# Chromatography

# Chromatography

- Chromatography is an area of science that studies the processes based on the movement of the zone of substance along the sorbent layer in the flow of the mobile phase and associated with repeated sorption and desorption acts.
- In any variant of chromatographic methods, a combination of fixed (stationary) phase (FP) and a mobile phase (MP) is used.
- The mobile phase (gas, liquid) in the process of chromatography continuously moves along the stationary phase (solid, liquid), so that the particles of chromatographed substances, transported together with MP, can repeatedly convert from the mobile phase to the stationary phase and back.
- The separation of substances by chromatography is based on different affinities of the separated substances to the mobile and stationary phases. The variety in affinity leads to a difference in the speed of the particles of the movement of the separated substances together with the mobile phase and eventually to their separation.

# Conditions

The following conditions must be fulfilled to perform a chromatographic analysis:

1. the presence of MP and FP;
2. repeated acts of sorption and desorption of the separated components moving together with MP along the FP;
3. the balance of sorption/desorption should be established quickly enough.

# Classification by the mechanism of separation of substances.

- a) Adsorption chromatography — based on the use of a different ability of the separated components to engage in a specific interaction with the surface of the adsorbent — FP — due to adsorption.
- b) Distribution chromatography — based on the use of differences in the distribution coefficients of the separated components between MP and FP, which is a liquid.
- c) Ion-exchange chromatography — based on the different ability of separated components ions in M (usually a liquid solution) to exchange with FP ions.

# Classification by the mechanism of separation of substances.

- d) Chemichromatography - based on the different ability of the components of the separated mixture to undergo certain chemical reactions with reagents contained in the FP. Sometimes ion-exchange chromatography is considered a special case of chemichromatography, taking into account that it is based on reversible chemisorption of ions in FP.
- e) Size-exclusion (molecular-sieve, penetrating) chromatography is based on the use of differences between the size (effective diameter) of the separated components particles and the pore size of the FP, which is a sorbent - porous substance
- f) Other chromatographic methods, such as electrochromatography (electrophoresis), based on the unequal ability of different ions in a solution to move under the influence of an external electric field.

# Classification based on the aggregation state of the phases.

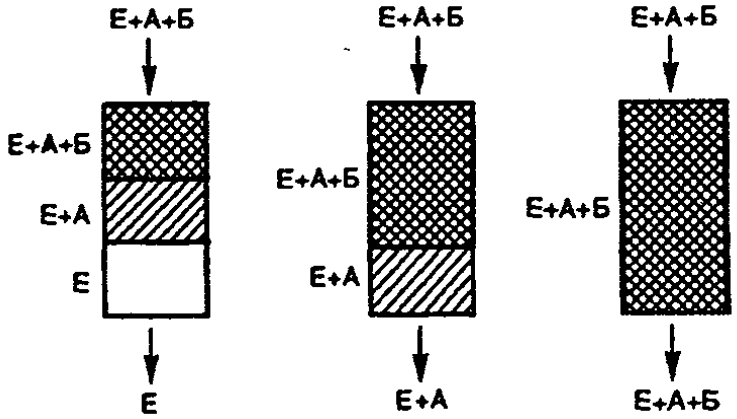
| FIXWD PFASE (FP) | MOBILE PHASE (MP) | METHOD |
|---|---|---|
| *ADSORPTION CHROMATOGRAPHY* | | |
| Solid | Liquid | Liquid adsorption chromatography |
| Solid | Gas | Gas adsorption chromatography |
| *PARTITION CHROMATOGRAPHY* | | |
| Liquid (thin film) | Liquid (not miscible with FP) | Liquid partition chromatography; high-performance liquid chromatography |
| Liquid (thin film) | Gas | Gas-liquid chromatography |

# Classification based on the method of relative phase displacement (on the method of obtaining a chromatogram).

- Frontal
- Eluent (elution development method)
- Displacement chromatography

# Frontal chromatography

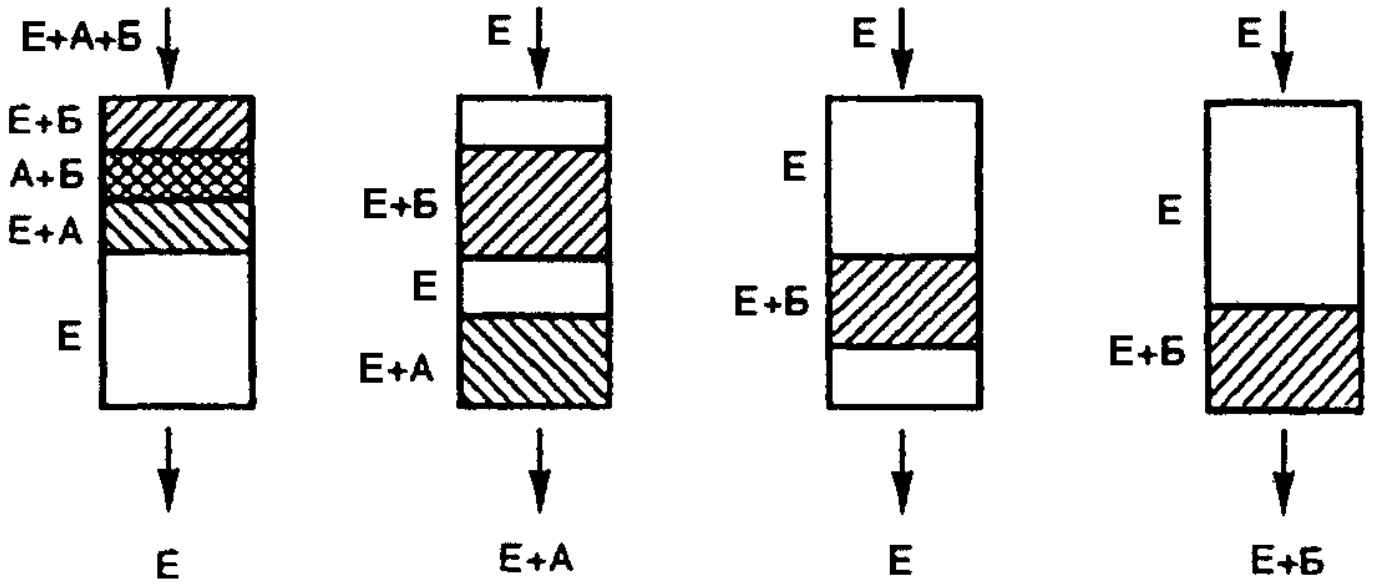
- In this chromatography method, the analyzed solution, containing the separated components A and B in addition to solvent E is continuously fed into the sorbent-filled chromatographic column, until the end of the chromatography process.
- At the beginning of chromatography pure solvent E exits the column. Component A, which has lower affinity for the sorbent (FP) than component B, moves faster than component B and exits before it. The solution of component A in solvent E exits the column. Component B “lags” behind the area of component A. Then, a mixture of solvent E with both components A and B exits the column
- Frontal chromatography allows separating only part of one component (in this case — component A), because a mixture of both components is continuously fed to the column.



