Semester IV

17BCU404B PROTEIN PURIFICATION TECHNIQUES 3H-3C

SCOPE

To introduce the students about the techniques of involved in protein purification.

OBJECTIVES

Students will understand the principles and techniques of various protein purification techniques like chromatographic, electrophoresis and dialysis.

Unit 1

Salting in and salting out- Principle, Debye-Huckel theory, Hofmeister series, Ionic strength, Ammonium sulfate precipitation, applications

Unit 2

Gel exclusion Chromatography- Principle, instrumentation and applications of gel exclusion chromatography, data analysis Advantages and disadvantages.

Unit 3

Polyacrylamide gel electrophoresis- Principle, instrumentation and applications of PAGE. Gel polymerization- APS, TEMED. Separation and determination of molecular weight of proteinsSDS, running gel, stacking gel, electrophoresis buffer.

Unit 4

HPLC -Principle, instrumentation and applications of HPLC. Preparation of column, adsorbent materials, void volume, efficiency factor. Van Deemter equation Applications- Manufacturing, legal, research and medical.

Unit 5

Dialysis- Principle and types- Hemodialysis, pediatric, intestinal and peritoneal dialysis. Dialyzable substances. Medical applications.

REFERENCES

Sheehan, D., (2010). Physical Biochemistry: Principles and Applications 2nd ed., Wiley Blackwell (West Sussex), ISBN: 978-0-470-85602-4 / ISBN: 978-0-470-85603-1.

Freifelder, D., (1982). Physical Biochemistry: Applications to Biochemistry and Molecular Biology 2nd ed., W.H. Freeman and Company (New York), ISBN:0-7167-1315-2 / ISBN:0-7167-1444-2.

Plummer D. T., (1998). An Introduction to Practical Biochemistry 3rd ed., Tata McGraw Hill Education Pvt. Ltd. (New Delhi), ISBN:13: 978-0-07-099487-4 / ISBN:10: 0-07-099487-0

KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed to be University)
(Established Under Section 3 of UGC Act 1956)
Coimbatore - 641021.
(For the candidates admitted from 2017 onwards)
DEPARTMENT OF BIOCHEMISTRTY

SUBJECT : PROTEIN PURIFICATION TECHNIQUES

SEMESTER : IV
SUBJECT CODE: 17BCU404-B CLASS : II B. Sc. BC

LECTURE PLAN
DEPARTMENT OF BIOCHEMISTRTY

S.No	Lecture Duration Hour	Topics to be Covered	Support Material/Page Nos
		UNIT-I	
1	1	Salting in and salting out- Principle	T1: 1-4
2	1	Debye-Huckel theory	T1:28-32
3	1	Hofmeister series	T1: 47-48
4	1	Ionic strength	T1: 98-99
5	1	Ammonium sulfate precipitation,	T1: 100-102
6	1	ASP - applications	T1: 103-105
	Total No Of Hours Planned For Unit 1=06		
		UNIT-II	
1	1	Gel exclusion Chromatography- Principle	T1: 462 -463
2	1	GEC - Instrumentation	T1: 463-464
3	1	Applications of gel exclusion chromatography	T1: 465
4	1	GEC - data analysis	T1: 466
5	1	GEC - Advantages and disadvantages.	T1: 467
	Total No Of Hours Planned For Unit II=05		
		UNIT-III	

1	1	Polyacrylamide gel electrophoresis- Principle	T1:399-401
2	1	Instrumentation of PAGE.	T1: 401-403
3	1	Applications of PAGE.	T1: 403-405
4	1	Gel polymerization- APS, TEMED.	T1:405-407
5	1	Separation and determination of molecular weight of proteins.	T1:417-419
6	1	Gel polymerization- SDS.	T1:329-330
7	1	Gel polymerization- running gel, stacking gel.	T1:405-407
8	1	Electrophoresis buffer.	T1:419-420
	Total No Of Hours Planned For Unit III=8		
		UNIT-IV	
1	1	HPLC - Principle	T1:446-447
2	1	Instrumentation and applications of HPLC.	T1:447-448
3	1	Preparation of column	T1:448-450
4	1	Adsorbent materials,	T1:450-451
5	1	Void volume,	T1:463-464
6	1	Efficiency factor.	T1:465-466
7	1	Van Deemter equation	T1:440-441
8	1	Applications- Manufacturing.	T1:453-454
9	1	Applications- legal.	T1:455-456
10	1	Applications- research and medical.	T1:456-457
	Total No Of Hours Planned For Unit IV=10		
		UNIT-V	
1	1	**Dialysis-** Principle	T2:140-142
2	1	**Dialysis-** types	T2:142-143
3	1	Hemodialysis	T2:143-144
4	1	Pediatric dialysis	T2:146-148
5	1	Intestinal dialysis	T2:149-151
6	1	Peritoneal dialysis.	T2:152-153

7	1	Dialyzable substances.	T2:153-154
8	1	Medical applications.	T2:154-155
	Total no of Hours Planned for unit V=7		
Total Planned Hours		**36**	

References:

T1: Wilson, K., and Walker, J., (2010). Principles and Techniques of Biochemistry and Molecular Biology, 7th Low Price Edition, Cambridge University Press, India.

T2: Donald Voet and Judith Voet; 2012, Biochemistry, 4th Edition, John Wiley and Sons. Inc

Signature of the Staff

UNIT-I

SYLLABUS

Salting in and salting out- Principle, Debye-Huckel theory, Hofmeister series, Ionic strength, Ammonium sulfate precipitation, applications.

Protein isolation and characterization - I

Introduction:

As we have seen in the past lectures, proteins are quite diverse in nature. Protein expression is strongly regulated for normal functioning of a cell or organism. To be able to understand protein structure and function in detail, they often need to be separated from several cellular components like lipids, nucleic acids, sugars, etc. and isolated to homogeneity. A given cell may have thousands of proteins. It is quite challenging to isolate a particular one from the huge number of proteins. Also after obtaining a protein to near homogeneity one has to ensure that it retains its native biological characteristics of structure as well as activity. Thus its purification and characterization, enzymatic activity and structural elucidation are essential for a complete understanding of the protein.

Proteins can be purified by exploiting specific properties that include solubility, size, charge, and binding affinity towards some specific agents. It must be remembered that protein purification strictly depends upon the precise nature of the protein.

General methods include

(i) Precipitation

(ii) Extraction and

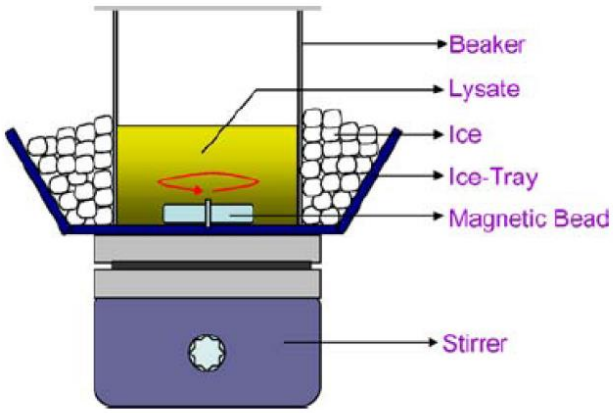
(iii) Chromatographic separation

Protein Precipitation

The most common source of proteins is microbial cells or tissue. Cytosolic proteins are highly water soluble and their solubility is a function of the ionic strength and pH of the solution. The most useful method of precipitating protein from its solution is to add salt to it. The commonly used salt for this purpose is ammonium sulfate, due to its high solubility even at lower temperatures. Both ions of ammonium sulfate are high in the Hofmeister series or lyotropic series which is a classification of ions in order of their ability to salt out or salt in proteins. The earliest step in any protein purification procedure needs to rupture cells; to release their proteins into a solution, often called a crude extract or lysate. Sometimes differential centrifugation may need to collects a specific sub cellular part or organelles.

Salting Out

Most proteins are less soluble at high salt concentrations, an effect called salting out. Proteins in aqueous solutions are highly hydrated, and with the addition of salt, the water molecules become more attracted to the salt than to the protein due to the higher charge. The addition of ammonium sulfate in the requisite amount can selectively precipitate a protein of interest while others remain in solution. For example, 0.8 M ammonium sulfate can precipitate fibrinogen, whereas a concentration of 2.4 M is needed to precipitate serum albumin. Salting out is very useful for concentrating dilute solutions of proteins, and has no adverse structural effects.

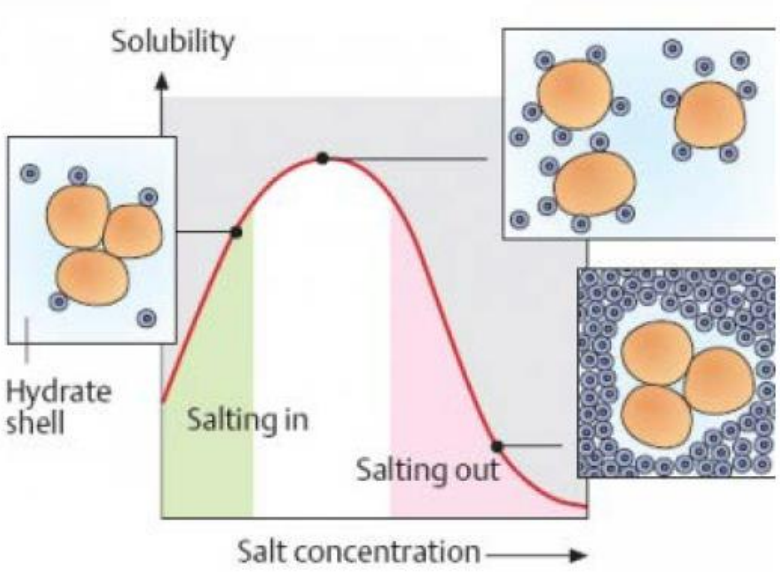


Protein precipitation using ammonium sulfate **(Needs to be drawn later)**

In this processes, the ammonium sulfate concentration is increased stepwise by adding solid ammonium sulfate in small quantities. The amount depends on the volume of the solution and the percentage saturation of the salt needed. It is possible to calculate how much is needed from available published nomograms. For protein purification, a step precipitation is carried out in which the precipitated protein is removed by centrifugation and the ammonium sulfate concentration increased to a value that will precipitate most of the protein of interest. In most cases this leaves out the protein contaminants in solution. The precipitated protein of interest is further recovered by the process of centrifugation and the pellet obtained dissolved in fresh buffer to be purified further. If required the above process is repeated usually with a different concentration of ammonium sulfate for precipitation of a different protein from the supernatant,

Salting In

As mentioned earlier, the solubility of proteins is strongly dependent on the salt concentration or ionic strength of the medium. Proteins are usually poorly soluble in pure water. Their solubility increases as the ionic strength increases, because more and more of the well-hydrated inorganic ions (blue circles) are bound to the protein's surface, preventing aggregation of the molecules (salting in).



Salting in and salting out (Needs to be drawn later)

AMMONIUM SULFATE PRECIPITATION

Principles

While several salts can be used as precipitants, AS has several properties that make it the most useful. It is very stabilizing to protein structure, very soluble, relatively inexpensive, pure material is readily available, and the density of a saturated solution (4.1 M) at 25° C (r¼1.235 g/cm3) is not

as high as another salting-out agent, potassium phosphate (3 M, r¼1.33 g/cm3). Figure shows a typical protein solubility curve where the log of the protein solubility is plotted as a function of AS concentration. The main features of this curve are a region at low salt where the solubility increases (called ‘‘salting in’’), and then a region where the log solubility decreases linearly with increasing AS concentration (called ‘‘salting out’’). The latter part of the curve can be described by the equation log10S¼b_Ks(G/2) where S in the solubility of the protein in mg/ml of solvent, G/2 is the ionic strength, and b and Ks are constants characteristic of the protein in question. Ks is a measure of the slope of the line and b is the log of the solubility if the salting-out curve is extrapolated to zero ionic strength. In general, most proteins have similar Ks values but vary considerably in their b value. Suppose that the curve in Figure is valid for your protein and that the concentration of your protein in a cell extract is 1 mg/ml. The upper

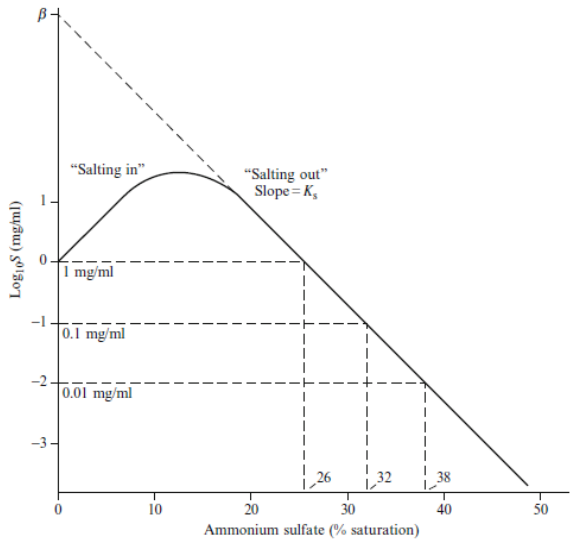


Figure - Ammonium sulfate solubility curve for a hypothetical protein. This represents the log solubility of a hypothetical protein as a function of percent saturation of ammonium sulfate. The ‘‘salting-out’’ line follows the relationship log S¼b_ Ks(G/2) as described in the text, where G/2 is the ionic strength, which here is given as ammonium sulfate percent saturation

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horizontal dotted line intercepts the solubility line at log S¼0 (S¼1 mg/ml) and at an AS percent saturation of 26%. This means that if you add AS to 26% saturation, all of your protein would be soluble. Now if you increased the AS to 32% saturation (the middle horizontal dotted line), the log S would be _1 (S¼0.1 mg/ml) so 90% of your protein would become insoluble and precipitate out. For this extract, an excellent strategy would be to make a 26–32% saturated AS cut: add AS to 26%, spin out insoluble material, and then make the supernatant 32% saturated and collect what precipitates, which would contain 90% of your protein. You would remove those proteins and cell components that precipitate at 26% saturation and those that fail to precipitate at 32% saturation. It is instructive to consider what would happen if you diluted the extract 10-fold with buffer. Now the initial concentration of your protein in the extract is 0.1 mg/ml or log S¼_1. You can add AS to 32% saturation and your protein will not precipitate. To achieve 90% precipitation of your protein, you would have to increase the AS to about 38% saturation (bottom horizontal dotted line) or carry out a 32–38% saturated AS cut. You would end up having to use more than 10 times as much AS with the diluted extract to obtain your protein. This illustrates how important it is to specify the concentration of your extract.You do not usually have a curve like that shown in Figure for your protein of interest so you have to determine the appropriate AS concentrations experimentally as described below.

Basic procedure

While there are numerous variations on AS precipitation, the most common ones are to add solid AS to a protein extract to give a certain percent saturation. Adding an amount of solid AS based on Table is convenient, reproducible, and practical.

1. Generally one determines a lower percent saturation at which the protein of interest just does not precipitate and a higher percent saturation that gives >90% precipitation as described in the section below.

2. Add solid AS to reach the lower value. Take care to add the AS slowly with rapid stirring so that the local concentration does not ‘‘overshoot’’ the target value. Some people carefully grind the solid AS with a mortar and pestle to a fine powder that dissolves rapidly. Once the AS is completely dissolved, allow the precipitation to continue for about 30 min. This is a compromise between waiting several hours as precipitation slowly approaches equilibrium and the desire to move along with the purification and not to introduce long delays in the procedure. Generally, one carries out all operations in an ice bucket or cold room.

3. Centrifuge at about 10,000_g for about 10 min in a precooled rotor to pellet the material that is insoluble.

4. Carefully pour off the supernatant and determine its volume. Determine the grams of AS from Table to go from the lower desired percent saturation to the final higher percent saturation. Again add the AS slowly with rapid mixing to avoid high local concentrations and let the solution sit for 30 min to allow precipitation to occur.

5. Centrifuge as above. Let the pellet drain for about 1 min to remove as much as possible of the supernatant. If you have carried out the test precipitation carefully, the pellet will contain 90% or more of your target protein. This protein can be dissolved in an appropriate buffer and after either dialysis, desalting, or dilution used in the next step of the purification.

Table 20.1 Final concentration of ammonium sulfate: Percentage saturation at 0 °C[a]

Initial concentration of ammonium sulfate (percentage saturation at 0 °C)	Percentage saturation at 0 °C																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulfate (g) to be added to 1 l of solution																
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	132	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

[a] Reprinted from England and Seifter (1990), which was adapted from Dawson et al. (1969).

Doing an ammonium sulfate precipitation test

Generally one can precipitate 90% of a given protein with a 10% increase in AS saturation so one should restrict the range of the ‘‘AS cut’’ to no more than 10% (the proteins that are just soluble at 30% saturation but precipitate at 40% saturation are referred to as the 30–40% AS cut). Figure illustrates a method to determine the optimal AS precipitation conditions using only two centrifugation steps. Basically you place a volume of cell extract, for example, 10 ml in each of five tubes. You add with mixing amounts of solid AS to give 20%, 30%, 40%, 50%, and 60% saturation based on Table, let sit 30 min to allow precipitation, and then centrifuge to p ellet the insoluble material. The pellets represent the 20%, 30%, 40%, 50%, and 60% saturated AS pellets. The volumes of the corresponding supernatants are determined and again solid AS is added to raise each to a 10% higher level of saturation. Again you mix, allow 30 min to precipitate, and then spin. The five pellets are the 20–30%, 30–40%, 40–50%, 50–60%, and 60–70% AS cuts. Each of these

is dissolved in buffer and assayed for enzyme activity and total protein and perhaps subjected to SDS gel analysis. Most of the activity should be in one of the cuts, but if, for example, half is in 30–40% cut and half is in the 40–50% cut, then perhaps a 35–45% cut would be optimal. While this test may seem onerous, it is really quite an efficient way to determine the optimal conditions that will result in higher enrichment in this important step.

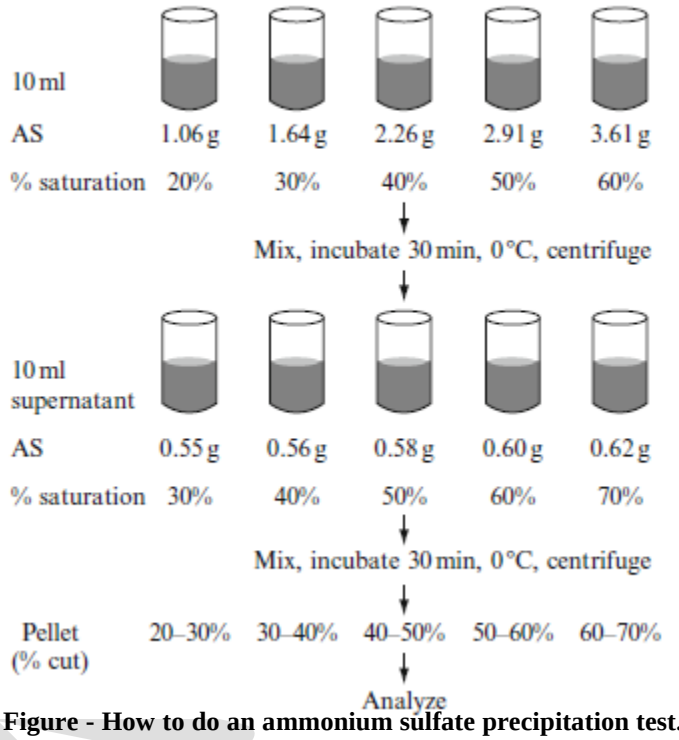


Figure - How to do an ammonium sulfate precipitation test. This test is carried out as described in the text and is self-explanatory.

Derivation of the Debye-Huckel equation for strong electrolytes

Establishing the Debye-Huckel potential

Strong electrolytes are entirely dissociated,*i.e.*, NaCl. Ions will be considered as hard spheres that are distributed in a continuum of dielectric constant ε_0, the solvent water, and yielding an average charge density ρ_e.

The Poisson equation states that the potential is given by:

$$\nabla^2\phi = \frac{-4\pi}{\varepsilon_0}\rho_e \tag{1}$$

Therefore, an ion will feel the electrical potential created by the other ions according to the above equation. Because of the spherical nature of the ions, it is best to use polar coordinates to express the Poisson equation. Hence,

$$\frac{1}{r^2}\frac{d}{dr}(r^2\frac{d\phi}{dr}) = \frac{-4\pi}{\varepsilon_0}\rho_e \tag{2}$$

With s ions of charge z_i for ion i. Electroneutrality implies:

$$\sum_{i=1}^{i=s} n_i z_i = 0 \tag{3}$$

Isolating ion J as a central ion, the objective is to determine the effect of all the other ions on J, and sum up the result over all other ions. This will yield the electrical potential energy that we will relate in a second step to the chemical potential and consequently the activity coefficients for individual ions.

We need to define a distance of closest approach, a_D, between the central ion J and a counter ion CI.

$$a_D = r_J + r_{CI} \tag{4}$$

where r_J = radius of ion J and r_{IC} = radius of the counter ion.

The electroneutrality of the solution entails that the summation of all charges around the central ion must be equal to the charge bore by the central ion J. Hence,

$$\int_{a_D}^{\infty} 4\pi r^2 \rho_e dr = -z_J e \tag{5}$$

With no electrical interactions, the average distribution of ions of type i is simply n_i, that is the number of ions per unit volume of solution. In this case particle interact electrostatically, therefore Debye-Huckel used the Boltzman distribution function instead. Hence,

$$n_i' = n_i exp(\frac{-E_e}{k_B T}) \tag{6}$$

where k_B is the Boltzman constant.

This is related to the probability of finding an ion i per volume element at the center of which the electrical potential energy is E_e. Note that when $E_e = 0$, then it results in a uniform distribution. In our case,

$$E_e = z_i e \phi_J \tag{7}$$

and

$$n_i' = n_i exp(\frac{-z_i e \phi_J}{k_B T}) \tag{8}$$

The thermal energy with increasing temperature smears out the distribution. This distribution function gives the probability of finding an ion i per volume element (it is therefore concentration related) at a distance r_J from the central ion. Now, we can just sum up this result over all other ions to express the charge density with respect to the central ion J.

$$\rho_J = \sum n_i \, z_i \, e \, exp(\frac{-z_i e \phi_J}{k_B T}) \tag{9}$$

The Poisson equation then becomes:

$$\frac{1}{r^2}\frac{d}{dr}(r^2 \frac{d\phi_J}{dr}) = \frac{-4\pi}{\varepsilon_0} \sum n_i \, z_i \, e \, exp(\frac{-z_i e \phi_J}{k_B T}) \tag{10}$$

In order to solve this equation, Debye-Huckel made **a very important simplifying assumption**. On the grounds that $z_i e \phi_J \ll k_B T$, or that the energy derived from electrical forces is small compared to the thermal energy, they expanded the exponential term in power series and neglected higher order terms, *i.e.*, terms of degree 2 and higher. Given that:

$$e^{-x} = 1 - x + \frac{x^2}{2!} - \frac{x^3}{3!} + ... \tag{11}$$

one obtains:

$$\rho_J = \sum n_i z_i e - \sum n_i \, z_i \, e(\frac{z_i e \phi_J}{k_B T}) \tag{12}$$

Since the first term is equal to zero, because of the electroneutrality condition, one finally gets:

$$\rho_J = -\sum \frac{n_i \, z_i^2 \, e^2 \phi_J}{k_B T} \tag{13}$$

Therefore,

$$\frac{1}{r^2}\frac{d}{dr}(r^2\frac{d\phi}{dr}) = \frac{4\pi}{\varepsilon_0}\sum\frac{n_i z_i^2 e^2 \phi_J}{k_B T} \tag{14}$$

or

$$\frac{1}{r^2}\frac{d}{dr}(r^2\frac{d\phi}{dr}) = \frac{4\pi e^2}{\varepsilon_0 k_B T}\sum n_i z_i^2 \phi_J \tag{15}$$

or, in a simpler manner:

$$\frac{1}{r^2}\frac{d}{dr}(r^2\frac{d\phi}{dr}) = \kappa^2 \phi_J \tag{16}$$

where

$$\kappa^2 = \frac{4\pi e^2}{\varepsilon_0 k_B T}\sum n_i z_i^2 \tag{17}$$

It turns out that κ is in reciprocal length (L^{-1}). A general solution of equation (16) that satisfies the fact that the potential remains finite when $r \to \infty$ is:

$$\phi_J = \frac{A}{r}e^{-\kappa r} \tag{18}$$

To obtain the value of the constant A, we need to state the electroneutrality of the solution. Therefore, using equations (9), (17), and (18) leads to :

$$\rho_J = \frac{-\kappa^2 \varepsilon_0}{4\pi} A \frac{e^{-\kappa r}}{r} \tag{19}$$

Using this equation in the expression of ρ_J, we can write a new expression for the electroneutrality, using (5), one gets:

$$A\kappa^2 \varepsilon_0 \int_{a_D}^{\infty} r e^{-\kappa r} dr = z_J e \tag{20}$$

after an integration by parts, one obtains the value of the constant A:

$$A = \frac{z_J e}{\varepsilon_0}\frac{e^{\kappa a_D}}{1+\kappa a_D} \tag{21}$$

Hence the expression of the potential is:

$$\phi_J = \frac{z_J e}{\varepsilon_0}\frac{e^{\kappa a_D}}{1+\kappa a_D}\frac{e^{-\kappa r}}{r} \tag{22}$$

This gives the expression of the potential at a distance r from the central ion J. The Poisson equation is only valid for static charges, this is not the case in solution. But we shall say that, since we are dealing with time averaged positions of the ions this assumption will hold.

Linking the Debye-Huckel potential to the activity coefficients

The potential due to ion J at a distance r form its center is

$$\phi_J'' = \frac{z_J e}{\varepsilon_0 r} \tag{23}$$

The total potential at a distance r is

$$\phi_J = \phi_J' + \phi_J'' \tag{24}$$

Then,

$$\phi_J' = \frac{z_J e}{\varepsilon_0 r}\left(\frac{e^{\kappa a_D}}{1+\kappa a_D} e^{-\kappa r} - 1\right) \tag{25}$$

The potential due to all the other ions upon the surface of the central ion J is simply:

$$\phi_J' = \frac{z_J e}{\varepsilon_0}\left(\frac{\kappa}{1+\kappa a_D}\right) \tag{26}$$

and if $a_D \ll \frac{1}{\kappa}$, like it is the case in a dilute solution, one gets:

$$\phi_J' = \frac{z_J e}{\varepsilon_0}\kappa \tag{27}$$

We can know calculate the change in electrical free energy of the solution at constant temperature and pressure when an ion J is added to the solution.

$$\Delta G_J = -z_J e \phi_J' \tag{28}$$

or

$$\Delta G_J = \frac{z_J^2 e^2}{2\varepsilon_0}\left(\frac{\kappa}{1+\kappa a_D}\right) \tag{29}$$

The number 2 occurs in the denominator because when all ions are considered J is counted twice. It is counted as the central ion and also as part of the atmosphere of other ions. We can relate now this change in free energy to the chemical potential. Assuming that 1 mole of ion is added to a large volume of solution, one obtains:

$$\Delta\mu_{e,J} = \frac{z_J^2 e^2 \mathcal{N}}{2\varepsilon_0}\left(\frac{\kappa}{1+\kappa a_D}\right) \tag{30}$$

Considering that μ has two contributions: $\mu_J = \mu_{ideal} + \Delta\mu_{e,J}$ and that $\mu_J = \mu_J^o + RT\,ln\,\gamma_J + RT\,ln\,[J]$ we obtain

$$ln\,\gamma_J = \frac{z_J^2 e^2}{2\varepsilon_0 k_B T}\left(\frac{\kappa}{1+\kappa a_D}\right) \tag{31}$$

HOFMEISTER SERIES

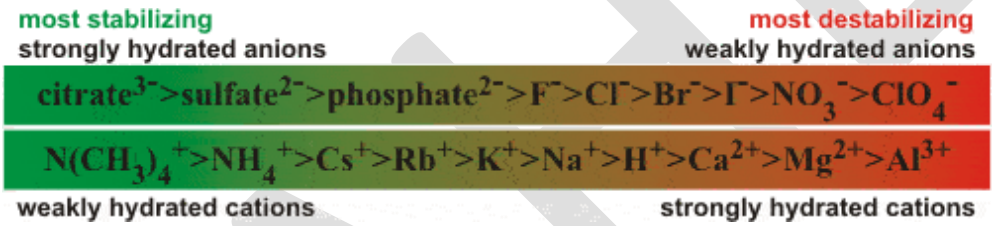
The effects of ions on biological and chemical processes in solution usually depend on the particular ions involved. These specific ion effects make up the Hofmeister phenomena. The Hofmeister series, below, originates from the ranking of various ions toward their ability to precipitate a mixture of hen egg white proteins.[a]

Anions: $SO_4^{2-} > HPO_4^{2-} > acetate^- > Cl^- > NO_3^-$

Cations: $Mg^{2+} > Li^+ > Na^+ = K^+ > NH_4^+$

Simplistically, this protein precipitation can be explained in terms of the extent of the ions binding to water (see also salting-out).[b] Thus the effective concentration of the proteins increases (in the remaining 'free' water) and they precipitate, so releasing low entropy surface water. The series has been shown to have a much more general utility with at least 38 observed phenomena (given in the comprehensive review, with the current state of play recently reviewed) including showing the graduated effects on the structuring or denaturation of biological macromolecules, effects on interfacial hydration and affecting pH measurements. Nowadays the Hofmeister series are usually given in terms of the ability of the ions to stabilize the structure of proteins.[3] A similar effect has been found with the salt-induced activation of lyophilized enzymes. They show opposite correlations for anions and cations with their degree of strong hydration. The relative positions (mostly corresponding to the degree of strong hydration) in the series should be thought of as indicative only as there will be variation with protein, pH and temperature, with acetate ions showing pronounced cation-specific effects. The relative order of cations may reverse with different anions (for example, NO^- rather than Cl^-) under some circumstances due to ion pair

effects. The relative order of anions may reverse dependent on the hydrophobicity/hydrophilicity and charge on the interacting surfaces; with $I^- > Cl^- > F^-$ on hydrophilic negatively charged and hydrophobic positively charged surfaces but $F^- > Cl^- > I^-$ on hydrophilic positively charged and hydrophobic negatively charged surfaces. Depending on the circumstances, NH_4^+ may occur out of order amongst the monovalent cations due to its hydrogen bonding capacity. Also, Li^+ often appears out of order as the water molecules within its first hydration shell are so tightly bound that its effective surface charge density is decreased by its hydration water.



Rationale

Although some data indicates that ions destroy the natural hydrogen bonded network of water, having effects similar to increased temperature or pressure (for example, by lowering the pressure required to form high pressure ice VII), other methodology indicates that ions cause negligible change to water's bulk structure; these differences may be due to the ionic concentration used, the sensitivity of the methods to the scale of potential structural changes in the bulk water, the difficulty in separating coexisting but opposite effects (that is, chaotropic and kosmotropic, see later), the precise meaning of 'bulk' water, and the importance of the presence of surfaces in stabilizing effects. In particular, effects of salts at lower concentrations may be smothered (in many studies) by the relatively large amount of unaffected 'bulk' water present whereas at high concentrations there may be insufficient water to properly show any specific effects. Some techniques do pick up the more extensive clustering effects expected; for example, Fourier transform infrared studies have shown four well-defined hydration spheres around a proton with an additional outer hydration layer and further more loosely bound water molecules.

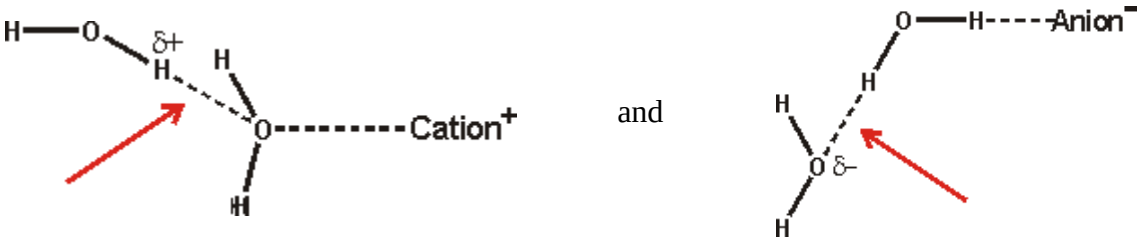
The effect of ions has been successfully approximated by the equivalent osmotic pressure and by the equivalent effect on water activity (4 molal NaCl is equivalent to 0.14 GPa). Ions that have the greatest such effect (exhibiting weaker interactions with water than water itself) are known as structure-breakers or chaotropes, whereas ions having the opposite effect are known as structure-makers or kosmotropes (exhibiting strong interactions with water molecules). Strongly hydrated ions considerably increase the difference between the hydrogen bond donating and accepting capacity of the linked water molecules resulting in the breakdown of the tetrahedral network.

Anions hydrate more strongly than cations for the same ionic radius as water hydrogen atoms can approach (about 0.8 Å) more closely than the water oxygen atoms (but note that most anions are larger than most cations), giving rise to greater electrostatic potential. Also, anions are far more polarizable than cations (compare Na^+ 0.12; K^+ 0.78; Cl^- 4.00 due to their more diffuse extra electron(s) and breaking hydrogen bonds round anions is relatively slow due to the difficulty in finding a new hydrogen-bonding partner. Anions are also thought more likely to promote the salting-out of amphiphiles. Although we put forward the surface charge density as being the important determinant of Hofmeister effects (as does, others state it is the polarizability that is important. However, a comprehensive study has shown the dominant role of charge density but no correlation of polarizability with thermal effects on either of an acidic or basic protein.

