Semester – V 17MBU503A INSTRUMENTATION AND BIOTECHNIQUES
(4H – 4C)

Instruction Hours / week:L: 4 T: 0 P: 0 **Marks:** Internal: **40**External: **60** Total: **100**
End Semester Exam: 3 Hours

COURSE OBJECTIVES
To develop skills related to

- Understand the principles of various instruments used in the life sciences
- Ability to operate the instruments
- Data analysis and interpretations

COURSE OUTCOME

- offers the students with an opportunity to gain knowledge on the bioinstrumentation and concepts of principles and applications.

Unit I
Brightfield and darkfield microscopy, Fluorescence Microscopy, Phase contrast Microscopy, Confocal Microscopy, Electron Microscopy (Scanning and Transmission Electron Microscopy).

Unit II
Principles and applications of paper chromatography (including Descending and 2-D), Thin layer chromatography. Column Chromatography - packing types (IEC, AC, SEC), fraction collection. GLC and HPLC.

Unit III
Principle and applications of native polyacrylamide gel electrophoresis, SDS- polyacrylamide gel electrophoresis, 2D gel electrophoresis, Isoelectric focusing, Zymogram preparation and Agarose gel electrophoresis.

Unit IV
Principle, Instrumentation and application of spectrophotometer, colorimeter and turdibometer.MALDI-TOF, FTIR, MS, NMR.

Unit V
Filtration types-Micro and Ultra filtration, Principles ofcentrifugations – RCF and sedimentation coefficient. Types of centrifuges – rotors - fixed angle and swinging bucket rotors. Types of Centrifugation – differential, density gradient and ultracentrifugation. Analytical centrifugation.

SUGGESTED READINGS

1. Wilson K and Walker J. (2010). Principles and Techniques of Biochemistry and Molecular Biology. 7th Ed., Cambridge University Press.
2. Nelson DL and Cox MM. (2008). Lehninger Principles of Biochemistry, 5th Ed., W.H. Freeman and Company.
3. Willey MJ, Sherwood LM & Woolverton CJ. (2013). Prescott, Harley and Klein's Microbiology. 9th Ed., McGraw Hill.
4. Karp G. (2010) Cell and Molecular Biology: Concepts and Experiments. 6th edition. John Wiley & Sons. Inc.

5. De Robertis EDP and De Robertis EMF. (2006). Cell and Molecular Biology. 8th edition. Lipincott Williams and Wilkins, Philadelphia.
6. Cooper G.M. and Hausman R.E. (2009). The Cell: A Molecular Approach. 5th Edition. ASM Press & Sunderland, Washington D.C., Sinauer Associates, MA.
7. Nigam A and Ayyagari A. 2007. Lab Manual in Biochemistry, Immunology and Biotechnology. Tata McGraw Hill.

LECTURE PLAN-UNIT-1			
S.NO	Lecture duration hour	Topics	Supporting materials
1	1	Introduction to Microscopy	T1 1-16
2	1	Bright field microscopy	T1 19-20
3	1	Dark field microscopy	T1-21
4	1	Fluorescence microscopy	T1 25-26
5	1	Phase contrast microscopy	T1 22-25
6	1	confocal microscopy	R1 84-86
7	1	electron microscopy-Scanning	T1 30-32
8	1	Transmission electron microscopy	T1 32-34
9	1	Unit Revision	
Textbooks:		T1- Microbiology. Prescot, Harley & Klein.6th Edition	
Journals:		-	
Website:		-	
Reference books:		R1-Principles and techniues of biochemistry and molecular biology-7th edition	

LECTURE PLAN-UNIT-2			
S.NO	Lecture duration hour	Topics	Supporting materials
1	1	Introduction to Chromatography	T1 185
2	1	Principles and application of paper Chromatography	R2 433,T1-186-191
3	1	Thinlayer chromatography	T1 192-193
4	1	Coloumn chromatography	T1 193-196
5	1	Packing types	T1 203-215
6	1	Fraction collection	T1 196
7	1	GLC	T1 228-226
8	1	HPLC	T1 227-237
9	1	Unit Revision	
Textbooks:		T1- Bioinstrumentation-L.Veerakumari	
Journals:		-	
		-	
Website:			
Reference books:		R1-Principles and techniues of biochemistry and molecular biology-7th edition Wilson and walker	

LECTURE PLAN-UNIT-3			
S.NO	Lecture duration hour	Topics	Supporting materials
1	1	Principles and application of native gel electrophoresis	T1239-241
2	1	SDS page	T1243-254
3	1	2D Gel electrophoresis	T1 255-256
4	1	Isoelectric focusing	T 257
5	1	Zymogram preparation	T1 258-260
6	1	Agarose gel electrophoresis	T1 247
7	1	Unit Revision	
8	1	Unit Revision	
Textbooks:		T1- Bioinstrumentation-L.Veerakumari	
Journals:		-	
		-	
Website:			
Reference books:			

LECTURE PLAN-UNIT-4			
S.NO	Lecture duration hour	Topics	Supporting materials
1	1	Introduction to spectrophotometer	T1 306
2	1	Principleof spectrophotometer	T1 307-308
3	1	Application of spectrophotometer	T1 309
4	1	Colorimeter	T1 302-305
5	1	Turbidometer	T1 317-321
6	1	Unit Revision	R2 583-585, R2
7	1	Unit Revision	
Textbooks:		T1- Bioinstrumentation-L.Veerakumari	
Journals:		-	
Website:		-	
Reference books:			

LECTURE PLAN-UNIT-5			
S.NO	Lecture duration hour	Topics	Supporting materials
1	1	Introduction to centrifugation	T1 113
2	1	Principles of centrifugation	T1 113
3	1	Types of centrifugesRotor- fixed angle	T1 119-122
4	1	Swinging bucket rotors	T1 123-125
5	1	Types of centrifugation	T1 127
6	1	Gradient and ultracentifugation	T1 116-117
7	1	Analytical centrifugation	T1 128-130
8	1	Unit Revision 1	
9	1	Unit Revision 2	
Textbooks:		T1- Bioinstrumentation-L.Veerakumari	
Journals:		-	
		-	
Website:			
Reference book			

Syllabus: Brightfield and darkfield microscopy, Fluorescence Microscopy, Phase contrast Microscopy, Confocal Microscopy, Electron Microscopy (Scanning and Transmission Electron Microscopy).

Microbiology usually is concerned with organisms so small they cannot be seen distinctly with the unaided eye. Because of the nature of this discipline, the microscope is of crucial importance. Thus it is important to understand how the microscope works and the way in which specimens are prepared for examination. To understand how a light microscope operates, one must know something about the way in which lenses bend and focus light to form images. When a ray of light passes from one medium to another, **refraction** occur that is, the ray is bent at the interface. The **refractive index** is a measure of how greatly a substance slows the velocity of light, and the direction and magnitude of bending is determined by the refractive indexes of the two media forming the interface. When light passes from air into glass, a medium with a greater refractive index, it is slowed and bent toward the normal, a line perpendicular to the surface As light leaves glass and returns to air, a medium with a lower refractive index, it accelerates and is bent away from the normal.

Thus a prism bends light because glass has a different refractive index from air, and the light strikes its surface at an angle.Lenses act like a collection of prisms operating as a unit. When the light source is distant so that parallel rays of light strike the lens,a convex lens will focus these rays at a specific point, the **focal point** The distance between the center of the lens and the focal point is called the **focal length** Our eyes cannot focus on objects nearer than about 25 cm or 10 inches. This limitation may be overcome by using a convex lens as a simple magnifier (or microscope) and holding it close to an object. A magnifying glass provides a clear image at much closer range, and the object appears larger.Lens strength is related to focal length; a lens with a short focallength will magnify an object more than a weaker lens having a longer focal length.

The Light Microscope

Microbiologists currently employ a variety of light microscopes in their work; bright-field, dark-field, phase-contrast, and fluorescence microscopes are most commonly used. Modern microscopes are all compound microscopes. That is, the magnified image formed by the objective lens is further enlarged by one or more additional lenses.

The Bright-Field Microscope

The ordinary microscope is called a **bright-field microscope** because it forms a dark image against a brighter background. The microscope consists of a sturdy metal body or stand composed

Of a base and an arm to which the remaining parts are attached **2.3**). A light source, either a mirror or an electric illuminator, is located in the base. Two focusing knobs, the fine and coarse adjustment knobs, are located on the arm and can move either the stage or the nosepiece to focus the image.The stage is positioned about halfway up the arm and holds microscope slides by either simple slide clips or a mechanical stage clip. A mechanical stage allows the operator to move a slide around smoothly during viewing by use of stage control knobs.The **substage condenser** is mounted within or beneath the stage and focuses a cone of light on the slide. Its position often is fixed in simpler microscopes but can be adjusted vertically in more advanced models. The curved upper part of the arm holds the body assembly, to which a nosepiece and one or more **eyepieces** or **oculars** are attached.More advanced microscopes have eyepieces for both eyes and are called binocular microscopes. The body assembly itself contains a series of mirrors and prisms so that the barrel holding the eyepiece may be tilted for ease in viewing The nosepiece holds three to five **objectives** with lenses of differing magnifying power and can be rotated to position any objective beneath the body assembly. Ideally a microscope should be **parfocal**—that is, the image should remain in focus when objectives are changed.

The objective lens forms an enlarged real image within the microscope, and the eyepiece lens further magnifies this primary image. When one looks into a microscope, the enlarged specimen image, called the virtual image,appears to lie just beyond the stage about 25 cm away. The total magnification is calculated by multiplying the objective and eyepiece magnifications together. For example, if a 45Xobjective is used with a 10X eyepiece, the overall magnification of the specimen will be 450X

The Dark-Field Microscope

Living, unstained cells and organisms can be observed by simply changing the way in which they are illuminated. A hollow cone of light is focused on the specimen in such a way that unreflected and unrefracted rays do not enter the objective. Only light that has been reflected or refracted by the specimen forms an image .The field surrounding a specimen appears black, while the object itself is brightly illuminated because the background is dark, this type of microscopy is called **dark-field microscopy.**

Considerable internal structure is often visible in larger eucaryotic microorganisms . The dark-field microscope is used to identify bacteria like the thin and distinctively shaped *Treponema pallidum*

The Phase-Contrast Microscope

Unpigmented living cells are not clearly visible in the brightfield microscope because there is little difference in contrast between the cells and water. Thus microorganisms often must be fixed and stained before observation to increase contrast and create variations in color between cell structures. A **phase-contrast microscope** converts slight differences in refractive index and cell density into easily detected variations in light intensity and is an excellent way to observe living cell).The condenser of a phase-contrast microscope has an annular stop, an opaque disk with a thin transparent ring, which produces a hollow cone of light . As this cone passes through a cell, some light rays are bent due to variations in density and refractive index within the specimen and are retarded by about 14 wavelength. The deviated light is focused to form an image of the object. Undeviated light rays strike a phase ring in the phase plate, a special optical disk located in the objective, while the deviated rays miss the ring and pass through the rest of the plate. If the phase ring is constructed in such a way that the undeviated light passing through it is advanced by 14 wavelength, the deviated and undeviated waves will be about 1 2 wavelength out of phase and will cancel each other when they come together to form an image . The background, formed by undeviated light, is bright, while the unstained object appears dark and well-defined. This type of microscopy is called **dark-phase-contrast microscopy.**

Color filters often are used to improve the image.Phase-contrast microscopy is especially useful for studying microbial motility, determining the shape of living cells,and detecting bacterial components such as endospores and inclusion bodies that contain poly- -hydroxybutyrate, polymetaphosphate, sulfur, or other substances.These are clearly visible because they have refractive indexes markedly different from that of water. Phasecontrast microscopes also are widely used in studying eucaryotic cells.

The Differential Interference Contrast Microscope

The **differential interference contrast (DIC) microscope** is similar to the phase-contrast microscope in that it creates an image by detecting differences in refractive indices and thickness. Two beams of plane polarized light at right angles to each other are generated by prisms. In one design, the object beam passes through the specimen,while the reference beam passes through a clear area of the slide. After passing through the specimen, the two beams are combined and interfere with each other to form an

image. A live, unstained specimen appears brightly colored and three-dimensional Structures such as cell walls, endospores, granules,vacuoles, and eucaryotic nuclei are clearly visible.

The Fluorescence Microscope

The microscopes thus far considered produce an image from light that passes through a specimen. An object also can be seen because it actually emits light, and this is the basis of fluorescence microscopy. When some molecules absorb radiant energy, they become excited and later release much of their trapped energy as light. Any light emitted by an excited molecule will have a longer wavelength (or be of lower energy) than the radiation originally absorbed. **Fluorescent light** is emitted very quickly by the excited molecule as it gives up its trapped energy and returns to a more stable state.The **fluorescence microscope** exposes a specimen to ultraviolet, violet, or blue light and forms an image of the object with the resulting fluorescent light. A mercury vapor arc lamp or other source produces an intense beam, and heat transfer is limited by a special infrared filter. The light passes through an exciter filter that transmits only the desired wavelength. A darkfield condenser provides a black background against which the fluorescent objects glow. Usually the specimens have been stained with dye molecules, called **fluorochromes,** that fluoresce brightly upon exposure to light of a specific wavelength, but some microorganisms are autofluorescing. The microscope forms an image of the fluorochrome-labeled microorganisms. from the light emitted when they fluoresce. A barrier filter positioned after the objective lenses removes any remaining ultraviolet light, which could damage the viewer's eyes, or blue and violet light, which would reduce the image's contrast.The fluorescence microscope has become an essential tool in medical microbiology and microbial ecology. Bacterial pathogens (e.g., *Mycobacterium tuberculosis*, the cause of tuberculosis) can be identified after staining them with fluorochromes or specifically labeling them with fluorescent antibodies using immunofluorescence procedures. In ecologicalstudies the fluorescence microscope is used to observe microorganisms stained with fluorochrome-labeled probes or fluorochromes such as acridine orange and DAPI (diamidino-2-phenylindole, a DNA-specific stain). The

the danger of artifacts because the cells are frozen quickly rather than being subjected to chemical fixation, dehydration, andplastic embedding.

The Scanning Electron Microscope

The previously described microscopes form an image from radiation that has passed through a specimen. More recently the **scanning electron microscope (SEM)** has been used to examine the surfaces of microorganisms in great detail; many instruments have a resolution of 7 nm or less. The SEM differs from other electron microscopes in producing an image from electrons emitted by an object's surface rather than from transmitted electrons.Specimen preparation is easy, and in some cases air-dried material can be examined directly. Most often, however, microorganisms must first be fixed, dehydrated, and dried to preserve surface structure and prevent collapse of the cells when they are exposed to the SEM's high vacuum. Before viewing, dried samples are mounted and coated with a thin layer of metal to prevent the buildup of an electrical charge on the surface and to give a better image.The SEM scans a narrow, tapered electron beam back and forth over the specimen. When the beam strikes a particular area, surface atoms discharge a tiny shower of electrons called secondary electrons, and these are trapped by a special detector.Secondary electrons entering the detector strike a scintillator causing it to emit light flashes that a photomultiplier converts to an electrical current and amplifies. The signal is sent to a cathode-ray tube and produces an image like a television picture,which can be viewed or photographed. The number of secondary electrons reaching the detector depends on the nature of the specimen's surface. When the electron beam strikes a raised area, a large number of secondary electrons enter the detector; in contrast, fewer electrons escape a depression in the surface and reach the detector. Thus raised areas appear lighter on the screen and depressions are darker. A realistic three-dimensional image of the microorganism's surface with great depth of focus results The actual in situ location of microorganisms in ecological niches such as the human skin and the lining of the gut also can be examined.

Confocal Microscopy

A conventional light microscope, which uses a mixed wavelength light source and illuminates a large area of the specimen, will have a relatively great depth of field. Even if not in focus, images of bacteria from all levels within the field will be visible. These will include cells above, in, and below the plane of focus As a result the image can be murky, fuzzy, and crowded.The solution to this problem is the **confocal scanning laser microscope (CSLM)** or confocal microscope. Fluorescently stained

specimens are usually examined. A focused laser beam strikes a point in the specimen. Light from the illuminated spot is focused by an objective lens onto a plane above the objective. An aperture above the objective lens blocks out stray light from parts of the specimen that lie above and below the plane of focus. The laser is scanned over a plane in the specimen (beam scanning) or the stage is moved (stage scanning) and a detector measures the illumination from each point to produce an image of the optical section. When many optical sections are scanned, a computer can combine them to form a three-dimensional image from the digitized signals. This image can be measured and analyzed quantitatively.

The confocal microscope improves images in two ways.First, illumination of one spot at a time reduces interference from light scattering by the rest of the specimen. Second, the aperture above the objective lens blocks out stray light as previously mentioned.Consequently the image has excellent contrast and resolution.A depth of 1 _m or less in a thick preparation can be directly observed. Special computer software is used to create high-resolution, three-dimensional images of cell structures and complex specimens such as biofilms .

Scanning Probe Microscopy

Although light and electron microscopes have become quite sophisticated and reached an advanced state of development, powerful new microscopes are still being created. A new class of microscopes, called **scanning probe microscopes,** measure surface features by moving a sharp probe over the object's surface. The **scanning tunneling microscope,** invented in 1980, is an excellent example of a scanning probe microscope. It can achieve magnifications of 100 million and allow scientists to view atoms on the surface of a solid. The electrons surrounding surface atoms tunnel or project out from the surface boundary a very short distance. The scanning tunneling microscope has a needlelike probe with a point so sharp that often there is only one atom at its tip. The probe is lowered toward the specimen surface until its electron cloud just touches that of the surface atoms. If a small voltage is applied between the tip and specimen, electrons flow through a narrow channel in the electron clouds. This tunneling current, as it is called, is extraordinarily sensitive to distance and will decrease about a thousandfold if the probe is moved away from the surface by a distance equivalent to the diameter of an atom.The arrangement of atoms on the specimen surface is determined by moving the probe tip back and forth over the surface while keeping it at a constant height by adjusting the probe distance to maintain a steady tunneling current. As the tip moves up and down while following the surface contours, its motion is

recorded and analyzed by a computer to create an accurate three dimensional image of the surface atoms. The surface map can be displayed on a computer screen or plotted on paper. The resolution is so great that individual atoms are observed easily. The microscope's inventors, Gerd Binnig and Heinrich Rohrer, shared the 1986 Nobel Prize in Physics for their work, together with Ernst Ruska, the designer of the first transmission electron microscope.The scanning tunneling microscope will likely have a major impact in biology. Recently it has been used to directly view DNA. Since the microscope can examine objects when they are immersed in water, it may be particularly useful in studying biological molecules.

More recently a second type of scanning probe microscope has been developed. The **atomic force microscope** moves a sharp probe over the specimen surface while keeping the distance between the probe tip and the surface constant. It does this by exerting a very small amount of force on the tip, just enough to maintain a constant distance but not enough force to damage the surface. The vertical motion of the tip usually is followed by measuring the deflection of a laser beam that strikes the lever holding the probe. Unlike the scanning tunneling microscope, the atomic force microscope can be used to study surfaces that do not conduct electricity well. The atomic force microscope has been used to study the interactions between the *E. coli* GroES and GroEL chaperonin proteins, to map plasmids by locating restriction enzymes bound to specific sites, and to follow the behavior of living bacteria and other cells.

POSSIBLE QUESTIONS

UNIT-1

1.Define resolution

2.Define microscope

3.What is the principle of fluorescence microscopy

4.illustrate about electron microscopy

5.Explain about the of electron microscopy

6.Expand TEM and SEM.

7.Demonstrate theprinciple of confocal microscopy

8.What is magnification

9.Sketch on types of microscopy

SINO	QUESTIONS	Option 1	Option2	Option 3	Option 4	answer
1	When the power of occular lens is 10X and the objective lens is 20X the magnification is	20X	200X	2000X	20,000X	200X
2	Which of the following is suitable for maximum resolution	red	green	blue	orange	blue
3	All the followings are components of microscope except	Stage clip	electron gun	eye piece	fine adjustment	electron gun
4	which of the following is suitable for surface view of object	SEM	TEM	both a and b	compound microscope	SEM
5	The resolving power of a microscope can be increased by	Increasing the wavelength of light used for illumination	Decreasing the wavelength of light used for illumination	Decreasing the numerical aperture of the lens	both a and c	both a and c
6	The working distance is the shortest for which one of the following objectives	Scanning	low power	highpower	oil immersion	Scanning

7	The refractive index of air is	**1**	1.25	1.5	0.5	1
8	the resolving power of unaided human eye is	1cm	100um	500nm	400nm	100um
9	A detailed image of the specimen's surface features can be obtained using ---------- microscope.	Bright field	Dark feild	phasecontrast	scanning electron	Scanning electron
10	Which of the following stains is routinely used in electron microscopy specimen preparation?	Crystal violet	Acridine orange	Safranin	**Lead citrate**	**Lead citrate**
11	Which of the following is used to visualise live cells	SEM	TEM	phasecontrast	all of the above	phase contrast
12	the instrument used to draw the coloured magnifying sketches of object under microscope is	compound microscope	light microscope	camera lucida	camera attached stereomicroscope	camera lucida

13	which of the following about dark field microscopy	adding disc called stop to the condensor will make bright field to dark field	stop disc prevent the entry of light from the cental field and object is illuminated with beam of light	the lights get reflected on the sides of the specimen and appear bright on the dark background	all of the above	
14	the microscope examination is begin with the power objective	low	high	100X	none of the above	low
15	what must be done to increase the contast of the structure viewed	illuminated	thinly slliced	highly stained	placed under cover slip	illuminated
16	system consist of camera and video screen	illuminating	phase contast	viewing and recording	imaging	phase contrast
17	microscope that are ---------- will remain focused after the low power objective	monocular	parafocal	paracentred	properly adjusted	parafocal

	lens is changed to high power objective lens					
18	at high power objective the ----------knob is used to adjust on focussing the image	fine	coarse	diaphragm	none of the above	fine
19	objectives gives great depth of field is	100x	high	low	depend on the specimen stained	depend on the specimen stained
20	objectives gives great field of view is	100x	high	low	depend on the specimen stained	100x
21	When the power of occular lens is 10X and the objective lens is 100X the magnification is	10X	43X	430X	1000X	1000X
22	The field view of microscope is with a 10X ocular and a 4X objective is 5mm.the field of view of 10X objective is	3.14mm	2mm	20mm	0.2mm	2mm

23	which of the following specimen used in dissect cope	E.coli	human skin cells	insects mouth parts	none of the above	human skin cells
24	the substage condensor is	changes the wavelength of the light reaching the specimen	focuses light on the specimen	decreases the amount of light reaching the specimen	none of the above	focuses light on the specimen
25	A 40X objective and an 10X ocular produce a total magnification of	50	400	90	100	400
26	Living, unstained cells and organisms can be observed best using	fluorescent microscopy	phase contrast microscopy	SEM	TEM	phase contrast microscopy
27	Scanning electron microscopy (SEM) is best used to study	small cell structures.	surface morphology.	internal cells	all of the above	surface morphology.
28	A microscope in which an image is formed by passing an electron beam through a specimen and focusing the	TEM	SEM	phasecontrast	none of the above	TEM

	scattered electrons with magnetic lenses is called a					
29	Resolution is the ability of a lens to distinguish between small objects close together. What approximate resolution can be obtained with a lower power (10X, N.A. 0.25) objective lens?	0.2 microns	0.3 microns	0.25 microns	0.9 microns	0.9 microns
30	Fixation is the process by which the structures of the cells are preserved and fixed in position. An advantage of chemical fixation over heat fixation is that it	magnifies the specimen	not magnifies the specimen	does not destroy internal structures	none of the above	magnifies the specimen
31	Monochromatic (one color) light is sometimes used to increase the resolution of light microscopes. Light of which color below would give you the best resolution?					
32		red	blue	orange	green	blue

33	Which of the following objectives would give you the best resolution of small objects?	10x air, N.A. 0.25	40x air, N.A. 0.65	64x oil, N.A. 1.4	100x oil, N.A. 1.25	64x oil, N.A. 1.4
34	Transmission electron microscopy is best for high magnification viewing of	internal structure of fixed cells.	internal structure of live, motile cells.	surface structure of fixed cells.	surface membranes of live, motile cells.	internal structure of fixed cells.
35	Which of the following statements is most correct about Atomic Force Microscopy (AFM)?	AFM can visualize protein bound to DNA molecules.	AFM can visualize unfixed specimens in water or buffer.	AFM moves a very sharp tip over the surface of the specimen to "feel" its shape.	all of the above	all of the above

36	AFM moves a very sharp tip over the surface of the specimen to "feel" its shape.	The specimen must be stained with osmium or other heavy metal.	The specimens are placed in a high vacuum for viewing.	The specimens must be sliced very thin, 20-100 nm in thickness.	The beam is focused by electromagnetic lenses.	The specimen must be stained with osmium or other heavy metal.
37	Phase Contrast microscopy	Continuously changes the phase of the incident light from the condenser to improve contrast in the specimen.	Uses circular filters in the condenser and objective to give contrast to parts of the cell with different refractive indices.	Uses special lenses to distinguish between solid and liquid phases of the cell.	Uses special lenses to change the color of light passing through them.	Uses circular filters in the condenser and objective to give contrast to parts of the cell with different refractive indices.

