Chromatography

Chromatographies are physico-chemical separation methods, the essence of which is the distribution of the components of the sample mixture between two phases, namely the stationary phase (stationary phase) and the mobile phase (mobile phase). These two phases are distinguished from each other by some basic **physico-chemical** property, e.g. polarity. Along with the moving mobile phase, the sample is also entrained through the system. The separated components of the sample (analytes) interact to varying degrees with the stationary and mobile phases. Analytes that bind more to the stationary phase move more slowly and are retained longer than analytes that bind less to the stationary phase. Based on this principle, the components of the mixture are divided.

See also Chromatography in the Czech Wikipedia and. Chromatography in the English Wikipedia

Division of chromatographic methods

According to the physicochemical principle of division

- Adsorption chromatography
- Partition chromatography
- Ion exchange chromatography
- Gel chromatography
- Affinity chromatography

According to the state of the mobile phase

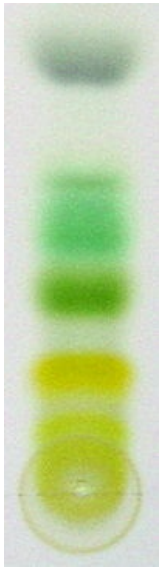
- Liquid chromatography
- Gas chromatography

According to the arrangement of the stationary phase

- Column chromatography
- Capillary chromatography
- Thin Layer Chromatography (Thin Layer Chromatography)
- Chromatography on paper

According to the purpose of use

- Analytical chromatography
- Preparative chromatography



Chromatograph

Basic chromatographic terms

- **Analyte** – a substance to be determined/isolated using an analytical method
- **Eluent** – mobile phase used for analyte separation
- **Elution solution/reagent** – the liquid with which we wash the chromatographic column during the actual separation in column chromatography in order to wash the sample fractions from the column. The elution agent is always a mobile phase, but not all mobile phases can be considered as an elution agent (e.g. the sample itself or the original solution in the column before the actual separation).
- **Effluent/eluate** – mobile phase flowing out of the column
- **Chromatograph** – a device enabling chromatography. It consists of several components. A liquid chromatograph always contains a device for storing and transporting the mobile phase (high-pressure pump = pump), a device for dosing the sample (doser, autosampler), a device for separating substances (chromatographic column) and a device for detecting substances. Optional components of the chromatograph are a mobile phase degasser, a column thermostat, a fraction collector, and a mobile phase mixer for gradient elution. The signal from the detector is usually fed to the integrator in the PC.
- **Chromatogram** – graphic output from the detector created during the chromatographic process expressing the dependence of the signal (y axis) on the elution time or volume (x axis)
- **Mobile phase** – a liquid or gas that carries the components of the separated mixture through the so-called stationary phase.
- **Carrier** – a solid substance, as far as possible inert from the point of view of division, with which the anchored phase is connected. The carrier thus ensures that the phase anchored on it will be an immobile stationary phase.

- **Retardation factor**– is the ratio of the distance of the front from the start and the distance of the center of the spot of the monitored substance from the start in area chromatography. However, reproducibility is questionable with flat chromatographs and therefore we do not encounter R_F tabulation. R_F values can range from $R_F = 0$ for substances not entrained by the developing agent under the given conditions (remaining at the start) to $R_F = 1$ for substances completely not retained by the stationary phase and entrained with the front.
- **Retention/elution time** or **volume** - the time from the start of elution that is required for a given sample fraction to reach the detector behind the column, or the volume of eluent that flows past the elution time

