
بسم الله الرحمن الرحيم

كروماتوغرافيا الطور السائل

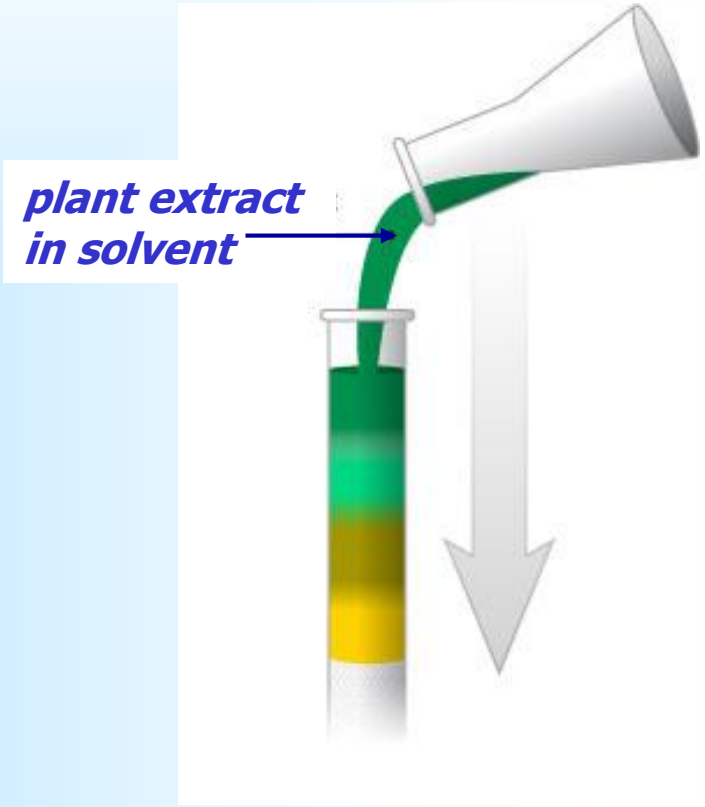
Liquid Chromatography

Liquid chromatography

- It is the first ever described chromatographic method (by Tswett in 1903)
- Unlike gas chromatography, the sample in liquid chromatography must not be vaporized so, almost all kinds of compounds can be analysed by liquid chromatography
- The development of instrumental liquid chromatography was later than for gas chromatography because of the higher pressure needed for the former
- HPLC is considered to be the major chromatographic technique available today for non-volatile or heat-sensitive substances.

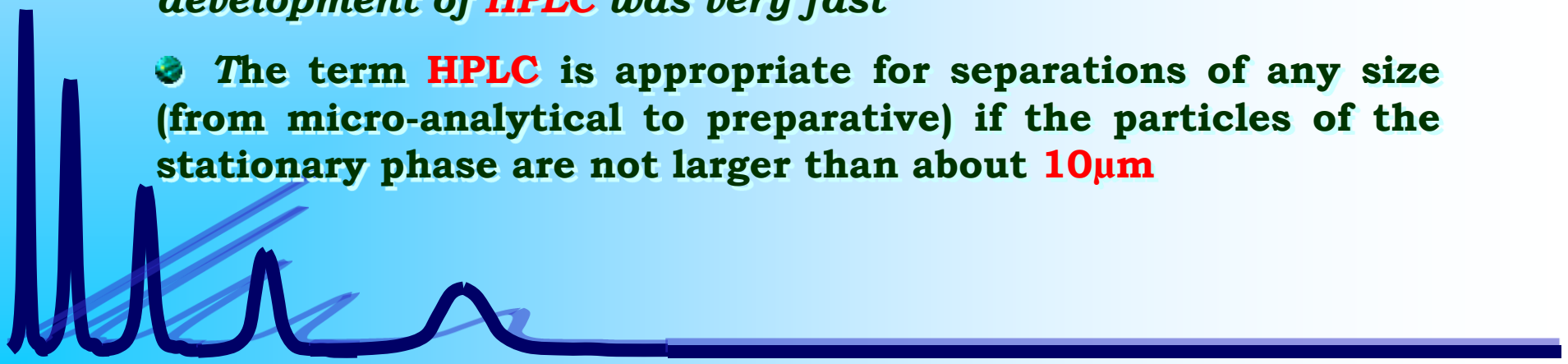
A little history:

- *The official date of birth of chromatography is the* **21 March 1903** *in Warsaw when* **Mikhail Semenovitch TSWETT** *has presented at the Congress of the* **Polish Natural Sciences Society** *a communication entitled: «* **A new class of adsorption phenomena and their applications in biochemical analysis** *» about the separation and purification of vegetal pigments (a mixture of chlorophylls and xantophylls) on a chalk column*



A little history:

- *1938 : REICHSTEIN proposes a theory for the elution and separation of solutes on a column*
- *1952 : application of gradient elution*
- *1967 : beginning of HPLC after the works of HUBER and HUZSMAN, this technique was first named « High Speed Liquid Chromatography » then « High Pressure Liquid Chromatography » and finally « High Performance Liquid Chromatography »*
- *1969 : after the 5th International Symposium International «Advances in Chromatography» the development of HPLC was very fast*
- **The term HPLC is appropriate for separations of any size (from micro-analytical to preparative) if the particles of the stationary phase are not larger than about 10µm**



Fundamental definitions (to IUPAC nomenclature)

- ***Chromatography:*** a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction

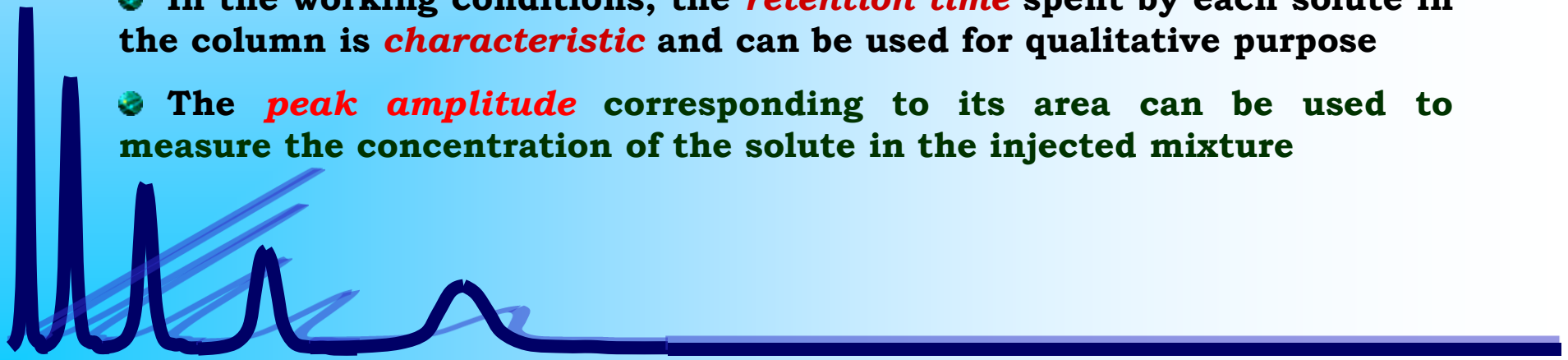
- ***Chromatogram:*** *a* graphical or other presentation of detector response, concentration of analyte in the effluent or other quantity used as a measure of effluent concentration versus effluent volume or time

- ***Stationary Phase:*** one of the two phases forming a chromatographic system. It may be a solid, a gel or a liquid. If a liquid, it may be distributed on a solid. This solid may or may not contribute to the separation process. The liquid may also be chemically bonded to the solid *(bonded phase:* covalently bonded to the support particles or to the inside wall of the column tubing*)* or immobilized onto it *(immmobilized phase)*

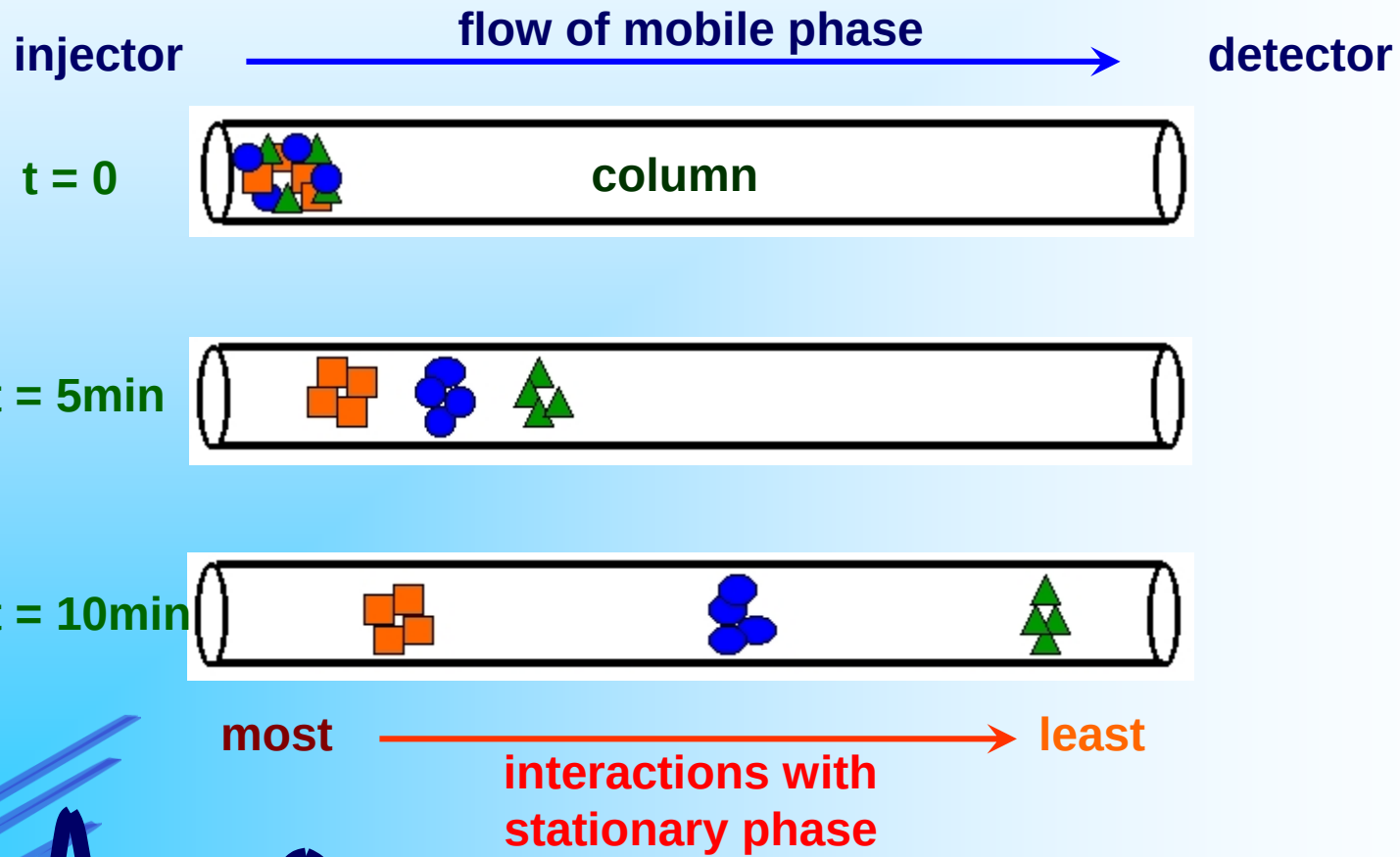
- ***Mobile Phase:*** *a* fluid which percolates through or along the stationary bed, in a definite direction. It may be a liquid *(liquid chromatography)* or a gas *(gas chromatography)* or a supercritical fluid *(supercritical-fluid chromatography)*

