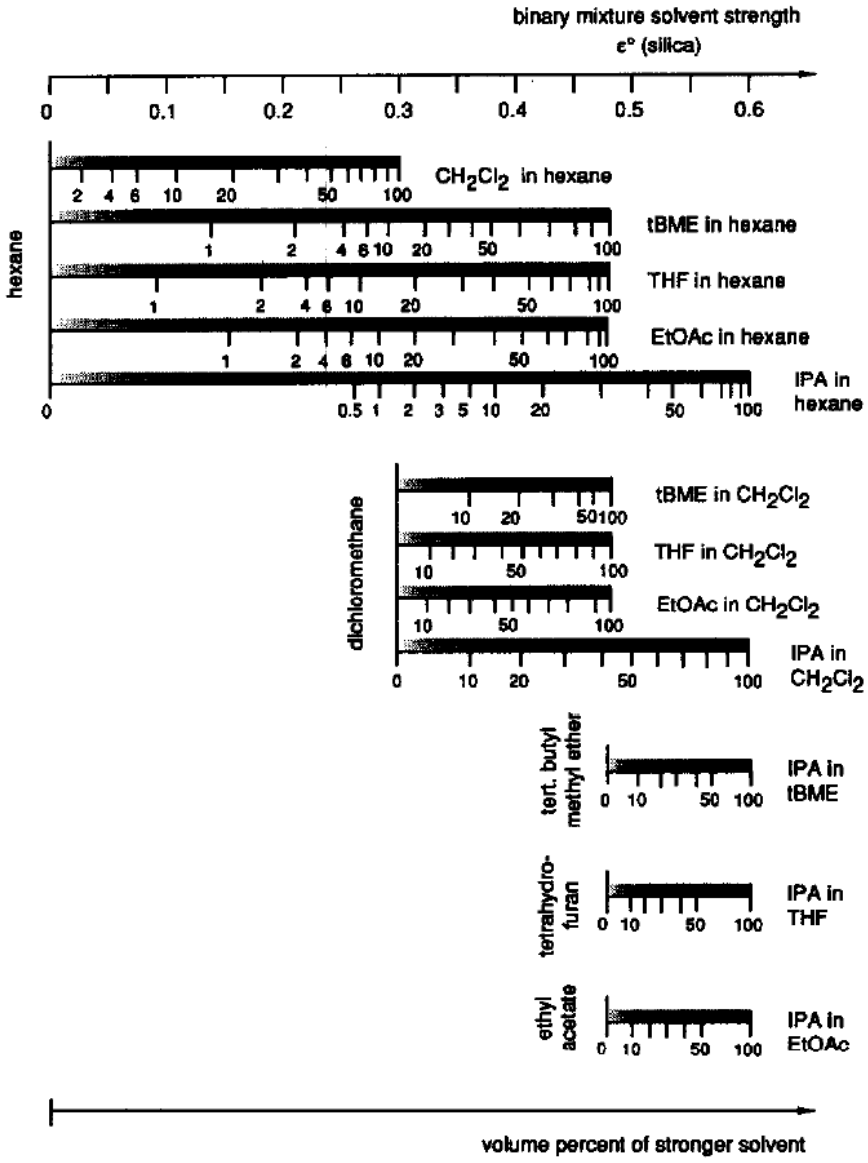




Normal Phase Chromatography

Elution strength of binary mixture

- Elution strength of the various solvents is determined empirically (ε°).
- Same elution strength could be achieved with solvents of different selectivity.



Reversed Phase Chromatography

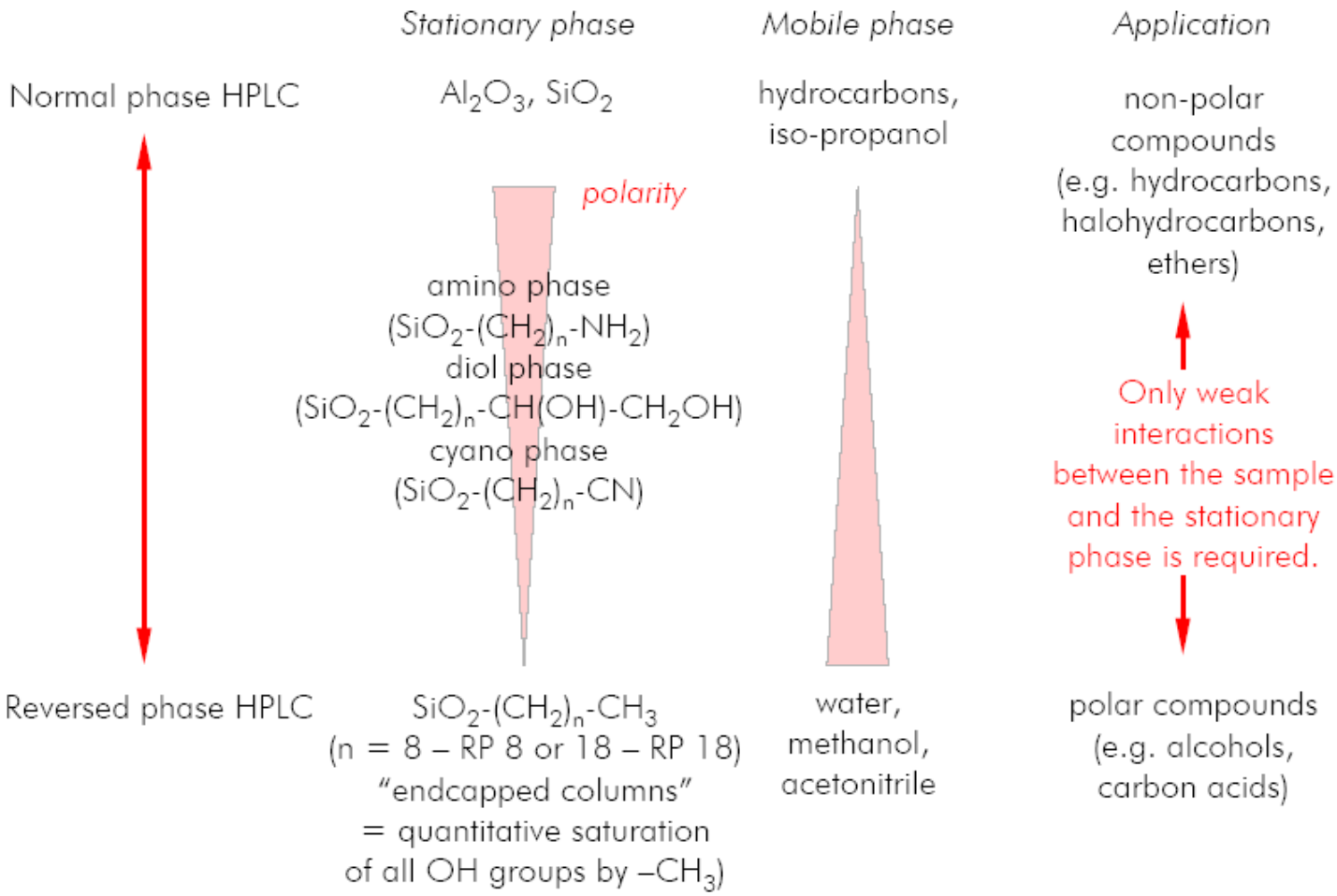
- Partition type of chromatography
- Main type of interaction is hydrophobic (van Der Waals interaction), but in real is the retention mechanism complex.
- Elution order:

  Strong Lewis acids (carboxylic acids) < Weak Lewis acids (alcohols, phenols) < Strong Lewis bases (amines) < Weak Lewis bases (ethers, aldehydes, ketones) < permanent dipoles ($CHCl_3$) < induced dipoles (CCl_4) < aliphatics
- Retention increases also with the number of carbon atoms in molecule:

  … Pentan < Hexan < Heptan…
- Branched-chain isomers are eluted earlier than linear form.

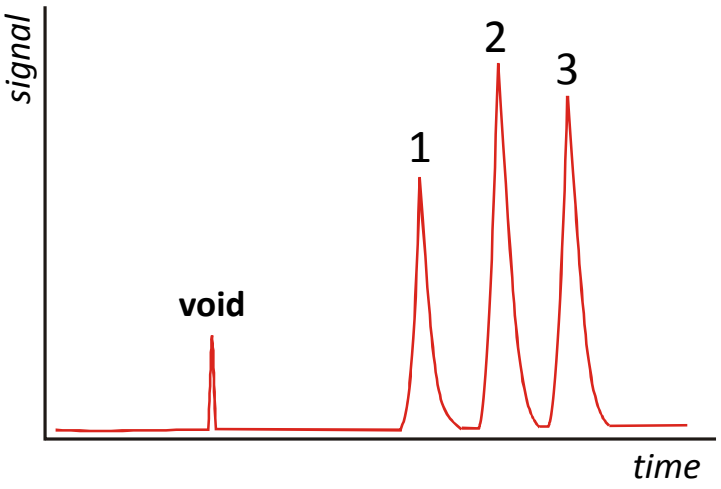
Reversed Phase Chromatography

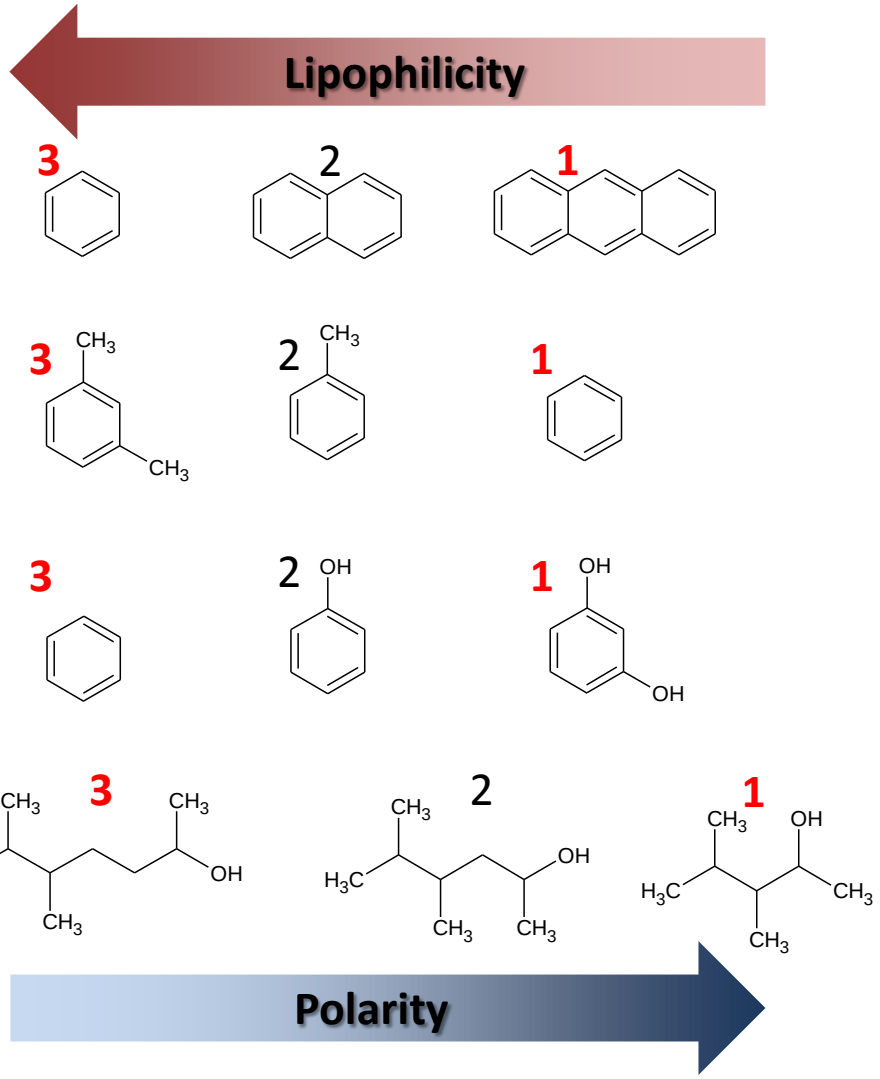
Comparison of normal and reversed phase chromatography



Reversed Phase Chromatography

Polar compounds are eluted **earlier** than the non-polar.





Reversed Phase Chromatography

Mobile phase selection in RP-HPLC

- Mobile phase generally consist of mixtures of water and water-miscible organic solvent:

Methanol
Acetonitrile
Ethanol
Isopropanol
Dimethylformamide
Propan-1-ol
Dioxane
Tetrahydrofuran

Decreasing polarity

Increasing elution power

- Non-aqueous eluents are used in RP-HPLC for elution of highly non-polar compounds.
- The mixtures with water have higher viscosity (produced higher back pressure) than the pure solvents.

Reversed Phase Chromatography

Mobile phase selection in RP-HPLC

- Polarity is only one parameter to change separation.
- Another parameter is selectivity:

$$R = \frac{(\alpha - 1)}{\alpha} \cdot \frac{k'_2}{k'_2 + 1} \cdot \frac{\sqrt{N}}{4}$$

Selectivity term **Polarity term**

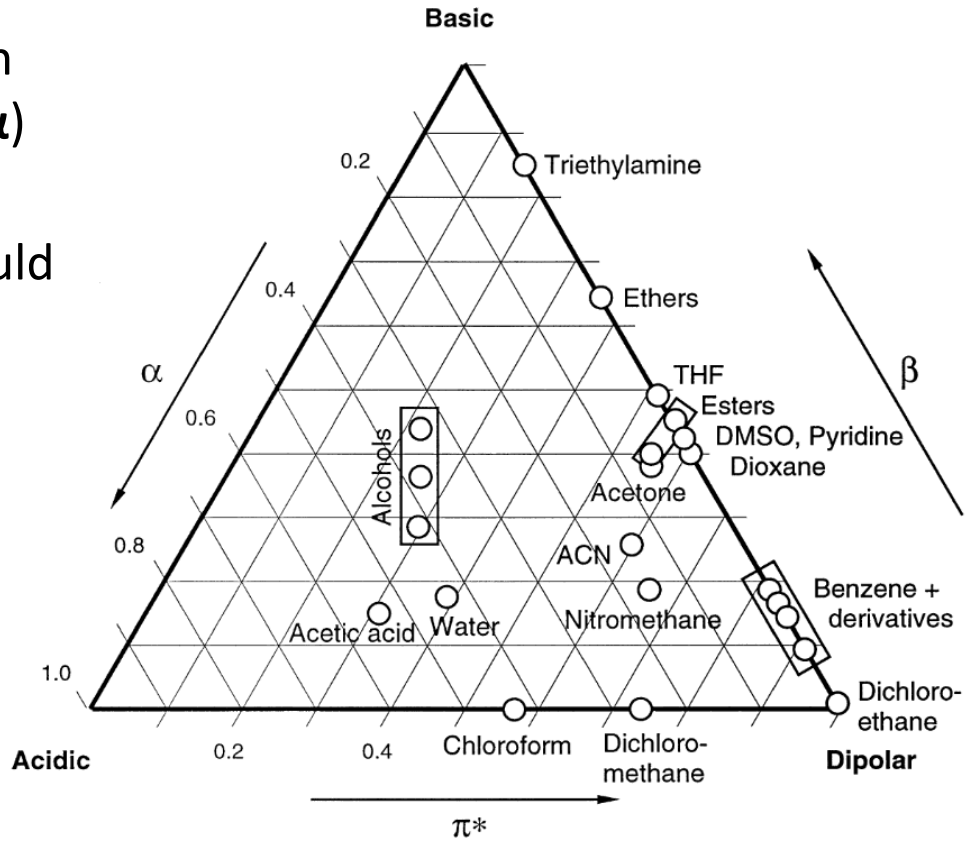
- Not all solvents could be used (possible chemical interaction, miscibility issue, toxic, flammable, volatile).

Reversed Phase Chromatography

Mobile phase selection in RP-HPLC

Triangle of selectivity

- Comparison of different solvents in terms of their **dipolar (π)**, **acidic (α)** and **basic (β)** properties.
- Largest difference in selectivity could be expected for solvents with the most different properties.

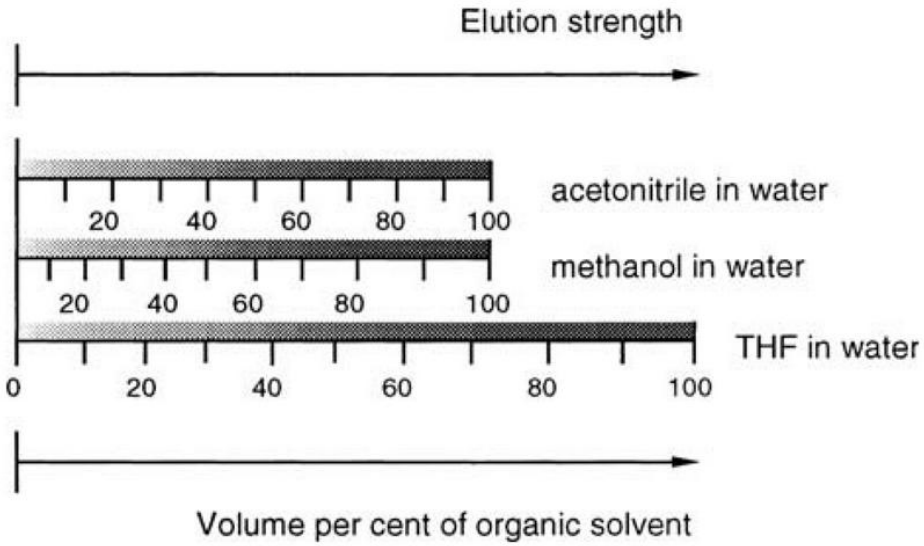


Reversed Phase Chromatography

Common solvents in RP-HPLC

- Methanol – acids
- Acetonitrile – bases
- Tetrahydrofuran – strong dipole
- Water – polarity adjustment

- Miscible
- Low viscosity
- Available in the highest purity
- Cheap



Reversed Phase Chromatography

Stationary phases

- C18 modified silica is the most common stationary phase, providing high retention (other phases are C8, phenyl, CN, diol, NH2 – providing lower retention and alternative selectivity).
- **Carbon load:** Retention strenght for C18 could be estimated from „carbon load“ – more carbon means thicker stationary phase and consequebntly higher retention (for non-polar analytes, columns with lower carbon load could be recommended).
- **Pore size** (Å, Ångström) determines suitability of the phase for small or large molecules – small pore size providing better capacity, but it is not for large molecules molecules.