# Eluent chromatography

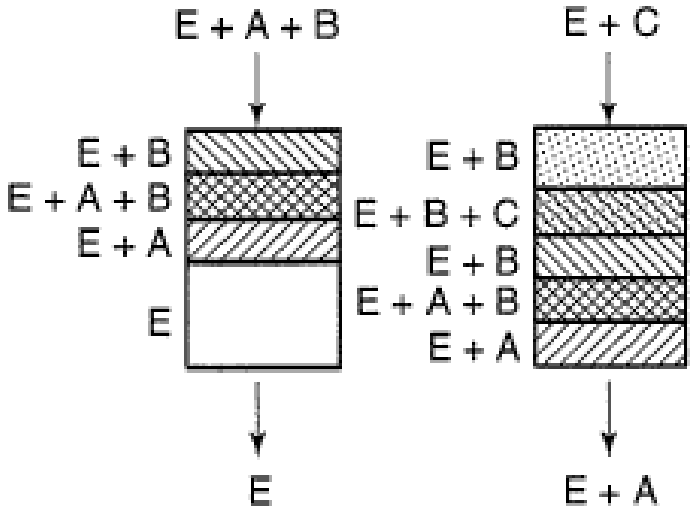
- A solution of the separated substances A and B in solvent E is introduced into the chromatographic column filled with the sorbent. Then these substances are washed out with a pure solvent (eluent) E.
- When eluding with a pure solvent E, components A and B move along the sorbent (NF) at different speeds. Component A, which has a lower affinity for NF, is transferred faster than component B, which has a higher affinity for NF. With a sufficient column length, components A and B are completely separated: first, the zone of component A is eluted, then the zone of pure solvent E, and finally the zone of component B.
- The process of washing out the components is called elution.
- The solvent (or solution) used for elution is called an eluent, and the solution (or solvent) coming out of the column is called an eluate.
- Eluent chromatography allows almost complete separation of the components of the analyzed mixture. The disadvantages of the method include dilution of components A and B with solvent E at the exit from the chromatographic column, which leads to a decrease in their concentration in the eluate.

# Eluent chromatography



# Displacement chromatography.

- The eluent, not a pure solvent E is used, but a certain substance B (for example, its solution in E), which has an affinity for the sorbent (NF) greater than that of components A and B. Substance B thus plays the role of a displacer: it displaces components A and B from the NF.
- A mixture of the separated components A and B with solvent E is introduced into the chromatographic column, after which a solution of the displacer B in solvent E is added.
- As the elution proceeds, the eluate first receives a pure solvent E, then successively E + A, E + A + B, E + B, E + B + C, and E + B. Thus, along with the zones of separated components A and B (diluted with solvent E), the solvent-diluted zones of the mixture of components A + B and B + B also pass into the eluate.

