## Phytochemistry

# Backgrounds of Separation Methods

CHROMATOGRAPHY

## Principles of Chromatography

- Distribution of compounds between two phases:
  - Mobile phase – motile, moving
  - Stationary phase – stable, not-moving
  - Separation based on the way, which distributes the compounds between phases.
  - Distribution coefficient: $D = \frac{c_{stat.phases}}{c_{mob.phases}}$
  - Compounds in constant and dynamic balance between both phases.
  - For a target compound is valid: a position in system is generated by an interaction with stationary phase and a competition of interaction with mobile phase.



Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)

- **Stationary phases:**
  - Solid
  - Liquid
- **Mobile phases:**
  - Liquid
  - Gassy

**Classification of chromatographic methods**

- **According to the alignment:**
  - Column chromatography – scale from capillary analytical methods to large preparative columns
  - Planar chromatography – different forms of TLC
- **According to the modus of separation:**
  - Adsorption
  - Partitioning
  - According to the charge:
    - Ion exchange
    - Ion pair
  - Based on size exclusion
  - Bio-affinity

---

- Dynamic balance of compounds distribution in stationary and mobile phase – different molecules spend different time both in mobile and stationary phase.
- When compounds distributed in mobile phase only, they move in the same speed and time.
- Stationary phase makes the retention.
  - The rate of retention could be described by:
    - Retention time.
    - Retention volume.
    - Capacity factor.



Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)

- Retention time $t_R$:
  - Time between injection of sample into column and detected elution of compound.
  - Depends directly on flow rate of mobile phase in column.
  - Sum of time which compound spends on stationary phase and time spended in mobile phase $t_M$
  - Corrected retention time: $t'_R = t_R - t_M$
- Retention volume: $V_R = F \cdot t_R$

  *F… flow rate*
- Capacity factor $k'$: $k' = \dfrac{t_R - t_0}{t_0}$

  $t_0$… *retention time of non-retained compound*
- Retention time of bounded compound: $t_R = L / v \times (1 + k')$

- Column Efficiency:
  - Repeated formation of balanced state.
  - Not in steps, but dynamic process.
  - Number of theoretical plates of column *N*:
    - How many times the balance is established.
    - Higher number → better separation
    - Could be used for description of separation efficiency.
    - Can describe how broad will be zones of separated compounds.
    - Depends on the column length. Longer column → better separation.



Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)

- Separation and selectivity α:
  - Different compounds → different distribution coefficients.
  - Compounds retained selectively → different retention times.

$$\alpha = K_B / K_A = k'_B / k'_A = t_{RA} / t_{RB}$$

- Resolution $R_S$:
  - Two parameters for calculation:
    - Distance of peak apexes.
    - Width of peaks at base.