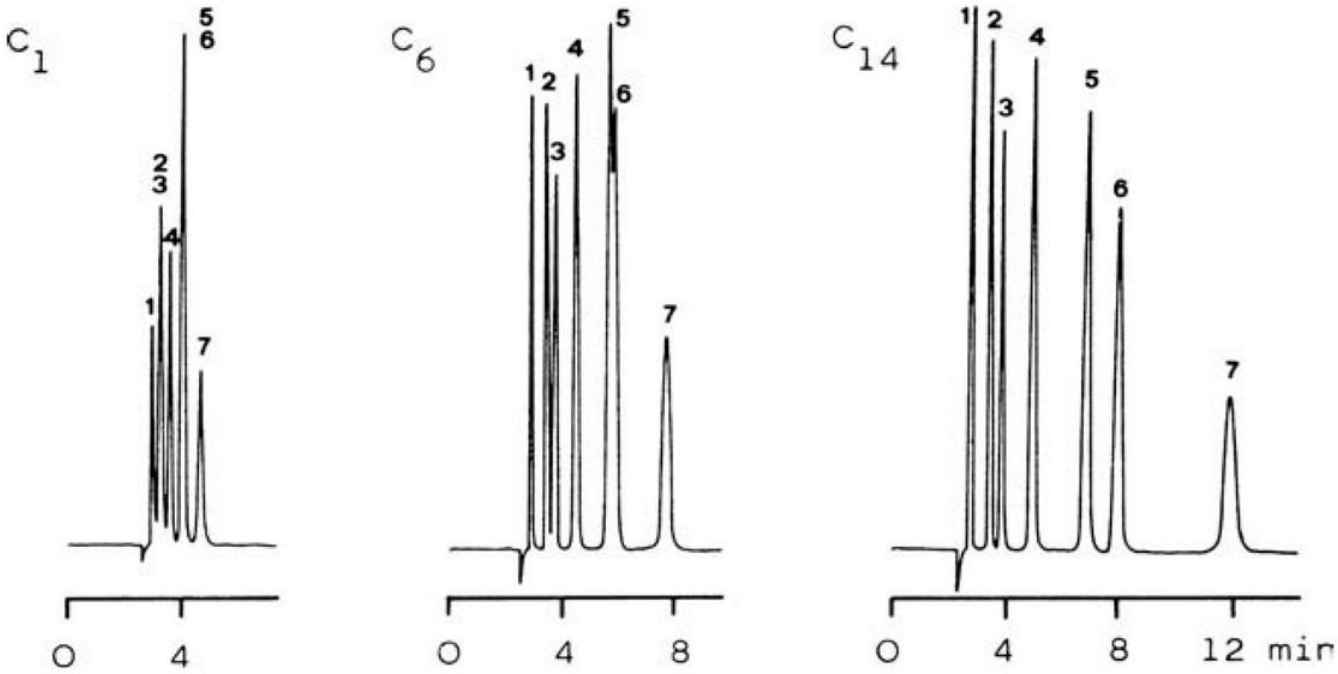
  1Å = 0.1 nm (1×10^{-10} meter)
- **Silanol activity** – it is not possible to derivatize all silanols for sterical reasons. Silanol groups could be endcapped or shielded stericaly. Silanol activity provides different selectivity of the column.

Reversed Phase Chromatography

Stationary phases

- Effect of chain lenght on retention.

1. Acetone
2. p-methoxyphenol
3. Phenol
4. m-cresol
5. 3,5-xylenol
6. Anisole
7. p-phenylphenol



Longer chain provides higher retention.

Reversed Phase Chromatography

Stationary phases

- Separation of the most polar compounds needs water-rich mobile phase.
- Since high hydrophobicity of C18 phase, such mobile phase can colapse.

Normal conditions, the solvents and sample have full acces to the stationary phase.

Collapsed phase due to high water mobile phase.

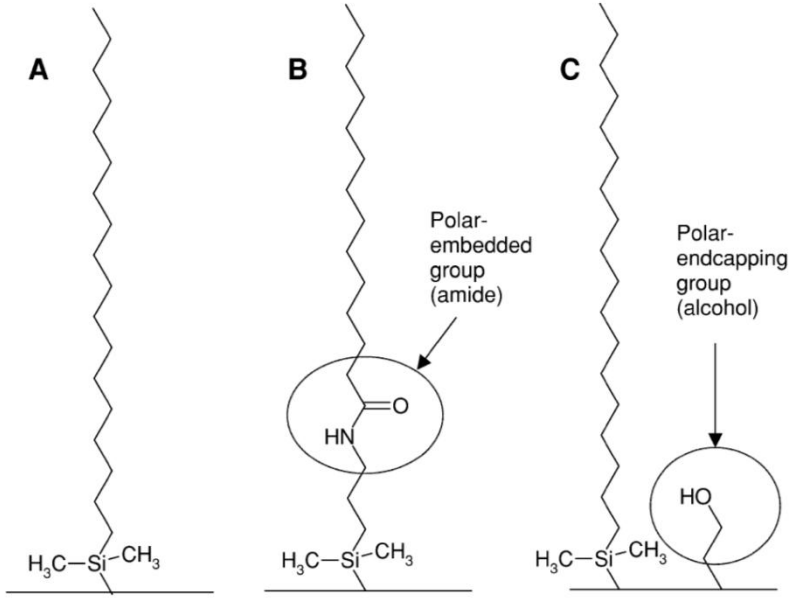
- New phases developed for separation of polar compounds and 100% water mobile phase compatibility.

Reversed Phase Chromatography

Stationary phases

- Introduction of polar (hydrophilic) groups stabilise the stationary phase even 100% water mobile phase is used.
- **Polar-encapped phase** – Hydrophobic interaction silmilar to the traditional phase, stronger hydrogen bonding and silanol activity.
- **Polar-embedded phase** – Opposite behaviour, reduction of the hydrophobic intercation, reduced silanol activity.

A. Common C18 phase
B. C18 + polar-embedded group
C. C18 + polar-encapping



Reversed Phase Chromatography

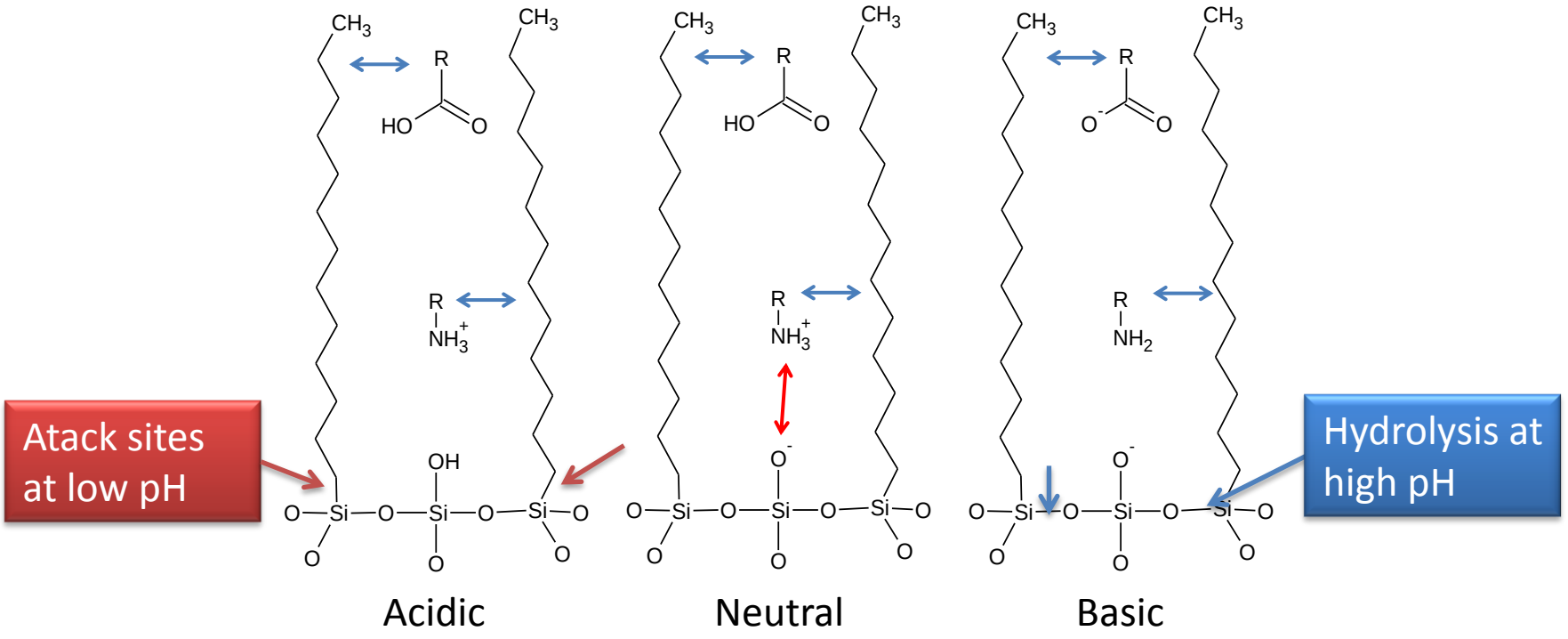
Separation of ionic compounds

- Ionic compounds should be analysed in the non-dissociated forms by adjusting pH.
- Use acidic mobile phase for acid analysis and basic mobile phase for bases.
  - pH should be 2 units above or under the analyte pK_A.
- For separation of basic compound, special endcapped or shielded phases with low silanole activity should be used.
- pH should be in the operation range of the column (usually pH 2-7)
  - Stationary phase is hydrolysed at low pH.
  - Silica support is hydrolysed at high pH.

Reversed Phase Chromatography

Separation of ionic compounds

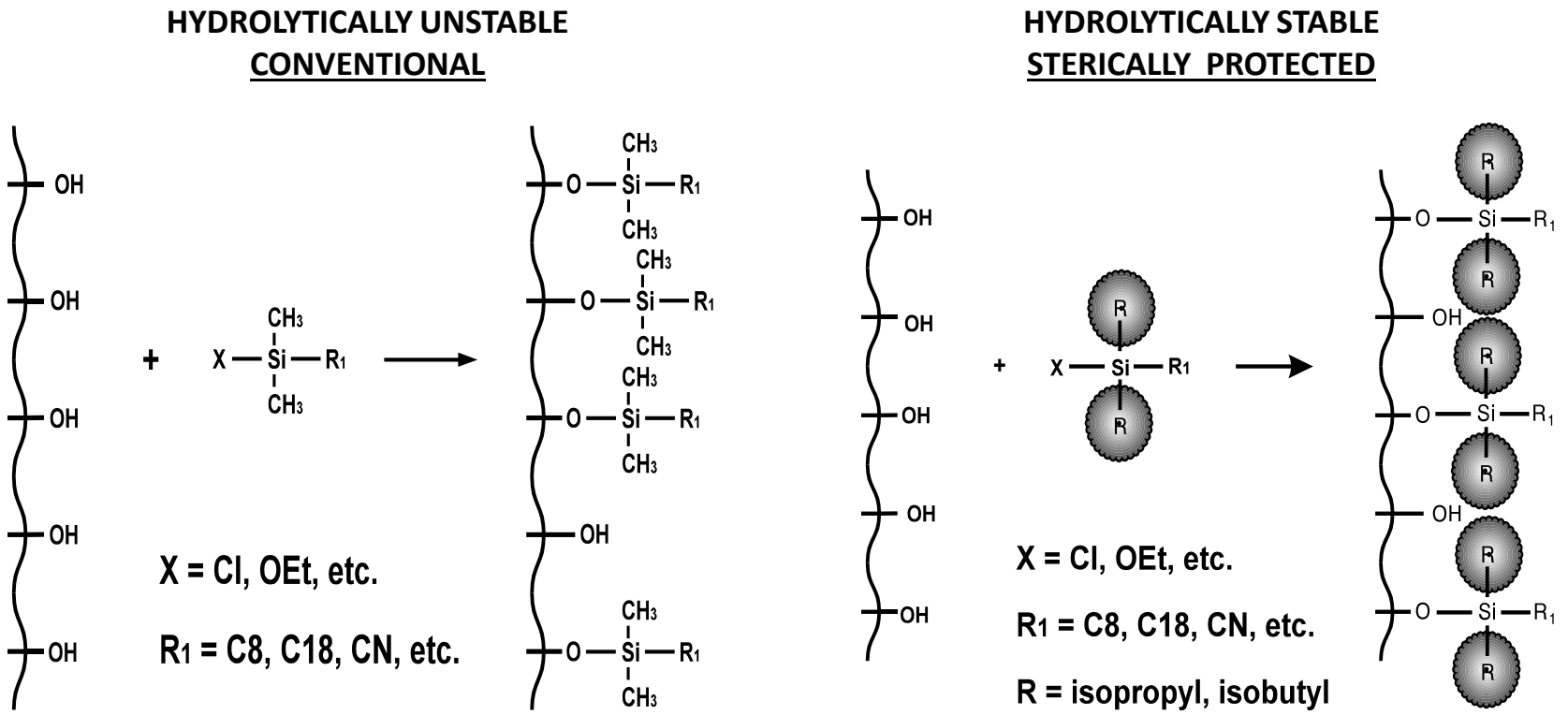
- Free silanol groups are charged in pH > 4. Except the hydrophobic, also ion exchange separation mechanism is employed and it can cause tailing of basic analytes (acids are not influenced).



Reversed Phase Chromatography

Separation of ionic compounds

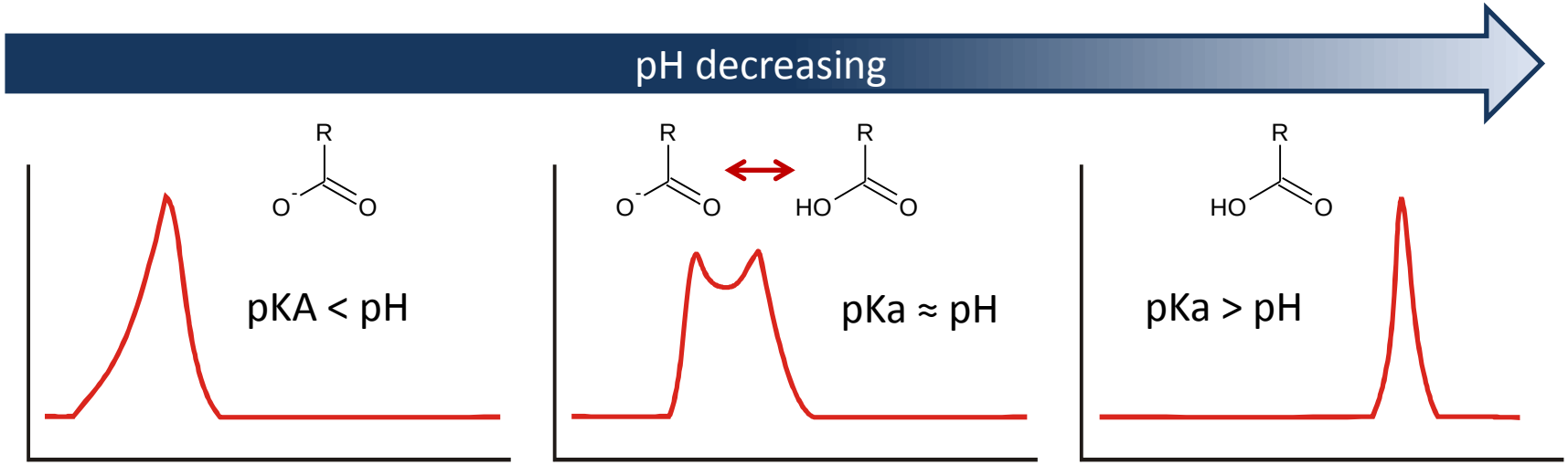
- Special stationary phases were developed to improve low pH column stability.
- The Si-C bond is sterically protected.



ICT PRAGUE

Reversed Phase Chromatography

Separation of ionic compounds - acids

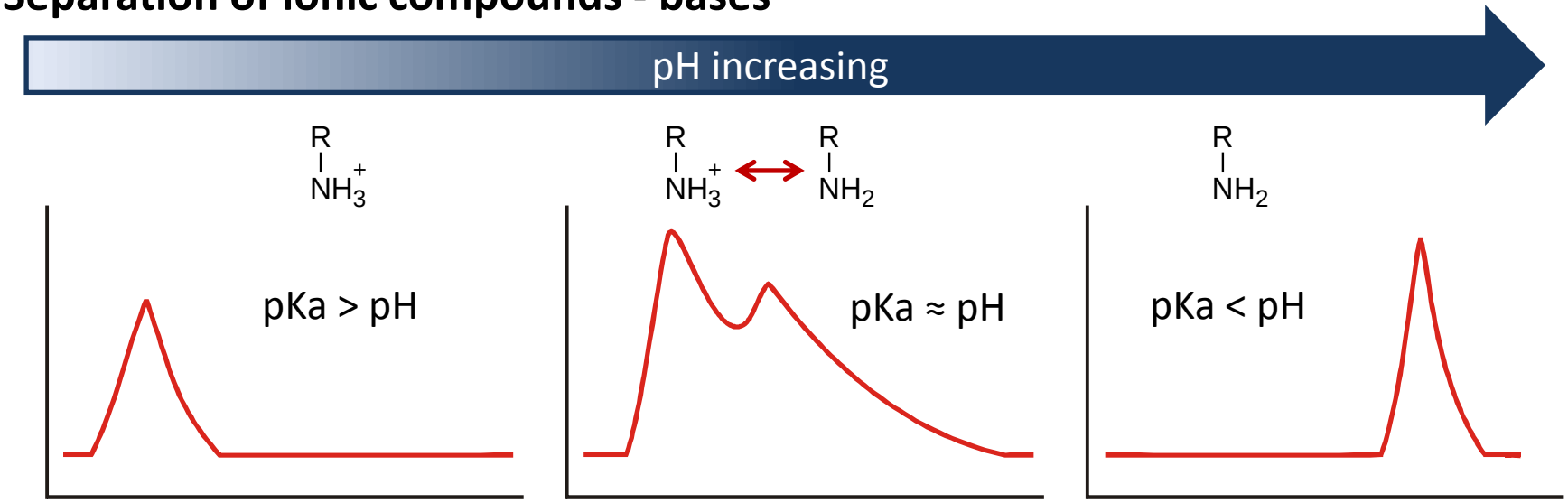


- Dissociated (polar) analyte provides poor retention and peak shape.
- At pH similar to analyte pKa both, disociated and non-disociated forms are present. The peak is splitted and wide.
  WORST CASE!
- Non-disociated analyte provide better retention and good peak shape.