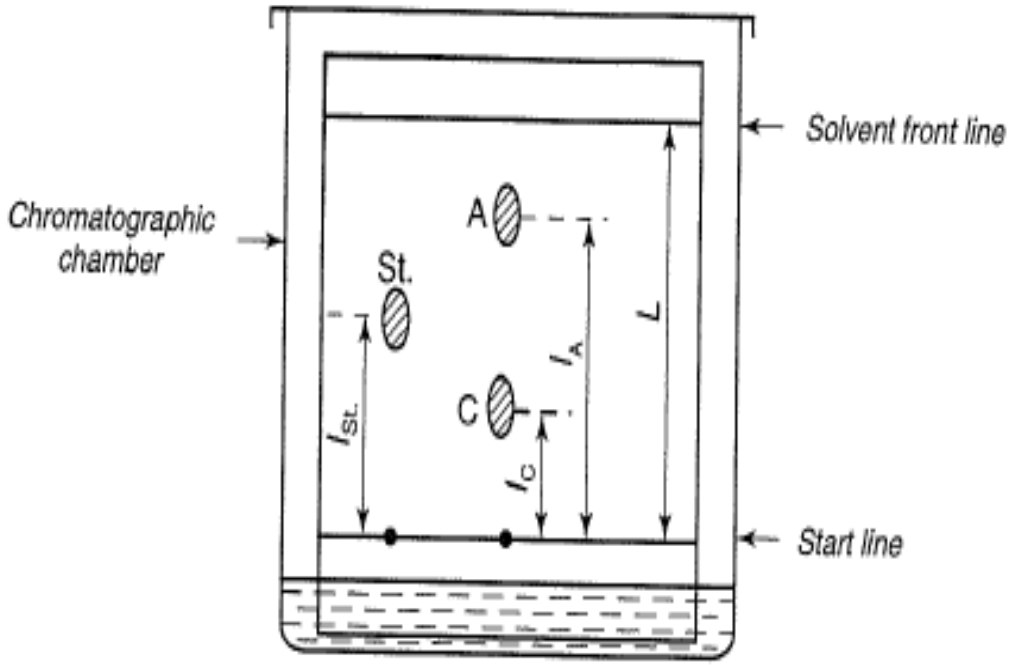


# ADSORPTION CHROMATOGRAPHY. THIN-LAYER CHROMATOGRAPHY.

- Thin-layer chromatography-abbreviated TLC-is a type of planar chromatography, in which the adsorbent is used as a thin layer on the plate (a plate glass, metal, plastic).
- The size of the plate can be different (length and width - from 5 to 50 cm, although this is not strictly limited). Ë start line (at a distance of 2-3 cm from the bottom edge of the plate) and ú finish line of the solvent are marked (for example, with a pencil) on the plate surface carefully, so as not to damage the sorbent layer.
- The sample a small amount of liquid containing a mixture of substances to be separated, such as two substances A and B in a suitable solvent is applied by a micro-syringe, capillary to the start line of the plate. The solvent is allowed to evaporate, then the plate is immersed in a chromatographic chamber in liquid MP, which is a specially selected for this case solvent or a mixture of solvents. Under the action of capillary forces, MP
- A small amount of liquid containing a mixture of separable substances (A and B) is applied to the start line of the plate (with a micro — syringe, capillary). Allow the solvent to evaporate, after which the plate is immersed in a chromatographic chamber in the liquid phase of PF,

# THIN-LAYER CHROMATOGRAPHY.

Under the action of capillary forces, MP spontaneously moves along the FP from the starting line to the solvent front line, carrying the components A and C of the sample, which move at different speeds. In the considered case, the affinity of component A to FP is less than the affinity to the same phase of component C, so component A moves faster than component C. After the solvent front line is reached by the mobile phase (solvent) during the time r, chromatography is stopped, the plate is removed from the chromatographic chamber, dried in air and the position of the spots of substances A and C on the surface of the plate is determined. Spots (zones) are usually oval or round-shaped. In the considered case, the spot of component A moved from the start line to a distance *la*, the spot of component C moved to a distance *lc*, and the solvent has moved to a distance L.



# To characterize the shared components in the system enter $R_f$ mobility coefficient (or $R_f$-factor):

$$R_f = V_i/V_E = (l_i/t)/(L/t) = l_i/L$$

where $V_i = l_i/t$ and $V_E = L/t$ — are respectively the speeds of the i component and the solvent E movement; $l_i$ and L are the paths travelled by the i component and the solvent, respectively; t is the time required to move the solvent I mm the start line to the solvent front line. Distances $l_i$ are counted from the start line to the center of the spot of the corresponding component.

**The mobility coefficient** depends on a number of factors:

- nature and quality of the solvent, its purity;
- nature and quality of the sorbent (thin layer), the uniformity of its particle size, the thickness of the layer;
- the activity of the sorbent (moisture content in it);
- experimental techniques (sample weight, length L of the solvent run); teser's skill, etc.

# The relative mobility coefficient $R_S$

To mitigate the influence of the process conditions a relative mobility coefficient Rs is introduced:

$$\mathbf{R_s = l/l_{st} = R_f/R_{f(st)}}$$

where Rf= *l*/L, Rst = *lst* /L; *lst* — the distance from the start line to the center of the standard spot.

The relative mobility coefficient Rs is a more objective characteristic of the mobility of a substance than the mobility coefficient $R_f$

With the use of the standard, the value of Rs usually lies within Rs= 0.1-10, the optimal limits are about 0.5-2.

# degree (criterion) of separation R( A/C):

To characterize the separation of the two components A and C under these conditions, the degree {criterion) of separation R( A/C):

$$\mathbf{R(A/B) = \Delta \mathit{l}/[\mathit{a}(A)/2 + \mathit{a}(B)/2] = 2\ \Delta \mathit{l}\ /[\mathit{a}(A) + \mathit{a}(B)],}$$

where Δl is the distance between the centers of the spots of components A and B; a(A) and a(B) are the diameters of the spots A and B on the chromatogram.

The greater the value of R (A/B) is , the more clearly the separated are the spots of components A and B on the chromatogram.

To essess the selectivity of the separation of two substances A and B, the separation coefficient α is used:

$\alpha = l_B/l_A$

If α = 1, then the components A and B are not separated.

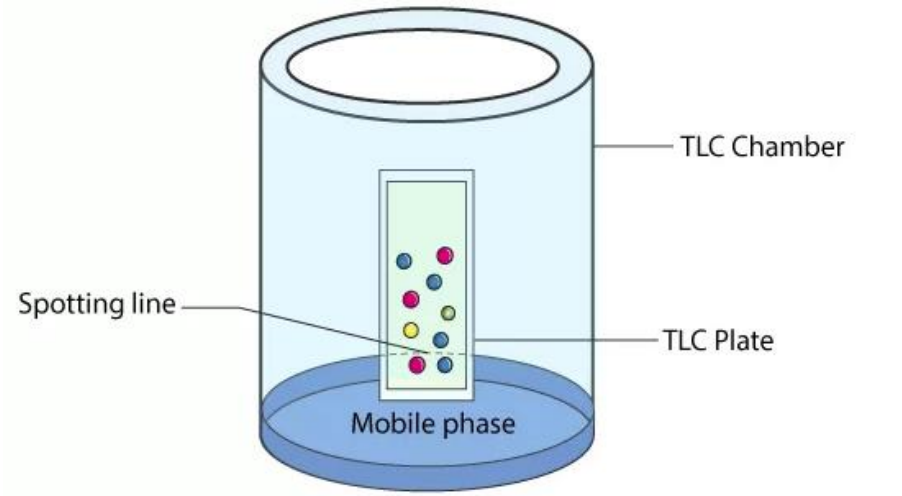
# sorbents

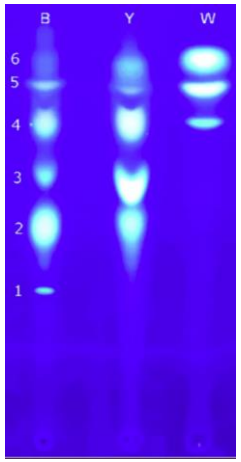
- The most important characteristic of the sorbent is its **activity**, i.e., the ability to sorb (retain) the components of the separated mixture. The activity of the sorbent depends on the nature of active centers and their concentration on the surface of the sorbent, the degree of dispersion of the sorbent particles, the size of the sorbent surface and water content, the nature of the MP interacting with the sorbent.
- The more water the sorbent contains, the less its activity, because water molecules block active centers of the sorbent by binding to them.
- As sorbents, silicon dioxide — silica gel $SiO_2$ and aluminum oxide $A1_2O_3$ file most often used, as well as some other materials (activated carbon, sucrosc, calcium carbonate, cellulose, talc, polyamide resins, etc.).