$$R_S = \frac{t_{RB} - t_{RA}}{1/2 \times (wA + wB)}$$

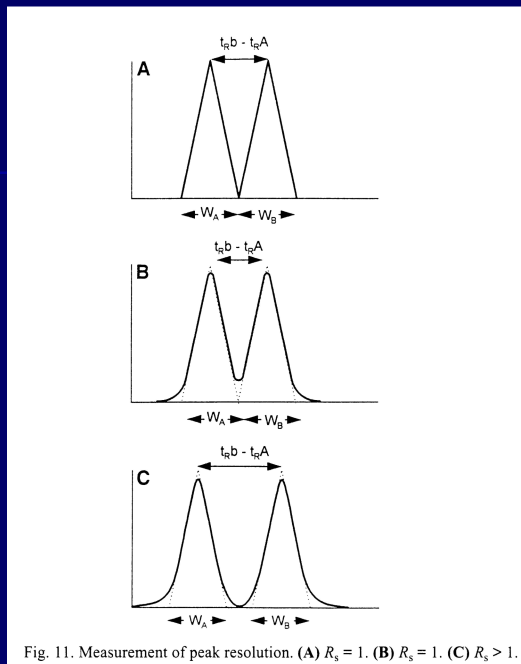
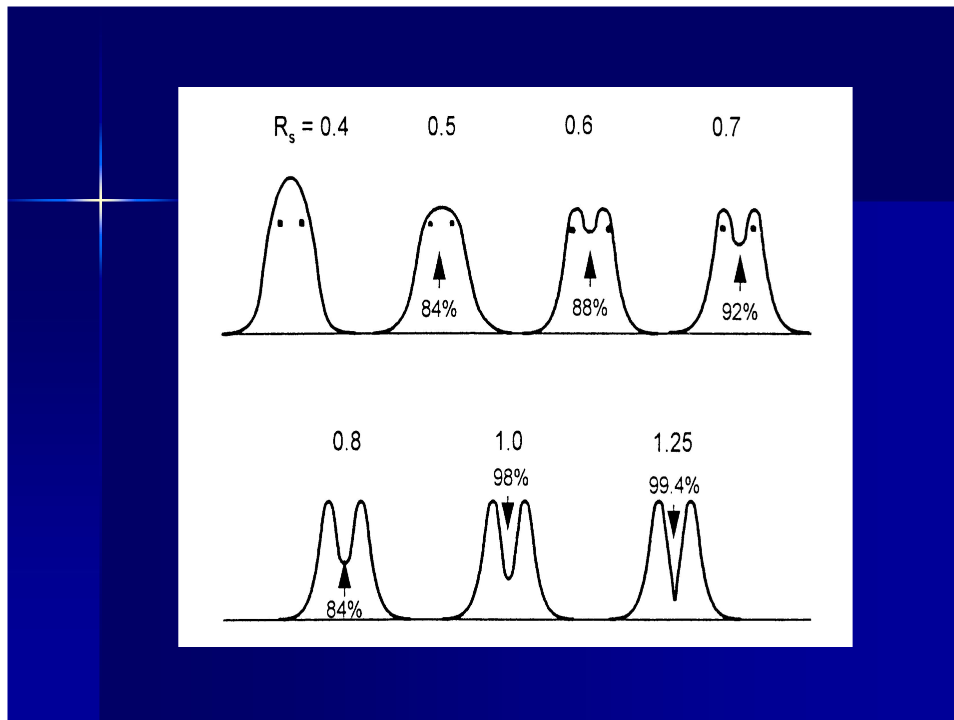



Fig. 11. Measurement of peak resolution. **(A)** $R_s = 1$. **(B)** $R_s = 1$. **(C)** $R_s > 1$.



Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)




## How to read a chromatogram to get an information.

1. **Identification (quality)**
   - Retention time- each compound displays the characteristic retention time under stable and defined conditions.
   - The same retention time $t_R$ for two samples ⟶ probably the same compound.
   - Method of simultaneous injection.
   - Verification with help of detection method.
2. **Physico-chemical character of compound**
   - Non-polar compounds ⟶ slow elution on reversed phase
     ⟶ fast elution on normal phase
   - Polar compounds ⟶ fast elution on reversed phase
     ⟶ slow elution on normal phase
3. **Amount (quantification)**
   - For target compound is the AUC (Area Under Curve) direct proportional to amount of compound.
   - Standard with known concentration ⟶ calibration curve.
   - Estimation of quantity according to the peak height.



Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)

## Quantitative analysis

### *Method of internal normalization*

- Basic condition – all components of sample must be eluted.
- Detector must give linear response depending on concentration of all analyzed compounds
- Percentual composition of mixture is derived from AUC of all peaks.
- Method is simple. Problems when detector response different or not linear for all analyzed compounds.

### *Method of absolute calibration*

- Based on injection of known amounts of sample and standard under the same experimental conditions.
- Content is evaluated from calibration curve or from direct comparison of AUC of standard and sample peaks.
- At least two injections on column (sample and standard).

## Quantitative analysis

### *Method of internal standard*

- The mixture of different weight ratio is prepared from standard and pure analyte, the analysis is then carried out.
- Calibration curve is based on ration AUC (sample)/ AUC (standard) and weight (sample)/weight (standard)
- When a sample with unknown concentration is analyzed, this sample is added to standard and from the ration of AUC is possible to calculate the amount of target compound.
- Advantage – it is not necessary to know the exact amount of injected sample.
- Disadvantage – difficult to find good standard.