Principles of liquid chromatography

- A liquid used as *mobile phase* moves along a tube used as *column*. This column is packed with a solid support which plays the role of *stationary phase*
- If the *stationary phase* and the *mobile phase* were correctly selected, the constituents of mixture are unequally retained along the column
- This phenomenon called *retention* means that the injected solutes move *slower* than the mobile phase with *different speeds*. They are thus *eluted* successively from the column and *separated*
- The *detector* connected at the column outlet gives a signal corresponding to each solute which is recorded as the *chromatogram*
- In the working conditions, the *retention time* spent by each solute in the column is *characteristic* and can be used for qualitative purpose
- The *peak amplitude* corresponding to its area can be used to measure the concentration of the solute in the injected mixture



Simplified scheme of the chromatographic separation process



Main modes in liquid chromatography

There are several modes in high performance liquid chromatography

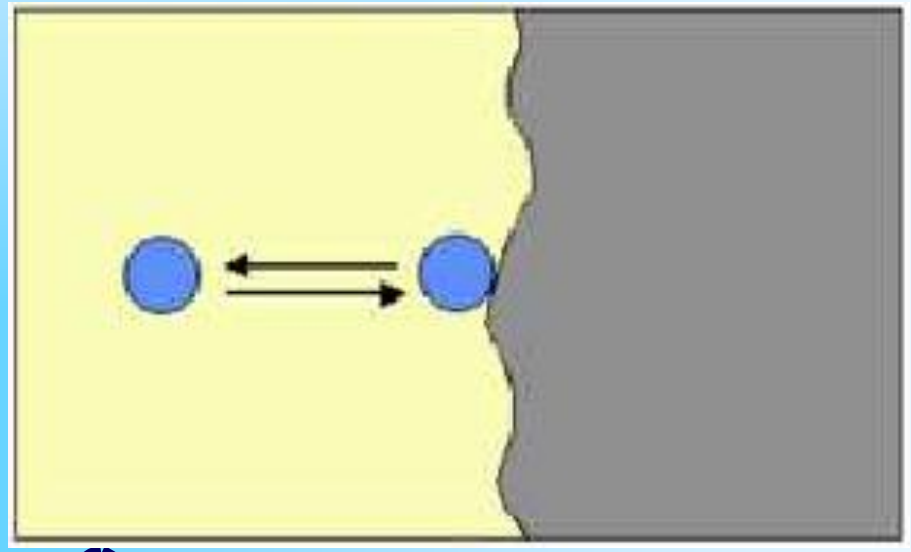
They are classified according to the mechanism of separation

Each mode corresponds to a given kind of *interaction*:

- ***surface adsorption***
- ***solvent partitioning***
- ***ion exchange***
- ***size exclusion***

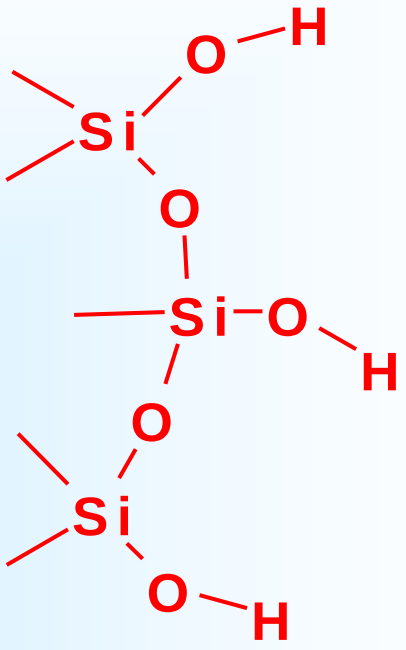
Adsorption chromatography

- *the stationary phase is a solid adsorbant*
- *retention is due to a series of adsorption / desorption steps*
- *separation is based mainly on differences between the adsorption affinities of the sample components for the surface of the active solid (liquid solid chromatography)*



Adsorption chromatography

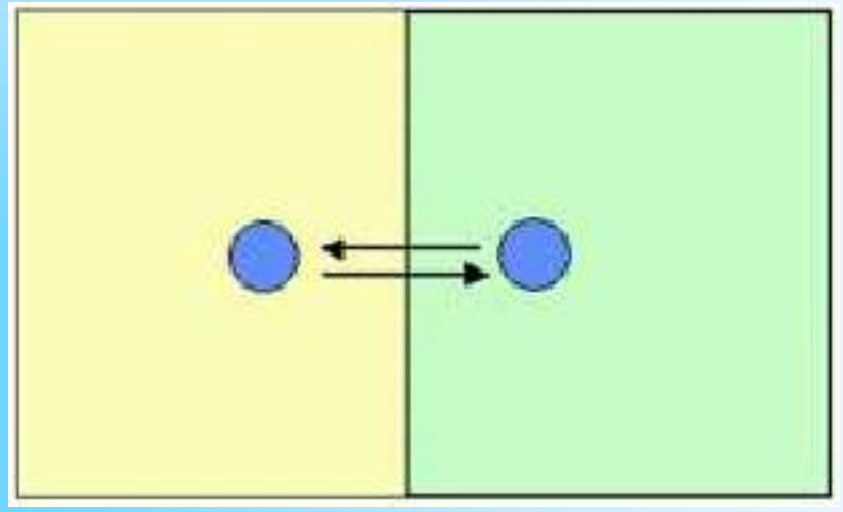
- *silica and alumina are the most used stationary phases*
- *both solute and solvent can be attracted by the active sites at the surface of the stationary phase*
- *the molecules are retained by the interaction of their polar functional groups with the surface functional groups such as silanols of silica*
- *if solutes have different interactions with the adsorbing sites, the separation can occur*



Silanol groups Si-OH at the surface of silica

Partition chromatography

- *the stationary phase is a liquid coated or linked to a solid support*
- *retention is due to the partitioning of the solute between the two liquid phases (relative solubility)*
- *separation is based mainly on differences between the solubilities of the components in the mobile and stationary phases (liquid – liquid chromatography)*



Partition chromatography

- *the most retained species is that having the highest affinity (solubility) for the liquid stationary phase, relatively to the mobile phase (eluent)*
- *separation is based on the differences in relative solubility*

There are two modes in liquid chromatography

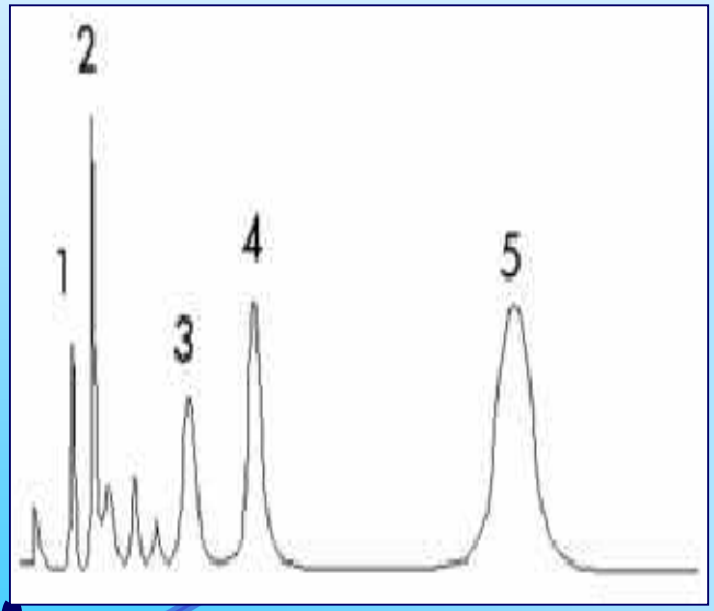
- *« normal » mode: polar stationary phase and non-polar mobile phase (the first mode described). In this procedure, the stationary phase is more polar than the mobile phase. This term is used in liquid chromatography to emphasize the contrast to reversed-phase chromatography*
- *« reversed-phase » mode: non-polar stationary phase and polar mobile phase (the most used mode). In this procedure the mobile phase is significantly more polar than the stationary phase, e.g., a microporous silica-based material with chemically bonded alkyl chains*

Partition chromatography

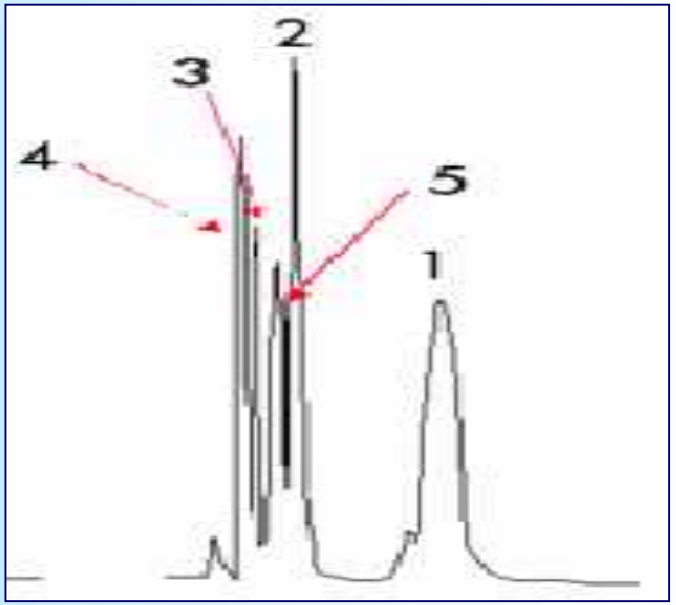
The elution order of solutes can be inverted with the same column used either in « normal » or « reverse » phase

Example:

« normal » mode

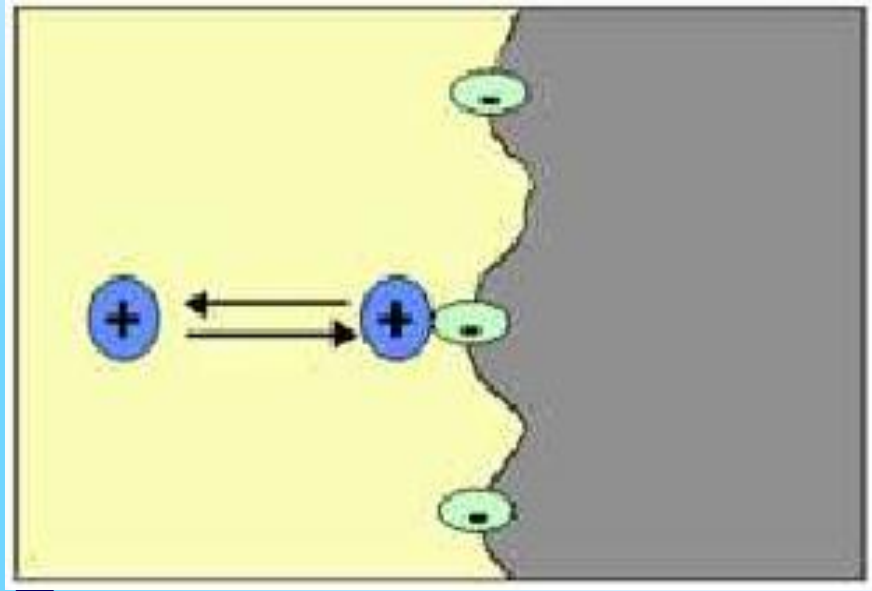


« reverse » mode



Ion exchange chromatography (IEC)