through the column. If well-defined columns are used (e.g. some commercial columns for HPLC or for capillary gas chromatography) under prescribed conditions, retention times or volumes for different separated substances can be tabulated, as they are well reproducible.
- **Stationary phase** – a solid substance or a coating of liquid immovably connected to it, which is itself an effective component of the chromatographic device (columns or flat layers).
- **Theoretical floor** – the height part of the column in which, on average, theoretically, one equilibrium separation step of a physicochemical phenomenon occurs (e.g. adsorption/desorption in sorption chromatography, dissolution of a substance in an anchored phase and its re-dissolution or evaporation into a mobile phase, etc.). Does not apply to ion exchange and affinity chromatography. However, this concept has little to do with reality and is really only "theoretical".
- **Column efficiency** – is proportional to the number of theoretical floors of the given column. For ion exchange and affinity chromatography, instead of efficiency, we are talking about column capacity . However, we are talking about column capacity for all preparative column chromatography. On the other hand, especially with analytical column chromatography, we are interested in the resolving power of the column, which we characterize by the so-called **separation number** , which is, simply put, the number of distinguishable peaks between two selected cut-offs.
- **Developing solution/reagent** – mobile phase in surface chromatography.
- **Anchored phase** – a layer of liquid relatively firmly connected to a solid substance. It forms its own stationary phase in partition chromatography.

Adsorption chromatography

It is based on different adsorption of substances on the surface of the sorbent, forming the stationary phase. Substances that are more strongly bound by sorption forces under the given conditions are more often and longer adsorbed in individual sections (e.g. in the theoretical layer) than other substances. Macroscopically, they are more delayed by the stationary phase and thus more slowly entrained by the mobile phase. In column methods, they are therefore characterized by higher elution times or larger elution volumes, and in surface techniques by smaller values of the retention factor.

Sorbents , used in adsorption chromatography as a stationary phase, differ from each other in their polarity or acidity.

Activated carbon is an extremely **non-polar** sorbent, or modern carbon sorbents.

Of **the polar acid sorbents** at least **silica gel** (hydrated silicon dioxide SiO_2.xH^2O) should be mentioned.

Examples of **polar basic** sorbents are hydrated aluminum and magnesium oxides:Al_2O_3.xH_2O a MgO.xH_2O.

Often, organic (co)polymers of various compositions and thus physical and chemical properties are used as sorbents.

We choose the mobile phase from the so-called **eluotropic series** of solvents, which are arranged in it according to their increasing polarity: pentane, hexane, chloroform , ethanol , acetic acid, water. However, various mixtures of these solvents often prove to be more suitable than pure solvents alone.

In gas adsorption chromatography, the mobile phase (carrier gas) is mostly nitrogen or helium (better, but considerably more expensive).

Task

Adsorption chromatography of leaf dyes on a thin layer of silica gel – pdf

Partition chromatography

It is based on the different solubility of the separated substances in two different liquids, i.e. on the different values of the **partition coefficient A**; = c_s/c_m**.**

One of the liquids used is the mobile phase, the other is then anchored on some carrier and thus forms the stationary phase. If the liquids used are mutually immiscible, a non-bonding interaction with the support surface is usually sufficient to anchor one of them. Otherwise, we have to anchor this liquid with a chemical bond. In gas chromatography, the mobile phase is a gas instead of a liquid. The anchored liquid must then either exhibit minimal volatility at the temperatures used in the separation (it must have a high boiling point), or we must also chemically bind it to the carrier.

Separated substances, which under the given conditions show a greater ratio between their solubility in the anchored phase and their solubility in the mobile phase, i.e. a higher value of the partition coefficient, are more delayed by the stationary phase, ""spend" in it longer than other substances and are therefore mobile phases drifted more slowly. Therefore, they show higher elution times or larger elution volumes, then lower values of the retention factor in surface techniques.

If the anchored phase is water, we speak of so-called chromatography with a **normal phase**. However, we often encounter anchored organic liquids with usually low polarity, and then we speak of the so-called **reversed phase** . The liquids used are anchored on carriers of various chemical compositions. We probably most often encounter carriers made of silicon dioxide, glass, various (co)polymers, starch, cellulose, etc.