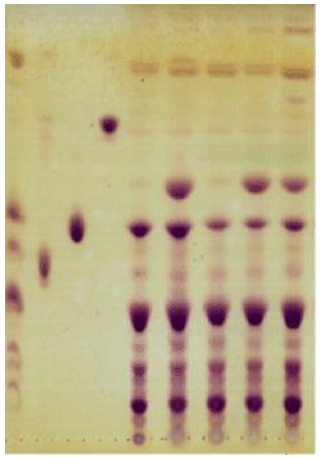
# solvents

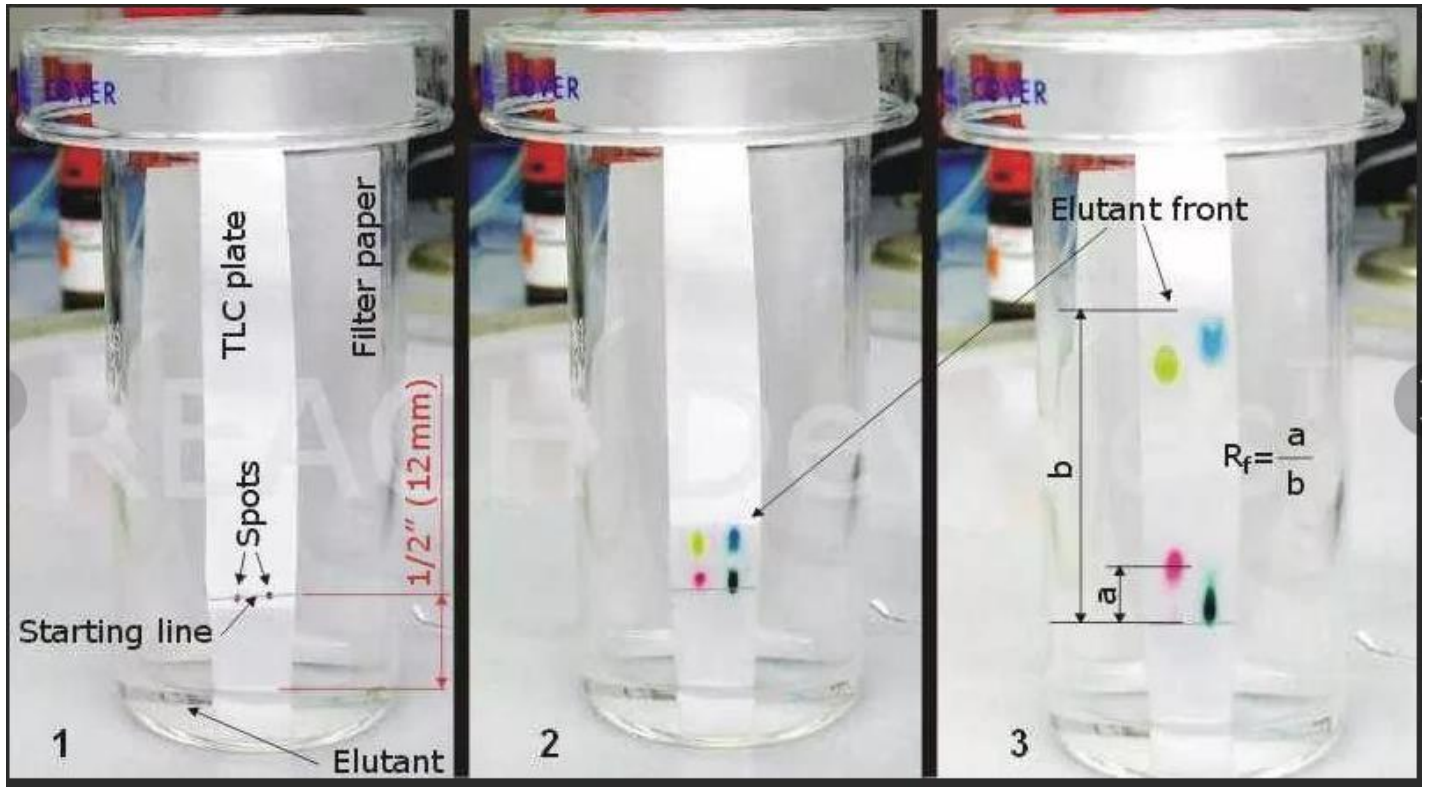
- The choice of solvent in the TLC method is determined by the nature of the sorbent and the properties of the analyzed mixture. Mixtures of several solvents are often prepared as the MP.
- When choosing solvents their eluting ability, i.e., the ability to displace compounds sorbed on FP is taken into account. It depends on the combination of solvent and FP properties. There exist eluotropic ranges for a given sorbent, facilitating to some extent the choice of solvent for TLC
- As an example, we provide the eluotropic range by Trappe. in this range, the solvents are arranged in order of increasing of their eluent capacity, in general - in order of increasing of their polarity (dielectric permittivity): cyclohexane, carbon tetrachloride, trichloroethylene, toluene, benzene dichloroethane, chloroform, diethyl ether, ethyl acetate, acetone, propanol, ethanol, methanol, water.
- The solvent system used as MP is selected by mixing two solvents from the beginning and end of the eluotropic range.