- *the stationary phase has ionically charged groups at the surface*
- *the retention is due to the attractive interactions between ionic solutes and the opposite charged stationary phase*
- *separation is based mainly on differences in the ion exchange affinities of the sample components*
- *this technique is now often referred to as Ion Chromatography (IC)*



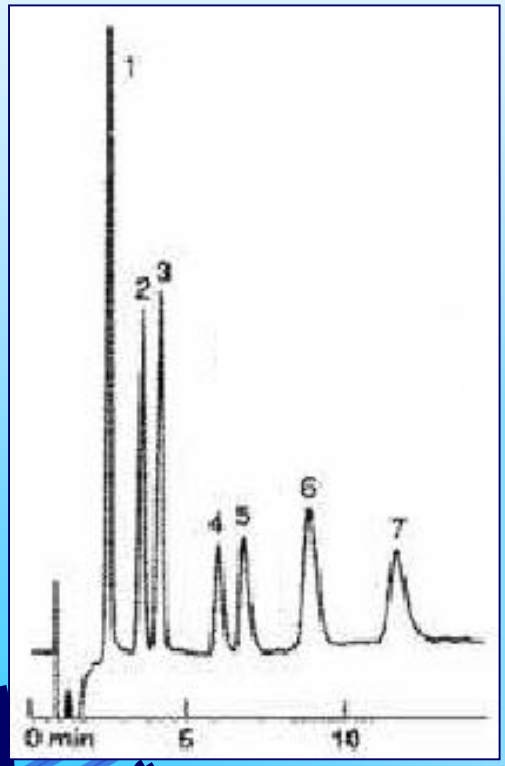
Ion exchange chromatography

- *in this mode, weak exchange resins are usually used as stationary phase*
- *they are obtained by linking charged groups to the solid support*

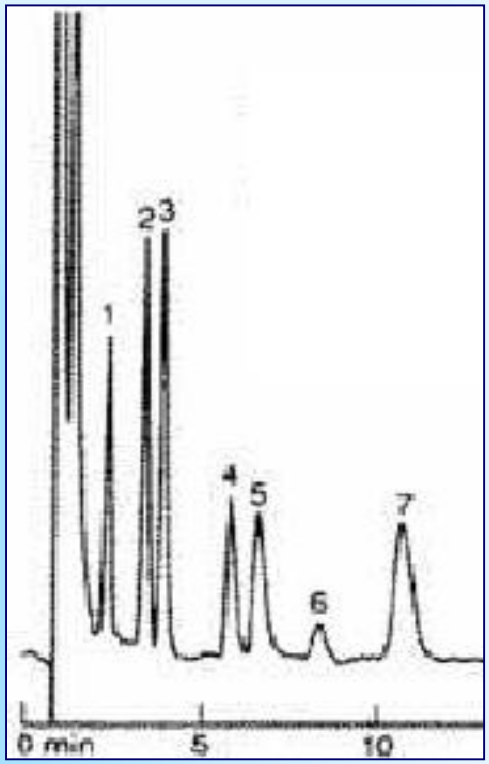
Ion exchange chromatography

Example of separation: separation of inorganic anions

UV detection



conductimetric detection



1- fluoride F^-

2- chloride Cl^-

3- nitrite NO_2^-

4- bromide Br^-

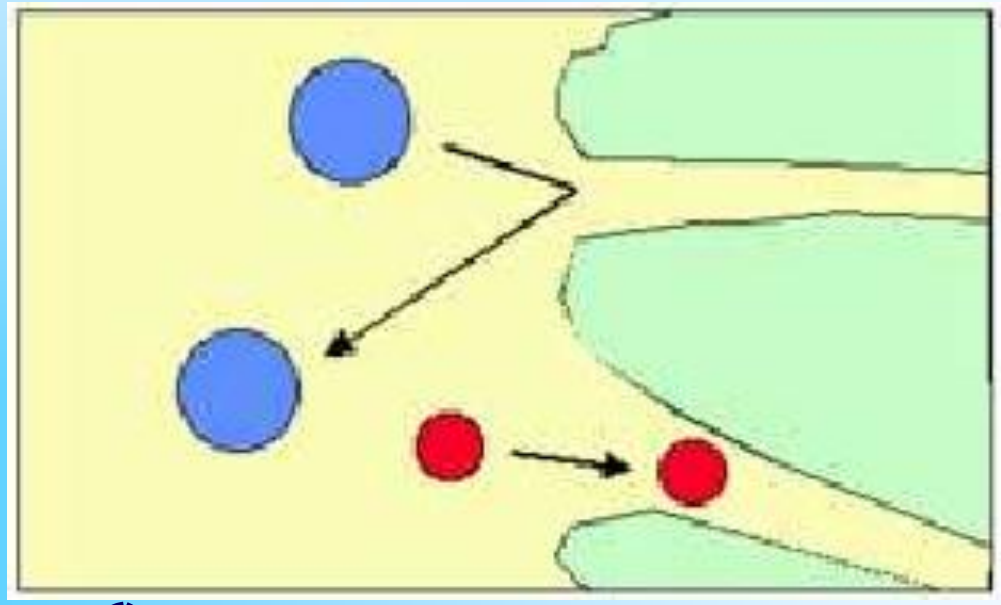
5- nitrate NO_3^-

6- phosphate PO_4^{3-}

7- sulfate SO_4^{2-}

Size exclusion chromatography (SEC)

- *the stationary phase is a porous material having controlled pore size*
- *separation is based mainly on exclusion effects, such as differences in molecular size and/or shape*
- *the terms Gel Filtration and Gel-Permeation Chromatography (GPC) were used earlier to describe this process*

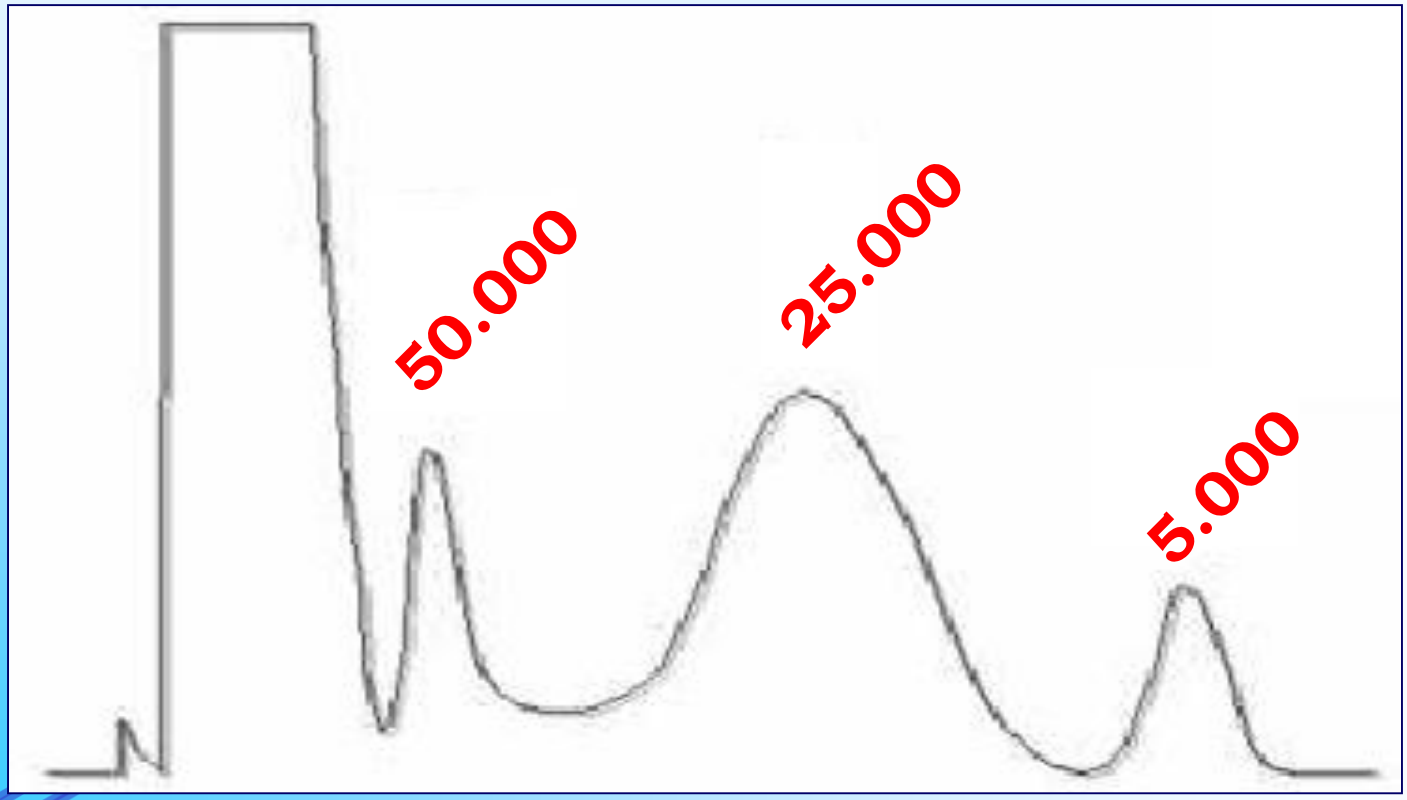


Size exclusion chromatography (SEC)

- *In this mode, each column can separate solutes having specific size range*
- *separation mechanism is sieving*
- *the larger species cannot enter all the pores and will elute first because they have a shorter path in the column*
- *this mode is very useful for the determination of molecular size of macromolecules (polymers, proteins,...)*
- *large molecules excluded from pores - not retained, first eluted*
- *intermediate molecules: retained, intermediate elution times*
- *small molecules permeate into pores: strongly retained, last eluted*

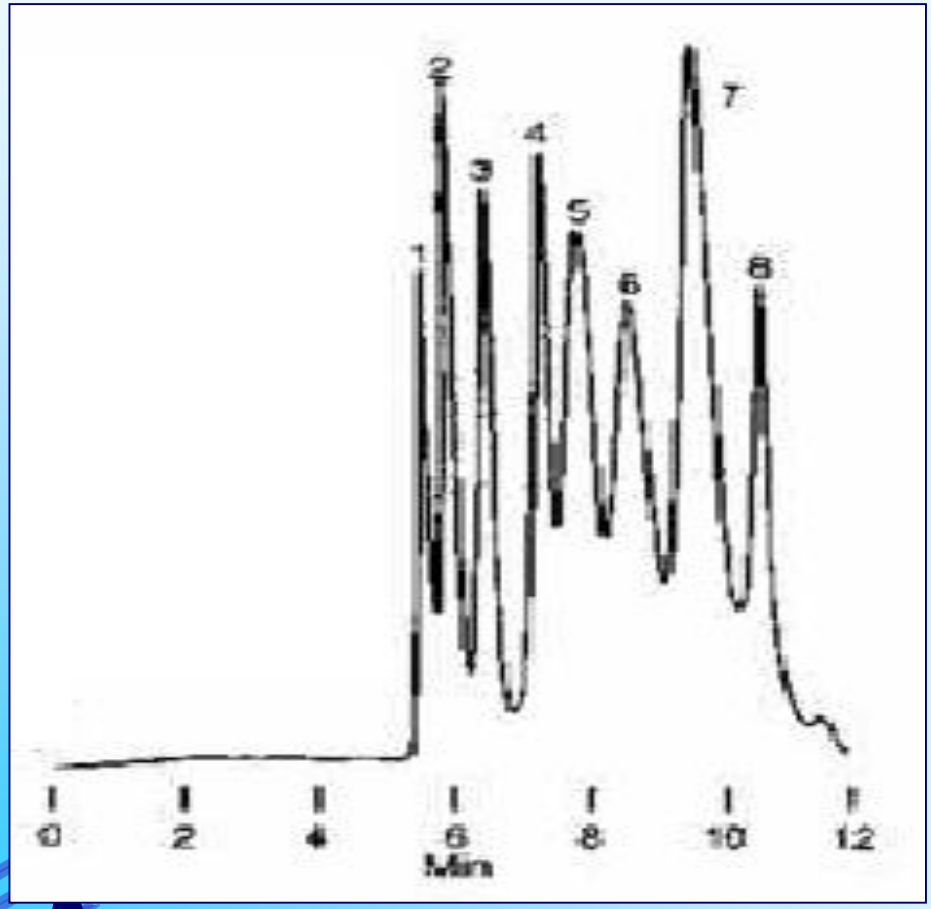
Size exclusion chromatography (SEC)

