Separation techniques

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Dialysis

Macromolecules (proteins, polysaccharides, nucleic acids, etc.) in solution can be separated from low-molecular-mass substances by natural or artificial **semi-permeable membranes** that allow diffusion of small molecules but not macromolecules. This process of differential diffusion is called **dialysis**.

Small molecules diffuse across the membrane according to the concentration gradient, system enters steady-state (i.e., no transport across the membrane occurs – or to be accurate – diffusion of small molecules across the membrane is equal in both directions) as soon as concentrations of small molecules are equal on both sides of dialysis membrane. To speed up the process (or to remove small molecules from dialyzed solution completely) you need to maintain the concentration gradient. You can either use large volume of dialysis solution (related to the volume of dialyzed solution) or change dialysis solution frequently (before equilibrium is achieved). The rate of dialysis depends also on the surface of the membrane – the bigger the surface, the faster the dialysis.

An important characteristic of dialysis membrane is "cut-off" value (MWCO-molecular weight cut-off). It indicates which molecules are considered to be small (i.e. can freely diffuse across given membrane) and which are considered as big (cannot pass through). The vast majority of molecules with relative molecular mass exceeding MWCO should be retained by given membrane. There is nothing definite about this parameter, it is to be considered rather as a rule of thumb: not only "size" (relative molecular weight) of the molecule, but also its shape plays an important role in diffusion across the membrane (You cannot pass through the door with opened umbrella, but you should have no troubles in entering the building when you close it. But it is still the same umbrella.).

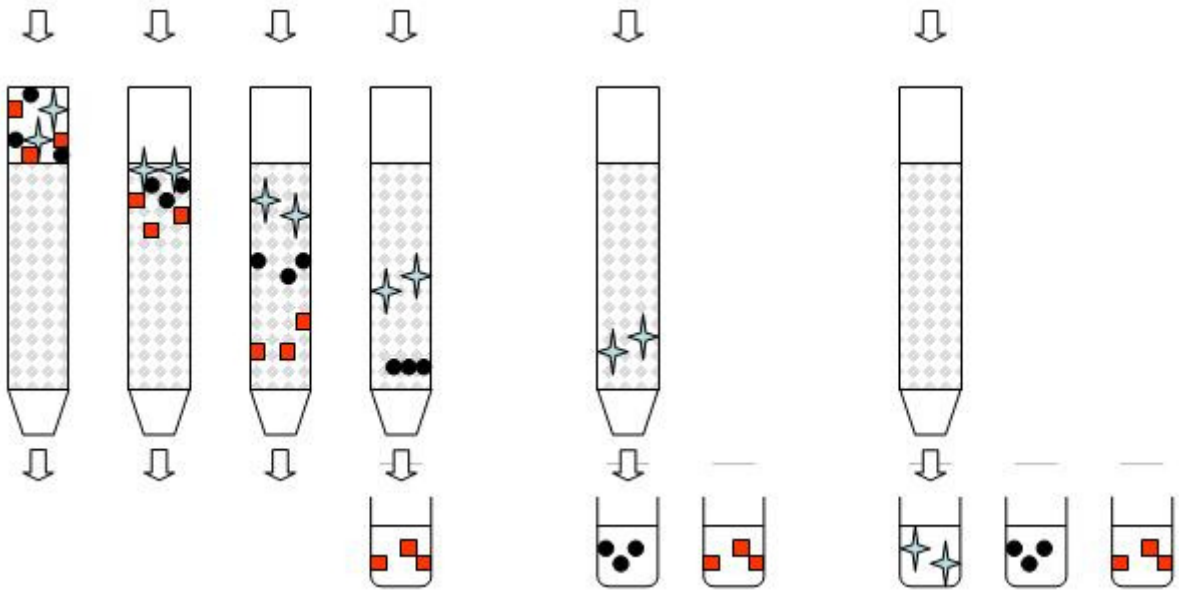
In the body, in fact, all the cellular and subcellular membranes are semi-permeable in complex ways, i.e. they allow flow of water and small molecules to various degrees, but not flow of proteins. In research laboratory, dialysis is a simple method of choice if, for instance, the task is to remove excess of salt from a protein solution, or to transfer proteins into a different buffer. In clinical practice, a widely used method of kidney function replacement is called **haemodialysis**, since it is based on the same principle: blood of a patient flows along semi-permeable membranes (Apart from special artificial membranes that are used outside of patient´s body, peritoneum can be used – in that case, patient´s peritoneal cavity is washed with dialysis solution = peritoneal dialysis.) that allow equilibration with the solution at the other side of the membrane (and removal of the waste metabolic products) while plasma proteins and blood cells are retained. Keep in mind that not all small molecules are waste products – if you forget to include for example glucose, Na^+, K^+, etc., in the dialysis solution your patient can die quickly.