38	Differential Interference Contrast microscopy	compares two identical specimens on the same microscope.	illuminates the specimen with light of two different colors.	illuminates the specimen with light of two different phases.	illuminates the specimen with both reflected and transmitted light	illuminates the specimen with light of two different phases.
39	Which of the following is NOT equivalent to 10 micrometers.	0.0001 cm	0.01 mm	10,000 nm	100,000 Angstroms	0.0001 cm
40	"Parfocal" refers to microscopes with multiple objectives where	objectives are used in pairs for stereoscopic effects.	each objective has the same working distance above the specimen.	each objective is positioned to be in focus at the same stage height.	sequential objectives increase power by a factor of two.	each objective is positioned to be in focus at the same stage height.
41	Working principle in a light microscope is using	UV light	visible light	spectrum light	white light	visible light
42	Resolution of electron microscope is	0.1nm	0.2nm	0.3nm	10nm	0.2nm

43	Magnification power of transmission electron microscope	250000	200000	450000	350000	250000
44	magnification power o flight microscope	1500X	1200X	1400X	1800X	1500X
45	A photograph which is taken from microscope is ----------	micrograph	diagraph	pictograph	none of the above	micrograph
46	What would you see using a dark-field microscope on bacteria that transmit light without reflecting it into the objective lens?	dark bacteria on dark background	bright bacteria on bright background	bright bacteria on dark background	flourescent bacteria on dark background	bright bacteria on bright background
47	If you wish to change an immunofluorescence stain so it stains a different type of microorganism than it did before, what would you do?	use a different type of microscope	switch from epiflourescence to transmitted flourescence	change the dye from flourescent to a non flourescent dye	use different type of antibody	use different type of antibody

48	SEM is used to reveal	surface structures	internal structure	either surface or internal structure	none of the above	internal structure
49	A microscope that exposes specimens to ultraviolet and forms an image with the resulting light emitted at a different wavelength is called a _____ microscope.	SEM	TEM	flourescence	phase contrast	flourescence
50	microscope which will produce image in visible light	brightfield	darkfield	flourescence	both b and c	both b and c
51	which microscope is usually good in unstained specimen	Phasecontrast	SEM	TEM	brightfield	phasecontrast

52	what do phase contrast and darkfield microscope in common	they increase contrast between specimen and their surroundings without staining	they make specimen flourescence	they make specimen bright in dark background	none of the above	they increase contrast between specimen and their surroundings without staining
53	Which of the following is defined as the ability to distinguish or separate two adjacent objects or points from one another in a microscopic specimen?	resolving power	image	illuminates the specimen with light of two different phases.	none of the above	resolving power
54	The compound light microscope can be used to observe:	aminoacids,bacteria,RBC	virus,bacteria and RBC	bacteria cell organelles,RBC	atoms,proteins,RBC	bacteria cell organelles,RBC
55	Two glass lenses are used in	compound microscope	light microscope	electron microscopr	simplemicroscope	light microscope

56	Object and lens is placed in the	vacuum chamber	electron gun	projector	none of the above	vacuum chamber
57	Lenses which enlarges image on photographic film are known as	electron microscope	electron gun	electromagnetic film	negative	electromagnetic film
58	Name of scientist who made first microscope is	James Watson	Zacharias Janssen	Leeuwenhoek	David Baltimore	Zacharias Janssen
59	eyepiece contains a	1X or 5X power lens	10X or 15X power lens	12X or 15X power lens	10X or 12X power lens	10X or 15X power lens
60	Working principle in a light microscope is using	UV light	visible light	spectrum light	white light	visible light
61	Microorganism first viewed in microscope by	Van Leeuwenhoek	Robert cook	Robert Hooke	Jefrey Raisman	Van Leeuwenhoek
62	Live cell can be viewed through---- microscope	Phase contrast	fluorescent	Electron microscope	Telescope	Phase contrast
63	Time-lapse video recording of cell movement done by using	Light microscope	SEM	Confocal microscope	TEM	Confocal microscope
64	Detailed image of the specimens surface features can be	Phase contrast	Bright field	SEM	TEM	SEM

	obtained using ----- microscope					
65	Which is not the parts of microscope?	Objective lens	Stage	Charger	Eye piece	Charger
66	To view fluorescent image, ----- are necessary	Exciter filter	flurophore	Mercury lamp	Exciter filter, flurophore, mercury lamp	Exciter filter, flurophore, mercury lamp

Unit II

Principles nd applications of paper chromatography (including Descending and 2-D), Thin layer chromatography. Column Chromatography - packing types (IEC, AC, SEC), fraction collection. GLC and HPLC.

Chromatography

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

BASIC PRINCIPLES

The basis of chromatography is to place substances to be separated into a system with two phases: a **mobile phase** and a **stationary phase**. Substances are then separated based upon their differential interaction with these two phases as the mobile phase moves across the stationary phase. In the case of liquid chromatography the mobile phase is a solvent. Molecules of interest (called the **solute**) are dissolved in the solvent and the solvent then flows across a solid matrix (i.e., the stationary phase). Solutes interact with the stationary phase by reversibly binding to the stationary phase. The strength of the binding between the solute and the stationary phase will determine how fast the solute is carried by the mobile phase. For example, substances which do not bind or interact with the solid phase will be carried unimpeded by the solvent. Whereas substances that interact with the solid phase will be temporarily retained. Therefore two substances that interact with the solid phase to different degrees can be separated from one another.

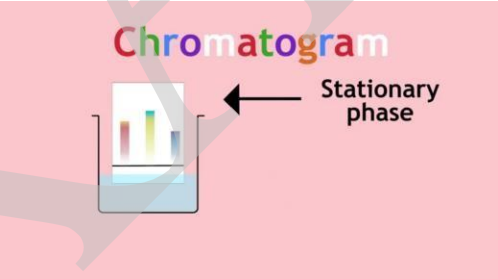
In most applications the mobile phase is liquid. The major exception is gas-liquid chromatography in which the mobile phase is a gas and the stationary phase is a liquid absorbed to a solid support. In liquid chromatography, the stationary phase can be in a column configuration or in a thin layer. The column is probably the most common way to hold the solid support and is especially convenient for preparative work such as the isolation of the solute. In particular, proteins are generally

isolated by column chromatography. The column is a cylinder or tube which holds the solid phase matrix and the liquid phase is passed through this column. There are several distinct types of solid phases used in the isolation and analysis of proteins (see Table). These various types of solid supports will separated proteins based upon different chemical and physical properties.

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

Introduction to paper chromatography

Paper chromatography is a chromatography technique used to separate mixture of chemical substances into its individual compounds. Paper chromatography is used to teach TLC or other chromatography as it is very similar to TLC.



Principles of paper chromatography

All chromatography follow the same principle. Paper Chromatography consists of two phases: one mobile phase and one contiguous stationery phase. The stationery phase a paper and the mobile gas is solvent. The compound mixture moves along with the mobile phase through stationery phase and separates depending on the different degree of adhesion (on the paper) of each component in the sample or the compound mixture.

Explanation

The stationery phase

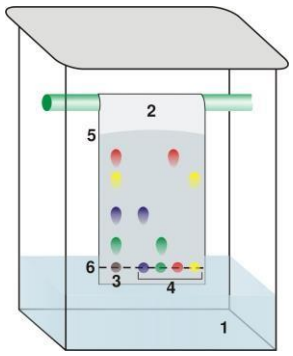
The paper chromatography is very similar to Thin layer chromatography. Difference is, instead of using a thin layer of silica on metal, it uses a special type of chromatography paper as stationery phase. This paper is made of cellulose. Cellulose is a polymer of simple sugar, glucose.

Cellulose contains -OH group similar to the silica or alumina on the TLC plate. The surface of cellulose is thus very polar. So the compounds can form hydrogen bond or can interact by van der waals dispersion forces and dipole dipole forces.

Process

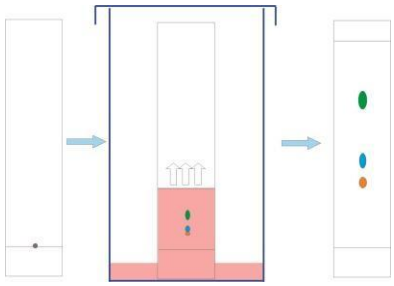
Paper chromatography works in few steps:

Step 1: A horizontal line is drawn near one end (about 1.5 cm from the bottom edge) of the paper. In figure below 6 is the horizontal line.



Step 2: The sample needs to be separated is placed as a small drop or line on to the paper using capillary tube. Labelling the drop by a pencil with an alphabet or number help to identify the compound later. In figure above 3 and 4 are the drops labelled. The drops are then soaked on the paper and dried.

Step 3: The paper is then placed into a sealed container with a swallow layer of suitable solvent. The solvent level must be lower than the pencil line or drop on it. The container need to be covered to stop the solvent to evaporate.

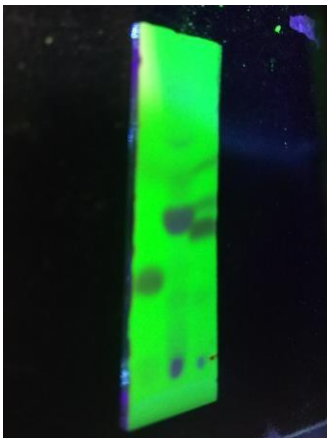


Step 4: The solvent rises up the paper chromatography taking each component of the sample with it. The components travel with the solvent depends on three things:

- The polarity of the sample molecule. The non polar components travel faster than the polar component.
- The attraction between the sample molecule and the solvent or solvent mixture.
- The attraction between the sample and the silica.

Suppose any sample compound mixture contains three colored molecules green, blue and red. According to their polarity, the order of these compounds is green<blue<red. Thus the most non polar green will travel first along with the mobile phase. Then blue and at last most polar compound the red one.

Step 5: When the solvent rises near the end of the paper then the paper should be taken out from sealed container and air dried. The paper with separated bands of components are then observed under UV-light.



R_f value

The compounds in the sample travels along with solvent to give separate bands on the paper. The distance travelled by same compound with respect to the solvent is always constant. Thus the ratio of the distance that the compound travelled and the distance that the solvent travelled is denoted as R_f. And mathematically expressed as:

Rf=distance/quadtravelled/quadby/quadcompounddistance/quadtravelled/quadby/quadsolvent

Summary

- Paper chromatography is an chromatography technique used to separate mixture of chemical substances into its individual compounds.
- Paper chromatography consists of two phases: one mobile phase and one contiguous stationery phase.
- Paper used in paper chromatography is made of cellulose.

- A suitable solvent (mobile phase) is moved along with a compound mixture through the paper according to the polarity and the degree of adhesion of each component on the stationery phase.
- The ratio of the distance that the compound travelled and the distance that the solvent travelled is denoted as R_f.

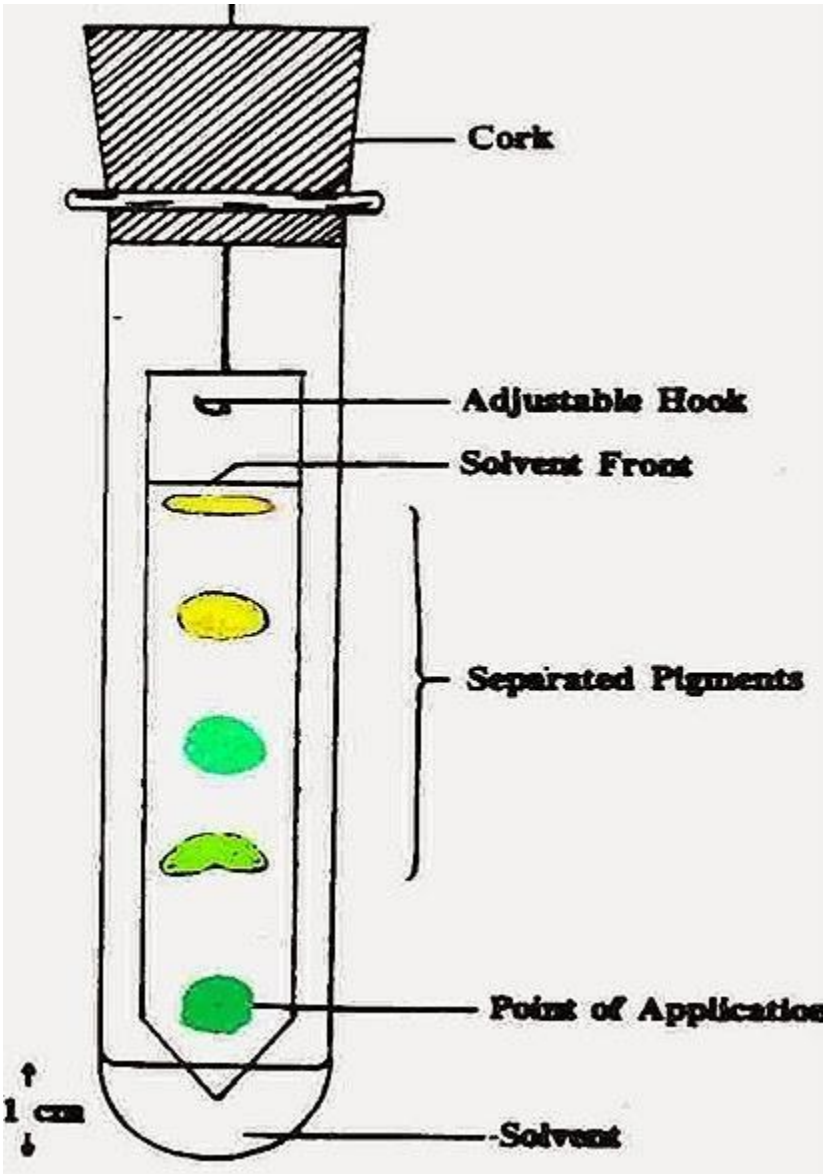
TYPES OF PAPER CHROMATOGRAPHY

Based on the moving direction of mobile phase the paper chromatography is classified into following types:

(I) ASCENDING PAPER CHROMATOGRAPHY
(II) DESCENDING PAPER CHROMATOGRAPHY
(III) HORIZONTAL PAPER CHROMATOGRAPHY OR RADIAL PAPER CHROMATOGRAPHY

i) Ascending paper chromatography: In this process, solvent is present in a dish in the base. The liquid moves toward upside of the paper by capillary movement against to gravitational pull.

Ascending Paper Chromatography Procedure: a filter paper is taken and a pencil line is drawn along the width of the paper above 5 cm from one end. This is called base line.



Then the solution mixture is employed on the center of the base line very densely. The filter paper is suspended in a glass tank containing the developing solvent so that the base line dips in the solvent to a depth of 2 cm. Due to capillary action the solvent moves up. The movement is fast in the beginning and it progressively slows down after few hours and lastly it stops. This is the solvent front.

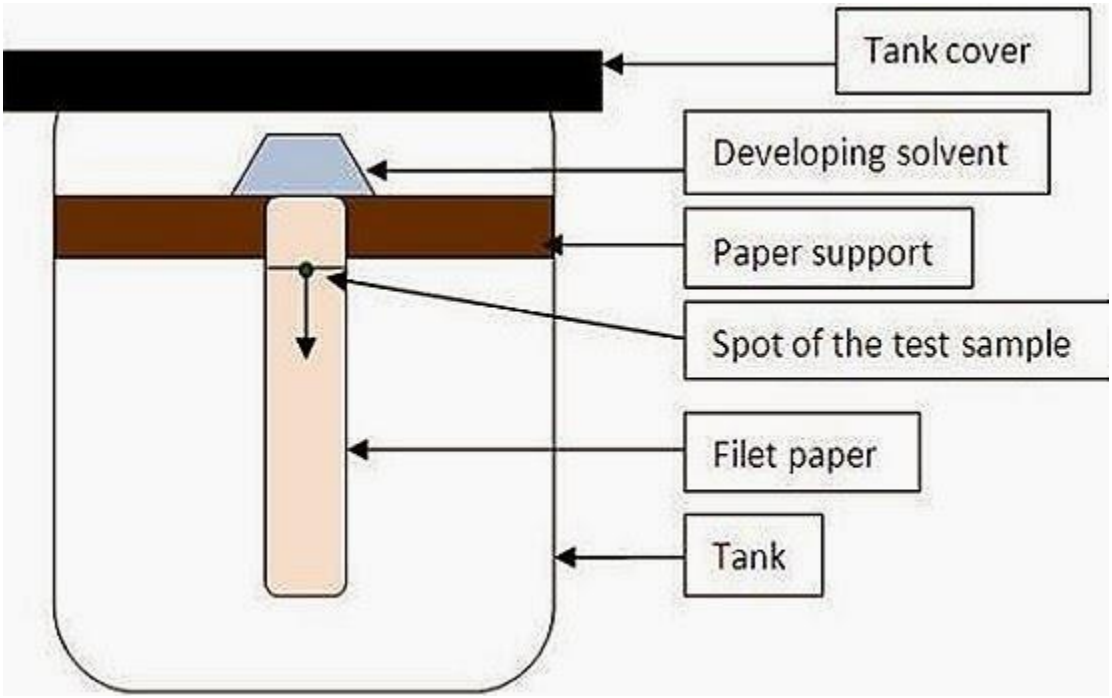
The movement stops totally due to a balance is formed between the capillary strength and downward gravitational pull.

The solvent front is marked with a pencil. Paper is removed from the tank without delay and dried. The paper now having different spots is called chromatogram. If the paper is placed in the tank for more time, the spots may diffuse on the developed Chromatogram.

Ascending chromatography Limitations: this procedure is not suitable for slow moving solvents. That means those have low RF values are not suitable for this method.

(II) DESCENDING PAPER CHROMATOGRAPHY: in this technique the solvent moves towards downside:

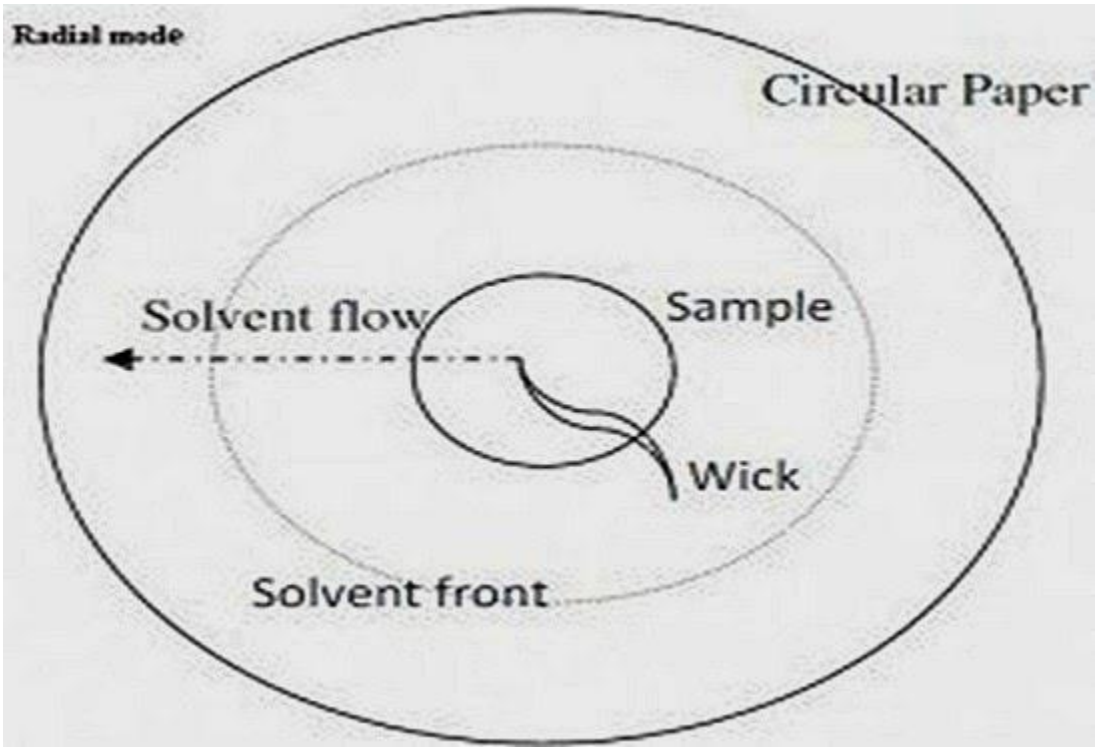
In this technique, the paper is dipped into the solvent (present in trough) near the top of the tank and a heavy glass rod maintains the paper in its alignment. The paper weighs on a other glass rod positioned horizontally parallel to the trough. The mixture of separation (dissolved in the solvent) is put on the paper at an outside spot (outside the trough) in a way that the solvent creates an uniformly moving front" prior to when it reaches the spot. In order to get even dripping and uniform movement, the base of the paper is cutting into a fitting edge.

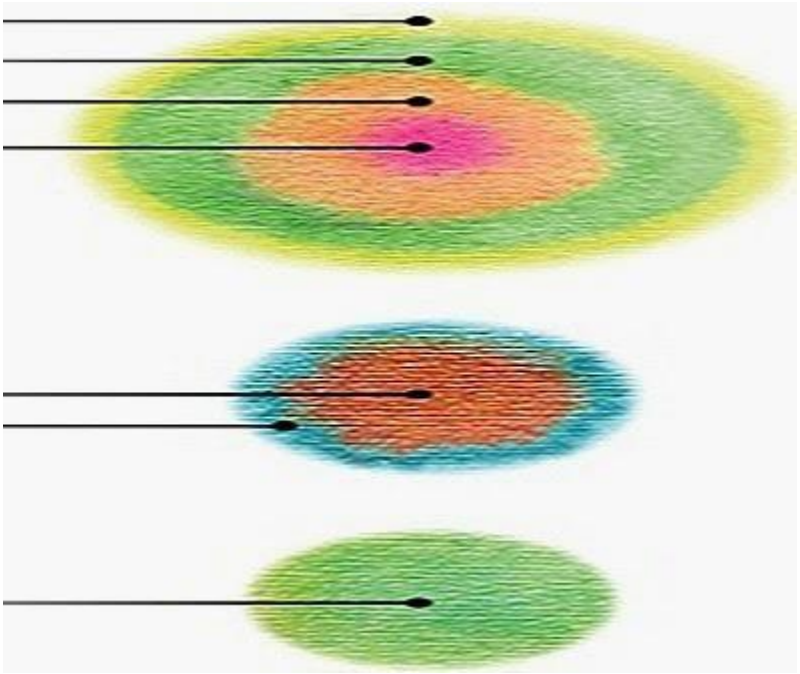


DESCENDING CHROMATOGRAPHY LIMITATIONS: Calculation of Rf value is not possible in this process. So standards are run simultaneously and the distances moved by various constituents of the mixture are in comparison.