Small ions are strongly hydrated, with small or negative entropies of hydration, creating local order and higher local density. Small cations do not bind directly to polar surfaces but small anions, which have lower surface charge density than the cations, may bind through ion pairing. Large singly charged ions such as I^-, SCN^- or Cs^+, with more positive entropies of hydration, act like hydrophobic molecules, binding to surfaces dependant not only on charge but also on van der Waals forces. The large anions interact with the polypeptide backbone via a hybrid binding site that consists of the peptide nitrogen and the adjacent α-carbon. These amide backbone binding sites for weakly hydrated anions are the most significant locations for the salting-in of uncharged polypeptides. They may additionally be pushed on by strong water-water interactions and certainly induce a change in the surface hydration and interfacial

aqueous clustering. Such large ions possess low surface charge density (*e.g.* for comparison, F^-, Cl^-, Br^- and I^- have surface charge densities of $-8.98x10^{-21}$, $-4.42x10^{-21}$, $-3.64x10^{-21}$, and $-2.84x10^{-21}$ Coulombs/$Å^2$ respectively) and are able to sit comfortably within dodecahedral water clathrate shells and produce the lowest apparent[c] density for the solution water. Less large ions (for example, Rb^+, K^+, Br^-) cause the partial collapse of such clathrate structures through puckering. These ions allow rotations of the water molecule dipole towards the oppositely charged ions, through weak interactions, that would be prevented at truly hydrophobic surfaces and hence produce greater localized water molecule mobility ('negative' hydration). Larger ions, such as the tetramethylammonium cation, form clathrate structures but do not allow these rotations in the surface water surface ('hydrophobic' hydration).

The collapse, through puckering, of the water clathrate structures surrounding the smallest ions (for example, Na^+, Li^+, F^-) is tightly formed as these ions hold strongly to the first shell of their hydrating water molecules and hence there is less localized water molecule mobility (strong or 'positive' hydration) and higher apparent[c] density for the solution water. There is also a less complete cluster structure, due to the hydrogen-bonding defects[d] caused by the inward-pointing primary hydrogen-bonding to anions or disoriented lone-pair electrons and electrostatic repulsion together with weakened hydrogen bonding reducing inward-pointing secondary hydrogen bond donation near cations or acceptance near anions. Generally, the water surrounding anions tends to retain favorable water-water hydrogen bonding whereas that surrounding small cations does not. Higher charge density anions, such as sulfate, are exceptions to this generalization. That is, these H-bonds possess reduced strength



It can be noted that such strong hydration round both anions and cations costs the equivalent of two hydrogen bonds (~ 46 kJ mol^{-1}; that is, one replaced by the ion plus one chain of H-bonding with severely reduced strength) and is only found to form round ions where at least this enthalpic contribution is released.

Thermodynamic properties

Water cluster binding studies have shown that the ions possessing high charge density bind larger water clusters more strongly, see molar ionic volumes below. The entropies of hydration correlate with the tendency for the ion to accumulate in low-density water (LDW, for example, ES; see values below) such that a gain in entropy of the ion on solution is countered by a loss in entropy of the water. Another correlation is with the Jones-Dole viscosity ***B*** coefficient (see below).[e] Ions that are weakly hydrated exhibit a smaller change in viscosity with concentration, having negative ***B*** coefficients (chaotropes), than strongly hydrated ions that have positive ***B*** coefficients (kosmotropes). Such negative ***B***coefficients are not shown by ions in any other solvent, except D_2O where they are even more negative [304]. They lose this effect under pressure. Similar effects are seen with the ionic surface tension increments, $k_i = d\gamma/dc_i$ (mN m^{-1} M^{-1}) (see table below), where more positive values indicate ions most repelled from the aqueous interface. Some exceptions exist where ions have ion-specific interfacial effects, such as the hydrogen ion.

Key properties of aqueous ions

Cations	Ionic volume[i]	ΔS[ii]	Jones-Dole[iii]	k_j[iv]	Anions	Ionic volume[i]	ΔS[ii]	Jones-Dole[iii]	k_j[iv]
Al^{3+}	-58.7	-396	+0.67	2.65	$Citrate^{3-}$	+77[v]	na	+0.27[vi]	na
Mg^{2+}	-32.2	-174	+0.385	2.25	SO_4^{2-}	+25	-126	+0.206	0.55
Ca^{2+}	-28.9	-132	+0.298	2.10	HPO_4^{2-}	na	na	+0.382	0.70
H^+	-5.5	na	+0.068	-1.05	F^-	+4.3	-70	+0.127	0.80
Na^+	-6.7	-5	+0.085	1.20	Cl^-	+23.3	+6	-0.005	0.90
K^+	+3.5	+34	-0.009	1.10	Br^-	+30.2	+28	-0.033	0.55
Rb^+	+8.6	+52	-0.033	0.95	I^-	+41.7	+55	-0.073	-0.05
Cs^+	+15.8	+59	-0.047	0.80	NO_3^-	+34.5	+9	-0.045	0.15
NH_4^+	+12.4	+5	-0.008	0.70	ClO_4^-	+49.6	+30	-0.061	-0.70
$N(CH_3)_4^+$	+84.1	na	+0.123	-0.10	Data from	[128]	[128]	[128]	[1981]

i Molar aqueous ionic volume, $cm^3\ mol^{-1}$, 298.15 K; negative values indicates contraction in volume. **ii** Entropy of hydration, kJ mol^{-1}, 298.15 K; standard molar entropy less the entropy of the primary hydrating water (that is, its immobilization). **iii** Viscosity Jones-Dole *B*-coefficient,[e] $dm^3\ mol^{-1}$, 298.15 K; results from the degree of water structuring by the ions. The accuracy and precision of this data may be overstated but the values are indicative. **iv** Ionic surface tension increments, $k_i = d\gamma/dc_i$ (mN m^{-1} M^{-1}) over the range 0.1 M - 1.0 M , values from [1981]. **v** Trisodium citrate value from [743] less Na^+ values from this table.**i** Calculated from [743]. [Back] na not available

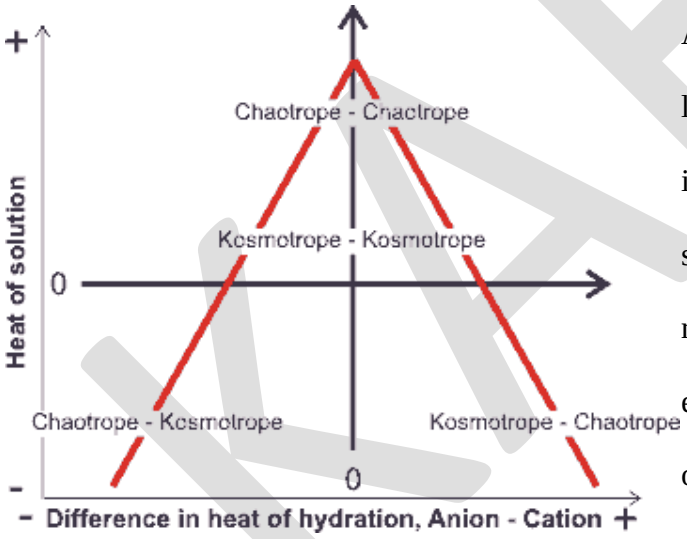
Effect on physical properties

As weakly hydrated ions accumulate in low-density (ES) water, ions can fit into the icosahedral network without affecting its hydrogen bonding and, therefore, with less effect on its viscosity as the concentration increases. Pressure shifts the ES CS equilibrium towards CS formation, so reducing this viscosity effect. It is clear from the experimental data that although the weakly hydrated ions encourage ES formation the resultant structure is not more-strongly hydrogen bonded (as expected in supercooled water) or else the viscosity would be expected to be higher (larger *A* coefficients), which is not found; the low viscosity being at least partially due to the lower density reducing non-bonded inter-molecular attractions. Similar correlations are seen with surface tension changes (more strongly hydrated ions increasing the surface tension more) and dissolved gas concentrations (more strongly hydrated ions reducing the solubility more); both effects are due to the shift to CS formation. The increased van der Waals dispersion forces of large ions also increase the pull on the gas/liquid surface and confuse the surface tension effects. As such dispersion forces correlate well with lower charge density for similarly charged ions, it may be difficult to separate these effects. A theory has been presented which takes into account both the ionic hydration and the polarizability to allow calculation of the surface tensions of various sodium salts.

Effect on solubility

Arrangement of water around ions is not restricted to the first hydration layer (for example, magnesium ions, see also the ion puckered clusters) except at high concentrations (the apparent number of hydrating water molecules and their rates of exchange depending not only on the ion but also the method of determination and the ionic concentration; a review is available).

Oppositely charged salt ions generally show two energy minima in their radial separation, one of which will be the global minimum. These involve close contact and solvent separated contact. The relative energy of these minima depends on the ions, their concentration, other solutes and the prevailing physical conditions. The type of ions present in solution control their overall properties; inner sphere ion pairs (that is, close contact) may be formed between two small ions of high charge density (for example, CaF_2), where the strong ionic attraction overcomes the hydration shells, or between two large ions of small charge density (for example, AgI; monovalent cations >1.06 Å, monovalent anions > 1.78 Å), where there are no strong hydration shells (i.e. when the ions have similar water affinities).



An equivalent but alternative way of looking at this (see 'volcano plot' opposite) is that when the anion and cation have similar affinities for water they are able to remove the water from each other most easily, to become ion-paired. A small ion of high charge density plus a large counter-ion of low charge density forms a highly soluble, solvent-separated hydrated but clustered ion pair as the large ion cannot break through its counter-ion's hydration shell (for example, CaI_2, AgF and LiI) but prefers to sit within the disturbed hydrogen bonding at intermediate distance between the ordered but poorly hydrogen bonded strongly-held first hydration shell and the more disordered but strongly

hydrogen bonded bulk phase water; an effect that also enhances strongly- hydrating ion-hydrophobe interactions. The tendency to form inner-sphere ion pairs between is reflected in the salt's solubility; the solubility of CaF_2, AgI, CaI_2 and AgF being 0.016, 0.0000016, 2090 and 1820 g/kg water respectively.[f] Such ion pair effects also cause partial raising ($Cl^- > Br^- > I^-$) of the homogeneous nucleation temperature of ice that has been lowered by increasingly hydrated (smaller) alkali cations ($Cs^+ < Rb^+ < K^+ < Na^+ < Li^+$).

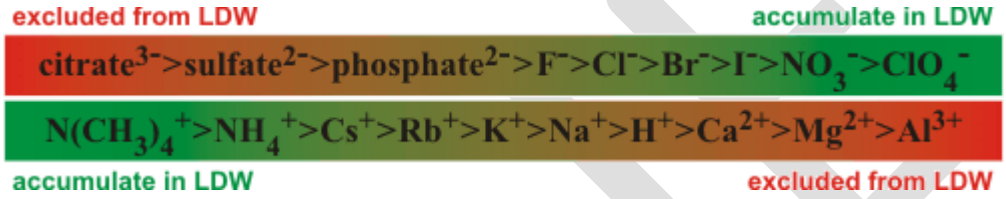
Stabilization of proteins

Proteins are most stable in solution when they are surrounded by fully hydrogen-bonded water molecules, as water molecules with spare hydrogen bonding capacity have higher entropy and are more aggressive. Such reactive water behaves in a similar way to that on raising the temperature and is the cause of the denaturing behavior of proteins on heating. Salt ions must be evenly distributed in solution as their distribution is controlled by osmotic gradients. Thus thechaotropic ions (with their weak aqueous interactions) should be closer to the protein and the kosmotropic ions (with their strong aqueous interactions) in the bulk. Thus ammonium sulfate is often a good salt for stabilizing protein structure and bioactivity.

Hydrophobic and hydrophilic associations

The Hofmeister series also shows the promotion of hydrophobic associations. The ions are in order of stabilizing structured low-density water that, in turn, stabilizes both the hydrophobic interactions of large molecules and the solubility of small hydrophobic molecules. Note that ionic chaotropes prefer low-density water, which is not required to break hydrogen bonds to accommodate them, but ionic kosmotropes are attracted to aqueous environments providing

more- available hydration sites (CS like) and are excluded from low-density water (and hence also much of the protein's surface). Ionic chaotropes therefore tend to higher concentrations next to a protein's hydrophobic surfaces whereas ionic kosmotropes tend to higher concentrations near protein residues that break up the local water structure such as typical carboxylates.



Hydrophilic polymers in solution are surrounded by water with varying LDW content; for example, proteins may be surrounded by LDW out to about 15 Å from the surface, equivalent to the radius of a complete icosahedral water cluster. Generally this LDW acts to separate such molecules but this process is also dependent on the ions present. Ions that only weakly interact with water (ionic chaotropes) partition into LDW, so stabilizing it and structures that depend on it. However, LDW is labile and may be abolished by solutes it accumulates due to the micro-osmotic gradients that may be fleetingly established. If both ions accumulate then micro-osmosis destroys the LDW and oscillations may occur. NH_4I and NH_4NO_3 destabilize LDW because both ions favor LDW. $MgSO_4$ destabilizes LDW because both ions favor unstructured normal-density water (followed by a tendency to form ion-pairs). $(NH_4)_2SO_4$ stabilizes LDW, as the ions are distributed evenly, and hence also stabilizes proteins and hydrophobic junction zones. This behavior has been shown to also enhance the activity of

lyophilized enzymes for use in organic liquids, where the formation of enhanced clathrate structuring may encourage a more fluid surface environment given the restricted water content. Net LDW formers increase the viscosity of poly-electrolyte solutions due to the increased LDW created between the polymers whereas netLDW destroyers (for example, NaCl, $MgCl_2$) reduce this viscosity. It should be noted that strongly hydrated salts alone, without polymer, have the opposite effect and increase viscosity, due to their tightly bound high-density ordered water. Ions distribute in aqueous two-phase systems according to their affinity for the more-LDW phase. Thus iodide ions prefer the polyethylene glycol (PEG) phase so producing a charged interface in PEG/dextran systems.

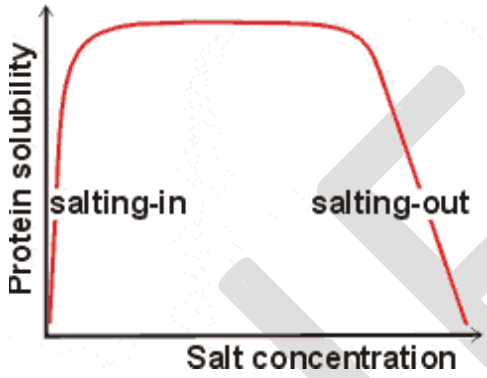
Footnotes

[a] Note (i) that the ability to precipitate protein(s) depends on the protein(s) present, (ii) that some proteins exhibit opposite Hofmeister series behavior to others (which may be due to the protein's net charge and the ionic strength of the solution; ovalbumin being negatively charged in the key experiments) and (iii) the order of some of the ions may be reversed in some series depending on the application, the counter ions used, the pH and any ion-specific factors present. Always note the application, conditions and method of comparison when examining published 'Hofmeister series'. Experiments into Hofmeister (ion-specific) effects have been criticized in that they often neglect the (possibly) confounding effects of the concentration of the ions (including counter-ions) present, the presence of other ions such as buffers, pH and the temperature. Clearly such effects must be taken into account if the relative effectiveness of the ions in any process is to be compared. The complete original series, given below in terms of molar effect, used a mixture of

egg white proteins and did not control for pH changes.

Anions: $citrate^{3-} > SO_4^{2-} = tartrate^{2-} > HPO_4^{2-} > CrO_4^{2-} > acetate^{-} > HCO_3^{-} > Cl^{-} > NO_3^{-} > ClO_3^{-}$

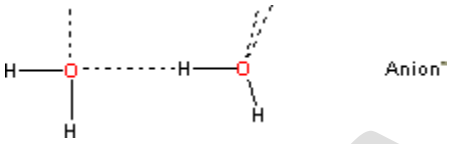
Cations: $Mg^{2+} > Li^{+} > Na^{+} = K^{+} > NH_4^{+}$



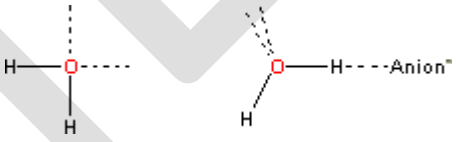
[b] Protein 'salting out' results from interfacial effects of strongly hydrated anions near the protein surface so removing water molecules from protein solvation and dehydrating the surface. The greatest effect is due to the most strongly hydrated anions. Protein 'salting in' (solubility increase on the addition of low levels of salt) results from protein-counter ion binding and the consequent higher net protein charge and solvation. Salting-in occurs where the protein has little net charge near its isoelectric point. In this case the greatest effect is due to the most weakly hydrated anions. Salting-in and salting-out processes are reviewed. Salting out follows the Hofmeister series above the isoelectric point for a number of proteins including lysozyme, but follows the reverse order below the isoelectric point. Many 'insoluble' proteins can be dissolved in pure water so long as the pH is away from the isoelectric point. Here the charged protein molecules repel each other, with hydrophobic areas covered with an organised low density water network so avoiding protein-protein interactions. In these cases, addition of ions reduce the repulsions and water organisation, so resulting in protein precipitation. [c] The

apparent density for the solution water is the weight of the water in the solution (that is, the weight of the solution less the weight of the salt) divided by the volume of the water in the solution (that is, the volume of the solution less the volume of the salt). The conclusion is irrespective of whether the 'dead space' around the ions is included (as in) or not (as in). [d] Cluster defects are water molecules, (labeled b-type previously) within the icosahedral cluster structure, with only 3 rather than 4 hydrogen bonds as the fourth site cannot accept/donate a hydrogen bond from/to either (a) a water molecule already possessing 4 hydrogen bonds;

that is, the fourth site has the arrangement



rather



than

or (b) a bound atom that already possesses 3 hydrogen bonds such as the oxygen atoms in SO_4^{2-}.

[e] The viscosity (η) of an aqueous salt solution (up to about 0.1 M), relative to the viscosity of water at the same temperature (η_0), varies with the salt concentration (c) according to the Jones-Dole expression:

$$\eta/\eta_0 = 1 + \boldsymbol{A}c^{0.5} + \boldsymbol{B}c$$

A is always positive and is greater for strongly hydrated ions (except H^+ and OH^-). It is associated with counter ion screening and only appreciably affects the viscosity at low concentrations (< 0.05 M) [304]. The ***B*** coefficient results from the degree of water structuring by the ions relative to bulk water interactions; also understood as the relative strength of the ion water interactions and varying linearly with the solute-water distance for monovalent electrolytes

[1764]. Ions with negative ***B*** coefficients have appreciably more negative ***B*** coefficients in D_2O but those with positive ***B*** coefficients show little change in D_2O. At higher concentrations (that is, above about 0.5 M), a further term ($\boldsymbol{D}c^2$), or terms, may be added to the right hand side as the viscosity increases more rapidly with concentration. ***A***, ***B*** and ***D*** all depend on temperature; ***B*** particularly so with ***B*** generally increasing with increasing temperature for the ionic chaotropes and reducingwith increasing temperature for the ionic kosmotropes. Salts where both *Jones-Dole* ***B*** coefficients are negative show a reduction in viscosity with increasing concentrations at higher concentrations; for example, KI solutions at 20 °C show reductions in viscosity with increasing concentrations above 2 mM reaching a minimum relative viscosity of ~0.888 at ~2.7 M. When used with non- electrolytes both the ***A*** and ***D*** coefficients are often neglected and ***B*** depends on the shape and solvated molar volume. [f] An equivalent but alternative viewpoint is that salts that are exclusively kosmotropic or chaotropic (that is, kosmotropiccation with kosmotropic anion or chaotropic cation with chaotropic anion) are less soluble than salts formed from ions with differing properties (for example, CsI (1.7 M, 0 °C) and LiF (0.1 M, 18 °C) are both less soluble than CsF (24 M, 18 °C) despite Li^+ and F^- ions generally hydrating most strongly), due to the energy cost of hydration shifting the ES CS equilibrium.

Questions	opt1	opt2	opt3	opt5	opt6	Answer
Most widely used salt for precipitation of protein is	A. Ammonium sulphate	B. Sodium chloride	C. Sodium sulphate	D. none of the above		**A. Ammonium sulphate**
It is observed that at low concentration of the salt, solubility of the proteins usually increases slightly. This is termed as	A. Salting out	B. Salting wide	C. Salting in	D. Salting narrow		**C. Salting in**
Protein can be precipitated by	A. Salt	B. Organic solvent	C. Organic polymer	D. All of the above		**D. All of the above**
PEG is	A. anionic and water soluble	B. Cationic and water soluble	C. Anionic and water insoluble	D. Cationic and water insoluble		**A. anionic and water soluble**
When the pH of the medium is less than the isoelectric point of a protein, the protein molecules will carry a net	A. Positive charge	B. Negative charge	C. Zero charge	D. Negative and positive both		**A. Positive charge**
Nucleic acids can be precipitated by	A. ethanol,	B. isopropanol	C. PEG	D. All of the above		**D. All of the above**
At isoelectric point the charge on protein is .	A. Zero	D- Ribose	C. negative	D. None of the above		**A. Zero**
Important criteria which should be considered when using organic solvent as a precipitation is as follows	A. Solvent should be completely miscible with water	B. Solvent should be available in highest purity	C. Solvent should not react with proteins	D. All of the above		**D. All of the above**
Which of the following can be used to precipitate nucleic acid?	A. CTAB	B. TCA	C. Polyethylenamine	D. All of the above		**D. All of the above**
The salt which produces salting out effect during extraction of proteins .	a) NH_4 SO_4	b) (NH4)2 SO4	c) (NH4)3 SO4	d) NaCl		**b) (NH4)2 SO4**
Protein purification refers to the	a) Purification of proteins	b) Separation of proteins from other biomolecules	c) Separation of a particular protein from other contaminating proteins	d) all of these		**c) Separation of a particular protein from other contaminating proteins**
Protein separation techniques are often based on the following properties except	a) solubility of the protein	b) viscosity of the protein	c) charge of protein	d) specific binding affinity of the protein		**b) viscosity of the protein**
Primary steps in protein purification includes	a) Homogenization	b) Differential centrifugation	c) Solubilisation	d) all of these		**d) all of these**
Which of the following detergent is commonly used to release integral proteins from its membranes?	a) urea	b) dimethyl sulphoxide	c) triton X 100	d) cyanogen bromide		**c) triton X 100**
Salting out process involves	a) precipitation of proteins using ammonium sulphate	b) precipitation of proteins using copper sulphate	c) precipitation of proteins using sodium chloride	d) none of these		**a) precipitation of proteins using ammonium sulphate**
Which of the following separation method is suited for a protein sample with large differences in molecular mass	a) dialysis	b) salting out process	c) density gradient centrifugation	d) rate zonal centrifugation		**d) rate zonal centrifugation**
In which of the following separation method where proteins are separated on the basis of their net charge	a) Affinity chromatography	b) Ion exchange chromatography	c) dialysis	d) gel filtration chromatography		**b) Ion exchange chromatography**
Which of not a method of protein precipitation?	a) Salting out with metals l	b) Acetone & alcoho	c) Changing pH other than iso-electric pH	d) Tri-Chloro-acetic acid		**c) Changing pH other than iso-electric pH**
Precipitation of proteins is done by all these except	a) Trichloro-acetate	b) Salts of heavy metals	c) Alcohol and acetone	d) Above or below the iso-electric pH		**d) Above or below the iso-electric pH**
Protein is purified using ammonium sulfate by	a) Salting out	b) Ion exchange chromatography	c) Mass chromatography	d) Molecular size exclusion		**a) Salting out**
A reducing disaccharide containing glucose is	maltose	lactose	trehalose	Furanose		**trehalose**

The reagent used for distinguishing a reducing monosaccharide from a reducing disacchride	Benedict's reagent	Barfoed's reagent	Fehling's reagent	Seliwanoff's reagent	**Benedict's reagent**
The arrangements of sugars into D&L configuration is based upon their resemblance toD&L	Glyceraldehydes	lactic acid	glucose	Ribose	**Glyceraldehydes**
Starch is composed of repeating unit of	Maltose	Glucose	Cellobiose	Fructose	**Maltose**
β (1-4) linkage is present in	Starch	Glycogen	cellulose	Amylose	**cellulose**
Amylose contains glucose units	100-200	200-300	300-400	500-600	**300-400**
Each branch of amylopectin is an interval of glucose units	14-20	24-30	34-40	44-50	**24-30**
The end product of hydrolysis of strach by amylase is	Soluble starch	Glucose	dextrin	maltose	**Soluble starch**
The component present in starch that gives blue colour with iodine is	Amylase	amylopectin	amylose	Fructose	**Amylase**
Amylopectins are present in	Hyaluronic acid	strach	sucrose	Glycogen	**Hyaluronic acid**
Erythrodextrin gives colour with iodine	blue	violet	red	no colour	**red**
Cellulose is made up of	α-glucose	β-glucose	fructose	mannose	**β-glucose**
Hyaluronidase is the enzyme, which acts on	homopolysaccharide	heteropolysaccharide	disaccharide	trisaccharide	**heteropolysaccharide**
In place of glucuronic acid chondroitin sulphate B contains	gluconic acid	glucamic acid	iduronic acid	Sulphonic acid	**iduronic acid**
Heparin has a molecular weight of about	14,000	15,000	16,000	17,000	**17,000**
Blood group subtances consists of	lactose	maltose	fucose	Mucosa	**fucose**
The component of cartilage & cornea is	Keratosulphate	chondroitin sulphate	cadmium sulphate	antimony sulphate	**Keratosulphate**
The compound which is an acid mucopolysacchride	dicoumarol	EDTA	Hyaluronic acid	Glycogen	**Hyaluronic acid**
A polymer of N-acetylated glucosamine is	dextran	heparin	chitin	dextrin	**chitin**
Change in optical rotation is	specific rotation	mutarotation	epimerism	Anomerism	**mutarotation**
The polymer of fructose is	Strach	glycogen	cellulose	insulin	**insulin**
The glycosaminoglycan which acts as an anticoagulant is	Heparin	Hyaluronic acid	dextrin	dermatin sulpharte	**Heparin**
The glycosaminoglycan which is present in synovial fluid	Heparin	Hyaluronic acid	dextrin	dermatin sulpharte	**Hyaluronic acid**
2 carbon epimer of glucose	Fructose	galactose	Mannose	Ribulose	**Mannose**
The mirror images are	Enantiomers	Anomers	Epimers	All	**Epimers**
Chitin is composed of	fructofuranose	D-glucuronic	N-acetyl glucoseamine	Fructose + glucose	**N-acetyl glucoseamine**
the mucopolysaccharide which serve as lubricant and shock absorbent in joints is	heparin	chondroitin sulphate	hyaluronic acid	cellulose	**hyaluronic acid**
All carbohydrates contain carbon. This can be shown by heating with	sodium hydroxide solution	burning in air	heating with conc. Sulphuric acid	Hydrochloric acid	**heating with conc. Sulphuric acid**
The buffer acting in the osazone reaction is	phenyl hydrazine hydrochloride+	acetic acid +phenyl hydrazine	sodium acetate +acetic acid	sodium acetate	**phenyl hydrazine hydrochloride+**
The monosaccharide units of hyaluronic acid are	glucuronic acid & N-acetyl D-glucosamine	glucuronic acid & N-acetyl galactosemine	iduronic acid & N-Acetyl galactoseamine	iduronic acid & N-acetyl d-Glucosemine	**glucuronic acid & N-acetyl D-glucosamine**
NASA has launched a satellite named ____ to study the effect of water in various forms on climate.	*Aqua*	*Hydro*	*Hi*	*Water*	***Aqua***
Water is	Tasteless	Odorless	both a and b	low boiling point	**both a and b**
The epimers of glucose	Fructose	galactose	ribose	deoxy ribose	**galactose**
The dissacharide which does not show mutarotation	sucrose	lactose	maltose	cellobiose	**sucrose**
Polysaccharides produce more than ___ monosaccharides on hydrolysis;	Eight	Ten	Six	five	**Ten**
Stereoisomer classified into	Two	Three	Four	Seven	**Two**
D isomer rotate the plane polarized light to	Left	Right	DL mixture	Interchange	**Right**
Isomers formed by the interchange of H and OH groups on Carbon atom2, 3 and 4 are known as	Monomer	Dimer	Epimers	Tetramer	**Epimers**
Sugars forming six member ring are known as	Furanose	racemixture	mannose	Pyranose	**Pyranose**

Maltose is composed of	two glucose molecules	three glucose molecules	four glucose molecules	six glucose molecules	**two glucose molecules**

UNIT-II

SYLLABUS

Gel exclusion Chromatography- Principle, instrumentation and applications of gel exclusion chromatography, data analysis Advantages and disadvantages.

Size Exclusion (Gel Filtration) Chromatography

Size exclusion chromatography is used for semi-preparative purifications and various analytical assays. It is a separation technique which takes the advantage of the difference in size and geometry of the molecules. The molecules are separated based on their size. Grant Henry Lathe and Colin R Ruthven was the pioneer of size exclusion chromatography who started this technique for separation of analytes of different size with starch gels as the matrix, later Jerker Porath and Per Flodin introduced dextran gels. Other gel filtration matrices include agarose and **polyacrylamide.**

Note: Unlike ion exchange chromatography, gel filtration does not depend on any chemical interaction with protein, rather it is based on a physical property of the protein - that being the effective molecular radius (which relates to mass for most globular proteins).

Principle: Size exclusion chromatography (SEC) is the separation of mixtures based on the molecular size (more correctly, their hydrodynamic volume) of the components. Separation is achieved by the differential exclusion or inclusion of solutes as they pass through stationary phase consisting of heteroporous (pores of different sizes) cross linked polymeric gels or beads. The process is based upon different permeation rates of each solute molecule into the interior of gel particles. Size exclusion chromatography involves gentle interaction with the sample, enabling high retention of biomolecular activity. For the separation of biomolecules in aqueous systems, SEC is referred to as gel filtration chromatography (GFC), while the separation of organic polymers in non-aqueous systems is called gel permeation chromatography (GPC).

Supplement 1: Gel filtration resin can be thought of as beads which contain pores of a defined size range. Large proteins which cannot enter these pores pass around the *outside* of the beads. Therefore, the volume of the column appears smaller to a large molecule. Smaller proteins which can enter the pores of the beads have a larger volume that they can explore, thus the volume of the column appear larger to a small molecule. Both large and small molecules experience the same flow rate of mobile phase (i.e. L/min).Thus, a sample of proteins passing through a gel filtration column will

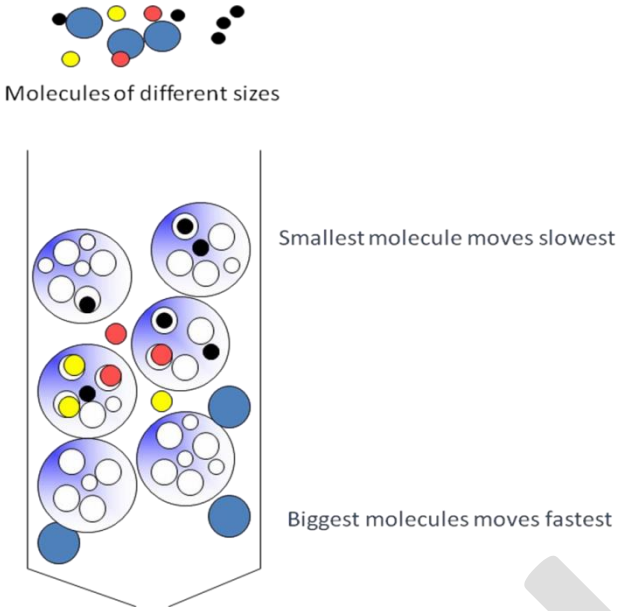


Figure 1: Theory of size exclusion chromatography. Please read details in supplement 1.

The basic principle of size exclusion chromatography is quite simple. A column of gel particles or porous matrix is in equilibrium with a suitable mobile phase for the molecules to be separated. Large molecules are completely excluded from the pores will pass through the space in between the gel particles or matrix and will come first in the effluent. Smaller molecules will get distributed in between the mobile phase of in and outside the molecular sieve and will then pass through the column at a slower rate, hence appear later in effluent (Fig. 1)

There are two extremes in the separation profile of a gel filtration column. There is a critical molecular mass (large mass) which will be completely excluded from the gel filtration beads. All solutes in the sample which are equal to, or larger, than this critical size will behave identically: they will all eluted in the excluded volume of the column. There is a critical molecular mass (small mass) which will be completely included within the pores of the gel filtration beads. All solutes in the sample which are equal to, or smaller, than this critical size will behave identically: they will all eluted in the included volume of the column Solutes between these two ranges of molecular mass will elute between the excluded and included volumes (Fig. 2) Thus, while deciding a size exclusion matrix for protein purification, included and excluded range should be considered. For

example: Sephadex G 75 matrix has fractionation range 3-80. This tells that the matrix has included volume range 3 kDa and excluded volume range 80kDa. If protein of interest and impurities both are close to 80 kDa or above they are likely to co-elute in excluded volume. Thus purification will not work. Now you can think what is the use of a size exclusion matrix Sephadax G25 (range 1- 5kDa)? This is generally used for desalting as all proteins are above 5kDa and comes in excluded volume and salts are eluted late in included volume.