Chromatography

Chromatography is one of the most powerful and most commonly used separation techniques. It was described by a Russian botanist Mikhail Tsvet in 1901. He used it for separation of plant pigments – since then it is called chromatography (chroma – Greek expression for color).

The fundamental question in this chapter is "Why?". Why do we need to separate different compounds? The answer is obvious in preparative separation – we need to isolate sufficient amount of pure substance for further use. However, in analytical chromatography (which is more relevant for us) the answer might not be so straightforward. It is important to realize that samples to be analyzed are often very complex mixtures (such as serum), consisting of thousands of compounds. One particular compound – e.g. estradiol (steroid hormone) cannot be quantified directly using for example spectrophotometry, as other compounds (cholesterol, vitamin D and other steroids) have similar absorption maxima and would contribute to the overall absorbance of the sample (interfere), thus overestimating estradiol concentration. For accurate results all interfering substances must be separated from the target compound prior measurement. Principle of separation is described in following paragraph.

Chromatographical methods rely on a system of two inmiscible phases - mobile and stationary. Compounds to be separated are carried by mobile phase through the stationary phase and separated according to their affinity to mobile/stationary phases. Analytes with higher affinity to the mobile phase tend to pass through the system quickly (in the extreme case are not retarded at all), whereas analytes „preferring" the stationary phase are slower (in the extreme case are bound to the stationary phase and do not move). Analytes leaving the system at different time points can be either collected or measured.



Complex mixture is loaded on a stationary phase (gray field) and eluted using a mobile phase (open arrow). Analyte (red square) with the highest affinity to the mobile phase is eluted out of the stationary phase first.

A great number of chromatographical techniques available can be assorted using various criteria:

A) Intended use

- **Preparative chromatography**: One or more compounds are to be isolated from a complex mixture in a pure form. Usually adapted to process large amount of sample, to increase yield of target compound.
- **Analytical chromatography**: Compounds are separated from a mixture just to be detected/quantified, separated compounds are not collected. Small amounts of sample are usually processed.

B) Physical state of mobile phase

- **Liquid chromatography**: Mobile phase is in liquid state, sample must be either soluble or miscible with mobile phase. The most commonly used chromatography.
- **Gas chromatography**: Gas serves as a mobile phase. Sample must be in gaseous state – this is the limiting factor. Usually it is performed at high temperatures (over 100°C) to evaporate volatile sample. Additionally, chemical modification of functional groups of target molecule (derivatization) can be used, to facilitate evaporation.

C) Arrangement of stationary phase

- **Planar chromatography**: Stationary phase either placed on a solid support (glass plate, aluminium foil, etc.) – TLC (thin layer chromatography) or has planar shape itself (chromatographical paper) – paper chromatography. Planar chromatography is currently used just in a few applications (like in toxicology) and it is likely to be completely replaced with column chromatography.
- **Column chromatography**: Stationary phase is packed in a tube (column) of various dimensions (diameter typically within the range of micro to centimeters, length usually centimeters to meters). The most common type of chromatography – often requires sophisticated and relatively expensive equipment; however its performance is very good.