(III) RADIAL CHROMATOGRAPHY OR HORIZONTAL CHROMATOGRAPHY: This technique is suitable for fast isolations. In this technique, the spot of the mixture is employed at the centre of a round paper held in the flat surface. The solvent in fed at the centre. As the solvent spreads by the

usual capillary force the components of the mixture are separated by radial development in the form of concentric arcs of circles.





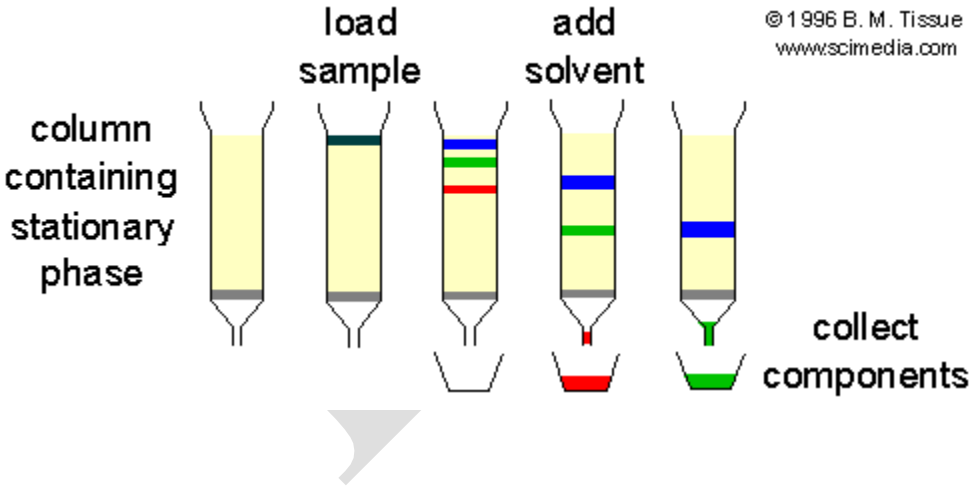
COLUMN CHROMATOGRAPHY

Invention of column chromatography a critical event in biochemistry, because it was the basis for development of procedures for obtaining pure proteins.

Different kinds of chromatographic separations based on one of the following:

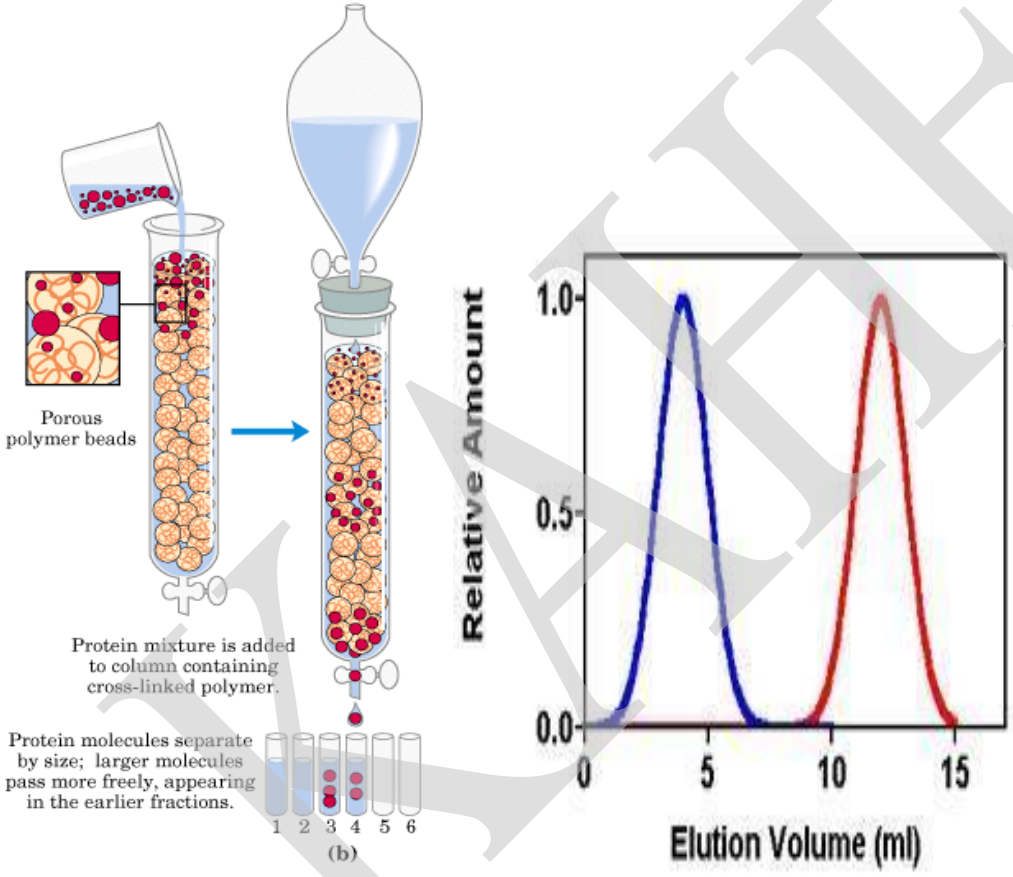
Size of protein (molecular sieve chromatography = gel filtration = size exclusion chromatography), or net charge of protein (ion exchange chromatography), or specific ligand binding properties of protein (affinity chromatograIn column chromatography a solid phase ("matrix", "resin", generally some kind of polymer, often a polysaccharide)(see below) is placed in a glass tube, the *column*.

Either due to molecular size differences or different binding affinities for column matrix, some proteins are retained longer on the column (e.g., some bind more tightly than others), and so elute later. Binding properties obviously depend on what type of stationary phase (column matrix) is used by repeating this procedure with several different adsorbents, pure protein can be obtained.



adsorbent: solid material/matrix, a "stationary phase" that some molecules *bind to* (*adsorb to*)

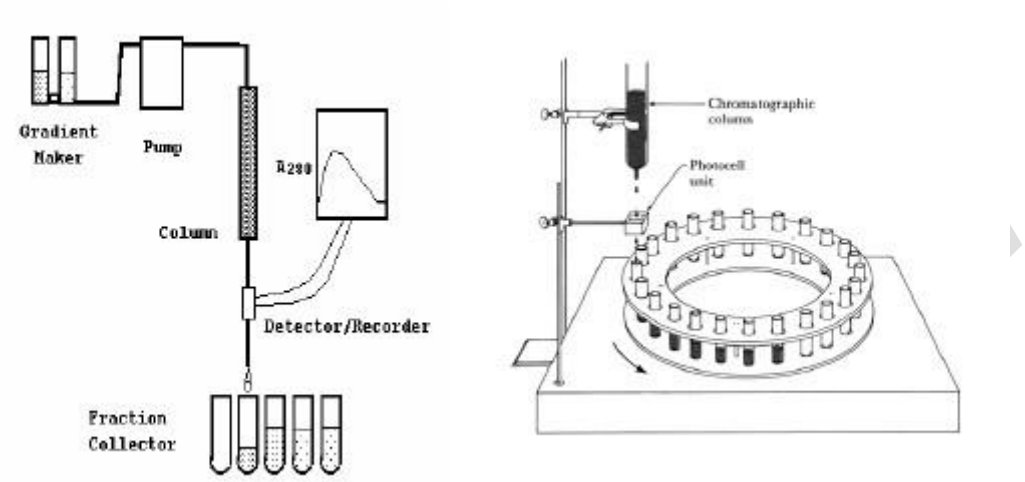
elution: the process of washing something off an adsorbent (with an ***eluting buffer***; the solution coming off the column is the ***eluate***.) Protein mixture is passed into the column.



EQUIPMENT

The equipment needed for column chromatography can range from expensive workstations to pasteur pipettes. The central component is the **column**. A **pump** may be needed to control the flow rate of buffers through the column. However, gravity can also be used. The solvent used in the mobile phase

will often need to gradually change. This gradual change is accomplished by a **gradient maker**. The elution of substances can be monitored during chromatography with an in line spectrophotometer which measures the absorbance of material coming off the column. In the case of protein chromatography the **detector/recorder** monitors the A280. Detectors to record radioactivity or fluorescence during chromatography are also available.



If chromatography is used as part of a protein purification scheme a **fraction collector** is needed. A fraction collector is a device that will automatically collect the liquid flowing from the column in separate tubes. Most fraction collectors will allow fractions to be collected per unit time or per unit volume (i.e., number of drops). In either case, the end result is a series of tubes containing approximately equal volumes. The tubes can then be evaluated for the substances of interest. The amounts of the substances being measured are often plotted against either the fraction number, volume or time. These three variables can be easily inconverted from the flow rate and volume of the individual fractions. Some fraction collectors can also be interfaced with the detector/recorder and programmed to collect only the peaks.

Calibrate the column:

Determine elution volumes of proteins with known molecular weights construct a **calibration curve** relating(known) molecular weight to (measured) elution volume *specifically for **that** column.* Such a calibration curve can then be used to estimate the molecular weight of an unknown protein.

AFFINITY

GEL FILTRATION CHROMATOGRAPHY ("Molecular Sieve" chromatography, or "Size Exclusion" chromatography)

Stationary phase (column matrix) = "beads" of a polysaccharide material that separates proteins based on **size** and **shape**.

Different column packing materials (hydrated, porous beads of carbohydrate polymer (e.g. dextran or agarose) or polyacrylamide) available, with wide range of molecular exclusion limits, for separating proteins of all sizes.

Solution of mixture of proteins, small molecules, etc. "filters" through the beads:

Large molecules can't get into the smaller pores in the beads and move more rapidly through the column, emerging (eluting) sooner.

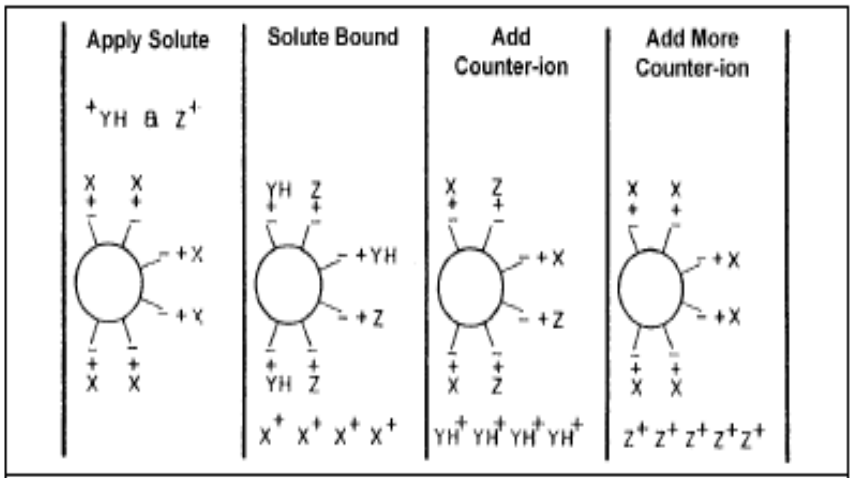
Smaller molecules and ions can enter all the pores in the beads with the buffer, and thus have more space to "explore" on their way down the column, and elute later.

For any particular column dimensions and material, **volume of buffer required to elute a specific protein** depends mostly on **molecular weight of the protein** (but shape plays an important role also -- separation is *really* based on differences in ***hydrodynamic volume***). Thus, one can separate proteins by **size**.

ION EXCHANGE CHROMATOGRAPHY

Ion exchange resins have charged groups covalently attached to the stationary phase (adsorbent, matrix), either positive or negative. Obviously, if ionizable groups are *weak* acids or bases, the **pH** of the buffer determines the charge state of the matrix.

Proteins bind to the matrix by electrostatic interactions.



Basic Principal of IEC. A solution containing the solutes of interest (^{+}YH & Z^{+}) is applied to the ion exchanger. The solutes bind to the exchanger and displace the counter-ions (X^{+}) already bound to the column. The solute is removed from the stationary phase by increasing the concentration of counter-ions or by changing the pH to affect the charge of the solute. Weakly charged solutes (^{+}YH) will be displaced from the stationary phase with lower concentrations of counter-ions than more highly charged solutes (Z^{+}). This results in separation of solutes based upon their net charges

Strength of these interactions depends on net charge on the protein (a function of buffer pH and the nature of the ionizable groups on that protein, reflected in the pI of the protein), and salt concentration of the buffer (high salt concentrations reduce the interaction and can be used to *elute* the proteins by competing with the protein groups for binding to the charged groups on the matrix).

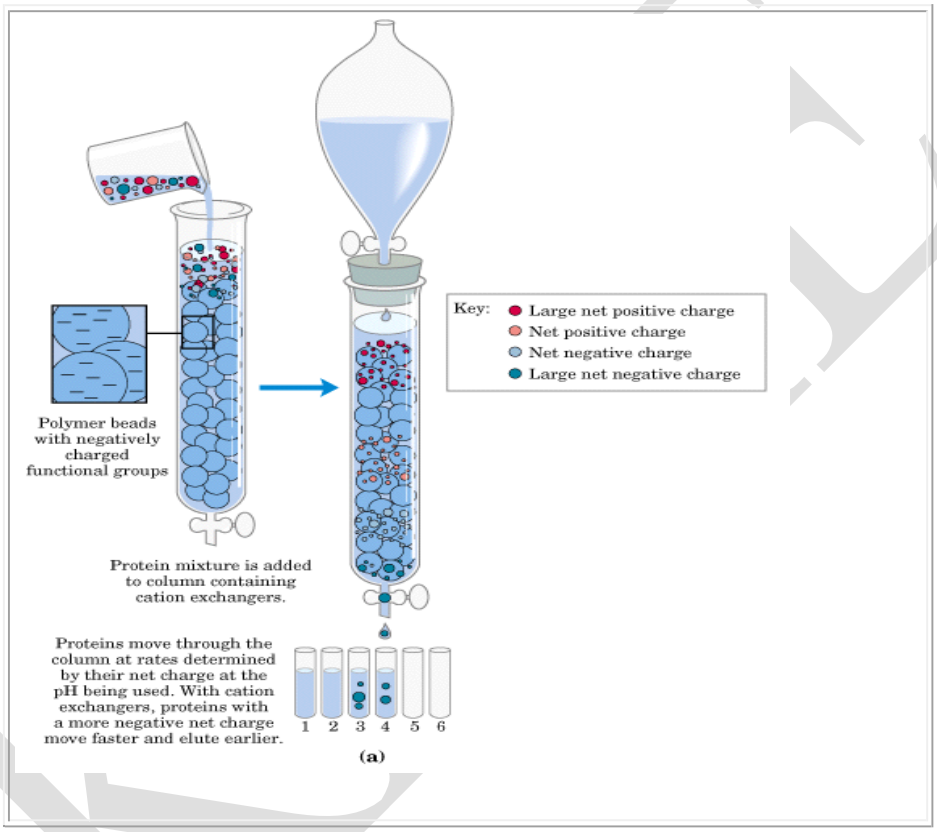
The higher the net charge on the protein *at the pH of the environment on the column*, the more tightly it sticks to an oppositely charged matrix, and the higher the salt concentration required to elute it from the column.

The further the "working pH" is from the isoelectric point (pI) of a protein, the greater the net charge on the protein, and the more tightly it will stick to an ion exchanger of opposite charge.

By proper choice of eluting buffer (often a *gradient* with *increasing salt concentation*, or changing the pH), specific proteins can be eluted from the column and separated from other proteins in the mixture.

Example in figure is *cation exchange chromatography* -- column packing beads have covalently attached negatively charged groups.

Negatively charged solutes move down the column more or less without sticking, so they elute first. Positively charged solutes bind, and the higher the positive charge on a molecule, the tighter it binds, so the later it elutes.



HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The acronym *HPLC*, coined by the late Prof. Csaba Horváth for his 1970 Pittcon paper, originally indicated the fact that high pressure was used to generate the flow required for liquid chromatography in packed columns. In the beginning, pumps only had a pressure capability of 500 psi [35 bar]. This was called *high pressure* liquid chromatography, or HPLC. The early 1970s saw a tremendous leap in technology. These new HPLC instruments could develop up to 6,000 psi [400 bar] of pressure, and incorporated improved injectors, detectors, and columns. HPLC really began to take hold in the mid-to

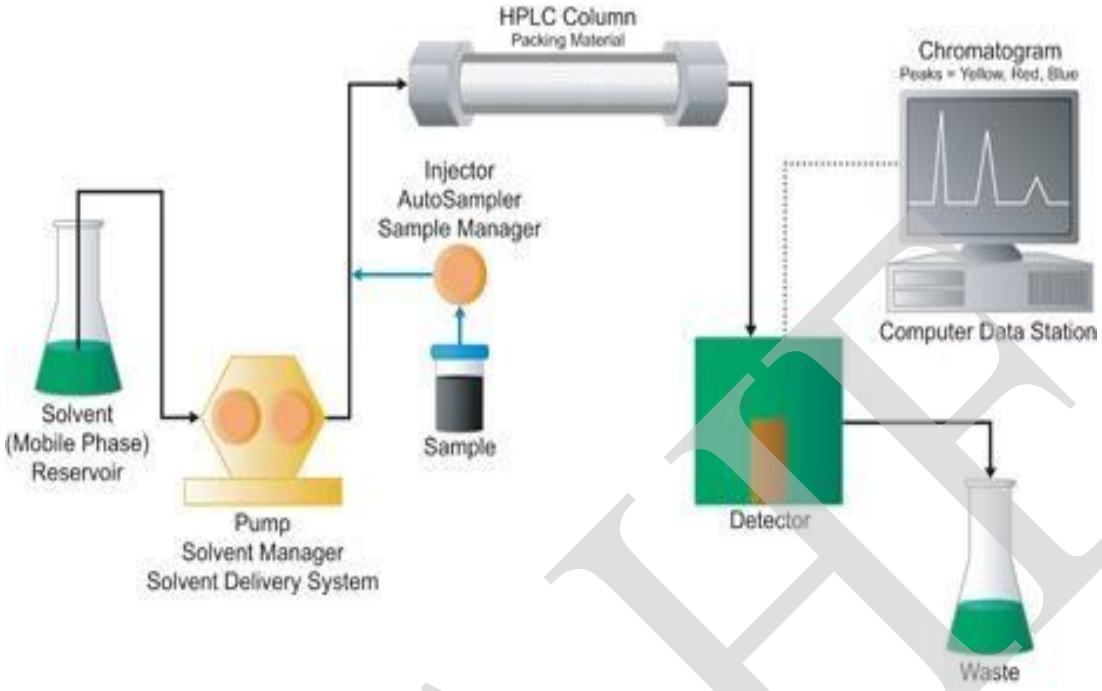
late-1970s. With continued advances in performance during this time [smaller particles, even higher pressure], the acronym HPLC remained the same, but the name was changed to *high performance* liquid chromatography.

High performance liquid chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantitate the compounds that are present in any sample that can be dissolved in a liquid. Today, compounds in trace concentrations as low as *parts per trillion* [ppt] may easily be identified. HPLC can be, and has been, applied to just about any sample, such as pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples, and industrial chemicals.

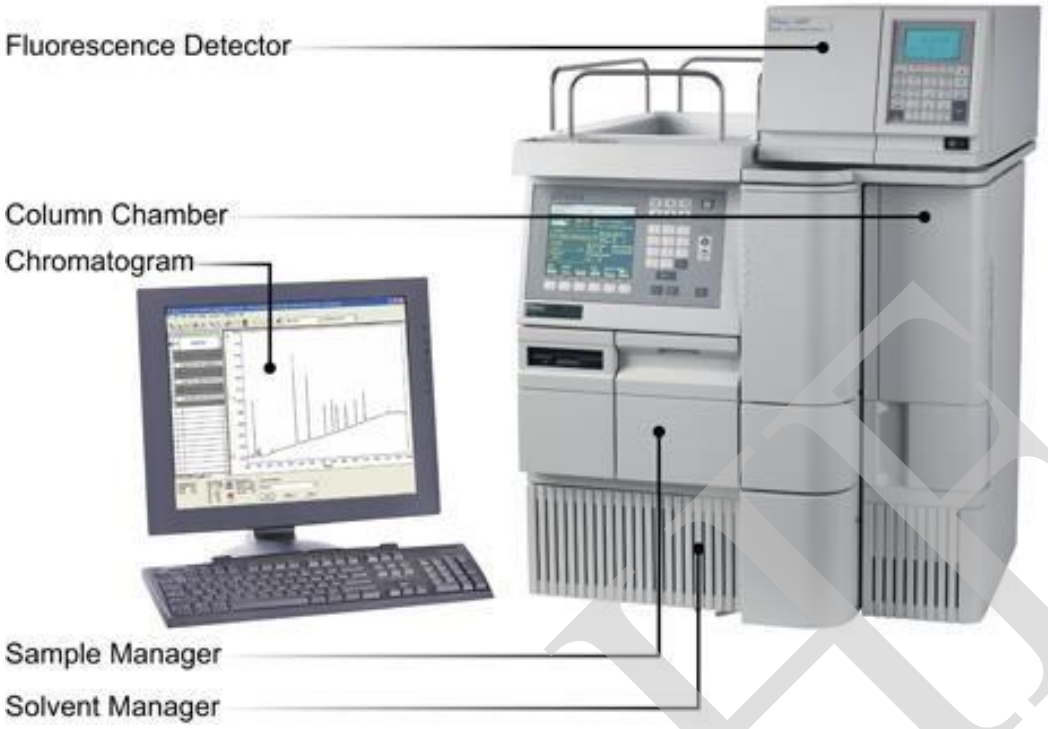
HPLC Column

The components of a basic high-performance liquid chromatography [HPLC] system are shown in the simple diagram in Figure . A reservoir holds the solvent [called the mobile phase, because it moves]. A high-pressure pump [solvent delivery system or solvent manager] is used to generate and meter a specified flow rate of mobile phase, typically milliliters per minute. An injector [sample manager or autosampler] is able to introduce [inject] the sample into the continuously flowing mobile phase stream that carries the sample into the HPLC column. The column contains the chromatographic packing material needed to effect the separation. This packing material is called the stationary phase because it is held in place by the column hardware. A detector is needed to *see* the separated compound bands as they elute from the HPLC column [most compounds have no color, so we cannot see them with our eyes]. The mobile phase exits the detector and can be sent to waste, or collected, as desired. When the mobile phase contains a separated compound band, HPLC provides the ability to collect this fraction of the elute containing that purified compound for further study. This is called preparative chromatography .

Note that high-pressure tubing and fittings are used to interconnect the pump, injector, column, and detector components to form the conduit for the mobile phase, sample, and separated compound bands.