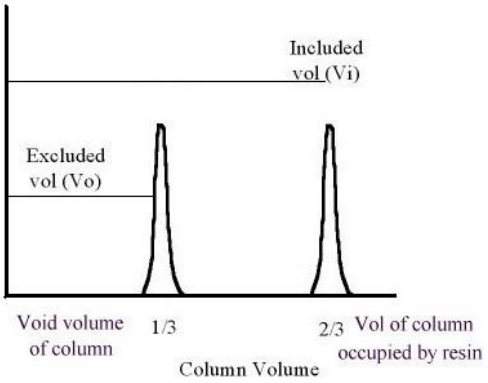


Figure 2: The excluded volume (Vo) is approximately equal to one third of the column volume, the included volume is approximately equal to two thirds of the column volume.

In gel filtration the resolution is a function of column length (the longer the better). However, one drawback is related to the maximum sample volume which can be loaded. The larger the volume of sample loaded, the more the overlap between separated peaks. Generally speaking, the sample size one can load is limited to about *3-5% of the total column volume*. Thus, gel filtration is best saved for the end stages of a purification, when the sample can be readily concentrated to a small volume. Gel filtration can also be used to remove salts from the sample, due to its ability to separate "small" from "large" components. Finally, gel filtration can be among the most "gentle" purification methods due to the lack of chemical interaction with the resin.

Mechanism of Size Exclusion Chromatography: Size exclusion (also known as gel filtration chromatography) is a case of liquid-liquid partition chromatography, in which the solute molecules are get distributed in between two liquid phases, (i) liquid in the gel pores and (ii) liquid outside the gel. The size exclusion may be explained by Steric Exclusion Mechanism. As the gel particles contains range of pore sizes, small molecules can enter in large number of pores while the large

molecules will get small number of pores into which they can enter. Thus the different fractions of total pore volume are accessible to molecules of different sizes. Thus, molecules with different sizes will differ in distribution coefficient between these two liquid phases [As the small molecules can enter in more pores while larger molecules can enter in pores only larger than the molecular size]

The total volume (V_t) of a column packed with a gel that has been swelled by solvent is given by

$$V_t = V_g + V_I + V_o$$

Where V_g is the volume occupied by the solid matrix of gel, V_i is the volume of solvent held in the pores or interstices and V_o is the free volume outside the gel particles. When mixing or diffusion occurs, the diffusion equilibrium and the retention volume (V_R) of the given species is given by

$$VR = V(int.) + Kd\ V(int.)$$

where distribution coefficient (K_d) is given by

$$K_d = Vi_{(acc)} / V_{(total)}$$

where $Vi_{(acc)}$ is the accessible pore volume. $V_{(total)}$ is the total pore volume and $V_{(int.)}$ is the interstitial volume.

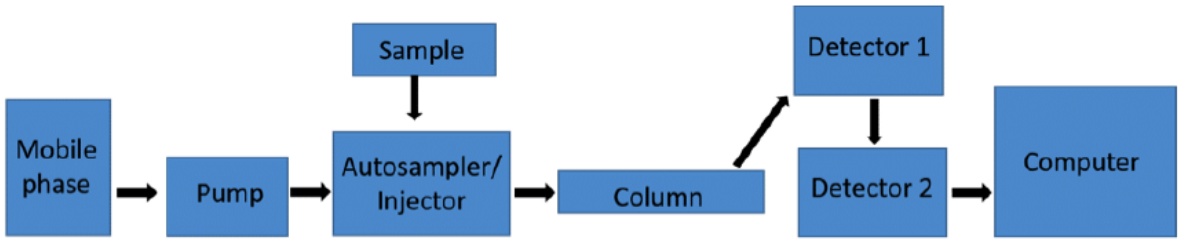
The other proposed mechanism is Secondary Exclusion Mechanism. This mechanism states that when a sample containing a mixture of small and large molecules is applied to a gel filtration column, the small molecules diffuse rapidly into the pores of gel, whereas large molecules will find relatively few unoccupied pores and move further down the column till they find the unoccupied pores. This results in the enhancement of separation of small and large molecules.

Applications:

- Purification. Desalting. Protein-ligand binding studies. Protein folding studies.
- Concentration of sample. Copolymerisation studies.
- Relative molecular mass determination

How to Measure Molecular Weight with Gel Permeation Chromatography (GPC)

So you have a new polymer, unknown protein, or material combination but don't know its molecular weight? An easy way to measure molecular weight of your sample is gel permeation chromatography (GPC). GPC is an analytical technique that separates molecules in polymers by size and provides the molecular weight distribution of a material. GPC is also sometimes known as size exclusion chromatography (SEC). So how exactly does GPC work?



Briefly, How GPC Works

Before you can use GPC to determine the molecular weight of your sample you must dissolve it. In what solvent depends a bit on your material to be studied. Once dissolved in appropriate solvent you can inject your sample into continuously flowing mobile phase of the same solvent. Your dissolved sample and the mobile phase is then pumped through a column. This finally leads to a detector and the data system. But component of GPC is equally important to your final result, the molecular weight of your sample components, so let's take a closer look at them. Solvent Choosing an appropriate solvent for your sample is very important. There are a wide range of solvents used in GPC, from non-polar to aqueous. The critical part is to select a solvent that fully dissolves your studied material. The solvent must also be compatible with the column being used. A common non-polar solvent is tetrahydrofuran (THF), while even water can be used as an aqueous solvent.

Injector

Most GPCs have an autosampler with an injector. Using an autosampler, you can set up a very large run and let it run overnight, while you are working on other tests, or (and I would never do this!) while you check Facebook. Obviously the important part of using an autosampler is to keep track of the order of your samples. Trust me, you don't want that confusion after you've run a test**!**

Column

A GPC column (Figure 3) is made up of the mobile phase and a stationary phase of porous particles all packed together nto a column. You can select your needed pore size and a wide range exists for GPC. In order to select an appropriate pore size, you must have some idea of a molecular weight range of your sample. Otherwise, you are stuck with finding the correct pore size via trial and error. And you might ruin a few perfectly good columns in the process.

GPC columns separate material by size. And how they do so is a little counter-intuitive. In other techniques like gel electrophoresis we are used to the smaller material moving faster than larger material. But in GPC, higher molecular weight material passes through the column first. While lower molecular weight material enters the pores and takes longer to elute out of the column. You can imagine that these smaller particles "get stuck" in the small pores, while large molecules simply pass by. Many different types of GPC columns exist and column selection should be based on your sample. A good column would have the absence of ionizing groups and a low affinity for the samples being studied. You don't w ant your sample sticking and clogging the column (they are expensive- often around $2,000 or $3,000).

Pump

The pump pushes the mobile phase and your samples through the column. Pump pressure and flow rate are two very important variables in GPC. Too much pressure and you can damage your sample and the column, but too little pressure and your sample may never elute out of the column. Flow rate is also critical. A flow rate that is too slow makes the test unnecessarily slow, while a flow rate that is too fast can provide inaccurate measurements.

Detector

Numerous types of detectors also exist for GPC, including ultraviolet, refractive index, infrared, density, etc. Normally two detectors are used in conjunction with each other, so that molecular weight can be directly calculated. The actual chromatogram from GPC shows the amount of material that exited the column at a certain time. Using a calibration curve of polymers with known molecular weights, the molecular weight distribution of your chromatogram can be calculated.

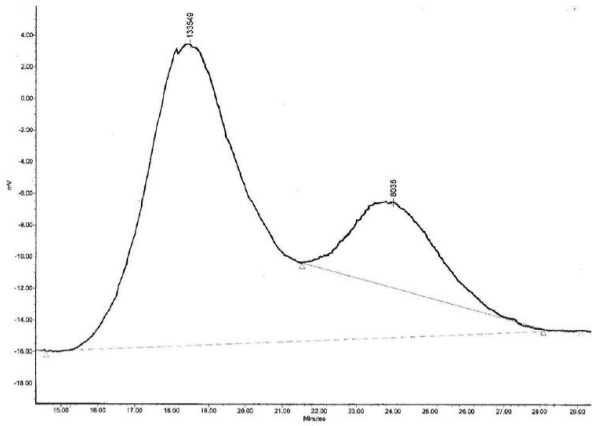


Figure shows the chromatogram output of GPC, before molecular weight is calculated. In this case, the two peaks would translate to different molecular weights. The peak on the left would have a higher molecular weight, while the peak on the right would have a lower molecular weight.

Molecular Weight

So what exactly can GPC tell you about molecular weight? GPC can provide number average molecular weight (M), weight average molecular weight (M), z average molecular weight (M), molecular weight distribution (MWD), and the polydispersity index (PDI). A molecular weight distribution typically looks like a bell curve, with one end indicating high molecular weight and the other end indicating low molecular weight. A broad molecular weight distribution peak indicates that there are many different molecules with different molecular weights – that the sample is very polydisperse. A sharp molecular weight distribution peak indicates a sample that has mostly one narrow molecular range – also known as a monodisperse sample.

Sample Insights

Besides obtaining an overall molecular weight for a sample, GPC also provides insight into the different components of a sample. Because the process separates different sized molecules from

each other, you can learn a lot about a sample with multiple parts. For instance, even if you already know an overall molecular weight, you can determine the size distribution of the molecules.

GPC Limits

One thing to keep in mind is that, while GPC can provide much needed insight into sample molecular weight, samples often have overlapping molecular weights. In these cases, you might not be able to get an accurate molecular weight of a certain component of your sample. Also, some studied samples are more finicky with GPC than others when it comes to solvent and column selection. There can be a small learning curve with GPC, but once you get the hang of it, it's actually a very easy test to run and very hands off after setup. Multitasking at its finest!

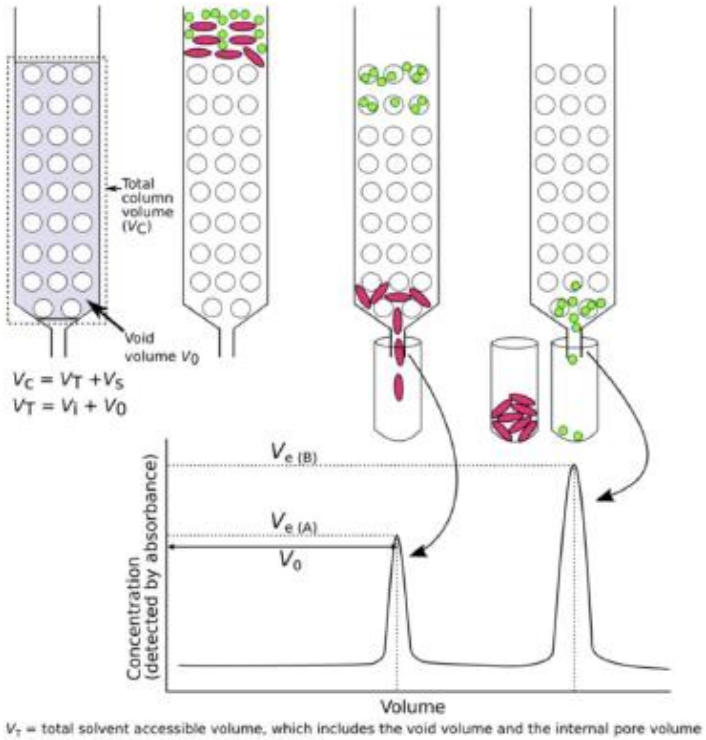


Figure 1

Using a Gel Filtration Chromatogram to Estimate Molecular Weight

Gel filtration chromatography (also known as size exclusion chromatography, molecular sieve chromatography, or gel permeation chromatography) is based on the differential distribution of the components in a sample between the mobile and stationary phases Specifically, in gel filtration chromatography, this differential distribution depends on the size and shape of the components.

Here, we take a more in-depth look at how the column fractionates the components in your sample and how the gel filtration chromatogram is used to determine molecular weight.

The Gel Filtration Column is not Your Typical Sieve In gel filtration chromatography, the stationary phase is comprised of porous beads packed into a column. The mobile phase is the running buffer or other solvent. Sample components partition between the stationary and mobile phases based on their size-based accessibility to the pores of the matrix beads. The smaller the size of the molecule or particle, the greater access it has to the pores of the matrix (or the accessible stationary phase), and the slower it moves through the column. Very large particles, which are completely excluded from the pores, elute first from the column at the void volume (V). Analytes of intermediate sizes somewhat permeate into the pores and elute next. Very small molecules with high access to the pores, elute last just before one column volume of buffer has passed through the column (called total accessible volume [V]). Therefore, although a gel filtration column separates particles by size, it is not a conventional sieve, which has pores of a uniform size.

The separated components are visualized as a plot of the volume of the mobile phase eluted through the column versus the detector signal. This plot, called a chromatogram, shows the location of the individual peaks and the quality of resolution of these peaks.

Take a Look at an Example

In Figure 1, there are two well-separated peaks corresponding to the two components (A and B) in the sample. Component A (red) requires solvent of volume V to elute at its maximum concentration. Given that component A is completely excluded from the stationary phase (beads' pores), V = V . Component B (green) has near complete access to the pores of the matrix. Therefore, its flow through the column is retarded. It elutes just before one column volume worth of the solvent can pass through the column (i.e., its elution volume, V , is almost equal to V). The location of the peaks, their heights, and widths convey a great deal of information about the sample components as well as the column. Importantly, you can use peak locations to determine the molecular weights of the sample components. Let's see how this is done.

How to Estimate the MW using a Gel Filtration Chromatogram

The distribution of an analyte between the stationary and mobile phases depends on its size. It is described by a parameter called its equilibrium distribution coefficient (K). K is the ratio of the

concentrations of the component in the stationary and the mobile phases. For component A, K = 0, since it cannot access the pores of the stationary matrix. For component B, K can be written as

$$K_{d\,(B)} = [B]_{\text{stationary phase}}/[B]_{\text{mobile phase}}$$

$$K_{d\,(B)} = [B]_{V_i}/[B]_{V_0}$$

where V is the total solvent accessible volume of the beads' pores.

The smaller the particle, the more it permeates into the beads' pores, and therefore, the larger the K value. So, K depends on particle size; therefore, it is essential to determine K to estimate particle size. Finding K It is difficult to determine the concentrations of an analyte in the stationary and mobile phases. Therefore, you express K in terms of the easily measurable parameters, V and V . We know that the flow of component B through the column is retarded and it elutes at volume V . The volume (V – V) is the 'extra' stationary phase volume that B is able to access by virtue of its small size. This 'extra' volume is a component of the internal pore volume V , and the partition coefficient of B can be expressed as a fraction of V . Therefore,

$$\frac{(V_{e\,(B)} - V_0)}{V_i} = K_{d\,(B)}$$

$$\frac{(V_{e\,(B)} - V_0)}{V_T - V_0} = K_{d\,(B)}$$

V is the total solvent accessible volume, a parameter that is difficult to measure. Therefore, substitute V by the term V , which is the total geometric volume of the column:

$$\frac{(V_{e\,(B)} - V_0)}{V_c - V_0} = K_{av\,(B)}$$

where K is not a true partition coefficient but is easily determined.

That was not too difficult, was it? Nothing works like quantification to help clear concepts!

From K to Molecular Weight There is a linear relationship between the K of molecules and the logarithms of their molecular weights over a considerable size range. You can exploit this

relationship to determine the molecular weight of an unknown sample. First, determine the K values for a set of standards of known molecular weights. Second, plot these K values against the logarithmic values of the corresponding molecular weights to get a "selectivity curve." For example, Figure 2 shows a selectivity curve for a popular, pre-packed gel filtration column determined using a set of commercially available protein standards. You can then use the calibration curve to determine the molecular weight of your protein of interest.

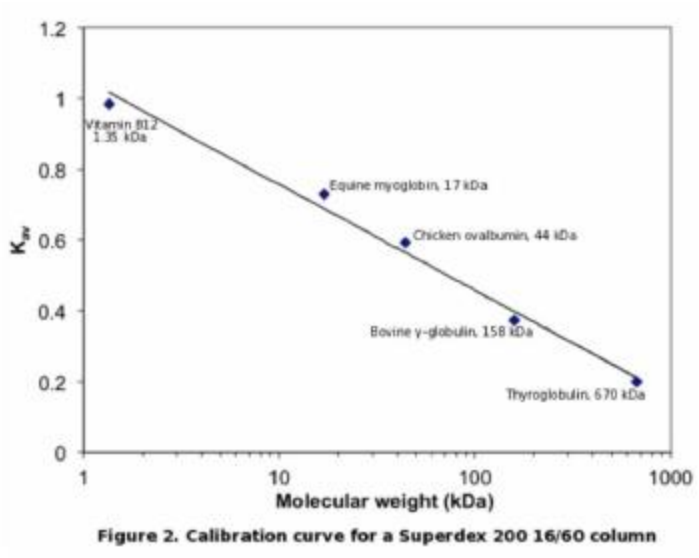


Figure 2. Calibration curve for a Superdex 200 16/60 column

Additionally, bear in mind, this assumes that the molecular shapes of the standards and your samples are the same. Commercially available protein standards are largely globular in shape. If your protein has an elongated shape, then the determined molecular weight is likely to be inaccurate. In such cases, use analytical ultracentrifugation or gel filtration coupled with multiple angle light scattering for molecular weight determination.

The eluent is the solvent that carries	analyte	substrate	product	antigen	analyte
The …….. is the analyte, the eluted solute	analyte	*eluite*	substrate	product	eluite
An …. is a stationary phase that is immobilized on the support particles	immobilized phase	analyte phase	substrate	product	immobilized phase
The ……..is the mobile phase leaving the column.	eluate	substrate	analyte phase	antigen	eluate
A ……...is equipment that enables a sophisticated separation	immobilized phase	chromatograph	substrate	antigen	chromatograph
The mobile phase is the phase that moves in a definite direction	immobilized phase	gas chromatography	Capillary Electrochromatography (CEC)	product	Capillary Electrochromatography (CEC)
The …….is the phase that moves in a definite direction	analyte phase	mobile phase	substrate	product	mobile phase
……. is used to purify sufficient quantities of a substance	CE chromatography	Preparative chromatography	Analytical chromatography	GC chromatography	Preparative chromatography
…….refers to the sample components in partition chromatography.	substrate	Solute	product	antigen	Solute
……..refers to any substance capable of solubilizing another substance	product	solvent	solute	substrate	solvent
……..refers to the instrument used for detection of analytes	solvent	detector	solute	antigen	detector
……..is the matter analyzed in chromatography	solvent	sample	antigen	solute	sample
……...is the characteristic time it takes for a particular analyte	resolution	retention time	substrate	antigen	retention time
The matrix support…….	gaseous molecule	paper	net polymer	substrate	paper
The stationary phase in TLC is a layer of adsorbent	aluminium oxide	gel	net polymer	source	aluminium oxide
Two-dimensional chromatography	paper chromatography	gas chromatography	centrifugation	capillary chromatography	paper chromatography
The mobile phase is generally mixture of	polar organic solvent wit	polar organic solvent	non polar organic solvent with water	non polar organic solvent	polar organic solvent with water
Circular chromatography is	linear	radial	ascending	descending	radial
Two-dimensional chromatography is	rectangular paper	tubular paper	linear paper	porous paper	rectangular paper
The thickness of the absorbent layer is typically around for analytical purposes	0.1 – 0.25 mm	0.11 – 0.25 mm	0.01 – 0.25 mm	0.15 – 0.25 mm	0.1 – 0.25 mm
The thickness of the absorbent layer is typically around for preparative TLC	0.15 – 2.0 mm	0.25 – 2.0 mm	0.5 – 2.0 mm	1.5 – 2.0 mm	0.5 – 2.0 mm
Many compounds to be visualized by	X Rays	UV light	white light	cosmic rays	UV light
Silica gel grades in the former technique is mesh	40 – 63 µm	20 – 63 µm	70 – 63 µm	50 – 63 µm	40 – 93 µm
Bound proteins are eluted out by utilizing a gradient of linearly	increasing salt concentratio	very low salt concentration	isotonic salt concentration	decreasing salt concentration	increasing salt concentration
Proteins that have a …….. will be eluted	low net charge	high net charge	very low net charge	lowest net charge	low net charge
A simple device can be used to create……..	a gradiant	a salt gradient.	analyte phase	product phase	a salt gradient.
The stationary phase used in TLC is	cellulose	silica gel	agarose	polyacrylamide	
Stationary phase used in paper chromatography is	filter paper	silica gel	polyacrylamide	agarose	
Forces involved in paper chromatography is	capillary forces	van der Waals forces	disulphide bridges	hydrogenbonds	
Stationary phase used in TLC is for separation of plant pigments is	cellulose	silica gel	Kieselguhr G	polyacrylamide	
Solvent system of amino acids in paper chromatography is	petroleum ether	acetic acid	hexane, water	butanol, acetic acid, water	
Molecules with higher solubility will migrate to	lesser than Rf	greater than Rf	equal to RF	RF-1	
Usually low moleculalr weight compounds are separated using …….. chromatograph	partition	adsoprtion	column	thin layer	
Impurities present in paper are removed by washin with	0.1 N HCl	1 N HCl	0.01 N HCl	0.001N HCl	
In paper chromatography, amino acids are viewed in purple or blue by spraying	ninhydrin	bromine water	methanol	ethanol	
The stationary phase used in column chromatography is known as	gradient	filter paper	gel matrix	axis	
Gel matrix cellulose has ……. units.	β-1,4 linked fucose	β-1,4 linked galactose	β-1,4 linked arabinose	β-1,4 linked glucose	
Gel matrix dextran has ……. units.	α-1,6 linked galact ose	α-1,6 linked gluc ose	α-1,6 linked fuc ose	α-1,6 linked arabinose	
Gel matrix agarose has ……. units.	D- galact ose	D-glucose	L-fucose	L-arabinose	
The stationary phase silica is made up of	sulphuric acid	acetic acid	orthosilicic acid	hydrochloric acid	
The peaks obtained during column chromatography is	EEG	electrophoretogram	ECG	chromatogram	
Ion exchange chromatography is as process based on	neutrally charged	oppositely charged particles	only positive	only negative	
When a gel matrix exchanges positive ions, it is called as	cation exchanger	anion exchanger	matrix without charges	void volume	
Example of strong cationic exchanger is ……..	epoxyamine	cellulose	polystyrene	starch	
Removal of sample from solid matrix using solvent is ……...	bed volume	effluent	retention	elution	
In chromatography, volume of mobile phase is	bed volume	void volume	retention	elution	
Time taken for each material to emerge from coumn is ……	bed volume	void volume	retention time	elution	
Column development using single solvent as mobile phase is ……	bed volume	void volume	retention time	isocratic elution	
Column chromatography involves ……. phenomenon.		3	2	4	5
Adsorption chromatography was developed by …….	D.T. Day	Sorensen	Richard	Edwin	
Adsorption chromatography is used mainly for separation of …….	clinical samples	soil samples	plant pigments	animal cells	
Scientit who used adsorption chromatography for separating plant pigment is ……	M.S.Tswett	Sorensen	Richard	Edwin	
Powdered charcoal can be prepared using ……..	coal	nuts	tar	coconut	
Fuller's earth is mixture of …… from clay deposits	vitamins	minerals	gel matrix	salt	
Hydroxyapatite is ……	MgCl2	KCL	calcium phosphate	sodium chloride	
Adsorption chromatography is used for separation of ……	chlorides	proteins	calcium	geometrical isomers	
Commerical name of strong cationic exchanger is ……	AG 3	Sephadex AG 50	Bio-Rex 70	AG 1	
Commerical name of weak cationic exchanger is ……	AG 3	Sephadex AG 50	Bio-Rex 70	AG 1	
Commerical name of strong anionic exchanger is ……	AG 3	Sephadex AG 50	Bio-Rex 70	QAE-Sephadex	
Commerical name of weak anionic exchanger is ……	AG 3	Sephadex AG 50	Bio-Rex 70	QAE-Sephadex	

UNIT-III

SYLLABUS

Polyacrylamide gel electrophoresis- Principle, instrumentation and applications of PAGE. Gel polymerization- APS, TEMED. Separation and determination of molecular weight of proteinsSDS, running gel, stacking gel, electrophoresis buffer.

Electrophoresis

Principle

Principles of Gel Electrophoresis Electrophoresis are a technique used to separate and sometimes purify macromolecules - especially proteins and nucleic acids - that differ in size, charge or conformation. As such, it is one of the most widely-used techniques in biochemistry and molecular biology.

As with all forms of gel electrophoresis, molecules may be run in their native state, preserving the molecules' higher-order structure, or a chemical denaturant may be added to remove this structure and turn the molecule into an unstructured linear chain whose mobility depends only on its length and mass-to-charge ratio. For nucleic acids, urea is the most commonly used denaturant. For proteins,sodium dodecyl sulfate (SDS) is an anionic detergent applied to protein samples to linearize proteins and to impart a negative charge to linearized proteins. This procedure is called **SDS-PAGE**. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis. Proteins that have a greater hydrophobic content, for instance many membrane proteins, and those that interact with surfactants in their native environment, are intrinsically harder to treat accurately using this method, due to the greater variability in the ratio of bound SDS.

Sample preparation

Samples may be any material containing proteins or nucleic acids. These may be biologically derived, for example from prokaryotic or eukaryotic cells, tissues, viruses, environmental samples, or purified proteins. In the case of solid tissues or cells, these are often first broken down mechanically using a blender (for larger sample volumes), using a homogenizer (smaller volumes), by sonicator or by using cycling of high pressure, and a combination of biochemical and mechanical techniques – including various types of filtration and centrifugation – may be used to separate different cell compartments and organelles prior to electrophoresis. Synthetic biomolecules such as oligonucleotides may also be used as analytes.

The sample to analyze is optionally mixed with a chemical denaturant if so desired, usually SDS for proteins or urea for nucleic acids. SDS is an anionic detergent that denatures secondary and non–disulfide–linked tertiary structures, and additionally applies a negative charge to each protein in proportion to its mass. Urea breaks the hydrogen bonds between the base pairs of the nucleic acid, causing the constituent strands to separate. Heating the samples to at least 60 °C further promotes denaturation.

In addition to SDS, proteins may optionally be briefly heated to near boiling in the presence of a reducing agent, such as dithiothreitol (DTT) or 2-mercaptoethanol (beta-mercaptoethanol/BME), which further denatures the proteins by reducing disulfide linkages, thus overcoming some forms of tertiary protein folding, and breaking up quaternary protein structure (oligomeric subunits). This is known as reducing SDS-PAGE.

A tracking dye may be added to the solution. This typically has a higher electrophoretic mobility than the analytes to allow the experimenter to track the progress of the solution through the gel during the electrophoretic run.

Preparing acrylamide gels

The gels typically consist of acrylamide, bisacrylamide, the optional denaturant (SDS or urea), and a buffer with an adjusted pH. The solution may be degassed under a vacuum to prevent the formation of air bubbles during polymerization. Alternatively, butanol may be added to the resolving gel (for proteins) after it is poured, as butanol removes bubbles and makes the surface smooth. A source of free radicals and a stabilizer, such as ammonium persulfate and TEMED are added to initiate polymerization. The polymerization reaction creates a gel because of the added bisacrylamide, which can form cross-links between two acrylamide molecules. The ratio of bisacrylamide to acrylamide can be varied for special purposes, but is generally about 1 part in 35. The acrylamide concentration of the gel can also be varied, generally in the range from 5% to 25%. Lower percentage gels are better for resolving very high molecular weight molecules, while much higher percentages are needed to resolve smaller proteins.

Gels are usually polymerized between two glass plates in a gel caster, with a comb inserted at the top to create the sample wells. After the gel is polymerized the comb can be removed and the gel is ready for electrophoresis.

Electrophoresis

Various buffer systems are used in PAGE depending on the nature of the sample and the experimental objective. The buffers used at the anode and cathode may be the same or

different.

An electric field is applied across the gel, causing the negatively charged proteins or nucleic acids to migrate across the gel away from the negative electrode (which is the cathode being that this is an electrolytic rather than galvanic cell) and towards the positive electrode (the anode). Depending on their size, each biomolecule moves differently through the gel matrix: small molecules more easily fit through the pores in the gel, while larger ones have more difficulty. The gel is run usually for a few hours, though this depends on the voltage applied across the gel; migration occurs more quickly at higher voltages, but these results are typically less accurate than at those at lower voltages. After the set amount of time, the biomolecules have migrated different distances based on their size. Smaller biomolecules travel farther down the gel, while larger ones remain closer to the point of origin. Biomolecules may therefore be separated roughly according to size, which depends mainly on molecular weight under denaturing conditions, but also depends on higher-order conformation under native conditions. However, certain glycoproteins behave anomalously on SDS gels.

Further processing

Following electrophoresis, the gel may be stained (for proteins, most commonly with Coomassie Brilliant Blue R-250; for nucleic acids,ethidium bromide; or for either, silver stain), allowing visualization of the separated proteins, or processed further (e.g. Western blot). After staining, different species biomolecules appear as distinct bands within the gel. It is common to run molecular weight size markers of known molecular weight in a separate lane in the gel to calibrate the gel and determine the approximate molecular mass of unknown biomolecules by comparing the distance traveled relative to the marker.

For proteins, SDS-PAGE is usually the first choice as an assay of purity due to its reliability and ease. The presence of SDS and the denaturing step make proteins separate, approximately based on size, but aberrant migration of some proteins may occur. Different proteins may also stain differently, which interferes with quantification by staining. PAGE may also be used as a preparative technique for the purification of proteins. For example, **q**uantitative **p**reparative **n**ative **c**ontinuous **p**oly**a**crylamide **g**el **e**lectrophoresis (QPNC- PAGE) is a method for separating native metalloproteins in complex biological matrices.

Polyacrylamide gel (*PAG)* had been known as a potential embedding medium for sectioning tissues as early as 1964, and two independent groups employed PAG in electrophoresis in

1959. It possesses several electrophoretically desirable features that make it a versatile medium. It is a synthetic, thermo-stable, transparent, strong, chemically relatively inert gel, and can be prepared with a wide range of average pore sizes. The pore size of a gel is determined by two factors, the total amount of acrylamide present (%T) (T = Total concentration of acrylamide and bisacrylamide monomer) and the amount of cross-linker (%C) (C = bisacrylamide concentration). Pore size decreases with increasing %T; with cross-linking, 5%C gives the smallest pore size. Any increase or decrease in %C from 5% increases the pore size, as pore size with respect to %C is a parabolic function with vertex as 5%C. This appears to be because of non-homogeneous bundling of polymer strands within the gel. This gel material can also withstand highvoltage gradients, is amenable to various staining and destaining procedures, and can be digested to extract separated fractions or dried for autoradiography and permanent recording.

Components

- **Chemical buffer** Stabilizes the pH value to the desired value within the gel itself and in the electrophoresis buffer. The choice of buffer also affects the electrophoretic mobility of the buffer counterions and thereby the resolution of the gel. The buffer should also be unreactive and not modify or react with most proteins. Different buffers may be used as cathode and anode buffers, respectively, depending on the application. Multiple pH values may be used within a single gel, for example in DISC electrophoresis. Common buffers in PAGE include Tris, Bis-Tris, or imidazole.
- **Counterion** balance the intrinsic charge of the buffer ion and also affect the electric field strength during electrophoresis. Highly charged and mobile ions are often avoided in SDS- PAGE cathode buffers, but may be included in the gel itself, where it migrates ahead of the protein. In applications such as DISC SDS-PAGE the pH values within the gel may vary to change the average charge of the counterions during the run to improve resolution. Popular counterions are glycine and tricine. Glycine has been used as the source of trailing ion or

  slow ion because its pKa is 9.69 and mobility of glycinate are such that the effective mobility can be set at a value below that of the slowest known proteins of net negative charge in the pH range. The minimum pH of this range is approximately 8.0.
- **Acrylamide (C_3H_5NO; mW: 71.08).** When dissolved in water, slow, spontaneous autopolymerization of acrylamide takes place, joining molecules together by head on tail fashion to form long single-chain polymers. The presence of a free radical-generating

system greatly accelerates polymerization. This kind of reaction is known as Vinyladdition polymerisation. A solution of these polymer chains becomes viscous but does not form a gel, because the chains simply slide over one another. Gel formation requires linking various chains together. Acrylamide is a neurotoxin. It is also essential to store acrylamide in a cool dark and dry place to reduce autopolymerisation andhydrolysis.

- **Bisacrylamide (*N*,*N*′-Methylenebisacrylamide) ($C_7H_{10}N_2O_2$; mW: 154.17).** Bisacrylamide is the most frequently used cross linking agent for polyacrylamide gels. Chemically it can be thought of as two acrylamide molecules coupled head to head at their non-reactive ends. Bisacrylamide can crosslink two polyacrylamide chains to one another, thereby resulting in a gel.
- **Sodium Dodecyl Sulfate (SDS) ($C_{12}H_{25}NaO_4S$; mW: 288.38).** (only used in denaturing protein gels) SDS is a strong detergent agent used to denature native proteins to unfolded, individual polypeptides. When a protein mixture is heated to 100 °C in presence of SDS, the detergent wraps around the polypeptide backbone. It binds to polypeptides in a constant weight ratio of 1.4 g SDS/g of polypeptide. In this process, the intrinsic charges of polypeptides become negligible when compared to the negative charges contributed by SDS. Thus polypeptides after treatment become rod-like structures possessing a uniform charge density that is same net negative charge per unit weight. The electrophoretic mobilities of these proteins are a linear function of the logarithms of their molecular weights.

  Without SDS, different proteins with similar molecular weights would migrate differently due to differences in mass-charge ratio, as each protein has an isoelectric point and molecular weight particular to its primary structure. This is known as Native PAGE. Adding SDS solves this problem, as it binds to and unfolds the protein, giving a near uniform negative charge along the length of the polypeptide.