We choose the mobile phase from the so-called **mixotropic series** of solvents, which are arranged in it according to their decreasing polarity (in contrast to the eluotropic series in adsorption chromatography): water, ..., acetic acid, ... ethanol, ... chloroform, ... hexane, pentane. Even here, however, various mixtures of these solvents are often more suitable than the pure solvents themselves. In gas partition chromatography, nitrogen or helium is again used as the mobile phase (carrier gas).

Ionex chromatography

JIt is based on the coulombic attraction of opposite charges. The stationary phase has charge-bearing chemical groups on its surface. If ions of the opposite charge or molecules present in the flowing mobile phase are strongly dipole, they are attracted by electrostatic forces and held firmly enough on the surface of the stationary phase. Unlike the previous two types of chromatography, they are usually not just slowed down, but stopped completely. In order to release them from the column, we have to change the character of the elution agent so that either the split ions are displaced or their charge changes, and this is done gradually. The interaction of an ion or dipole with an ionex increases with its charge, or its polarity and decreases with its size, as can be deduced even from the idea of Coulomb's law.

Materials used as stationary phases in ion exchange chromatography are called **ion exchanges**. Ionexes are divided according to charge into **annexes** carrying a positive charge on the surface and thus attracting anions and **cations** with negatively charged groups and attracting cations from the solution.

The most common groups chemically bound on the surface of annexes are (in order of increasing anion binding strength): primary amines $–NH_2$, secondary amines –NHR, tertiary amines $–NR_2$ and quaternary ammonium bases $–N^+R_3$.

On the surface of cathexes, we then usually encounter acidic groups (again according to increasing cation binding strength): phenolic group –OH, carboxyl group –COOH, phosphate group $–PO(OH)_2$ and sulfate group $–SO_3H$. The ionex skeleton itself is either organic (e.g. dextrans, cellulose, copolymers of styrene and divinylbenzene) or inorganic (zeolites, phosphates, molybdates, phosphomolybdates, etc.).

The mobile phase is almost always aqueous solutions, whether it is a sample solution or an eluent. As mentioned above, the ions from the sample are usually tightly bound by the ionex and eventually have to be released. Two types of elution are used for this: displacement and charge change (recharging). In both cases, in order to elute the individual components from the original sample gradually and thus separate them from each other, we use gradient elution. This means that we change the composition of the elution solution over time.

In displacement elution, we use the aforementioned fact that the strength of the electrostatic bond between an ion and an ionex decreases with the size of the ion. Small ions(e.g. Na^+ or Cl^-) in sufficient concentration are therefore able to displace larger ions (e.g. amino acid ions). During displacement elution, we gradually increase the ionic strength of the elution solution arriving at the column. Elution by changing the charge (overcharging) can be applied when the substances separated by us are amphoteric and change their charge by changing the pH. An example can be the ionex separation of a mixture of amino acids or proteins. At a sufficiently high pH, all the components we require are captured on the annex in the form of anions. We elute with a solution whose pH gradually decreases. If the pH in the column drops to the value of the isoelectric point (pI) of one of the components, it loses its charge, the coulombic forces are practically canceled and the elution solution takes it with it. With a further decrease in pH, that component receives a negative charge and is even repelled from the surface of the annex. Later, the pH drops to the pI value of the next component and it is similarly eluted from the column after the first one. This can then be repeated for other components of the original mixture and we are thus able to separate them from each other.

Gel permeation chromatography

It is based on the **different permeability** of the holes and hollow niches on the particles of the stationary phase for different sized particles of the partitioned mixture. The **stationary phase** consists of small spheres in which there are many "tunnels" and "caves" of various sizes.

However, some component of the divided mixture may have such large particles that it **does not fit** into any of the mentioned recesses , and thus has the possibility of only flowing around the balls of the separating material and **flowing** through the cracks between them. Therefore, it actually has the smallest space available during entrainment of the mobile phase and is thus entrained the **fastest**.

Another component may have particles of such a size that they already **fit into at least some** of the openings in the beads of the separation material. Thus, during the flow, these particles have available not only the space around the balls, but also the space of depressions of sufficient size, where they can diffuse freely. As a result, their speed (with a constant mobile phase flow rate, of course) is slightly slower than that of the large particles of the first considered component.