The detector is wired to the computer data station, the HPLC system component that records the electrical signal needed to generate the chromatogram on its display and to identify and quantitate the concentration of the sample constituents. Since sample compound characteristics can be very different, several types of detectors have been developed. For example, if a compound can absorb ultraviolet light, a UV-absorbance detector is used. If the compound fluoresces, a fluorescence detector is used. If the compound does not have either of these characteristics, a more universal type of detector is used, such as an evaporative-light-scattering detector [ELSD]. The most powerful approach is the use multiple detectors in series. For example, a UV and/or ELSD detector may be used in combination with a mass spectrometer [MS] to analyze the results of the chromatographic separation. This provides, from a single injection, more comprehensive information about an analyte. The practice of coupling a mass spectrometer to an HPLC system is called LC/MS.



Mobile phase enters the column from the left, passes through the particle bed, and exits at the right. Flow direction is represented by green arrows. First, consider the top image; it represents the column at time zero [the moment of injection], when the sample enters the column and begins to form a band. The sample shown here, a mixture of yellow, red, and blue dyes, appears at the inlet of the column as a single black band. [In reality, this sample could be anything that can be dissolved in a solvent; typically the compounds would be colorless and the column wall opaque, so we would need a detector to see the separated compounds as they elute.]

After a few minutes [lower image], during which mobile phase flows continuously and steadily past the packing material particles, we can see that the individual dyes have moved in separate bands at different speeds. This is because there is a competition between the mobile phase and the stationary phase for attracting each of the dyes or analytes. Notice that the yellow dye band moves the fastest and is about to exit the column. The yellow dye likes [is attracted to] the mobile phase more than the other dyes. Therefore, it moves at a *faster* speed, closer to that of the mobile phase. The blue dye band likes the packing material more than the mobile phase. Its stronger attraction to the particles causes it to move significantly *slower*. In other words, it is the most retained compound in this sample mixture. The red dye

band has an intermediate attraction for the mobile phase and therefore moves at an *intermediate* speed through the column. Since each dye band moves at different speed, we are able to separate it chromatographically.

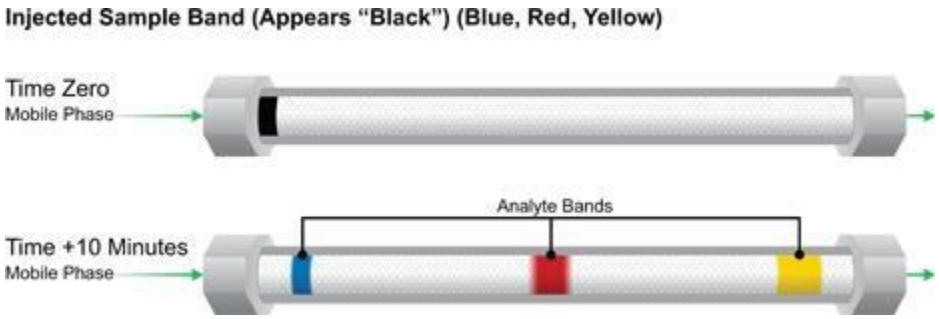


Figure : Understanding How a Chromatographic Column Works – Bands

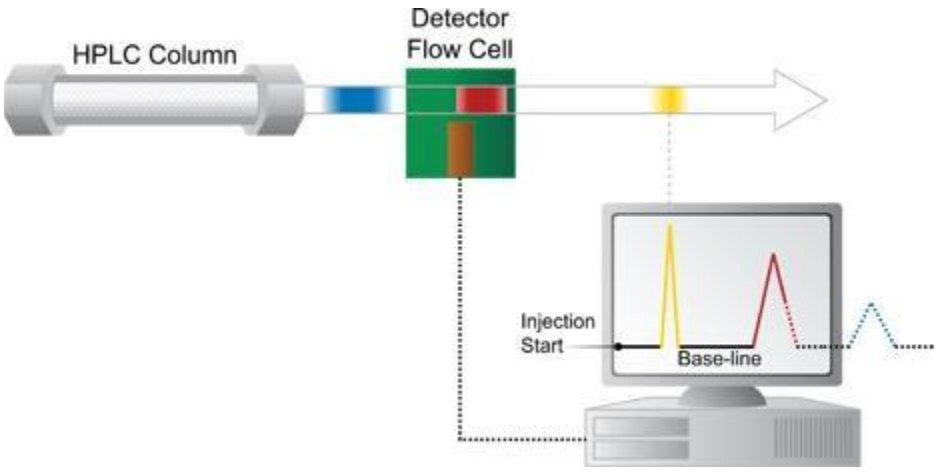
Detector

As the separated dye bands leave the column, they pass immediately into the detector. The detector contains a flow cell that *sees* [detects] each separated compound band against a background of mobile phase [see Figure H]. [In reality, solutions of many compounds at typical HPLC analytical concentrations are colorless.] An appropriate detector has the ability to sense the presence of a compound and send its corresponding electrical signal to a computer data station. A choice is made among many different types of detectors, depending upon the characteristics and concentrations of the compounds that need to be separated and analyzed, as discussed earlier.

Chromatogram

A chromatogram is a representation of the separation that has chemically [chromatographically] occurred in the HPLC system. A series of peaks rising from a baseline is drawn on a time axis. Each peak represents the detector response for a different compound. The chromatogram is plotted by the computer data station.

Peaks created



The yellow band has completely passed through the detector flow cell; the electrical signal generated has been sent to the computer data station. The resulting chromatogram has begun to appear on screen. Note that the chromatogram begins when the sample was first injected and starts as a straight line set near the bottom of the screen. This is called the baseline; it represents pure mobile phase passing through the flow cell over time. As the yellow analyte band passes through the flow cell, a stronger signal is sent to the computer. The line curves, first upward, and then downward, in proportion to the concentration of the yellow dye in the sample band. This creates a peak in the chromatogram. After the yellow band passes completely out of the detector cell, the signal level returns to the baseline; the flow cell now has, once again, only pure mobile phase in it. Since the yellow band moves fastest, eluting first from the column, it is the first peak drawn.

A little while later, the red band reaches the flow cell. The signal rises up from the baseline as the red band first enters the cell, and the peak representing the red band begins to be drawn. In this diagram, the red band has not fully passed through the flow cell. The diagram shows what the red band and red peak would look like if we stopped the process at this moment. Since most of the red band has passed through the cell, most of the peak has been drawn, as shown by the solid line. If we could restart, the red

band would completely pass through the flow cell and the red peak would be completed [dotted line]. The blue band, the most strongly retained, travels at the slowest rate and elutes after the red band. The dotted line shows you how the completed chromatogram would appear if we had let the run continue to its conclusion. It is interesting to note that the width of the blue peak will be the broadest because the width of the blue analyte band, while narrowest on the column, becomes the widest as it elutes from the column. This is because it moves more slowly through the chromatographic packing material bed and requires more time [and mobile phase volume] to be eluted completely. Since mobile phase is continuously flowing at a fixed rate, this means that the blue band widens and is more dilute. Since the detector responds in proportion to the concentration of the band, the blue peak is lower in height, but larger in width.

Identifying and Quantitating Compounds

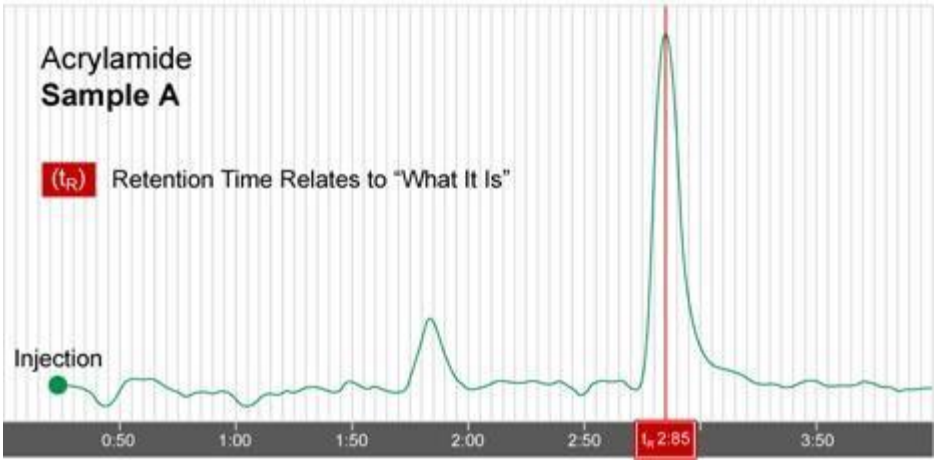
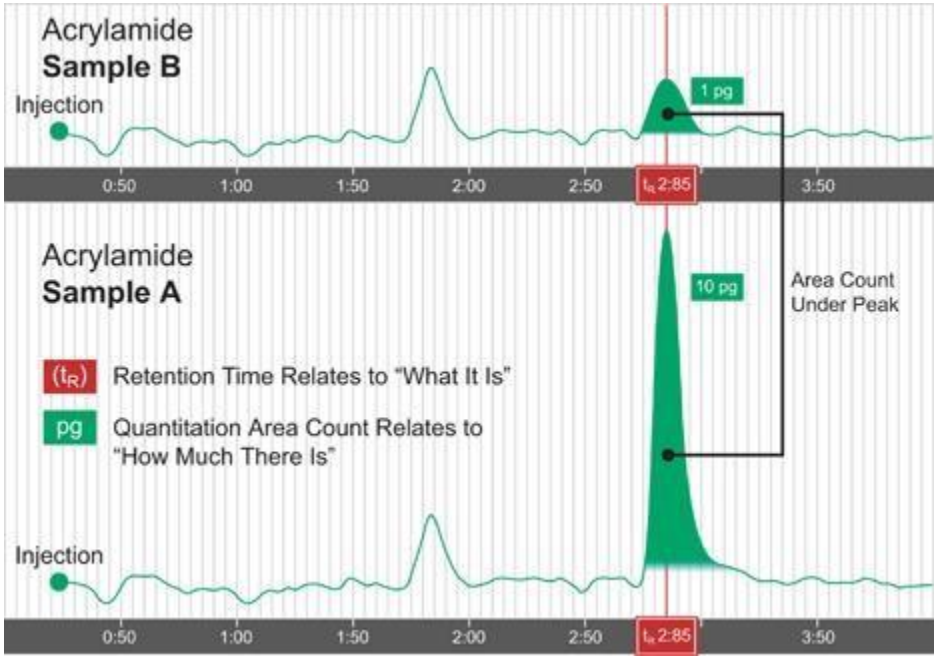


Figure: Identification

In Figure , three dye compounds are represented by three peaks separated in time in the chromatogram. Each elutes at a specific location, measured by the elapsed time between the moment of injection [time zero] and the time when the peak maximum elutes. By comparing each peak's retention time [tR] with that of injected reference standards in the same chromatographic system [same mobile and stationary phase], a chromatographer may be able to identify each compound.

In the chromatogram shown in Figure, the chromatographer knew that, under these LC system conditions, the analyte, acrylamide, would be separated and elute from the column at 2.85 minutes [retention time]. Whenever a new sample, which happened to contain acrylamide, was injected into the LC system under the same conditions, a peak would be present at 2.85 minutes.

Once identity is established, the next piece of important information is how much of each compound was present in the sample. The chromatogram and the related data from the detector help us calculate the concentration of each compound. The detector basically responds to the concentration of the compound band as it passes through the flow cell. The more concentrated it is, the stronger the signal; this is seen as a greater peak height above the baseline.



Identification and Quantitation

Chromatograms for Samples A and B, on the same time scale, are stacked one above the other. The same volume of sample was injected in both runs. Both chromatograms display a peak at a retention time [t_R] of 2.85 minutes, indicating that each sample contains acrylamide. However, Sample A displays a much bigger peak for acrylamide. The area under a peak [peak area count] is a measure of the

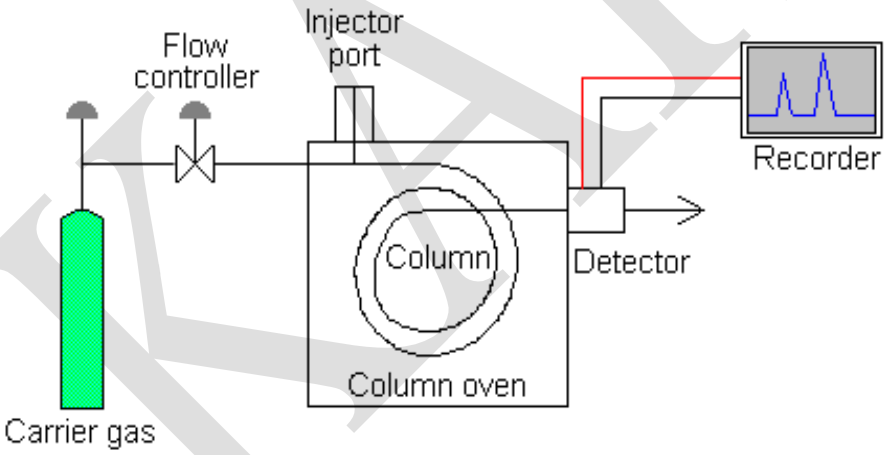
concentration of the compound it represents. This area value is integrated and calculated automatically by the computer data station. In this example, the peak for acrylamide in Sample A has 10 times the area of that for Sample B. Using reference standards, it can be determined that Sample A contains 10 picograms of acrylamide, which is ten times the amount in Sample B [1 picogram]. Note there is another peak [not identified] that elutes at 1.8 minutes in both samples. Since the area counts for this peak in both samples are about the same, this unknown compound may have the same concentration in both samples.

GAS CHROMATOGRAPHY

Principle

Gas Chromatography is a technique for separating chemical substances that relies on differences in partitioning behaviour between a flowing mobile phase and a stationary phase to separate the components in a mixture. The sample is carried by a moving gas stream through a tube packed with a finely divided solid or may be coated with a film of a liquid.

Diagram



Instrumentation

First, introducing the test mixture or sample into a stream of an inert gas, commonly helium or argon, that acts as carrier. Liquid samples are vaporized before injection into the carrier stream. The gas stream is passed through the packed column, through which the components of the sample move at velocities that are influenced by the degree of interaction of each constituent with the stationary nonvolatile phase. The substances having the greater interaction with the stationary phase are retarded to a greater extent and consequently separate from those with smaller interaction. As the components elute from the column they can be quantified by a detector and/or collected for further analysis.

Types

Two types of gas chromatography are encountered:

- gas-solid chromatography (GSC) and
- gas-liquid chromatography (GLC).

Gas-solid chromatography is based upon a solid stationary phase on which retention of analytes is the consequence of physical adsorption. Gas-liquid chromatography is useful for separating ions or molecules that are dissolved in a solvent. If the sample solution is in contact with a second solid or liquid phase, the different solutes will interact with the other phase to differing degrees due to differences in adsorption, ion-exchange, partitioning or size. These differences allow the mixture components to be separated from each other by using these differences to determine the transit time of the solutes through a column.

The major applications are:

1. **Pharmaceuticals**
   - Residual solvents in intermediate and finished products
   - Related substances and volatile impurities
   - Drug assays
2. **Foods and Beverages**
   - Residual pesticide, herbicides etc.
   - Food adulteration

- Analysis of Fragrances and flavors
- Fatty acids profiling of fats and oils
- Fatty Acids Methyl Esters (FAME) analysis

3. Environmental Analysis

- Ambient air and stack emissions monitoring
- Analysis of Volatile components in waste water
- Residual pesticide in agricultural produce, vegetables, fruits, soils and drinking water
- Analysis of dioxins, polynuclear aromatic hydrocarbons, polychlorinated biphenyls (PCB's), dibenzofurans, etc.

POSSIBLE QUESTIONS

UNIT-II

1. **Define chromatography.**
2. **Write the principles of chromatography**
3. **What is retention factor.**
4. **Write the types of Chromatography.**
5. **Sketch a note on TLC**
6. **Expand HPLC.**
7. **Sketch a note on ion exchange chromatography**
8. **What is SEC?**
9. **Expand AC and GLC**

Unit-2						
SNO	**Question**	**Option 1**	**Option2**	**Option 3**	**Option 4**	**answer**
1	Purification of proteins can be done by.....Chromatography.	SEC	ion-exchange	paper	thin layer	Ion exchange chromatography
2	Series of symmetric peaks in chromatography is	spectrum	chromatogram	elution volume	retention time	chromatogram
3	The Rf value is always	more than 1	more than 2	less than 1	less than 2	less than 1
4	Silica gel is the stationary phase in	GLC	PAGE	HPLC	TLC	TLC
5	The stationary phase used in TLC is	silica gel	cellulose	agarose	polyacrylamide	silica gel
6	Stationary phase used in paper chromatography is	silica gel	filter paper	polyacrylamide	agarose	filter paper
7	Forces involved in paper chromatography is	van der Waals forces	capillary forces	disulphide bridges	hydrogenbonds	capillary forces
8	Stationary phase used in TLC is for separation of plant pigments is	silica gel	cellulose	Kieselguhr G	polyacrylamide	Kieselguhr G
9	Solvent system of amino acids in paper chromatography is	acetic acid	petroleum ether	hexane, water	butanol, acetic acid, water	butanol, acetic acid, water
10	Molecules with higher solubility will migrate to	greater than Rf	lesser than Rf	equal to RF	none of the above	greater than Rf
11	Usually low moleculalr weight compounds are separated using …….. chromatography	adsoprtion	partition	column	thin layer	partition
12	Impurities present in paper are removed by washin with	1 N HCl	0.1 N HCl	0.01 N HCl	0.001N HCl	0.001N HCl
13	In paper chromatography, amino acids are viewed in purple or blue by spraying	bromine water	ninhydrin	methanol	ethanol	ninhydrin

14	The stationary phase used in column chromatography is known as	filter paper	gradient	gel matrix	none of the above	gel matrix
15	Gel matrix cellulose has ……. units.	β-1,4 linked galactose	β-1,4 linked fucose	β-1,4 linked arabinose	β-1,4 linked glucose	β-1,4 linked glucose
16	Gel matrix dextran has ……. units.	α-1,6 linked gluc ose	α-1,6 linked galact ose	α-1,6 linked fuc ose	α-1,6 linked arabinose	α-1,6 linked gluc ose
17	Gel matrix agarose has ……. units.	D-glucose	D- galact ose	L-fucose	L-arabinose	D- galact ose
18	The stationary phase silica is made up of	acetic acid	sulphuric acid	orthosilicic acid	hydrochloric acid	orthosilicic acid
19	The peaks obtained during column chromatography is	electrophoretogra m	EEG	ECG	chromatogram	chromatogram
20	Ion exchange chromatography is as process based on	oppositely charged particles	neutrally charged	only positive	only negative	oppositely charged particles
21	When a gel matrix exchanges positive ions, it is called as	anion exchanger	cation exchanger	matrix without charges	all the above	cation exchanger
22	Example of strong cationic exchanger is ……..	cellulose	epoxyamine	polystyrene	all the above	polystyrene
23	Removal of sample from solid matrix using solvent is ……...	effluent	bed volume	retention	elution	elution
24	In chromatography, volume of mobile phase is	void volume	bed volume	retention	elution	void volume
25	Time taken for each material to emerge from coumn is ……	void volume	bed volume	retention time	elution	retention time
26	Column development using single solvent as mobile phase is ……	void volume	bed volume	retention time	isocratic elution	isocratic elution
27	Column chromatography involves ……. phenomenon.	2	3	4	5	4
28	Adsorption chromatography was	Sorensen	D.T. Day	Richard	Edwin	D.T. Day

	developed by …….					
29	Adsorption chromatography is used mainly for separation of ……..	soil samples	clinical samples	plant pigments	animal cells	plant pigments
30	Scientit who used adsorption chromatography for separating plant pigment is …….	Sorensen	M.S.Tswett	Richard	Edwin	M.S.Tswett
31	Powdered charcoal can be prepared using ……..	nuts	coal	tar	coconut	coconut
32	Fuller's earth is mixture of ……. from clay deposits	minerals	vitamins	gel matrix	none of the above	minerals
33	Hydroxyapatite is …….	KCL	MgCl2	calcium phosphate	sodium chloride	calcium phosphate
34	Adsorption chromatography is used for separation of ……	proteins	chlorides	calcium	geometrical isomers	geometrical isomers
35	Commerical name of strong cationic exchanger is ……	Sephadex AG 50	AG 3	Bio-Rex 70	AG 1	Sephadex AG 50
36	Commerical name of weak cationic exchanger is ……	Sephadex AG 50	AG 3	Bio-Rex 70	AG 1	Bio-Rex 70
37	Commerical name of strong anionic exchanger is ……	Sephadex AG 50	AG 3	Bio-Rex 70	QAE-Sephadex	QAE-Sephadex
38	Commerical name of weak anionic exchanger is …….	Sephadex AG 50	AG 3	Bio-Rex 70	QAE-Sephadex	AG 3
39	To separate metallic ions ……. exchangers are used.	cationic	anionic	resin	none of the above	resin
40	Exchangers used for separation of proteins / polysaccharides is	CM-Sephadex	Dowex 50	both 1 & 2	cellulose	cellulose
41	Resin used to prepare deionized water is …….	QAE-Sephadex	Mixed-bed resin	AG 1	Bio-Rex 70	Mixed-bed resin
42	Matrix used in affinity chromatography is ……...	Bio-Rex 70	QAE-Sephadex	Bio-Gel P	Dowex 50	Bio-Gel P

43	Matrix used in affinity chromatography is ……...	Bio-Rex 70	QAE-Sephadex	AG 1	Sepharose	Sepharose
44	Matrix used in affinity chromatography is ……...	Sephacryl S	Bio-Rex 70	QAE-Sephadex	Dowex 50	Sephacryl S
45	in affinity chromatography, gel is linked with arms called ……..	matrix	ligands	gel	compounds	ligands
46	For isolation of lipoprotein …… serves as ligand.	NAD	NADP	heparin	avidin	heparin
47	For isolation of immunoglobulins …… serves as ligand.	NAD	NADP	heparin	Protein A & G	Protein A & G
48	For isolation of biotin containing enzymes..... serves as ligand.	avidin	heparin	NADPH	Protein A & G	avidin
49	For isolation of coagulation factors …… serves as ligand.	heparin	cibacron blue	NADPH	Protein A & G	cibacron blue
50	High Performance Liquid Chromatography is …….	TLC	HPTLC	GLC	HPLC	HPLC
51	_____pump is cost effective for use in HPLC.	displacement pump	isocratic pump	pneumatic pump	filter	pneumatic pump
52	Ratio between the distance travelled by solute and solvent in a stationary phase is called_____.	Rf	RT	K	Rt	
53	Chromatography was discovered by _____.	Louis	Johnson	Michael	Mikhail Tswett	
54	The carrier gas used as mobile phase in chromatography is _____.	oxygen	nitrogen	helium	carbondioxie	
55	The material used to make preparative column chromatography is _____.	glass	plastic	polyurethane	steel	

56	_____used to make heating element of the column in gas chromatography.	iron	copper	aluminium	boxite	
57	Adsoption of liquid sample to a semisolid stationary phase is used in _____chromatography	HPLC	TLC	Paper	LPLC	
58	Adsoption of solid sample to a solid stationary phase with ionic surface is used in _____chromatography.	HPLC	TLC	Paper	LPLC	HPLC
59	Which is polar solvent?	Benzene	Hexane	Ethanol	chloroform	Ethanol
60	Which is non-polar solvent ?	Ethanol	Methanol	Benzene	water	Benzene
61	In size exclusion chromatography, ---- size particle elute first	Small	Medium	Bigger	Tiny	Bigger
62	In gas-liquid chromatography (GLC), …….. is used as inert gas	Sodium	Potassium	Nitrogen	calcium	Nitrogen

Unit III

Principle and applications of native polyacrylamide gel electrophoresis, SDS- polyacrylamide gel electrophoresis, 2D gel electrophoresis, Isoelectric focusing, Zymogram preparation and Agarose gel electrophoresis.