Example of separation of a polymer by SEC: 3 groups of macromolecules are characterized, they are eluted in order of decreasing molecular mass



Size exclusion chromatography (SEC)

Example: separation of polystyrene standards having different molecular masses



1- 1.800.000

2- 300.000

3- 100.000

4- 35.000

5- 17.500

6- 9.000

7- 2.000

8- toluene

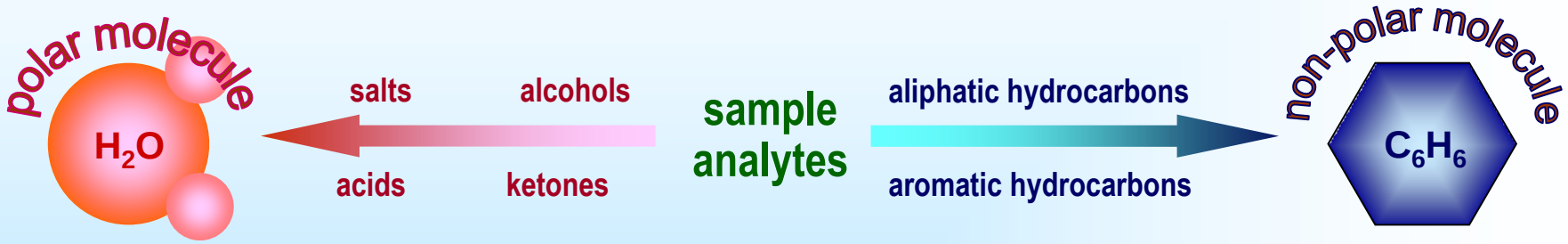
Importance of polarity in HPLC

- ***polarity* plays a fundamental role in HPLC**
- **all chemicals have a unique and characteristic behaviour related to their molecular structure and electron charge distribution**
- **they can be described as being " *polar* " or " *non-polar* ", with a range of polarities between the most polar and most non-polar**
- ***water* is a good example of a very polar liquid, and *paraffin* based oil is a good example of a very non-polar liquid**
- **this " *polarity* " characteristic of chemicals allows to explain the chromatographic " *retention mechanisms* " that are used to create many HPLC separations**
- **a simple rule describes this behavior for polarity-based retention mechanisms:**

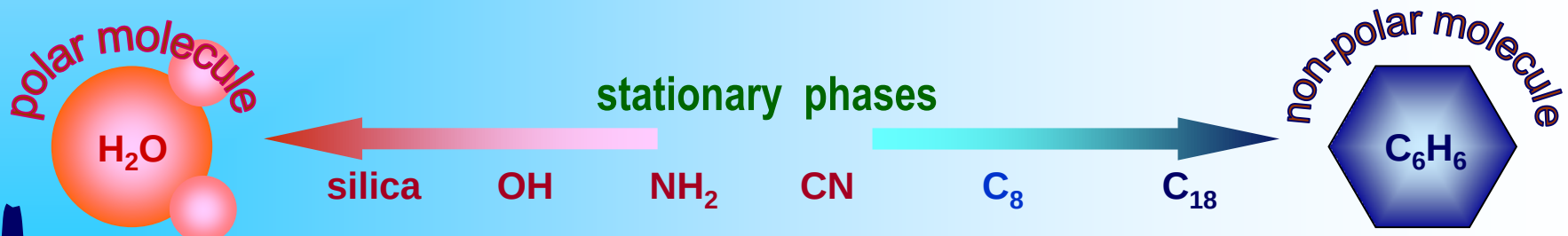
"Like Attracts Like, and Opposites are Not Attracted"

Importance of polarity in HPLC

- *thus sample analytes can be classified according to their polarity:*



- *on the other hand, a similar classification can be done also for solvent eluents and stationary phases*



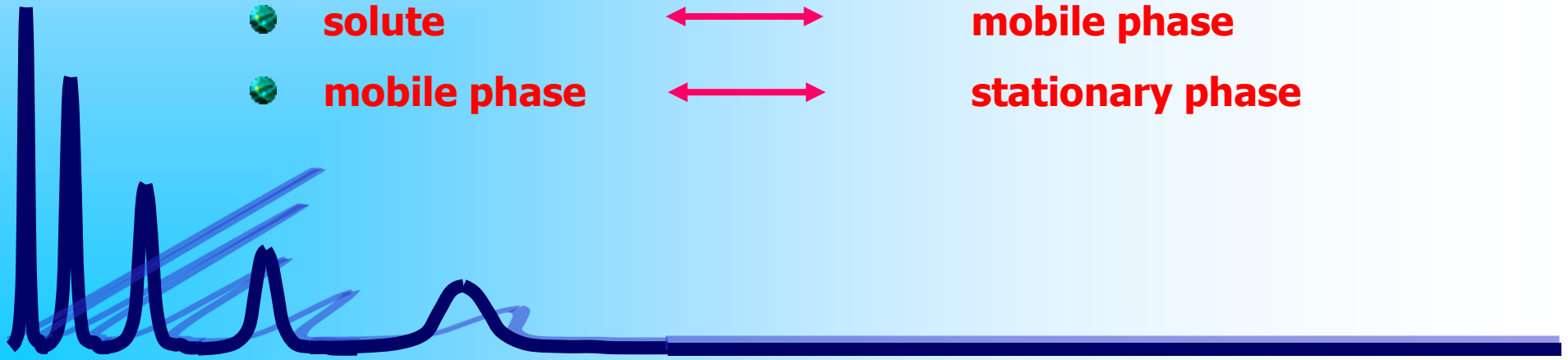
Main HPLC techniques

- ***SEC: size exclusion chromatography (GPC)***
- ***IEC: ion exchange chromatography (IC)***
- ***HIC: hydrophobic interaction chromatography***
- ***LSC: liquid-solid chromatography***
- ***RPC: reverse phase chromatography***
- ***BPC: bonded phase chromatography (LLC)***
- ***IPC: ion pair chromatography***

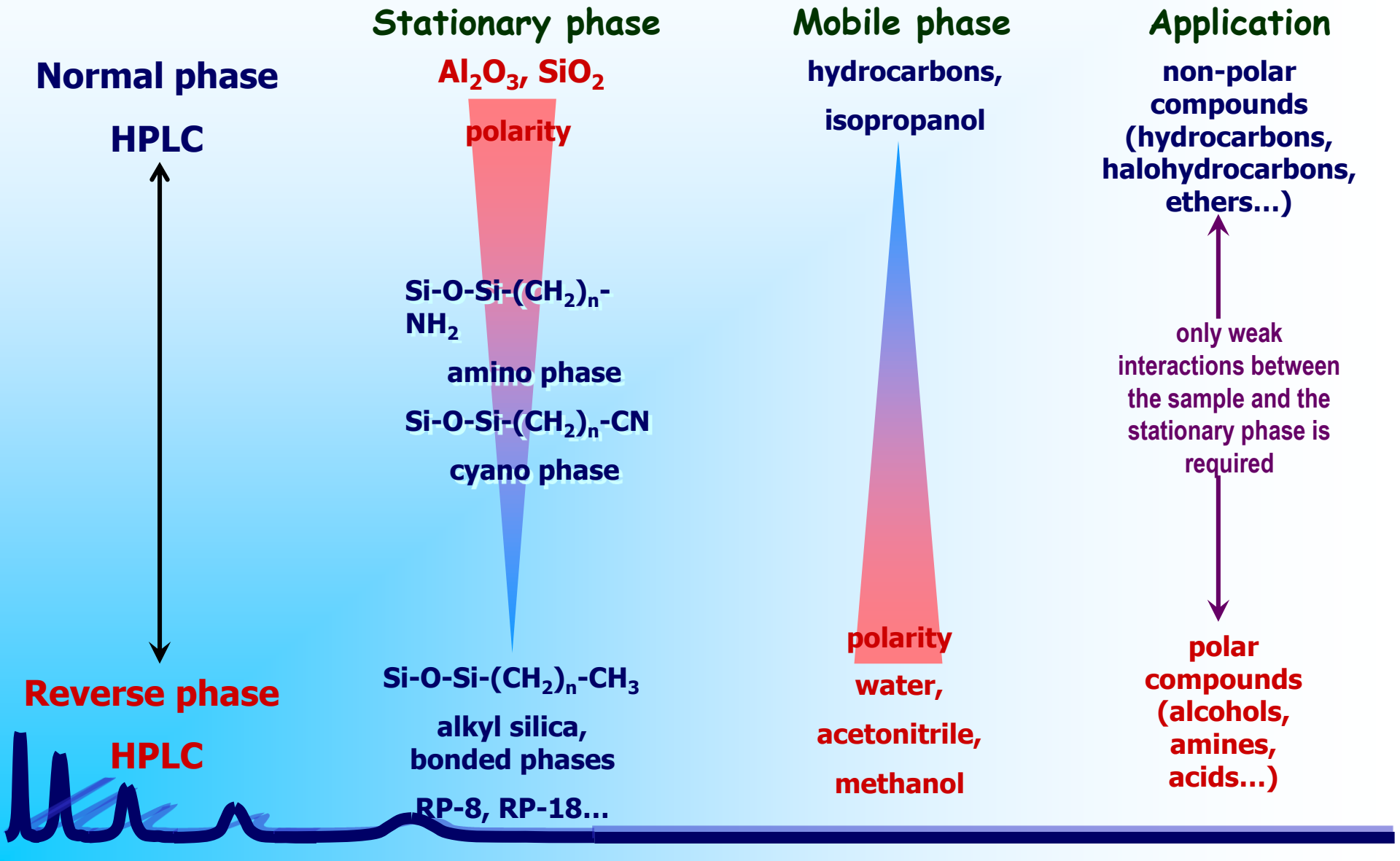
The solvent in liquid chromatography

- ***the properties of mobile phase are the fundamental parameters in high performance liquid chromatography***
- ***unlike gas chromatography, the interactions involved in HPLC are different:***

- **solute ⟷ stationary phase**
- **solute ⟷ mobile phase**
- **mobile phase ⟷ stationary phase**



Normal and reverse phase HPLC



Mode selection in HPLC

- *the first step is to select the suitable mode:*

normal or *reverse?*

This is a general and simple rule:

- **choose column with similar polarity to analyte for maximum interaction**
- *if the solute is water insoluble or non-polar:*

⇒ *Use the normal mode*

- *if the solute is water soluble or polar:*

⇒ *Use the reverse mode*

- *analyte polarity:*

hydrocarbons < ethers < esters < ketones < aldehydes < amines < alcohols

N.B.: in practice, the situation is not as simple and clear, but this rule gives an acceptable starting point

- **the reverse mode is much more used because it uses cheaper aqueous solvents and allows larger applications**

Solvent selection in HPLC:

- *it is unusual to find a single pure solvent acceptable as mobile phase*
- *generally, it is necessary to use a mixture of at least two solvents in order to achieve an acceptable separation*

What are the factors to consider?