# Tlc chromatography





# PARTITION CHROMATOGRAPHY. PAPER CHROMATOGRAPHY (CHROMATOGRAPHY ON PAPER)

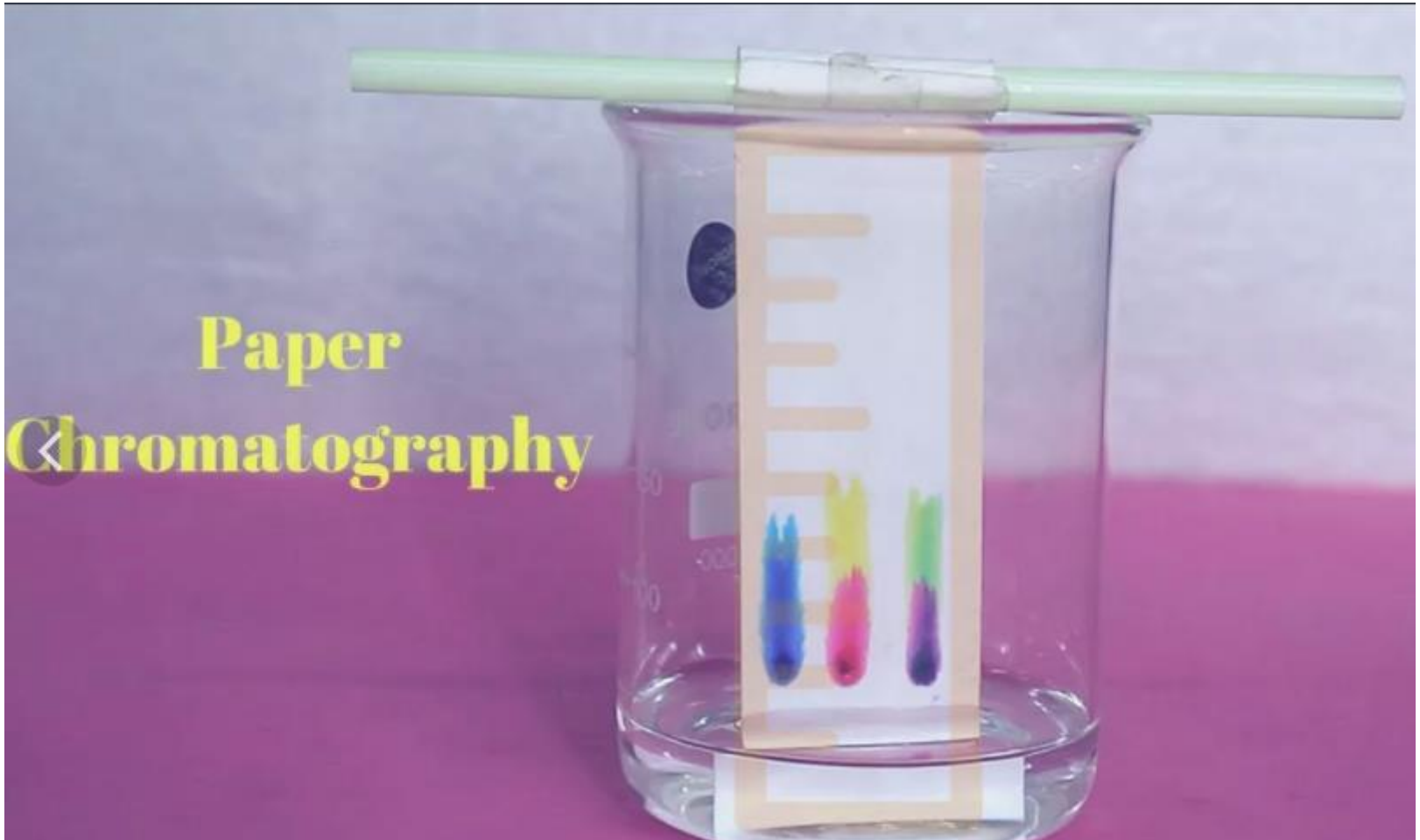
- Paper chromatography (or chromatography on paper) in its usual variants refers to partition chromatography. In this method, instead of plates with a thin layer of sorbent, used in TLC, a special chromatographic paper, through which the impregnating liquid MP moves during chromatography from the start line to the finish line of the solvent, is used.
- There are **normal-phase** and **reversed-phase** paper chromatography.

# Special chromatographic paper

It must meet a number of requirements:

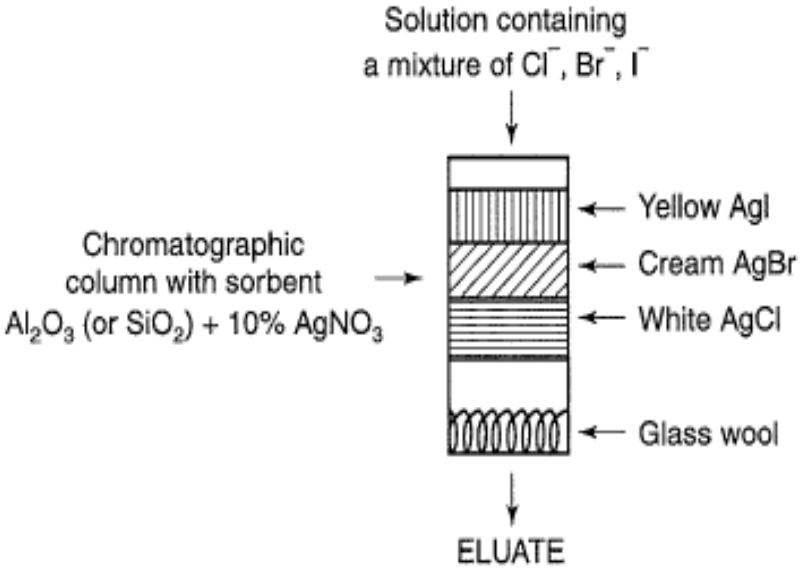
1. Prepared from high-quality fibrous grades of cotton
2. Homogeneous by density and thickness, by the direction of fibers orientation
3. Chemically pure and inert in relation to FF and the separated components.

# paper chromatography



# The precipitation chromatography method

- The precipitation chromatography method is generally used for separation and identification of inorganic ions contained in mixtures.
- The essence of the method. Precipitation chromatography is based on using of precipitation chemical reactions of the components to be separated from a mixture with a precipitant reagent, contained in the FP. The separation takes place due to the unequal solubility of the forming compounds, which are transferred by the mobile phase with different speeds: less soluble substances are transferred with the MP slower than more soluble.