D) Phenomenon responsible for separation (retardation of analyte flow)

- **Adsorption chromatography**: Probably the most common type - analytes are adsorbed to a solid stationary phase.
- **Ion-exchange chromatography**: Based on electrostatic interactions, stationary phase is ion-exchange resin (either anionic or cationic). Separated compound must be at least partially charged under given conditions.
- **Affinity chromatography**: Stationary phase can specifically bind separated compound. Typical example can be antibody purification using antigen as a stationary phase (or vice versa), ligand receptor interaction, etc.
- **Gel permeation chromatography** (gel filtration, size-exclusion): Compounds are separated according their size.

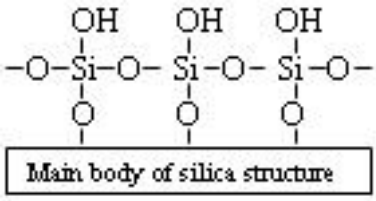
The list of chromatographical techniques is far from complete; however, it should be sufficient for basic orientation.

Three types of chromatography are described in somewhat extended form below. Thin layer chromatography and gel filtration – techniques you will use in practical lesson and liquid column chromatography as the most commonly used technique.

Thin layer chromatography (TLC)

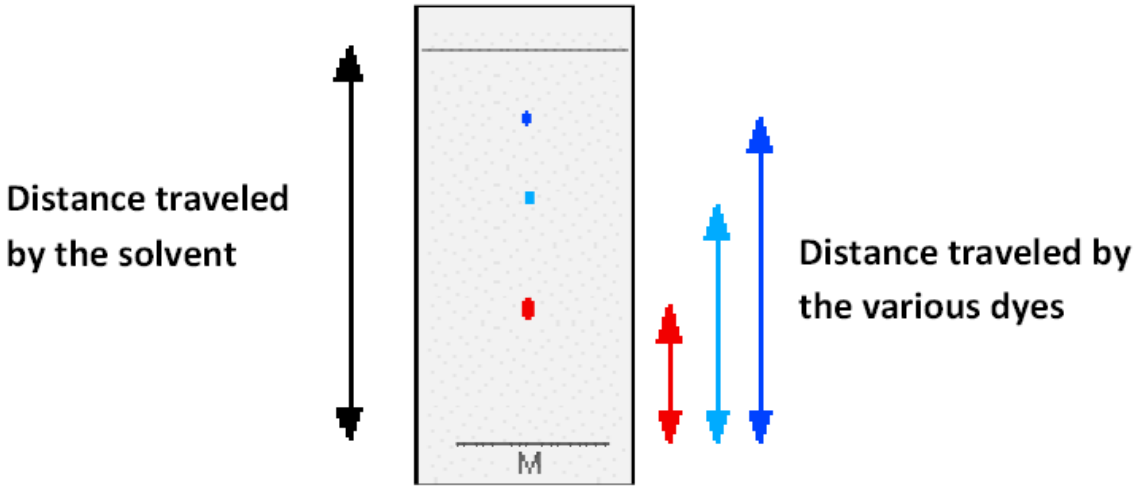
Thin layer chromatography is exactly what it says – a thin layer of a stationary phase (silica gel, alumina etc.) coated on a glass, aluminium or plastic sheet. In the practical lesson, we will use aluminium sheets coated with silica gel. **Silica gel** is a form of silicon dioxide,

where silicon atoms are cross-linked via oxygen atom and free –OH groups are exposed on the surface. The whole structure looks as follows:



Because of –OH groups, the surface of the plate is polar and can form hydrogen bonds and other non-covalent interaction with suitable compounds. **Mobile phase** is a mixture of solvents we will pour into a chromatographic chamber. We will place the plate into the thin layer of solvent, so the sample is above the solvent level. Mobile phase rises up the plate through the stationary phase and carries the components of the mixture with it. How fast the compounds get carried up the plate depends on the solubility of the sample in the mobile phase and a degree of interaction with the stationary phase. The stronger is the affinity of the sample to the stationary phase, the slower it travels up. When the solvent gets far enough (mostly more than 3/4 of the plate), we take the plate out of the chromatographic chamber and mark the position of the solvent front with a pencil. Then we measure the distance of individual spots from the start and calculate so called **retention (retardation) factor Rf.** The Rf is constant (and characteristic) for each compound of the analyzed mixture in the given system of mobile and stationary phases. Thus, Rf can be used for an identification of compounds in an unknown sample.