To select the mobile phase, the concept of solvent strength and polarity is utilized. A strong solvent is one which causes a sample to elute rapidly from the column. Various measures of solvent strength are used:

- *solvent strength parameter (E°): it gives a measure of its relative polarity (ability to displace a given solute). It is based on the adsorption energies of the solvent on silica or alumina*
- *solvent polarity parameter (P'): based on experimental solubility data which reflects the proton acceptor, proton donor and dipole interactions of the solvent molecule*
- *Hildebrand solubility parameter (δ): which measures dispersion and dipole interactions, and hydrogen acceptor and donor properties*

Properties of some common solvents used in HPLC

Solvent	Solvent strength* E°	Polarity index P'	Viscosity (cP)	Refractive index	UV cutoff (nm)
n-pentane	0.00	~0.0	0.23	1.36	210
n-hexane	0.01	0.1	0.30	1.372	210
carbone tetrachloride	0.18	1.6	0.97	1.47	265
toluene	0.29	2.4	0.59	1.50	285
diethyl ether	0.38	2.8	0.32	1.35	220
tetrahydrofuranne	0.45	4.0	0.46	1.41	220
butanone	0.51	4.7	-	1.38	330
acetonitrile	0.65	5.8	0.37	1.34	210
ethanol	0.88	4.3	1.08	1.359	210
methanol	0.95	5.1	0.60	1.33	210
water	large	10.2	0.89	1.333	205

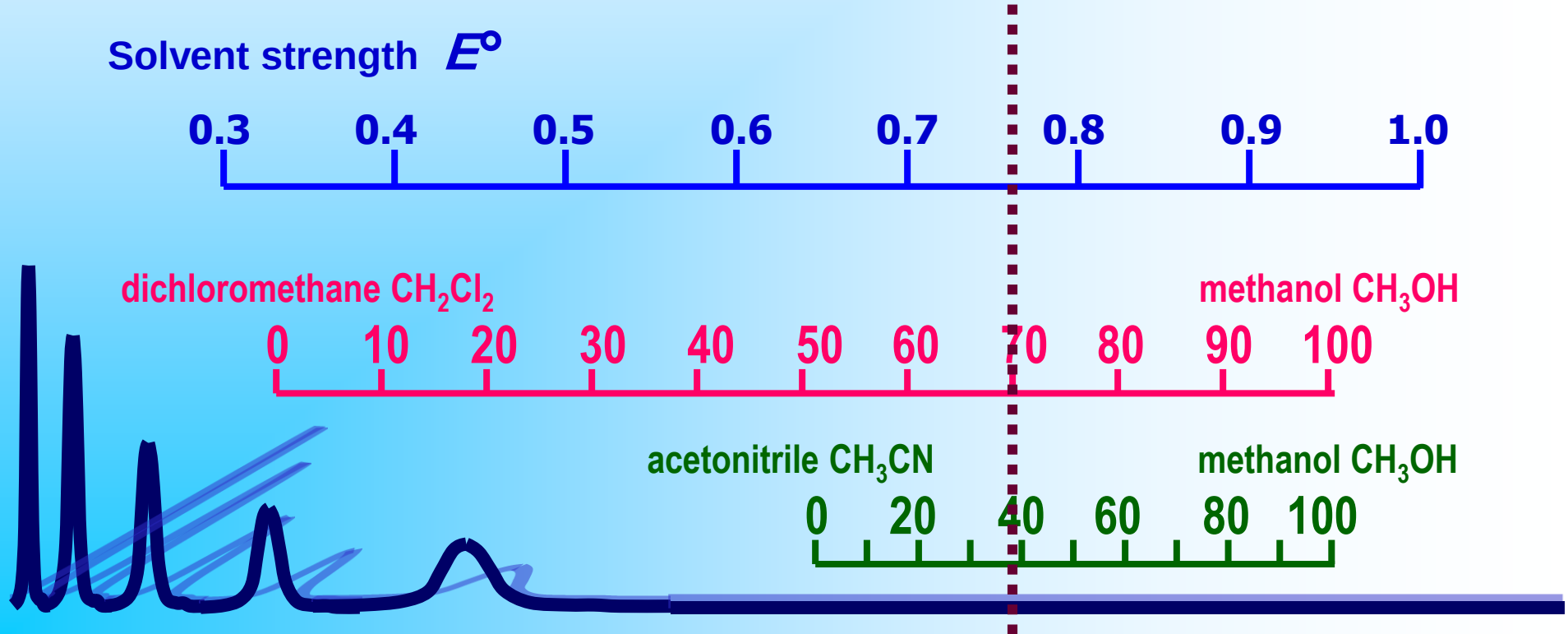
* E° given for alumina ($E^\circ(Al_2O_3)$ x 0.8 = $E^\circ(SiO_2)$)

Solvent mixture in HPLC:

- *it is possible to optimize the solvent mixture in terms of solvent strength or polarity, by evaluating the composition of the mixture*
- **for the solvent polarity parameter the value of *P'* for a binary mixture (*AB*) is given by:**

$$P'_{AB} = \phi_A \cdot P'_A + \phi_B \cdot P'_B$$

where ϕ_A and ϕ_B are volume fractions of solvents *A* and *B*.



Solvent limitations:

When a solvent mixture cannot be used in HPLC:

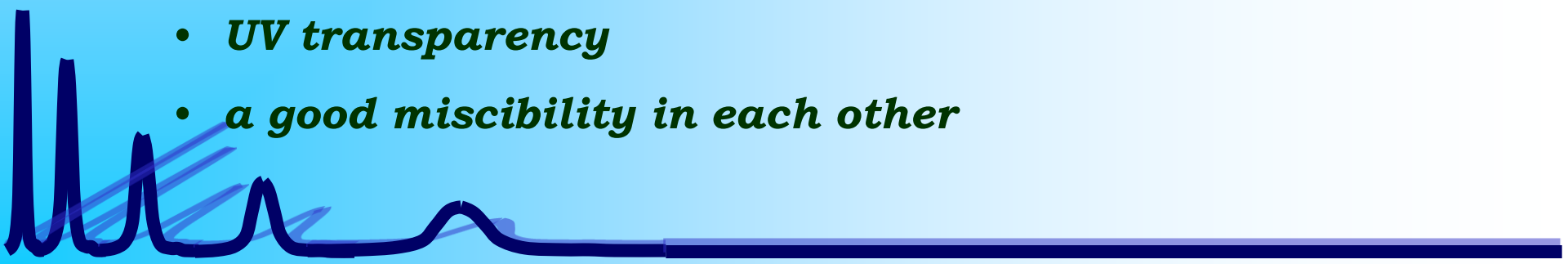
- *if they are not miscible at any proportion*
- *in case of chemical reaction between the solvents*
- *in case of excessive UV absorbance*
- *if the viscosity is too high*
- *highly toxic solvent*
- *highly volatile and flammable*
- *too expensive solvent*

Most used solvents in HPLC:

- **methanol**: *acid character*
- **acetonitrile**: *basic character*
- **tetrahydrofuran**: *polar character*
- **water**: *allows to adjust the solvent polarity*

These solvents have convenient properties:

- *a low viscosity*
- *a good availability with high purity*
- *UV transparency*
- *a good miscibility in each other*

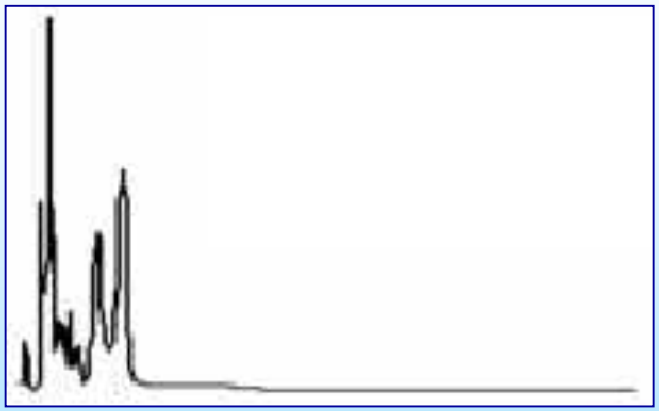


Mobile phase composition:

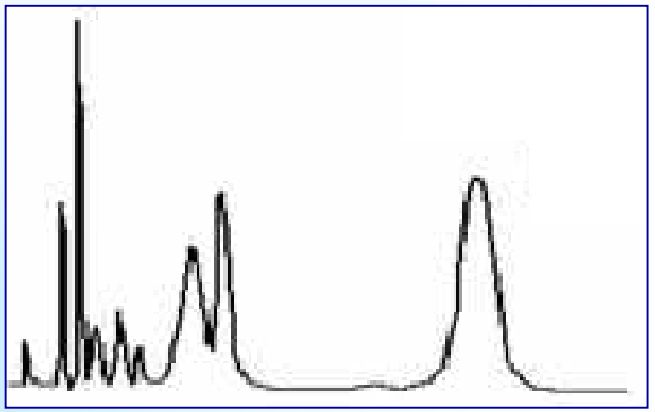
- ***isocratic analysis**: in this procedure the composition of the mobile phase remains **constant** during the elution process*

- ***gradient elution**: in this procedure the composition of the mobile phase is **changed continuously** or **stepwise** during the elution process*