### *Method of standard addition*

- Based on addition of defined amount of analyzed compound to a sample.
- Two analyzes: 1. without addition, 2. with addition.
- Increase of AUC of compound peak is directly proportional to an amount of added analyte.



Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)

## How to improve a chromatographic separation?

- Capacity factor
- Theoretical plate number
- Selectivity

**Capacity factor *k′***

- $k' = 0$ compound not retained
- $k' > 20$ $t_R$ not effective (too long)

How to affect:

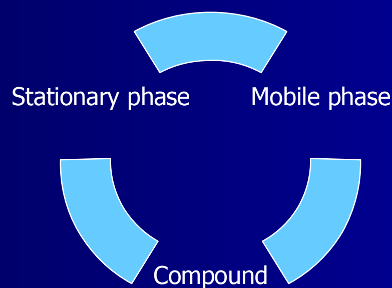
1. Good choice of mobile phase.
2. Using of gradient elution.

**Efficacy**

- Increase of $N$ affects the peak width and quality of resolution, but did not affect the principle of separation. Relative $t_R$ stays unchanged.
- Affect: stationary phase particle size.

Selectivity

3 groups of molecular interactions





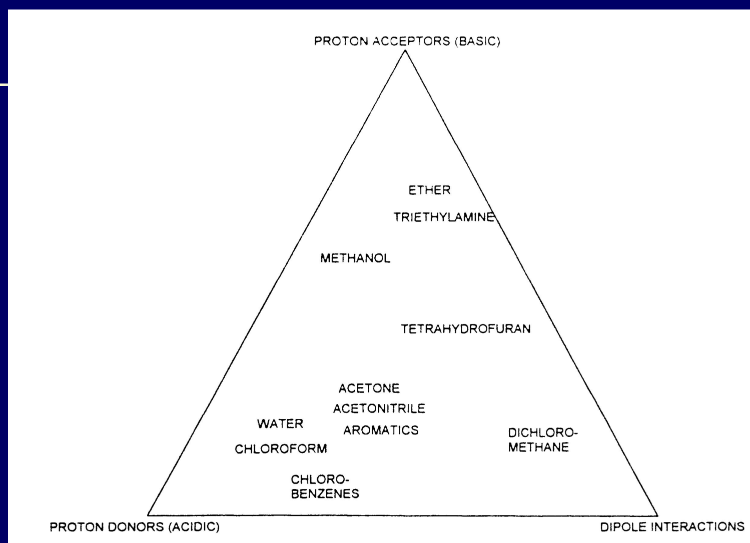

Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)

# Eluotropic series

**Eluotropic Series of Solvents**

| Solvent | $E^0(Al_2O_3)$ | Boiling pt., °C | Viscosity, $mN.s.m^{-2}$(20°C) | UV Cutoff, nm |
|---|---|---|---|---|
| Pentane | 0 | 36 | 0.24 | 210 |
| Cyclohexane | 0.04 | 69 | 0.98 | 210 |
| $CCl_4$ | 0.18 | 77 | 0.97 | 265 |
| Toluene | 0.29 | 111 | 0.59 | 286 |
| Diethyl ether | 0.38 | 35 | 0.25 | 218 |
| Chloroform | 0.40 | 62 | 0.57 | 245 |
| Dichloromethane | 0.42 | 40 | 0.44 | 235 |
| Tetrahydrofuran | 0.45 | 66 | 0.55 | 220 |
| 2-Butanone | 0.51 | 80 | 0.32 | 330 |
| Acetone | 0.56 | 56 | 0.32 | 330 |
| 1,4-Dioxane | 0.56 | 107 | 1.44 | 215 |
| Ethyl acetate | 0.58 | 77 | 0.45 | 255 |
| Diethylamine | 0.63 | 115 | 0.33 | 275 |
| Acetonitrile | 0.65 | 82 | 0.37 | 190 |
| 2-Propanol | 0.82 | 82 | 2.50 | 210 |
| Ethanol | 0.88 | 78 | 1.20 | 210 |
| Methanol | 0.95 | 64 | 0.59 | 210 |
| Water | 1.00 | 100 | 1.0 | – |