Rf= Distance traveled by given compound/ Distance traveled by solvent

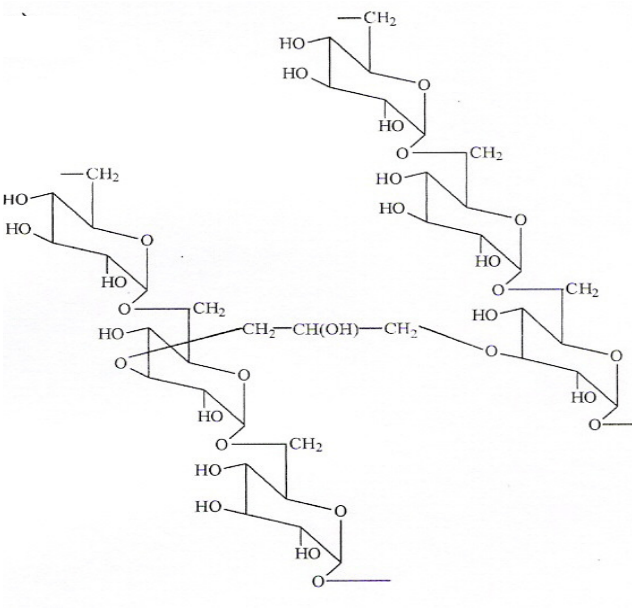


If the substances are colorless, it is necessary to visualize them first. There are several methods available, from the presence of the fluorescent dye directly in the plate, when we see the black spots under the UV light, to chemical detection, when the developed chromatographic plate is sprayed with a chemical solution (ninhydrin for amino acids etc.) that reacts with the spots and creates colored complexes.

Gel filtration

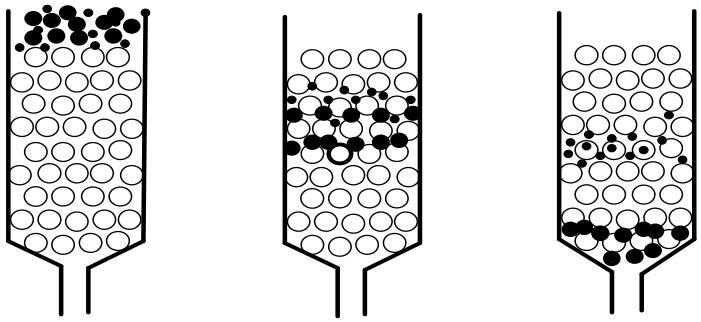
Gel filtration separates molecules according their size – in practice, it is used to purify proteins (or other macromolecules) from low molecular weight contaminants (salts, buffers, etc.), to size-fractionate sample or to estimate molecular weight of unknown compound.

Various types of gel (synthetic or natural, hydrophobic or hydrophilic) are used as stationary phase. The most commonly used gels are soft hydrophilic polysaccharides (such as Sephadex). Polysaccharide molecules are cross-linked with epichlorhydrine to form a 3-D net. The more epichlorhydrine is used, the denser the net is, foming smaller pores. Sephadex is supplied in a form of porous beads (with defined size of pores).