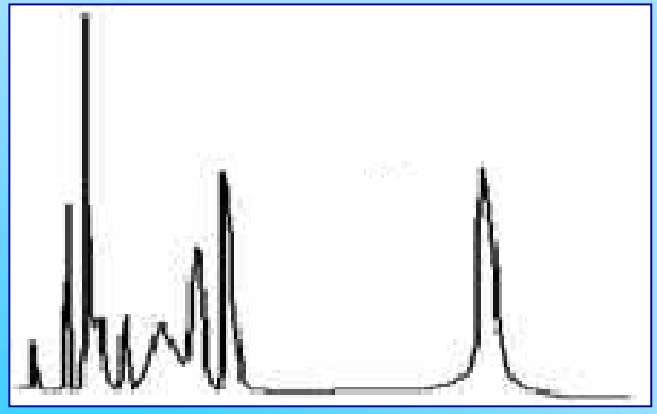
Example of mobile phase optimization



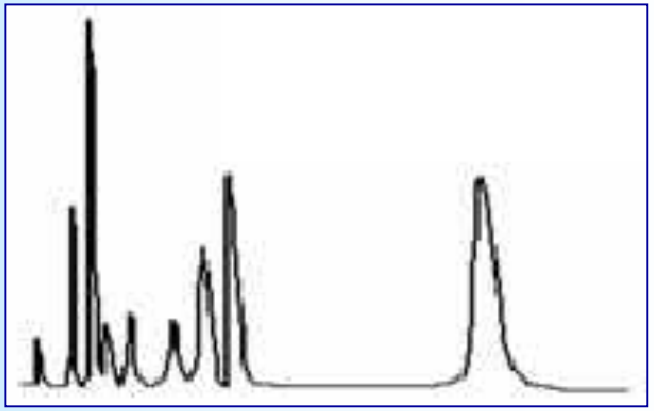
41% CH_3CN – 59% H_2O k' = 5



30% CH_3CN – 70% H_2O k' = 10



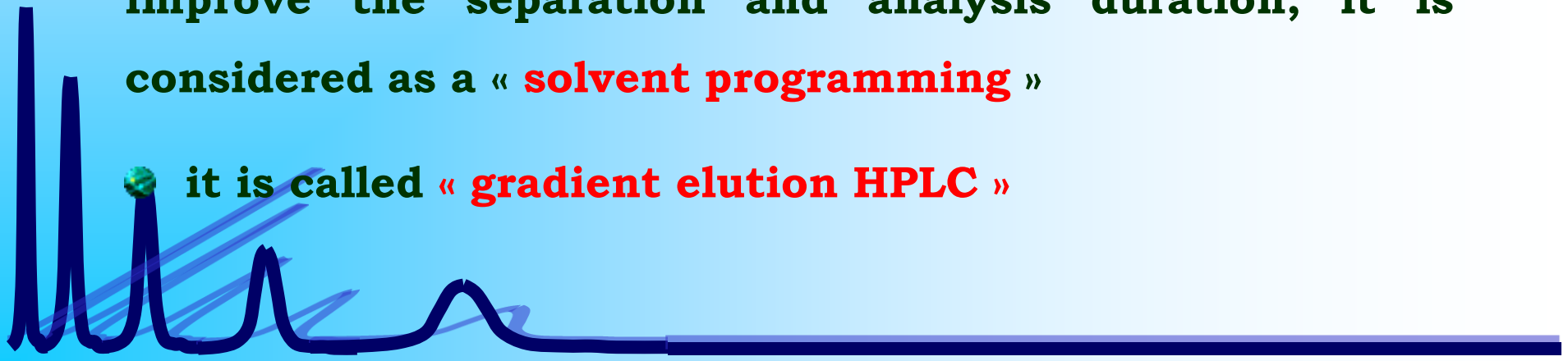
21% CH_3OH – 79% H_2O k' = 10



11% CH_3CN – 12% CH_3OH – 77% H_2O k' = 10

Gradient elution in HPLC:

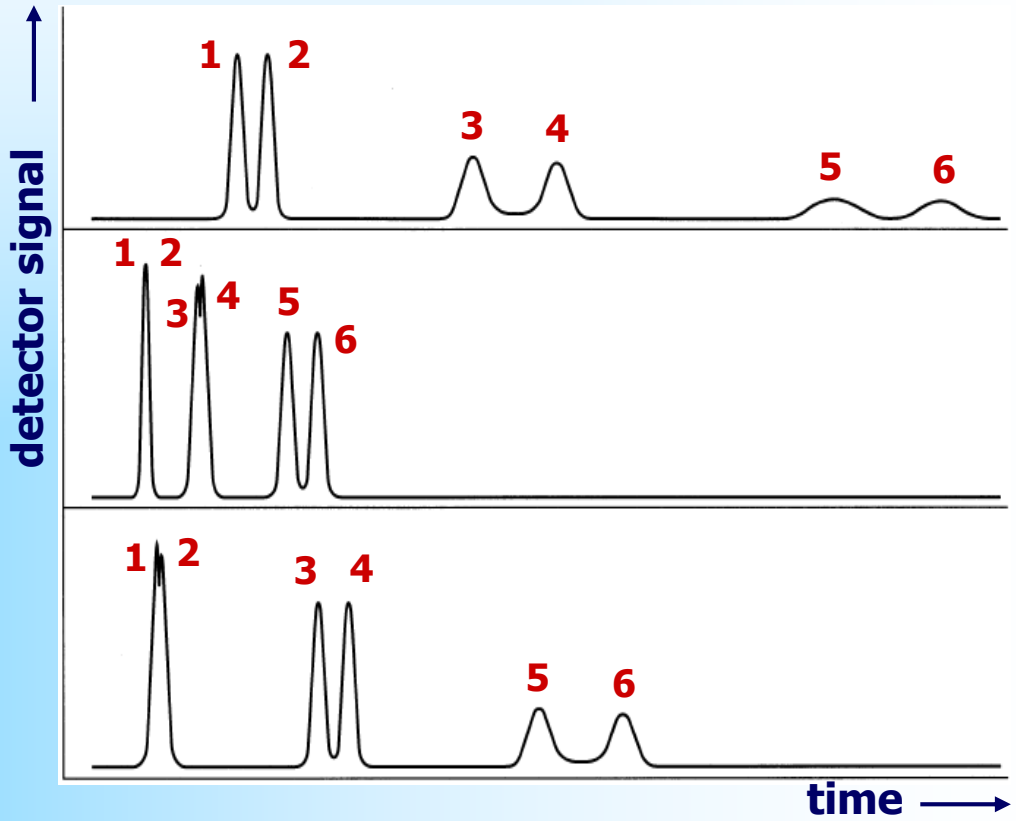
- unlike gas chromatography, the **temperature** has only limited effect on the retention and separation in HPLC
- on the other hand, the **solvent polarity** has a great influence on both retention and separation
- for this reason, a **progressive modification** of the mobile phase composition can adjust its polarity and improve the separation and analysis duration; it is considered as a « **solvent programming** »
- it is called « **gradient elution HPLC** »



Example of a mixture separation:

For the separation of the constituents in a mixture, conditions are rarely optimum for all components:

- ***under isocratic conditions, acceptable resolution of all peaks results in long retention times***
- ***on the other hand, acceptable retention times for last eluting peaks results in poor resolution of first eluting peaks***
- ***in order to optimize the separation, the best solution is to gradually change the mobile phase composition during analysis:***



gradient elution

Gradient elution in HPLC:

Advantages

- **analysis time reduction**
- **improvement of the peaks resolution all over the chromatogram**
- **optimisation of the peak shape**
- **higher sensitivity (higher peaks)**

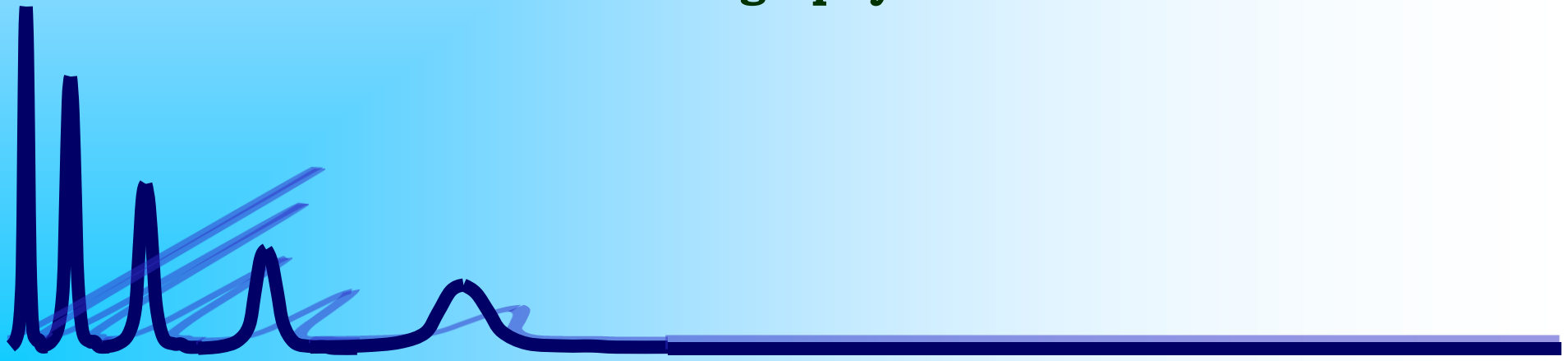
Disadvantages

- ***reproducibility more delicate to obtain***
- ***baseline drift***
- ***difficult gradient programming in certain cases***

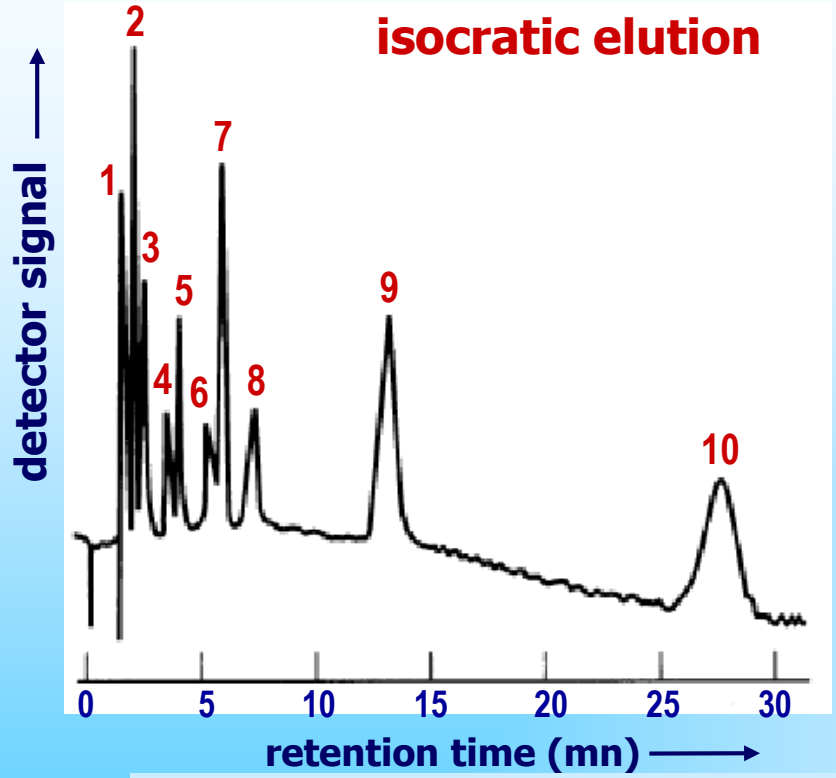
Application of gradient elution in HPLC:

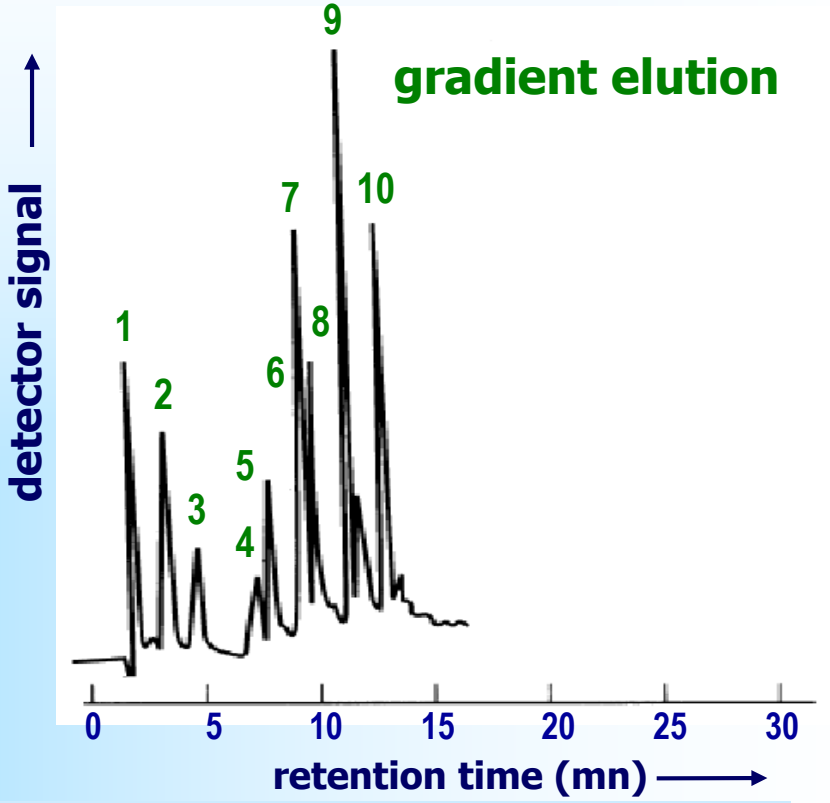
The gradient elution cannot be used for all modes in high performance liquid chromatography