Sensitivity in ESI- conditions (polarity in which most acids provide ions) could be lowered, when low pH mobile phase is used.

Reversed Phase Chromatography

Separation of ionic compounds - bases



- Highly polar (dissociated) analyte provides poor retention and peak shape.
- At pH similar to analyte pKa both, disociated and non-disociated forms are present, also ion interaction causes peak tailing. The peak is splitted and wide.

WORST CASE!

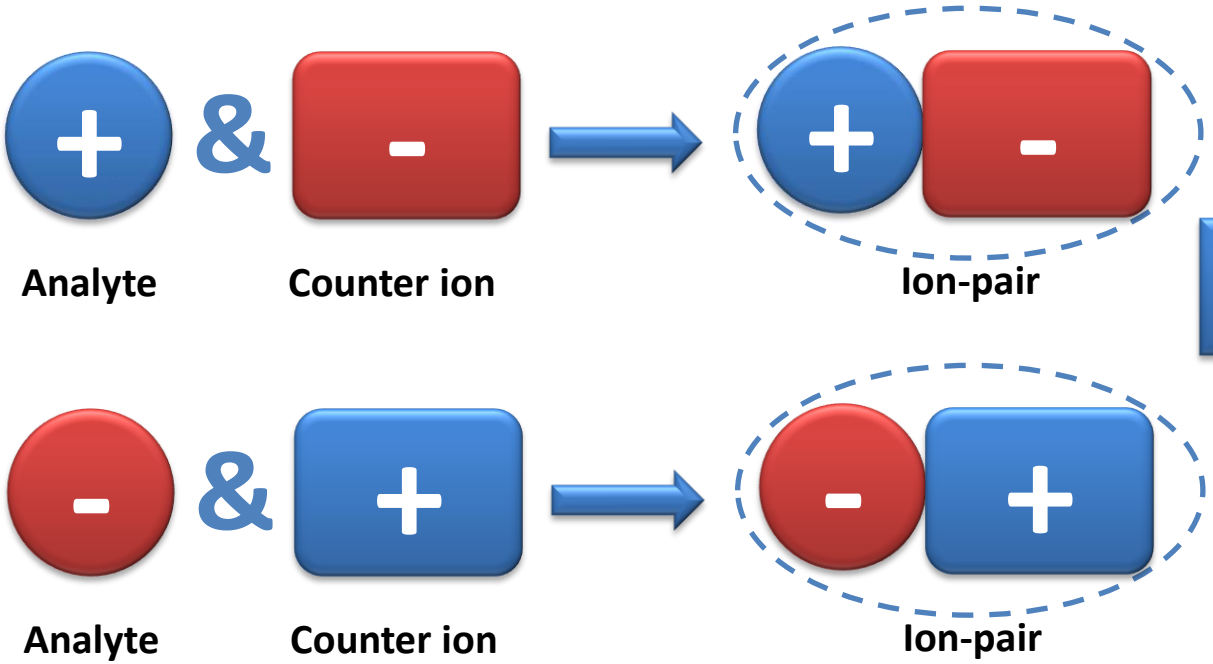
- Non-disociated analyte provide better retention weak ion interaction still plays role (peak slightly tails).

Sensitivity in ESI+ conditions (polarity in which most bases provide ions) could be lowered when high pH mobile phase is used!

Reversed Phase Chromatography

Separation of ionic compounds – Ion-Pair Chromatography

- Method of choice, when neutral and ionic compounds have to be analysed togehter.
- Reversed-phase chromatography with counter ion in mobile phase (neutral compounds are not influenced).



Ion-pairs are separated as neutral molecules.

Reversed Phase Chromatography

Separation of ionic compounds – Ion-Pair Chromatography

- Common ion-pair agents:

Counter ion	Suitable for
Quarternary amines (tetramethylammonium, tetrabutylammonium, palmityltrimethylammonium)	Strong and weak acids, suphonated dyes, carboxylic acids
Tertiary amines (trioctylamine)	Sulphonates
Alkyl- and arylsulphonates (methanesulphonate, heptanesuphonate)	Strong and weak bases, benzalkonium salts, catecholamines.
Perchloric acids	Strong ion pairs with basic compounds
Perfluoric acids	Strong ion pairs with basic compounds

Ion-Pair chromatography is not suitable for LC-MS applications, since stable ion-pairs do not provide ions and sensitivity is significantly compromised.

HILIC

- **HILIC – Hydrophilic Interaction Chromatography**
  - Term coined in 1990 to distinguish from normal-phase

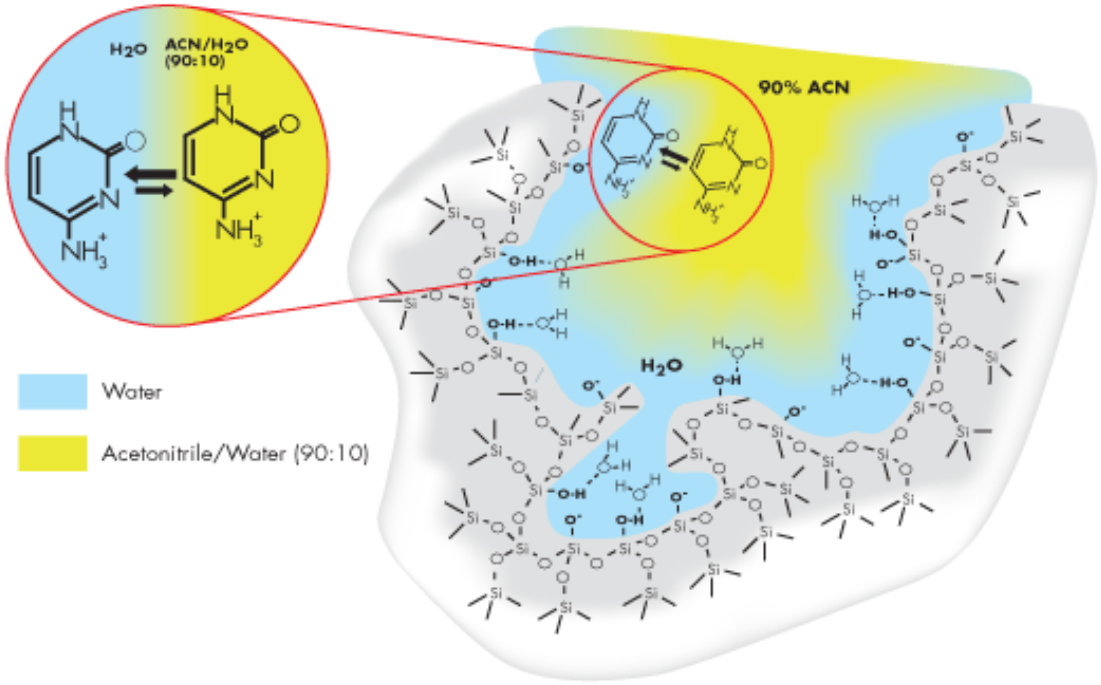
- HILIC is a variation of normal-phase chromatography without solvents that are not miscible in water. Also called „Reversed reversed-phase“ or „Aqueous normal-phase“.

- Stationary phase is POLAR: Silica, cyano, amino, diol
- The mobile phase is highly organic (> 80%) with a smaller amoutn of aqueous mobile phase.
  - Water (or the polar solvent) is the strong, eluting solvent.

HILIC

Principle of retention

- Polar analyte partitions into and out of adsorbed water layer.
- Charged polar analyte can undergo cation exchange with charged silanol groups.



Benefits of HILIC

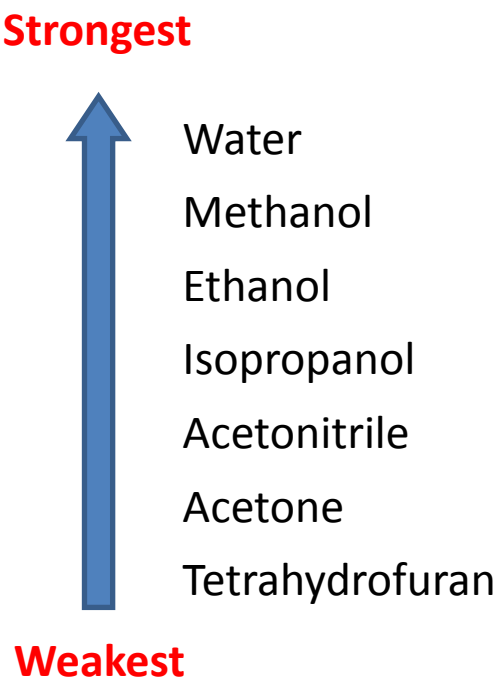
- Retention of highly polar analytes not retained by reversed-phase
- Complementary selectivity to reversed phase
- Enhanced sensitivity in mass spectrometry
  - High organic mobile phase promotes enhanced ESI MS response
- Shorter sample preparation, elimination of evaporation/reconstitution step by directly injecting the organic phase.

HILIC

Mobile phases

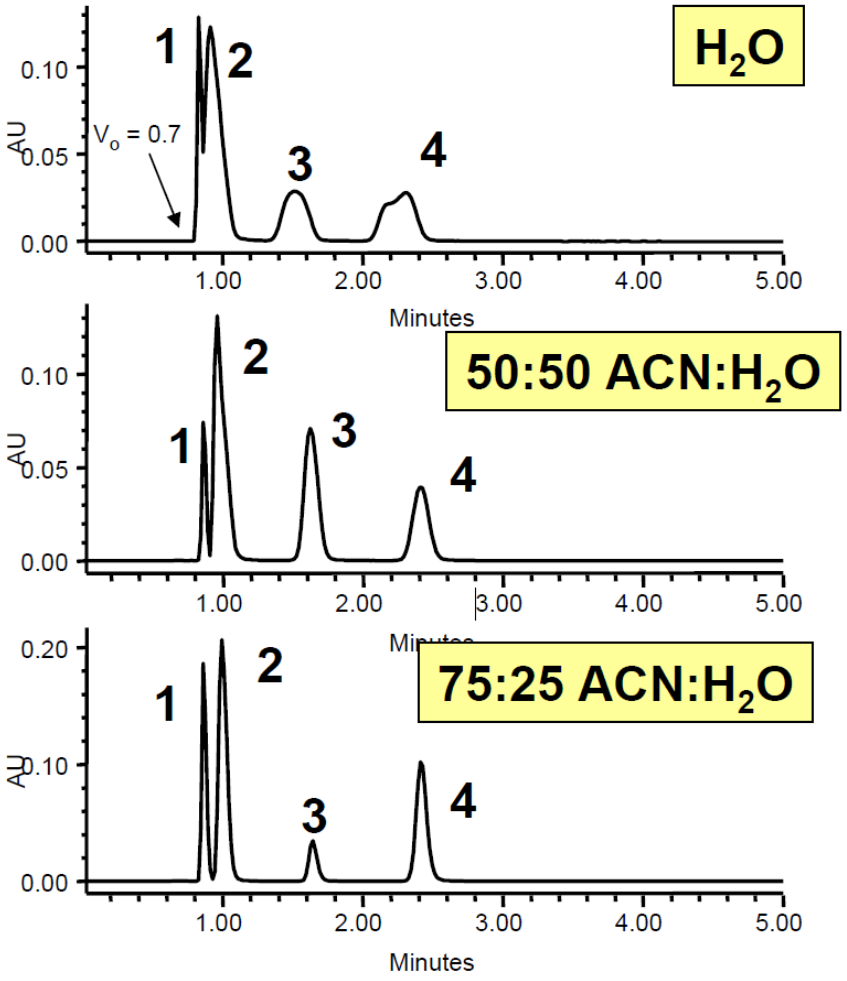
- Phosphate buffers are not recommended due to recipitation in high organic mobile phase.
- Ammoniom formate (pH 3); ammonium acetate (pH 5); 0.2% formic acid (pH 2.5), 0.2% phosphoric acid (pH 1.8).
- For optimum performance and reproducibility it is recommended concentration of 10 mM buffer or 0.2% of an additive ON COLUMN.
- To increase analyte retention, replace some of the water with another polar solvent (methanol, isopropanol).

Solvent strenght



HILIC

Influence of sample dilluent on peak shape

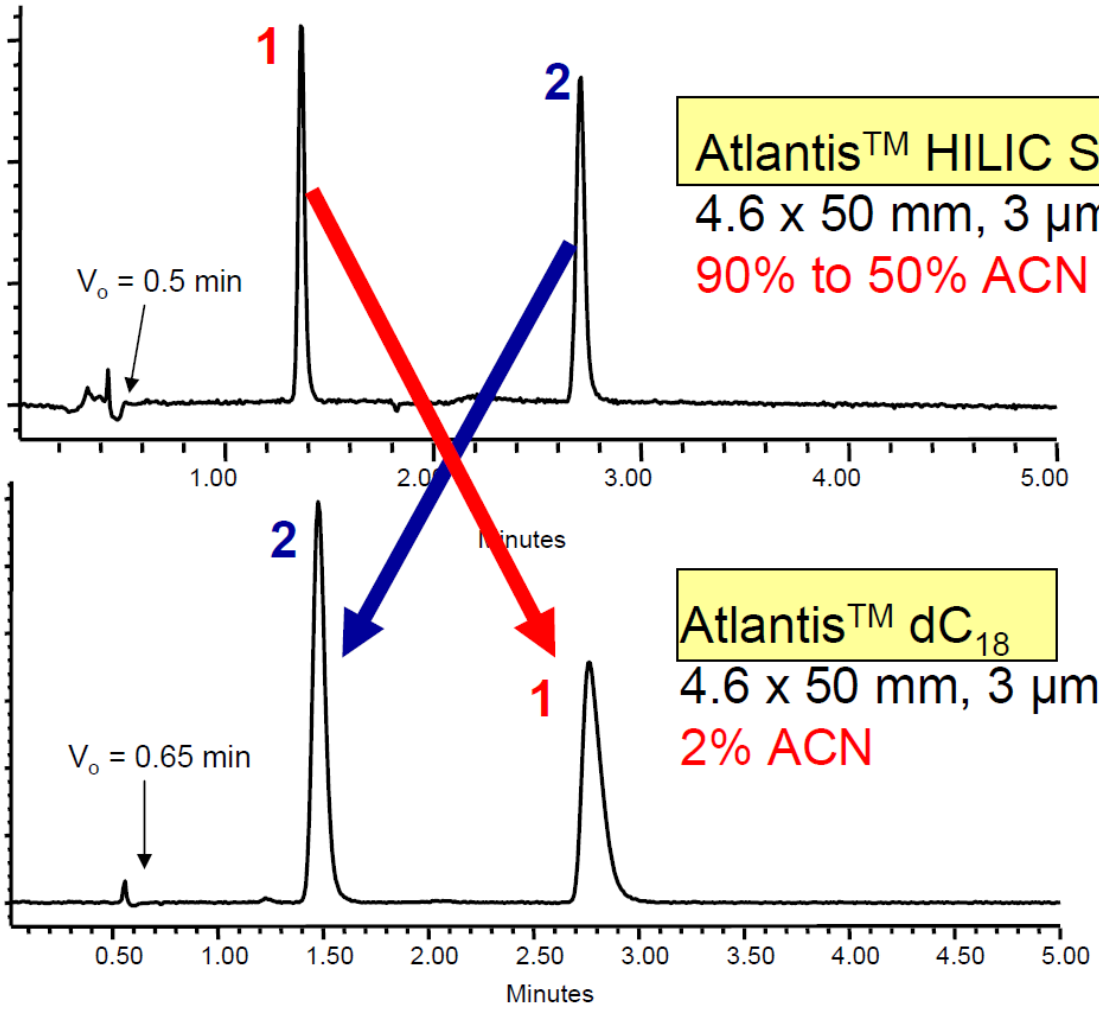


1. 5-Fluorouracil
2. Uracil
3. 5-Fluorocytosine
4. Cytosine

Peak shape improves as % ACN in the diluent increases, but solubility can suffer. Replacing of the aqueous portion of the diluent with a polar solvent can solve this problem.