# Snyder triangle






Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)

# Liquid chromatography

- The most universal and most often used method in natural compounds separation

| Mechanisms of Separation | Stationary phase example |
| --- | --- |
| Adsorption | Silica, aluminium oxid, polyamides |
| Rozdělování | RP- materials (C2, C8, C18), celullose |
| Ion pair | RP- materials (C18) |
| Ion exchange | Ionex, catex |
| Complexation | catex with $Ag^{I}$ |
| Chiral separation | Chiral phases or mobile phases with chiral modifier |
| Gel filtration, size exclusion | Gels, Sephadex LH-20 |

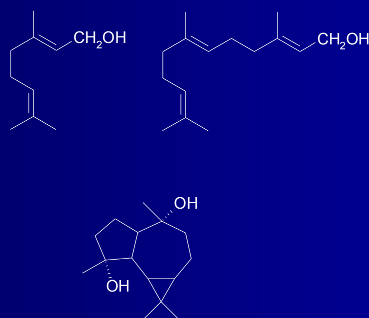
## *Adsorption liquid chromatography*

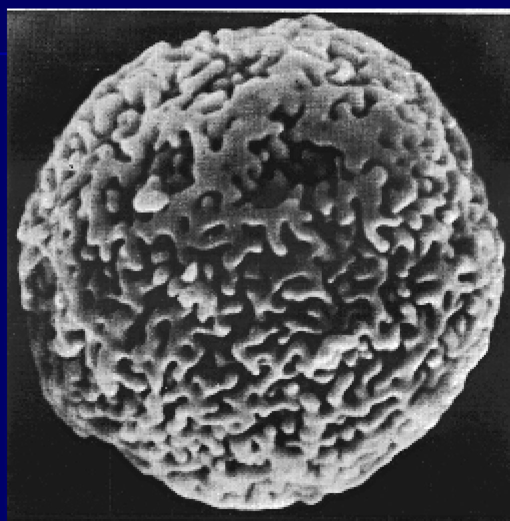
- Interaction of analytes with stationary phase is based on adsorption
- Distribution constant based on series of parameters (volume of monomolecular layer of solvent, activity of adsorbent, elution power of solvent)
- The interaction should be physical, not chemical.
- The separation should be carried out in linear part of adsorption isotherm. In non-linear part of isotherm we will get „tailing" peaks.
- Most common adsorbents used:
  - Silikagel or aluminium oxid (alumina)
  - Carbon organic adsorbents
- Most common mobile phases:
  - Non-polar carbohydrate + polar organic modifier



Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)

- Silikagel
  - Gel of silic acid
  - Adsorption chromatography
  - Normal phase
  - Polar characteristics – presence of –OH functional groups.
  - Silikagel is weakly acidic (used at pH = 2 to 8)
  - Under normal circumstances – separation of compounds which differ in polar functionalities. Differences in non-polar parts – no efficient separation.





Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)

## Aluminium oxid

- Aluminium oxid suitable for separation of basic compounds
  - Acidic
  - Neutral
  - Basic

## Chromatography on bonded phases

- Different functional groups are bonded on carrier (usualy silikagel)
- The most common – chemically bonded phases of siloxane type (prepared by reaction of silikagel with chlorsilanes)
- Disadvantage of this sorbent type is limited pH range (2 to 8, different pH can cause the „bleeding" of stationary phase)
- Typical example of chemically bonded phase: C8, C18, CN-, phenyl, chiral compound
- Non-reacted (free) –OH groups of silikagel should be deactivated
- According to the bonded functional group can be separated different compounds
  - For example: bonded chiral phases can separate optical izomers
- Exact description of separation process is difficult
  - Process is basic on solvophobic (hydrophobic) effect
  - Important is dissolution of compound in mobile and stationary phase and competition



Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)

## *Chromatography on bonded phases*

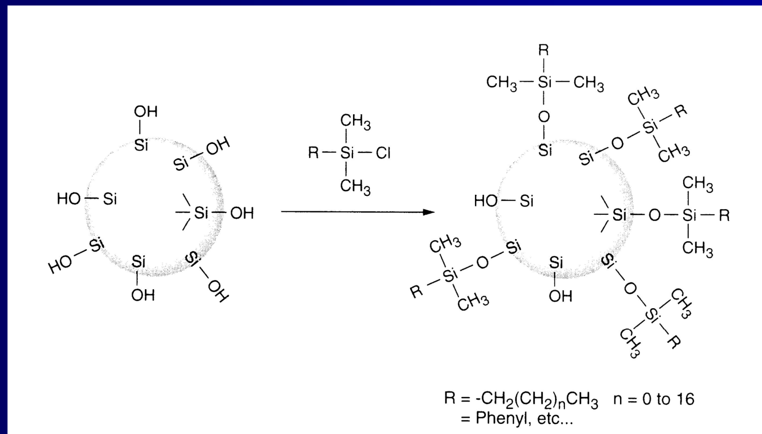
- Stationary phases used are of non-polar character
- Mobile phase : water with addition of polar organic solvents (alcohols, acetonitril, dioxan, tetrahydrofuran, acetone)
- Selectivity is strongly affected by mobile phase composition
- Elution power of mobile phase rises with decreasing polarity
- Chromatography on reversed phase is suitable of separation of homologic compounds
- RP HPLC rules modern separation
- Nomenclature "reversed phases" is used from historical reasons

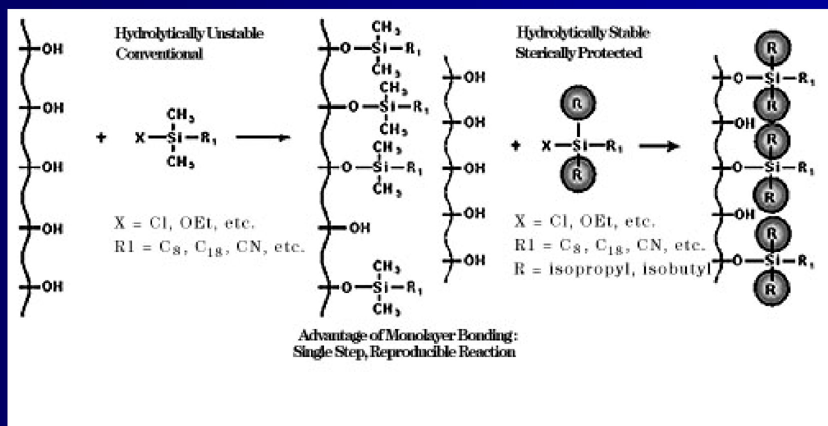
## Modified silica

- Silikagel + carbohydrate side-chain
- C2, C8, C18
- Distribution chromatography
- Non-polar character
- Reversed phase
- Changes of non-polar part of analyzed molecule will affect separation.
- Limited pH range.



Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)






Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)

## *Chromatography of ion pairs*

- Often used for ion compounds, combination with chemically bonded phases (C18)
- Mobile phase contains ion pair reagent with opposite charge, than is the charge of analyzed compound
  - For anion analysis tetraalkylamonium salts are often used
  - For cation analysis dodecyl sulphate is often used
- Incurred ion pair displays properties of polar organic molecule and pass from mobile phase to stationary phase.