- **Urea ($CO(NH_2)_2$; mW: 60.06).** Urea is a chaotropic agent that increases the entropy of the system by interfering with intramolecular interactions mediated by non-covalentforces such as hydrogen bonds and Vander Waals forces. Macromolecular structure is dependent on the net effect of these forces, therefore it follows that an increase in chaotropic solutes denatures macromolecules,
- **Ammonium persulfate (APS) ($N_2H_8S_2O_8$; mW: 228.2)**. APS is a source of free radicals and is often used as an initiator for gel formation. An alternative source of free radicals is riboflavin, which generated free radicals in a photochemical reaction.
  - **TEMED (*N*, N, N′, N′-tetramethylethylenediamine)** ($C_6H_{16}N_2$; mW: 116.21).

TEMED stabilizes free radicals and improves polymerization. The rate of polymerization and the properties of the resulting gel depend on the concentrations of free radicals. Increasing the amount of free radicals results in a decrease in the average polymer chain length, an increase in gel turbidity and a decrease in gel elasticity. Decreasing the amount shows the reverse effect. The lowest catalytic concentrations that allow polymerization in a reasonable period of time should be used. APS and TEMED are typically used at approximately equimolar concentrations in the range of 1 to 10 mM.

Chemicals for processing and visualization

The following chemicals and procedures are used for processing of the gel and the protein samples visualized in it:

- **Tracking dye**. As proteins and nucleic acids are mostly colorless, their progress through the gel during electrophoresis cannot be easily followed. Anionic dyes of a known electrophoretic mobility are therefore usually included in the PAGE sample buffer. A very common tracking dye is Bromophenol blue (BPB, 3', 3", 5', 5" tetrabromophenolsulfonphthalein). This dye is coloured at alkali and neutral pH and is a small negatively charged molecule that moves towards the anode. Being a highly mobile molecule it moves ahead of most proteins. As it reaches the anodic end of the electrophoresis medium electrophoresis is stopped. It can weakly bind to some proteins and impart a blue colour. Other common tracking dyes are xylene cyanol, which has lower mobility, and Orange G, which has a higher mobility.
- **Loading aids**. Most PAGE systems are loaded from the top into wells within the gel. To ensure that the sample sinks to the bottom of the gel, sample buffer is supplemented with additives that increase the density of the sample. These additives should be non-ionic and non-reactive towards proteins to avoid interfering with electrophoresis. Common additives are glycerol and sucrose.
- **Coomassie Brilliant Blue R-250 (CBB) ($C_{45}H_{44}N_3NaO_7S_2$; mW: 825.97)**. CBB is the most popular protein stain. It is an anionic dye, which non-specifically binds to proteins. The structure of CBB is predominantly non-polar, and it is usually used in methanolic solution acidified with acetic acid. Proteins in the gel are fixed by acetic acid and simultaneously stained. The excess dye incorporated into the gel can be removed by destaining with the same solution without the dye. The proteins are detected as blue bands on a clear background. As SDS is also anionic, it may interfere

with staining process. Therefore, large volume of staining solution is recommended, at least ten times the volume of the gel.

- **Ethidium bromide (EtBr)** is the traditionally most popular nucleic acid stain.

Silver staining. Silver staining is used when more sensitive method for detection is needed, as classical Coomassie Brilliant Blue staining can usually detect a 50 ng protein band, Silver staining increases the sensitivity typically 50 times. The exact chemical mechanism by which this happens is still largely unknown. Silver staining was introduced by Kerenyi and Gallyas as a sensitive procedure to detect trace amounts of proteins in gels. The technique has been extended to the study of other biological macromolecules that have been separated in a variety of supports. Many variables can influence the colour intensity and every protein has its own staining characteristics; clean glassware, pure reagents and water of highest purity are the key points to successful staining. Silver staining was developed in the 14th century for colouring the surface of glass. It has been used extensively for this purpose since the 16th century. The colour produced by the early silver stains ranged between light yellow and an orange-red. Camillo Golgi perfected the silver staining for the study of the nervous system. Golgi's method stains a limited number of cells at random in their entirety.

- **Western Blotting** is a process by which proteins separated in the acrylamide gel are electrophoretically transferred to a stable, manipulable membrane such as anitrocellulose, nylon, or PVDF membrane. It is then possible to apply immunochemical techniques to visualise the transferred proteins, as well as accurately identify relative increases or decreases of the protein of interest. For more, see Western Blot.

Isoelectric focusing takes place in a pH gradient. The charged molecules move towards the anode or the cathode until they reach a position in the pH gradient where their net charges are zero. This pH value is the "**isoelectric** point" (pI) of the substance.

Principle of Isoelectric Focusing

The use of isoelectric focusing is limited to molecules which can be either positively or negatively charged. Proteins, enzymes and peptides are such amphoteric molecules. The net charge of a protein is the sum of all negative and positive charges of the amino acid side chains, but the three-dimensional configuration of the protein also plays a role.

Isoelectric focusing takes place in a pH gradient. The charged molecules move towards the anode or the cathode until they reach a position in the pH gradient where their net charges are zero. This pH value is the isoelectric point of the substance. Since it is no longer charged, the

electric field does not have any influence on it.

The method of separating proteins according to their isoelectric points in a gradient is called isoelectric focusing. This technique was discovered by H. Svensson and Sweden. This method has a high resolution power because ordinary paper electrophoresis resolves plasma proteins into six bands whereas isoelectric focusing resolves them into 40 bands.

In conventional electrophoresis, the pH between anode and cathode is constant and the positively charged ions migrate towards the cathode and negatively charged ions migrate towards the anode. But in isoelectric focusing, a stable pH gradient is arranged. The pH gradually increases from anode to cathode. When a protein is introduced at a pH which is lower than its isoionic point, it will possess a net positive charge and will migrate in the direction of the cathode. Due to the presence of pH gradient, the net charge of the molecule changes due to ionization as it moves is zero, it will stop migrating. This is the isoelectric point of protein. Each protein present in the mixture will migrate to its isoelectric point and stop its migration at that point. Thus, once a final, stable focusing is reached, the resolution will be retained for a long time.

Uses

Isoelectric focusing is widely used for the separation and identification of serum proteins. It is used in the food and agriculture industry, forensic and human genetic laboratories, for research in enzymology, immunology and membrane biochemistry, etc.

Immuno electrophoresis

Immunoelectrophoresis is a general name for a number of biochemical methods for separation and characterization of proteins based on electrophoresis and reaction with antibodies. All variants of immunoelectrophoresis require immunoglobulins, also known as antibodies, reacting with the proteins to be separated or characterized. The methods were developed and used extensively during the second half of the 20th century. In somewhat chronological order: Immunoelectrophoretic analysis (one-dimensional immunoelectrophoresis *ad modum*Grabar), crossed immunoelectrophoresis (two-dimensional quantitative immunoelectrophoresis *ad modum* Clarke and Freeman or *ad modum* Laurell), rocket-immunoelectrophoresis (one-dimensional quantitative immunoelectrophoresis *ad modum* Laurell), fused rocket immunoelectrophoresis *ad modum* Svendsen and Harboe, affinity immunoelectrophoresis *ad modum* Bøg-Hansen.

Agarose as 1% gel slabs of about 1 mm thickness buffered at high pH (around 8.6) is traditionally preferred for the electrophoresis as well as the reaction with antibodies. The agarose was chosen as the gel matrix because it has large pores allowing free passage and separation of proteins, but provides an anchor for the immunoprecipitates of protein and

specific antibodies. The high pH was chosen because antibodies are practically immobile at high pH. An electrophoresis equipment with a horizontal cooling plate was normally recommended for the electrophoresis.

Immunoprecipitates may be seen in the wet agarose gel, but are stained with protein stains like Coomassie Brilliant Blue in the dried gel. In contrast to SDS-gel electrophoresis, the electrophoresis in agarose allows native conditions, preserving the native structure and activities of the proteins under investigation, therefore immunoelectrophoresis allows characterization of enzyme activities and ligand binding etc. in addition to electrophoretic separation.

Crossed immunoelectrophoresis is also called two-dimensional quantitative immunoelectrophoresis *ad modum* Clarke and Freeman or*ad modum* Laurell. In this method the proteins are first separated during the first dimension electrophoresis, then instead of the diffusion towards the antibodies, the proteins are electrophoresed into an antibody-containing gel in the second dimension. Immunoprecipitation will take place during the second dimension electrophorsis and the immunoprecipitates have a characteristic bell-shape, each precipitate representing one antigen, the position of the precipitate being dependent on the amount of protein as well as the amount of specific antibody in the gel, so relative quantification can be performed. The sensitivity and resolving power of crossed immunoelectrophoresis is than that of the classical immunoelectrophoretic analysis and there are multiple variations of the technique useful for various purposes. Crossed immunoelectrophoresis has been used for studies of proteins in biological fluids, particularly human serum, and biological extracts.

Rocket immunoelectrophoresis is one-dimensional quantitative immunoelectrophoresis. The method has been used for quantitation of human serum proteins before automated methods became available.

Fused rocket immunoelectrophoresis is a modification of one-dimensional quantitative immunoelectrophorsis used for detailed measurement of proteins in fractions from protein separation experiments.

Affinity immunoelectrophoresis is based on changes in the electrophoretic pattern of proteins through specific interaction or complex formation with other macromolecules or ligands. Affinity immunoelectrophoresis has been used for estimation of binding constants, as for instance with lectins or for characterization of proteins with specific features likeglycan content or ligand binding. Some variants of affinity immunoelectrophoresis are similar to affinity chromatography by use of immobilized ligands.

The open structure of the immunoprecipitate in the agarose gel will allow additional binding of

radioactively labeled antibodies to reveal specific proteins. This variation has been used for identification of allergens through reaction with IgE.

Two factors determine that immunoelectrophoretic methods are not widely used. First they are rather work intensive and require some manual expertise. Second they require rather large amounts of polyclonal antibodies. Today gel electrophoresis followed by electroblotting is the preferred method for protein characterization because it's ease of operation, its high sensitivity, and its low requirement for specific antibodies. In addition proteins are separated by gel electrophoresis on the basis of their apparent molecular weight, which is not accomplished by immunoelectrophoresis, but nevertheless immunoelectrophoretic methods are still useful when non-reducing conditions are needed.

Pulsed field gel electrophoresis is a technique used for the separation of large deoxyribonucleic acid (DNA) molecules by applying to a gel matrix an electric field that periodically changes direction.

The procedure for this technique is relatively similar to performing a standard gel electrophoresis except that instead of constantly running the voltage in one direction, the voltage is periodically switched among three directions; one that runs through the central axis of the gel and two that run at an angle of 60 degrees either side. The pulse times are equal for each direction resulting in a net forward migration of the DNA. For extremely large molecules (up to around 2 Mb), switching-interval ramps can be used that increases the pulse time for each direction over the course of a number of hours—take, for instance, increasing the pulse linearly from 10 seconds at 0 hours to 60 seconds at 18 hours.

This procedure takes longer than normal gel electrophoresis due to the size of the fragments being resolved and the fact that the DNA does not move in a straight line through the gel.

Applications

PFGE may be used for genotyping or genetic fingerprinting. It is commonly considered a gold standard in epidemiological studies of pathogenic organisms. Subtyping has made it easier to discriminate among strains of *Listeria monocytogenes* and thus to link environmental or food isolates with clinical infections.

Capillary electrophoresis

Capillary electrophoresis (**CE**) is a family of electrokinetic separation methods performed in submillimeter diameter capillaries and in micro- and nanofluidic channels. Very often, CE refers to capillary zone electrophoresis (CZE), but other electrophoretic techniques including

capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), capillary isotachophoresis and micellar electrokinetic chromatography (MEKC) belong also to this class of methods.[1] In CE methods, analytes migrate through electrolytesolutions under the influence of an electric field. Analytes can be separated according to ionic mobility and/or partitioning into an alternate phase via Non-covalent interactions. Additionally, analytes may be concentrated or "focused" by means of gradients inconductivity and pH.

The instrumentation needed to perform capillary electrophoresis is relatively simple. A basic schematic of a capillary electrophoresis system. The system's main components are a sample vial, source and destination vials, a capillary, electrodes, a high voltage power supply, a detector, and a data output and handling device. The source vial, destination vial and capillary are filled with an electrolyte such as an aqueous buffer solution. To introduce the sample, the capillary inlet is placed into a vial containing the sample. Sample is introduced into the capillary via capillary action, pressure, siphoning, or electrokinetically, and the capillary is then returned to the source vial. The migration of the analytes is initiated by an electric field that is applied between the source and destination vials and is supplied to the electrodes by the high-voltage power supply. In the most common mode of CE, all ions, positive or negative, are pulled through the capillary in the same direction by electroosmotic flow. The analytes separate as they migrate due to their electrophoretic mobility, and are detected near the outlet end of the capillary. The output of the detector is sent to a data output and handling device such as

an integrator or computer. The data is then displayed as an electropherogram, which reports detector response as a function of time. Separatedchemical compounds appear as peaks with different retention times in an electropherogram. Capillary electrophoresis was first combined with mass spectrometry by Richard D. Smith and coworkers, and provides extremely high sensitivity for the analysis of very small sample sizes. Despite the very small sample sizes (typically only a few nanoliters of liquid are introduced into the capillary), high sensitivity and sharp peaks are achieved in part due to injection strategies that result in concentration of analytes into a narrow zone near the inlet of the capillary. This is achieved in either pressure or electrokinetic injections simply by suspending the sample in a buffer of lower conductivity (*e.g.* lower salt concentration) than the running buffer. A process called field-amplified sample stacking (a form of isotachophoresis) results in concentration of analyte in a narrow zone at the boundary between the low-conductivity sample and the higher-conductivity running buffer.

To achieve greater sample throughput, instruments with arrays of capillaries are used to analyze many samples simultaneously. Such capillary array electrophoresis (CAE) instruments with 16 or 96 capillaries are used for medium- to high-throughput capillary DNA sequencing, and the inlet ends of the capillaries are arrayed spatially to accept samples directly from SBS-standard footprint 96-well plates.

Separation by capillary electrophoresis can be detected by several detection devices. The majority of commercial systems use UV or UV-Vis absorbance as their primary mode of detection. In these systems, a section of the capillary itself is used as the detection cell. The use of on-tube detection enables detection of separated analytes with no loss of resolution. In general, capillaries used in capillary electrophoresis are coated with a polymer (frequently polyimide or Teflon) for increased flexibility. The portion of the capillary used for UV detection, however, must be optically transparent. For polyimide-coated capillaries, a segment of the coating is typically burned or scraped off to provide a bare window several millimeters long. This bare section of capillary can break easily, and capillaries with transparent coatings are available to increase the stability of the cell window. The path length of the detection cell in capillary electrophoresis (~ 50 micrometers) is far less than that of a traditional UV cell (~ 1 cm). According to the Beer-Lambert law, the sensitivity of the detector is proportional to the path length of the cell. To improve the sensitivity, the path length can be increased, though this results in a loss of resolution. The capillary tube itself can be expanded at the detection point, creating a "bubble cell" with a longer path length or additional tubing can be added at the detection point. Both of these methods, however, will decrease the resolution of the separation. Post-column detection utilizing a sheath flow configuration has also been described.

Fluorescence detection can also be used in capillary electrophoresis for samples that naturally fluoresce or are chemically modified to contain fluorescent tags. This mode of detection offers high sensitivity and improved selectivity for these samples, but cannot be utilized for samples that do not fluoresce. Numerous labeling strategies are used to create fluorescent derivatives or conjugates of non-fluorescent molecules, including proteins and DNA. The set-up for fluorescence detection in a capillary electrophoresis system can be complicated. The method requires that the light beam be focused on the capillary, which can be difficult for many light sources. Laser-induced fluorescence has been used in CE systems with detection limits as low as 10^{-18} to 10^{-21} mol. The sensitivity of the technique is attributed to the high intensity of the incident light and the ability to accurately focus the light on the capillary.[2] Multi-color fluorescence detection can be achieved by including multiple dichroic mirrors and bandpass filters to separate the fluorescence emission amongst multiple detectors (*e.g.*, photomultiplier tubes), or by using a prism or grating to project spectrally resolved fluorescence emission onto a position-sensitive detector such as a CCD array. CE systems with 4- and 5-color LIF detection systems are used routinely for capillary DNA sequencing and genotyping ("DNA fingerprinting") applications.

In order to obtain the identity of sample components, capillary electrophoresis can be directly coupled with mass spectrometers or Surface Enhanced Raman Spectroscopy (SERS). In most systems, the capillary outlet is introduced into an ion source that utilizes electrospray

ionization (ESI). The resulting ions are then analyzed by the mass spectrometer. This set-up requires volatile buffer solutions, which will affect the range of separation modes that can be employed and the degree of resolution that can be achieved. The measurement and analysis are mostly done with a specialized gel analysis software.

For CE-SERS, capillary electrophoresis eluants can be deposited onto a SERS-active substrate. Analyte retention times can be translated into spatial distance by moving the SERS-active substrate at a constant rate during capillary electrophoresis. This allows the subsequent spectroscopic technique to be applied to specific eluants for identification with high sensitivity. SERS-active substrates can be chosen that do not interfere with the spectrum of the analytes.

Application

Capillary electrophoresis may be used for the simultaneous determination of the ions NH_4^+, Na^+, K^+, Mg^{2+} and Ca^{2+} in saliva

Polyacrylamide gel electrophoresis (PAGE)

About the PAGE method in general

As mentioned previously, polyacrylamide gels can be used for the separation and analysis of proteins and relatively small nucleic acid molecules. For example, when it was first invented, Sanger's DNA sequencing method (see in details in Chapter 10) applied PAGE to separate linear single-stranded DNA molecules based on their length. The resolution of the PAGE method is so high that, in the size range of about 10-1000 nucleotide units, it is capable of separating DNA molecules that differ in length only by a single monomer unit. In the case of single-stranded DNA, individual molecules are separated solely based on their length. This is due to the fact that, in the case of DNA (or RNA), the number of negative charges is a simple linear function of the number of monomer units (i.e. the length of the molecule). In other words, the specific charge (number of charges per particle mass) is invariant, i.e. it is the same for all DNA molecules. It is so because each monomer unit has one phosphate moiety that carries the negative charge. When an appropriate denaturing agent, such as urea, is added to the DNA sample and the gel is heated, the shape of the varying-length linear DNA molecules becomes identical. As a consequence, denatured molecules will be separated exclusively based on their size. (We will see the same

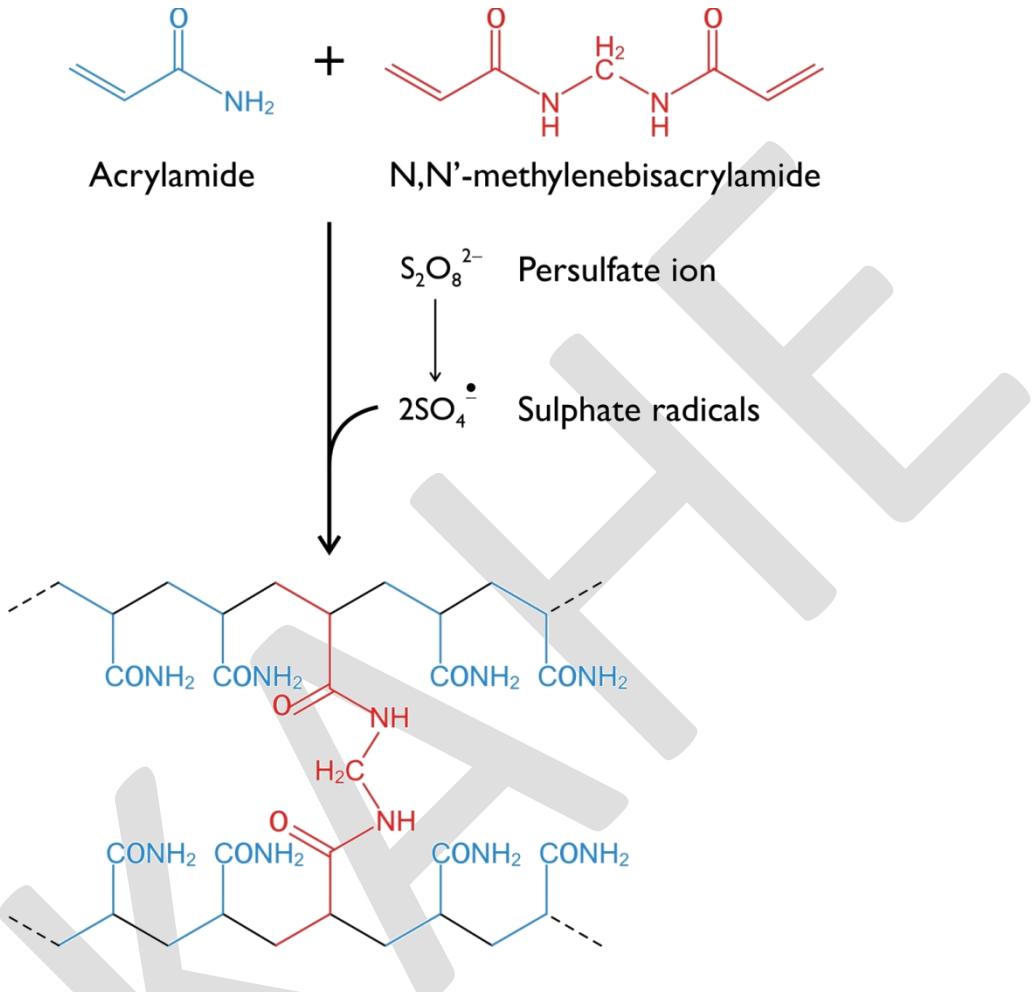
principle at the SDS-PAGE method that separates denatured proteins almost exclusively based on their size (molecular weight)). There are several PAGE methods (SDS-PAGE, isoelectric focusing, 2D PAGE) that can be applied mostly for the separation of proteins based on distinct molecular properties.

At a given pH, different proteins carry different amounts of electric charge. Moreover, different proteins have different shapes and sizes, too. Consequently, during electrophoresis, proteins are separated by a complex combination of their charge, shape and size. PAGE separation of proteins provides high resolution. However, as three independent molecular properties simultaneously influence electrophoretic mobility, it will provide limited room for precise interpretation. For example, when two proteins are compared, it remains hidden what makes one of them migrate faster: a larger number of electric charges, a smaller size, or a more spherical shape. Nevertheless, even the simplest PAGE method, which will be referred to as native PAGE, provides many particular advantages (see below).

In order to increase the analytical applicability of the PAGE technology, several variations of the method have been established to separate proteins based on a single molecular property. As we will see, SDS-PAGE separates proteins based primarily on molecular weight, while isoelectric focusing separates proteins exclusively based on isoelectric point.

In the presence of suitable initiator and catalyst compounds, acrylamide can readily polymerise in a radical process. (Acrylamide is harmful by inhalation or skin contact, and thus it should be handled with care.) This reaction would lead to very long polyacrylamide chains, yielding a highly viscous liquid instead of a gel. As already mentioned, these long chains need to be cross-linked to form a three-dimensional network. This is achieved by mixing *N*,*N*′-methylenebisacrylamide into the acrylamide solution. In essence, *N*,*N*′-methylenebisacrylamide is composed of two acrylamide molecules covalently interconnected via a methylene moiety. When, during the polymerisation reaction, the acrylamide groups of *N*,*N*′-methylenebisacrylamide molecules become incorporated in the long polyacrylamide chains,

cross-links are formed between the polyacrylamide chains leading to a gel (Figure 7.2). In the course of electrophoresis, ions (proteins or nucleic acids) are separated in this gel.



Molecular structure of the polyacrylamide gel. The three-dimensional molecular network comes into being by a radical polymerisation of acrylamide monomers and cross-linking *N*,*N*′-methylenebisacrylamide components.

Without any modification, polyacrylamide electrophoresis separates macromolecular ions based on a combination of charge, size and shape. Size (and shape) separation is due to the molecular sieving property of the gel. The size range in which molecules can be separated is dictated by the average pore size of the gel. In the case of polyacrylamide gels, this can be controlled through the concentration of the acrylamide monomer and the proportion of the cross-linking *N*,*N*′-

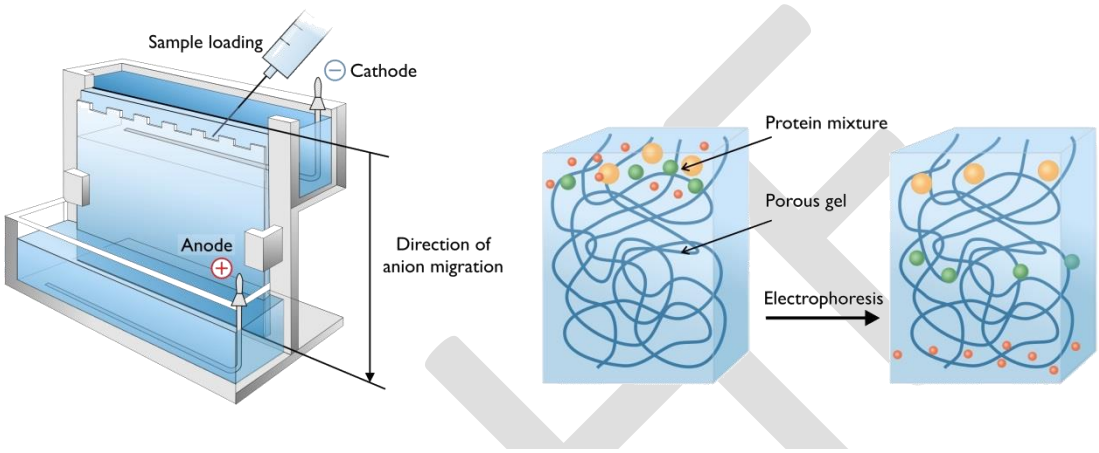
methylenebisacrylamide. The acrylamide concentration can be set in the range of about 4-20 % as this is the range in which the mechanical properties of the gel are appropriate. Below this range the gel will be too soft and it will not keep its shape, while above this range it will be too rigid and prone to break. The optimal proportion of the *N*,*N*′-methylenebisacrylamide component is 1-3 % relative to the acrylamide component. The polyacrylamide gel possesses all advantageous properties necessary for a good electrophoresis medium, i.e. it is hydrophilic, free of electric charges and chemically stable. A further very important property of the polyacrylamide gel is that it does not participate in any non-specific or specific binding interaction with proteins. Furthermore, the polyacrylamide gel does not interfere with common protein staining reactions.

When electrophoresis is performed under native (non-denaturing) conditions, such as near neutral pH and ambient or lower temperature, many enzymes retain their native conformation and, in turn, their enzymatic activity. This way, many enzymes can be separated and specifically detected in the gel after electrophoretic separation.

In the course of creating the gel, a buffer with a properly chosen pH is mixed into the acrylamide/*N*,*N*′-methylenebisacrylamide solution. Radical polymerisation is subsequently triggered by suitable catalyst and initiator compounds. The catalyst is usually ammonium persulfate, which spontaneously decomposes in aqueous media, thereby generating free radicals. These free radicals in themselves cannot efficiently cleave the double bonds of the acrylamide molecule, but are able to excite the electrons of the initiator molecules. This leads to the generation of free radicals, originating from the initiator molecules, that are able to trigger radical polymerisation of acrylamide monomers. The most frequently used initiator is tetramethylethylenediamine (TEMED).

There are two types of gels according to their geometry. In early gel electrophoretic applications, gel tubes were used that allowed only a single sample to be run. Gel slabs were later introduced, allowing for many samples to be run at the same time in the same gel in parallel. Gel slabs became much more common than gel tubes. Gel slabs are created by pouring the gel-forming

solution between two parallel glass sheets prior to polymerisation (Figure 7.3). Besides its higher throughput, this gel geometry provides another important advantage over gel tubes: samples are loaded side by side on such slabs and are run in the same gel at the same time. This allows for a more reliable comparison of the samples, facilitating the interpretation of experimental results.



. Separation of proteins in a polyacrylamide gel. As illustrated in the left panel, several samples can be run in parallel in a slab gel. Ions can move between the two electrodes only through the gel interconnecting the two chambers. The gel acts as a molecular sieve. The larger the molecule, the larger the drag force exerted on it by the gel.

Proper selection of pH and acrylamide concentration is instrumental for successful electrophoresis. For protein electrophoresis, the pH is set usually higher than the pI value of the proteins in the sample. At such a pH, all proteins will be negatively charged and will move towards the anode. The buffer in the medium serves two purposes. One is to set and maintain the proper pH during electrophoresis. The other function of the buffer is to establish the electric current in the medium.

The majority of the electric current is carried by the ions of the buffer. Normally, the protein-ions that are separated by electrophoresis have only a negligible contribution to the current. In other words, proteins have a low ion transport number. However, if the buffer concentration is set too low, the contribution of proteins to carrying the current will increase, and the protein molecules

will migrate rapidly. This usually leads to smearing of the bands of migrating proteins. On the other hand, if the buffer concentration is set too high, the mobility of the proteins will be too low. In this case the electrophoresis process would take a very long time. Unnecessary lengthening of the process provides excess time for diffusion, which lowers the resolution of separation.

According to the applied buffer system, gel electrophoretic methods can be classified into two types: continuous and discontinuous. Continuous methods apply the same buffer in the gel and in the two buffer chambers containing the electrodes. The only advantage of this method lies in its simplicity. More complex discontinuous methods were introduced to provide higher resolution. SDS polyacrylamide gel electrophoresis (see later) is usually associated with such a discontinuous system.

The discontinuous system applies two gels of different pore size and three different buffers. One of the gels, the resolving gel, is polymerised at a higher acrylamide concentration. The pore size of this gel is set according to the size range of the proteins to be separated. Another gel, the stacking gel is created on top of the resolving gel. (The gels are mounted in a vertical format.) The stacking gel is polymerised from a more dilute acrylamide solution to provide larger pores. This pore size does not provide a molecular sieving effect.

As mentioned above, there are three buffers: different ones in each of the two gels and a third one, the so-called ‘running buffer’ in the buffer chambers containing the electrodes. In the gel buffers, the anion originates from a strong acid; it is usually chloride ion. Dissociation of strong acids does not depend on the pH: these acids always fully dissociate. Consequently, chloride ion is never protonated in the solution: its ionisation state is independent of the pH. On the other hand, the anion component of the running buffer is the conjugate base of a weak acid. Consequently, the ionisation state of this ion depends on the pH of the buffer. Glycinate ion is one of the most frequently used compounds for this purpose. The pH in the running buffer is set to 8.3.

The protein sample is layered on the top of the stacking gel. When an electric field is generated by the power supply, the protein ions and the ions of the running buffer enter the stacking gel. The pH in the stacking gel is set to 6.8. This value is only slightly higher than the pI value of glycine (6.5). At this pH, most glycine molecules are in a neutral zwitterionic state, and only a small portion of the molecules carry a net negative charge. In this state, glycine has a low electrophoretic mobility and a corresponding low transport number. The local sparsity of ions elevates the local electric resistance of the medium. As the electric current must be of the same magnitude at any segments of the electric circuit (there is no macroscopic charge separation), the voltage will increase according to Ohm's law. Due to this effect, the migration speed of the proteins will be relatively high and the protein front will reach the chloride front in the stacking gel. The ion concentration in the chloride front is high and, therefore, here the electric resistance and the voltage are low. This slows down the protein front. This effect results in a very sharp protein front, with the protein molecules being crowded right behind the chloride ion front.

The protein sample will thus enter the resolving gel in a sharp band. The pH in the resolving gel is set to about 8.8. At this pH, almost all glycinate molecules are in the anionic state. Thus, the electric mobility of glycinate increases, and the concentrating effect applied by the stacking gel ends in the resolving gel. Different proteins will be separated in the resolving gel according to their charge, size and shape.

In most electrophoretic methods, a tracking dye is mixed in the sample. Usually, this dye is chosen to have a higher electrophoretic mobility than any of the components of interest (proteins or nucleic acids) in the sample. The function of the tracking dye is to visualise the running front and, in turn, the completeness of the run. The most popular tracking dye is bromophenol blue.

The following sections review the various PAGE methods listed from the simplest to the most complex one.

Native PAGE

Native PAGE is an electrophoresis method to separate native proteins. The conditions are set such that the migrating proteins are kept in their native state. The buffers provide a non-denaturing, native-like milieu, and the electrophoresis is performed at low temperature in order to dissipate heat. Many enzymes retain their native conformation and their enzymatic activities while running in the gel. If certain conditions apply, these enzymes can be highly selectively detected within the gel through a specific 'staining' reaction even in the presence of a large excess of 'contaminating' proteins. After completion of electrophoresis, the gel is soaked in a solution containing the substrate of the enzyme. As the substrate is usually a small molecule, it quickly diffuses into the gel while the large enzyme molecules do not diffuse out. In an optimal case, the natural product of the enzymatic reaction is a coloured and insoluble compound that precipitates inside the gel and marks the exact location of the enzyme. Of course, most enzymes do not have such natural substrates. However, once the molecular mechanism of catalysis is revealed, synthetic substrates can be designed that, on the one hand, mimic natural substrates and, on the other hand, lead to colourful insoluble products.

Native PAGE is also a useful method for checking the uniformity of the isolated protein. Even if the purified protein sample contains only a single type of protein, the sample might not be uniform. Some of the molecules might be unfolded or have undergone chemical modifications. Unfolding changes the overall shape of the molecule, while most chemical modifications change the electric charge of native molecules. These alterations can be detected after traditional staining of the purified sample. If no such side products are present, protein molecules will run in a single sharp band. Otherwise, multiple bands or smearing of the band is expected.