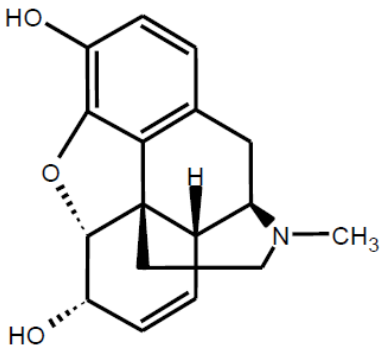
HILIC

Complementary selectivity to Reversed-Phase

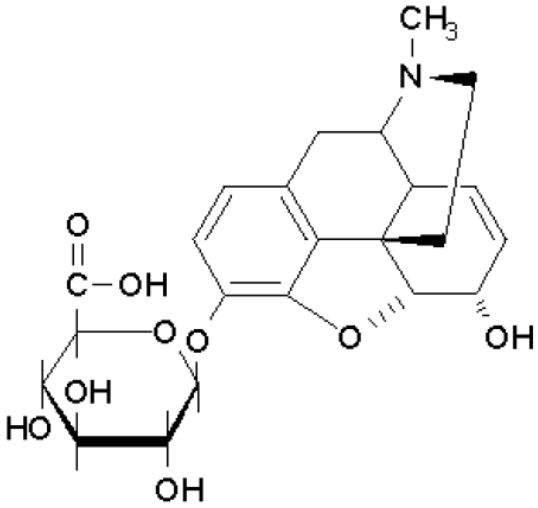


Atlantis™ HILIC Silica
4.6 x 50 mm, 3 µm
90% to 50% ACN

Atlantis™ dC_{18}
4.6 x 50 mm, 3 µm
2% ACN



1. Morphine



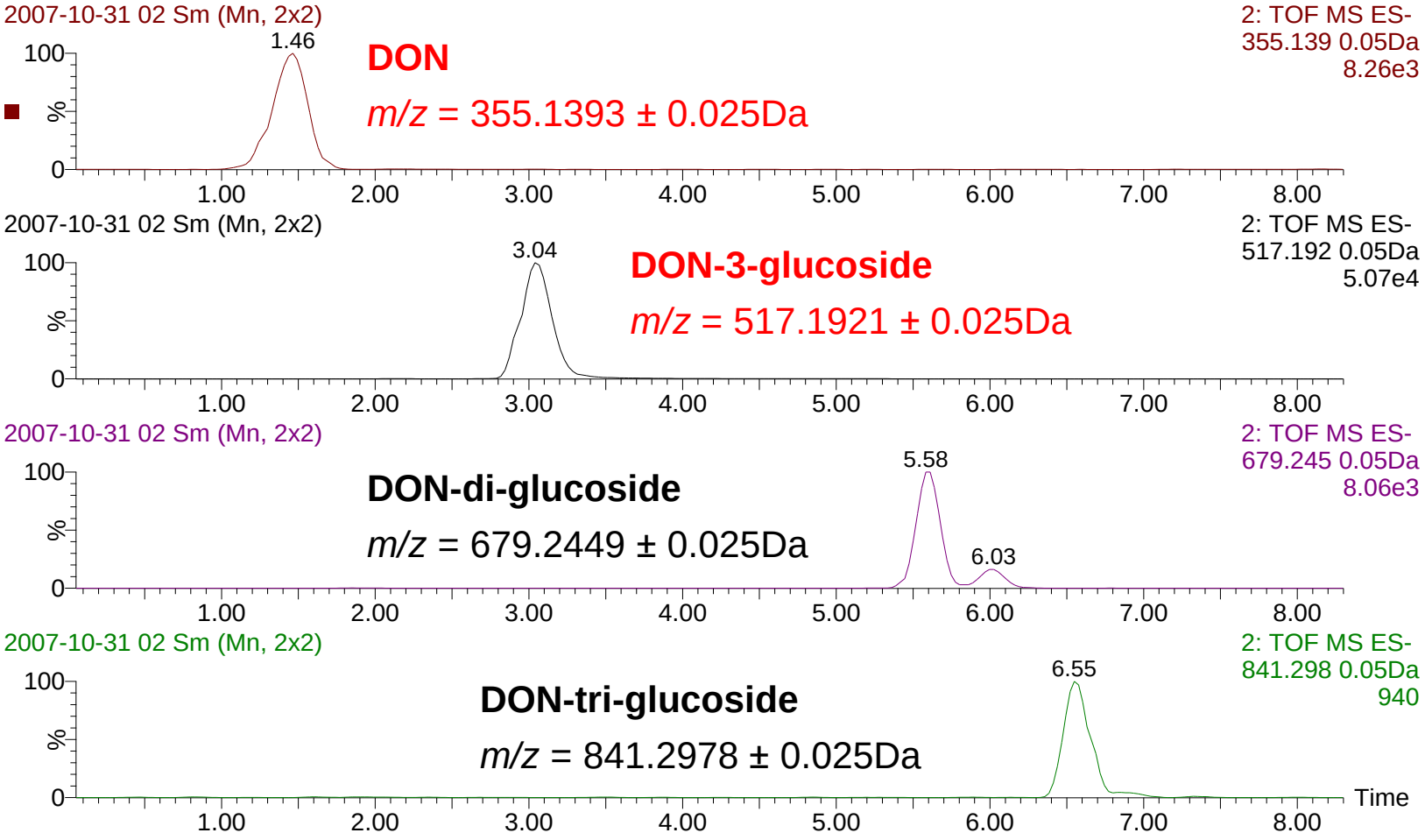
2. Morphine 3-ß-D-Glucuronide

ICT PRAGUE

HILIC

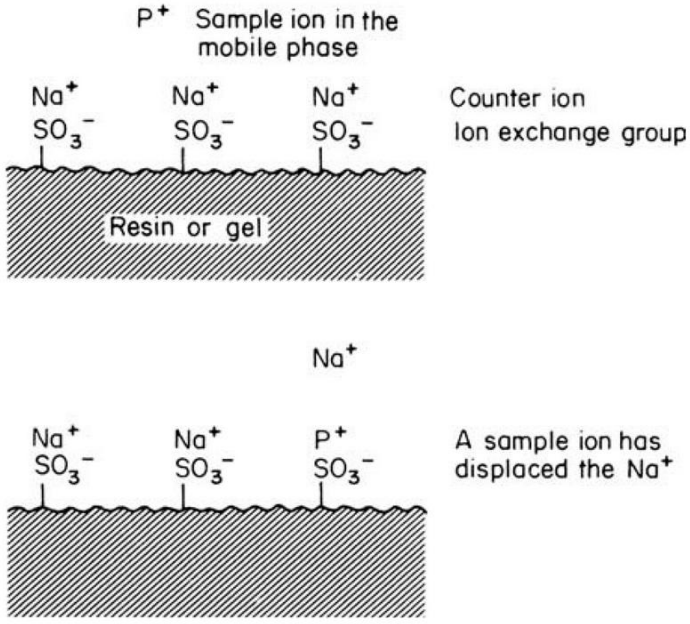
Example of aplication: Separation of DON and its conjugates

(apHera NH2 Polymer 150×2mm; 5µm)



Ion-Exchange Chromatography

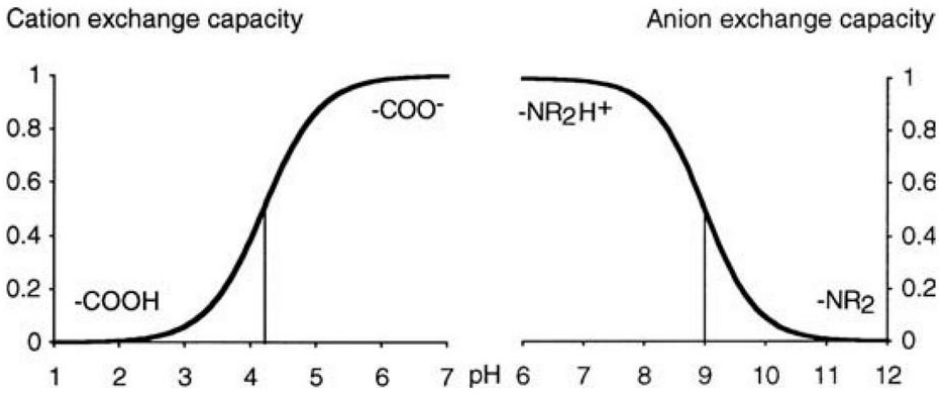
- Principle: Similar to adsorption chromatography, the sample and the solvent molecules compete with each other for active sites.
- The main principle is ionic interaction.
- Stationary phase capable to retain ions: SO_3^{2-}, COO^-,NH_3^+ or NR_3^+
  - Negatively charged sorbents interact with cations (catex).
  - Possitively charged exchanger forms bond with anionts (anex).
- Mobile phase usually water with couner-ion. The elution is based on increasing of ion strenth (concentration of the conter ion).



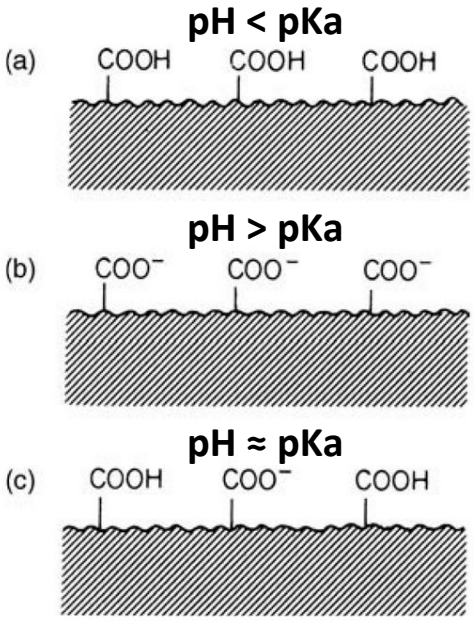
Ion-Exchange Chromatography

Stationary phase:

- Usually based on polymers (silica is not suitable, since very wide range of pH is used).
- Strong exchangers (anion and cation) are charged over the full range pH (not affected by pH of mobile phase.
- Capacity of weak exchangers is pH dependent. Only dissociated active sites can interact with analyte.

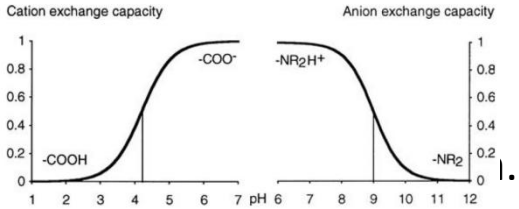


Profile of capacity for cation exchange and anion exchange.



a) Undissociated cation exhcanger
b) Dissociated cation exchanger
c) Partly dissociated cation exchanger

Ion-Exchange Chromatography

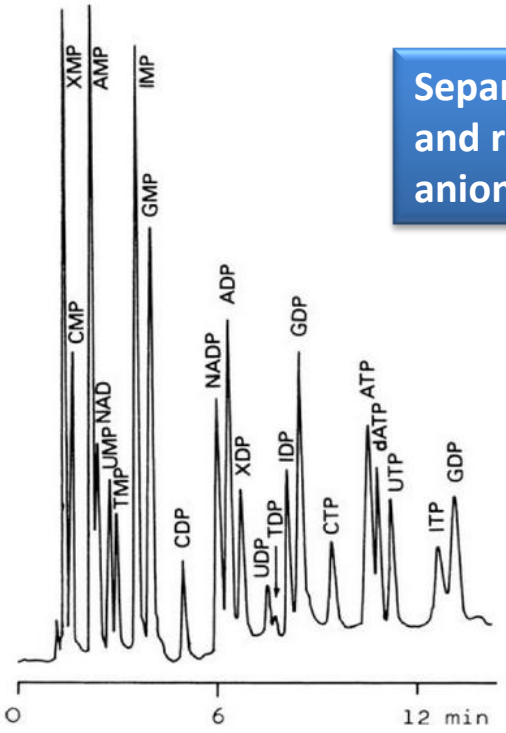


Mobile phase:

- Mobile phase usually consist from water (up to 50% o l.
- Retention is decreased with higher mobile phase ion strength (concentration of the conter ion).
  - An increase in pH reduces retention time in cation exchange.
  - Conversly, an decrease in pH reduces retention time in anion exchange.

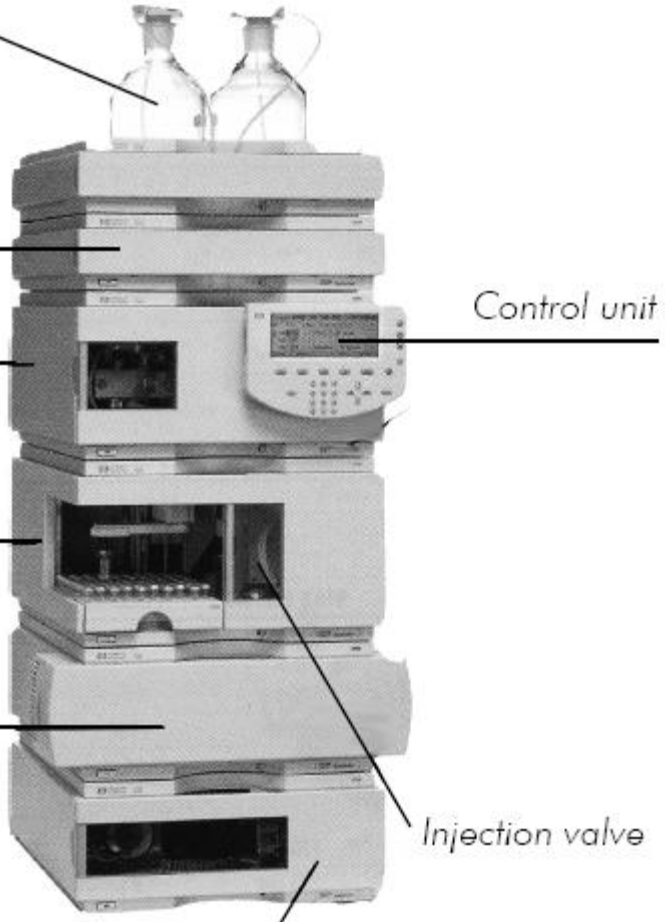
Applications

- *Weak exchangers are used for separation of the strong ions, strong exchangers for separation of weak ions*
- Analysis of very polar pesticides (glyphosate, ethephone, quarternary amines)
- Separation of biomolecules



Separation of 22 nucleotides and reated compounds by anion exchange.

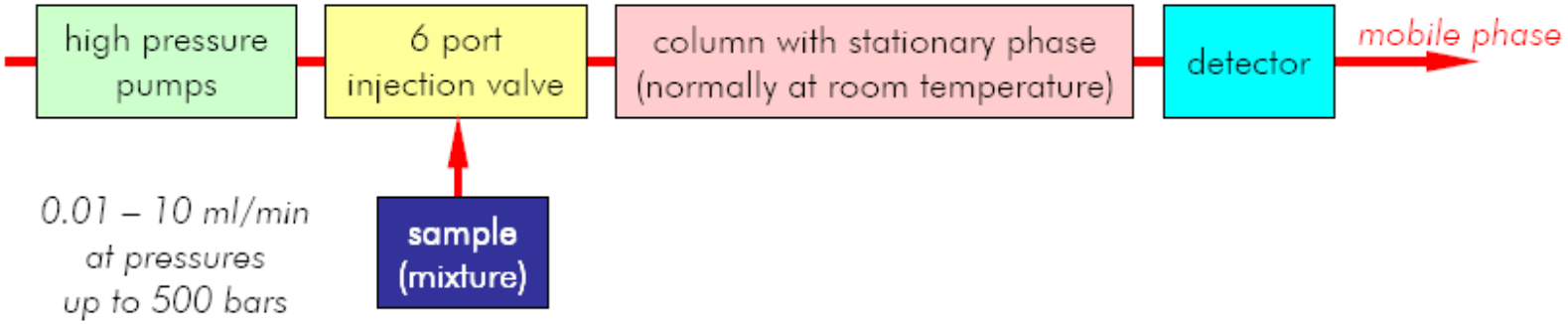
HPLC system



Operation modes:

- isocratic: constant eluent composition
- gradient: continuous variation of eluent mixture during analysis

HPLC System



Samples:
- liquid samples
- limitations: solubility in the mobile phase, no thermal restrictions
- sample preparation: filtration, extraction

Duration of an analysis:
- 5 ... 60 min

Application:
- purity control, quality management and certification (wide application)
- environmental and pharmaceutical analysis
- analysis of main and trace components (% to ppm)

	Mobile Phase	*Stationary Phase*
Normal Phase HPLC	non-polar (e.g. hydrocarbons)	polar
Reversed Phase HPLC	polar (H_2O, buffer solutions, alcohols, acetonitrile and mixtures of them)	non-polar

HPLC System

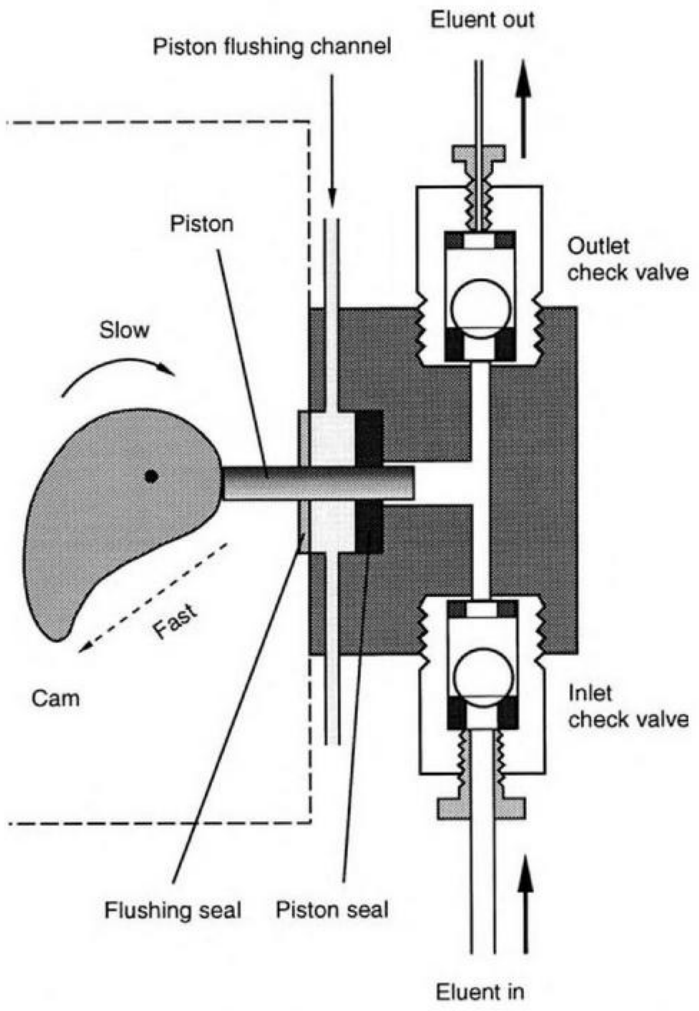
Pumps

Requirements:

- Stable flow rate in the range 0.05 – 2 (5) ml/min.
- Generating high pressure up to 1000 bar.
- Pressure ripple less than 0.5 %.

The Short-Stroke Piston piump

- The Cam guides the piston to its forward and backward direction.
- Irregular shape of the Cam causes fast filling of the piston and the slow delivery period.