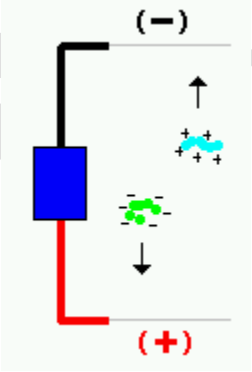
ELECTROPHORESIS

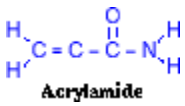
Gel electrophoresis

Electrophoresis is 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.

When charged molecules are placed in an electric field, they migrate toward either the positive or negative pole according to their charge. In contrast to proteins, which can have either a net positive or net negative charge, nucleic acids have a consistent negative charge imparted by their phosphate backbone, and migrate toward the anode.

Proteins and nucleic acids are electrophoresed within a matrix or "gel". Most commonly, the gel is cast in the shape of a thin slab, with wells for loading the sample. The gel is immersed within an electrophoresis buffer that provides ions to carry a current and some type of buffer to maintain the pH at a relatively constant value.





The gel itself is composed of either agarose or polyacrylamide, each of which have attributes suitable to particular tasks:

Agarose is a polysaccharide extracted from seaweed. It is typically used at concentrations of 0.5 to 2%. The higher the agarose concentration the "stiffer" the gel. Agarose gels are extremely easy to prepare: you simply mix agarose powder with buffer solution, melt it by heating, and pour the gel. It is also non-toxic.

Agarose gels have a large range of separation, but relatively low resolving power. By varying the concentration of agarose, fragments of DNA from about 200 to 50,000 bp can be separated using standard electrophoretic techniques.

Polyacrylamide is a cross-linked polymer of acrylamide. The length of the polymer chains is dictated by the concentration of acrylamide used, which is typically between 3.5 and 20%. Polyacrylamide gels are significantly more annoying to prepare than agarose gels. Because oxygen inhibits the polymerization process, they must be poured between glass plates (or cylinders).

Acrylamide is a potent neurotoxin and should be handled with care. Wear disposable gloves when handling solutions of acrylamide, and a mask when weighing out powder. Polyacrylamide is considered to be non-toxic, but polyacrylamide gels should also be handled with gloves due to the possible presence of free acrylamide.

Polyacrylamide gels have a rather small range of separation, but very high resolving power. In the case of DNA, polyacrylamide is used for separating fragments of less than about 500 bp. However, under appropriate conditions, fragments of DNA differing is length by a single base pair are easily resolved. In contrast to agarose, polyacrylamide gels are used extensively for separating and characterizing mixtures of proteins.

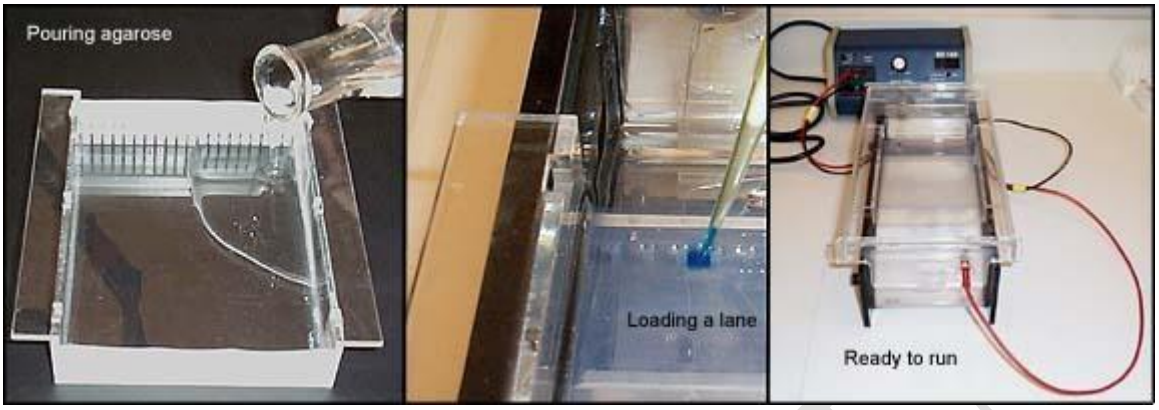
Preparing and Running Standard Agarose DNA Gels

The equipment and supplies necessary for conducting agarose gel electrophoresis are relatively simple and include:

- An electrophoresis chamber and power supply
- Gel casting trays, which are available in a variety of sizes and composed of UV-transparent plastic. The open ends of the trays are closed with tape while the gel is being cast, then removed prior to electrophoresis.
- Sample combs, around which molten agarose is poured to form sample wells in the gel.
- Electrophoresis buffer, usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE).
- Loading buffer, which contains something dense (e.g. glycerol) to allow the sample to "fall" into the sample wells, and one or two tracking dyes, which migrate in the gel and allow visual monitoring or how far the electrophoresis has proceeded.
- Ethidium bromide, a fluorescent dye used for staining nucleic acids. NOTE: Ethidium bromide is a known mutagen and should be handled as a hazardous chemical - wear gloves while handling.
- Transilluminator (an ultraviolet lightbox), which is used to visualize ethidium bromide-stained DNA in gels. NOTE: always wear protective eyewear when observing DNA on a transilluminator to prevent damage to the eyes from UV light.

To pour a gel, agarose powder is mixed with electrophoresis buffer to the desired concentration, then heated in a microwave oven until completely melted. Most commonly, ethidium bromide is added to the gel (final concentration 0.5 ug/ml) at this point to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature or, if you are in a big hurry, in a refrigerator.

After the gel has solidified, the comb is removed, using care not to rip the bottom of the wells. The gel, still in its plastic tray, is inserted horizontally into the electrophoresis chamber and just covered with buffer. Samples containing DNA mixed with loading buffer are then pipeted into the sample wells, the lid and power leads are placed on the apparatus, and a current is applied. You can confirm that current is flowing by observing bubbles coming off the electrodes. DNA will migrate towards the positive electrode, which is usually colored red.



The distance DNA has migrated in the gel can be judged by visually monitoring migration of the tracking dyes. Bromophenol blue and xylene cyanol dyes migrate through agarose gels at roughly the same rate as double-stranded DNA fragments of 300 and 4000 bp, respectively.

When adequate migration has occured, DNA fragments are visualized by staining with ethidium bromide. This fluorescent dye intercalates between bases of DNA and RNA. It is often incorporated into the gel so that staining occurs during electrophoresis, but the gel can also be stained after electrophoresis by soaking in a dilute solution of ethidium bromide. To visualize DNA or RNA, the gel is placed on a ultraviolet transilluminator. Be aware that DNA will diffuse within the gel over time, and examination or photography should take place shortly after cessation of electrophoresis.

Migration of DNA Fragments in Agarose

Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the $\log_{10}$ of their molecular weight. In other words, if you plot the distance from the well that DNA fragments have migrated against the $\log_{10}$ of either their molecular weights or number of base pairs, a roughly straight line will appear.

Circular forms of DNA migrate in agarose distinctly differently from linear DNAs of the same mass. Typically, uncut plasmids will appear to migrate more rapidly than the same plasmid when linearized. Additionally, most preparations of uncut plasmid contain at least two topologically-different

forms of DNA, corresponding to supercoiled forms and nicked circles. The image to the right shows an ethidium-stained gel with uncut plasmid in the left lane and the same plasmid linearized at a single site in the right lane.

Several additional factors have important effects on the mobility of DNA fragments in agarose gels, and can be used to your advantage in optimizing separation of DNA fragments. Chief among these factors are:

Agarose Concentration: By using gels with different concentrations of agarose, one can resolve different sizes of DNA fragments. Higher concentrations of agarose facilite separation of small DNAs, while low agarose concentrations allow resolution of larger DNAs.

The image to the right shows migration of a set of DNA fragments in three concentrations of agarose, all of which were in the same gel tray and electrophoresed at the same voltage and for identical times. Notice how the larger fragments are much better resolved in the 0.7% gel, while the small fragments separated best in 1.5% agarose. The 1000 bp fragment is indicated in each lane.

Voltage: As the voltage applied to a gel is increased, larger fragments migrate proportionally faster that small fragments. For that reason, the best resolution of fragments larger than about 2 kb is attained by applying no more than 5 volts per cm to the gel (the cm value is the distance between the two electrodes, not the length of the gel).

Electrophoresis Buffer: Several different buffers have been recommended for electrophoresis of DNA. The most commonly used for duplex DNA are TAE (Tris-acetate-EDTA) and TBE (Tris-borate-EDTA). DNA fragments will migrate at somewhat different rates in these two buffers due to differences in ionic strength. Buffers not only establish a pH, but provide ions to support conductivity. If you mistakenly use water instead of buffer, there will be essentially no migration of DNA in the gel! Conversely, if you use concentrated buffer (e.g. a 10X stock solution), enough heat may be generated in the gel to melt it.

Effects of Ethidium Bromide: Ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids and allows very convenient detection of DNA fragments in gels, as shown by all the images on this page. As described above, it can be incorporated into agarose gels, or added to samples of DNA

before loading to enable visualization of the fragments within the gel. As might be expected, binding of ethidium bromide to DNA alters its mass and rigidity, and therefore its mobility.

Electrophoresis of macromolecules can be carried out in solution. However, the ability to separate molecules is compromised by their diffusion. Greater resolution is achieved if electrophoresis is carried out on semi-solid supports such as polyacrylamide or agarose gels. Gels are formed by cross-linking polymers in aqueous medium. This will form a 3-dimensional meshwork which the molecules must pass through. Polyacrylamide is a common gel for proteinelectrophoresis whereas agarose is more commonly used for nucleic acids .

Agarose gels have a larger pore size than acrylamide gels and are better suited for larger

macromolecules. However, either type of gel can be applied to either nucleic acids or proteins depending on the application. Gels are formed from long polymers in a cross-linked lattice (Figure). The space between the polymers are the pores. Higher concentrations of the polymer will result in smaller average pore sizes. Polyacrylamide gels are formed by covalently cross-linking acrylamide monomers with bis-acrylamide with a free radical like persulfate ($SO4\cdot$). The cross-linking of the acrylamide polymers results in 'pores' of a defined size. The total acrylamide concentration and the ratio of bis-acrylamide to acrylamide will determine the average pore size. The polyacrylamide solution is poured into a mold and polymerized. This mold can be a cylindrical tube, but is usually a 'slab' poured between two glass plates.

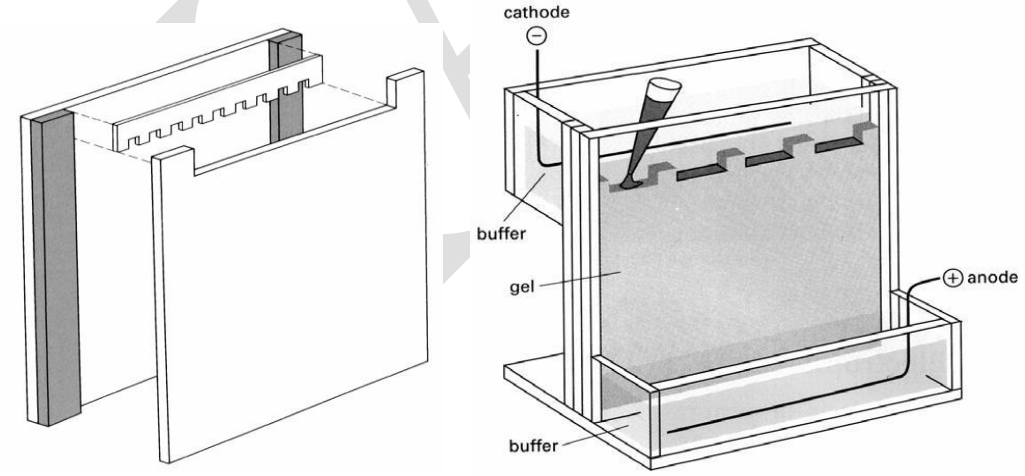
Since the gel is solid with respect to the mold, all molecules are forced through the gel. Smaller molecules will be able to pass through this lattice more easily resulting in larger molecules having a lower mobility than smaller molecules. In other words, the gel acts like a molecular sieve and retains the larger molecules while letting the smaller ones pass through. (This is opposite of gel filtration where the larger molecules have a higher mobility because they to not enter the gel.) Therefore, the frictional coefficient is related to how easily a protein passes through the pores of the gel and size will be the major determinant of the mobility of molecules in a gel matrix. Protein shape and other factors will still affect mobility, but to a lesser extent. Substituting size for the frictional coefficient results in:

mobility $\cong$ (voltage)(charge)/(size)

In other words, the mobility of a protein during gel electrophoresis is primarily a function of its charge/mass ratio.

Equipment

Equipment to conduct gel electrophoresis is relatively simple. They consist of a mold to form the gels, an apparatus to hold the gel and contain buffers, and a power supply capable of delivering the required voltage or current. There are many types of apparatus for carrying out electrophoresis depending on the application. Gels can be either in a vertical or horizontal configuration. Polyacrylamide gels are run in a vertical fashion and agarose gels tend to be run in a horizontal position. Gels can either be formed as cylinders by using glass tubing as a mold (often called tube gels) or formed as rectangular slabs. These slab gels are formed by polymerizing the acryamide solution between glass plates separated by spacers (Figure).



Typically the gel is 0.75-1.5 mm thick. At the top a 'comb' is used to form sample wells. Slab gels allow multiple samples to be compared on the same gel, thus eliminating gel-to-gel variations. The formed gel is placed into the apparatus to that the top and bottom of the gel are in contact with chambers containing buffer (Figure). These chambers contain electrodes which are connected to a power supply. Thus an electric field is generated across the gel when a voltage is applied. The buffer in the chambers is generally different that the buffer making up the gel for protein electrophoresis and in some applications the buffers in the lower and upper chambers may be different. In most applications the buffers are such that the protein has a negative charge and therefore the anode (positve pole) will be in the lower chamber and the cathode (negative pole) will be in the upper chamber. However, there are applications in which the proteins of interest may be positively charged and therefore the electrodes will be reversed.

Discontinuous or "disc" electrophoresis. The Laemmli discontinuous buffers are extensively used in gel electrophoresis. Discontinuous gels consist of two distinct gel regions referred to as stacking gel and separating gel and a Tris-glycine tank buffer. The stacking gel has a lower acrylamide concentration, a lower pH and a lower ionic strength than the separating gel.

The lower ionic strength of the stacking gel results in a greater local electric field strength than in the separating gel. The field strength difference combined with the lower acrylamide concentration results in proteins having a higher mobility in the stacking gel than in the separating gel. In addition, the glycine in the tank buffer has a higher mobility in the separating gel than in the stacking gel because of the pH differences. Therefore, proteins will migrate faster than the glycine in the stacking gel. When proteins reach the separating gel their mobility is decreased because of the increased acrylamide concentration and decreased field strength, whereas the increase in pH results in glycine having a higher mobility. All of these factors result in the proteins becoming compressed at the interface between the two gels and thus increasing resolution (Figure). Resolution in non-discontinuous electrophoresis depends partially on the volume of the sample. However, stacking also occurs at the interface of the sample and gel, especially if a high voltage is applied.

SDS-PAGE

1. Pour separating gel.

2. Pour stacking gel.

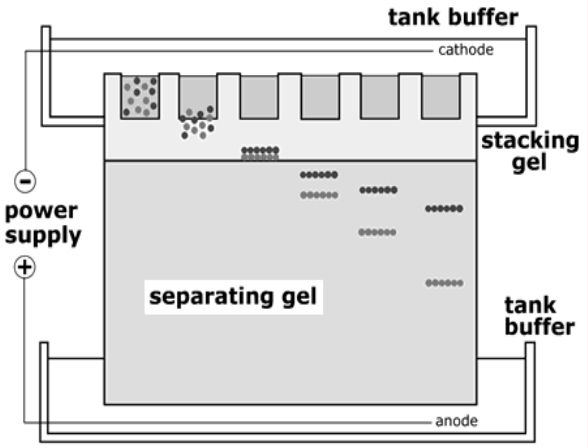
3. Load samples.

4. Apply electric field.

5. Stain or process gel.

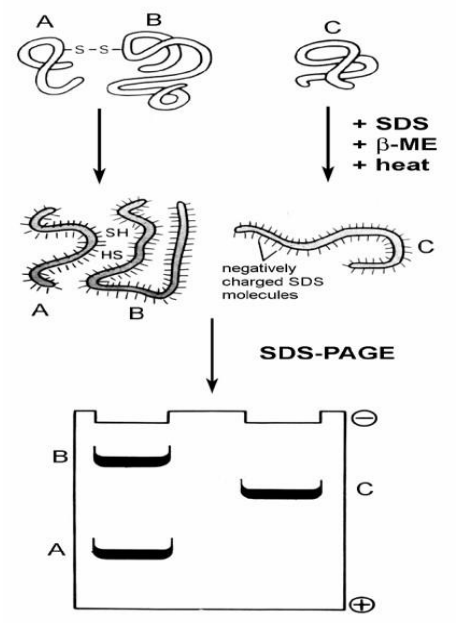
Polyacrylamide gel electrophoresis in the presence of SDS (sodium dodecyl sulfate) is the most common form of protein gel electrophoresis. SDS completely disrupts protein-protein interactions and denatures almost all proteins resulting in a complete unfolding of proteins. In addition, ß-mercaptoethanol (or other reducing agents) is often used to break disulfide bonds. The SDS binds to the unfolded proteins giving all proteins a similar shape (i.e., random coil or extend conformation) and an uniform charge-to-mass ratio. In other words, coating proteins with a negatively charged detergent minimizes the effects of a protein's net charge. Therefore, during electrophoresis in the presence of SDS the mobility of a protein now depends primarily upon its size (i.e., mobility is inversely proportional to protein mass). Mobility in SDS gel electrophoresis is expressed as a relative mobility (Rf). The distance the protein migrated is compared to the length of the gel, or: **Rf = distance protein migrated ÷ gel length**

The length of the gel is often defined by the migration of a substance which is not impeded by the matrix such a small molecular weight tracking dye (eg., bromophenol blue). This mobility can then be used to calculate the size of proteins. Protein standards of known size are used to generate a standard curve by plotting the log of the molecular weight against the Rf values. The molecular weight of an unknown protein can be extrapolated from its Rf value (see Appendix 1). Such a calculated molecular weight is designated as Mr to indicate that it is a relative molecular weight based on comparisons to other proteins. For some proteins, though, this estimated molecular weight can differ from the actual molecular weight. In particular, highly charged proteins behave anomalously during SDS gel electrophoresis. In addition, some proteins are not completely denatured by SDS and this retention of some structure will lower the mobility.



The first step in electrophoresis is to pour the separating gel (Box). Prepoured gels are also commercially available. Separating gels will typically contain 6-20% acrylamide. The size range of the proteins being separated, the desired resolution and the amount of sample being applied are factors to consider when choosing an acrylamide concentration Gradient gels can be used in situations where it is necessary to examine both high and low molecular weight proteins on the same gel. The stacking gel is poured after the separating gel polymerizes and just before electrophoresis to minimize diffusion between the two gels. Proteins to be analyzed by SDS-PAGE are solubilized in a sample buffer that typically contains 2% SDS and 5% β-mercaptoethanol and then boiled. The reducing agent is omitted in situations where disulfide bonds need to be preserved. In situations where an enzyme activity will be measure following electrophoresis a lower SDS concentration is used and the sample is not boiled. The amount of protein that can be loaded onto a gel is limited. Overloading the gels results in the pores becoming plugged and has an adverse effect on the electrophoresis. After loading the samples into the wells of the gel an electric field is applied across the gel. The mobility of proteins in an electric field is proportional to field strength (E/d), or simply the voltage (E) since the distance (d) is determined by the electrophoresis apparatus. Electrophoresis will proceed faster, and therefore finish sooner, at higher

voltages. However, electrophoresis generates heat in proportional to the amount of power, which is the product of voltage and current (P = EI).



Excessive heating may result in proteins precipitating within the gel or have other deleterious effects on proteins depending on the nature of the protein sample and total protein concentration. These electrical parameters change during electrophoresis because the ions migrate to the anode and cathode buffers, and therefore lead to an increase in resistance (R). Resistance affects the voltage and current (E = IR) depending upon which variable is held constant. Most power supplies are capable of delivering constant voltage (E), constant current (I), or constant power (P). Electrophoresis is usually carried out under constant voltage or constant power to minimize the resulting increase in heating that occurs during electrophoresis. A tracking dye (bromophenol blue) is included in the sample. When this dye reaches the bottom of the gel or some predetermined time afterwards the power is turned off and the proteins detected. A common way to detect proteins after electrophoresis is to stain the gel with Coomassie blue, a dye that binds proteins. Gels are usually 'fixed' before staining with an acetic acid and methanol solution which precipitates proteins into the acrylamide matrix.

Electrophoresis gel staining

Coomassie Blue Staining Following Electrophoresis

G-250 Method (quick)

1. Fix the proteins by incubating the gel in the destain solution (10% methanol and 7% acetic acid) for at least 15 minutes. Gels can be left in fixative indefinitely.

2. Briefly rinse the gels with water.

3. Stain the gels for 20 minutes with 0.1% Coomassie-blue G-250 in 3.5% perchloric acid. Gels thicker than 0.75 mm may need to be stained longer.

4. Destain the gel with several changes of the destain solution (10% methanol and 7% acetic acid) over the next 30-60 minutes. (Works best if the first 1-2 washes carried out in the first 5-10 minutes.)

R-250 Method (slow)

1. Fix the proteins by incubating the gel in the destain solution (10% methanol and 7% acetic acid) for at least 15 minutes. Gels can be left in fixative indefinitely.

2. Stain the gels for 3 hours with 0.1% Coomassie-blue R-250 in 50% methanol + 10% acetic acid.

3. Destain the gel with several changes of the destain solution (10% methanol and 7% acetic acid) over the next 6-24 hours.

Notes

• For optimal results, the G-250 should be added to the perchloric acid. Water should then be slowly added while stirring. Continue stirring for at least one hour and then filter the solution over filter paper.

• The R-250 solution should also be filtered after its preparation.

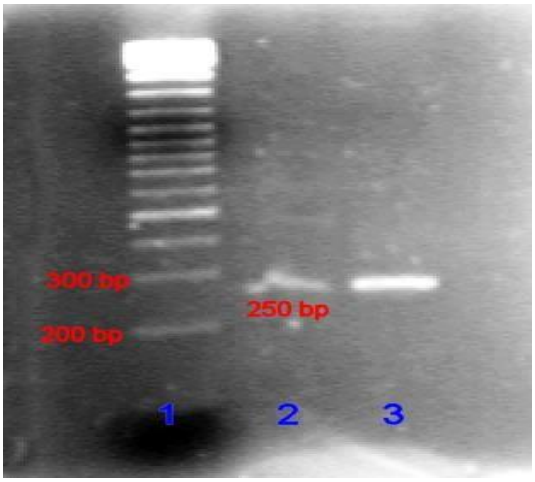
• Other concentrations of methanol and acetic acid also work in the fixative and/or destaining solutions.

Application

- Estimation of the size of DNA molecules following restriction enzyme digestion, e.g. in restriction mapping of cloned DNA.
- Analysis of PCR products, e.g. in molecular genetic diagnosis or genetic fingerprinting
- Separation of restricted genomic DNA prior to Southern transfer, or of RNA prior to Northern transfer.