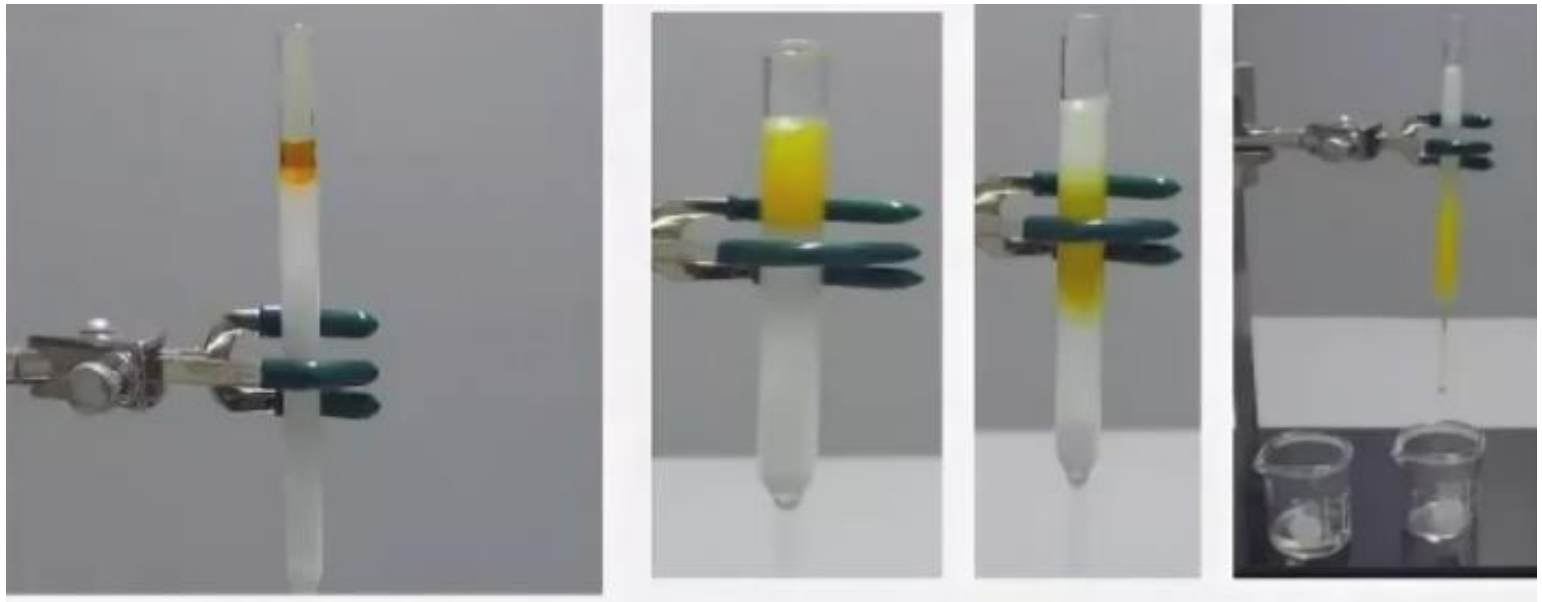


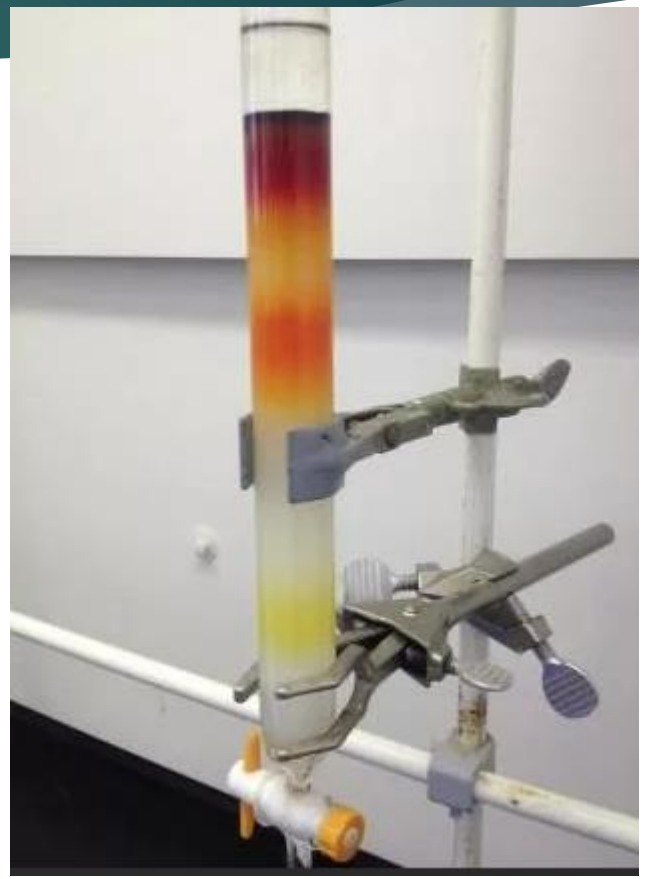
# Classification of precipitation chromatography methods

- Classification of precipitation chromatography methods based on the experiment technique:

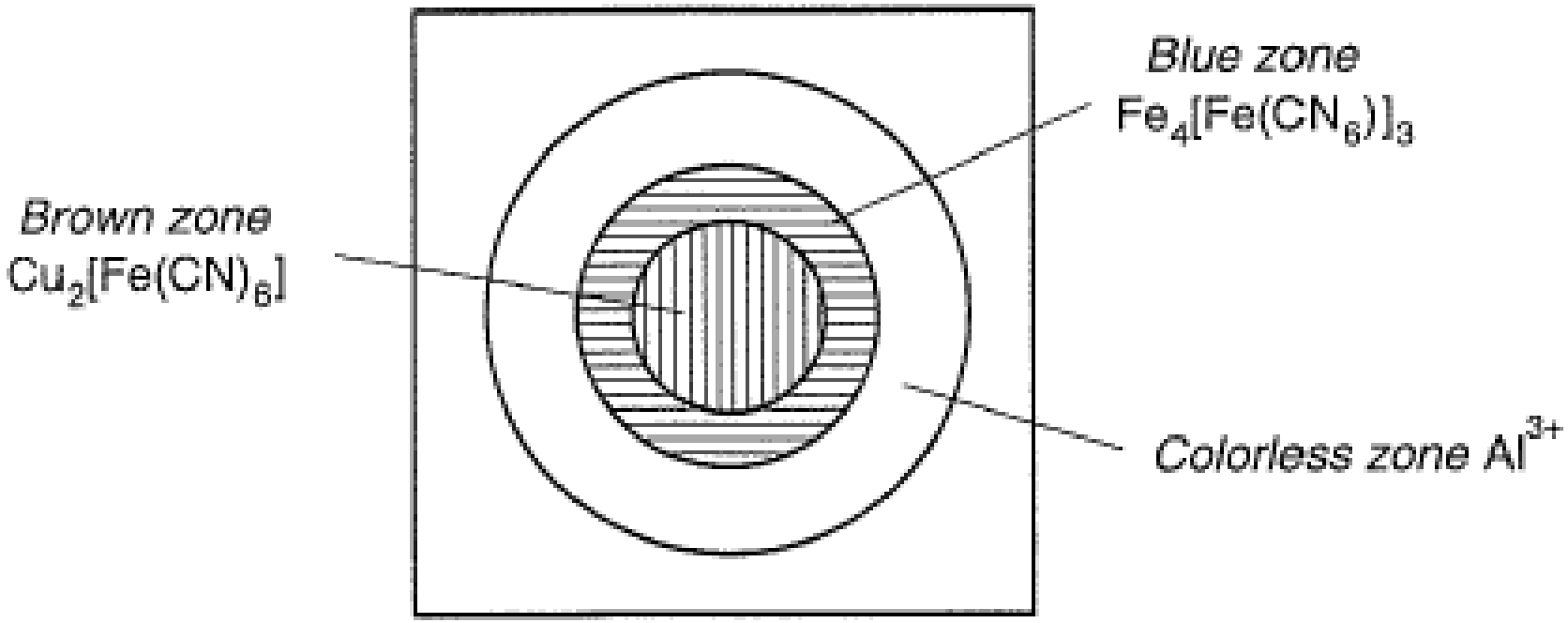
1. ***Column precipitation chromatography***, performed in chromatographic columns,
2. ***Planar precipitation chromatography***, performed on paper or in a thin layer of sorbent are distinguished.

# Column chromatography





# Precipitation chromatography on paper.



# MOLECULAR-SIEVE (SIZE-EXCLUSION) CHROMATOGRAPHY CONCEPT. GEL CHROMATOGRAPHY

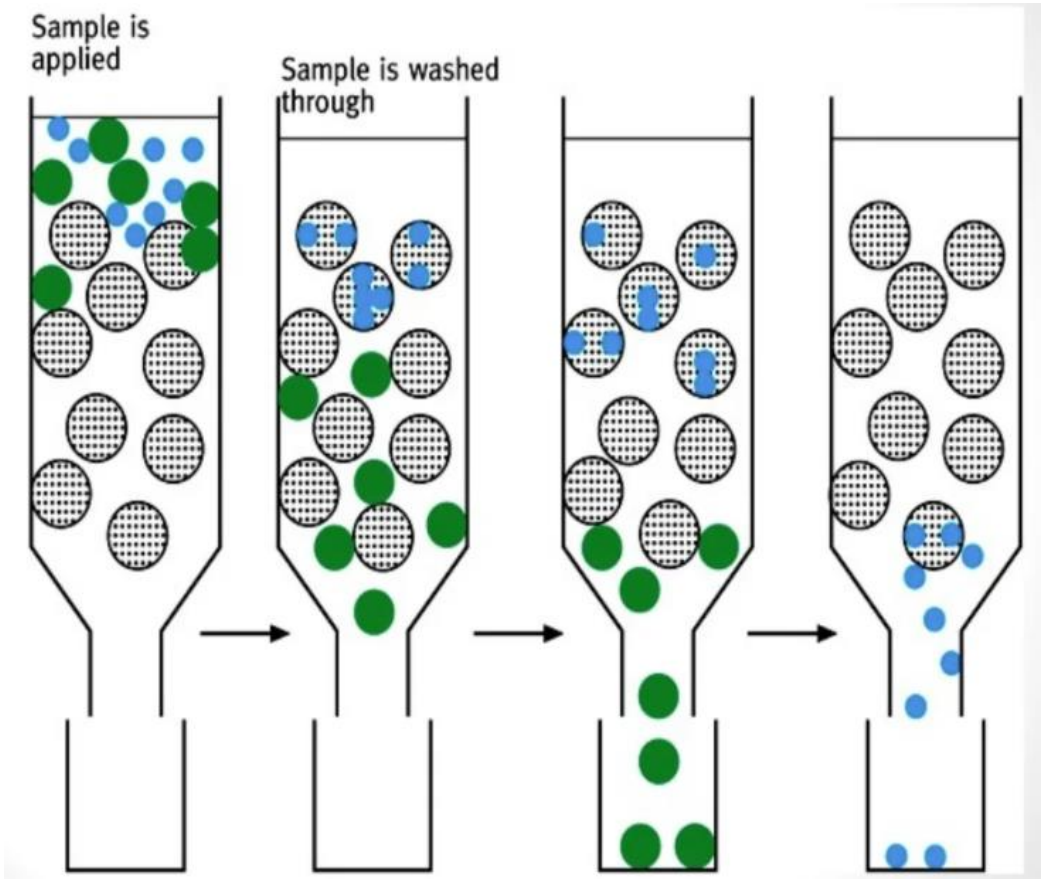
- The method of molecular-sieve (size-exclusion) chromatography is one of the variants of liquid partition chromatography.
- It is based on the use of porous substances — so-called molecular sieves, the pore sizes of which can be larger or smaller than the particle sizes of the separated components, as the FP. Particles smaller than the pore size of the sorbent penetrate with the MP solvent into these pores and can be retained in them, whereas larger particles cannot penetrate the pores due to their size and are carried away with the MP. A separation of small and large particles occurs. Large particles are thus eluted first. Smaller particles trapped in the FP pores are eluted after larger particles.
- Method of molecular-sieve chromatography allows to separate high molecular and low molecular substances, to perform desalting of solutions, to remove impurities from gases, liquids, etc.