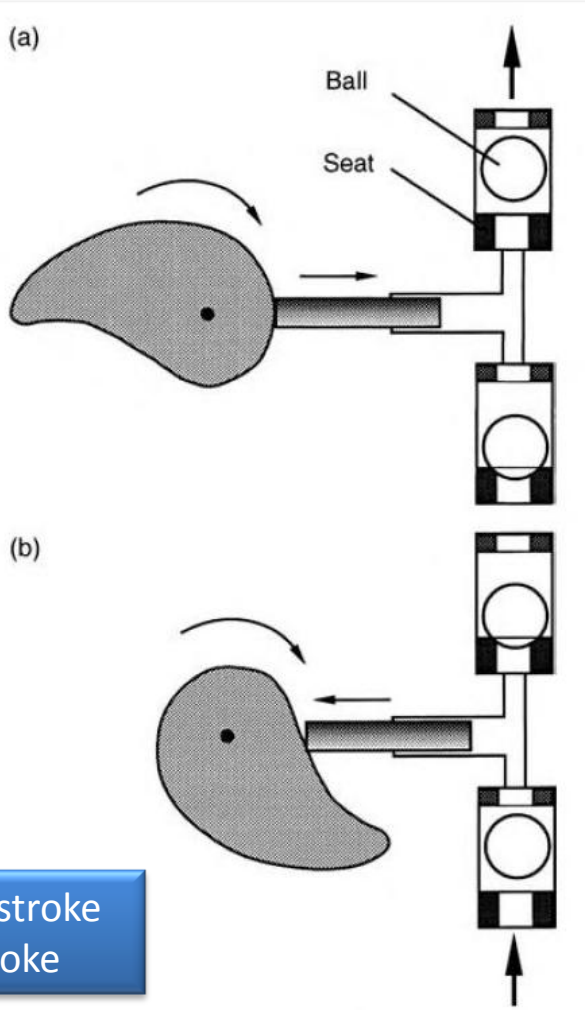


HPLC System

Pumps

Tandem piston pump:

- Current design of HPLC and UHPLC pumps.
- Combination of two strokes, the first fills the second stroke
- The flowrate is smooth as much as possible.
- Compensation of mobile phase compresibility.
- Suppress cavitation (formation of bubbles during piston filling).



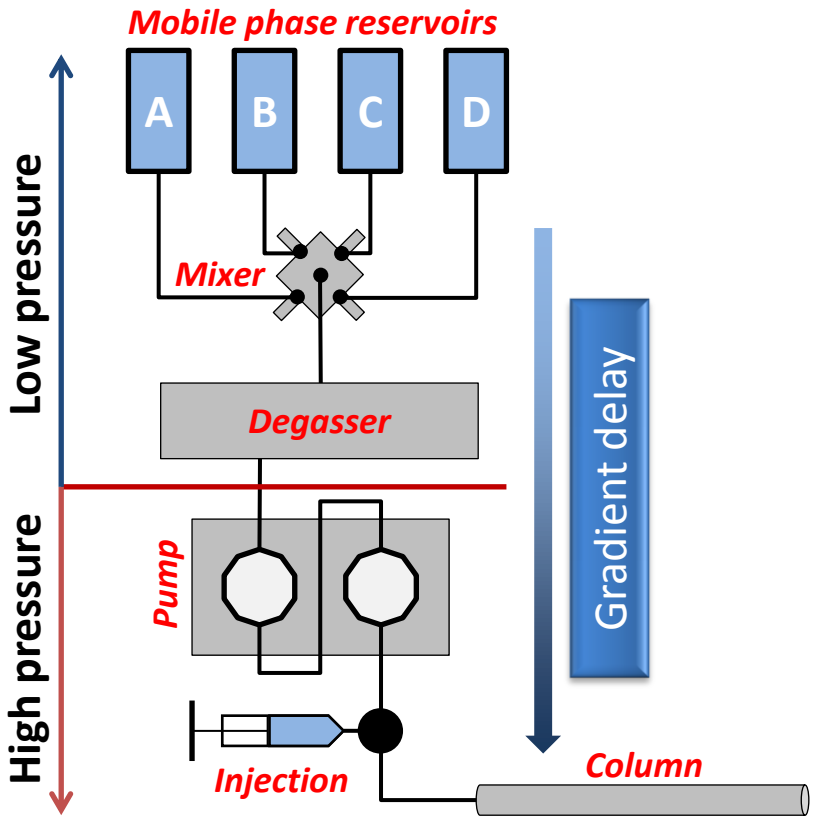
a) Delivery stroke
b) Filling stroke

HPLC System

Gradient systems

Quarternary pump (*quarternary gradient, low pressure gradient*)

- Mixing of up to 4 different solvents
- Cheaper instrumentation (one degasser, one pump).
- Higher gradient delay volume (compared with the binary pump)
- Gradient is mixed in the mixer and it is based on opening of the valves.

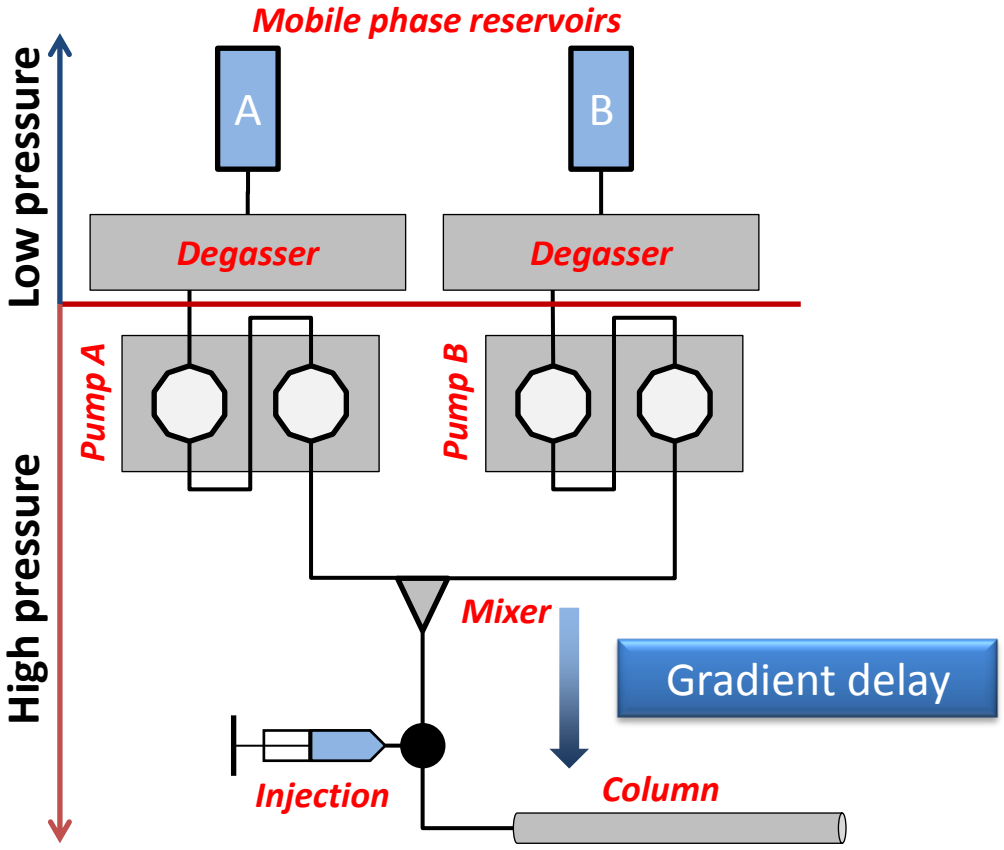


HPLC System

Gradient systems

Binary pump (binary *gradient, high pressure gradient*)

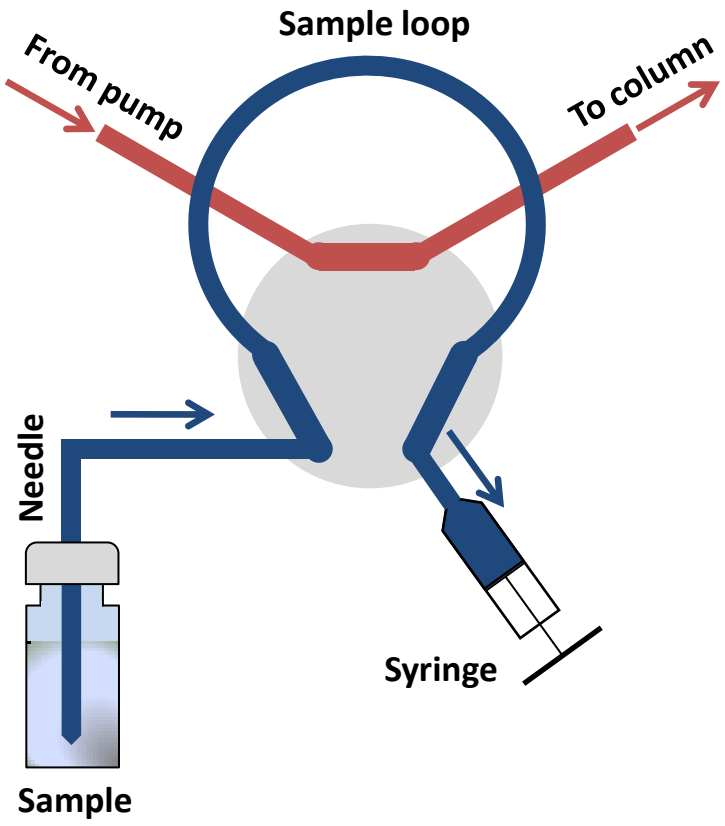
- Mixing only of two solvents
- Expensive, sofisticated instrumentation (two degassers, two pumps, control unit for pump synchronization).
- Very small gradient delay.
- Superb gradient reproducibility.
- Gradient is achieved by controll of flow rate of the pump.



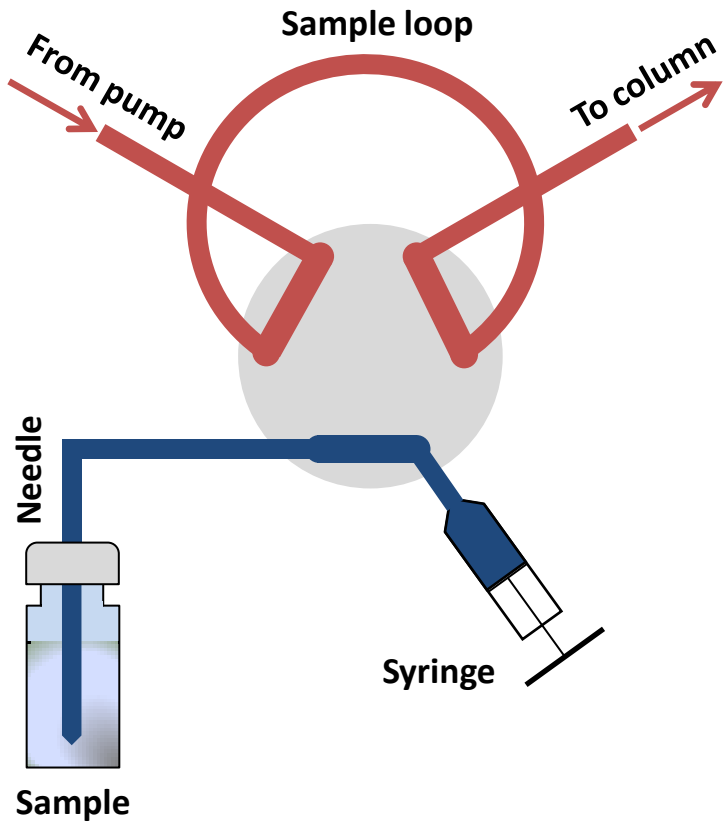
HPLC System

Injection – six port valve

Sample load



Sample inject



HPLC System

Column

Column is the most important for chromatographic separation:

Stationary phase selection depends on sample/analyte properties (solubility, polarity, etc.)

Factors for the selection of appropriate column and sorbent:

- Separation efficiency, peak symetry
- Column lifetime, stability of the bonded phase
- Reproducibility
- Time of analysis
- Selectivity

Column lenght:

- Resolution
- Separtion efficiency
- Back pressure
- Mobile phase consumption
- Analysis time

→ **Increase with lenght**

Column diameter:

- Back pressure
- Mass sensitivity
- Separtion efficiency

→ **Decrease with diameter**

- Column capacity
- Mobile phase consumption

→ **Increase with diameter**

HPLC System

Columns and Stationary Phases

History of the HPLC particles development

Year	Particle size		Number of plates (15 cm)
1950s		100 µm	200
1967		50 µm (pellicular)	1000
1972		10 µm	6000
1985		5 µm	12000
1992		3–3.5 µm	22000
1996*		1.5 µm (pellicular)	30000
1999		5.0 µm (poroshell)	8000**
2000		2.5 µm	25000
2003		1.8 µm	32500

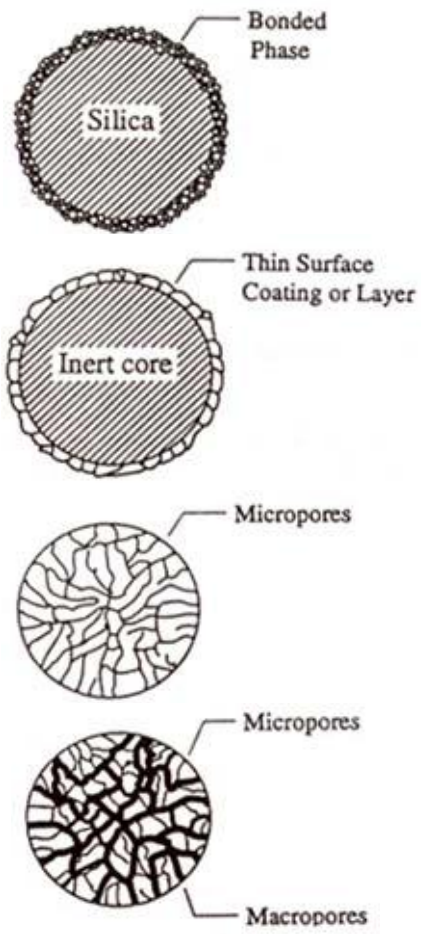
Particle parameters:

- Size (diameter),
- Shape (regular, irregular)
- Type (porous, non-porous)
  - Pore diameter
- Active surface area

HPLC System

Columns and Stationary Phases

Types of particles



Bonded Phase Silica

- Silica bead containing silanol (Si-OH) are bonded with hydrocarbon groups.
- The nature of the bonded phase determines the chromatographic behavior.

Pellicular Packing

- An inert core provides physical support
- A thin layer of coating on the core provides functional groups for the separation of analytes

Microporous

- Gel-type resin consisting of cross-linked polymers

Macroporous

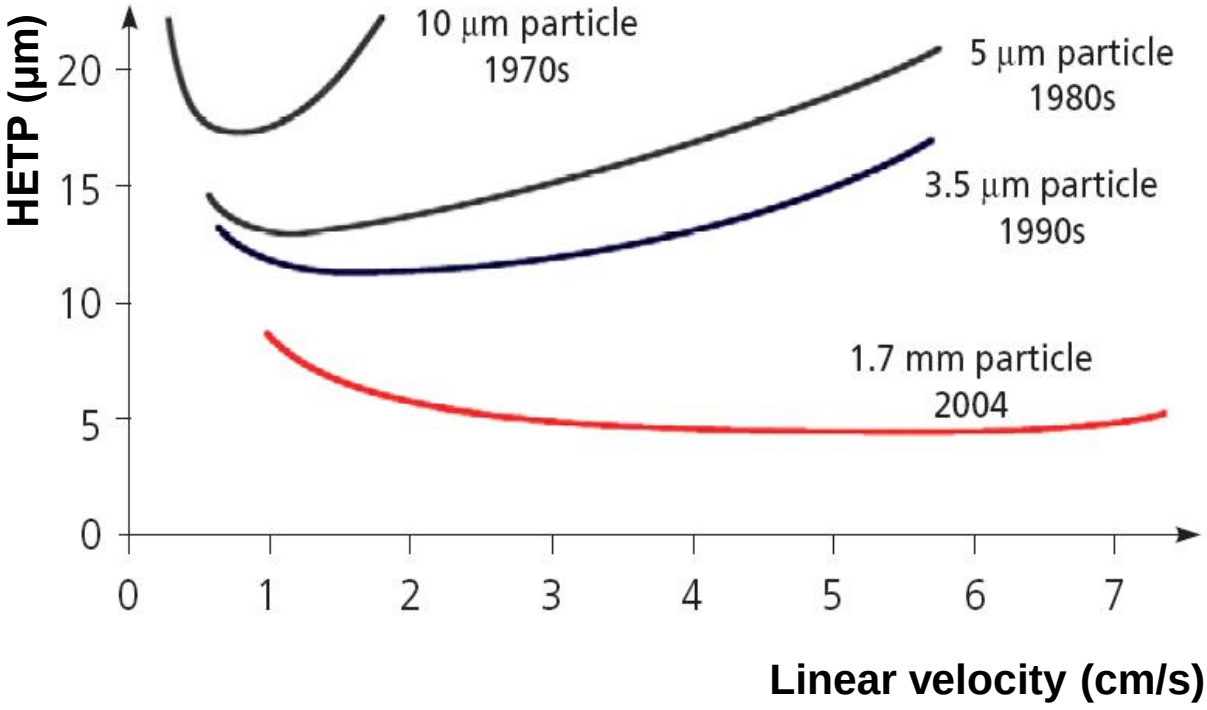
- Highly cross-linked (>50%) resin
- Stable from pH 1 to 14
- Available in a variety of particle and pore sizes

HPLC System

Columns and Stationary Phases

Particle size and separation efficiency

- Smaller particle diameter allows to achieve more separation plates
- Back pressure significantly increase when small particles are used.



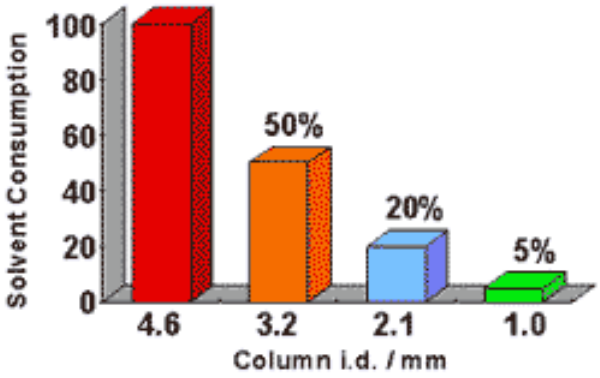
- Flow rate significantly influeneces the column efficiency and analysis time.
- Columns with small particles provides optimal separation in the wide range of flowrate, which allows to use higher flowrates.