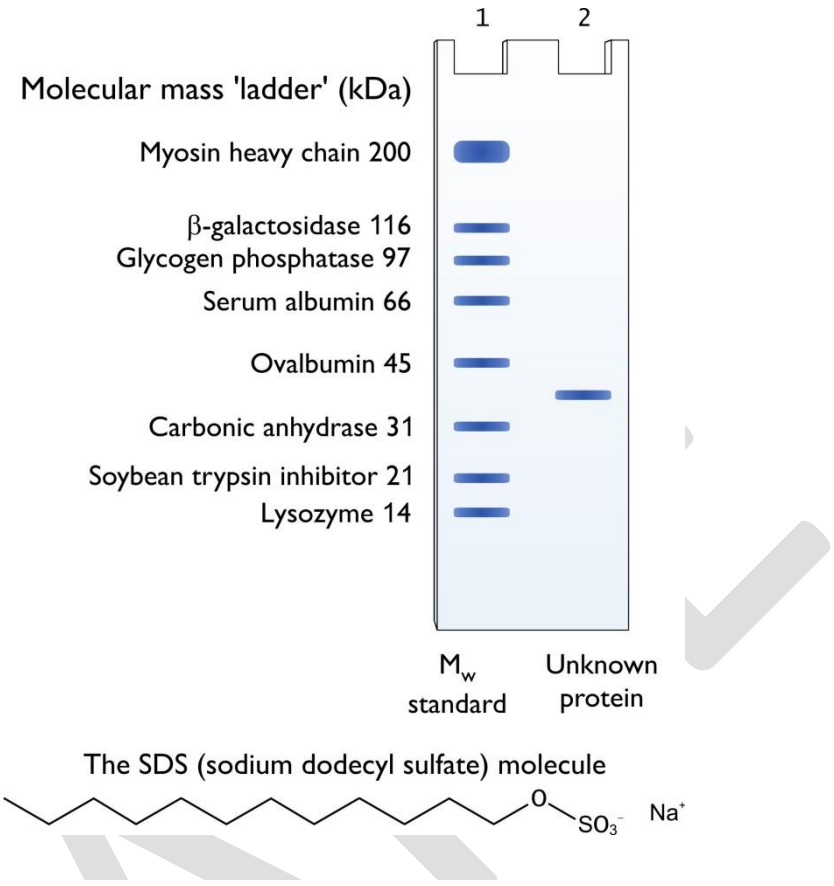
In addition, native PAGE can also be used to detect complex formation between proteins. If two (or more) proteins (or proteins and non-proteinous ligands) form a complex, the complex can be detected as an extra band in the gel. This is because in native-like conditions, many non-covalent (subunit-subunit, receptor-ligand, enzyme-inhibitor) interactions are maintained and the complex migrates apparently as a single molecule.

In the course of native PAGE, it is highly important to pay attention to the relationship of the pI values of the proteins or protein complexes and the pH of the gel buffer, as this will determine where individual proteins will migrate in the gel.

SDS-PAGE

SDS-PAGE is an electrophoresis method to separate proteins. However, unlike in the case of native PAGE, here the proteins migrate in their denatured state. As it was mentioned in the general introduction to traditional (native) PAGE, the migration velocity of proteins is a function of their size, shape and the number of electric charges they carry. As the velocity is a complex function of these properties, native PAGE cannot be used to estimate the molecular mass of proteins. The traditional native PAGE method is similarly unable to assess whether a purified protein is composed of a single subunit or multiple subunits. Even a multi-subunit protein may migrate in a single sharp band.

SDS-PAGE (Figure 7.4) was introduced to analyse such cases and to allow the estimation of the molecular mass of single-subunit proteins or those of individual subunits of multi-subunit proteins. SDS-PAGE is the most prevalent PAGE method currently in use.



SDS polyacrylamide gel electrophoresis. SDS (sodium dodecyl sulphate) is an anionic detergent that unfolds proteins and provides them with extra negative charges. The amount of the associated SDS molecules—and therefore the number of charges—is proportional to the length of the polypeptide chain. The SDS gel separates individual polypeptide chains (monomeric proteins and subunits of multimeric proteins) according to their size. The velocity of the proteins is an inverse linear function of the logarithm of their molecular mass. Proteins of known molecular mass can be used to establish a calibration curve (a descending line) along which the unknown molecular mass of other proteins can be estimated.

SDS (sodium dodecyl sulphate) is an anionic detergent. When proteins are treated with SDS at high temperature, radical conformational changes occur. The treatment breaks all native non-covalent intermolecular (inter-subunit) and intramolecular interactions. The subunit structure of multi-subunit proteins disintegrates and the proteins unfold. If the native structure is stabilised by

disulfide bridges, reducing agents are also added to open up these connections. SDS molecules bind to unfolded proteins in large excess, providing extra negative charges to the molecules.

The amount of the bound SDS molecules is largely independent of the amino acid sequence of the polypeptide chain and it is roughly a linear function of polypeptide length—i.e. the molecular mass of the protein. Therefore, upon SDS-treatment, the specific charge (the charge-to-mass ratio) of different proteins will become roughly identical. Another result of the treatment is that the shape of the different proteins becomes similar. The negatively charged SDS molecules repel each other, which lends a (presumably) rod-like shape to the SDS-treated proteins. These factors together result in a situation analogous to the one already discussed in this chapter for the PAGE separation of linear single-stranded (denatured) DNA molecules. Instead of being separated simultaneously by charge, shape and size, SDS-treated proteins—just like denatured linear DNA molecules—will be separated solely based on their size. As size is a linear function of mass, SDS-PAGE ultimately separates proteins based on their molecular mass.

SDS-PAGE is the most popular cost-effective method to estimate the molecular mass of protein subunits with considerable accuracy. The relative mobility (i.e. the running distance of the protein divided by the running distance of the tracking dye) of the SDS-treated protein is in inverse linear proportion to the logarithm of the molecular mass of the protein. By running several proteins of known molecular mass simultaneously alongside the protein of interest, a log molecular mass – relative mobility calibration curve (a descending linear graph) can be created. Based on the calibration curve, the estimated molecular mass of the protein in question can be easily calculated.

Table below shows the useful separating range of polyacrylamide gels as a function of acrylamide concentration. In the useful range, the log molecular mass – relative mobility relationship is linear.

Acrylamide concentration	Linear range of separation

(%)	(kDa)
15	12-43
10	16-68
7.5	36-94
5.0	57-212

Table Relation between acrylamide concentration and the molecular mass of optimally separated molecules

SDS-PAGE is a standard method for assessing whether the sample of an isolated protein is homogeneous. Besides that, SDS-PAGE is a robust method for the analysis of large supramolecular complexes such as multi-enzyme complexes or the myofibril, as discussed below. SDS-PAGE separates and denatures individual subunits of these complexes. Thus, all polypeptide chains will migrate separately in the gel. Via various staining procedures, all subunits can be visualised and the relative amounts of these proteins (subunits) can also be determined. This allows for the identification of each subunit of a complex and provides a good estimate of the stoichiometry of subunits, too.

Isoelectric focusing

In the course of isoelectric focusing, the conditions are set in a way that proteins will be separated exclusively based on their isoelectric point (Figure 7.5). The two termini and many side chains of proteins contain dissociable groups (weak acids or bases). The dissociation state of these groups is a function of the pH of the environment (as described quantitatively by the Henderson-Hasselbalch equation, see Chapter 3). Isoelectric focusing is based on the pH-dependent dissociation of these groups. Due to this pH-dependent phenomenon, the net electric charge of a protein molecule will be a function of the pH of the medium. If, in a given protein, the number of acidic residues (Asp, Glu) exceeds that of the basic ones (Arg, Lys, His), the protein will have a net negative charge at neutral pH. The isoelectric point (pI) of the protein—

i.e. the pH at which the net charge of the protein is zero—will be in the acidic pH range. Such proteins are often denoted as acidic proteins. If the number of basic residues exceeds that of the acidic ones, the protein will be positively charged at neutral pH, and its pI value will be in the basic pH range. These proteins are often called basic proteins.

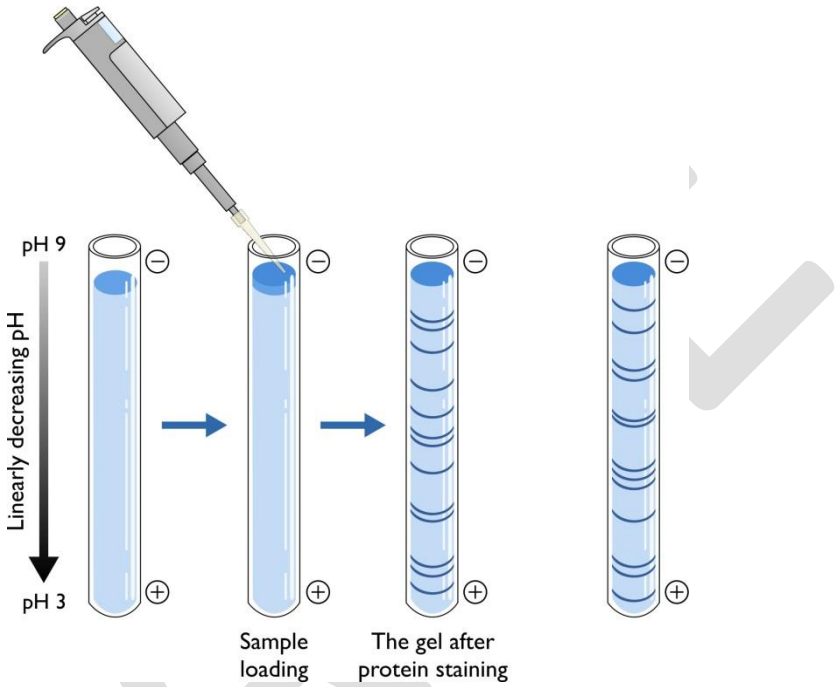


Figure Isoelectric focusing. In the course of isoelectric focusing, a pH gradient is created in the gel (usually made of polyacrylamide, less frequently agarose). Upon electrophoresis, various proteins will accumulate in different narrow regions of the gel where the pH equals their individual pI value. At this pH, the number of positive charges equals that of the negative charges on the protein—the net charge will thus be zero. Consequently, no resultant electric force is exerted on the protein.

Isoelectric focusing is an efficient high-resolution method because the pI values of various proteins are spread across a broad range. If the pH is lower than the pI of the protein, the protein will be positively charged and will move towards the cathode during electrophoresis. If the pH is higher than the pH of the protein, the protein will be negatively charged and will migrate towards

the anode. If the pH equals the pI value, the net charge of the protein will be zero and the protein will not migrate in the gel any further.

In the course of isoelectric focusing, proteins are placed in a gel representing a special medium in which the pH gradually decreases by going from the negative cathode towards the positive anode. As the protein migrates, it encounters a gradually changing pH and its net charge will also change accordingly. If it has a net negative charge and therefore moves towards the cathode, it will encounter a gradually decreasing pH, i.e. a more and more acidic environment. Consequently, the protein will take on more and more protons—up to a level where its net charge will be zero. This state is reached when the protein reaches a location where the pH equals its pI value. At this point, the protein will stop moving because no electric force will be exerted on it. If it spontaneously diffused further towards the anode, it would take on more protons, would become positively charged and would turn back to migrate towards the cathode. Following the same line of thinking, if a positively charged protein moves towards the cathode, it will encounter increasing pH and lose more and more protons. It will migrate to the place where the pH equals its pI value and will thus stop. If it diffused further towards the cathode, it would become negatively charged and would turn back towards the anode. As one can see, by performing electrophoresis in a medium in which the pH decreases from the cathode towards the anode, each protein will "find its place" according to its pI value and will become sharply focused at that location. In addition, it does not matter where exactly the proteins were introduced in the medium between the cathode and the anode.

A decisive component of this method is the usually linear pH gradient created inside the gel. There are two methods to create such a gradient. One of them applies carrier ampholytes (ampholyte is an acronym from the words amphoteric and electrolyte). Ampholytes or zwitterions are molecules that contain both weakly acidic and weakly basic groups. Just like in the case of proteins, the net charge of ampholytes is a function of the pH. In the course of isoelectric focusing, a mixture of various ampholytes is used such that the pI of the various ampholyte components will cover a range in which the pI values of the "neighbouring" ampholytes differ only slightly. This ampholyte mixture is soaked in the gel and an appropriate

electric field is generated by a power supply. This leads to a process analogous to the one already explained for proteins. Each ampholyte will migrate to the location where its net charge becomes zero. As soon as this steady-state is achieved, ampholytes will function as buffers and keep the pH of their immediate environment constant. This establishes the pH gradient in which the proteins can be separated.

The other, more sophisticated method applies special ampholytes that can be covalently polymerised into the polyacrylamide gel. The appropriate ampholyte gradient is created before the gel is polymerised. This way, the gradient will be covalently fixed in the gel, providing an immobilised pH gradient. The appropriate pH range provided by the ampholyte mixture should be selected based on the pI values of the proteins to be separated.

Regardless of how the pH gradient was created, once the proteins reach the location in the gel where the pH equals their pI, they finally stop moving and the system reaches a steady-state.

One of the potential technical difficulties encountered during isoelectric focusing originates from the fact that the solubility of proteins is lowest at their pI value (see Chapter 5). This can lead to the precipitation of some proteins in the gel. To prevent this unwanted process, urea is most often applied in the gel as an additive. Urea denatures proteins and keeps denatured proteins in solution. As the pI value of proteins is largely independent of their conformational state, this modification does not compromise the method. The solubility of membrane proteins can be further promoted by the addition of non-ionic detergents.

Isoelectric focusing is aimed at separating proteins based exclusively on their pI value—thus, independently of their size. Therefore, the molecular sieving property of the gel in this method should be avoided. The only function of the gel is to prevent free convectional flows in the medium. Accordingly, for isoelectric focusing, polyacrylamide gels are made at very low acrylamide concentrations, and sometimes even agarose gels are applied when very large pores are needed. Isoelectric focusing is usually performed in a horizontally-mounted electrophoresis apparatus and by applying intense cooling.

Two-dimensional (2D) electrophoresis

The various separation methods are all aimed at separating complex systems to individual components. Separation is always based on at least one physicochemical property that shows diversity among the components. The general problem encountered in the case of complex mixtures is that not all components differ significantly from all other components when only one property is considered. Accordingly, separation based on a single property rarely results in single-component fractions. Some components will be efficiently separated from all others, while some other components will remain in the mixture.

The remaining mixtures can be further fractionated by another separation technique that relies on a different physicochemical property. The most effective separation can be achieved if the combined consecutive separation steps rely on absolutely independent physicochemical properties. A good example of this is the very high-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) that combines two already discussed electrophoresis methods, isoelectric focusing and SDS-PAGE.

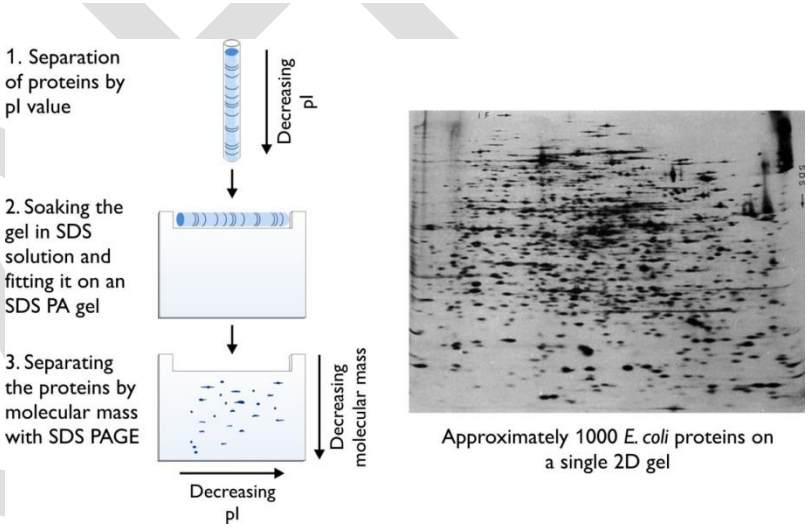


Figure Two-dimensional (2D) electrophoresis. 2D electrophoresis is the combination of isoelectric focusing and SDS-PAGE. Proteins are first separated based on their pI values and then based on their molecular mass. As these properties are completely independent, the combination of the two separation methods provides much higher resolution than either of the two methods alone.

As the first step of 2D gel electrophoresis, isoelectric focusing is performed to separate proteins based on their pI values. Only a single sample is loaded on a gel strip in this step. The

sample is separated in one dimension both in a primary and in a figurative sense. In a primary sense because the components are separated along a single line, and in a figurative sense as the separation is based on a single well-defined property, the pI value.

After the first separation step has been completed in the first dimension, the gel strip is soaked in an SDS solution and is fitted tightly to one side of a "classical" SDS polyacrylamide gel. The second separation step is traditional SDS-PAGE, which separates proteins based on their molecular mass. This second step represents a second dimension in both a primary and a figurative sense. The second separation is performed in a second dimension in a direction rectangular to that of the first separation, and the property utilised in the second step (molecular mass) is completely independent of the one utilised in the first step (pI).

If, after the first step, some gel regions contain different proteins that coincidentally have identical pI values, these proteins will be separated from each other in the second step if their molecular mass is different. Note that every aspect discussed for SDS-PAGE also applies to the second separation step of 2D-PAGE. Van der Waals interactions that might have held protein subunits together in the course of isoelectric focusing will break and individual subunits will become separated. If disulfide bridges need to be opened up, some kind of reducing agent needs to be added. Accordingly, in the second separation step, single polypeptide chains will migrate in the gel. If isoelectric focusing collects a multimeric protein at a certain gel location, the second electrophoresis step will dissect it into individual chains. If the multimer contains subunits of different sizes, these subunits will be separated from each other in the second separation step.

POSSIBLE QUESTIONS

UNIT-IV

PART-A (20 MARKS)

(Q.NO 1 TO 20 Online Examination)

PART-B (2 MARKS)

1. What are the factors affecting the electrophoresis techniques
2. What are the uses of SDS-PAGE?
3. Write about PAGE.
4. Comment on gel documentation.
5. How are DNA and RNA separated?

PART-C (8 MARKS)

1. Explain separation of DNA using agarose gel electrophoresis
2. Explain the principle of gel formation in polyacrylamide gel electrophoresis
3. How do proteins get separated in SDS-PAGE?
4. Explain capillary electrophoresis
5. Explain the separation of molecule based on its isoelectric point.
6. Write the applications of pulse field gel electrophoresis and capillary electrophoresis.

Electrophoresis is based on	solubility	molecular mass	absoption	filtration	molecular mass
Electrophoretic movement of particles can be influenced by the following factor:	density	electrical charge	magnetic filed	TCA	electrical charge
Forensic science involves ……	paper chromatography	GLC	immunoelectrophoresis	TLC	immunoelectrophoresis
This cannot be used in gel electrophoresis	starch	agar	polyacrylamidealbumin	albumin	albumin
Electrophoresis involves migration of …… molecules.	neutral	both charged	negatively charged	positively	both charge
Buffer not used in electrophoresis is	calcium	citrate	formate	phosphate	calcium
In electrophoresis use of cellulose acetate paper was introduced in	1959	1960	1958	1957	1958
Migration of charge particles is called as	GC	TLC	centrifugation	electrophoresis	electrophoresis
Molecular weight can be determined by ……	immunoelectrophoresis	SDS-PAGE	agarose gel electophoresis	Cetrifugation	SDS-PAGE
Principle of electrophoresis is based on …….	charged ions	solar energy	colour	UV	charged ions
Better resolution is obtained in cellulose acetate thatn paper because	less hydrophobic	more hydrophilic	less hydrophilic	aal	less hydrophilic
Paper used in electrophoresis is made up of	mannose	fucose	galactose	cellulose	cellulose
When serum is subjected to electrophoresis, the fastest moving fraction is	albumin	alpha globulin	beta globulin	gamma globulin	alpha globulin
In PAGE, movement of protein depends on ……. molecule.	charge	size	size & charge	weight	size & charge
During electrophoresis of proteins in an alkaline medium, they	act as anions and move towards anode	act as cations and move towards cathode	do not move	disappear	act as anions and move towards anode
Electrophoresis using acrylamide gel is known as ……..	TLC	chromatography	PAGE	gel electrophoresis	PAGE
Polymerization of acrylamide to polyacrylamide is due to addition of	SDS	ammonium persulphate	beta mecaptoethanol	urea	ammonium persulphate
Polyacrylamide is cross-linked with	n-N"methylene bis acrylamide	agarose	styrene	TCA	n-N"methylene bis acrylamide
Subunits of oligomeric proteins are linked by	hydrogenbonds	van der Waals forces	disulphide bridges	carbons atoms	disulphide bridges
SDS stands for	sodium disulphite	synthetic dihydrogen sodium	sodium dihydrogen phosphate	sodium dodecyl sulphate	sodium dodecyl sulphate
In SDS-PAGE, SDS serves as	inititator of polymerization	an anioinc detergent	cationic detergent	neutralizing agent	an anioinc detergent
In SDS-PAGE, the fast moving protein will have	highest charge	low energy	lowest charge	no charge	highest charge
Ammonium per sulphate and TEMED initiate ……. Polymerization	agarose	agar	acrylamide	agarobiose	acrylamide
The role of mercaptoethanol in electrophoresis is	break hydrogenbonds	pH maintenance	impart negative charge to proteins	break S-S bonds	break S-S bonds
Among proteins, ……. carries largest charge and moves faster.	globulin	albumin	keratin	hemoglobin	albumin
Staining method for protein electrophoretogram is	silver stain	methylene blue	Ponceau-S	ethidium bromide	silver stain
Cross linking agents in PAGE is	ammonium per sulphate	SDS	bisacrylamide	TEMED	bisacrylamide
Polymerisation in PAGE is initiated by	bisacrylamide	acrylamide	TEMED	CBB	TEMED
Buffer with a pH of ……. is used for separation of proteins in paper electrophoresis	8.1	8.6	6	7.5	8.6
SDS-PAGE cannot be used for	enzymes	proteins	vitamin A	nucleic acids	protein
In electrophoresis, lipoproteins can be detected by staining with	methylene blue	acrydine orange	vinyl orange	Sudan black	Sudan black
Proteins possesing more than one polypepetide chain are known as	disulphide bridges	oligomeric proteins	alpha chain	beta chain	oligomeric proteins
Subunit of oligomeric proteins can be separated by	solubilizers	glycols	detergents	emulsifiers	solubilizers
In rocket immunoelectrophoresis antibodies are	mixed with buffer	mixed with agar	applied in well	sprayed on gel plate	applied in well
Agarose is produced from	animal oils	metals	plants	algae	algae
Nucleic acids are detected by	ninhydrin	ethidium bromide	ninhydrin	Coomassie blue	ethidium bromide
Ampholytes contain	both positive & negative groups	neutral charges	positive groups	negative groups	both positive & negative groups
Iso electric focusing separates proteins that differ by	3 charge units	4 charge units	2 charge units	one charge unit	one charge unit
Disulphide bonds in proteins are broken by …… in SDS-PAGE.	SDS	Beta-ME	APS	Coomassie blue	Beta-ME
pH at which net charge of the protein becomes neutral is called	alkaline pH	acidic pH	isoelectric pH	nutral pH	isoelectric pH
Electrophoresis was first discovered by ……..	Faraday	Michael	DuBois	Alexander Reuss	Alexander Reuss
Agar gel used for immunoelectrophoresis was intorduced by ...	Faraday	Graber & Williams	DuBois	Alexander Reuss	Graber & Williams
Biosensor recognition component is	Bioreceptor	b) biorecognition	Biotransducer	nanomaterial	Bioreceptor
Immunosensor utilizes a specific	substrate	antibody	product	compounds	antibody
Antigen and antibody recognition can be distrupted by	chaotropic substance	acids	alkaline	neutral salts	chaotropic substance
the artificial binding proteins are	smaller than Ab	larger than Ab	smaller than antigens	larger than the antigens	smaller than Ab
The biorecognition antigens are	limited stability	highly stable	low molecular weight	low density	limited stability
Small binding scaffolds are called as	Antigen binding proteins	Antibody	smaller proteins	haptens	Antigen binding proteins
In vitro display techniques is	phage display	animal display	plant display	cell display	phage display
the artificial binding proteins are	less than 100 aminoacids	less than 10 aminoacids	more than 100 aminoacids	less than 1000 aminoacids	less than 100 aminoacids
Enzymes in the biosensors	catalyze many reactions	involves product utilization	involves substrate utilization	involve coenzyme utilization	catalyze many reactions
Antibodies have a high binding constant	excess of 10^8 L/mol	less than 10^8 L/mol	excess of 20^8 L/mol	excess of 100^8 L/mol	excess of 10^8 L/mol
The artificial binding proteins are	Double bonds	lack disulphide bons	lack saturated bonds	hydrogen bonds	lack disulphide bons
Concanavalin A may function as affinity receptor exhibiting a binding constant of	4x10^2 L/mol	8x10^2 L/mol	14x10^2 L/mol	40x10^2 L/mol	4x10^2 L/mol
Use of affinity binding receptors for purposes of biosensing has been proposed by	Schultz and Sims	Faraday	Michel & John	Oscar & Lewis	Schultz and Sims
Use of affinity binding receptors for purposes of biosensing has been proposed bySchultz and Sims in	1879	1979	1934	1999	1979
Fluorescent assay for measuring glucose in the relevant physiological range between	4.6 and 6.8 mmol/L	4.5 and 6.1 mmol/L	4.4 and 6.1 mmol/L	7.4 and 6.0 mmol/L	4.4 and 6.1 mmol/L
Biosensors that employ nucleic acid interactions	Bioinducers	Geosensors	Bioanalyte	Biorecognition site	Geosensors
The recognition process is based on	affinity	repulsion	complementary base pairing	diffraction	Complementary base pairing
The hybridization probes can then base pair	target sequence	target compounds	product	analyte	target sequence

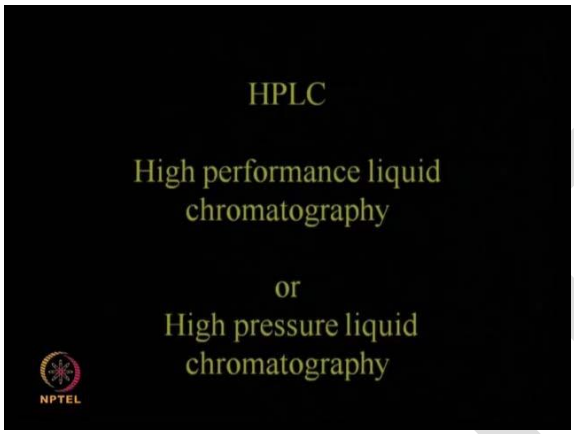
UNIT-IV

SYLLABUS

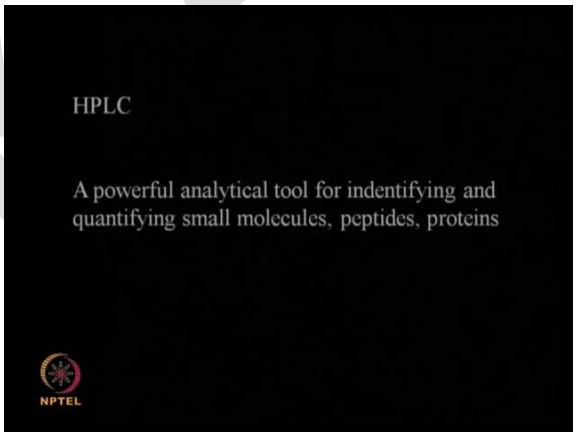
HPLC -Principle, instrumentation and applications of HPLC. Preparation of column, adsorbent materials, void volume, efficiency factor. Van Deemter equation Applications- Manufacturing, legal, research and medical.

HPLC

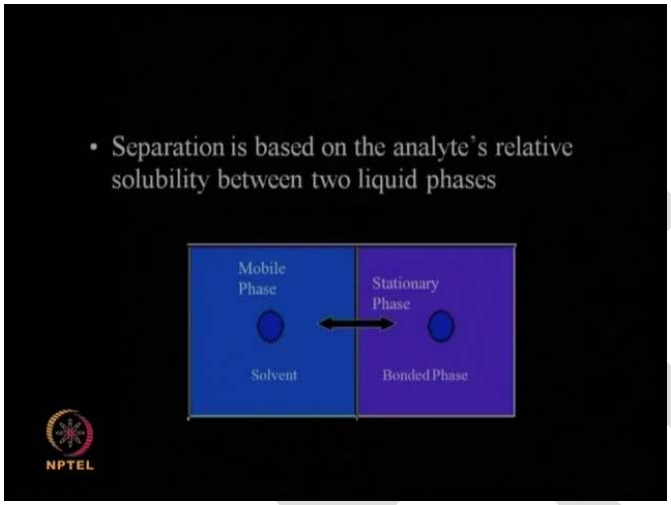
HPLC is an analytical tool, which is used for identifying unknown metabolite or a protein or a peptide as well as can be used for quantification. So, HPLC has been the short form of high performance liquid chromatography or high pleasure liquid chromatography.



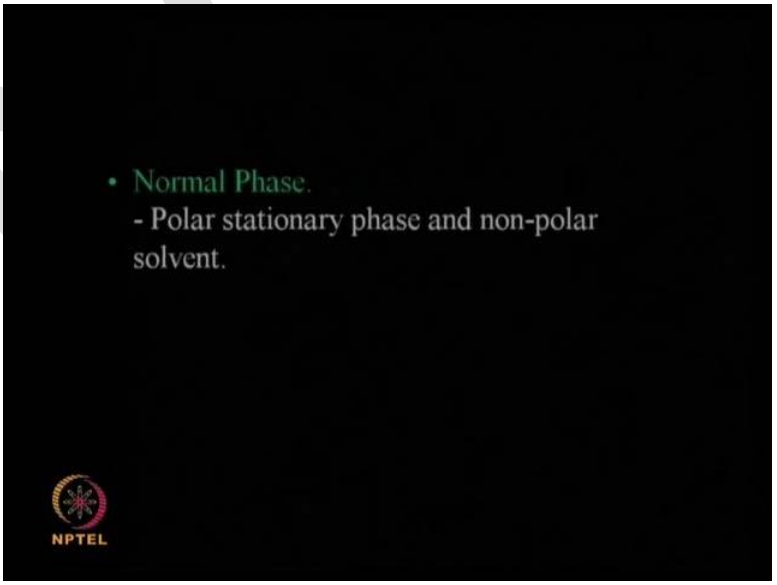
So, HPLC also uses the techniques of chromatography. So, you have different types of HPLC's which operate using different principles as we discussed the various principles of chromatography. So, the separation is achieved either through hydrophobic interactions or polar interactions or ionic forces and so on actually. So, we are going to look at the HPLC today which is a very important tool which can be used for both small molecules as well as large protein as well.



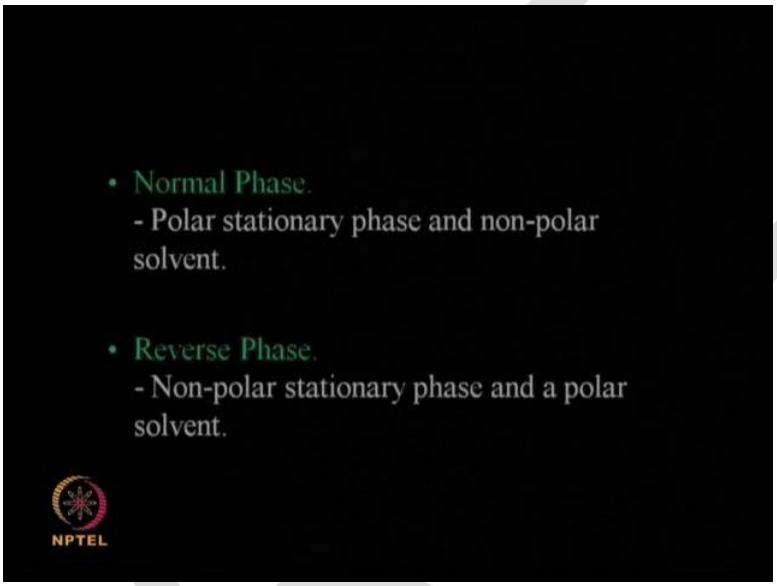
So, it is a very powerful analytical tool for identifying and quantifying small molecules, peptides, proteins, metabolides, drugs, natural chemicals, natural products, phyto chemicals and so on actually. It is extremely powerful and you achieve very good separation by changing the composition of the mobile phase for changing the stationary phase and so on actually.



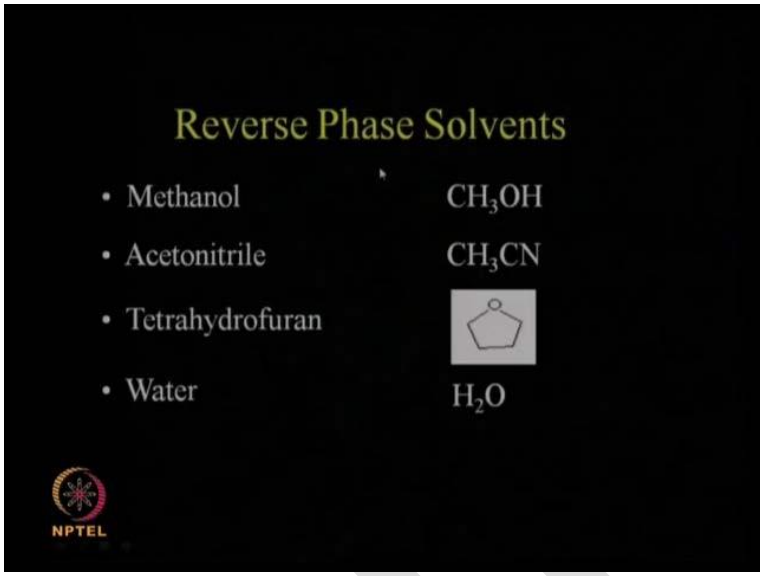
So, what happens in HPLC? You have a set of a solutes and solute partitions between the the stationary phase and the mobile phase. So, it is basically relate to solubility between two phases actually. So, the solvent which is you are a continuous phase or the mobile phase and you have the bonded phase of the stationary phase.