- **liquid-liquid chromatography:** **difficult**
- **bonded phase chromatography:** **yes**
- **adsorption chromatography:** **yes**
- **ion exchange chromatography:** **yes**
- **size exclusion chromatography:** **no**



Comparison of isocratic and gradient elution
Separation of chlorobenzenes

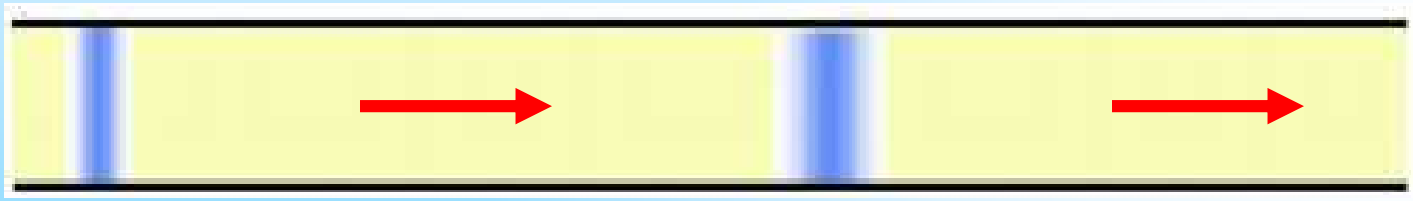




1 benzene
2 monochlorobenzene
3 orthodichlorobenzene
4 1,2,3-trichlorobenzene
5 1,3,5-trichlorobenzene
6 1,2,4-trichlorobenzene
7 1,2,3,4-tetrachlorobenzene
8 1,2,4,5-tetrachlorobenzene
9 pentachlorobenzene
10 hexachlorobenzene

Elution process:

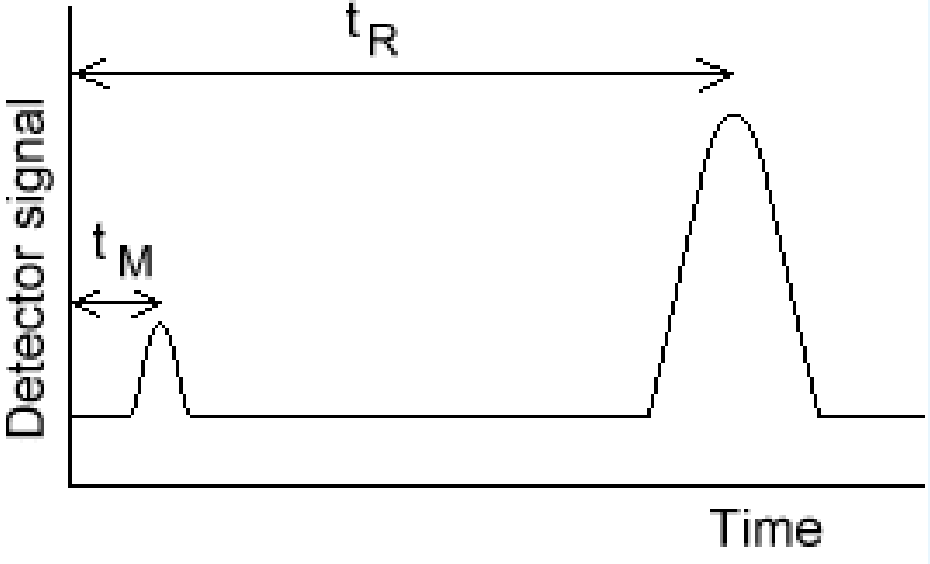
- During the solute transfer in the column, it shows a typical broadening due to the diffusion phenomena (transversal and longitudinal)



- The solute band width increases with the retention time giving a typical peak broadening

The chromatogram:

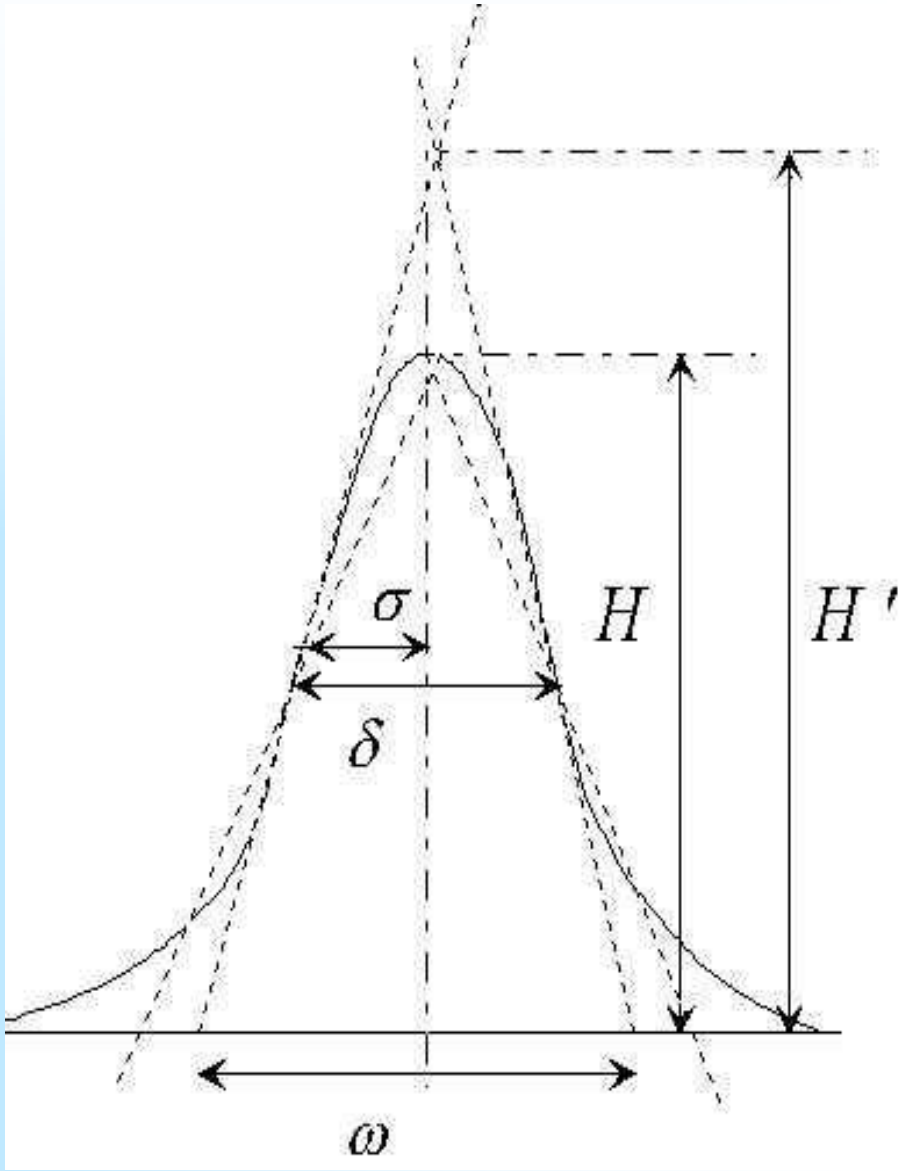
characteristic parameters



- t_M : dead time (for a « non-retained solute »)
- t_R : retention time, characteristic of each solute

Chromatographic peak:

- portion of a differential chromatogram when a *single* component is eluted If separation is incomplete, two or more components may be eluted as one *unresolved peak*
- The measure of band *broadening* (or *bandspreading*) is band width
- if the peak is supposed gaussian, then:
- σ: standard deviation
- δ: width at half-height
- ω: baseline width
- Since is often difficult to accurately evaluate the beginning and end of the peak, most equations use the *width at half-height* δ which can be easily measured on the chromatogram



Some fundamental equations:

- Corrected retention time: $t'_R = t_R - t_M$
- average linear velocity ($\bar{u}$) is measured from the retention time of an unretained substance (t_M) which moves at the same velocity as the mobile phase:

$$\bar{u} = L / t_M$$

- Retention factor (or capacity ratio) k : corresponds to a relative retention:

$$k = t'_R / t_M = (t_R - t_M) / t_M$$

- since: $t_M = L / \bar{u}$

we can write: $t_R = (1 + k) . t_M = (1 + k) . L / \bar{u}$

Hence the retention time is directly proportional to the column length L and inversely proportional to the linear flow rate of the mobile phase $\bar{u}$

- when k is $\leq$ 1.0, separation is poor
- when k is > 30, separation is slow
- when k is 2-10, separation is optimum

Column efficiency:

The chromatographic peaks being supposed gaussian, the peak broadening can be related to the separation and the column efficiency which is evaluated by the number of theoretical plates of the column N which is a number indicative of column performance.

For a gaussian peak, N can be calculated by one of the following equations:

$$N = (t_R / \sigma)^2$$ (σ: standard deviation of the peak)

$$N = 16\,(t_R / \omega)^2$$ (ω: width at baseline)

$$N = 5{,}54\,(t_R / \delta)^2$$ (δ: width at half- height)

In order to compare columns having different lengths, one calculate the plate height or height equivalent to a theoretical plate HETP:

$$H = L/N$$ (L : column length)

H may vary from centimeters (packed columns) to several microns (high resolution capillary columns)

Column efficiency:

- **Column selectivity:**

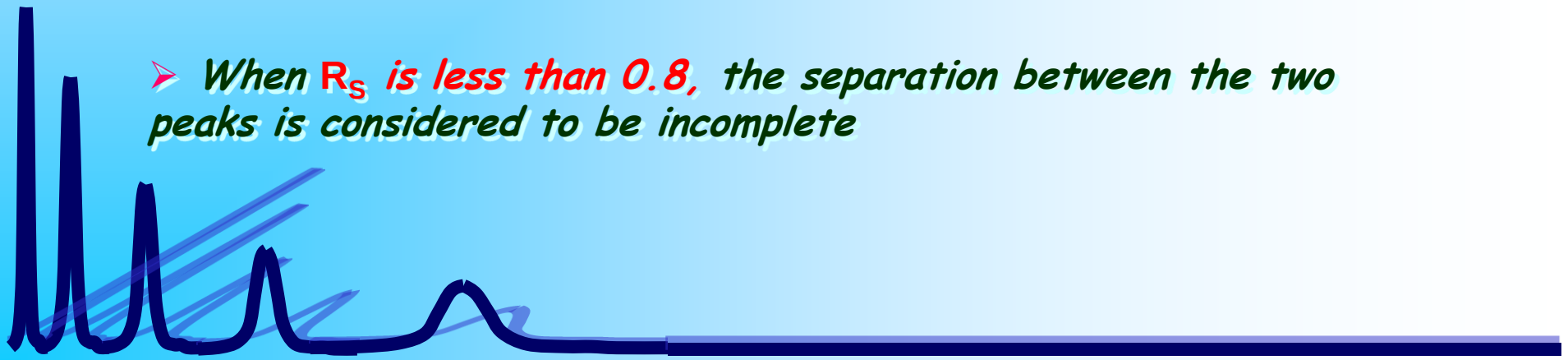
$$\alpha = t'_{R2} / t'_{R1} = k_2 / k_1$$ (separation occurs only if $\alpha > 1$)

