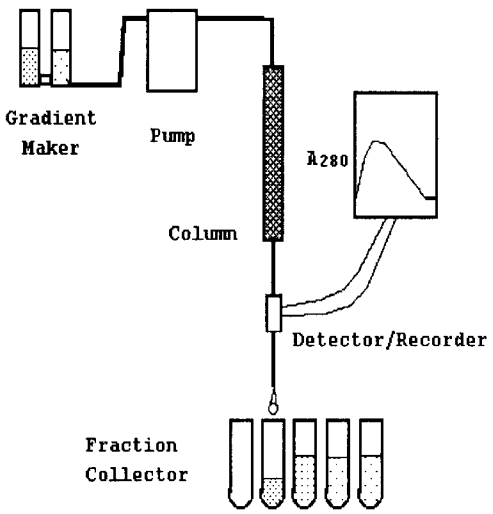
CHROMATOGRAPHY

Grossly dissimilar molecules are relatively easy to separated. For example, lipids, proteins and DNA can usually be separated from one another based on differences in solubility in various solvents. Separation of substances with similar chemical and physical properties is more complex and subtle. Although individual proteins are all unique in terms of their structures, the overall chemical and physical properties are somewhat similar in that they are all polymers of amino acids. Chromatography provides a means to separate substances that exhibit minor differences in their overall properties.

BASIC PRINCIPAL AND EQUIPMENT



A **solute** (i.e., the molecule of interest) is placed into a system containing two phases: a **mobile phase** (solvent) and a **stationary phase** (solid matrix). The solute partitions between these two phases and the degree to which it adsorbs or interacts with the stationary phase will determine how fast the solute is carried by the mobile phase. Therefore two substances which interact with the solid phase differently can be separated.

Proteins are generally separated on columns. The equipment needed for column chromatography can range from expensive workstations to pasteur pipettes. The central component is the **column**. The column is a cylinder which holds the solid phase matrix. A **pump** is needed to control the flow rate of buffers through the column. However, gravity can also be used. The solvent used in the mobile phase will often need to gradually change. This gradual change is accomplished by a **gradient maker**. The elution of substances can be monitored during chromatography with an in line spectrophotometer which measures the absorbance of material coming off the column. In the case of protein chromatography the **detector/recorder** monitors the A_{280}. Detectors to record radioactivity or fluorescence during chromatography are also available. If chromatography is used as part of a protein purification scheme a **fraction collector** is needed. Most fraction collectors will allow fractions to be collected per unit time or per unit volume (i.e., number of drops). Fraction collectors can also be interfaced with the detector/recorder and programmed to collect peaks.

- adsorption
- ion exchange
- gel filtration
- affinity
- hydrophobic
- HPLC/FPLC
- GLC

ADSORPTION CHROMATOGRAPHY

There are many different types of stationary phases used in chromatography (Box). The basic principal discussed above refers specifically to adsorption chromatography. Thin-layer chromatography (TLC) and paper chromatography are examples of

adsorption chromatography. These techniques are usually used to separate small molecules such as nucleotides, amino acids, lipids, simple carbohydrates. Adsorption chromatography is not widely used in protein purification. Hydroxyapatite is an example of a stationary phase that is sometimes used to analyze either proteins or nucleic acids.

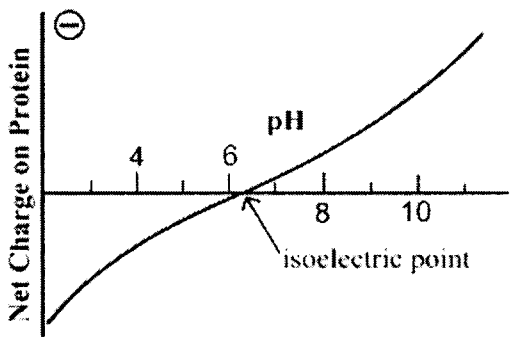
ION EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography (IEC) is a specific type of adsorption chromatography based upon charge-charge interactions. The stationary phase consists of fixed charges on a solid support. The fixed charges on the stationary phase can be either negative or positive and are respectively referred to as **cation exchange** or **anion exchange** chromatography. Counter ions will interact with the fixed charged groups and can 'exchange' with the solute. In other words, substances to be separated will replace the counter ions on the chromatography medium and stably bind to the exchanger.

Solute binding is dependent on net charge of the solute at the particular pH and the ionic strength of mobile phase. The solute is removed from the stationary phase by increasing the concentration of counter ions or by changing the pH to affect the charge of the solute. Weakly charged solutes will be displaced from the stationary phase with lower concentrations of counter ions than more highly charged solutes. This results in separation of solutes based upon their net charges. For example, mixtures of adenine nucleotides (i.e., adenosine, AMP, ADP and ATP) can be separated by IEC. Adenosine, which is uncharged does not bind to the anion exchanger. As an increasing concentration of formate is applied to the column, AMP is the first to elute followed by ADP and then ATP. The order of elution corresponds to the overall negative charge of the nucleotides.