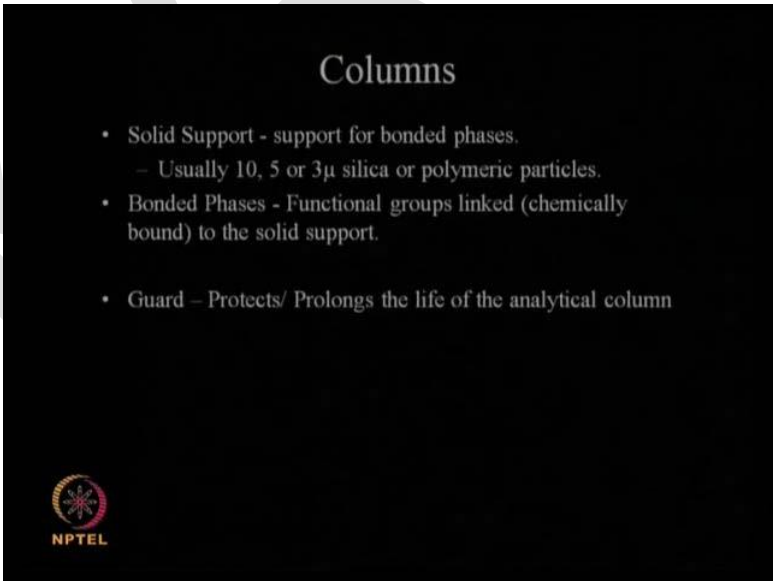
So, you're compound gets equilibrated between these two phases. And the separation happens because of forces that act on the solute and hence you have a equilibrium process taking place. So, we have two types of HPLC's, the normal phase and the reverse phase. So, what is a normal phase? Normal phase has a polar stationary phase whereas here reverse phase has a non polar stationary phase. So, in a normal phase the stationary phase is a polar or hydrophilic compound and the solvent is a non polar.



Whereas in reverse phase you have a non polar or hydrophobic compound on the stationary phase and we use the polar solvent. So, nowadays the reverse phase chromatography has become very popular whereas, originally we had the normal phase chromatography being that popular. That means in normal phase you have hydrophilic stationary phase. So, hydrophilic compounds get adsorbed onto the hydrophilic stationary phase. Whereas in a reverse phase hydrophobic compounds get adsorbed or get more partitioned and hydrophilic compounds travel faster.

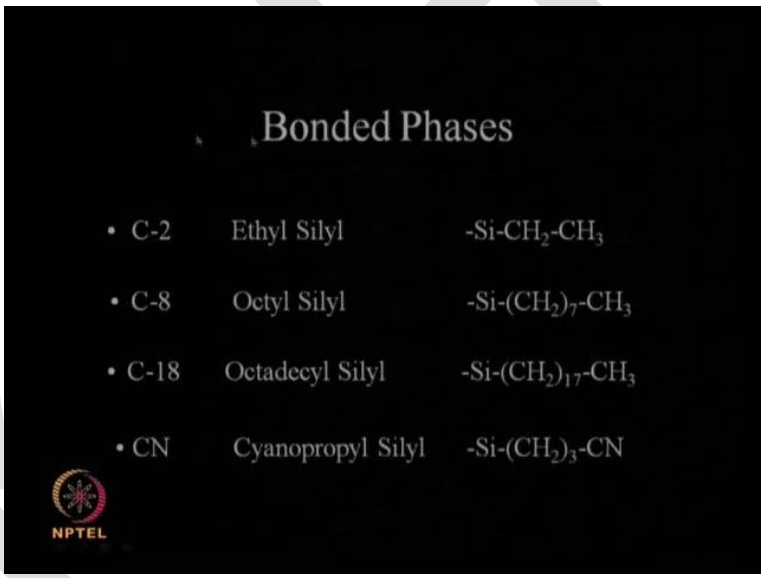


So, in reverse phase what are the solvents used? As I said in reverse phase the stationary phase is a hydrophobic compound. So, this the continuous phase is a hydrophilic for a polar. So, you use methanol, you use acetonitrile, you can use that tetrahydrofuran. Tetrahydrofuran is also called THF or you use water or even combinations of these two. Sometimes we use acetonitrile water combination if you want play around with the certain dielectric constant or other parameters.



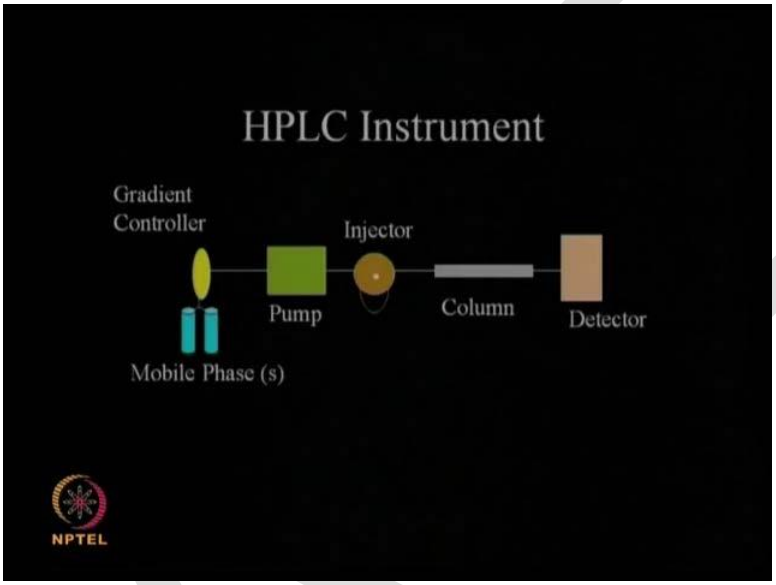
So, if we look at this solvent in a reverse phase, predominantly you will be using water and water is very cheap. That is why reverse phase chromatography has become very popular

whereas, if we use a normal phase chromatography, this stationary phase is polar like silica for example then you have to use a hydrophobic solvent like hydro carbon. So, that is very expensive and normally in this type of HPLC systems the amount of solvent which we use is quite a lot. So, the solvent cost is extremely high and then generally we try to recover the solvent. In a reverse phase chromatography we use acetonitrile water and so you can use predominantly water and small amount of acetonitrile. So, you will loose only small amount of acetonitrile. So, operating cost may reverse phase chromatography is much less when compared to the operating cost in a normal phase chromatography. And sometimes we also use three solvents. As I said if you want to play around with the dielectric constant in separations we sometimes use say methanol, acetonitrile, water and so on actually. We will look at some of those solvents. As we proceed in scores. So, what are the columns used? Suppose, you have solid support usually about 10, 5 or 3 micron silica or polymeric particles.



So, on top of that we have bonded phases, functional groups linked. The functional groups are covalently linked to the solid support. You also have a guard column; this is a small column, which is placed before the original large column. So, this guard column acts as a filter so it captures particles, it captures polymeric materials and other unwanted materials. Thereby, it is prolonging the life of the analytical column. After sometime after about 10,000 hours of use something you can throw away the guard column, replace it with another guard column.

So, what are the bonded phases we use in a reverse phase chromatography? We use the ethyl silyl. So, the ethyl is a hydrophobic or you can use octyl silyl or you can use octadecyl silyl that is C 18 or we can even use cyanopropyl silyl. So, what we have? We have ethyl or octyl or octadecyl, so as we go down the series we are making it more hydrophobic, unhydrophobic. So, that is the advantage of it and C 18 type of column having become extremely popular and it is become (( )) that means it is used in quite a lot of applications practically, separation of many fighter chemicals or even organic metabolites.

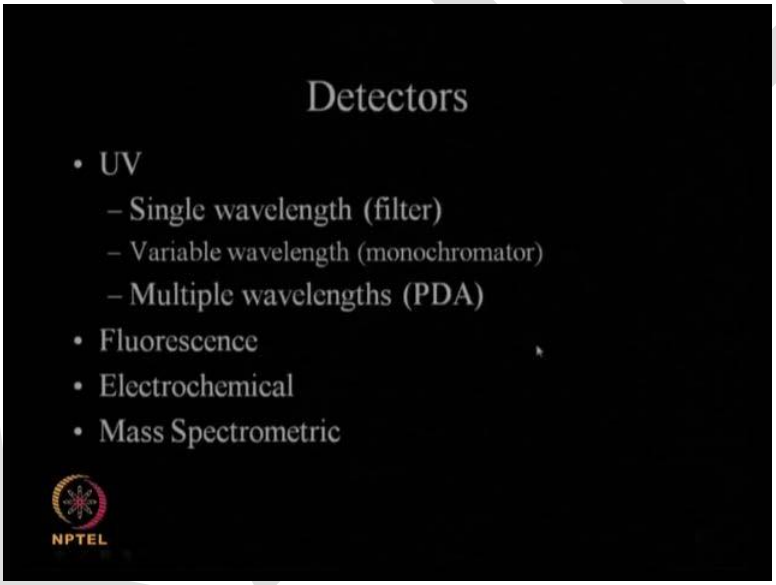


So, typical set up of an HPLC. So, we have the column here. Column will be in the order of several meters then we have a pump here and we have several tanks having various mobile phases. Either, we can run one single mobile phase that is called an isocratic system or we can have two mobile phases. We can shift from one mobile phase to another over a period of time

So, that we get some sort of a gradient which can improve the separation efficiency. So, there may be one pump if it is a isocratic or we may have two pumps pumping two different solvents. So, we have the pump and then there is a injector, that is where you are in injecting you're solute mixture. Then finally, you have the detector. There are different types of detectors that are used in HPLC. Depending upon the type of component you are separating we can have refracting index detector, we have UV detectors and we have diode array detectors, light scattering detector, even mass spectrometric detectors.

So, you can have a HPLC mass spectrometric connected to each other, that is called LCMS. So, mass spectrometric will detect the mass of the anilide that is coming out. And the LC or the HPLC does the real separation of the various components. So, there is a typical setup.

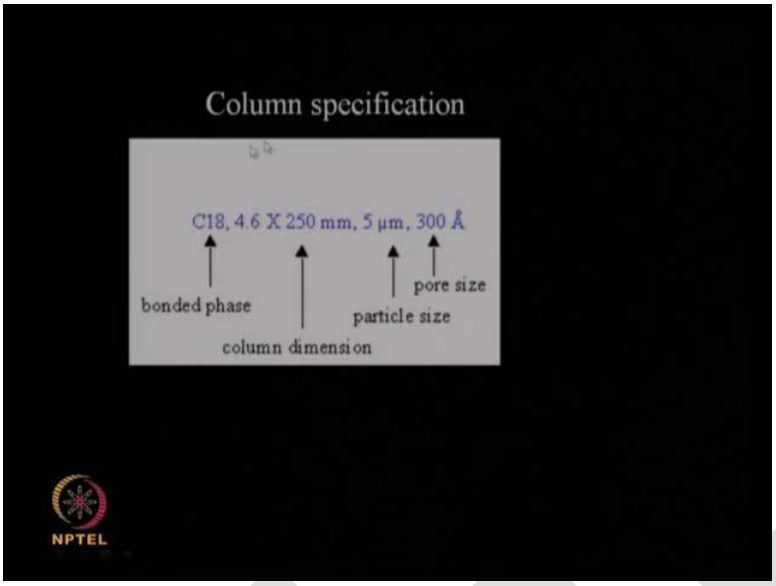
The pump is the most important in the sense it has to deliver very high pressure may in HPLC the pressures are extremely high 30 40 50 bars because you are using very fine particles in micron size. So, the back pressure developed is also very large. So, we are going to look at different types of detectors, there advantages and disadvantages during the course of this lecture.



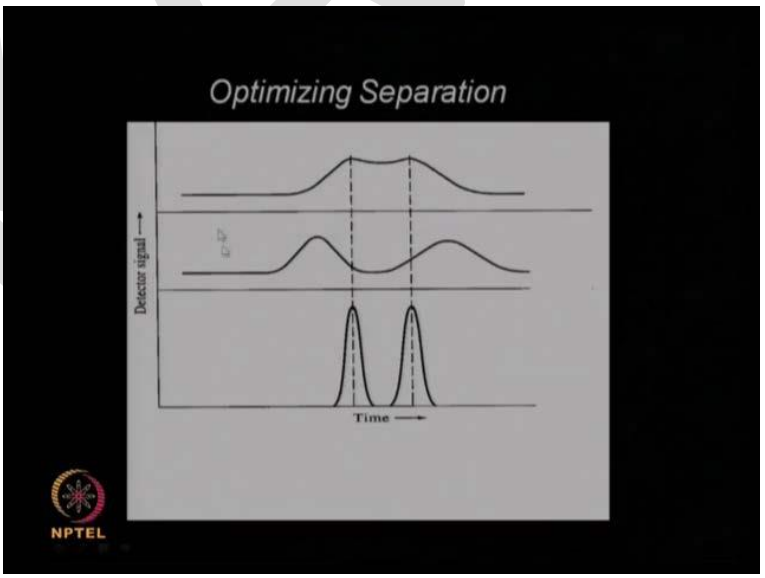
So, you may have a UV detector that means a single wavelength UV detector. Exactly matching one particular wavelength, if we know the components and come at this particular wavelength we just have a single UV detector. It is very cheap or we can have a variable wavelength detector using a monochromator or you have a multiple wavelength detector.

So, these are more expensive than a single wavelength. You can have a fluorescence detector if you know the compounds are fluorescence. We can have a electrochemical detector, we can have a mass spectrometric detector. So, lot of detectors depending upon

the type of compounds and how and they behave.



Typically, a column specification look like this, you know the C 18 column. This is the column dimension which are 4.6 meter long column, 255 which are particle size 5 micron and we have 30 angstrom pore size. That means the pore size of the particles which are supported are 300 angstrom's. This is the typical specification for a column.



Now, the output from HPLC will be a chromatograph. It will be like a Gaussian distribution or normal distribution and so on. But if you have multiple components and if you're separation is not very good you may have peaks coming out like this. This is a peak, this is another peak, but both are over lapping so much we cannot differentiate the area of this peak or area of this peak or you may have a reasonable separation. But still this is not good separation.

Again, you have some overlap in this region and this is the best separation one hope for. You get a very good base line separation these two components are well separated.

So, we can measure the area under this curve, we can measure the area under curve independently without any error. So, we can determine what is the concentration of this species or what is the concentration of this species. So, ideally one would like to have the this type of separation, but one may end up like this and this is extremely poor. And imagine if you have any components you are going to have many peaks overlapping, some of the overlapping, some of them well spread out.

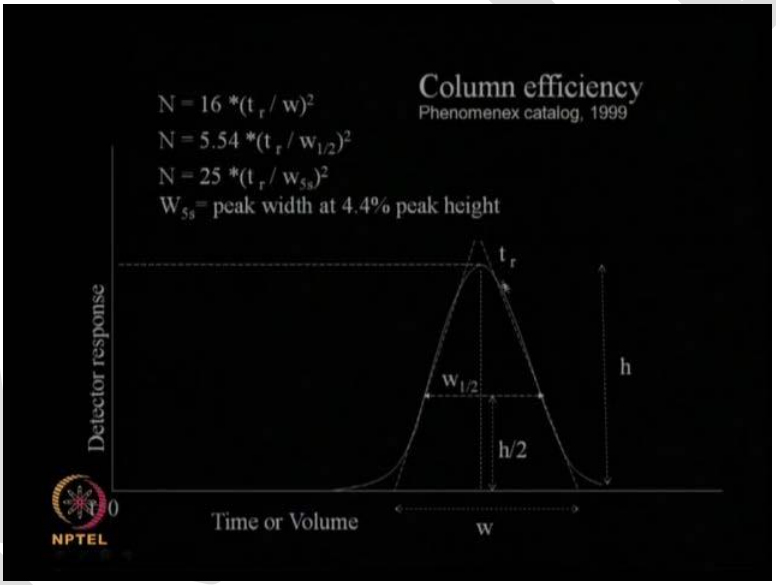
So, you the challenge is to separate each one of the component and clearly at the base line, so that you can measure the area under the curve and hence, the composition of the particular component of interest. So, the x axis you have the time and the y axis will be your detection signal. So, this is called the retention time that is the time at which we get the maximum of this particular peak.

So, the retention time is very characteristic of a particular component for a given HPLC hardware as well as given solvent and solvent fluoride. If we change solvent fluoride you are going to change your retention time or if I use different column I am going to get different retention time.

So, the retention time is very, very characteristic in a HPLC system. So, if I want to detect a compound and if I know what the compound probably is, what do I do? I get a pure authentic sample from a manufacturer injected in HPLC and see the retention time and then I inject the mixture and I see compound coming out at the same retention time and I can tell probably this particular compound is present in that mixture.

So, that is how we do identification of components. So, suppose we are looking at 3 or 4 and what we know and what we expect, it will be there in my mixture. I will buy those 3 or 4 authentic samples from a manufacturer, inject each one of the independently and check its retention time. And when I inject the mixture and if I get those 3 or 4 peaks at those retention times then I can tell that my mixture contains these components. So, that is the identification part in HPLC. How about quantification? In a quantification what we do? We inject authentic sample at certain concentration and we identify the area under the curve.

So, using that relationship between area under the curve and concentration I can tell how much concentration of another component is present in the mixture by measuring the area under the curve.



So, it typically the output from HPLC will look like this. You will have nice looking Gaussian distribution. We talked quite a lot about Gaussian distribution and the type of equation a normal distribution will have and so on. So, we can apply those knowledge and those equations in HPLC as well.

So, normally you will get a nice looking Gaussian distribution and this is called the retention time and this is called the height of the peak and this is called the half the height and this is called the width at half height. So, here this is called the width of the peak which

is done by drawing two parallel lines.

You get the width at the base and the width at half the height. That means you will check what is the half the height of this and then measure the width. That is called width at half by. Now, there is a parameter called theoretical plates just like we studied theoretical plates of the chromatography. Here also, you have the concept of theoretical plates and the theoretical plates could be estimated by measuring the retention time as well as either the width at the base or width at the half maximum.

Theoretical plates is a measure of the efficiency of the HPLC column. So, if a column has large number of theoretical plates as against another column which has small number of theoretical plates we can say the one which has large number of theoretical plates to be more efficient in separating components.

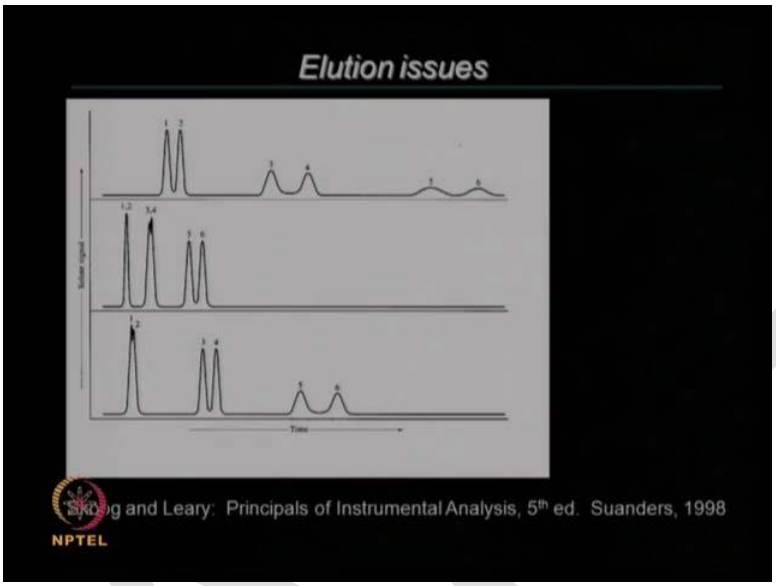
So, these are some formulae which can be used to measure the theoretical plates. N is the theoretical plates, t r is the retention time, w is the width at the base. So, you have N equal 16 into t r by w whole square or we can use these equation if you are measuring w per half.

That means if you are measuring the width at half maximum then we use instead of 16 here you will get 5.54 or there is another equation, this called n equal to 25 into t r by w 5 s whole square where w 5 s is the peak width at 4.4 percent of the peak height. If this is the peak height 4.4 will come somewhere there. So, this width will be like this, you know big width.

So, if you have that then you have to multiply by 25. So, w 5 s will be width somewhere here, whereas w is the width when you draw two tangents to this Gaussian distribution. So, we can use different equations to calculate the number of theoretical plates and there will be small differences depending upon the equation, which we use. Because although we say this is a very Gaussian looking distribution which never show, there will be small differences in its normal distribution shape.

So, the N which will be calculate from this equation or this equation or this equation will be slightly different. But we can use the same equation, if you want to compare different column. So, that we do not get confused with the value of N which we get. So, all you have

to do is inject the sample, determine its retention time, determine its width at half maximum, use this equation and calculate your number of theoretical plates. Then if you take another column do the same job, get the number of theoretical plates. So, we can usethis equation to compare quantitatively between two different columns and say which column is more efficient in separating a mixture and which column is less efficient.



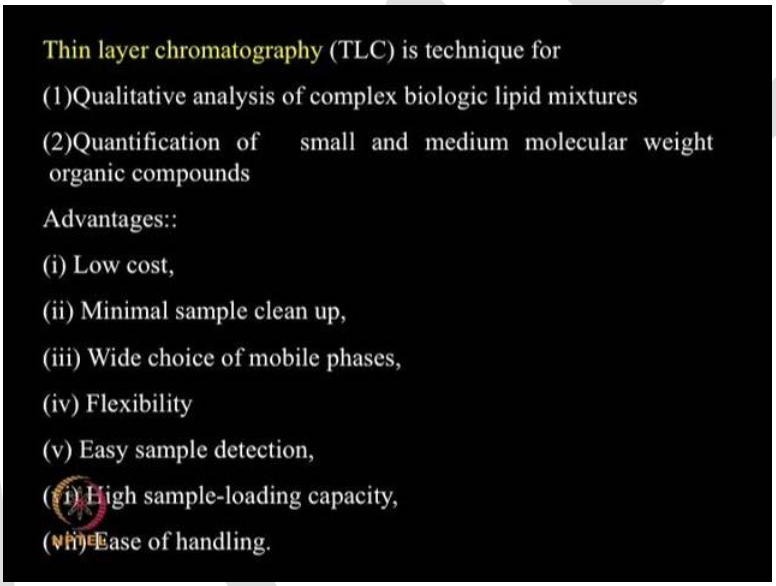
Now, there are many issues if you are performing in HPLC, it is not so easy. This is, this example is taken from a book, Skoog and Leary Principles of Instrumental Analysis. You see we are having six components. So, we are getting two nice peaks and after sometime we get slightly broader peak and after a very very long time we get extremely shallow peaks, peak number 5 and 6.

So, these peaks maybe coming at very long time, number one. Number two, there are so broad, area under the curve when we measure there will be lot of errors. So, to overcome the problem I may change the solvent conditions that means I may change type of solvents.

When we do that I may get 5 and 6 closer, these are nice and sharp peaks. But then you see 1 and 2 has overlapped and 3 and 4 has overlapped. We cannot differentiate 1 and 2, but we can slightly differentiate 3 and 4. So, again we play around with the solvent mixture and so on. 5 and 6 look reasonably good, 3 and 4 very good separations, but still 1 and 2 is

not very very good separation because there is a large overlap. So, if you are measuring the area under curve for 1 and 2 we are going to have large error. So, again this is also not very good.

So, we need to again work on the solvent optimization until we get reasonably good base line separation for 1 and 2. So, these are some issues we call it elution issues. So, we need to play around with the various solvents in the continuous phase. That is very very important. There is another chromatography which we use quite a lot in synthetic organic chemistry and I also talk about it for the past 10 lectures. That is called thin layer chromatography.

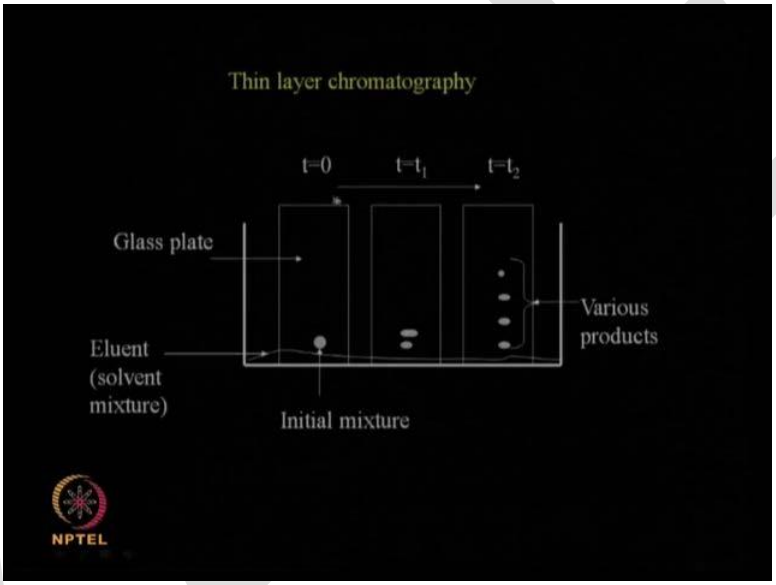


So, we can use it for qualitatively looking at mixture of biological or small molecule, chemical components. The advantages are it is a low cost, you need very little sample for that, we can use different mobile phases to perform, it is very flexible, very easy simple detection.

We can even go for high sample loading. Ease of handling is all good. So, if I am doing synthetic organic chemistry where I am performing a reaction a plus b and I want to know whether c is formed or d is formed, all I have to do is random thin layer chromatography and see weather I get new spots. So, thin layer chromatography is based on spots which I find on the plate, chromatographic plate. So, that is the main advantage.

We can monitor a reaction. Suppose, I have a raw material and I am slowly getting a product. Initially raw material will give one spot, as the product is formed I will get two spots. One for the raw material as well as one for the product, after sometime when the raw material completely gets converted and the raw material spot will disappear and I

will have only the product spot. So, beautifully we can monitor reactions. So, organic chemists use this TLC technique very extensively, practically every day for all their synthetic organic chemistry and synthesis work.

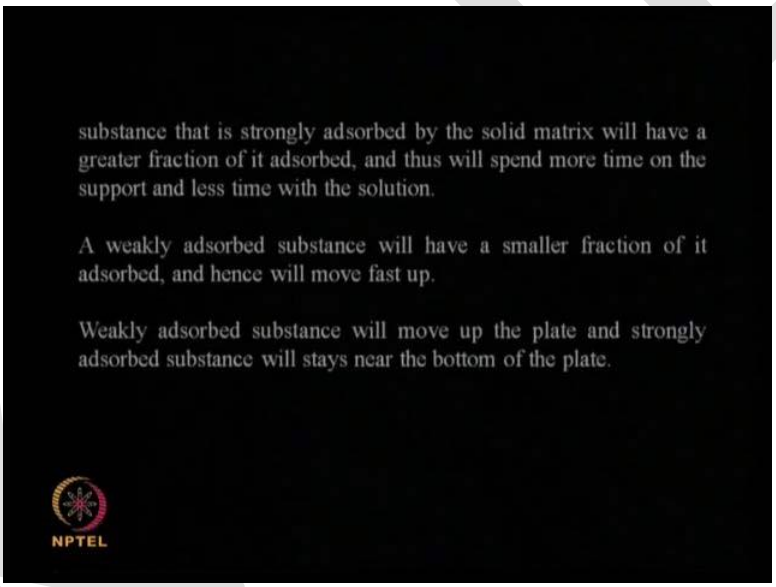


So, how does it work? So, you may have a glass plate and a coated with silica on the glass plate. Then I put a initial mixtures spot and then take a solvent which is the eluent. So, now it is a silica. So, it is a normal phase chromatography, silica is a hydrophilic compound or a polar compound. So, I have another solvent which moves as it travels upward due to capillary effect. It carries components with it. Component which are tightly bound silica remain at the bottom, component which are loosely bound will start traveling.

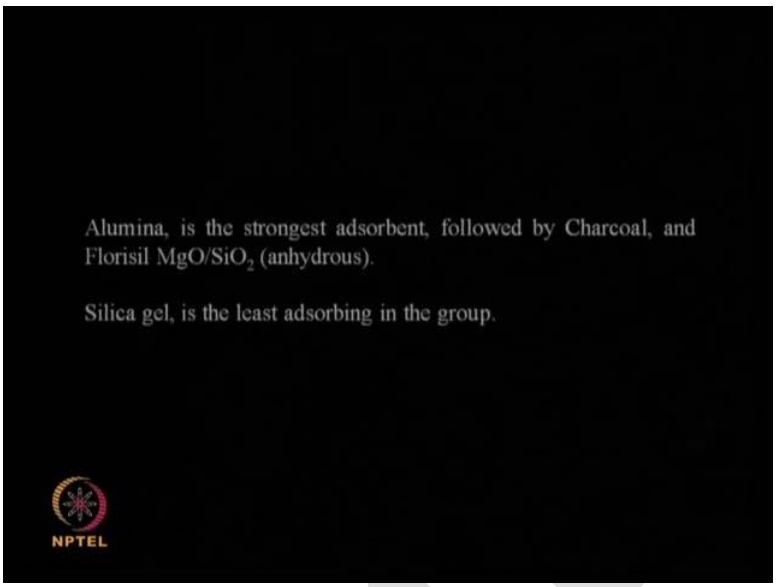
So, over a long period of time I am going to get various products in the mixture as various parts. Ideally I would like to get clean sharp separated spots, but sometimes what happens is you may get over lap of spots. So, generally simple system all we need is a glass plate and I coat just silica. And I put it in a solvent mixture. So, I can play around with different

types of solvent mixture. So, that I change the polarity and hence, the movement of the solute from bottom to top. This layer is called thin layer chromatography and it is as I said widely used in synthetic organic chemistry.

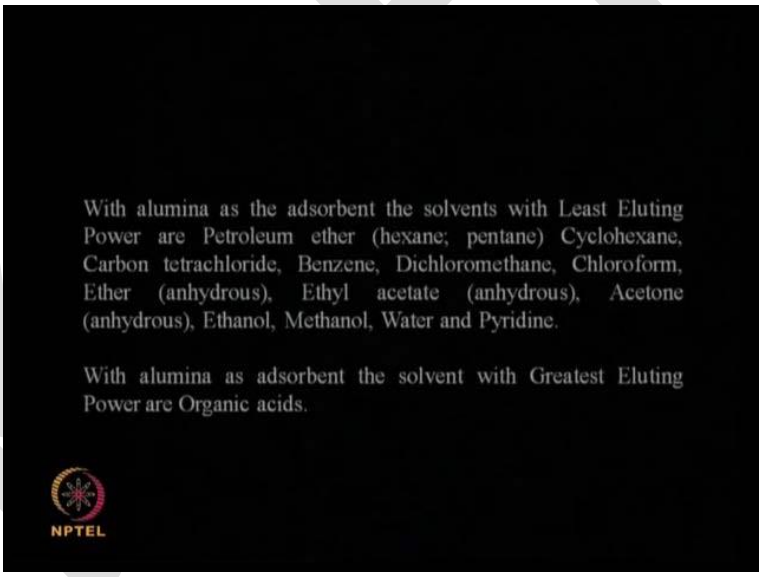
It is also used in downstream processing if I am looking at separation of small peptides or bio molecules. So, how does it happen? You have a capillary action. Just so the solvent and the solution mixture, solute mixture flows upwards. So, based on the partition coefficient solid will absorb, fraction of each component of the mixture. Reminder, will be in the solution will be travelling up. So, if it is slightly bound and silica it try, becomes very hard for it to travel will upward whereas if it is loosely bound and silica it becomes much easy for it to go upwards.



So, substance that is strongly adsorbed by the solid matrix will have a greater fraction of it adsorbed. So, it will spend more time on the support and less time with the solution, whereas weakly adsorbed substance will have a smaller fraction of it adsorbed. Hence, it will move fast. So, weakly adsorbed substance will move up the plate and strongly adsorbed substance will stay near the bottom of the plate. So, and from the location of the various spots we can tell components which are strongly adsorbed on silica, components which are weakly adsorbed on silica.

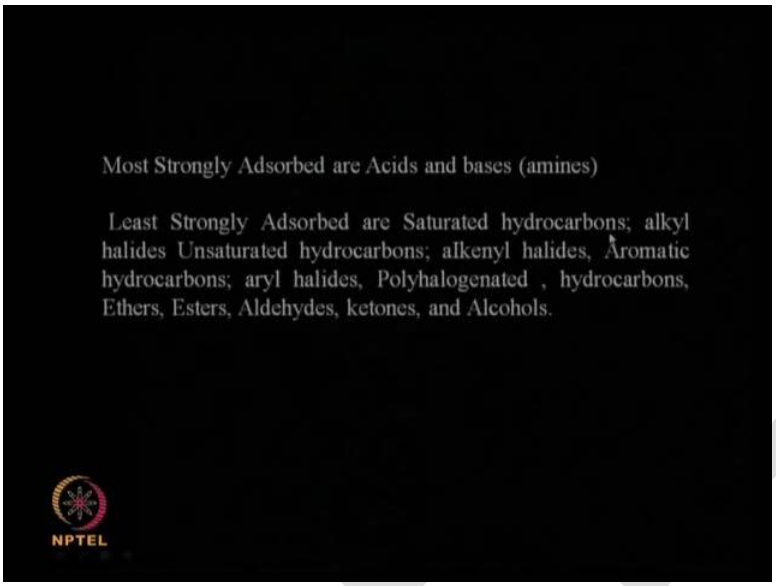


So, alumina is the strongest adsorbent followed by charcoal and florisil, magnesia, silica anhydrous and so on. Silica gel is the least adsorbing in this particular group of components actually.