## *Ion exchage chromatography*

- Technique suitable for separation of ionic nature based on electrostatic interaction
- Analyte during the separation migrates in the direction of ion exchanger, than diffunds through the exchanger where ion exchange takes place.
- Proper choice of ion exchange can affect parameters of separation.
  - Different types of catexes and anexes
  - Parameters of separation are based on size of molecules, size of and polarity of charge, on ionic power of mobile phase, degree of solvatation…
- Affinity of compound separated rises with increasing charge of ionex
- When parameters of charge and size of different molecules separated are the same, superiority is given to compound in higher concentration

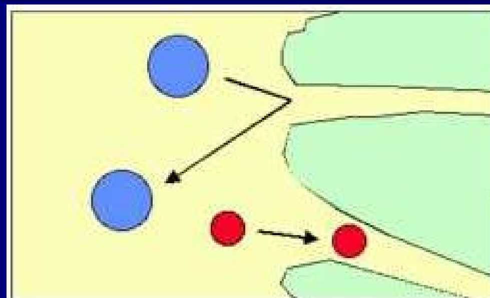


Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)

## *Size exclusion - gel permeation chromatography*

- Separation based on size and shape of compound particles
- Mobile phase just carry the separated compounds through the column, it does not participate on separation process directly.
- Huge molecules (macromolecules) do not permeate into pores of gel and elute from the column as first
- Small molecules permeate into pores of gel, their movement through the column slows.
- Exclusion limit = size of particles which will not permeate inot pores and therefore will not be separated.
  - Can be used for determination of molecular weight
- Material
  - agarose and dextran gels
  - organic polymers (co-polymer styrene + divinylbenzene)
  - Use of poresic, rigid glasses and silikagel
- Mobile phase must dissolve analytes, but should not react with analytes, must wet surface of stationary phase and must be compatible with a mode of detection.
- Often used: water and water buffers, isopropanol.
- ***Analyses of macromolecules, proteins, peptides, alergens***

- Sephadex LH-20
- Hydroxypropyldextrane gel
  - According to conditions:
    - Adsorption
    - Partitioning
    - Gel filtration
    - Combination





Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)

## *Bio-affinity chromatography*

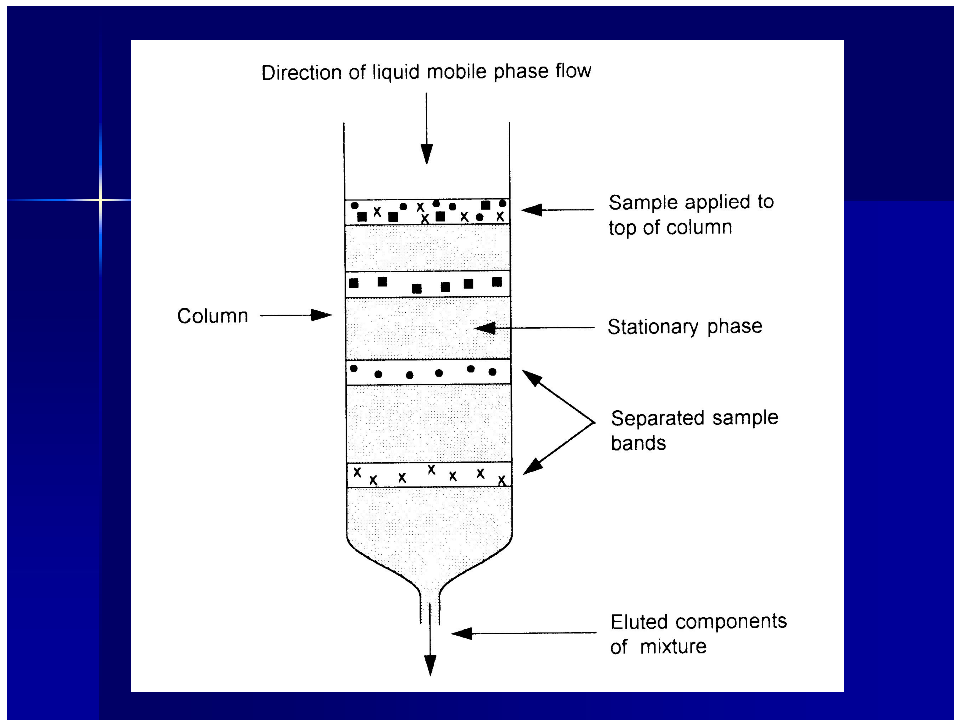
- It is based on specific interaction between biological active compounds and their „anti-compounds"
- Examples of such as specific interation:
- *Antibody- Antigen*
- *enzyme – substrate*
- *lecithine – glycoprotein*
- Bio-affinity chromatography is used for separation, isolation, and purification of sample
- Stationary phase must be first at first bonded on suitable inert carrier.
- Ligand (stationary phase) must have high affinity for determined compound
- Bond of ligand and analyte can be disturbed by change of experimental conditions or using a deforming buffer.