Finally, we can have a component whose particles are so small that they have the space of all the openings in the balls of the separating material available for their movement . This component will therefore move the **slowest** in the direction of the mobile phase flow .

In the **final effect**, theoretically, the first component with the largest particles and the last component with the smallest particles will flow out of the chromatographic column. Therefore, gel permeation chromatography is used to **separate mixtures of high-molecular substances** (mainly proteins) according to their **molecular weights** . But a necessary condition is that the **material of the separation medium** is completely **inert** to all the separated components and does not specifically retain any of them.

If we want to separate a mixture as well as possible, we must choose a stationary phase with the most appropriate range of particle sizes. Such selection therefore depends on the expected size range (molecular weight) of the components of the split mixture. Fortunately, we have a very wide range of commercial materials at our disposal, and we can normally separate mixtures of relatively low-molecular substances with a molecular weight of the order of thousands to tens of thousands of daltons as well as mixtures of very high-molecular substances with a molecular weight of hundreds of thousands to a million.

According to the **polarity of the stationary and mobile phases, we distinguish between hydrophilic and hydrophobic** systems . In the vast majority, however, we encounter the first ones, i.e. hydrophilic systems, and among them the **system of polysaccharide** skeletons as the stationary phase and **aqueous solutions** as the mobile phase completely prevails.

Gel permeation chromatography is sometimes incorrectly called **molecular filtration** or **molecular sieve filtration** . As the true nature of gel permeation chromatography implies, these names are misleading because there is no filtration involved.

Affinity chromatography

It is based on specific interactions of a usually non-binding nature. One of the partners is firmly chemically bound as a **ligand** to a suitable carrier and thus forms a stationary phase together with it. The second of the partners is contained in the sample and, under suitable conditions, is specifically bound to the column by non-binding interactions to that particular ligand. After thoroughly washing the column with a suitable solution (e.g. physiological, etc.) to remove all components non-specifically (and thus very weakly) retained on the column, the desired substance is eluted with an appropriately selected solution - an elution agent.

For a number of suitable systems, the appropriate stationary phase with already bound ligand can be purchased, as it is commercially available. Those unavailable (or too expensive for the workplace) can be prepared in the laboratory. A suitable carrier is first chemically activated and the sorbent itself is prepared by pouring the ligand solution through the activated carrier. After thorough washing and deactivation of reactive group residues, it is ready for isolation of the relevant component from the sample. The most common type of carriers are polysaccharide gels and these are activated, for example, with CNBr cyanide bromide. The nitrile groups formed on the carrier by activation then react easily with –OH or –NH 2 groups of the ligand to form classic covalent bonds.

Elution agents are most often buffers with a low or high pH. Such an acidic or basic environment will change the conformation and possibly also the charge of the isolated component and/or ligand, which will cancel the original non-bonding interactions and the isolated component will be carried out of the column by the mobile phase. After isolation, it is usually sufficient to reconstitute the ligand on the column and the isolated components only by transferring them to a buffer with a suitable "physiological" pH. Other elution options – that is, elution by displacement by a competitive ligand or a competitive substance – are rarely used, among other things, due to the necessity of complex reverse reconstitution.

Affinity chromatography can be used, for example, for systems antigen-antibody, lectin-glycoprotein, enzyme-substrate, receptor-hormone, immunoglobulins-protein A or G, albumin-Blue Sepharose, etc.

Column liquid chromatography

The separation medium – the stationary phase – is placed in glass or metal tubes, in so-called *chromatographic columns* . We inject the sample through the upper end of the column and add any washing solution and, of course, the eluent. If the elution solution flows only due to gravity, we are talking about so-called low-pressure column chromatography . If we force the solutions to flow through the column under increased pressure using pumps, we speak of high-pressure chromatography. A much finer stationary phase material can then be used, and the columns become significantly more efficient for separation, with a higher number of theoretical floors (for adsorption or partition chromatography), and more capacious due to the larger surface of the stationary phase.