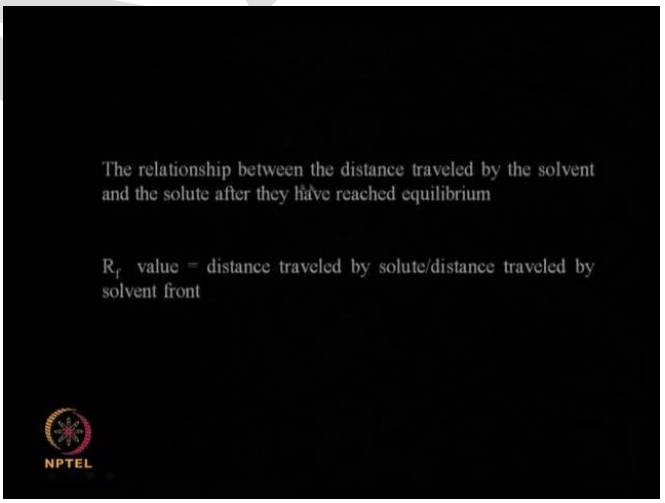


With the alumina as the adsorbent we can use different type of solvents depending upon whether I want least eluting power or whether I want strong eluting power. So, with the alumina is the adsorbent, solvent with least eluting power are petroleum ether like hexane, pentane and then go cyclohexane, then goes carbon tetrachloride, then goes benzene and dichloromethane, chloroform, ether, ethyl acetate, acetone, ethanol, methanol, water and pyridine.

So, with alumina as adsorbent the solvent with greatest eluting power will be organic acids. So, if I am using alumina I need to play around with the solvents depending upon whether I want least eluting or I want a greatest eluting power.

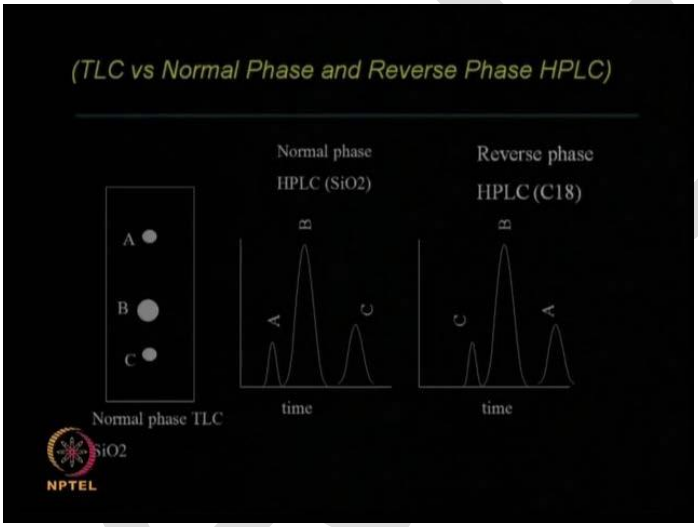


So, most strongly adsorbed are acids and bases and least strongly adsorbed are saturated hydrocarbons because you are using a stationary phase like silica or alumina which are polar or hydrophilic. So, least strongly adsorbed are saturated hydro carbons, alkyl halides, unsaturated hydro carbon, alkaline halides, organic hydro carbons, aryl halides, polyhalogenated, then comes hydro carbons, ethers, esters, aldehydes, ketones and alcohols. So, alcohols because they are hydrophilic they will get strongly adsorbed,



whereas, hydro carbons which are extremely hydrophobic are leastly adsorbed.

So, the relationship between the distance the solutes travel because of the solvent after the each equilibrium is given something called R f value. So, R f value is characteristic of the compound for a given stationary phase. So, R f value is the distance traveled by the solute divided by distance traveled by the solvent front. So, it is distance travelled by the solute divided by distance travelled by the solvent. So, if the R f value is very very large obviously it, the compound travels fast or very far after it has achieved equilibrium. If the



R f value is very small then you can say that compound has not travelled very far in the system.

So, for example, I have TLC then I go to reverse phase HPLC. How do I connect the spots I get on TLC with the peaks that I get in the HPLC. So, that depends upon the type of HPLC whether I am using normal phase in HPLC or whether I am using a reverse phase HPLC. Now, in a TLC we use generally silica. So, it is almost like a polar stationary phase. Now, if I take a normal phase chromatography I will again use the polar stationary phase. So, the order of the components in a TLC which uses silica as against your normal phase chromatography will be the same.

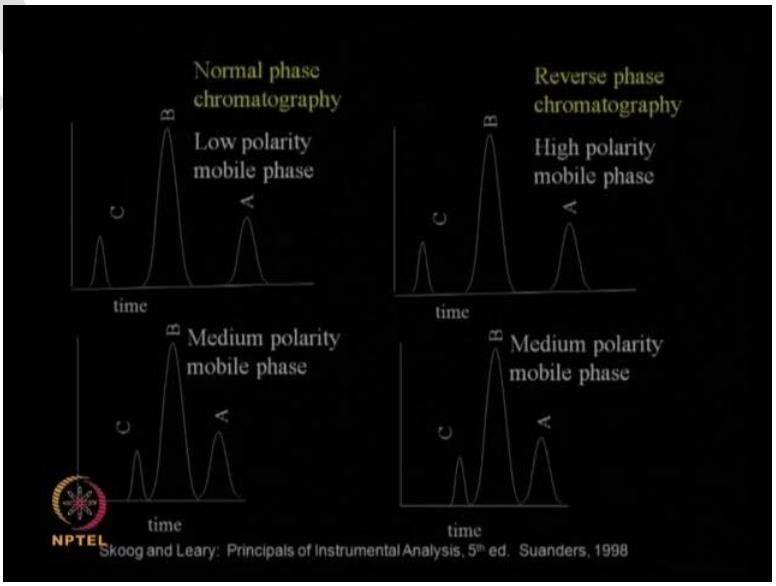
So, if I take a TLC and I see a spot C which is right at the bottom and then I see B and then I see A; that means here C is very very polar and A is the least polar. So, in a normal phase what will happen? A will come out first, B will come out second, C will come out third.

That means A will have the least elution time or retention time. B will have the next retention time and C will have the highest retention time. So, in a normal phase HPLC we are using silica. So, that is also a polar. So, they will behave in the same fashion as a normal phase TLC which are also uses silica whereas, if I use a reverse phase HPLC where I am using a hydro carbon like a C 18 as the stationary phase.

Now, this stationary phase is hydrophobic or lipophilic. Then what happens? So, in a hydrophobic reverse phase chromatography I use a polar as the mobile phase. So, what will happen? C which is more polar will come out first, B which is next will come and finally, A which is hydrophobic or non polar or lipophilic will come out last. So, you will see the order of way these three component A B C come in a normal phase, these, all these reverse phase are very very different. So, you should not get confused. You should know if I get in a TLC three components A traveling the furthest, B in the middle, C travelling the least, if you are using a normal phase you will have A coming out first.

That means A having the lowest retention time followed by B and C will have the highest retention time, whereas if I use reverse phase chromatography C will have the least retention time and A will have the highest retention time. So, we see that there is a completely reverse happening in the normal phase, in the reverse phase chromatographies.

So, you need to understand this concept very very well, so that if I do TLC curing initial

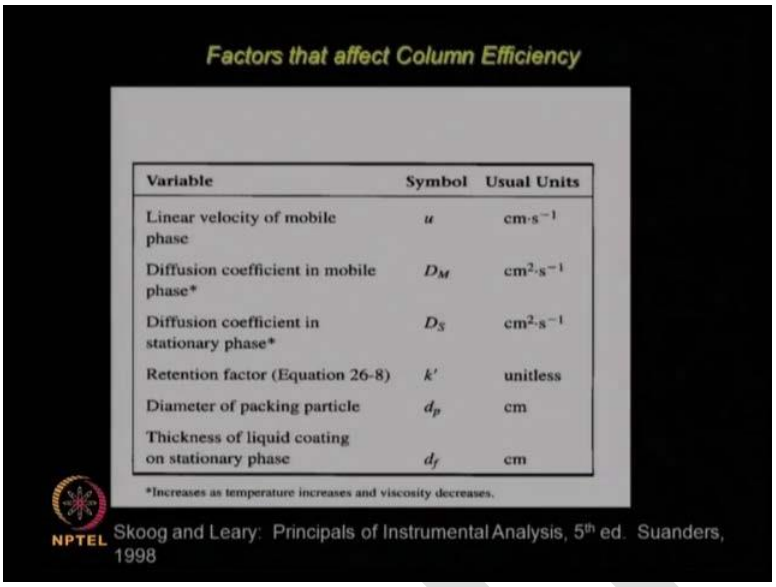


downstream processing and the later on I am going to HPLC, I should be able to tell depending upon the type of HPLC I am using when each of the components will come with respect to the spots which I observe in my TLC.

Let us go forward. So, you have a normal phase chromatography. I have a low polarity mobile phase. It is a normal phase chromatography. So, I am in the stationary phase is polar, do not forget that. So, I have components like this C B A. So, if I increase the polarity what happens? I am having a polar stationary phase and I am increasing the polarity. So, obviously these three will come together. So, that means if I am using normal phase chromatography and if the peaks are very close I want to move peaks further apart, I reduce the polarity.

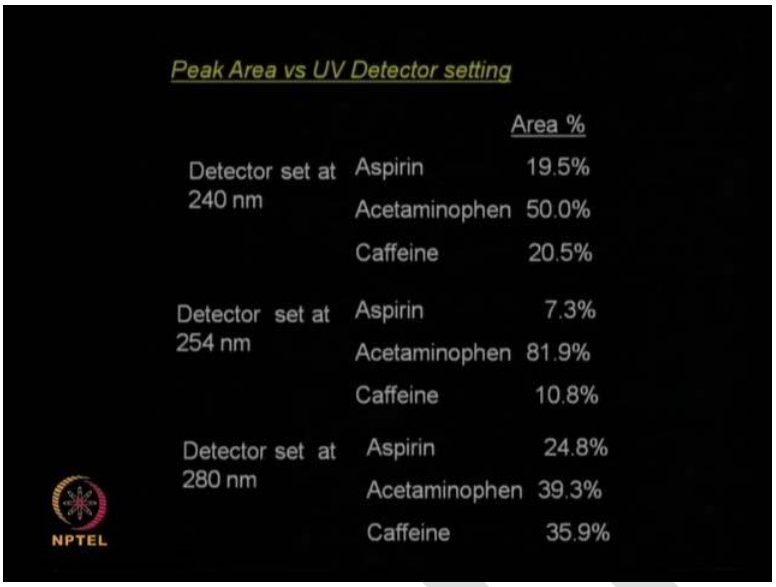
That means relatively make it more hydrophilic sorry hydrophobic. So, a normal phase chromatography if the peaks are very closed and I want to move the peaks further apart I use a slightly more hydrophobic system that means I reduce the polarity of these mixtures. Now, let us go to reverse phase. Suppose, I have a reverse phase chromatography and my peaks are like this, C B and A. Now, my solvent mixture or mobile phase is a high polarity, if I reduce to polarity to medium then they will come closer.

So, in a reverse phase chromatography if the peaks are very very close, all I have to do is increase the polarity of the mobile phase then the peaks will get separated out. So, in a normal phase chromatography if the peaks are closer I reduce the polarity for the peaks to separate out, whereas in reverse phase chromatography if the peaks are closer I increase the polarity, so that the components get separated out. So, you see the strategy which you adapt in a normal or a reverse phase changes dramatically. So, if you want move from a close to a further separated system.



So, what are the various factor that affect the column efficiency? Factors which affect the column efficiency or the linear velocity of the mobile phase. So, if the velocity is high then obviously my retention time goes down relatively where diffusion coefficient in the mobile phase, diffusion coefficient in the stationary phase. That means how the components diffuse inside the pores of the stationary phase. Then we have the retention factor that means how much of the solute is taken up by the stationary phase.

Diameter of the packing material, thickness of liquid coating on the stationary phase, so if you have thick material you are going to have mass transfer resistances, if you have very thin coating we are going to have less mass transfer resistance. So, all these factors affect the column efficiency. So, you can play around with many other factors, but once I select my column when some of the factors get fixed, whereas I can still play around with the solvent fluoride or I can play around with mixtures of solvent. So, that I affect my retention factor as well as I will affect my linear velocity of the mobile phase.



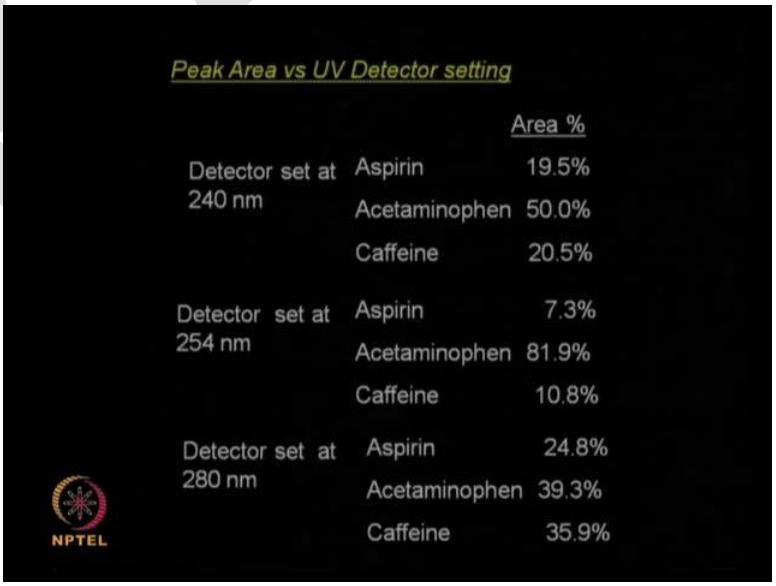
So, imagine I have three components and I am using UV detector that means the components are detector using a UV. So, if I set the detector to 240 nanometers and suppose I have three components like aspirin, acetaminophen and caffeine I may get area under the curve for aspirin 19.5, the acetaminophen as 50, caffeine as 20. Now, if I change the detector setting to 254 nanometers then this become 7.3, area under the curve goes down dramatically and this becomes very very large 81.9, caffeine becomes 10.8.

Now, if I set the detector to 280 nanometers aspirin becomes 24.8, acetaminophen become 39.3, caffeine becomes 35.9. Now, how much is the real amount? We do not know because depending upon detector setting I am getting different area under the curve, percentage area under the curve. Why is it so? That is because of the lambda max. If the lambda max for each of the component very different depending upon where I said the lambda max that particular component will show a very high area. So, that is another big challenge when you are doing HPLC.

So, I may get mistaken whether the component acetaminophen is very very large or it is equal to caffeine. So, you will see that I am able to get different area under the curves for aspirin depending upon the lambda max of my UV detector. So, I need to have some idea about the lambda max for the components with which I am measuring in my solution or in my solute mixture.

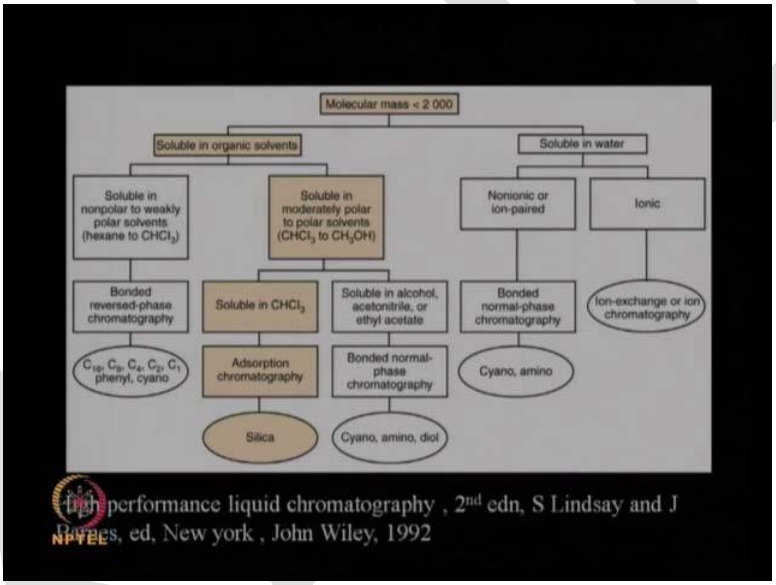
If I do not know then I may end up with this type of misunderstanding on the whole system.So, you see UV max for aspirin is 225 and 296 nanometer, acetaminophen is 248. So, at 254 I get a very very large number. So, when I go away from 248 you see the acetaminophen keeps going down. Now, for caffeine it is 272. So, as I keep increasing, see the caffeine percentage goes up.

So, that main problem here is the lambda max aspirin has low lambda max, caffeine has the next one, sorry acetaminophen as the next one and caffeine has the third largest.



So, the numbers keep grammatically changing. So, see the aspirin it goes down and again increases because aspirin has two different lambda maxes. So, there is one lambda max here that is why you get some number. As we move out from that particular number the values go down and again it goes up, it peaks up because aspirin has two lambda max. So, the first thing is to you will use variable system just to find out the lambda max for, of various component present in my mixture.

Then you decide which lambda max to use if I want to use constant UV detector. And then do on your standardization, measurements and so on actually. So, understanding the lambda max for each one of the component is very very important. That is what this particular example tells you. Otherwise, you can totally get mistaken.



Now, there are different types of liquid chromatography's we can use depending upon the type of molecule which you are trying to detect. This is taken from a book by Lindsay and Barnes from John Wiley publication. It is called High performance liquid chromatography.

Now, if it is small molecule like molecular mass less than 2000, it could soluble in water that means it is hydrophilic or it could be soluble in organic solvent. That means it is hydrophobic. Now, in soluble in water it could be ionizable or it could be nonionic or ion paired type of this system. So, with an ionic then I can use ionic exchange or ion chromatography.

It is a non ionic or ion paired then I can use bonded normal phase chromatography. So, it is a normal phase that means the stationary phase will be hydrophilic. Now, soluble in water, now let us go to the system. If the system in solvent organic solvents, that means it hydrophobic.

Now, here we can have two different things. One is soluble in non polar to weakly polar solvents, soluble in moderately polar to polar solvent. So, if it is non polar to weakly then we will go to reverse phase chromatography. Here, we can use C 18, C 8, C 4, C 2 or phenyl or cyano. This is completely hydrophobic material.

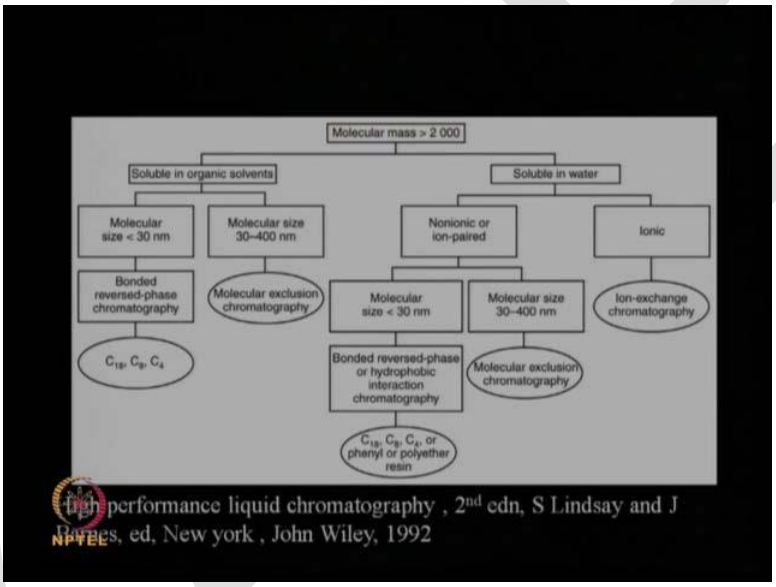
So, we go for reverse phase chromatography whereas it is moderately polar to polar solvents. So, it may be soluble in chloroform or methanol. So, if it is soluble in chloroform you go to adsorbtion type of chromatography that means the stationary phase forces are just based on adsorptive forces or it soluble in alcohol or acetonitrile or ethyl acetate.

So, here we use bonded on normal phase chromatography. Again, we use in normal phase chromatography base cyano or amino or diol as the stationary phase. So, depending upon, this is for small molecules, depending upon whether molecule is soluble in water or whether it is soluble in organic solvent. If it is soluble in water is it easily ionized or its nonionic or ion paired, we may have two different types of chromatography either ion exchange or normal phase.

Now, on the other side if it soluble in organic solvents then one could be soluble only in weak polar or completely non polar or the other one could be moderate polar solvents.

Completely non polar we may be using a reverse phase chromatography, whereas in a moderately polar we can have two systems, one is the chloroform soluble or other one is alcohol soluble or acetonitrile soluble.

So, in a chloroform soluble you may be using a silica based chromatography whereas in the other we may be using a normal phase chromatography like cyano, amino, diol and so on actually. Now, let us look at molecules which are larger than 2000 that means they are much larger molecules, whereas so far we looked at molecules which are smaller than 2000.



So, again this is taken from the same book High performance liquid chromatography, second edition, Lindsay and Barnes. Again, we have soluble in water, soluble in organic solvents. So, soluble in water again it can be ionic, it can be nonionic or ion paired. If it is ionic we can use a ionic exchange chromatography. If it is nonionic it could be small molecular size or large molecular size.

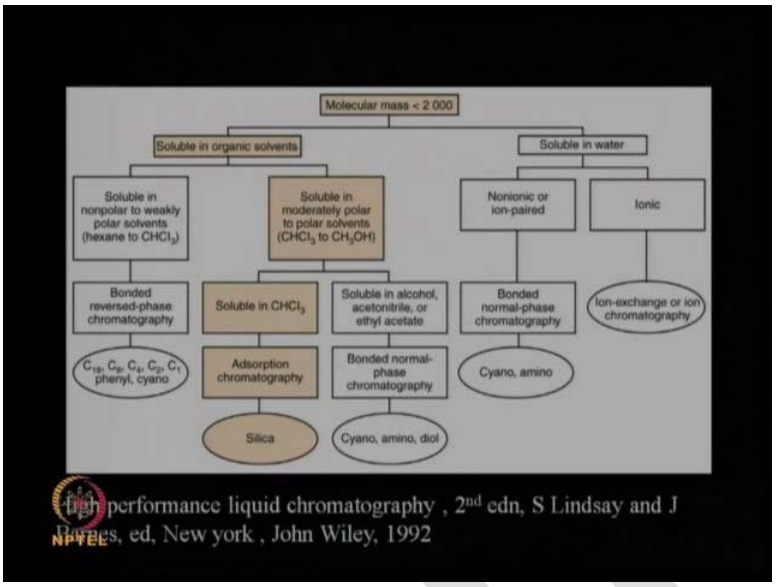
So, if it is a small molecular size we can use a reverse phase or hydrophobic interaction chromatography like different types of stationary phases C 18, C 8, C 4. If it is a large molecular size 30 to 400 nanometers then we can use a molecular exclusion chromatography. So, size exclusion chromatography we talked about it long time back. It is called size exclusion or gel permeation. So, it is based on size or molecular weight alone.

So, larger size polymer to smaller size polymer, larger size metabolize to smaller size metabolize or larger molecular weight metabolized to smaller molecular weight. Now, that is do with the solubility in water. What happens if they are not soluble in water, but they are soluble in organic solvents? Again, you have small molecules and large molecules. Small means less than 30 nanometers, large means 30 to 400 nanometers. So, large molecules we can again go for the exclusion chromatography like size exclusion or gel filtration chromatography, whereas small molecule we can go for reverse phase chromatography C 18, C 8, C 4.

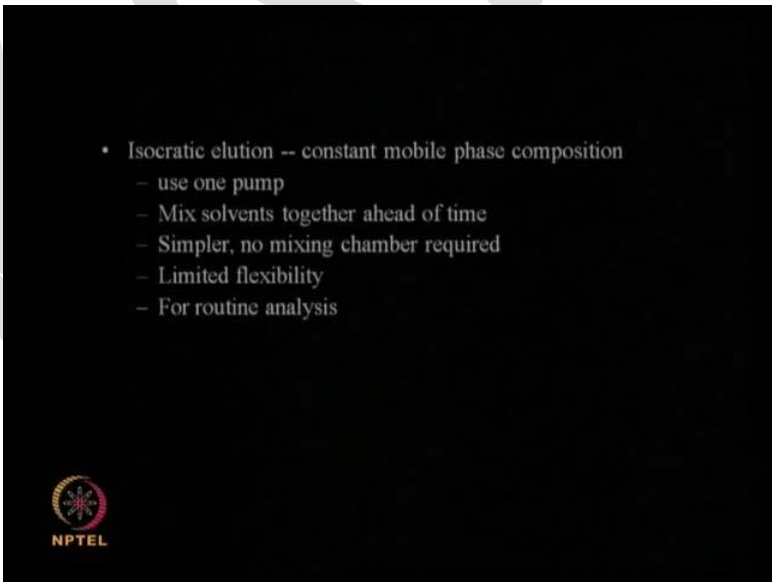
So, again large systems we again have water soluble large systems or solvent, organic solvent soluble large systems. If the size of the molecules or molecular weight of the molecules are very large we can go for gel permeation chromatography. Of course, if it is ionic we cannot use gel permeation chromatography because as you can see here we can use only ion exchange chromatography.

Gel permeation is good for nonionic or ion paired systems actually which is based on just play size separations. So, smaller molecules will get entrapped in the pores of your stationary phase whereas larger molecules do not get entrapped, so they travel faster.

So, larger molecules will come out of the column faster, whereas smaller molecules will take much longer time because they interact. So, this table gives you what type of chromatography it use depending upon whether it is a small molecule or whether it is a large molecule. So, if the molecule are large we can go for ion exchange chromatography or we can go for gel permeation chromatography or we can go for hydrophobic interaction or reverse phase chromatography.

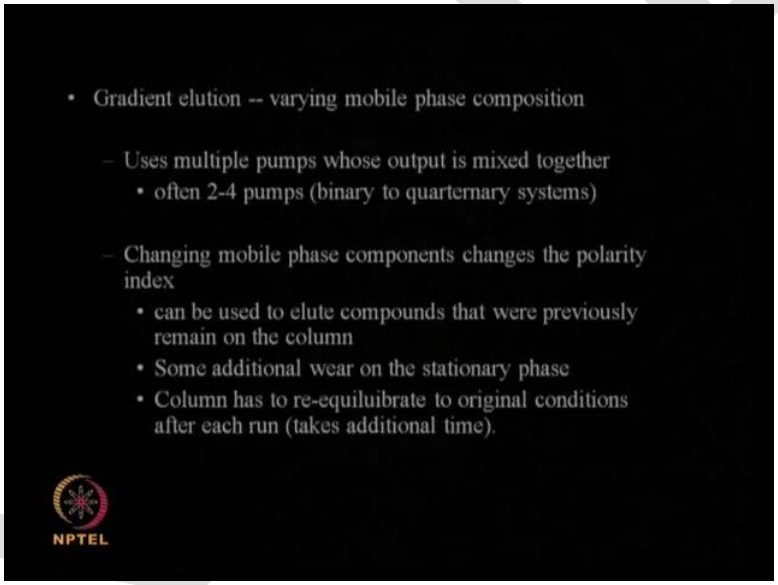


If the size of molecular sizes are very small, again we go for an exchange chromatography, normal phase chromatography, absorption chromatography and reverse phase chromatography. So, selections based on the ionizability, selection based on the size of the molecule, selections based on the solubility of the molecule.



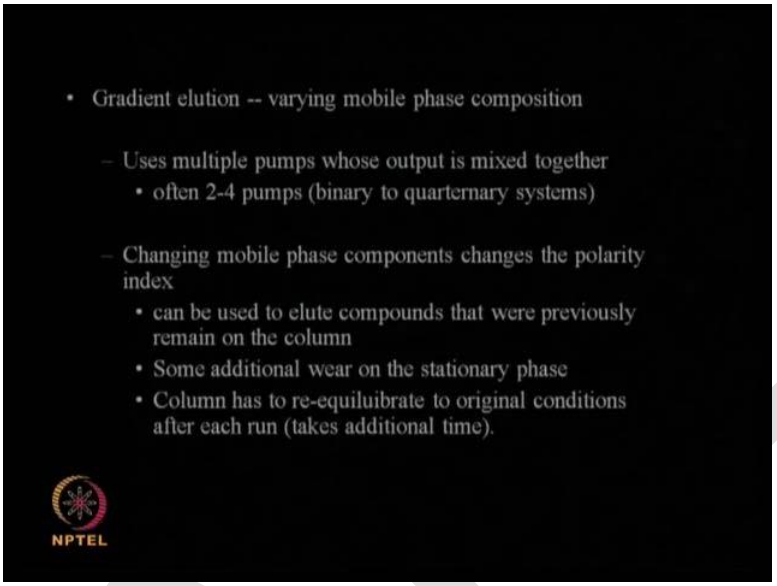
There is something called isocratic elution that means I use only one mobile phase. It is very simple, very cheap I use only one solvent, I use only one pump. The pump is very very expensive in a HPLC. Now, using only one pump and able to bring down the cost or if I want to use two solvents, I mix the solvents and make it in a container and use that mixed solvent for elusion.

But still I use only one pump. So, it is simpler. You do not need a mixing chamber. If I have two solvents then obviously I need a mixing chamber. But it has got limited flexibility because I cannot change the dielectric constant of the operation. It is good for routine operation. So, if I am going to do the same system like a (( )) where you are going to do the same thing day in and day out for very long time to come, this is the best system.



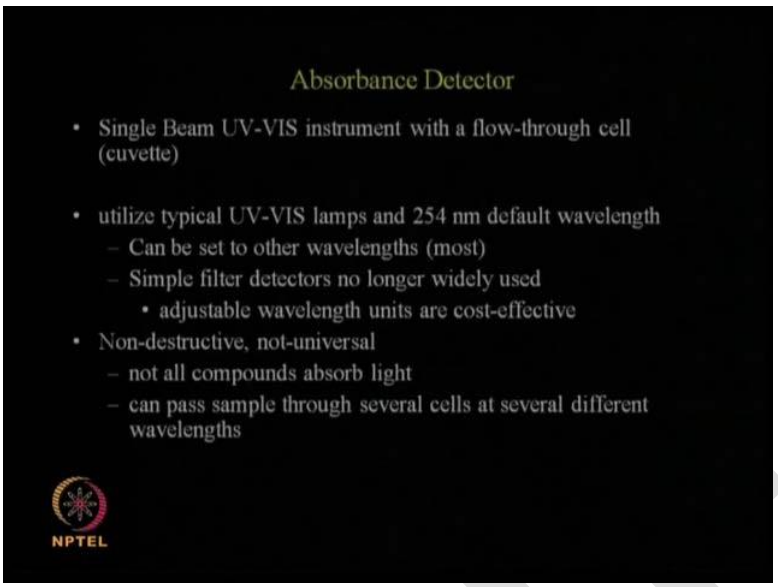
Gradient elution where the mobile phase compositions are changed as a function of time. So, initially you may start with one solvent. As time proceeds I change the solvent to something else. This is just like gas chromatography. In gas chromatograph either you can do the chromatography in one single temperature value that means the column is placed inside the oven and the temperature maintained constant or as a function of time I can change the temperature of the column. So, I may start with say 150 and in 10 minutes I may go to 250 and then maintain at the temperature.

So, exactly similar to changing the temperature in a gas chromatograph I am changing the solvent dielectric constant in a HPLC system. So, I may start with one solvent, I may go into another solvent or I may start with one solvent mixture then I move to some other solvent mixture. So, that is called the gradient elusion which is opposite to your isocratic.



So, you will be using multiple pumps. So, you can use two pumps or you can use four pumps that means it can be a binary system or it can be quarternary system. Of course, the cost is very high because pump costs are very high. So, more the pumps, more is the cost. So, when I change the mobile phase components I am changing the polarity index. So, I can use it to elute compounds that are not coming out of my column, they stick inside, they stay inside. So, they, but the problem is that there could be some additional wear on the stationary phase. After each run I have to again bring it back to the original condition.

So, each run will take longer. So, I do a run and then I will bring it back to the original condition which requires again several minutes. So, in a gradient elution normally the time taken is much longer than a isocratic elution because in isocratic we are not changing the composition of the solvent over a period of time. So, you do not have to again re equiluiberate the system. So, that is one of the disadvantages of gradient elution. It takes much longer time, but we can get excellent separations using two solvents or three solvents or mixtures of solvents. That is the main advantage of this type of gradient elution.



There are various types of detectors that are possible in HPLC depending upon the properties of the solutes, properties of the amino acids or proteins or peptides which are separating. And we are going to now look at some of the detectors, look at some of the advantages, disadvantages and so on actually. The most cheapest one is the single beam UV visible with the flow through cell, cuvette; that means you take your sample inside a cuvette and there is a UV which measures the absorbents of the solute mixture. Generally, it operates at 254 nanometer default wavelength, but we can also set it for other wavelengths also, know.