Gel electrophoresis is used in forensics, molecular biology, genetics, microbiology and biochemistry. The results can be analyzed quantitatively by visualizing the gel with UV light and a gel imaging device. The image is recorded with a computer operated camera, and the intensity of the band or spot of interest is measured and compared against standard or markers loaded on the same gel. The measurement and analysis are mostly done with specialized software.

Depending on the type of analysis being performed, other techniques are often implemented in conjunction with the results of gel electrophoresis, providing a wide range of field-specific applications.



An agarose gel of a PCR product compared to a DNA ladder.

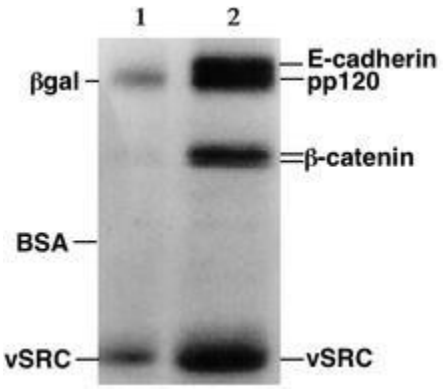
In the case of nucleic acids, the direction of migration, from negative to positive electrodes, is due to the naturally-occurring negative charge carried by their sugar-phosphate backbone. Double-stranded DNA fragments naturally behave as long rods, so their migration through the gel is relative to their size or, for cyclic fragments, their radius of gyration. Circular DNA such as plasmids, however, may show multiple bands, the speed of migration may depend on whether it is relaxed or supercoiled. Single-stranded DNA or RNA tend to fold up into molecules with complex shapes and migrate through the gel in a complicated manner based on their tertiary structure. Therefore, agents that disrupt the hydrogen bonds, such as sodium hydroxide or formamide, are used to denature the nucleic acids and cause them to behave as long rods again.[17]

Gel electrophoresis of large DNA or RNA is usually done by agarose gel electrophoresis. See the "Chain termination method" page for an example of a polyacrylamide DNA sequencing gel. Characterization through ligand interaction of nucleic acids or fragments may be performed by mobility shift affinity electrophoresis.

Electrophoresis of RNA samples can be used to check for genomic DNA contamination and also for RNA degradation. RNA from eukaryotic organisms shows distinct bands of 28s and 18s rRNA, the 28s

band being approximately twice as intense as the 18s band. Degraded RNA has less sharpely defined bands, has a smeared appearance, and intensity ratio is less than 2:1.

SDS-PAGE autoradiography – The indicated proteins are present in different concentrations in the two samples



SDS-PAGE autoradiography – The indicated proteins are present in different concentrations in the two samples.

Proteins, unlike nucleic acids, can have varying charges and complex shapes, therefore they may not migrate into the polyacrylamide gel at similar rates, or at all, when placing a negative to positive EMF on the sample. Proteins therefore, are usually denatured in the presence of a detergent such as sodium dodecyl sulfate/sodium dodecyl phosphate (SDS/SDP) that coats the proteins with a negative charge.[3] Generally, the amount of SDS bound is relative to the size of the protein (usually 1.4g SDS per gram of protein), so that the resulting denatured proteins have an overall negative charge, and all the proteins have a similar charge to mass ratio. Since denatured proteins act like long rods instead of having a complex tertiary shape, the rate at which the resulting SDS coated proteins migrate in the gel is relative only to its size and not its charge or shape.[3]

Proteins are usually analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), by native gel electrophoresis, by quantitative preparative native continuous polyacrylamide gel electrophoresis (QPNC-PAGE), or by 2-D electrophoresis.

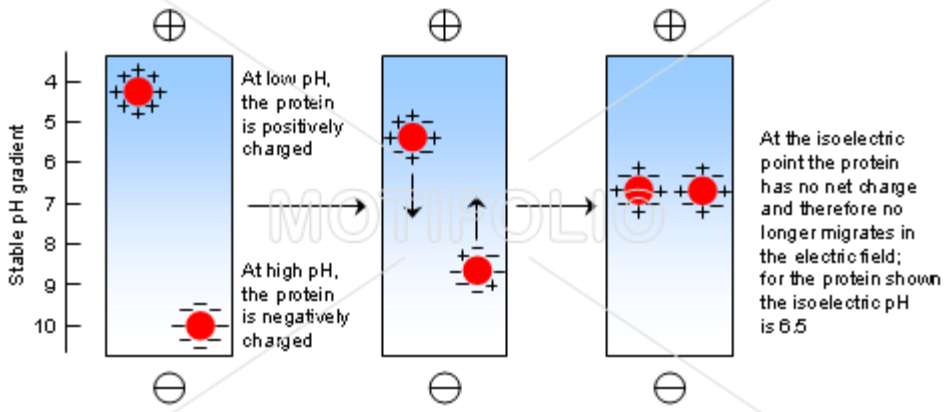
Characterization through ligand interaction may be performed by electroblotting or by affinity electrophoresis in agarose or by capillary electrophoresis as for estimation of binding constants and determination of structural features like glycan content through lectin binding.

Isoelectric Focusing (IEF)

Principle

IEF is an electrophoretic method for separating proteins based on their isoelectric point. The isoelectric point is the pH at which the net charge of the protein is zero. With the presence of a pH gradient in the IEF technique, the protein will migrate to the position in the gradient where its charge is zero. Proteins with a positive net charge will migrate toward the cathode until it meets its pI. Proteins with a negative net charge will migrate toward the anode until it meets its pI. If the protein diffuses away from its pI, it will regain its charge and migrate back. This focusing effect allows proteins to be separated based on very small charge differences. IEF is performed under high voltages (> 1000 V) until the proteins have reached their final position in the pH gradient. If IEF is performed under denaturing conditions very high resolution and cleanliness of sample can be obtained.

Separation of protein molecules by isoelectric focusing



Radioactive isotope, also called radioisotope, radionuclide, or radioactive nuclide, any of several species of the same chemical element with different masses whose nuclei are unstable and dissipate excess energy by spontaneously emitting radiation in the form of alpha, beta, and gamma rays.

Application

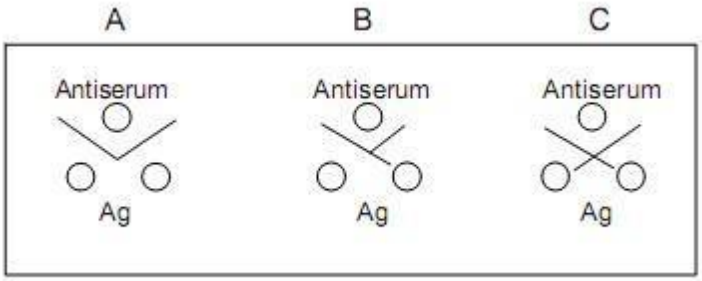
- Radioactive isotopes have a variety of applications as they are useful as the radioactivity of these isotopes can be detected or the energy released by them can be used.
- Carbon-14 isotopes are used in determining the steps in the process of photosynthesis in plants. Other use of radioisotope is for establishing age of various objects.
- Radiations that are emitted by some radioactive substances can be used to kill microorganisms in foodstuffs which enable to prolong the shelf life of these products.
- Agricultural products like tomatoes sprouts, mushrooms and berries are irradiated with emissions from radioisotopes like cobalt-60 or cesium-137. The process of irradiation kills a lot of bacteria that can cause spoilage of food.
- Radioactive isotopes are used in various medical applications. They are used in diagnosing and treating illness and diseases.
- Radioisotopes have extensive application in molecular biology. Radioisotopes can be incorporated into DNA, RNA and protein molecules both in vivo and in vitro conditions. The molecules of interest or the metabolic pathway can be traced or investigated.

- **Immunoelectrophoresis / Immunodiffussion**

- Immunodiffusion in gels encompasses a variety of techniques, which are useful for the analysis of antigens and antibodies. An antigen reacts with a specific antibody to form an antigen-antibody complex, the composition of which depends on the nature, concentration and proportion of the initial reactants.
- Immunodiffusion in gels are classified as single diffusion and double diffusion. In Ouchterlony double diffusion, both antigen and antibody are allowed to diffuse into the gel. This technique can be used to test the similarity between antigens, for example in a study of evolution. Antigens from different species are loaded into two wells and the known antibody is loaded in a third well located between and slightly below the antigen

wells to form a triangle. Depending on the similarity between the antigens, different geometrical patterns are produced between the antigen and antiserum wells. The pattern of lines that form can be interpreted to determine whether the antigens are same or different.

- **Diagram**



- **Pattern of Identity: A**
- The antibodies in the antiserum react with both the antigens resulting in a smooth line of precipitate. The antibodies cannot distinguish between the two antigens. i.e., the two antigens are immunologically identical.
- **Pattern of Partial Identity: B**
- In the 'pattern of partial identity', the antibodies in the antiserum react more with one of the antigens than the other. The 'spur' is thought to result from the determinants present in one antigen but lacking in the other antigen
- **Pattern of Non-Identity: C**
- In the 'pattern of non-identity', none of the antibodies in the antiserum react with antigenic determinants that may be present in both the antigens, i.e., the two antigens are immunologically unrelated as far as that antiserum is concerned.
- Scintillation counter is an instrument for detecting and measuring ionizing radiation. It consists of a scintillator which generates photons of light in response to incident radiation, a sensitive photomultiplier tube which converts the light to an electrical signal, and the necessary electronics to process the photomultiplier tube output.

POSSIBLE QUESTIONS

UNIT-3

1.Define electrophoresis

2.what is the principle of electrophoresis?

3.Define SDS

4.Expand PAGE.

5.Write the application of electrophoresis

6.What is isoelectric focusing.

7.Define agarose electrophoresis

8.What is isoelectric focusing.

unit-3

S.No	Question	Option 1	Option2	Option 3	Option 4	answer
1	Electrophoresis is the migration of charged particle in a solution under the influence of an_____	density	Electromagnetic	Magnetic field	Electric field	Electric field
2	Low voltage electrophoresis runs at_____	0-500 V	0-200 V	0-100 V	0-300 V	0-500 V
3	Silver staining is used for_____	detecting carbohydrates	detecting RNA	detecting DNA	detecting protein	detecting protein
4	Agarose gel is used for separation of	all the above	DNA	protein	RNA	all the above
5	_____will affect the electrophoresis separation process	none of the above	ionic strength of the buffer	pressure	vacuum	ionic strength of the buffer
6	High voltage electrophoresis can be operated under _____condition	high pressure	cold	vacuum	hot	cold
7	_____will affect the electrophoresis separation process	none of the above	size of the sample	pressure	vacuum	size of the sample
8	_____% w/v is used for polymerization	0.1-1.2	0.1-0.6	0.1-0.3	0.1-0.9	0.1-0.3
9	_____will affect the electrophoresis separation process	none of the above	electric field	pressure	vacuum	electric field

10	Usually_____% of gels are used for separating of proteins	10-25.0	0-5	1-15.0	0-10	1-15.0
11	SDS is used in electrophoresis for_____	none of the above	naturing of proteins	denaturing of proteins	both A and B	denaturing of proteins
12	% of gradient gel used for separation is_____	20-50	0-10	5-25.0	5-100	5-25.0
13	_____will affect the electrophoresis separation process	size of the gel	pressure	pH	vacuum	pH
14	Coomassive Brilliant Blue is used for_____	detecting carbohydrates	detecting RNA	detecting DNA	detecting protein	detecting protein
15	DNA can be detected by_____	sodium lamp exposure	tungsten lamp exposure	UV exposure	mercury lamp exposure	UV exposure
16	Low voltage electrophoresis runs at_____	TEMED	bisacrylamide	acrylamide	ammonium per sulphate	TEMED
17	In capillary blotting the gel is placed on a_____	Water	Urea and formamide	APS	Formalin	Urea and formamide
18	TEMED used in polyacrylamide gel electrophoresis_____	pH modifier	Inhibitor	Catalysts	Crosslinking agent	cross linking agent
19	Ampholytes are_____	gel	buffer	pH gradients	density	pH gradients
20	Capillary electrophoresis _____amount of samples are separated	kilograms	Milligrams	Nanograms	Centigrams	Nanograms

21	Acryl amide is a_____	verotoxic	cytotoxic	Nurotoxic	leucotoxic	Nurotoxic
22	_____is used in electrophoresis unit	aluminum	gold	platinum wire	copper	platinum wire
23	High voltage electrophoresis_____	500-2000 V	500-1000 V	500-10000 V	500-1500 V	500-10000 V
24	Power pack provides_____	none of the above	alternative current	direct current	both A and B	direct current
25	Photo polymerization can be done by using_____	Ammonium phosphate	Ammonium sulphate and TEMED	Rifoflavin	Both A and B	Both A and B
26	Bromophenol is used as a_____	gradient	electricity	Tracking dye	electrode	Tracking dye
27	SDS is a _____	cationic detergent	anionic detergent	anionic dye	cationic dye	anionic detergent
28	_____% of stacking gels used for separating proteins	16	8	4	12	4
29	Agarose is a product extracted from_____	water	Wood	Plant	Sea weed	Sea weed
30	Pulse field gel electrophoresis is used for separating_____	All the above	RNA	DNA	Chromosome	All the above
31	_____will affect the electrophoresis separation process	vacuum	pressure	temperature	all the above	temperature
32	Electrophoresis is based on	electrical field	temperature	charge	all the above	all the above
33	Electrophoretic movement of particles can be influenced by	electrical charge	solubility	size	all the above	all the above

	the following factor(s):					
34	Forensic science involves …….	GLC	paper chromatography	immunoelectrophoresis	none of the above	immunoelectrophoresis
35	This cannot be used in gel electrophoresis	agar	starch	polyacrylamidealbumin		albumin
36	Electrophoresis involves migration of.....molecules.	both charged	neutral	negatively charged	positively	both charge
37	Buffer not used in electrophoresis is	citrate	calcium	formate	phosphate	calcium
38	In electrophoresis use of cellulose acetate paper was introduced in	1960	1959	1958	1957	1958
39	Migration of charge particles is called as	TLC	GC	centrifugation	electrophoresis	electrophoresis
40	Molecular weight can be determined by ……	SDS-PAGE	immunoelectrophoresis	agarose gel electophoresis	none of the above	SDS-PAGE
41	Principle of electrophoresis is based on …….	solar energy	charged ions	colour	UV	charged ions
42	Better resolution is obtained in cellulose acetate thatn paper because	more hydrophilic	less hydrophobic	less hydrophilic	aal	less hydrophilic
43	Paper used in electrophoresis is made up of	fucose	mannose	galactose	cellulose	cellulose
44	When serum is subjected to electrophoresis, the fastest moving fraction is	alpha globulin	albumin	beta globulin	gamma globulin	alpha globulin
45	In PAGE, movement of protein depends on.....molecule.	size	charge	size & charge	none of the above	size & charge
46	During electrophoresis of proteins in an alkaline medium, they	act as cations and move towards	act as anions and move towards anode	do not move	none of the above	act as anions and move towards anode

		cathode				
47	Electrophoresis using acrylamide gel is known as ……..	chromatography	TLC	PAGE	gel electrophoresis	PAGE
48	Polymerization of acrylamide to polyacrylamide is due to addition of	ammonium persulphate	SDS	beta mecaptoethanol	urea	ammonium persulphate
49	Polyacrylamide is cross-linked with	agarose	n-N"methylene bis acrylamide	styrene	TCA	n-N"methylene bis acrylamide
50	Subunits of oligomeric proteins are linked by	van der Waals forces	hydrogenbonds	disulphide bridges	carbons atoms	disulphide bridges
51	SDS stands for	synthetic dihydrogen sodium	sodium disulphite	sodium dihydrogen phosphate	sodium dodecyl sulphate	sodium dodecyl sulphate
52	In SDS-PAGE, SDS serves as	an anioinc detergent	inititator of polymerization	cationic detergent	neutralizing agent	an anioinc detergent
53	In SDS-PAGE, the fast moving protein will have	low energy	highest charge	lowest charge	no charge	highest charge
54	Ammonium per sulphate and TEMED initiate ……. Polymerization	agar	agarose	acrylamide	agarobiose	acrylamide
55	The role of mercaptoethanol in electrophoresis is	pH maintenance	break hydrogenbonds	impart negative charge to proteins	break S-S bonds	break S-S bonds
56	Among proteins,..... carries largest charge and moves faster.	albumin	globulin	keratin	hemoglobin	albumin
57	Staining method for protein electrophoretogram is	methylene blue	silver stain	Ponceau-S	ethidium bromide	silver stain
58	Cross linking agents in PAGE is	SDS	ammonium per sulphate	bisacrylamide	TEMED	bisacrylamide
59	Chemical components in PAGE	acrylamide	bisacrylamide	TEMED	all the above	all the above

	includes					
60	Buffer with a pH of ..... is used for separation of proteins in paper electrophoresis	8.6	8.1	6	7.5	8.6
61	SDS-PAGE cannot be used for	proteins	enzymes	vitamin A	nucleic acids	vitamin A
62	In electrophoresis, lipoproteins can be detected by staining with	acrydine orange	methylene blue	vinyl orange	Sudan black	Sudan black
63	Proteins possesing more than one polypepetide chain are known as	oligomeric proteins	disulphide bridges	alpha chain	beta chain	oligomeric proteins
64	Subunit of oligomeric proteins can be separated by	glycols	solubilizers	detergents	emulsifiers	solubilizers
65	In rocket immunoelectrophoresis antibodies are	mixed with agar	mixed with buffer	applied in well	sprayed on gel plate	applied in well
66	Agarose is produced from	metals	animal oils	plants	algae	algae
67	Nucleic acids are detected by	ethidium bromide	ninhydrin	Ponceau-S	Coomassie blue	ethidium bromide
68	Ampholytes contain	neutral charges	both positive & negative groups	positive groups	negative groups	both positive & negative groups
69	Iso electric focusing separates proteins that differ by	4 charge units	3 charge units	2 charge units	one charge unit	one charge unit
70	Disulphide bonds in proteins are broken by .....in SDS-PAGE.	Beta-ME	SDS	APS	Coomassie blue	Beta-ME
71	pH at which net charge of the protein becomes neutral is called	acidic pH	alkaline pH	isoelectric pH	none of the above	isoelectric pH
72	Electrophoresis was first discovered by	Michael	Faraday	DuBois	Alexander Reuss	Alexander Reuss

Unit IV

Principle, Instrumentation and application of spectrophotometer, colorimeter and turdibometer.

SPECTROSCOPY

Properties of Electromagnetic Radiation

Electromagnetic radiation is a form of energy, sometimes called radiant energy or optical energy. The most familiar form of electromagnetic radiation is visible light. However, there are many other forms of electromagnetic radiation including:

- Visible Light
- X-rays
- Ultraviolet Light
- Radio Waves
- Microwaves
- Infrared Light

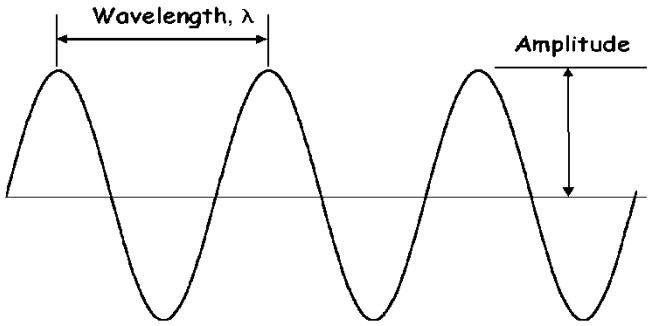
Our eyes are only sensitive to a small portion of the electromagnetic spectrum, but in a physical sense there is nothing that makes visible light unique from say the X-rays used at the hospital or the microwaves used in your oven.

What are some of the features common to all forms of electromagnetic radiation:

- They are all propagating (moving) forms of energy
- They all travel at the velocity (the speed of light)
- They all have wavelike characteristics

Lets expand upon this last point, what does it mean to behave as a wave? Waves have a periodic up/down motion to them. For example if a boat remains stationary while waves of water go past the height of the boat will alternately rise and fall, reaching a maximum at the peak of the wave and a minimum at the trough.

If we were now to take a picture of the wave, freeze the wave at one moment of time it would look something like the picture below:



This picture actually shows three cycles of the wave, that is it repeats itself three times in the above picture. The wave shown above is not moving, but both electromagnetic waves and water waves do not stand still, they move (propagate). Whereas, a stationary boat in the water is a good image for describing the periodicity of a wave, a surfer is a good image for describing the motion of a wave. A surfer travels with the wave, ideally staying at the same point on the wave as it moves (for example on just below the peak). Using these images and the picture above we can define the properties of a wave.

Wavelength The distance between any point on the wave and the corresponding point where the wave begins its next cycle (for example from peak to peak).

Velocity The speed with which any point on the wave moves through space (the speed of the surfer).

Frequency The number of cycles that pass by a stationary point per second (If you were sitting on a tower sticking out of the surf, the frequency would be the number of surfers that would go past you every second, assuming 1 surfer per wave). Units of frequency are Hertz, Hz (1 Hz = 1 cycle/sec = 1 s^{-1}).

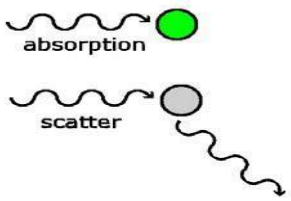
Amplitude The height of the wave (as measured from the middle of the wave to the peak).

Going back to the surfer riding the crest of wave, lets consider how changes in the frequency and wavelength affect his speed. If we increase the frequency, we increase the number of waves that go by every second and his velocity increases. On the other hand if we keep the frequency constant and increase the wavelength the wave has to move faster in order to keep the same number of waves going by per unit time, so the surfer's speed will increase.

Instrumentation and applications:

Spectrophotometer

Spectrophotometer is an instrument, which measures light absorbance at various wavelengths by producing a monochromatic light using a diffraction grating or glass prism. Light is passed through a monochromator to provide selection of the desired wavelength out of the spectrum to be used for the measurement. Slits are used to isolate a narrow beam of light and improve its chromaticity. The light is then passed through the cuvette, where a portion of the radiant energy is absorbed depending on the nature of the substances in a solution. Any light not absorbed is transmitted to a detector, which converts light energy to electrical energy. A monochromator is a system of isolating radiant energy of a desired wavelength and excluding that of other wavelengths. Spectral isolation can be accomplished by various means including the use of filters, prisms and diffraction grating. Method of producing the monochromatic light is different in spectrophotometers and absorptiometer. Filter photometer (absorptiometer) uses filter for wavelength isolation while a spectrophotometer isolates the light by a prism or diffraction grating system. The color intended to be measured should be due to the substance under investigation but not due to any of the reagents used. This is controlled by using reasent blank.



Principles of Spectrophotometer

Spectrophotometry is a versatile analytical tool. The underlying principle of spectrophotometry is to shine light on a sample and to analyze how the sample affects the light. Advantages of spectrophotometry are: 1) it is often non-destructive (i.e., can measure and recover sample), 2) it is

selective (often a particular compound in a mixture can be measured without separation techniques), 3) it has a short time interval of measurement (10-14 seconds).