Amino a.	pK_a
Asp, Glu	4.3-4.7
His	≈ 7
Lys, Arg	> 10

Proteins are complex **ampholytes** (have both - and + charges). Negative charges are due to aspartic acid and glutamic acid residues and positive charges are due arginine, lysine and histidine residues. (See Appendix of Introduction to Proteins for structure of side-chain groups.) Each of these side-chains functions as either a weak acid or weak base and has a pK_a value (see Table). The exact pK_a for the side-chain groups will depend on the position of the residue within the protein. The side-chains of aspartate and glutamate have a carboxylic acid group that will either be negatively charged or uncharged depending upon the pH. At pH values greater than the pK_a the carboxylic acid group will be deprotonated, or negatively charged, whereas below the pK_a the carboxylic acid will be protonated and thus have no charge. Similarly, the side-chains of the basic amino acids have an amine group that will be positively charged (i.e., protonated) at pH values below the pK_a or uncharged (i.e. deprotonated) at pH values above the pK_a.



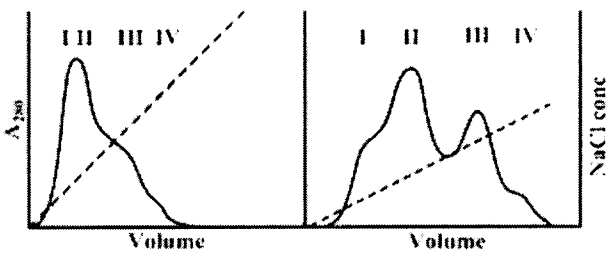
The combination of all of the charged side chains will give the protein a net charge which depends on the pH. The **isoelectric point** (Figure) of a protein is the pH at which

the net charge is zero (i.e., the number of positive and negative charges are equal). Therefore, proteins will have either a net negative charge or net positive charge depending upon the isoelectric point of the protein and the pH of the solution, and thus, it is possible to use either anion exchange or cation exchange chromatography. Anion exchange chromatography is generally carried out above the isoelectric point of the protein of interest and cation exchange chromatography is carried out below the isoelectric point.

The functional groups in IEC can be either strong or weak acids for cation exchange or strong or weak bases for anion exchange. A strong exchanger is used for the separation of weakly ionizable groups and a weak exchanger is usually used for more highly charged solutes. In addition, weak exchangers result in better resolution when the charge differences between solutes is small. IEC of proteins is most often carried out with weak exchangers. Several types of matrices are used for IEC. The matrix should not have ionizable groups or bind proteins. Dextran (trade name Sephadex), agarose (trade name Sepharose), and cellulose are common matrices used in protein chromatography.

The basic steps in column chromatography are to prepare the column, apply the sample to the column, and to elute the solute from the column. Prepared IEC columns can be purchased or alternatively the ion exchange media is purchased and prepared. The amount of preparation necessary will depend upon the form of the media and the manufacturer's instructions should be followed. In general, short wide columns work best for IEC and that the total column volume should be such that all of the protein binds in the top 1-2 cm of the column. The sample should be loaded under conditions (i.e., pH and ionic strength) which promote the binding of the protein of interest. Proteins that do not bind to the column are washed away using the same buffer.

Proteins are eluted by increasing the ionic strength or changing the pH. Increasing the ionic strength can be done batchwise by sequentially washing with buffers containing higher concentrations of salt or gradually using a gradient maker. Eluting in a 'steps' is generally simpler, but of lower resolution. A gradient maker will mix two buffers of different ionic strengths resulting in controlled increase in the ionic strength of the elution buffer. The slope and shape of the gradient will determine the resolution in regards to separating solutes. For example, decreasing the slope of the gradient will lead to a greater separation of the solutes (see Figure). However, as the slope decreases the proteins will be more dilute.



Chromatofocusing is a specialized form of ion-exchange chromatography which separates proteins according to their isoelectric points. This method takes advantage of the buffering capacity of the exchanger and generates a pH gradient across the column. The column is developed with a mixture of buffers called the 'polybuffer'. As these migrate down the column, the most acidic components will bind to the basic groups of the exchanger and lead to an increase in the local $[H^+]$. As elution progresses the pH as each point in the column is

gradually lowered due to the addition of more buffer components. The more acidic components will elute, migrate further down the column and bind to again to the basic exchanger groups. This will result in a pH gradient across the length of the column. The sample is loaded onto the column at a pH > than the pI of the solute of interest. As the pH drops the protein elutes and migrates down the column until the pH is greater than the isoelectric point and then reabsorbs. This will continue until the proteins elute at their isoelectric points.