- **Resolution between two neighboring peaks:**

$$R_S = 2\,(t_{R2} - t_{R1}) / (\omega_2 + \omega_1) = 1{,}18\,(t_{R2} - t_{R1}) / (\delta_2 + \delta_1)$$

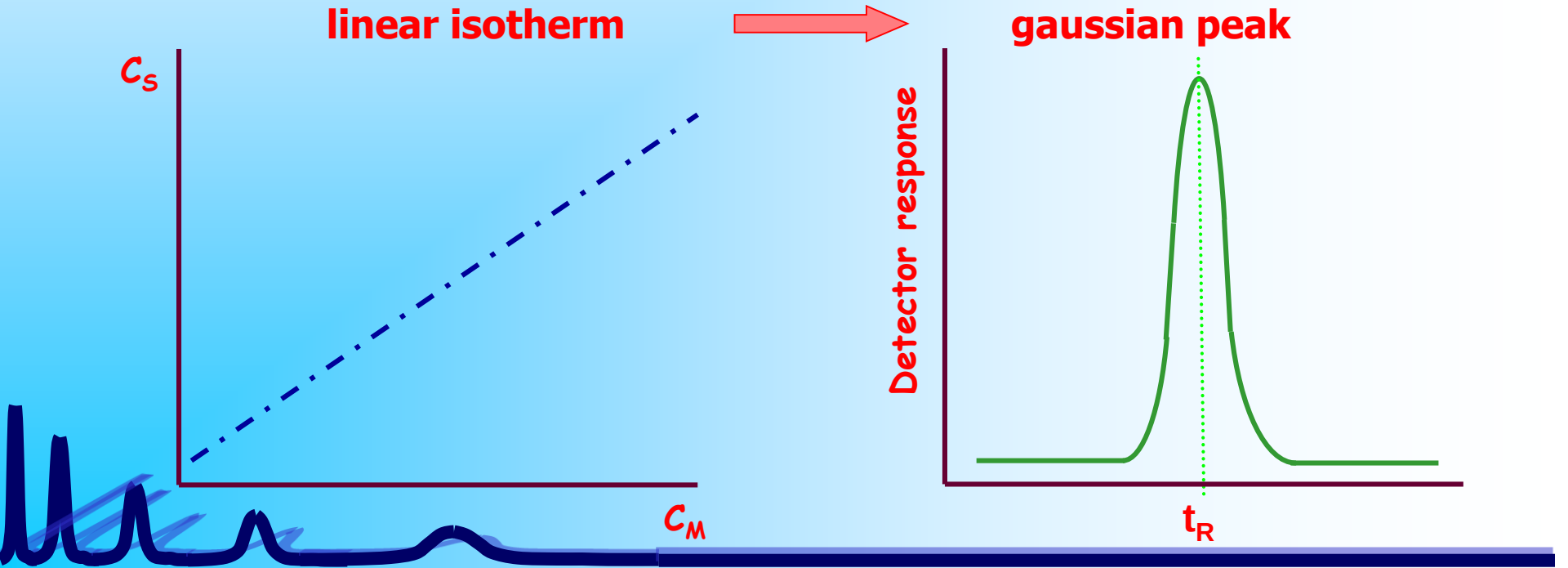
- *For two neighboring peaks, a resolution R_S higher than 1 means a complete separation (for $R_S = 1$, the overlapping peak surface is 2%)*

- *When R_S is less than 0.8, the separation between the two peaks is considered to be incomplete*



Peak shape and broadening

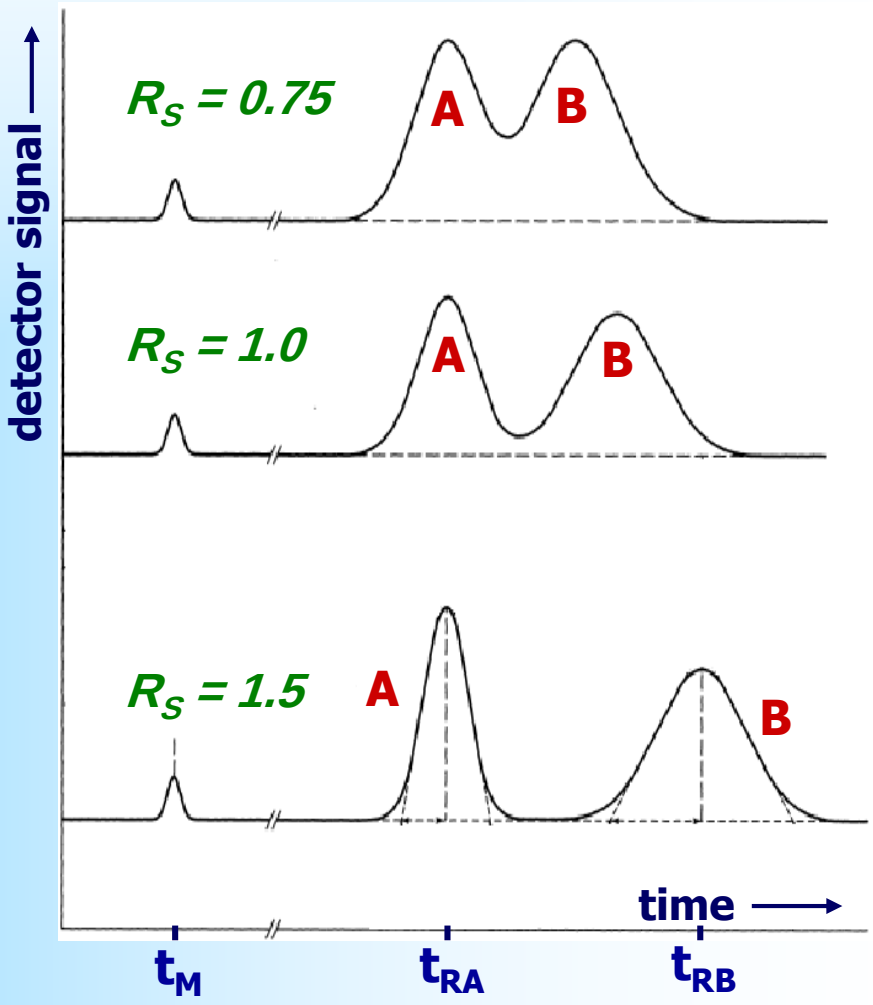
The variation of solute concentration in the stationary phase (C_S) with solute concentration in the mobile phase (C_M), at constant temperature, is known as the sorption isotherm. Simple chromatographic theory assumes a linear isotherm relationship, i.e. the distribution coefficient is constant. Under these conditions the retention time is independent of sample concentration and the peak moves with a constant speed. Given a peak profile with plug-shape distribution on injection, this shape should be maintained as the peak passes through the column to emerge at the exit. However, because of longitudinal diffusion in the direction of flow, the peak takes on a Gaussian distribution



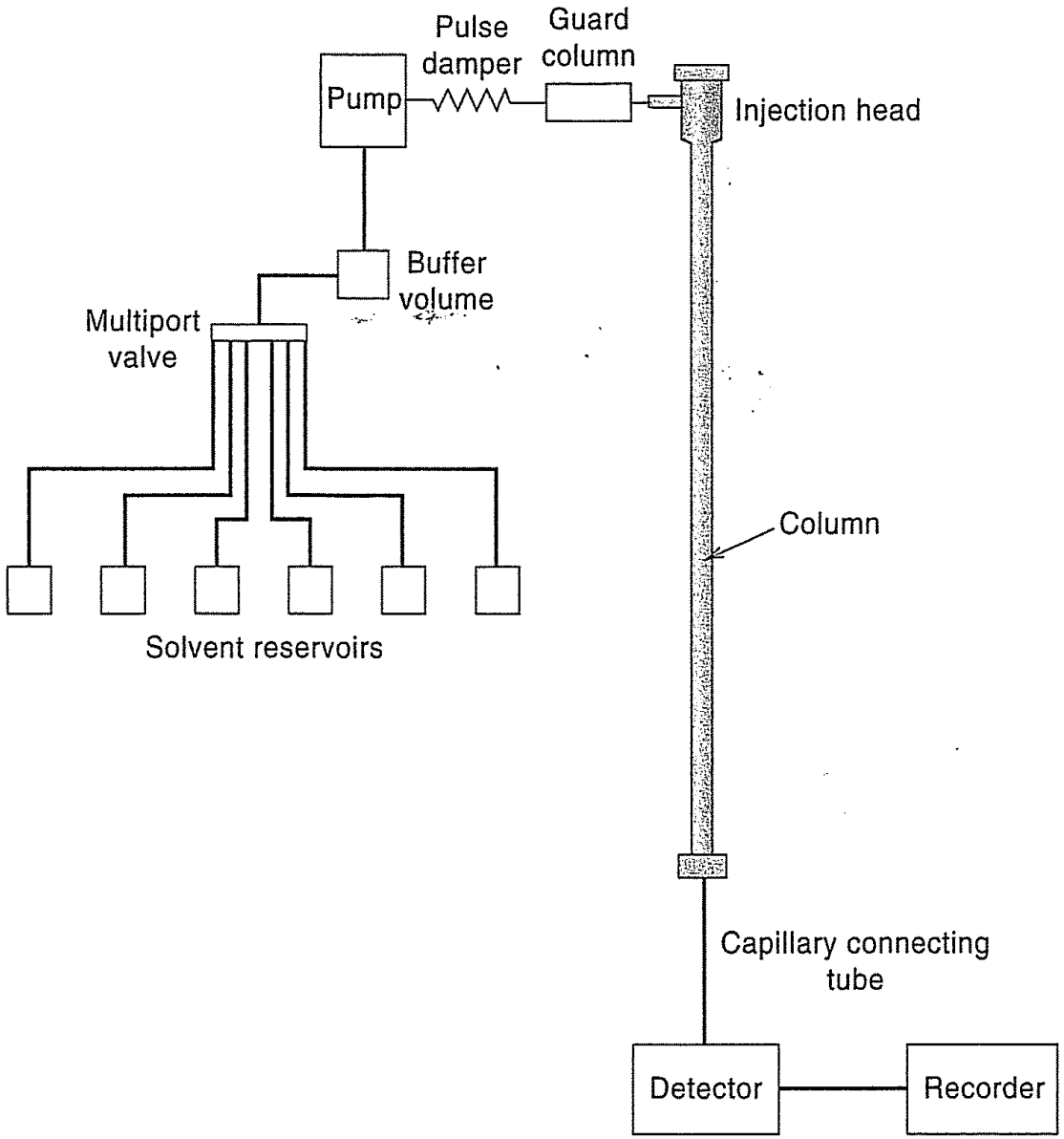
Optimization of column efficiency and resolution:

Resolution (and zone broadening) depends on:

- ū (linear flow rate): low flow favors increased resolution
- H (plate height) (or N number of plates): use smaller particles, lengthen column, viscosity of mobile phase (diffusion)
- α (selectivity factor): vary temperature, composition of column/mobile phase
- k (capacity factor): vary temperature, composition of column/mobile phase



ig. 21.1. Basic components of igh-performance liquid chromato-raph. (Adapted from Analabs, Inc. *esearch Notes*. Copyright © 1971. eproduced by permission.)



HPLC equipment