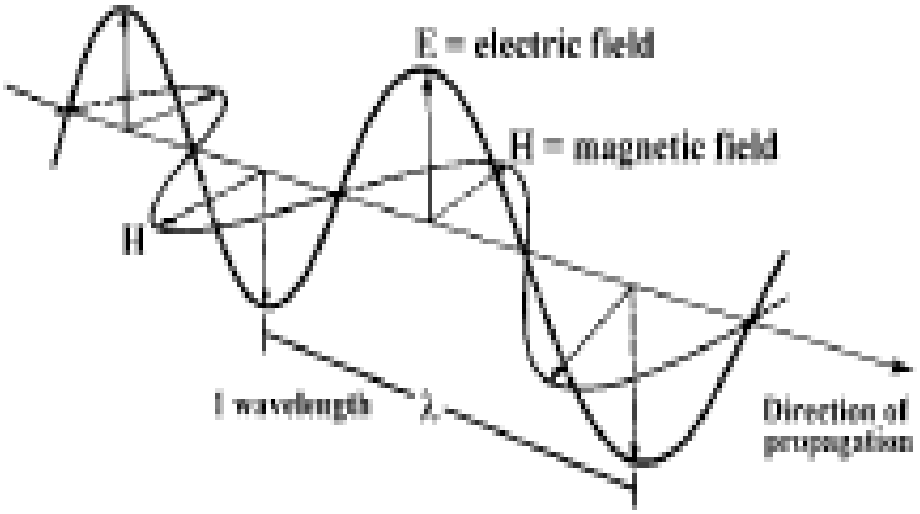
Theory of Spectrophotometry

Light can be described as a wave. This wave has an electric component and a magnetic component which are perpendicular to each other (Figure). Electromagnetic radiation exhibits a direction of propagation and wave-like properties (i.e., oscillations). The energy of electromagnetic radiation is defined as:

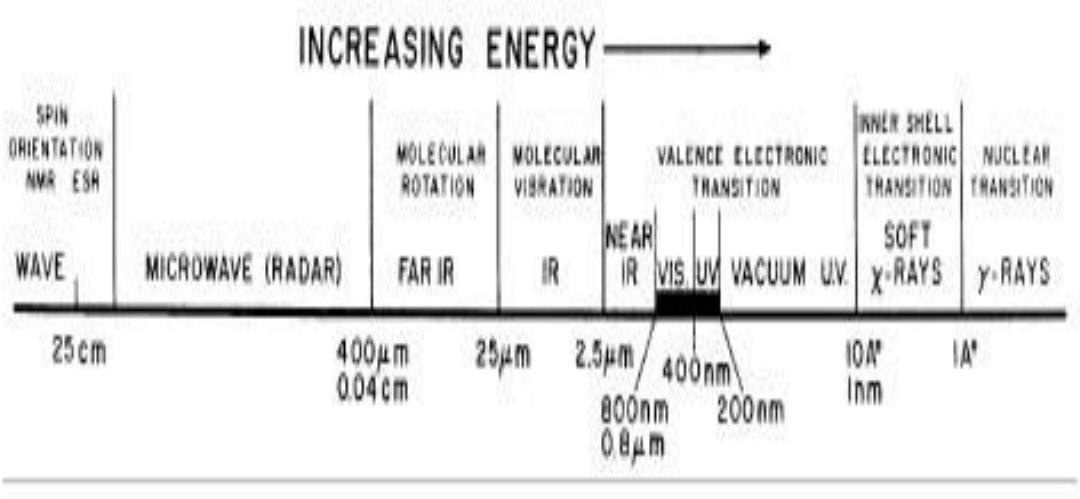
$$E = hc/\lambda = h\upsilon$$

where E = energy, h = Planck's constant, c = the speed of light, λ = the wave length, and

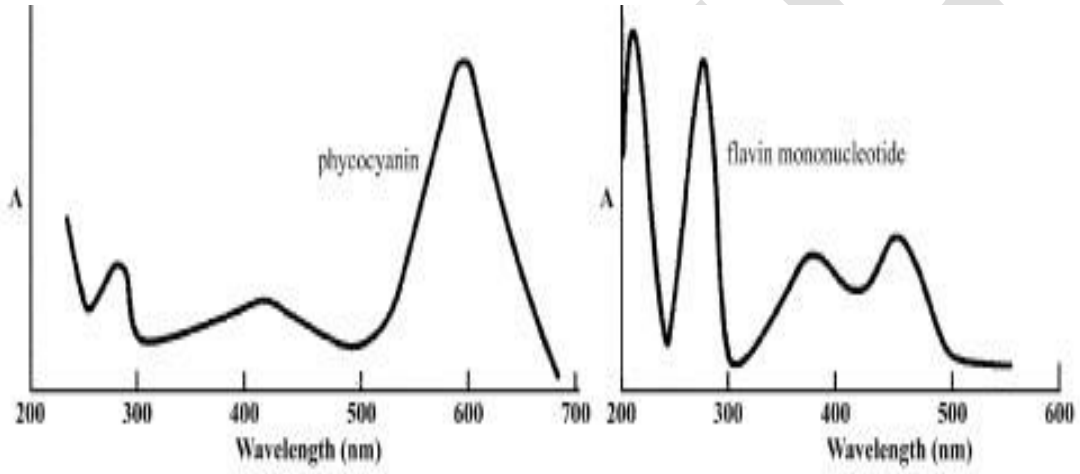
υ = frequency. Light behaves both as a wave and as a particle. The conceptual particle of light is called a **photon** and is represented by hυ. Electromagnetic radiation exhibits a wide spectrum and specific ranges of wavelengths have names (Figure). The energy of electromagnetic radiation is inversely proportional to its wavelength. When a light wave encounters a particle, or molecule, it can be scattered (i.e., direction changed), absorbed (energy transferred), or unaffected. Molecules only absorb discreet packets of energy, or quanta, and absorption occurs when the energy of the photon corresponds to differences between energy levels in that particular molecule.



These discrete energy levels, called electronic energy levels, are a property of the particular molecule and are determined by the spatial distribution of the electrons. Absorption of the energy from the photon elevates the molecule to an excited electronic state by causing an electron to move from one orbit to another. These electronic energy levels are further subdivided into vibrational levels. The vibrational levels correspond to stretching and bending of various covalent bonds. The transitions to the excited state can occur between different vibrational levels giving a range of energy that can be absorbed by the molecule.



A molecule or substance that absorbs light is called a **chromophore**. Chromophores exhibit unique absorption spectra (Figure) and can be defined by a wavelength of maximum absorption, or λmax, of a broad absorbtion band due to the vibrational levels. The absorption spectra can consists of several absorption maxima of various amplitudes. A large number of biological molecules absorb light in the visible and ultraviolet (UV) range.



The net affect of absorption is that the intensity of the light decreases as it passes through a solution containing a chromophore. The amount of light absorbed depends on the nature of the chromophore, the concentration of the chromophore, the thickness of the sample, and the conditions (eg., pH, solvent, etc.) under which absorption is measured.

Absorption is governed by the Beer-Lambert Law:

$I = Io10^{-\varepsilon dc}$ or $\log(I/Io) = -\varepsilon dc$

where I = final light intensity, Io = initial light intensity, ε = molar extinction coefficient, d = thickness, and c = molar concentration. Absorption (A) will be defined by:

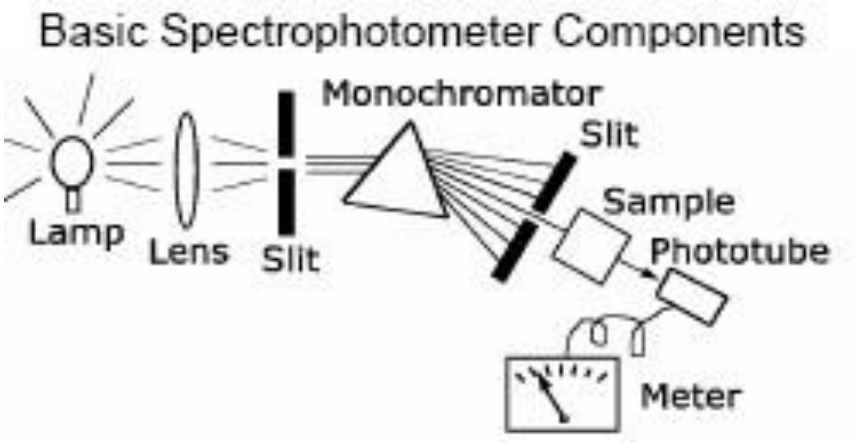
A = -log(I/Io) = εdc

The molar extinction coefficient (ε) is defined as the A of 1 M of pure compound under

standard conditions and reflects something about the nature of the chromaphore. The units of ε are liter/cm·mole. However, the extinction coefficient can be expressed in other units. For example, it can be expressed in terms of mM concentration. The thickness of the sample (d) is almost always 1 cm and therefore can be ignored in calculations. Sometimes, though, the extinction coefficient units is expressed in cm2/mole (by converting liters to cubic centimeters) and care should be taken in making calculations. In cases where the molecular weight of the substance is not known, or varies, E1% is used as the extinction coefficient. E1% is defined as the A of a 1% (w/v) solution. It is important to precisely record the units of ε when looking it up or determining it experimentally since these units will determine the concentration. It is also important to record the conditions (eg., pH, solvent, temperature, etc.) for an extinction coefficient

Instrumentation

Spectrophotometers produce monochromatic light and then accurately measure the light intensity. The major components of a spectrophotometer are the light source, a monochromator, sample holder, a light detector (phototube), and a meter. In most instruments a tungsten lamp is used for the visible range and either high pressure H2 or D2 lamps are used for UV range. Monochromatic light is generated by either 1) a movable prism, 2) a diffraction gradient, or 3) filters. Monochromatic light is projected through the sample and then measured by a



photomultiplier tube. A photomultiplier tube converts the energy of the light photons into electrons (see appendix). The voltage resulting from these electrons is measured by a meter and converted to an absorbance value. The Io (initial intensity) is determined by calibrating the instrument with a 'buffer blank'. The relative difference in the light intensity between the blank and the sample is then expressed as the absorbance (A). Spectrophotometers often include accessories such as chart recorders or microprocessors for data analysis.

Ultraviolet-visible spectroscopy (uv-vis frequency range)

Absorption spectroscopy

Absorption = transfer of energy from a photon (light) to a molecule *Chromophore* = a molecule or a group on a molecule that absorbs light Chromophores in proteins include

the peptide bond (maximum wavelength of absorbed light, λ_{max}, ~220 nm, "far" uv) aromatic a.a. residues (λ_{max} ~280 nm for Trp, "near" uv; aromatics also absorb ~220 nm) some prosthetic groups (tightly bound non-amino acid components in proteins, e.g., the heme in hemoglobin and myoglobin is red -- it absorbs visible light.)

USES of absorbance spectroscopy

Determine concentration (Beer's Law)

conformational changes (environment of chromophore affects λ_{max} and absorbance)

detect and quantitate ligand binding (e.g., O_2 binding to hemoglobin changes absorbance of the heme).

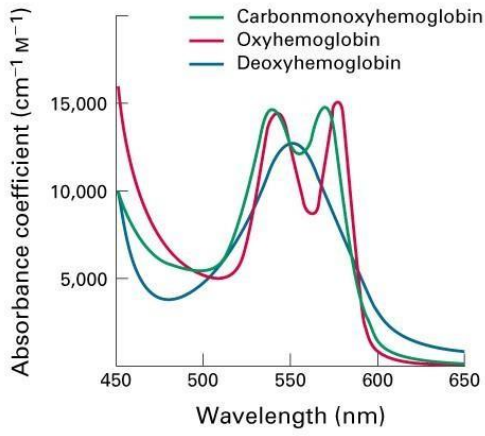
Example: Absorption spectra of deoxy- and oxyhemoglobin

The visible absorption spectrum of hemoglobin changes markedly upon binding of O_2 or CO

*Deoxy*Hb (blue) has single absorbance maximum ~550 nm.

*Oxy*Hb (red) has 2 absorbance peaks, at about 540 nm and 575 nm.

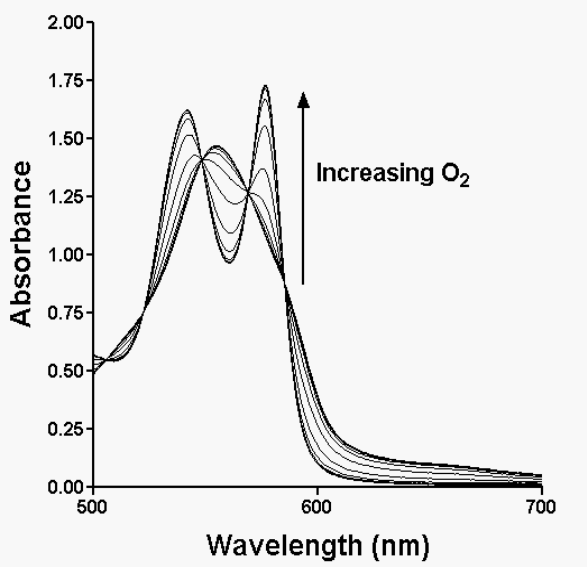
Maximum *difference* between deoxy and oxy spectra is seen at about 576 nm.



For a given Hb solution with no O_2 present, value of A_{576nm} indicates all "empty" sites (all *deoxy*, so ([occupied sites]/[total sites])

When $[O_2]$ has been increased to a concentration sufficient to essentially saturate the binding sites on the Hb ($\Box$ = 1), that maximal A_{576nm} indicates that all binding sites in the solution are "occupied".

As O_2 concentration increases from 0 to saturating, increases and can be monitored by the change in A_{576nm}, A_{576nm}, up to the maximum A_{576nm}, which occurs when all the sites have O_2 bound.



Applications of spectrophotometry

Determining the concentration of substances in solution is the most common use of the spectrophotometer. Exact concentrations can be determined in cases where ε are known and the measurement is carried out under the prescribed conditions. The substance being measured does not necessarily need to absorb radiation if it can scatter radiation. For example, measuring the A600 is a quick and easy way to monitor bacterial growth and determine the number of bacteria in cultures. In addition, since compounds exhibit unique absorption profiles, spectrophotometry can also be used to identify unknown compounds. Spectrophotometry is also a convenient method to measure enzyme activity in cases where the substrate and the product exhibit different λmax. Either the disappearance of substrate or the appearance of product over time is measured. The change in the Aλ per unit time (generally per minute) is calculated. The change in Aλ of a blank (= identical sample without enzyme source) is subtracted from this value. The enzyme activity in terms of amount of product formed per unit time per mg protein can be calculated by factoring in the amount of enzyme, dilution factors and the extinction coefficient (see example in appendix). A typical example of a formula for the calculation of enzyme activity is:

activity = (ΔAsample - ΔAblank)·volume·106/ε·Δtime·mg protein

where activity is expressed as μmoles product formed/min/mg protein; ΔA sample is the change in absorbance of the sample containing enzyme; ΔAblank is the change in absorbance of a sample containing everything except the enzyme; the volume in the cuvette expressed in the same units as ε; 106 μmoles per mole (assuming ε is expressed in moles); ε is the molar extinction coefficient; Δtime is the time in minutes the reaction was measured; and mg protein in the cuvette. In deriving such formulas it is important to match the units. The units of ε may also include the 1 cm thickness of the cuvette which is ignored in the calculations.

http://www.biologydiscussion.com/colorimetry/colorimetry-principle-and-instruments/57652

The colorimeter is a device that is mainly used in industries and laboratories for analyzing the color quality of the products along with color measurement.

What is Colorimeter?

The colorimeters are highly sensitive devices that can measure the concentration and intensity of a particular color that is used in a product. There are mainly two different types of colorimeters that are used in industries that are color densitometers and color photometers. The color densitometers measure the color density of primary colors in a color combination in a test sample. The color photometers are used for measuring the reflectance of a color as well as the transmission.

Principle of Colorimetry:

Colorimetry is a widely used technique applied in biological system. It involves the measurement of a compound or a group of compounds present in a complex mixture. The property of colorimetric analyses is to determine the intensity or concentration of compounds in coloured solution.

This is done by passing light of specific wavelength of visible spectrum through the solution in a photoelectric colorimeter instrument and observe the galvanometric reading of reflection sensitizing the quantity of light absorbed

Based on the nature of colour compounds, specific light filters are used. Three types of filters are available — blue, green and red — with corresponding light wavelength transmission rays from 470-490 nm, 500-530 nm and 620-680 nm, respectively.

There are two fundamental laws of absorption which are highly important in colorimetric estimation. These are Lambert's law and Beer's law. Lambert's law states that when monochromatic light passes through a solution of constant concentration, the absorption by the solution is directly proportional to the length of the solution.

In contrary, Beer's law states that when monochromatic light passes through a solution of constant length, the absorption by the solution is directly proportional to the concentration of the solution.

Thus both the laws can be expressed as:

Lambert's law: $\log_{10} I_0/I = K_1 I$

Beer's law: $\log_{10} I_0/I = K_2 I$

[where I_0 = Intensity of incident light (light entering a solution);

I = Intensity of transmitted light (light leaving a solution);

l = Length of absorbing solution;

c = Concentration of coloured substance in solution;

K_1 and K_2 = Constants.]

Both Beer-Lambert law are combined together for getting the expression transmittance (T).

$T = I/I_0$

(where I_0 is the intensity of incident radiation and I is the intensity of transmitted radiation).

A 100% value of 'T' represent a totally transparent substance, with no radiation being aborted, whereas a zero value of 'T' represents a totally opaque substance that, in effect, represents complete absorption. For intermediate value we can define the absorbance (A) or extinction (E) that is given by the logarithm (to base 10) of the reciprocal of the transmittance:

$A = E = \log_{10} (I/T) = \log_{10} (I_0/I)$

Absorbance used to be called optical density (OD) but continued use of this term should be discouraged. Also, as absorbance is a logarithm it is, by definition, unit-less and has a range of values from 0 (= 100% T) to cc (= 0% T).

Thus the variation of colour of the reaction mixture (or system) with change of substrate concentration forms the basis of colorimetric analysis.

The formation of colour is due to the reaction between substances and reagents in appropriate proportion. The intensity of colour observed is then compared with that of reaction mixture which contains a known amount of substrate. The optical spectrophotometry is based on identical principles of colorimetry.

Instruments of Colorimetry:

(A) Colorimeter:

The colorimeter instrument is very simple, consisting merely of a light source (lamp), filter, curette and photosensitive detector to collect the transmitted light. Another detector is required to measure the incident light; or a single detector may be used to measure incident and transmitted light, alternately.

The latter design is both cheaper and analytically better, because it eliminates variations between detectors. The filter is used here to obtain an appropriate range of wavelengths within the bands which it is capable of selecting.

Applications of Colorimeters

The Testronix's colorimeters can be used for measuring the carol concentration and intensity of a variety of materials such as:

- Food ingredients,
- Building materials,
- Textile products,
- Beverages,
- Chemical solutions and many others

http://www.testronixinstruments.com/blog/working-principle-applications-of-colorimeters/

Turbidimetry and Nephelometry

When particles are suspended in a solution in a cuvette, they make the solution unclear (turbid). Incident light entering the cuvette will be subjected to three reactions;

1- some of the light will be absorbed (blocked) by the particles

2- some will be transmitted through the cuvette

3- some will be scattered in various directions. Turbidimetry • Turbidimetry is involved with measuring the amount of transmitted light (and calculating the absorbed light) by particles in suspension to determine the concentration of the substance in question. Amount of absorbed light, and therefore, concentration is dependent on ; a) number of particles, and 2) size of particles. • Measurements are made using light spectrophotometers Clinical Applications

- Determination of the concentration of total protein in biological fluids such as urine and CSF which contain small quantities of protein (mg/L quantities) using trichloroacetic acid

- Determination of amylase activity using starch as substrate. The decrease in turbidity is directly proportional to amylase activity.

- Determination of lipase activity using triglycerides as substrate. The decrease in turbidity is directly proportional to lipase activity.

Nephelometry. Principle

• Nephelometry is concerned with measurement of scattered light from a cuvette containing suspended particles in a solution.

• The components of a nephelometer are the same as a light spectrophotometer except that the detector is placed at a specific angle from the incident light.

• The detector is a photomultiplier tube placed at a position to detect forward scattered light. Detectors may be placed at 90^o , 70^o or 37^o depending on the angle at which most scattered light are found.

• Since the amount of scattered light is far greater than the transmitted light in a turbid suspension, nephelometry offers higher sensitivity than turbidimetry.

• The amount of scattered light depends on the size and number of particles in suspension.

• For most clinical applications, the light source is a tungsten lamp giving light in the visible region

• For higher sensitivity and for applications that determine the size and number of particles in suspension, laser light nephelometers is used.

Clinical applications of nephelometry.

• Widely used to determine concentrations of unknowns where there is antigen-antibody reactions such as

Determination of immunoglobulins (total, IgG, IgE, IgM, IgA) in serum and other biological fluids

Determination of the concentrations of individual serum proteins; hemoglobin, haptoglobin, transferring, c-reactive protein, 1-antitrypsin, albumin (using antibodies specific for each protein)

Determination of the size and number of particles (laser-nephelometr} Turbidimetry & Nephelometry

Considerations in turbidimetry and nephelometry

• The reaction in turbidimetry & nephelometry does not follow Beer's Law

• Therefore, standard curves must be plotted and the concentration of the unknown is determined from the standard curve.

• Because the absorbance is dependent on both number and size of particles, the standard solution which is used for the standard curve must have similar size in suspension as unknown.

• Because some precipitation and settlement of particles may occur with time, in order to obtain good accuracy it is important to ; a) mix the sample well prior to placing the cuvette in the instrument, and, b) keep the same time for measurement of every sample throughout the measurement.

• Kinetic reactions (measurement of the progress of reaction with time) provides higher degree of accuracy, sensitivity, precision and less time than end-point reactions (measuring the reaction at the start and finish of the reaction)

1. Additionally in kinetic reactions there is no need for reagent blank since the previous reading is taken as the base-line for the next reading.

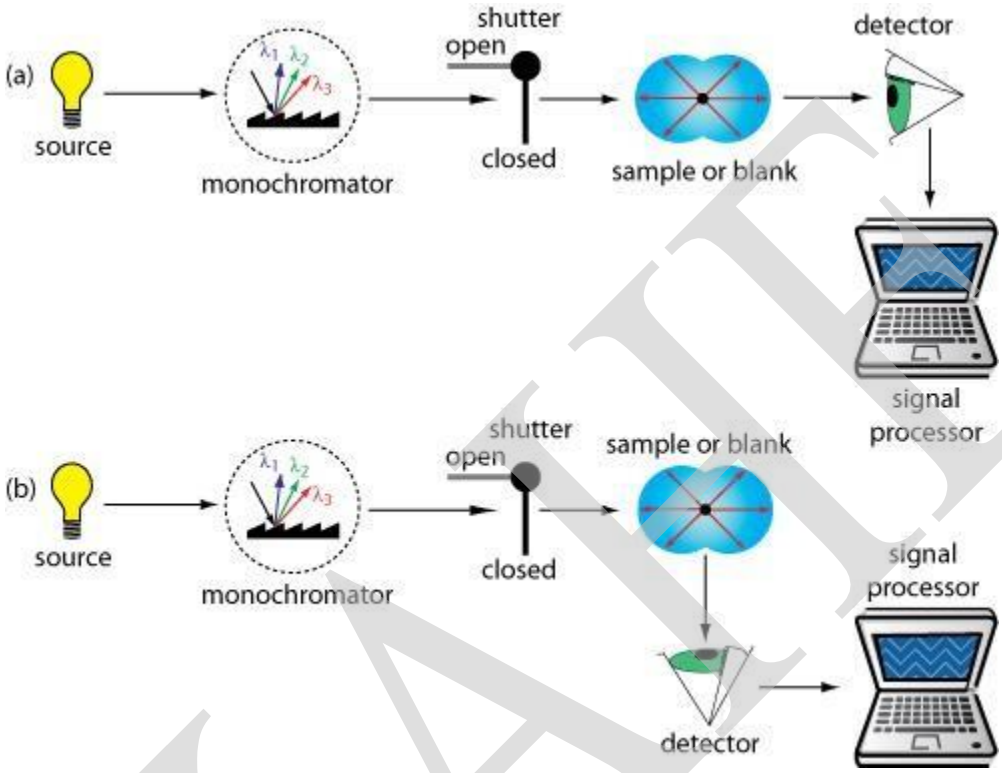
2. Kinetic reaction may be taken in 60, 90 or 120 seconds (taking readings at 10 seconds intervals), whereas endpoint reactions may take much longer time e.g. 15 - 120 minutes. • Selection of a wavelength

• If both solution and suspended particles are colorless, then use any wave length in the visible range

• If the solution is coloured but the particles are not coloured, then use a wave length that gives minimum absorption for the solution

• If the particles are coloured and the solution is colorless then use a wavelength that gives maximum absorption with the particles

• If both solution and particles are coloured then use two wavelengths; one that gives minimum absorbance for the solution and the other one maximum absorbance for the particles. Subtract the solution absorbance from the particles absorbance.