AFFINITY CHROMATOGRAPHY

The biological function of proteins often involves binding to or interacting with specific ligands, substrates, co-factors, inhibitors, other proteins, etc. Affinity chromatography takes advantage of these specific interactions between proteins and ligands. A protein mixture is passed over the column with an appropriate moiety covalently attached to a solid support. Only the protein which recognizes the ligand of interest will bind to the column and the other proteins will be washed away. Affinity chromatography can be very specific and may allow the isolation of a protein in a single step.

Affinity columns are usually prepared from cyanogen bromide (CNBr)-activated agarose. The CNBr group reacts with primary amines and covalently attaches the ligand to the solid support. In some cases, the ligand already bound to the matrix is commercially available. In addition, linker arms are often used to extend the ligand away from the matrix of the solid support. The matrix should not adsorb contaminating proteins and covalent attachment of ligand to matrix should not alter its binding to protein of interest. Binding of the protein to the ligand should be relatively tight (i.e., high affinity), but at the same time the binding should not preclude the ability to elute the solute from the column.

- affinity elution
- pH changes
- ionic strength
- chaotropic salts
- denaturing agents

Conditions which promote the dissociation of the protein and ligand are used to elute proteins from affinity columns (box). For example, high concentrations of the free ligand can compete with the bound ligand resulting in elution. In cases where affinity elution is not possible or does not work, it may be possible to change the pH or increase the ionic strength resulting in a destabilization of the protein-ligand interactions. Another possibility is to use denaturing or chaotropic agents to elute the protein which will result in the protein unfolding. However, these later methods can only be used if it is not important to recover protein in the native state.

HYDROPHOBIC CHROMATOGRAPHY

- OCTYL-AGAROSE
  - for weakly hydrophobic proteins
- PHENYL-AGAROSE
  - for intermediate hydrophobicity

Proteins can be separated according to differences in their hydrophobicities. Hydrophobicity is a chemical property which promotes the aggregation of nonpolar compounds with each other in an aqueous environment. These hydrophobic interactions are not an attractive force per se, but are forced upon nonpolar compounds in a polar environment. The media for hydrophobic chromatography is a support matrix with long chain hydrocarbons covalently bound. Two common examples are octyl-agarose (8 contiguous methyl groups) and phenyl-agarose (box). This will provide a highly hydrophobic surface for proteins to interact with

instead of aggregating with each other. Incidently, this phenomenon was initially observed with the linker arms used in affinity chromatography.

Proteins bind to hydrophobic columns under conditions that promote hydrophobic interactions and these conditions will determine the extent of binding. For example, raising the ionic strength increases hydrophobic interactions, or the 'salting out' effect (see chapter on Differential Solubility). Both anions and cations can be listed in a series in terms of either promoting hydrophobic interactions or increasing the chaotropic effect. **Chaotropic agents** disrupt the structure of H_2O by decreasing H-bonding and therefore decrease hydrophobic interactions. The most common salt used in hydrophobic chromatography is $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ concentration should not promote protein precipitation (eg., 20% saturated or approximately 1 M). Other salts can be substituted, but higher concentrations may be required (eg., 4 M NaCl).

- decrease ionic strength
- decrease solvent polarity
- chaotropic agents
- detergents

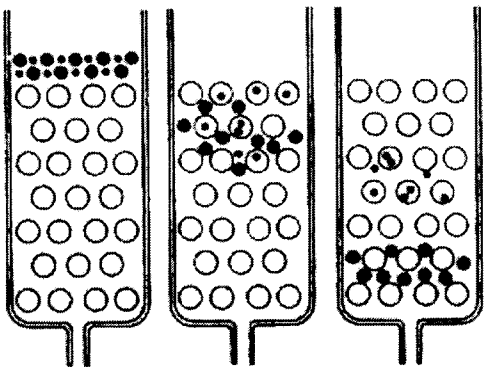
Conditions which decrease hydrophobic interactions are used to elute proteins from hydrophobic columns (Box). Decreasing the ionic strength is generally the preferred method since the other methods introduce substances which may be difficult to remove and/or denature the protein. Decreasing the ionic strength can be done in a step-wise fashion or with gradients as discussed for ion exchange chromatography. Substances which affect the polarity of the solvent (i.e., water) will also affect hydrophobic interactions. For example, a gradient of ethylene glycol will lead to the differential elution of proteins. Similarly, chaotropic agents can be use to elute proteins from hydrophobic columns. Detergents are more hydrophobic than proteins and will complete with the proteins for binding to the hydrophobic matrix.