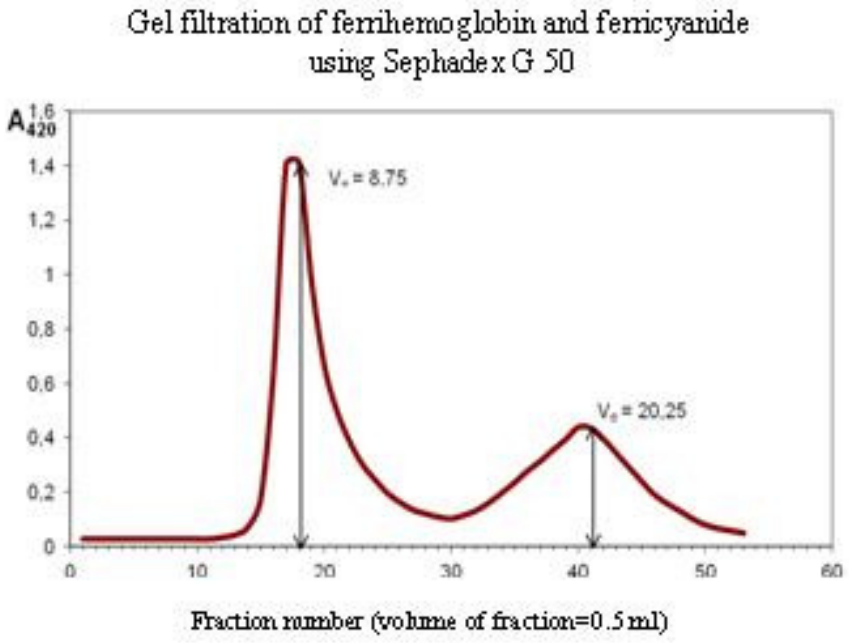
Structure of Sephadex

Separated molecules pass through the column – small molecules (smaller than diameter of pores) enter the beads and are retained inside. Larger molecules do not enter but rather by-pass the beads and are thus eluted faster.



Similarly as in TLC, the "rate of passage" through stationary phase is under given conditions characteristic for given compound (i.e. unknown compound can be identified). In TLC, separation is terminated before solvent front reaches the end of stationary phase – all analytes are present in stationary phase (at different positions). In gel filtration (and generally in all column chromatographies) sample is eluted out of the column – retardation factor can not be calculated, as all compounds traveled the same distance (length of the column). Time to elution would be more suitable for characterization of the "rate of passage", however mobile phase flow in gel filtration is not constant (driven by the gravity force only as gel beads are soft and would not withstand higher pressure, when pumps are used.) and time to elution would not be reliable. Therefore elution volume **Ve** ("volume to elution" – volume of mobile phase needed to elute given compound) is used instead.

Eluted analytes must be detected (and ideally quantified) – various detectors are used for this purpose – spectrophotometer is the most common one. If the signal from detector (absorbance in case of spectrophotometer) is plotted on y axis against volume of mobile phase on x axis elution curve (generally chromatogram) is created. Each peak represents one compound – position of the peak is characteristic for given compound, height of the peak (or area under the peak) corresponds to the concentration.



Example of elution curve

Liquid column chromatography (other than gel filtration)

System set-up is similar to gel filtration, stationary phase (usually non porous silica beads, often coated with hydrophobic molecules) is packed in a column and mobile phase flows through. On the other hand, mechanism of separation is mainly adsorption of sample to the bead surface. Efficacy of separation is to a certain extent dependent on the contact of sample and stationary phase. The higher the surface, the better is the separation. Columns for efficient separations used to be very long (e.g. 3 meters) to accommodate enough stationary phase, however separation of one sample took a long time (hours to days) and a large volume of mobile phase was needed. Evolution in chromatography led to invention of stationary phases composed of finer and finer beads (with relatively larger and larger surface) – today, we use stationary phase with particles smaller than 2 μm in diameter – columns can thus be much shorter (just several centimetres) and analyses much faster (minutes). Such fine particles require much sophisticated instrumentation – high pressure is needed to "push" the mobile phase through the column (pressures up to 400 bar (5800 psi) are often used).

Liquid chromatography is typically used as a complete system – with high pressure pumps, valves for sample injection, temperature controlled column compartment and one or more in-line detectors. In-line detector means that mobile phase with separated compounds flows from the stationary phase directly into detector and absorbance (or different parameters) are continually recorded. There is no need of fraction collection, all processes can be precisely monitored and regulated. In such precise system, the analyzed compound is characterized by "retention time" – time that given compound needs to reach detector. In principle, retention time has the same meaning as retardation factor in TLC or elution volume in gel filtration. When signal from detector is plotted against time, chromatogram is constructed. Similarly as in gel filtration – each peak represents one compound – position (retention time) says what compound it is, height of the peak (or area under the peak) says, how much of it is there.

Literature:

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