Flat panel liquid chromatography

The stationary phase is in the form of a loose layer. Either it is some powdery material on an inert support in thin-layer chromatography, or it is the chromatography paper itself (similar to filter papers) in paper chromatography. Thin-layer chromatography is still occasionally used for orientational analyzes of samples or for the preparation of fractions before another analytical method (e.g. for the separation of fatty acids from biological material before their detailed analysis by gas chromatography). For analytical use, the stationary phase layer is really thin, usually less than 1 mm. For preparations, however, the layer must be considerably thicker, up to about 1 cm, in order to contain a larger amount of the sample. Paper chromatography was once a very widespread chromatographic method, but today we probably no longer encounter it in practice.

The mobile phase, the so-called **developing agent** , is introduced to the separation medium in sheet chromatography either from below - the so-called ascending arrangement, usual in thin-layer chromatography, or from above - the so-called descending arrangement, usual in paper chromatography. In the ascending

configuration, the driving force is the mobile phase of the ascent, in the descending arrangement, gravity is added to the ascent forces. For both techniques, however, it is necessary that the entire separation medium be placed in a container (e.g., a chromatographic cuvette) with saturated vapors of the used developing solution during the separation, so that due to the evaporation of the developing agent from the relatively large area of the separation medium, there are no significant inhomogeneities in the flow of the mobile phase, and so to the deformations of the zones of divided fractions.

File:Rf.png

The ratio of the distance of the center of the concentration maximum of each zone from the start to the distance of the front of the mobile phase from the start is referred to as the **retardation factor** ($\boldsymbol{R_F}$).

Since there are a considerable number of factors (e.g. temperature, small variations in the composition of the chromatographic system, humidity) which to a certain extent influence the position of the zone and thus the R F value , we usually develop a sample of the standard (authentic substances) on the chromatogram at the same time. If the standard and the substance separated from the unknown sample reach the same R F value (at least in three different chromatographic systems), it is likely that the substances are chemically identical. The amount of substance in the zone can be estimated based on its area, quantitatively determined with a densitometer, or after washing the substance from the zone with a suitable solvent.

Task

Adsorption chromatography of leaf dyes on a thin layer of silica gel – pdf

Gas chromatography

Gas chromatographies (GC) use an inert gas as the mobile phase - for the sample and the stationary phase. This carrier gas is often nitrogen, helium has more favorable properties for division, but it is considerably more expensive. However, the use of gas as the mobile phase results in a limitation of GC: all components of the sample must be volatile enough not only to be gasified before entering the column, but also to eventually flow out of the column and not remain there permanently as a high-boiling condensate. If the chromatographic column is made up of a shorter or longer (in that case it is usually twisted into several turns) tube with a powder-like filling of the stationary phase, we are talking about a so-called packed GC. Depending on the type of filling, we can consider both adsorptive and distribution GCs for this GC. If we use columns of a larger diameter, semi-preparative use can also be considered,

However, we can also replace the classic column with a long glass or polycarbonate capillary (on the order of tens of meters, twisted into many turns), the inner surface of which is coated with a very thin layer of some organic high-boiling liquid (e.g. silicone oils, etc.). In that case, we are talking about the so-called capillary GC. It follows from the above that capillary GC can only be used in the case of partition chromatography and only in analytics. For preparations, the column is too thin and the sample can therefore be used for separation in an amount of the order of µl. However, when used analytically, capillary GC has one huge advantage over cartridge GC – a much higher separation efficiency and therefore higher resolution.

The separation of the sample components and their final exit from the GC column can occur at a constant temperature and is therefore an isothermal elution. However, more often, especially for more complex mixtures in the sample, we use a temperature gradient. We start the chromatography at a lower temperature and then gradually (continuously or jumps) increase it over time. Therefore, the entire columns are placed in thermostated and temperature-programmable chambers. The maximum usable temperatures depend on the properties of the stationary phase and the sample and usually do not exceed 300 °C.