# gel chromatography

- Molecular-sieve chromatography, in which gels are used as FP, is called gel chromatography (gel-penetrating chromatography).
- Gels are substances capable of swelling and possessing pores of different sizes. Depending on the tasks, hydrophilic and hydrophobic gels are used. **Hydrophilic gels include dextran** (Sephadexes, molselects), polyacrylamide (biogels), oxyalkylate (spherons) and some other gels. **Hydrophobic gels include some sefadexes**, polystyrene (styrogel, poragel), polyvinyl acetate gels, porous silica gel, porous glass (porasil).
- Depending on the tasks solving methods the following methods are sometimes distinguished:

1. ***Gel filtering (gel filtration***) — separation of very large molecules from small ones;
2. ***Gel chromatography*** as such -separation of a mixture of particles with unequal, but not extremely different sizes;
3. ***Determination*** of the molecular weight of polymers.

# gel chromatography

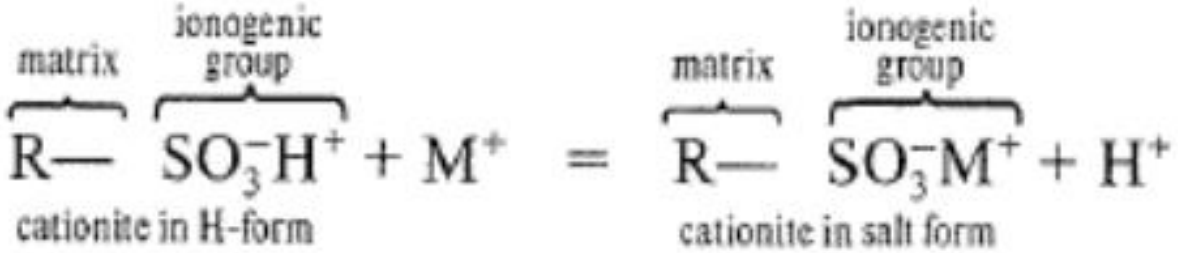


# ION-EXCHANGE CHROMATOGRAPHY

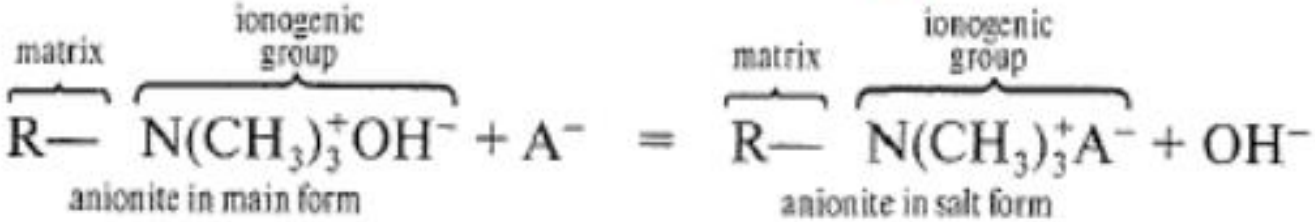
- The method of ion-exchange chromatography is based on using the phenomenon of ion-exchange between a stationary solid phase (ion exchange resin, sorbent) and mobile liquid phase (solution containing ions exchanged with sorbent ions).
- Ion exchange is a heterogeneous process in which the sorbent and the solution in contact with it reversibly and stoichiometrically exchange with similarly charged ions (ions of the same sign)
- Ion exchange resins (ionites), which are usually solid phases insoluble in water, are used as sorbents.

# ION EXCHANGE

## *Cation exchange*:




## *Anion exchange*



# USE OF ION-EXCHANGE CHROMATOGRAPHY

- Separation of electrolytes.
- Purification of electrolyte solutions from impurities.
- Concentrating diluted electrolyte solutions.
- Quantitative determination of electrolytes.
- Determination of drug products.

# GAS CHROMATOGRAPHY

**Gas chromatography** is the process of mixture components separation based on the difference in the equilibrium distribution of components between two phases: a carrier gas (mobile phase) and either solid phase or liquid deposited as a thin film on the surface of a solid phase or walls of a chromatographic column (liquid stationary, liquid stationary phase). In the first case, the method is called gas adsorption chromatography, in the second case, it is called gas-liquid (partition) chromatography. Of these two variants of gas chromatography, gas-liquid chromatography (GLC)

# USE OF GAS-LIQUID CHROMATOGRAPHY

- Separation of various mixtures, including optical isomers, identification of substances, their quantitative determination.
- In a pharmaceutical analysis, GLC is used in quality control of drug substances and drug dosage forms — more often for identification and determination of residual volatile solvents whose traces persist in drugs during their production.
- Determination of metabolic profiles of biological media — blood, urine, saliva.

# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

- **High-performance liquid chromatography**, or high-pressure liquid chromatography, is based on the same principles as GLC, but, instead of gas- carrier, a flow of liquid immiscible with a liquid stationary phase is used as a mobile phase of the chromatographic column. Thus, in HPLC both contacting phases — stationary phase and mobile phase are liquids. Separation of components is based on the difference of their separation factors between the stationary phase and the mobile phase.
- The chromatographic column may be at room temperature, which allows performing chromatography of proteins, amino acids and other thermally sensitive compounds. The molar mass of separated substances may reach 2000.

# USE OF HPLC

- Identification, separation and determination of various substances: optically active compounds, proteins, nucleic and amino acids, polysaccharides, dyes, explosive substances, biological matrices, drug products, etc.
- The method is used for specialized chromatographic analysis of medical and biological objects in cases of pathological deviations from the norm — so-called «pattern recognition technique».
- With technological and pharmacopoeial quality control of drug substances and drug dosage forms, HPLC has become one of the main methods for the determination of both pharmacologically active substances themselves and auxiliary components and foreign impurities