Reverse phase chromatography (RPC) also separates compounds based upon differences in hydrophobicities. RPC differs from hydrophobic interaction chromatography (HIC) in that the mobile phase is a nonpolar solvent such as hexane instead of an aqueous salt solution. HIC is usually performed under non-denaturing conditions and separates proteins according to differences in surface hydrophobicity. RPC is carried out under denaturing conditions and separates according to differences in the total hydrophobicity, since all of the amino acid residues are available for interaction with the stationary phase. The separation of small polypeptides and proteolytic fragments is a common application of RPC.

HIC vs. RPC

	Hydrophobic	Reverse Phase
Mobile Phase	Polar Solvent	Nonpolar Solvent
Conditions	Native	Denatured
Solute Properties	Surface Residues	Total Residues

GEL FILTRATION CHROMATOGRAPHY



Gel filtration, also called molecular sieve chromatography or size exclusion chromatography, separates proteins on the basis of molecular size. A protein solution is passed over a column made up of small beads composed of cross-linked polymers. The degree of cross-linking will defined a pore size. Solutes larger than this pore size are excluded from the matrix and pass through the column unimpeded. Smaller solutes will enter the gel matrix and are retained on the column longer. The retention time is inversely proportional to the size of the solute.

- Dextran (=Sephadex)
- Polyacrylamide
- Agarose (=Sepharose)

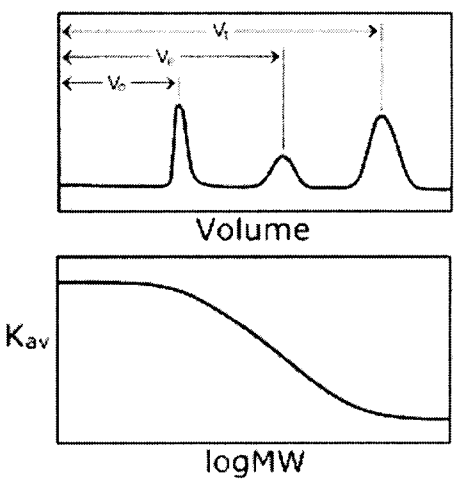
Several different polymers have been used as support matrices for gel filtration chromatography (Box). Different grades of gel filtration media with different pore sizes are available. The different pore sizes allow for separation of macromolecules with different size ranges. Gel filtration chromatography is generally carried out in buffers containing 0.15-1.0 M salt to prevent interactions of proteins with the support matrix. Unlike the other chromatographic methods the solute (eg., protein) does not bind to the stationary phase during chromatography. Therefore, gel filtration is a gentle technique and fragile proteins are not damaged by adsorption to the chromatographic support. Since proteins do not bind to the stationary phase, the resolution is dependent upon loading the smallest possible volume of sample.

Gel filtration can also be used to determine the molecular weight of a protein if the columns are calibrated by using molecular weight standards. Proteins of known size are passed over the column and the K_{av} is determined for each protein according to the following equation:

V_o = void volume
V_t = total volume
V_e = elution volume

$$K_{av} = V_e - V_o / V_t - V_o .$$

The void volume (V_o), also called the excluded volume, is the elution volume of a substance which is two large to enter the matrix of the support medium. This is experimentally determined and represents the solvent between the beads. The total volume is calculated from the volume of the column bead (πr^2 x length) and represents the elution volume of molecules completely included in the matrix. All substances, assuming there are no interactions with the matrix, will elute in this volume. The K_{av} values are plotted against the log of the molecular weight for each protein standard. The molecular weight of the unknown protein can then be determined from its



K_{av} and the standard curve.

Shape also affects the retention of macromolecules. For example, long rod shaped proteins elute from gel filtration columns at apparent molecular weights greater than their actual molecular weights.

Desalting. Gel filtration is often employed to remove salts or other small molecular weight solutes from protein solutions or to carry out buffer exchanges. The sample is loaded onto a gel filtration column that will exclude proteins (eg., G-10 or G-25) and the void volume collected. Desalting columns are much faster than dialysis. However, the sample is usually diluted 2-4 fold. Spin columns, widely used in DNA isolations, are another example of desalting columns.

HIGH PERFORMANCE (PRESSURE) LIQUID CHROMATOGRAPHY

HPLC is not a new chromatographic technique, but an advancement of technology. The flow rates in conventional chromatography is limited because of the compression of the support matrices used in the columns. These low flow rates result in diffusion and loss of resolution. New resins that can withstand packing and high flow rates allow for higher resolution have been developed. This allows for separations to be carried out under higher pressures (i.e., flow rates) resulting in increased resolution. All of the same types of chromatographic media are available for HPLC as in conventional chromatography.

CHROMATOGRAPHY	DISCRIMINATION
Ion Exchange	Charge
Gel Filtration	Size and Shape
Hydrophobic	Surface Hydrophobicity
Reverse Phase	Total Hydrophobicity
Affinity	Specific Amino Acids
Adsorption	Amino Groups?

http://www.science.uts.edu.au/subjects/91326/Section3/section3.html (good summary of chromatography)