Elution chromatography

The individual fractions of the sample separated in the chromatographic column must somehow eventually get out of the column. Washing the column with a liquid (so-called eluent) for this purpose is called elution . If we wash the column with an eluent of constant composition, we speak of so-called **isocratic elution** . However, we often have to change the composition of the elution agent during elution (e.g. its ionic strength, pH, ratio of polar and non-polar solvents, etc.) and then we talk about the so-called **gradient elution** . For this, it is necessary to preferably have two programmable pumps sequentially mixing two solutions (initial and final) in different ratios or a pump connected to a gradient mixer to which two reservoirs of different solutions are connected.

Displacement **elution** is rarely encountered, unless we include elution by gradient ionic strength in ion exchange chromatography or elution by displacement by a competitive ligand or competitive substance in affinity chromatography, as mentioned above.

Frontal elution is practically only possible for the preparation of so-called deionized water. In it, distilled or tap water flows through a column with an annex and cathexis, while all corresponding ions are picked up from it by the given ionex. The eluent here is actually the "sample" itself, i.e. purified water. Deionized water is thus obtained by this frontal elution until the capacity of the used ionex is exhausted.

Analytical chromatography

It serves to identify and quantify the individual components of the investigated mixture and generally works with a very small amount of sample.

Preparative chromatography

It has the task of separating the individual components of the mixture from each other in a sufficient (so-called viable) quantity so that they can be further processed and used. Therefore, the amount of sample is usually considerably larger than in analytical chromatography. The size and capacity of the chromatographic columns used must correspond to this when performed on a column, or the thickness of the so-called thin layer in thin-layer chromatography.

Detection techniques used in chromatography

Chromatographies are **always** associated with a certain detection technique based on the principle:

- Absorption spectrophotometry
- [[fluorescence spectrophotometry]
- Coulometry
- Amperometry
- Conductometry
- Mass spectrometry

In **column/capillary** techniques, the eluted analytes are detected continuously by measuring some of their measurable physicochemical properties (e.g. absorbance, fluorescence, redox potential, conductivity). The detector signal is usually processed by a computer program, which also serves to evaluate the separation. The signal from the detector, depending on time, gives a so-called **chromatogram** – a time record characterizing a given mixture of components. Individual peaks of the chromatogram correspond to individual components in the investigated mixture. The amount of a given analyte corresponds to the area/height under the elution curve (peak).

Detectors used in column liquid techniques

- UV-VIS spectrophotometers
- DAD spectrophotometers (Diode Array Detector)
- Fluorimeters
- Coulometers
- Amperometers
- Conductor meters
- Refractometers
- Radiometers
- Polarimeters

Detectors used in gas chromatography

- Thermal conductivity (TCD)
- Flame Ionization (FID)
- Thermoionization (TID)
- Helium Ionization (HeD)
- Electron Capture (ECD)
- Mass spectrometric (MS)

In **sheet** chromatography, if no radioactive substances were used, the spots can only be quantified with difficulty. Therefore, sheet chromatography is used more or less only for qualitative analysis.

If the separated components were not themselves colored or luminescent, we usually have to make them visible by a colored chemical reaction. Sometimes it is enough to heat a thin layer to a higher temperature, when oxidation leads to the formation of a colored substance or to the charring of separated substances, but mostly we use detection reagents. For organic substances, it is, for example, a yellow solution of phosphomolybdic acid, which with most of them turns blue to black when heated (reduction of Mo^{6+} to MoO_2).

Commercially available are also thin layers that contain a fluorescent dye sensitive to UV light of a suitable wavelength. If there are substances absorbing this wavelength in the sample, when irradiated with UV light, they shade the fluorescent dye and this is reflected in darker spots on the glowing thin layer. E.g. on Silufol UV 254, all aromatic compounds and a number of compounds with multiple conjugate bonds are detectable in this way.