## Types of liquid chromatography

- Classical column chromatography
  - Oldest, simplest.
    - Commonly normal phase (silikagel, aluminiumoxid).
    - Large particle size (60-200 μm)
    - First step of separation.
    - Big sample amounts.
    - Sample application:
      - Liquid sample (good solubility in mobile phase)
      - Solid sample (bad solubility, adsorption on silikagel 1:1)



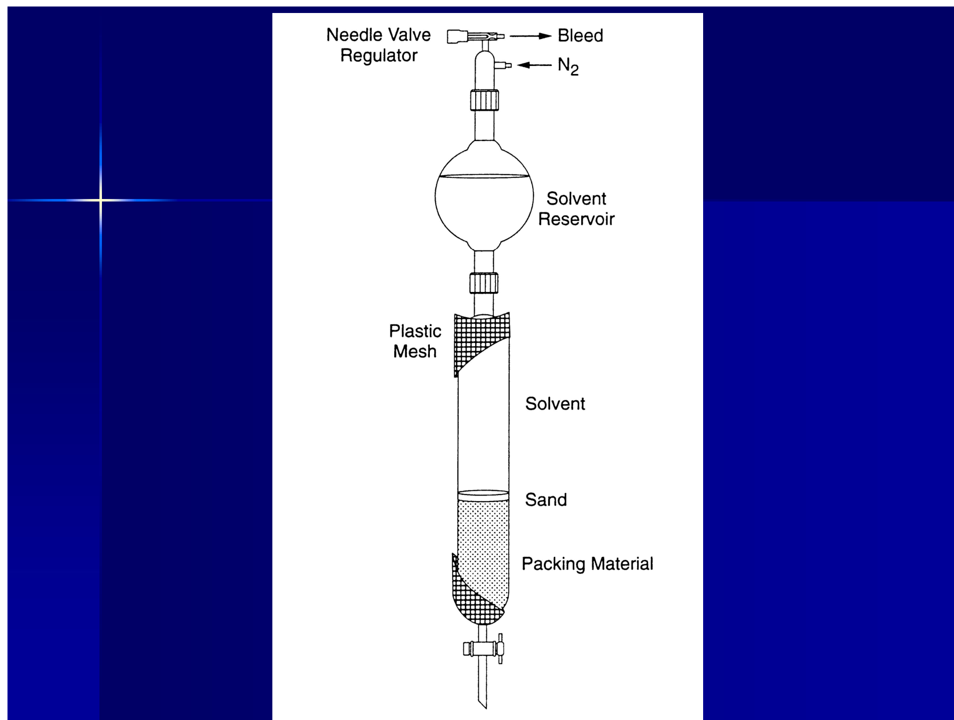
Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)




- Flash chromatography
  - The same principles as classical column.
  - Overpressure upon the mobile phase (air, $N_2$).
  - Higher pressure ⟶ higher velocity of mobile phase ⟶ lower sorbent particle size 40-60 µm.
  - Faster and/or better separation



Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)




- Low-pressure and medium pressure chromatography (LPLC a MPLC)
  - LPLC
    - Hybrid of flash chromatography and HPLC.
    - Mobile phase is pressurized to 1-6 atm.
    - Simple pump.
    - Particle size similar to flash chromatography.
    - Different column size, different types of sorbents.
  - MPLC
    - Sophisticated apparatus.
    - Smaller particles 10-40 μm.
    - Pressure 3-50 atm.



Create PDF files without this message by purchasing novaPDF printer (http://www.novapdf.com)