HPLC System

Columns and Stationary Phases

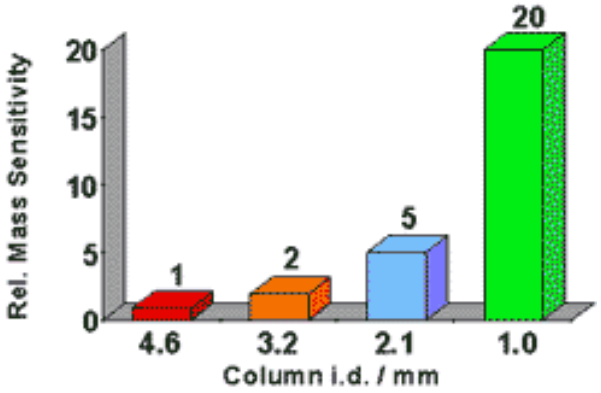
Solvent consumption:

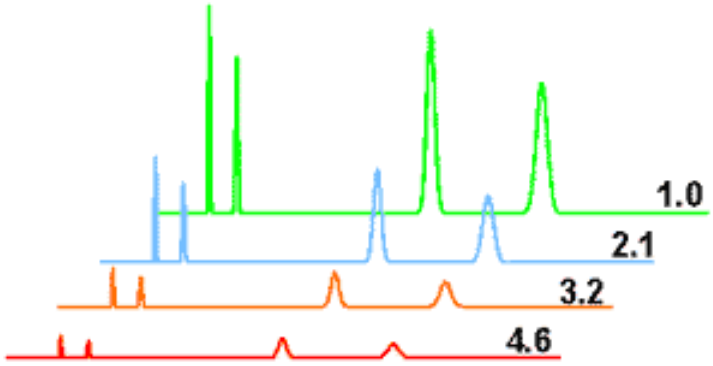
- Use of narrow column lead to the significant reduction of mobile phase consumption.



Mass sensitivity

- Narrow column provides higher sensitivity (with the same injection volume).





Injection of 1 µl of hydrocarbon mixture on columns with different diameter.

HPLC System

Detection

The ideal detector should:

- either be equally sensitive to all eluted peaks (*universal*) or record only those of the interest (*selective*).
- not be affected by changes in temperature or in mobile phase composition (*gradient elution*).
- be able to monitor small amounts of compound (trace analysis).
- not contribute to band broadening, hence the cell volume should be small.
- react quickly to pick-up correctly narrow peaks which pass rapidly through the cell.

HPLC System

Detection

Concentration or Mass sensitive:

- **Concentration-sensitive** detectors produce a signal, which is proportional to the concentration of the sample in the eluate.

$$S \propto c \; [g \cdot ml^{-1}]$$

- **Mass-sensitive** detectors produce signal which is proportional to tha mass flux (number of molecules or ions per unit time).

$$S \propto \frac{n}{\Delta t} \; [g \cdot s^{-1}]$$

Selectivity

- **Non-selective** detectors react to the bulk property of the solution passing through (Refractive index detector, RID, conductivity detector).
- **Selective detectors** utilizing specific properties of the compound (UV/VIS absorbance, fluorescence, redox potential, mass-to-charge ratio).

HPLC System

Detection

HPLC detectors:

	Detector	Selectivity	Sensitivity	Merits
Optical detection	UV/UV-VIS detector	2	3	A wide variety of substances can be detected that absorb light from 190 to 900 nm. Sensitivity depends strongly on the component.
	Diode array detector (DAD, PDA)	2	3	A wide variety of substances can be detected that absorb light from190 to 900 nm. Sensitivity depends strongly on the component. The spectrum can be confirmed for each component.
	Fluorescence (FL) detector	3	4	Components emitting fluorescence can be detected selectively with high sensitivity. This is often used for pre-column and post-column derivatization.
	Differential refractive index (RI) detector	1	1	Any component that differs in refractive index from the eluate can be detected, despite its low sensitivity. Cannot be used to perform gradient analysis.
	Evaporative light scattering detector (ELSD)	1	2	This detector atomizes the column eluate, and detects the scattered light of the resulting particulate components. Non-UV-absorbing components are detected with high sensitivity.
Electrical detection	Conductivity detector (CD)	2	3	Ionized components are detected. This detector is used mainly for ion chromatography.
	Electrochemical detector (ECD)	3	4	Electric currents are detected that are generated by electric oxidation-reduction reactions. Electrically active components are detected with high sensitivity.
	Corona® Charged Aerosol Detector® (Corona® CAD®)	1	3	This detector atomizes the column eluate and electrically detects the resulting particulate components treated with corona discharge. UV-nonabsorbing components can be detected with sensitivity higher than that of ELSD.

HPLC System

Detection

HPLC detectors:

Name	*Selectivity*	*Typical minimum detectable level [g/ml]*	*Linear dynamic range*
UV-VIS detector/ Diode array detector[1]	- for larger organic molecules and transition metal compound which absorb UV-VIS light - time resolved recording of UV-VIS spectra, possibility of deconvaluation of non-separated peaks	$5 \cdot 10^{-10}$	$5 \cdot 10^{4}$
Fluorescence detector[1]	- detects fluorescence radiation emitted by the sample compounds - specific for highly condensed organic molecules like PAH	10^{-10} ... 10^{-9}	$\sim 10^{3}$
Refraction index detector	- non-specific low-cost detector	$5 \cdot 10^{-10}$	10^{4}
Electric conductivity detector	- specific low-cost detector for compounds dissociated into ions (e.g. inorganic and organic salts, tensides, amino acids)	10^{-8}	10^{3}
Mass selective detector[1]	- most selective detector for HPLC - strong requirements for the interface (transition from the high column pressure to vacuum inside the MSD) - high costs	no data available	10^{5}

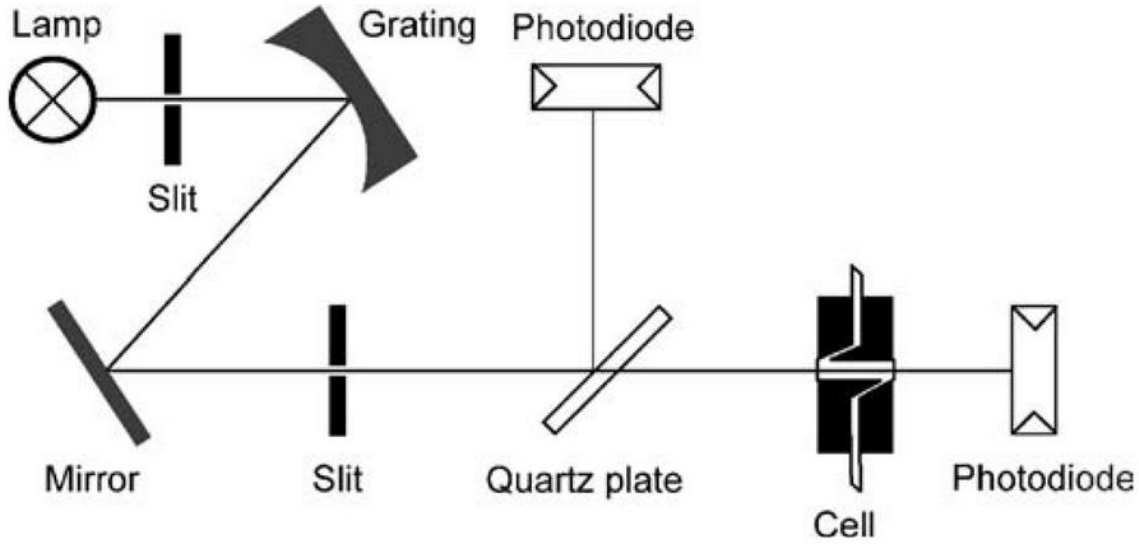
1 – suitable for gradient techniques

HPLC System

Detection – UV/VIS

- The most common detection technique HPLC detector.
- Detection of bromine, iodine, sulphur, carbonyl group, nitro group, conjugated double bonds, aromatic ring…etc.

- UV/VIS detector:

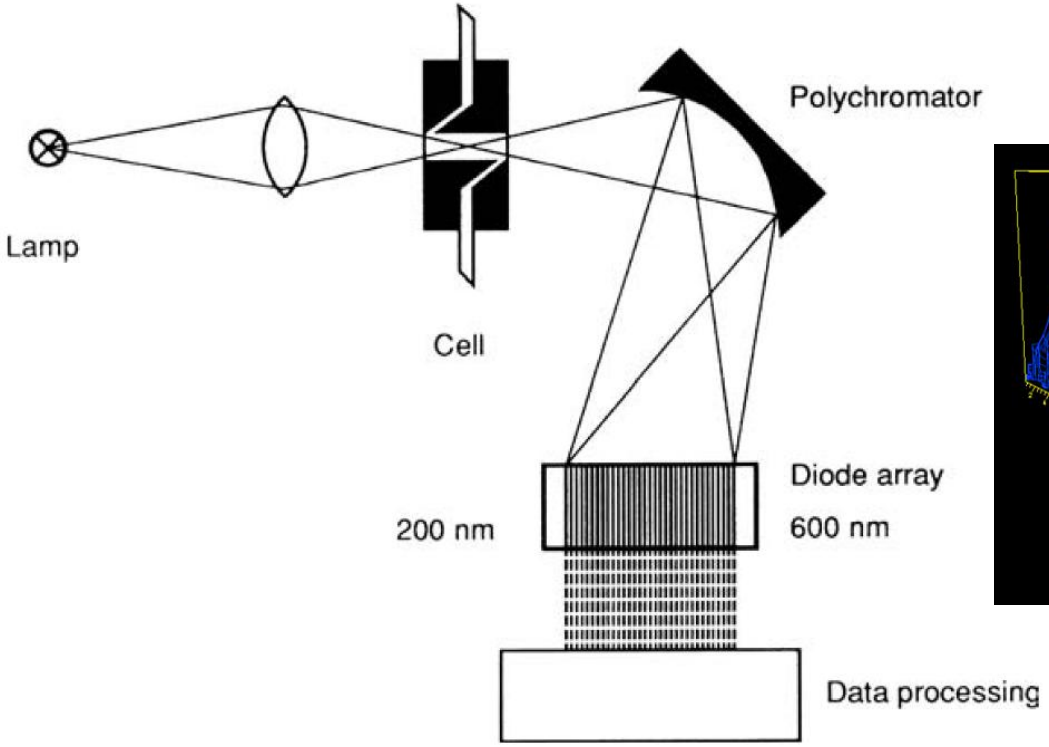


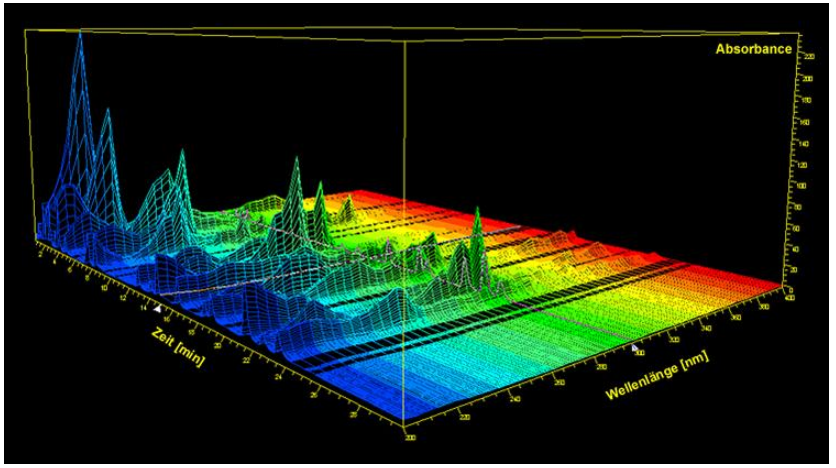
HPLC System

Detection – Diod array detector (DAD)

- Improved version of UV/VIS, full absorption spectrum.
- Detection of the same analytes.

Diod array detector:



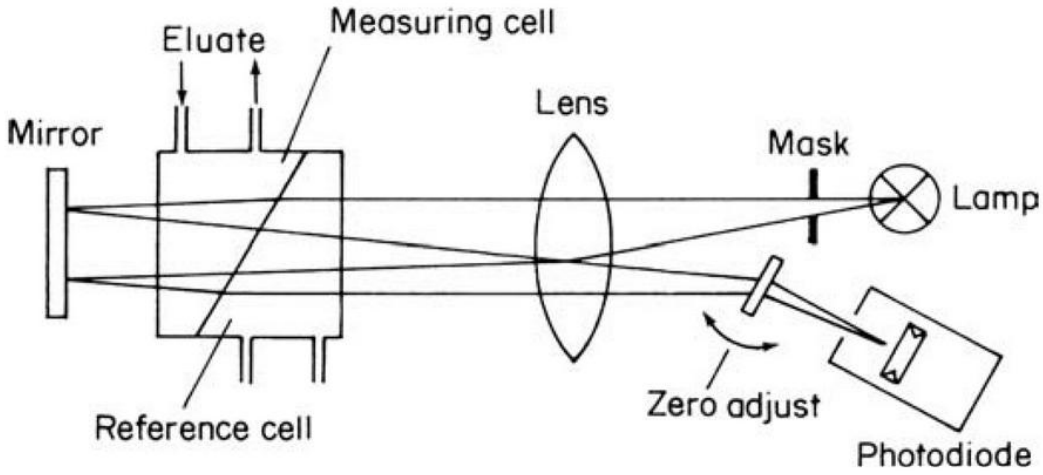


HPLC System

Detection – Refractive index detectors

- Approx 1000× less sensitive than UV detectors.
- Incompatible with gradient elution, sensitive on temperature.
- Detection based on the refraction of light in solution.
- Two types of construction – *Deflection refractometer* and *Interferometer.*

Deflection refractometer:



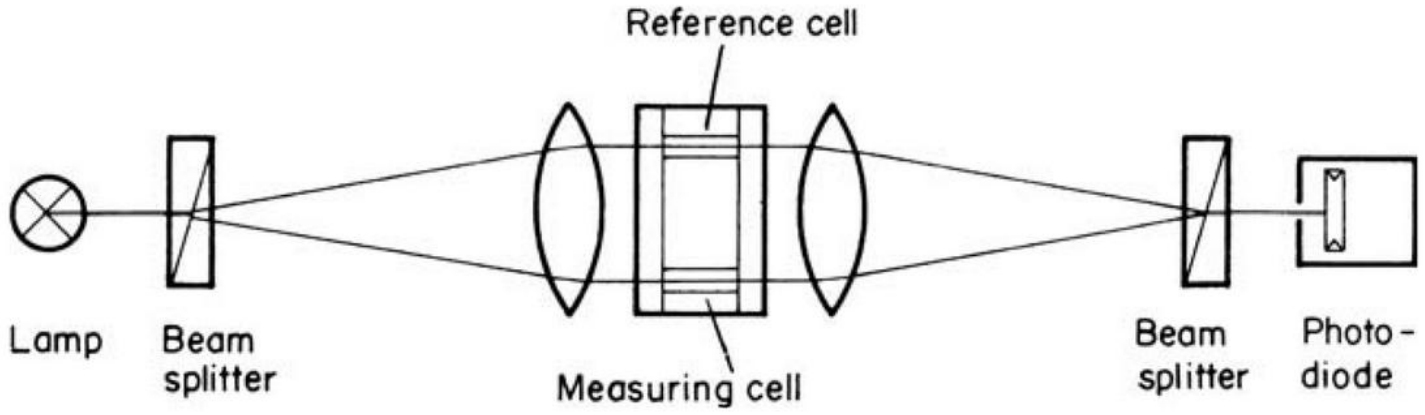
- When pure mobile phase is in measuring cell, the zero-glass is adjusted to direct the light on to the photodiode.
- Change of refrafctive index in measuring cell deflect the light beam and it decreases resistivity of the photodiode

HPLC System

Detection – Refractive index detectors

Interferometer:

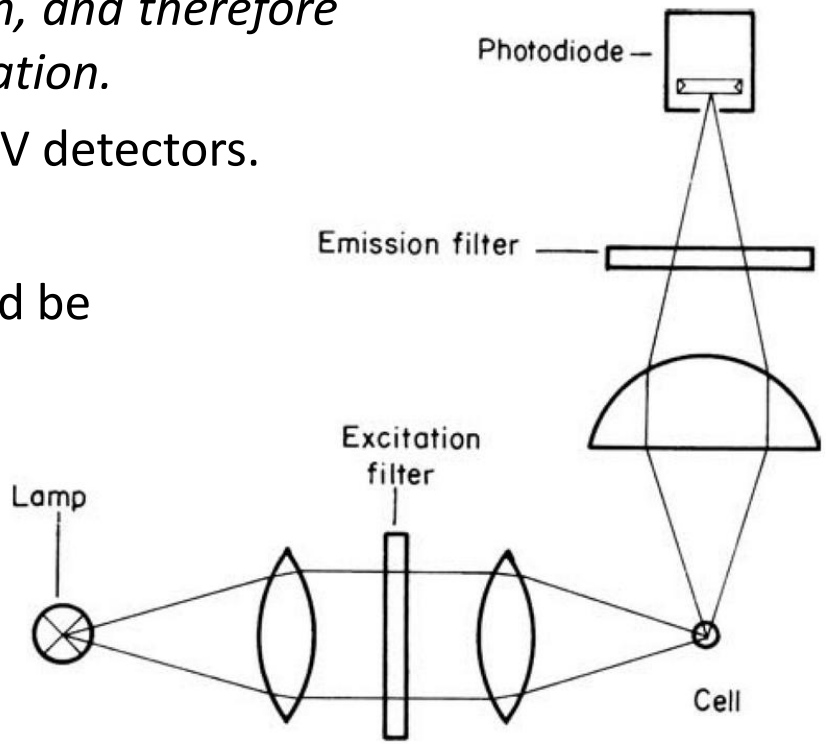
- 10× more sensitive than the deflection refractometer.
- The light passes through beam splitter and is divided into two beams of equal intensity, one passes through reference cell and the second through measuring cell. Two beams are superimposed in a second beam splitter.
- If refractive index in measuring cell is changed, the beams are not moving in the same velocity and are partly extinguished in the second beam splitter.