Some UV detectors you cannot set it then it is very cheap. There may be UV detectors where you can pre set it to some fixed value 3 4 different wavelengths or we can use even filters. We can also have adjustable wavelength units which are cost effective. The main advantage is the cost, it is very cheap. And then it is non destructive. So, the sample we can take it back and use it for some other purposes. So, especially if the sample amount is very small you cannot have, afford to have a sample which is totally destroyed after the analysis.

So, that is the main advantages. The disadvantage is not all compounds will absorb the light. So, you will not be able to detect a compound which is not UV detectable at all. So, that is a big problem or we can have several cells with different wavelengths, fixed wavelengths. So, we can look at various wavelengths in each one of the cell. So, that is

other approach by which we can slightly improve the flexibility of this type of UV detectors. Now, we will look at more detectors in the next classes and as I said depending upon the detector we can look at the compounds that are present in the mixture to very simple compounds to very complex compounds. So, we shall continue this particular topic in the next class.

Thin layer chromatography is	partition chromatograph	electrical mobility of ionic species	adsorption chromatography	migration of charged ion	**adsorption chromat**	analyte
Relative flow (Rf) value ranges from	0 to 1	0 to 2.0	+2 to -2	+1 to -1	**0 to 1**	eluite
Sucrose can be determined after silylation using which chromatographic technique	HPLC	Gel chromatography	Gas liquid chromatography	Paper chromatography	**Gas liquid chromat**	immobilized phase
The relationship between concentration, temperature & potential of a solution is given	Ilkovic equation	Henderson equation	Nernst equation	Hassalbach equation	**Nernst equation**	eluate
Ion exchange chromatography is based on the	electrostatic attraction	electrical mobility of ionic species	adsorption chromatography	partition chromatograph	**electrostatic attracti**	chromatograph
The locating agent of amino acids is	Diazo reagent	ninhydrin spray	Amphoteric oxides	neutral oxides	**ninhydrin spray**	Capillary Electrochromatography (CEC)
Chromatography can be used to	form mixtures	change mixture compositions	separate mixtures into pure substances	is not a separation techn	**separate mixtures in**	mobile phase
In gas chromatography, the basis for separation of the components of the volatile mat	partition coefficients	conductivity	molecular weight	molarity	**partition coefficient**	Preparative chromatography
Proteins can be visualized directly in gels by	staining them with the d	using electron microscope only	measuring their molecular weight	Spectrophotometer	**staining them with t**	Solute
Electrophoresis of histones and myoglobin under non-denaturing conditions (pH = 7.(	both proteins migrate to	histones migrate to anode and my	histones migrate to the cathode and myoglo	both proteins migrate to	**histones migrate to**	solvent
In isoelectric focusing, proteins are separated on the basis of their	relative content of positi	relative content of negatively char	size	relative content of positi	**relative content of p**	detector
In SDS-PAGE, the protein sample is first	treated with a reducing a	fractionated by electrophoresis the	treated with a oxidizing agent and then with	treated with acetic acid	**treated with a redu**	sample
SDS is a(n)	anionic detergent	cationic detergent	not an detergent	Chargeless	**anionic detergent**	retention time
Proteins are separated in an SDS-PAGE experiment on the basis of their	positively charged side c	molecular weight	negatively charged side chains	different isoelectric poin	**molecular weight**	paper
DNA possesses	No charge	a positive charge	a negative charge	a supercharge	**a negative charge**	aluminium oxide
The rate at which DNA migrates through the gel is mainly determined by	molecular size of the DN	salt levels	Protein	lipid	**molecular size of th**	paper chromatography
What is ethidium bromide?	buffer	dye	DNA solution	restriction enzyme	**dye**	polar organic solvent with water
Why do scientists load DNA of known sizes into the agarose gel?	It makes it easier to dete	To fill in all the slots on the gel so	To practice loading the DNA before you ge	So you know how long t	**It makes it easier to**	radial
The colour of cathode is	Red	Black	Blue	Green	**Black**	rectangular paper
The colour of anode is	Red	Black	Blue	Green	**Red**	0.1 – 0.25 mm
Which of the following is not required for SDS-PAGE	TEMED	APS	SDS	Tryptophan	**Tryptophan**	0.5 – 2.0 mm
Which amino acid gives yellow colour with ninhydrin	Proline	Alanine	glutamine	serine	**Proline**	UV light
Galactose is a constitutent of	Lactose	Agarose	lactose and agarose	not a constituent of lacto	**lactose and agarose**	40 – 93 µm
pH of stacking gel is	10	6.8	8.8	9	**6.8**	increasing salt concentration
pH of separating gel is	10	6.8	8.8	9	**8.8**	low net charge
Protein with less molecular weight moves faster during electrophoresis.This statemen	TRUE	FALSE	hypothetical	will not comment	**TRUE**	a salt gradient.
Electrophoresis technique was first developed in	1947	1950	1937	2000	**1937**	
Electrophoresis technique was first developed by	Tiselius	James	Watson	Crick	**Tiselius**	
Ampholytes contain	Positive charge	Negative charge	both positive and negative charge	no charge	**both positive and negative charge**	
Why glycerol is added in loading buffer	to give weight to sample	to give charge to sample	to give weight and charge	it is not added in loading	**to give weight to sample**	
Mercaptoethanol reduces	peptide bond	disulfide bond	Hydrogen bond	vander waals force	**disulfide bond**	
Which causes decomposition of persulphate ion to give free radical during SDS PAGE	APS	TEMED	SDS	acrylamide	**TEMED**	
Urea at 3 to 12 M concentration disrupts	peptide bond	disulfide bond	Hydrogen bond	vander waals force	**disulfide bond**	
EtBr intercalates DNA and is visible under	dark condition	UV	visible light	IR	**UV**	
Glycinate ion in stacking gel is	poorly ionized	completely ionized	not ionized	ionized	**poorly ionized**	
Which of the following is referred as “leading ion” in SDS PAGE	Chloride	Glycinate	APS	Protein	**Chloride**	
Which of the following is referred as “trailing ion” in SDS PAGE	Chloride	Glycinate	APS	Protein	**Glycinate**	
Amount of time an analyte stays in column is referred as	retention volume	retention ratio	retention time	Void	**retention time**	
Amount of solvent required for an analyte to come out of column is referred as	retention volume	retention ratio	retention time	Void	**retention volume**	
During gel chromatography the molecules are separated according to	Charge	Charge/mass ratio	Mass	applied electric field	**Mass**	
Dextran is a polysaccharide composed of	Glucose	Fructose	Galactose	Fucose	**Glucose**	
In chromatography, Rf is referred as	retention force	retardation factor	relative flow	random factor	**relative flow**	
Ion exchange chromatography uses	cationic exchanger	anion exchanger	no exchanger	cation and anion exchanger	**cation and anion exchanger**	
In TLC the plates are dried after applying stationary phase at	4 Celsius	40 Celsius	100 Celsius	55 Celsius	**40 Celsius**	
Antimony trichloride is used for the detection of	Steroids	Terpenoids	Carbohydrates	Steroids and terpenoids	**Steroids and terpenoids**	
Acridine orange is used for the detection of	Nucleic acids	Terpenoids	Carbohydrates	Steroids	**Nucleic acids**	
Anisaldehyde is used for the identification of	Carbohydrates	Proteins	Fats	Vitamins	**Carbohydrates**	
Weakly acidic cation is effective at pH	3	5	Zero	2	**5**	
DEAE-sephadex exchanger is an example for	strong cationic	weak cationic	strong anionic	weak anionic	**weak anionic**	
A good adsorbent used in chromatography should ideally be	Inert	Stable	Cheap	inert, stable and cheap	**inert, stable and cheap**	
Agarose gel electrophoresis is referred as	Horizontal	Vertical	Slanting	Running	**Horizontal**	
Electrophoresis can be done in paper. This statement is	TRUE	FALSE	Imaginary	Hypothetical	**TRUE**	
Identification of DNA is referred as	Western blotting	Northern blotting	Eastern blotting	Southern blotting	**Southern blotting**	
Identification of RNA is referred as	Western blotting	Northern blotting	Eastern blotting	Southern blotting	**Northern blotting**	
Identification of proteins is referred as	Western blotting	Northern blotting	Eastern blotting	Southern blotting	**Western blotting**	
Amount of SDS bound to protein per gram protein is	14 gram	2.5 gram	1.4 gram	8 gram	**1.4 gram**	
Generally, the loading dye used for agarose gel electrophoresis is available at	6X	10X	1X	8X	**6X**	
500 base pair DNA fragments are generally identified using	agarose gel electrophoresis	acrylamide electrophoresis	PAGE	Chromatography	**agarose gel electrophoresis**	
Bromophenol blue migration in agarose gel electrophoresis coincides with	300 bp	10 bp	500 bp	1000 bp	**300 bp**	
EtBr used in agarose gel electrophoresis	Mutagen	Neurotoxin	Safe	Nephrotoxin	**Mutagen**	

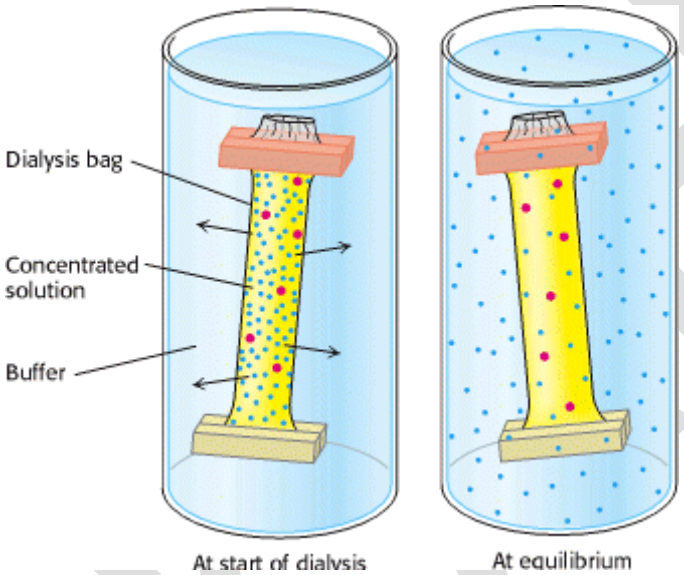
UNIT-V

SYLLABUS

Dialysis: Principle and types- Hemodialysis, pediatric, intestinal and peritoneal dialysis. Dialyzable substances. Medical applications.

Dialysis

A solution containing the protein of interest must be further altered before purification steps are possible. Proteins can be separated from small molecules by taking advantage of the larger size of the protein compared to other molecules. Dialysis through a semi permeable membrane (SEM), such as a cellulose membrane with well defined pores is often done to free a protein of choice from other contaminants. The partially purified protein solution is placed in a dialysis bag and the bag suspended in a much larger volume of buffer of appropriate ionic strength.



Protein molecules (red) are retained within the dialysis bag, whereas small molecules (blue) diffuse into the surrounding medium.

Proteins molecules having significantly greater dimensions than the pore diameter of the dialysis bag, whereas smaller molecules and ions cross the pores of such membranes and emerge in the dialysate outside the bag. This technique is useful for removing a salt or other small molecule, but it will not distinguish between proteins effectively. Dialysis bags of definite molecular weight cutoff are often employed to purify protein of definite

size. For example, a dialysis bag having a 12,000 molecular weight cutoff can be safely used to purify proteins having molecular weights of ~20,000 but may not be appropriate for proteins with a molecular weight of 10,000.

Dialysis and its Application

Dialysis is a renal replacement therapy that provides an artificial replacement for kidney disfunction, and it is a life support treatment but not treat kidney diseases. Dialysis is based on the principle of the diffusion of solutes along a concentration gradient across a semipermeable membrane. There are three main types of dialysis: hemodialysis, peritoneal dialysis and hemofiltration.

Kidney is an important organ in animal to remove waste from the body, such as potassium and urea, as well as free water from the blood. Under the healthy condition, kidneys remove waste products from the blood and also remove excess fluid in the form of urine. Disfunction kidney could loss these functions, which is a serious disease that damages many people's normal life and even be fatal. There are millions of people who are suffered from kideny disfunction. Dialysis is a renal replacement therapy that provides an artificial replacement for kidney dysfunction, and it is a life support treatment but not treat kidney diseases, which is based on the principle of the diffusion of solutes along a concentration gradient across a semipermeable membrane. In dialysis, blood passes on one side of a semipermeable membrane, and a dialysis fluid is passed on the other side. By altering the composition of the dialysis fluid, the concentrations of undesired solutes (potassium and urea, etc) in the fluid are low and desired solutes (such as sodium) are at their natural concentration as in healthy blood. The undesired solutes, i.e. waste, then diffuse across the membrane into

the dialysis fluid and are removed, and the desired solutes will be kept in the natural concentration. Dialysis may be used for very sick patients who have suddenly lost their kidney function or for quite stable patients who have permanently lost their kidney function. Dialysis treatments could play the functions to remove waste from the body, instead of the kidney's function. Dialysis lets the blood into the comparative normal condition for the renal dysfunctional patients. Bacterial kidney disease is a systemic disease that threatens the expansion of both cultured and wild salmonids worldwide. The progressive increase in the mean age of dialysis patients associated with increasing comorbidity factors such as the presence of cardiovascular disease and diabetes have significantly worsened patients' clinical status and tolerance to hemodialysis

Dialysis is based on the principle of the diffusion of solutes along a concentration gradient across a semipermeable membrane. In dialysis, blood passes on one side of a semipermeable membrane, and a dialysis fluid is passed on the other side. By altering the composition of the dialysis fluid, the concentrations of undesired solutes (potassium and urea, etc) in the fluid are low and desired solutes (such as sodium) are at their natural concentration as in healthy blood. The undesired solutes, i.e. waste, then diffuse across the membrane into the dialysis fluid and are removed, and the desired solutes will be kept in the natural concentration.

According to the techniques, there are three main types of dialysis: hemodialysis, peritoneal dialysis and hemofiltration.

(1) Hemodialysis

In hemodialysis, the patient's blood is passed through a tubing system to a

semipermeable membrane which has dialysis fluid running on the other side. Through dialysis, the cleansed blood is then returned through the circuit system back to the body. Ultrafiltration occurs by increasing the hydrostatic pressure of the blood in the dialysis circuit to cause water to cross the membrane down a pressure gradient. The dialysis process is very efficient, allowing the treatment to be undertaken intermittently, usually two or three times a week, about four hours each time. The dialysis is normally done in the hospital, even it can also be done in a patient's home as the home hemodialysis. When dialysis taken, the tubes are kept in patient, and the patients in the dialysis treatment will be a handicapped status. Also, dialysis is high cost.

In contrast to peritoneal dialysis, in which transport is between fairly static fluid compartments, hemodialysis relies on convective tranport and utilizes counter current flow, where the diasylate is flowing in the opposite direction to blood flow in the extracorporeal circuit. Counter-current exchanges maintain the concentration gradient across the membrane at a maximum and increase the efficiency of the dialysis. The efficiency of waste clearance during hemodialysis is much higher than in natural kidneys. Therefore, dialysis treatments do not have to be continuous and can be performed intermittently, typically two or three times per week, or less. Fluid removal (ultrafiltration) is achieved by altering the hydrostatic pressure of the dialysate compartment, causing free water to move across the membrane along a pressure gradient. The dialysis solution is a sterilized solution of mineral ions. Urea and other waste products, such as potassium and phosphate, diffuse into the dialysis solution. However, concentrations of most mineral ions (e.g. sodium) are similar to those of normal plasma to prevent loss (Yucha 2004).

A. Dialysis prescription - A prescription for dialysis by a physician will specify various parameters for setting up dialysis machines, such as time and duration of dialysis sessions. In the United States, 3-4 hours each time and 2-3 times per week are typical. There are also a small number of patients who undergo nocturnal dialysis for 8 hours per night 6 nights per week.

B. Side-effects and complications - Hemodialysis usually also involves the removal of extra fluid, because most patients with end- stage renal failure pass no urine. The sudden removal of fluid on dialysis may cause side effects, which are usually proportionate to the amount of fluid which is removed. These potential side effects include low blood pressure, fatigue, breeding, chest pains, leg-cramps and headaches. Hemodialysis may cause inflammation. Since hemodialysis requires access to the circulatory system, patients undergoing hemodialysis have a portal of entry for microbes, which could lead to septicemia or an infection affecting the heart valves (endocarditis) or bone (osteomyelitis). The risk of infection depends on the type of access used. Blood clotting in the tubing and dialyser is a frequent cause of complications until the routine use of anticoagulants. While anti-coagulants have improved outcomes, they can lead to uncontrolled bleeding. Occasionally, people have severe allergic reactions to anticoagulants. In this case dialysis is done without anticoagulation or the patient is switched to an alternate anticoagulant. Heparin is the most commonly used anticoagulant in hemodialysis patients, as it is generally well tolerated and can be quickly reversed with protamine. A common alternative to heparin is citrate, that is suitable fo the patients who are allergic to heparin.

C. Hemodialysis access - There are three primary modes of access to the blood in hemodialysis: an intravenous (IV) catheter, an arteriovenous (AV) Cimino fistula and synthetic graft. The type of access is influenced by factors such as the expected time course of a patient's renal failure and the condition of his/her vasculature. Patients may have multiple accesses, usually because an AV Cimino fistula or synthetic graft is maturing, and a catheter is still used.

D. Catheter - Catheter access, sometimes called a central venous catheter (CVC), consists of a plastic catheter with two lumens (or occasionally two separate catheters) which is inserted into a large vein (usually the vena cava, via the internal jugular vein or the femoral vein) to allow large flows of blood to be withdrawn from one lumen, to go into the dialysis circuit, and to be returned via the other lumen. However, the blood flow is almost always less than that of a well functioning fistula or graft. They are usually found in two general varieties, tunnelled and non-tunnelled.

Tunnelled catheter access involves a longer catheter, which is tunnelled under the skin from the point of insertion in the vein to an exit site some distance away. They are usually placed in the internal jugular vein in the neck and the exit site is usually on the chest wall. The tunnel acts as a barrier to invading microbes and as such tunnelled catheters are designed for short to medium term access, as infection is still a frequent problem.

Non-tunnelled catheter access is for short term access, up to about 10 days, but often for one dialysis session only, and the catheter emerges from the skin at the site of entry into the vein.

Aside from infection, venous stenosis is another serious problem with catheter access. The catheter is a foreign body in the vein, and often provokes an inflammatory reaction in the

vein wall, which results in scarring and narrowing of the vein, often to the point where it occludes. This can cause problems with severe venous congestion in the area drained by the vein and may also render the vein, and the veins drained by it, useless for the formation of a fistula or graft at a later date. Patients on longterm hemodialysis can literally 'run-out' of access, so this can be a fatal problem.

Catheter access is usually used for rapid access for immediate dialysis, for tunnelled access in patients who are deemed likely to recover from acute renal failure, and patients with end-stage renal failure, who are either waiting for alternative access to mature, or those who are unable to have alternative access. Catheter access is often popular with patients, as attachment to the dialysis machine doesn't require needles. However the serious risks of catheter access noted above mean that such access should only be contemplated as a long term solution in the most desperate access.

The hemodialysis machine performs the function of pumping the patient's blood and the dialysate through the dialyzer. The newest dialysis machines on the market are highly computerized and continuously monitor an array of safety-critical parameters, including blood and dialysate flow rates, blood pressure, heart rate, conductivity, pH, etc. If any reading is out of normal range, an audible alarm will sound to alert the nurse to se the patient condition.

E. Water system - An extensive water purification system is a basic equipment for hemodialysis. Since dialysis patients are exposed to vast quantities of water, which is mixed with the acid bath to form the dialysate, even trace mineral contaminants or bacterial endotoxins can filter into the patient's blood. Because the damaged kidneys are not able to perform their intended function of removing impurities, ions that are introduced into the blood

stream via water can build up to hazardous levels, causing numerous symptoms including death. For this reason, water used in hemodialysis is typically purified using reverse osmosis. It is also checked for the absence of chlorine ions and chloramines, and its conductivity is continuously monitored, to detect the level of ions in the water.

F. Dialyzer - The dialyzer, or artificial kidney, is the piece of equipment that actually filters the blood. One of the most popular types is the hollow fiber dialyzer, in which the blood is run through a bundle of very thin capillary-like tubes, and the dialysate is pumped in a chamber bathing the fibers. The process mimics the physiology of the glomerulus and the rest of the nephron. Pressure gradients are used to remove fluid from the blood. The membrane itself is often synthetic, made of a blend of polymers such as polyarylethersulfone and polyamide. Dialyzers come in many different sizes. A larger dialyzer will usually translate to an increased membrane area, and thus an increase in the amount of solutes removed from the patient's blood. Different types of dialyzers have different clearances for different solutes. I suggest that the dialyzer should be discarded after each treatment and not shared among patients.

G. Pre-dialysis - A dialysis machine should be available first. There are many models of dialysis machines, but typically in modern machines there will be a computer, CRT, a pump, and facility for disposable tubing and filters. The filters (the actual artificial kidneys) are cylindrical, clear plastic outside with the filter material visible inside. They are perhaps 15-18 inches long, and 2-3 inches thick. They have tubing connectors at both ends. The nurse will set up plumbing on the machine in a moderately complex pattern that has been worked out to move blood through the filter, allow for saline drip, allow for various other .

medications/chemicals to be administered. How the plumbing is set up may vary between models of machine and they types of filters.

The pump does not directly contact the blood or fluid in the plumbing — it works by applying pressure to the tubing, then moving that pressure point around. Think of a disk with a protrusion in it. Put this into a close fitting 270 degree enclosure. Put plastic tubing between the enclosure and the disk, entering and exiting in the 90 open degrees. Now imagine the disk turning. It will put pressure on the tubing, and the pressure point will roll around through the 270 degrees, forcing the fluid to move. It is characteristic of dialysis machines that most of the blood out of the patients body at any given time is visible. The patient arrives and is carefully weighed. Standing and sitting blood pressures are taken. Temperature is taken. Access is set up. For patients with a fistula this means inserting two large gauge needles into the fistula. This is painful and a local anaesthetic injection could be done. When access has been set up, the patient is then connected to the preconfigured plumbing, creating a complete loop through the pump and filter.

F.Dialysis - The pump and a timer are started. Hemodialysis is underway. Periodically (every half hour, nominally) blood pressure is taken. As a practical matter, fluid is also removed during dialysis. Most dialysis patients are on moderate to severe fluid restrictive diets, since kidney failure usually includes an inability to properly regulate fluid levels in the body. A session of hemodialysis may typically remove 2-5 kg of fluid from the patient. The amount of fluid to be removed is set by nurse according to the patient's estimated dry weight. This is a weight that the care staff believes represents what the patient should weight without fluid built up because of kidney failure. Removing this much fluid can cause or exacerbate low blood

pressure. Monitoring is intended to detect this before it becomes too severe. Low blood pressure can cause cramping, nausea, shakes, dizziness, lightheadedness, and unconsciousness. During dialysis, occasionally, patients have low blood pressure and lose consciousness. Often this is temporary and passes after the head is placed down for a short time.

G.Post-dialysis - At the end of the prescribed time, the patient is disconnected from the plumbing - blood lines. Needle wounds are bandaged with gauze, held for up to 1 hour with direct pressure to stop bleeding, and then taped in place. The process is similar to getting blood drawn, only it is lengthier, and more fluid or blood is lost. Temperature, standing and sitting blood pressure, and weight are all measured again. Temperature changes may indicate infection. BP discussed above. Weighing is to confirm the removal of the desired amount of fluid. Care staff verifies that the patient is in condition suitable for leaving. The patient must be able to stand, maintain a reasonable blood pressure, and be coherent. Different rules apply for in-patient treatment.

H.Post-dialysis washout. - Following haemodialysis, patients may experience a syndrome called "washout". The patient feels weak, tremulous, extreme fatigue. Patients report they "are too tired, too weak to converse, hold a book or even a newspaper." It may also vary in intensity ranging from whole body aching, stiffness in joints and other flu-like symptoms including headaches, nausea and loss of appetite. The syndrome may begin toward the end of treatment or minutes following the treatment. It may last 30 minutes or 12-14 hours in a dissipating form. Patients though exhausted have difficulty falling to sleep. Eating a light meal, rest and quiet help the patient cope with washout until it has 'worn away.

(2)Peritoneal dialysis - In peritoneal dialysis, a special solution is run through a tube into the peritoneal cavity, the abdominal body cavity around the intestine, where the peritoneal membrane acts as a semipermeable membrane. The fluid is left there for a period of time to absorb waste products, and then is removed through the tube. This is usually repeated a number of times during the day. Ultrafiltration occurs via osmosis in this case, as the dialysis solution is supplied in varying osmotic strengths to allow for some control over the amount of fluid to be removed. The dialysis process in this case is less efficient than hemodialysis and is carried out daily, but the ultrafiltration process is slower and gentler.

Peritoneal dialysis works on the principle that the peritoneal membrane that surrounds the intestine, can act as a natural semipermeable membrane, and that if a specially formulated dialysis fluid is instilled around the membrane then dialysis can occur, by diffusion. Excess fluid can also be removed by osmosis, by altering the concentration of glucose in the fluid.

Dialysis fluid is instilled via a peritoneal dialysis catheter, which is placed in the patient's abdomen, running from the peritoneum out to the surface, near the navel. This is done as a short surgery. Peritoneal dialysis is typically done in the patient's home and workplace, but can be done almost anywhere; a clean area to work, a way to elevate the bag of dialysis fluid and a method of warming the fluid are all that is needed. The main consideration is the potential for infection with a catheter; peritonitis is a commonest serious complication, and infections of the catheter exit site or tunnel are less serious but more frequent. Because of this, patients are advised to take a number of precautions against infection.

(3) Hemofiltration - Hemofiltration is a similar treatment to hemodialysis, but in this case, the membrane is far more porous and allows the passage of a much larger quantity of water and

solutes to pass across it. The fluid which passes across the membrane is discarded and the remaining blood in the circuit has its desired solutes and fluid volume replaced by the addition of a special hemofiltration fluid. It is a slow continuous therapy with sessions typically lasting 12-24 hours, usually daily. This, and the fact that ultrafiltration is very slow and thus gentle, makes it ideal for patients in intensive care units, where acute renal failure is common. A combination of hemofiltration and hemodialysis, called hemodiafiltration (incorporating a hemofilter to a standard hemodialysis circuit), is being used in some centres for chronic maintenance therapy.

Peritoneal dialysis (PD) has been used as a home dialysis therapy for renal replacement for more than 30 years. A high proportion of PD patients are overhydrated. Clinical assessment of dry weight in PD patients is difficult and further complicated by the paucity of signs and symptoms indicative of dehydration. Bioimpedance analysis technique has been considered as a potential tool to measure body fluid non-invasively, inexpensively and simply.

What does haemodialysis removes other than harmful wastes?	a) Protein	b) Salt	c) Insulin	d) Glycogen	**b) Salt**
Which part acts as a kidney in dialysis?	a) Dialyzer	b) Nephrolyzer	c) Kidneylyzer	d) Hemolyzer	**a) Dialyzer**
How many times a week dialysis must be done?	a) Every day	b) Once a week	c) Twice a day	d) Thrice a week	**d) Thrice a week**
Where is haemodialysis carried out?	a) Home	b) Hospital	c) Both dialysis cer	d) Dialysis centre	**c) Both dialysis centre and hospital**
What can be a side effect of haemodialysis?	a) Cramps	b) Weakness	c) Nausea	d) All of the mentioned	**d) All of the mentioned**
Which mineral must be consumed limitedly for a person undergoing dialysis?	a) K	b) Fe	c) Zn	d) Mo	**a) K**
Which of the following is a problem of peritoneal dialysis?	a) Nausea	b) Abdominal infecti	c) Insomnia	d) Respiration problems	**b) Abdominal infection**
What is the difference between diet for peritoneal dialysis and haemodialysis?	a) More calories	b) More proteins	c) More calcium	d) More carbohydrates	**b) More proteins**
What is the correct definition for dialysis?	a) Pumping of heart is e	b) Liver enzymes are	c) Waste materials	d) Stomach is implanted	**c) Waste materials are removed**
According to kidney dialysis, the space around the gut is called as:	a) Peritoneal cavity	b) Abdominal cavity	c) Vertebral cavity	d) Renal cavity	**a) Peritoneal cavity**
Hemodialysis rids your body of harmful wastes. What else does hemodialysis remove?	a. Extra protein	b. Extra sugar	c. Extra water	d. Extra insulin	**c. Extra water**
Peritoneal dialysis uses a _____ as the access for treatment.	a) Catheter	b) Fistula	c) Graft	d) Dialysis machine	**a) Catheter**
In hemodialysis (HD), blood is filtered through a _____.	a) Hemolyzer	b) Dialyzer	c) Cholesterol scre	d) Hemoglobin	**b) Dialyzer**
In peritoneal dialysis (PD), draining out the dirty fluid and putting in the clean fluid is call	a) A replacement	b) A hemo cleaning	c) An exchange	d) A dialysate	**c) An exchange**
A vascular access is a _____.	a) Piece of the dialysis n	b) Medicine taken by	c) Medical probler	d) Special blood vessel used for HD	**d) Special blood vessel used for HD**
Home HD is typically done by _____.	a) The patient and a par	b) The doctor and a r	c) Skilled nurses or	d) Dialysis technicians	**a) The patient and a partner**
Immunosuppressant drugs are taken _____.	a) With hemodialysis	b) With a kidney tra	c) With peritoneal	d) With major viruses	**b) With a kidney transplant**
Getting longer and/or more frequent HD treatments can:	a) Protect my heart	b) Allow fewer diet a	c) Give me more cı	d) All of the above	**d) All of the above**
Medical complications of peritoneal dialysis (PD) include	A) fluid overload.	B) protein loss.	C) glucose load.	D) all the above.	**D) all the above.**
Peritoneal dialysis replaces which of these functions of normal kidneys?	A) Metabolism	B) Active solute secr	C) Passive solute e	D) Hormone regulation	**C) Passive solute excretion**
Small, water-soluble drugs are more likely to be cleared from the blood of a PD patient v	A) a high-transport mer	B) a small-surface-ar	C) infrequent daily	D) long dialysate dwells.	**D) long dialysate dwells.**
The most common causative organism for PD peritonitis is	A) S. aureus.	B) S. epidermidis.	C) E. coli.	D) P. aeruginosa.	**B) S. epidermidis.**
The use of vancomycin in PD peritonitis	A) is endorsed for routir	B) produces adequat	C) does not increa	D) a and c only	**D) a and c only**
Intermittent IP antibiotics should be used with caution when	A) patients have residua	B) patients are funct	C) patients have p	D) a and c only	**D) a and c only**
Regarding intermittent versus continuous IP antibiotic therapy for PD peritonitis,	A) intermittent cefazolir	B) intermittent ther	C) intermittent am	D) a and c only	**D) a and c only**
Which of the following are markers of adequate peritoneal dialysis?	A) Kt/V > 2.1 per week	B) Weekly CrCl > 65	C) URR > 10 perce	D) a and b only	**D) a and b only**
In PD exit-site infections,	A) prophylactic IP vanco	B) topical or intrana	C) gram-negative I	D) a and b only	**B) topical or intranasal mupirocin is recommended for prophylaxis.**
With regard to IP heparin as an adjunctive therapy, which of the following is true?	A) It is intended to provi	B) It has direct actio	C) It may reduce o	D) It reduces the duration of treatment for exi	**C) It may reduce or prevent outflow obstruction.**
Appropriate intermittent IP antibiotic therapy should result in	A) sustained high dialys	B) dialysate concent	C) very low serum	D) a and b only.	**D) a and b only.**
Regarding the stability of drugs added to peritoneal dialysate,	A) assays measuring tot	B) chemical stability	C) combinations o	D) most single-antibiotic additives are stable f	**D) most single-antibiotic additives are stable for a week in a refrigerator.**

Kidney dialysis and kidney transplant are two treatments for	A. misbalance in glomerular filtrate	B. kidney failure	C. kidney stones	D. misbalance of osmoregulation	**B. kidney failure**
Causes of kidney failures are	A. hypertonic	B. diabetes	C. hypertension	D. both b and c	**D. both b and c**
Method of removal of kidney stones in which non-electrical shock waves are bombarded on stones is classified as	A. angioplasty	B. lithotripsy	C. endoscopy	D. angiography	**B. lithotripsy**
Considering kidney dialysis, space around gut is known as	A. peritoneal cavity	B. abdominal cavity	C. renal cavity	D. vertebral cavity	**A. peritoneal cavity**
Kind of dialysis in which blood of patient is pumped through dialyzer is classified as	A. hypotonic dialysis	B. peritoneal dialysis	C. abdominal dialysis	D. haemodialysis	**D. haemodialysis**

Concentration polarization can be reduced by	A) Pre filtering the solut	B) Reducing the flow	C) Back washing p	D) all of the above	**D) all of the above**
Cloth filter is generally made of	A) canvas	B) synthetic fabrics	C) metal or glass fi	D) all of these	**D) all of these**
The filtration involves the separation of large particles generally	A) dp>5µm	B)dp>10µm	C) dp>15µm	D) dp>20µm	**B)dp>10µm**
Continuous ambulatory peritoneal dialysis involves _____.	A)methods to allow regi	B)a anticoagulant to	C)an ambulance cr	D)placing the dialyzing fluid inside the periton	**D)placing the dialyzing fluid inside the peritoneal cavity to operate during normal activity**
Modern hemodialysis involves _____.	A)an all day process twi	B)a anticoagulant to	C)a complex nutrie	D)methods to allow regular hookup to the pat	**D)methods to allow regular hookup to the patient's bloodstream**