HPLC System

Detection – Fluorescence detector

- *Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation of a different wavelength. In most cases, emitted light has a longer wavelength, and therefore lower energy, than the absorbed radiation.*
- Approx 1000× MORE sensitive than UV detectors.
- Very specific and selective.
- Compouns without fluorescence could be derivatized.

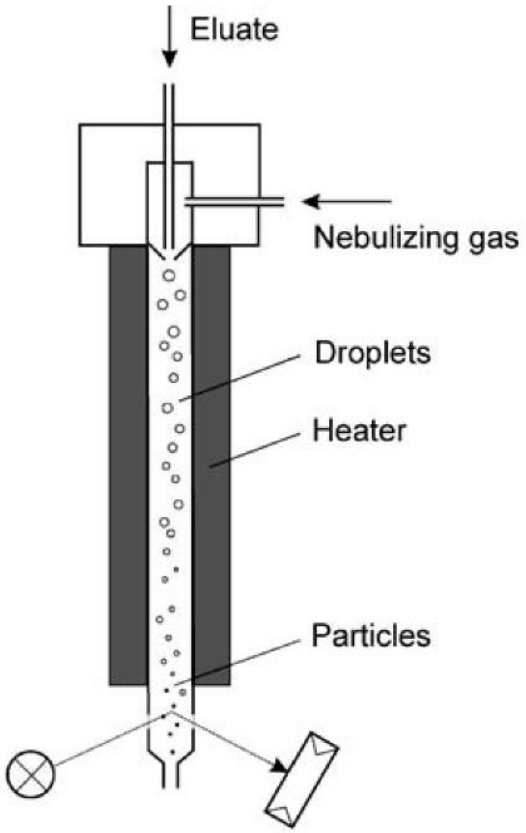


Light of the suitable wavelenght is passed through the cell and the longer wavelenght radiation emitted is detected in right-angled direction.

HPLC System

Detection – Evaporative Light Scattering Detector (ELSD)

- Non-selective detection of non-volatile analytes.
- The elueate is nebulized in a stream of gas (nitrogen), the droplets are then evaporated, thus producing solid particles which are passed through a laser beam. The scattered lighet is registering by photodiode.
- Mobile phase must be volatile (including buffers and additives).

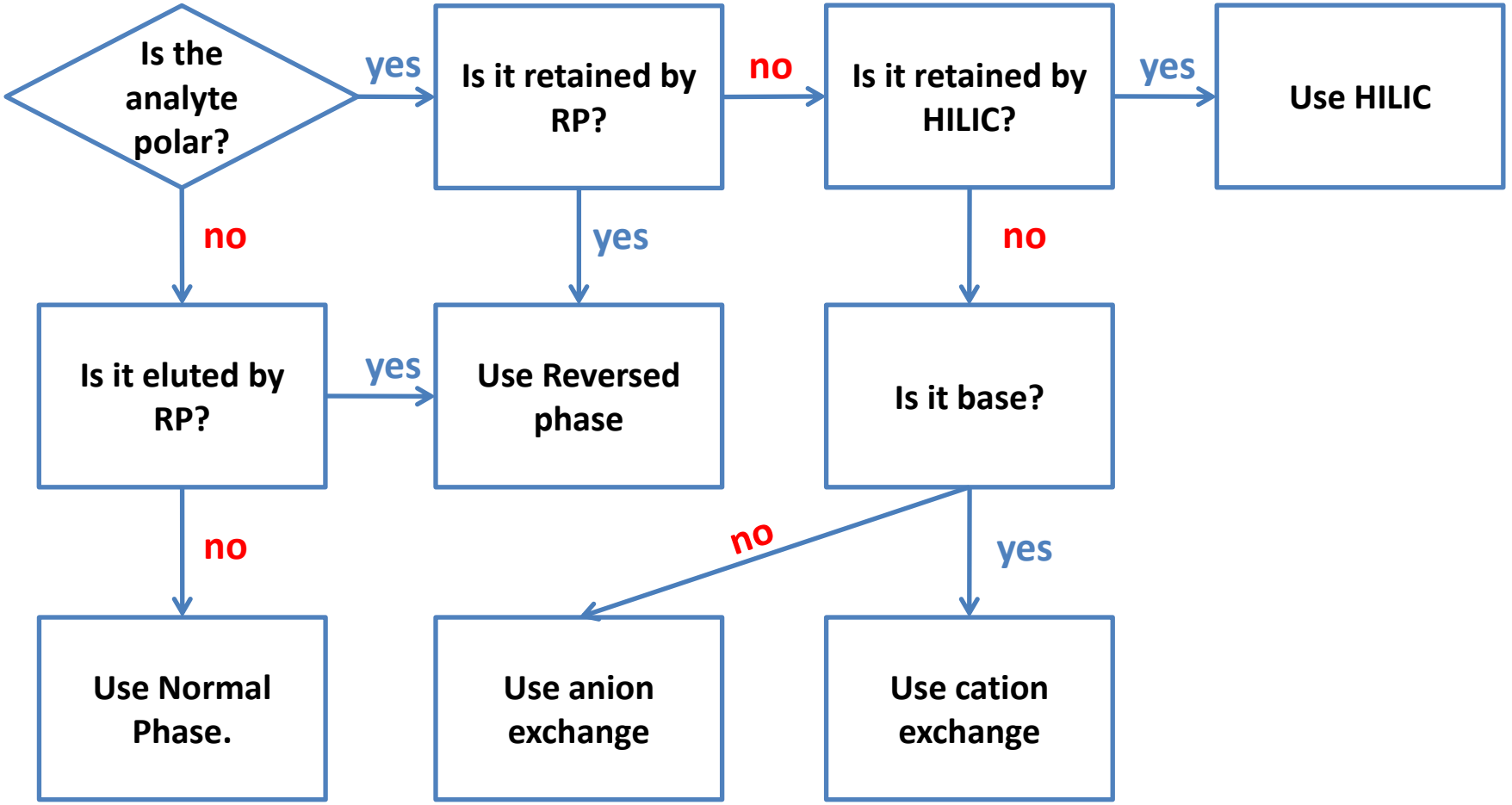


LC-MS method development

Different to compare with „traditional“ HPLC:

- Suitable buffers and solvents are limited.
- pH adjustment can influence ionization process (bases do not ionize in high pH, acids in low pH).
- Selectivity is provided by MS instrument.
- Non-intereferring matrix could cause „matrix effects“ (ion suppression or ionization enhancement), problematic quantitation.
- High water mobile phase decrease MS sensitivity.
- Some ionic compounds (Na^+, $HCOO^-$, Cl^-) provide strong adducts with analyte, not allowing further MS/MS fragmentation.

LC-MS method development



LC-MS method development

Mobile phase

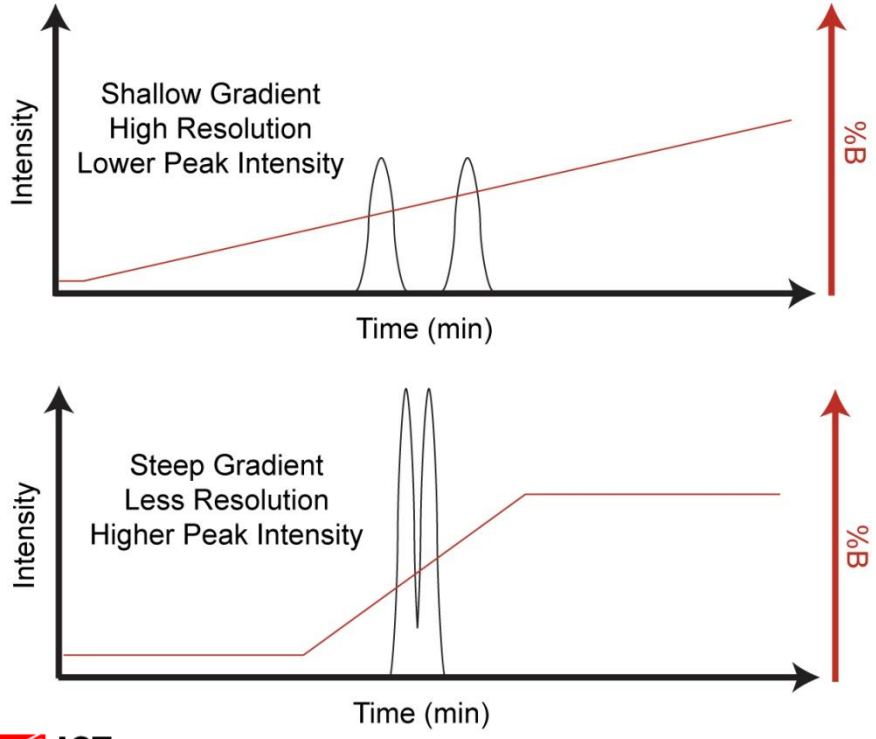
- Volatile buffers and acids (ammonium acetate, ammonium formate, volatile quarternary amines, formic acid, acetic acid, trifluoracetic acid).
- Water with resistivity 18 MΩ (without any ions).
- Highest purity of all solvents (HPLC or LC-MS grade).
- Solvents have to be compatible with the ion-source:

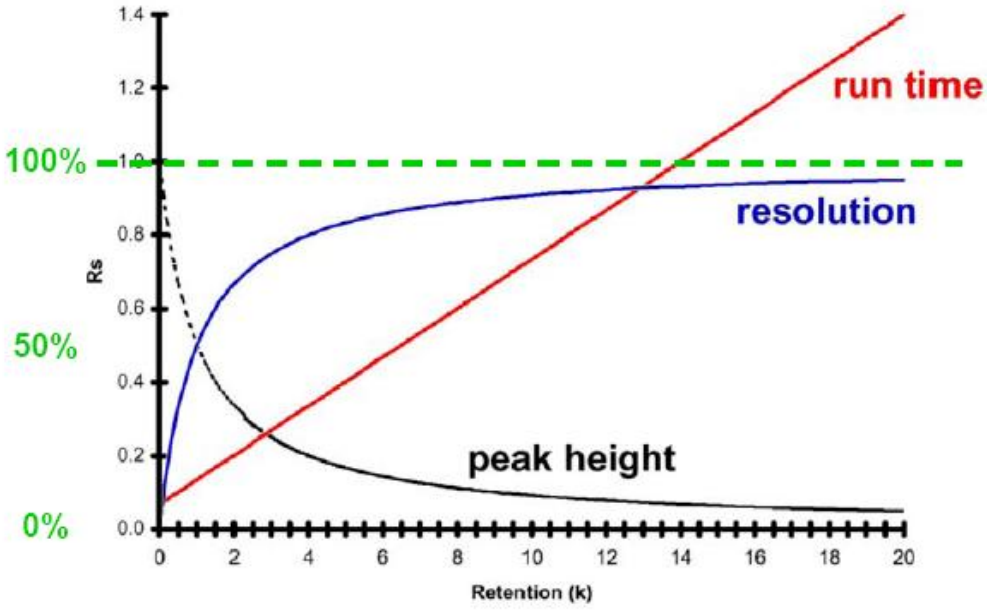
	Electrospray ionization (ESI)	Atmospheric pressure chemical ionization (APCI)	Atmospheric pressure photo ionization (APPI)
Analyte	Polar, ionic	Polar, non-polar	Aromatic, conjugate double bonds, non-polar
Mobile phase	Water, Methanol, Acetonitrile, Tetrahydrofuran	Water, Methanol, Acetonitrile, Tetrahydrofuran, Ethylacetate, Toluene	Water, Methanol, Acetonitrile, Tetrahydrofuran, Ethylacetate, Toluene, Hexane
Chromatographic system	Reversed phase, HILIC, Ion-Exchange	Reversed Phase, Normal Phase	Reversed Phase, Normal Phase

LC-MS method development

Gradient optimization

- Baseline separation of all analytes in multi-analyte methods is not required (possible). The functions of gradient are different:
  - Separation of the analytes to detect only limited analytes at once.
  - Separation of the matrix co-extracts to minimize matrix effects.

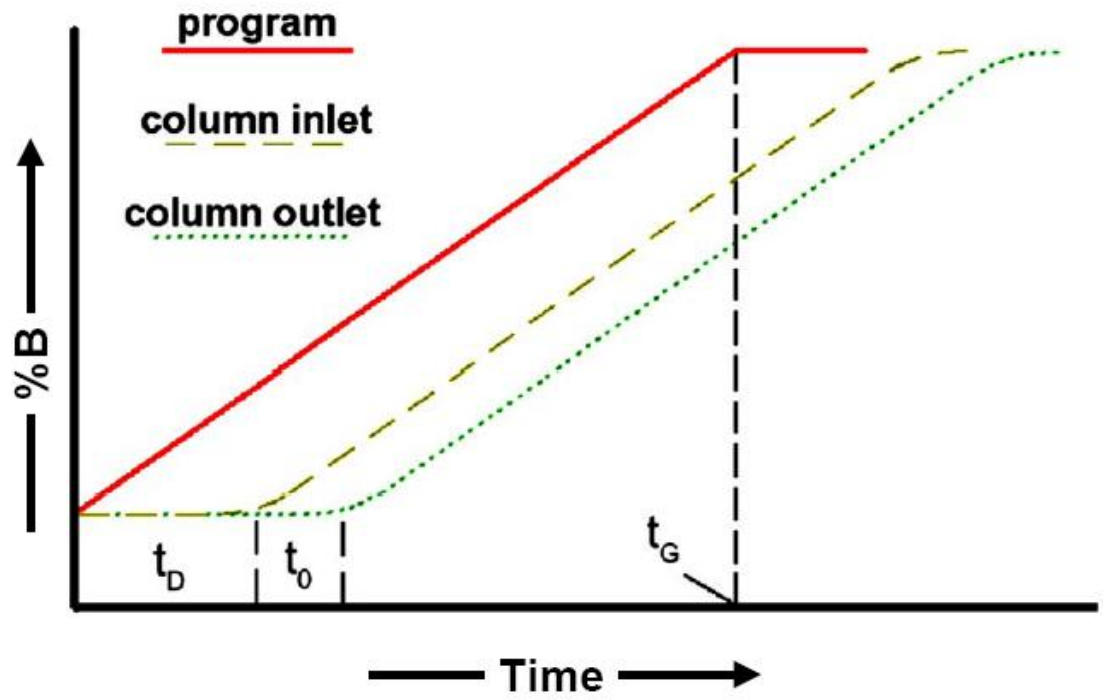




LC-MS method development

Gradient optimization

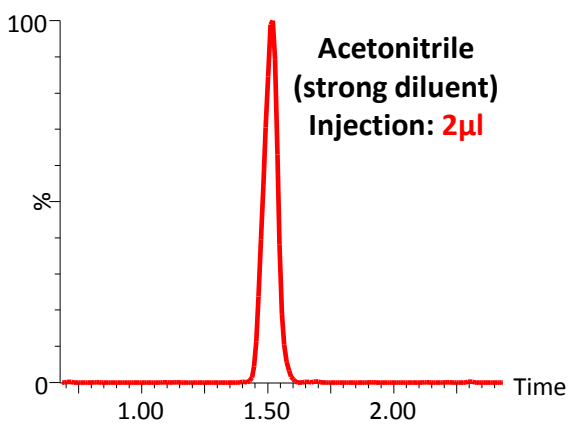
- The real gradient is always delayed after the program.
- Binary pump system produces more precise gradient, the gradient delay is lower than in quarternary system.

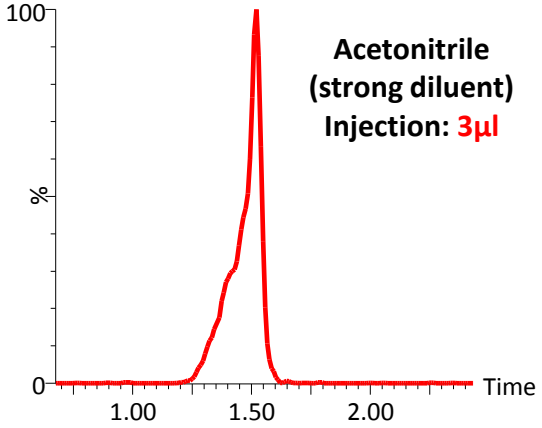


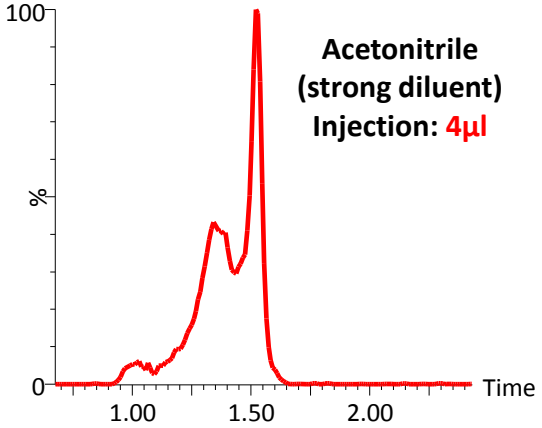
LC-MS method development

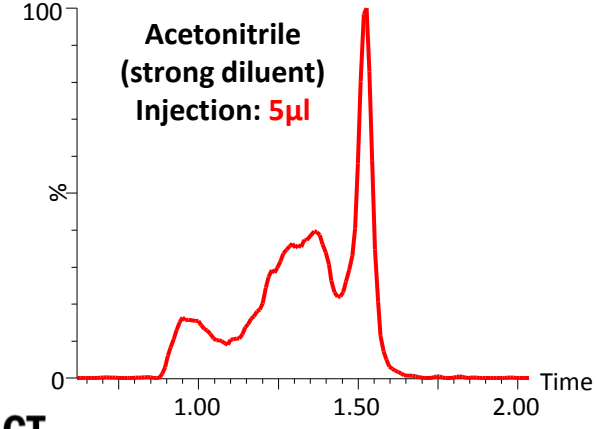
Gradient optimization

- Injection of strong sample diluent causes serious band broadening of less retained compounds:

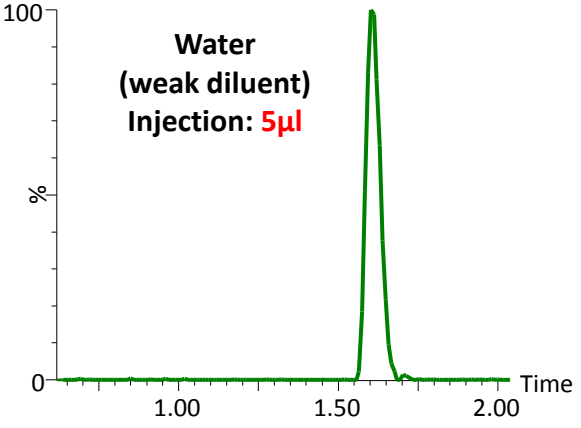








Change of dilluent

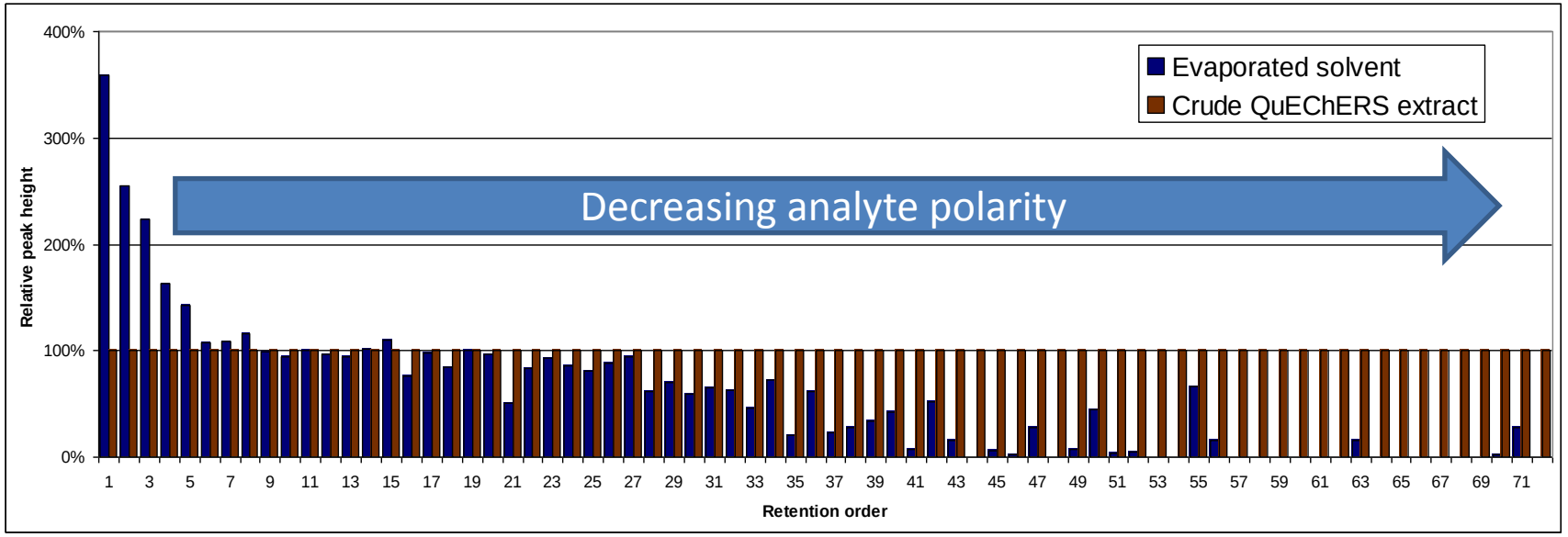


ICT PRAGUE

LC-MS method development

Gradient optimization

- In multi-analyte methods is needed to inject compounds with very different physico-chemical properties (also solubility). Injection in the weakest solvent is almost impossible due to sorption of some analytes.

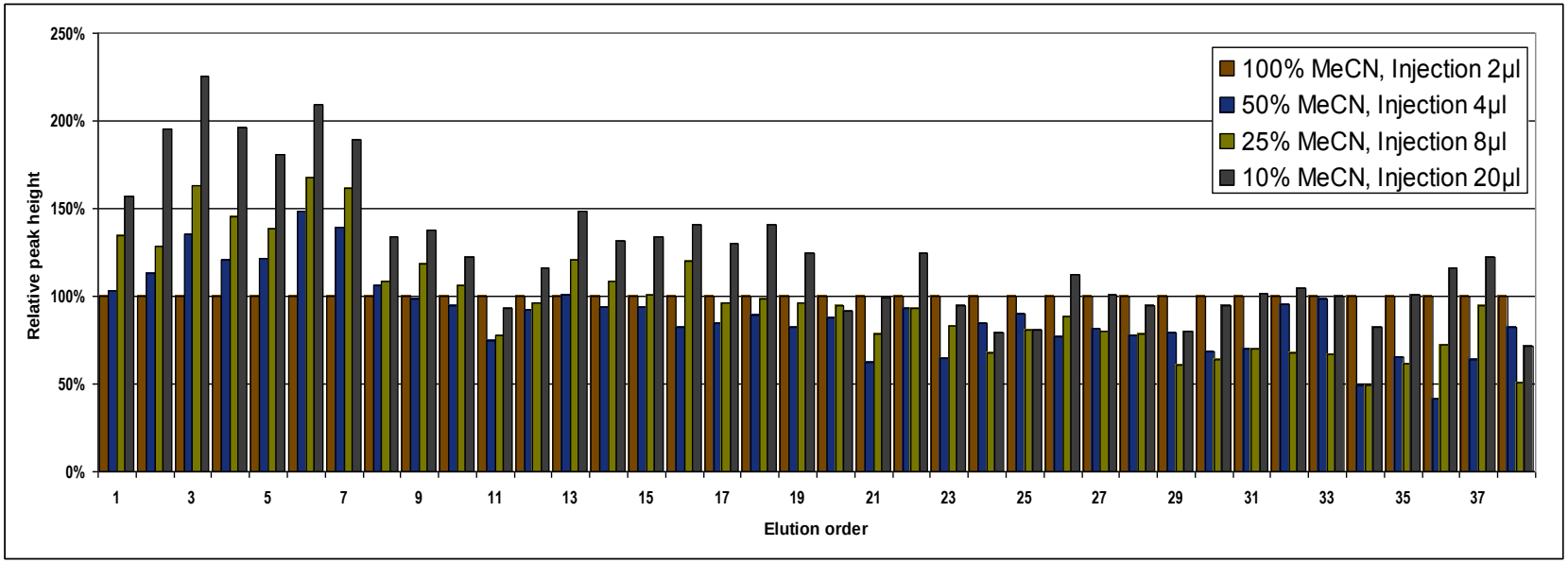


Change of the acetonitrile extract to the water (evaporation and reconstitution in water).

LC-MS method development

Gradient optimization

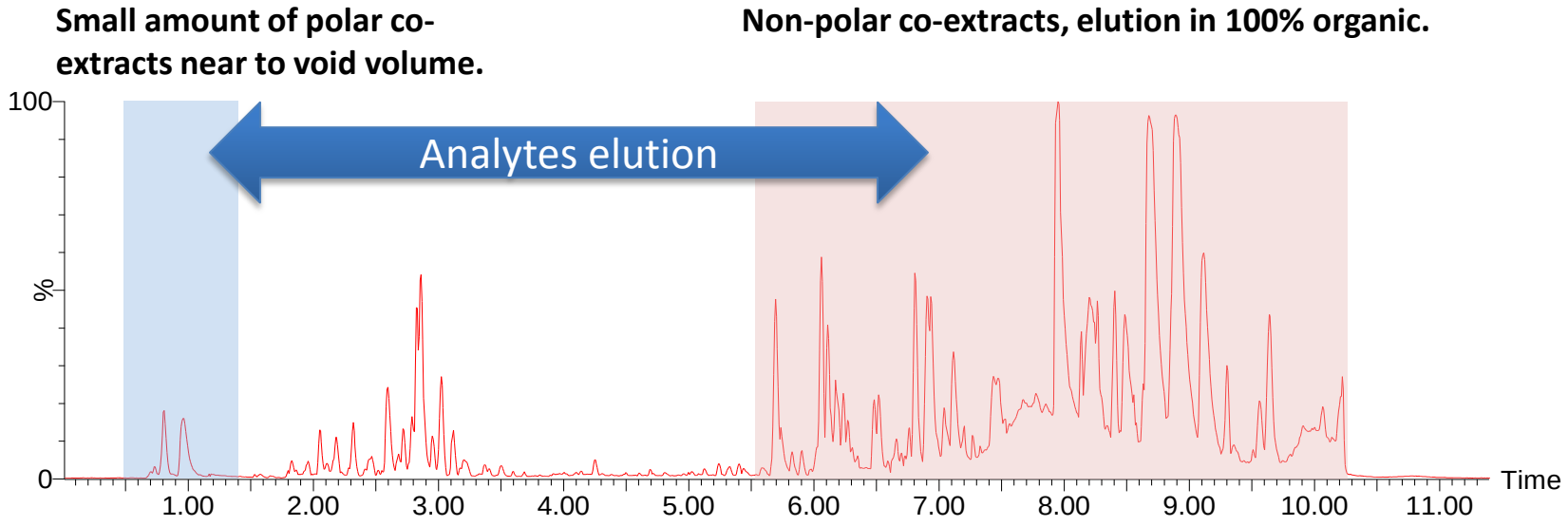
- Possible solution is dillution of extract by water and increasing of the injection volume.
- The concentration of organic in the sample have to be lower than in inital mobile phase.
- Risk of matrix precipitation or analyte hydrolysis in water.



LC-MS method development

Gradient optimization

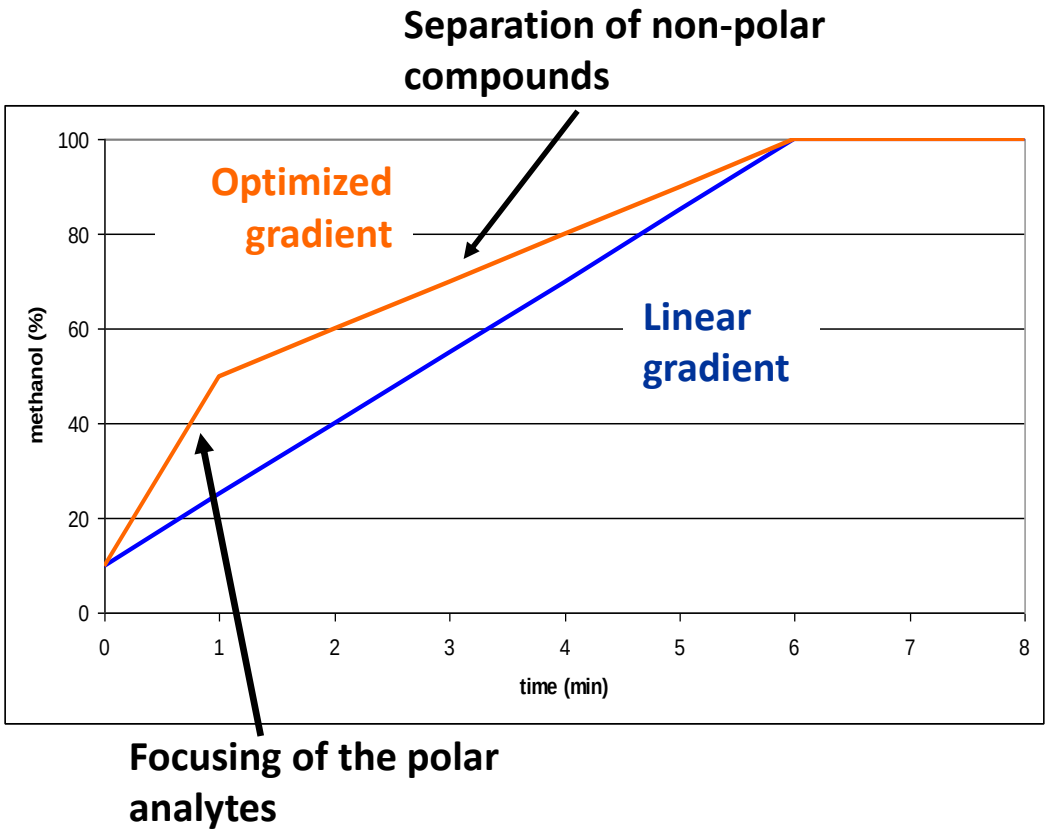
- Chromatographic profile of the matrix (UHPLC-Time-of-flight MS detector, *m/z* 50-1000)

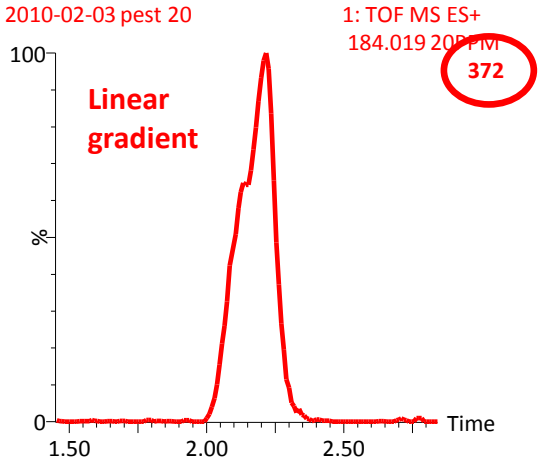


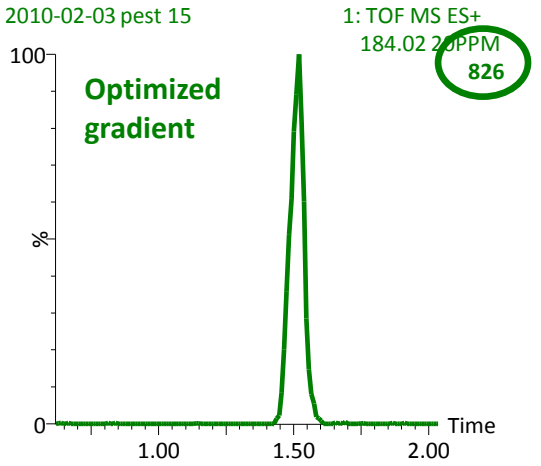
LC-MS method development

Gradient optimization

- Gradient tuning for injection of strong solvent in multi-analyte method with.







2.5× higher intensity

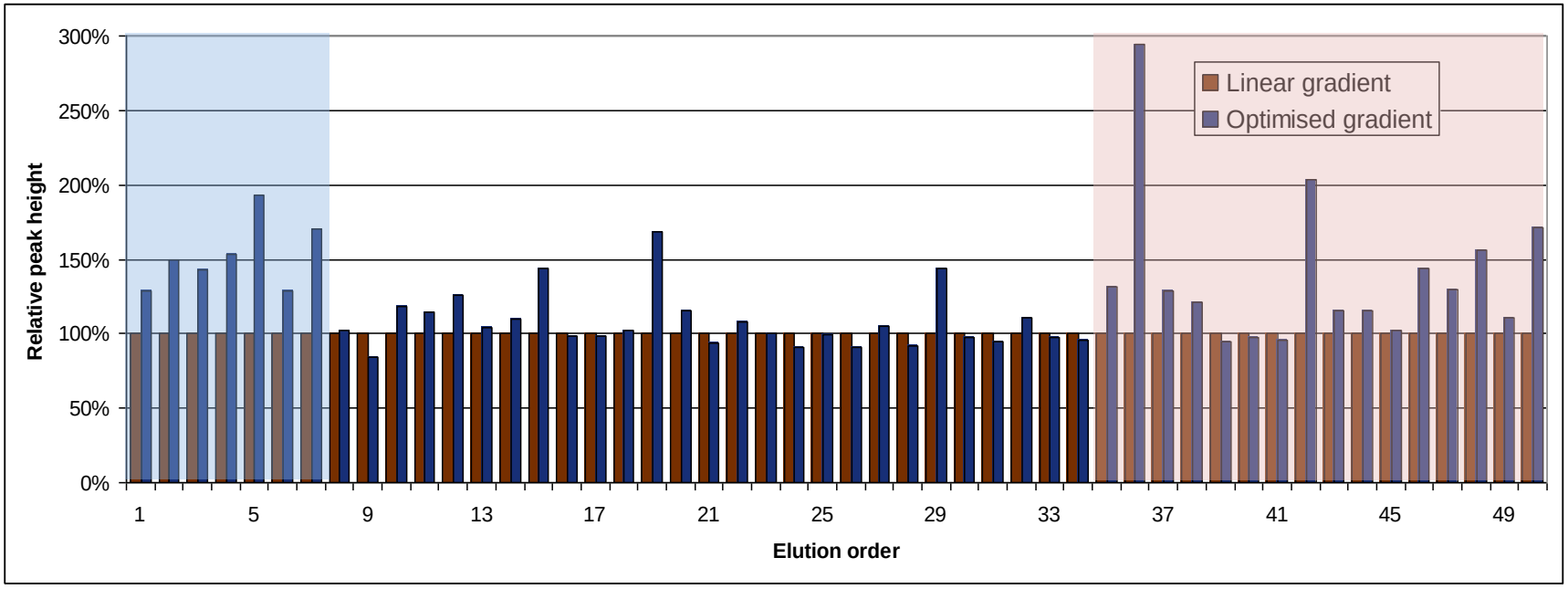
LC-MS method development

Gradient optimization

- Comparison of linear and optimized gradient.
- Separation takes the same time.
- Signal of some analytes is improved.
- No adverse effects.

Focusing of polar analytes.

Better separation of non-polar matrix reduce ion suppresion induced by coeluted matrix.



LC-MS method development

Summary

- Before the method development use fresh, high purity solvents and flush the system.
- Start in the weak mobile phase (5 or 10% of organic phase in reversed-phase chromatography). In the first phase of method development, use low pH mobile phase.
- Inject sample in mobile phase to avoid band broadening. If it is not possible, try to dilute sample with water and inject higher volume or inject only small volume (2-5µl of strong solvent, depends on the column capacity).
- Increase B eluent (organic) to 100% and keep it at least 3 column void volumes, to be sure, that all non-polar compounds are eluted from the column.
- Column equilibration needs 5-10 column void volumes. Do not forget calculate with the void volume of the gradient pump!
- Always wait for the second injection, the first injection is not reliable (column is not equilibrated properly).