Turbidimetry and nephelometry are two techniques based on the elastic scattering of radiation by a suspension of colloidal particles: (a) in turbidimetry the detector is placed in line with the source and the decrease in the radiation's transmitted power is measured.; (b) in nephelometry scattered radiation is measured at an angle of 90º to the source. The similarity of turbidimetry to the measurement of absorbance and of nephelometry to the measurement of fluorescence is evident in these instrumental designs . In fact, turbidity can be measured using a UV/Vis spectrophotometer and a spectrofluorimeter is suitable for nephelometry.

POSSIBLE QUESTIONS

UNIT-IV

1.Define Beer lamberts law.

2.Sketch a note on calorimeter.

3.Write the principles of calorimeter.

4.Sketch a note on spectrophotometer

5.illustrate the application of spectrophotometer

6.Write a note on turbidometer.

7.Give a note on uses of turbidometer.

8.Write the principle of turbidometer.

unit-4

S.No	Question	Option 1	Option2	Option 3	Option 4	answer
1	Intensity of the coloured solution is measured using	spectrophotometer	monochromator	colorimeter	fluorimeter	colorimeter
2	Wavelength of the ultra-violet rays range	380-800nm	180-380nm	150-320nm	290-370nm	180-380nm
3	When heated sodium metal emits …….. spectrum	absorption	x-rays	emission	visible	emission
4	Colorimeter uses.....as the light source	deuterium lamp	mercury lamp	sodium lamp	tungsten	tungsten
5	The electrical energy produced is converted and amplified by ……	galvanometer	Photomultiplier tube	filter	detector	Photomultiplier tube
6	Absorbance of unknown substance can be determined by using	monochromator	spectrophotometer	conductivity meter	colorimeter	spectrophotometer
7	Light from the tungsten lamp ranges between ----------	400-600nm	180-380nm	450-660nm	10-100nm	400-600nm
8	Colourless substance are converted to coloured substance stoichiometrically by adding----------group.	chemicals	chromophoric	dyes	auxometric	chromophoric
9	Lamp used for UV light in spectrophotometer is	tritium lamp	tungsten	mercury	deuterium	deuterium
10	Cuvettes are made up of ----------	glass	plastic	silica	all the above	all the above
11	Cuvettes made up of.....are used to transmit radiations below 110nm.	quartz	glass	lithium fluoride	silica	lithium fluoride
12	Cathode of photo multiplier tube is made up of ……..	cadmium	selenium	gelatin	germanium	selenium
13	The input power of the photo multiplier tube is----------	900v 600v	550v	600v	650v	900v
14	Concentration of both colour and colourless solutions can be measured using ----------	colorimeter	spectrophotometer	both a and b	conductometer	spectrophotometer

15	The resolving power of the grating is directly proportional to----------	intensity of the light	wave length of the light	both a and b	closeness of the lines	closeness of the lines
16	Oxygen also absorbs light at a wave length of---------	below 110nm	below 200nm	above200nm	below 100nm	below 200nm
17	In flourimeter---------- is used to select UV light but not visible light.	condensing lens	primary filter	secondary filter	photo cell	primary filter
18	In nuclear research .....is used to determine uranium in salts.	spectrophotometer	colorimeter	conductivity meter	fluorimeter	fluorimeter
19	On oxidation of thiamine (vitamin B1) it forms thiochrome which emits----------	blue	purple	red	yellow	blue
20	Both qualitative and quantitative analysis of sample can be done using …….	fluorimeter	spectrophotometer	both a and b	none of the above	both a and b
21	Based on Lambert's law the amount of light absorbed is directly proportional to ----------	concentration of the substance	length of the medium	both a and b	none of the above	length of the medium
22	When the number of light absorbing molecules increases in the medium, the intensity of light coming out of it will be -------	increased	decreased	decreased exponentially	increased exponentially	decreased exponentially
23	Beer Lambert's law is the principle followed in ……..	spectrophotometer	colorimeter	fluorimeter	all the above	all the above
24	A monochromator consists of ----------	prism	grating	both a and b	filter	both a and b
25	A quartz cuvette will have a optical path of …….	0.5cm	1cm	0.5mm	1mm	1cm
26	Cuvette which cannot be used for light measurement in UV region	silica	quartz	lithium fluoride	none of the above	none of the above
27	Half silvered mirror is used in---------- instrument	single beam spectroscopy	double beam spectroscopy	fluorimeter	none of the above	double beam spectroscopy
28	Light energy is converted to electrical	photomultiplier		filter	condensing lens	photomultiplier

	energy by …….	tube	monochromator			tube
29	In spectrophotometer, after passing through cuvette the transmitted light will fall on ……..	monochromator	photomultiplier tube	galvanometer	slit	photomultiplier tube
30	In electromagnetic spectrum..... will have the higher wavelength.	visible	X-rays	microwave	infra red	microwave
31	If a sample absorbs all wavelengths in the visible region of the spectrum, it will appear----------	white	blue	colourless	black	black
32	The color we see in a sample of solution is due to ……	absorption	adsorption	selective absorption	refraction	selective absorption
33	If a sample does not absorbs any wavelengths in the visible region of the spectrum, it will appear----------	white	colourless	a (or) b	black	colourless
34	A colorimeter will consist of ----------	filter	photocell	condensing lens	all the above	all the above
35	In a colorimeter monochromatic light is produced by	photo cell	filter	condensing lens	light source	filter
36	In a photomultiplier tube electrons produced is amplified by …….	cathode	amplifier	dynodes	anode	dynodes
37	Estimation of cadmium is done by ……..	colorimeter	fluorimeter	conductivity meter	spectrophotometer	fluorimeter
38	The wavelength of visible spectrum of light ranges between	576-580nm	600-720nm	400-550nm	380-800nm	380-800nm
39	Prisms which are made up of quartz is for----------	X-rays	gamma rays	UV light	infra red	UV light
40	The condensing lens renders light rays into..... beam before it falls on monochromator	parallel	perpendicular	condense	none of the above	parallel
41	Photo multiplier tube consist of ..... dynodes	6	4	9	10	9
42	The light source of fluorimeter is	tungsten	sodium	hydrogen	mercury	mercury

	…….. lamp					
43	In fluorimeter light from sample pass through .....before PMT	condensing lens	primary filter	secondary filter	photo cell	secondary filter
44	Grating is superior to prism because of …… of the spectrum	linear resolution	monochromatic light	perpendicular light	none of the above	linear resolution
45	Monochromatic light consists of---------- wave length	linear	single	different	condensed	single
46	The negative logarithm of transmittance with inverse relationship is ……	adsorption	concentration	absorbance	path length	absorbance
47	An instrument which separates electromagnetic radiation into wavelengths and selectively measures the intensity of radiation after passing through sample is ….	fluorimeter	spectrophotometer	both a and b	none of the above	spectrophotometer
48	Grating which comprises numerous equi-distant parallel lines ruled on a plane surface is …..	diffraction	refraction	condensing lens	all the above	diffraction
49	Light that cannot be separated into components is …….	linear resolution	monocromatic light	polychromatic light	none of the above	monocromatic light
50	A spectrophotometer with two dispersing elements is called ……..	single beam spectrophotometer	colorimeter	double beam spectrophotometer	None of the above	double beam spectrophotometer
51	The spectrum can be reunited to give the original white light by focusing the components back through ---------	grating	reversed prism	prism	monochromator	reversed prism
52	The wide range of wave length that the light source capable to produce is ……	intensity	polychromatic	monochromatic	spectrum	spectrum
53	On heating sodium metal emits …… colour.	yellow	blue	white	green	yellow
54	In an electromagnetic spectrum---------- have less wave length	infra red	microwave	gamma rays	UV rays	gamma rays

55	When a beam of light is incident on certain substance they emit visible light which is called as …..	fluorescence	luminescence	both a and b	none of the above	fluorescence
56	The optical density of a substance is affected by …….	concentration of the molecule	intensity of the light	amount of impurities	all the above	all the above
57	The light coming out of tungsten lamp will contain ……	monochromatic	polychromatic	single spectrum	none of the above	polychromatic
58	When the length of the medium is increased, then the optical density of the solution ……..	increases	decreases	decreases exponentially	increases exponentially	decreases exponentially
59	In the equation a = E x c x l, E stands for	extinction coefficient	exotic coefficient	electric coefficient	none of the above	extinction coefficient

Unit V

Principles of centrifugations – RCF and sedimentation coefficient. Types of centrifuges – rotors - fixed angle and swinging bucket rotors. Types of Centrifugation – differential, density gradient and ultracentrifugation. Analytical centrifugation.

Centrifugation is a process which involves the application of the centripetal force for the sedimentation of heterogeneous mixtures with a centrifuge, and is used in industrial and laboratory settings. This Process is used to separate two miscible substances, but also to analyze the hydrodynamic properties of macromolecules. More-dense components of the mixture migrate away from the axis of the centrifuge, while less-dense components of the mixture migrate towards the axis. Chemists and biologists may increase the effective gravitational force on a test tube so as to more rapidly and completely cause the precipitate (pellet) to gather on the bottom of the tube. The remaining solution (supernatant) may be discarded with a pipette.

Centrifugation is a technique used for the separation of particles using a centrifugal field. The particles are suspended in liquid medium and placed in a centrifuge tube. The tube is then placed in a rotor and spun at a definitive speed. Rotation of the rotor about a central axis generates a centrifugal force upon the particles in the suspension.Two forces counteract the centrifugal force acting on the suspended particles:

Buoyant force: This is the force with which the particles must displace the liquid media into which they sediment.

Frictional force: This is the force generated by the particles as they migrate through the solution.Particles move away from the axis of rotation in a centrifugal field only when the centrifugal force exceeds the counteracting buoyant and frictional forces resulting in sedimentation of the particles at a constant rate.

Particles which differ in density, size or shape sediment at different rates. The rate of sedimentation depends upon:

1. The applied centrifugal field
2. Density and radius of the particle.
3. Density and viscosity of the suspending medium.

Angular velocity = w radians / second;

since one revolution = 360o = 2p radians.

Basic principles of sedimentation

From everyday experience, the effect of sedimentation due to the influence of the Earth's gravitational field (g¼981 cm s–2) versus the increased rate of sedimentation in a centrifugal field (g>981 cm s–2) is apparent. To give a simple but illustrative example, crude sand particles added to a bucket of water travel slowly to the bottom of the bucket by gravitation, but sediment much faster when the bucket is swung around in a circle. Similarly, biological structures exhibit a drastic increase in sedimentation when they undergo Acceleration in a centrifugal field. The relative centrifugal field is usually expressed as a multiple of the acceleration due to gravity. Below is a short description of equations used in practical centrifugation classes.When designing a centrifugation protocol, it is important to keep in mind that:

• the more dense a biological structure is, the faster it sediments in a centrifugal field;

• the more massive a biological particle is, the faster it moves in a centrifugal field;

• the denser the biological buffer system is, the slower the particle will move in a centrifugal field;

• the greater the frictional coefficient is, the slower a particle will move;

• the greater the centrifugal force is, the faster the particle sediments;

• the sedimentation rate of a given particle will be zero when the density of the particle and the surrounding medium are equal.

Types of Centrifuges and their Uses:

There are threemajor types of centrifuges. They are:

1. Small Bench Centrifuges:

They are used to collect small amount of material that rapidly sediment like yeast cells, erythrocytes etc. They have maximum relative centrifugal field of 3000-7000 g.

2. Large Capacity Refrigerated Centrifuges:

They have refrigerated rotor chamber and have capacity to change rotor chambers for varying size. They can go up to maximum of 6500 g and use to sediment or collect the substances that sediment rapidly like erythrocytes, yeast cell, nuclei and chloroplast.

3. High Speed Refrigerated Centrifuges:

They can generate speed of about 60000g and are used to collect micro-organism,cellular debris, larger cellular organelles and proteins precipitated by ammoniumsulphate.

Design and Types of Rotors:

1. Swinging Bucket Rotors:

The swinging bucket rotor has buckets that start off in a vertical position but during acceleration of the rotor swing out to a horizontal position so that during centrifugation the tube and hence the solution in the tube, is aligned perpendicular to the axis of rotation and parallel to the applied centrifugal field, the tube returning to its original position during deceleration of the rotor.

2. Fixed Angle Rotors:

In fixed angles the tubes are located in holes in the rotor body set at a fixed angle between 14° and 40° to the vertical. Under the influence of centrifugal field, particles move radially outward and have only a short distance to travel before colliding with, and precipitating on, the outer wall of the centrifuge tube. A region of high concentration is formed that has a density greater than surrounding medium, with the result that the precipitate sinks and collects as a small compact
pellet at the outermost point of the tube.

3. Vertical Tube Rotors:

They are considered as zero angle fixed angle rotors in which the tubes are aligned vertically in the body of the rotors at all times.

4. Zonal Rotors:

The zonal rotors may be of the batch or continuous flow type. The former being more extensively used than the latter, and are designed to minimize the wall effect that is encountered in swinging- bucket and fixed angle rotors, and to increase sample size.

5. Elutriator Rotors:

The elutriator is a kind of continuous flow rotor that contains recesses to hold a single conical shaped separation chamber, the apex of which points away from the axis of rotation, and a bypass chamber on the opposite side of the rotor that serves as a counter balance and to provide the fluid outlet.

4. Ultra Centrifuges

(a) Analytical ultracentrifuge:

It is capable of operating at 500000 g. Three kinds of optical systems are available in analytical ultracentrifuges: a light absorption system, and the alternative Schlieren system and Rayleigh interferometric system, both of which detect changes in the refractive index of the solution.In an Analytical ultracentrifuge, a sample being spun can be monitored in real time through an optical detection system, using ultraviolet light absorption and/or interference optical refractive index sensitive system. This allows the operator to observe the evolution of the sample concentration versus the axis of rotation profile as a result of the applied centrifugal field. With modern instrumentation, these observations are electronically digitized and stored for further mathematical analysis. Two kinds of experiments are commonly performed on these instruments: sedimentation velocity experiments and sedimentation equilibrium experiments.Sedimentation velocity experiments aim to interpret the entire time-course of sedimentation, and report on the shape and molar mass of the dissolved macromolecules, as well as their sizedistribution.

The size resolution of this method scales approximately with the square of the particle radii, and by adjusting the rotor speed of the experiment size-ranges from 100 Da to 10 GDa can be covered. Sedimentation velocity experiments can also be used to study reversible chemical equilibria between macromolecular species, by either monitoring the number and molar mass of macromolecular complexes, by gaining information about the complex composition from multi-signal analysis exploiting differences in each components spectroscopic signal, or by following the composition dependence of the sedimentation rates of the macromolecular system,as described in Gilbert-Jenkins theory.

Sedimentation equilibrium experiments are concerned only with the final steady-state of the experiment, where sedimentation is balanced by diffusion opposing the concentration gradients, resulting in a time-independent concentration profile. Sedimentation equilibrium distributions in the centrifugal field are characterized by Boltzmann distributions. This experiment is insensitive to the shape of the macromolecule, and directly reports on the molar mass of the macromolecules and, for chemically reacting mixtures, on chemical equilibrium constants.

The kinds of information that can be obtained from an analytical ultracentrifuge include the gross shape of macromolecules, the conformational changes in macromolecules, and size distributions of Macromolecular samples. For macromolecules, such as proteins, that exist in chemical equilibrium with different non-covalent complexes, the number and subunit stoichiometry of the complexes and equilibrium constant constants can be studied.

(b) Preparative ultracentrifuge:

It can produce relative centrifugal force of about 600000g and its chamber is refrigerated, sealed and evacuated. It is employed for separation of macromolecules/ligands binding kinetic studies,separation of various lipoprotein fractions from plasma and deprotonisation of physiological fluids for amino acid analysis. Preparative ultracentrifuges are available with a wide variety of rotors suitable for a great range of experiments. Most rotors are designed to hold tubes that contain the samples. Swinging bucket rotors allow the tubes to hang on hinges so the tubes reorient to the horizontal as the rotor initially accelerates. Fixed angle rotors are made of a single block of material and hold the tubes in cavities bored at a predetermined angle. Zonal rotors are designed to contain a large volume of sample in a single central cavity rather than in tubes. Some zonal rotors are capable of dynamic loading and unloading of samples while the rotor is spinning at high speed.

Preparative rotors are used in biology for pelleting of fine particulate fractions, such as cellular organelles (mitochondria, microsomes, ribosomes) and viruses. They can also be used for gradient separations, in which the tubes are filled from top to bottom with an increasing concentration of a dense substance in solution. Sucrose gradients are typically used for separation of cellular organelles. Gradients of caesium salts are used for separation of nucleic acids. After the sample has spun at high speed for sufficient time to produce the separation, the rotor is allowed to come to a smooth stop and the gradient is gently pumped out of each tube toisolate the separated components.

Differential centrifugation

Differential centrifugation is a common procedure in microbiology and cytology used to separate certain organelles from whole cells for further analysis of specific parts of cells. In the process, a tissue sample is first lysed to break the cell membranes and mix up the cell contents. The lysate is then subjected to repeated centrifugations, each time removing the pellet and increasing the centrifugal force. Finally,purification may be done through equilibrium sedimentation, and the desired layer is extracted for further analysis.

Separation is based on size and density, with larger and denser particles pelleting at lower centrifugal forces. As an example, unbroken whole cells will pellet at low speeds and short intervals such as 1,000g for 5 minutes. Smaller cell fragments and organelles remain in the supernatant and require more force

and greater times to pellet. In general, one can enrich for the following cell components, in the separating order in actual application:Whole cells and nuclei; Mitochondria, chloroplasts, lysosomes, and peroxisomes; Microsomes (vesicles of disrupted endoplasmic reticulum); and Ribosomes and cytosol.

Sample preparation

Before differential centrifugation can be carried out to separate different portions of a cell from one another, the tissue sample must first be lysed. In this process, a blender, usually a piece of porous porcelain of the same shape and dimension as the container is used. The container is, in most cases, a glass boiling tube.The tissue sample is first crushed and a buffer solution is added to it, forming a liquid suspension of crushed tissue sample. The buffer solution is a dense, inert, aqueous solution which is designed to suspend the sample in a liquid medium without damaging it through chemical reactions or osmosis. In most cases, the solution used is sucrose solution; in certain cases brine will be used. Then, the blender, connected to a high-speed rotor, is inserted into the container holding the sample, pressing the crushed sample against the wall of the container.

With the rotator turned on, the tissue sample is ground by the porcelain pores and the containerwall into tiny fragments. This grinding process will break the cell membranes of the sample's cells, leaving individual organelles suspended in the solution. This process is called cell lysis. A portion of cells will remain intact after grinding and some organelles will be damaged, and these will be catered for in the later stages of centrifugation.

Ultracentrifugation

The lysed sample is now ready for centrifugation in an ultracentrifuge. An ultracentrifuge consists of a refrigerated, low-pressure chamber containing a rotor which is driven by an electrical motor capable of high speed rotation. Samples are placed in tubes within or attached to the rotor. Rotational speed may reach up to 100,000 rpm for floor model, 150,000 rpm for benchtop model (Beckman Optima Max-XP or Sorvall MTX150), and creating centrifugal speed forces of 800,000g to 1,000,000g. This force causes sedimentation of macromolecules, and can even cause non-uniform distributions of small molecules.

Since different fragments of a cell have different sizes and densities, each fragment will settle into a pellet with different minimum centrifugal forces. Thus, separation of the sample into different layers can be done by first centrifuging the original lysate under weak forces, removing the pellet, then exposing the subsequent supernatants to sequentially greater centrifugal fields.

Each time a portion of different density is sediment to the bottom of the container and extracted and repeated application produces a rank of layers which includes different parts of the original sample. Additional steps can be taken to further refine each of the obtained pellets.Sedimentation depends on mass, shape, and partial specific volume of a macromolecule, as well as solvent density, rotor size and rate of rotation. The sedimentation velocity can be monitored during the experiment to calculate molecular weight. Values of sedimentation coefficient (S) can be calculated. Large values of S (faster sedimentation rate) correspond to larger molecular weight. Dense particle sediments more rapidly. Elongated proteins have larger frictional coefficients and sediment more slowly to ensure accuracy.

POSSIBLE QUESTIONS

UNIT-5

1.Define centrifugation.

2.Explain Beer lamberts law.

3.What is RCF.

4.Define sedimentation coefficient.

5.What is rotors.

6.Sketch the types of rotors.

7.Illustrate the principle of differential centrifugation

8.Demonstrate the working of analytical centrifugation

9.Explain about ultra centrifugation

unit-5						
Sl.N	**Question**	**Option 1**	**Option2**	**Option 3**	**Option 4**	**answer**
1	Differential centrifugation is useful for separation of _____	protein	Nucleic acids	chloroplast	Both a and b	Both a and b
2	Analytical centrifuge is useful for _____	Detection of conformational changes	collecting of molecules	Separation of molecules	Pelleting of molecules	Detection of conformational changes
3	Ultracentrifuge rotor made up of	titanium alloy	steel	gold	metal	titanium alloy
4	Gradient centrifugation is useful for separation of different_____ molecules	density	plasmids	structure	mitochondria	density
5	Low spped cnetrifuge rotor made up of	aluminium	steel	copper	gold	aluminium
6	Rotor must be protected from	moisture	rain	temperature	heat	moisture
7	Large volume can be separated by_____rotors	vertical	Fixed angle	swinging bucket	Zonal	Zonal
8	Glass centrifuge tubes are usually used for_____centrifugation	Low speed	All the above	High speed	Ultra centrifuge	Low speed
9	Cell debris may separated at _____ rpm	1000	600	320	550	1000
10	Molecular weight of the macromolecules can be determined by using _____	Low speed centrifuge	Medium speed centrifuge	Ultra centrifuge	High speed centrifuge	Ultra centrifuge
11	Rotors are made up of _____material	brass and steel	copper	steel	silver	brass and steel
12	In fixed – angle rotors the tubes are located in holes in the rotor body set at a fixed angle of between	15^0 and 50^0	14^0 and 40^0 4	16^0 and 90^0	16^0 and 60^0	14^0 and 40^0 4

	_____ to the vertical					
13	Caesium chloride used in the preparation of _____	Enzyme solution	gradient solution	vitamin solution	protein solution	gradient solution
14	Mitochondria may separated at _____ rpm	10000	1500	2000	20000	20000
15	Laboratory centrifuges are _____	refrigerated	chiller	non-refrigerated	ultra centrifuge	refrigerated
16	Ultra centrifuge must be with _____	temperature control	vacuum control	speed control	refrigerated	vacuum control
17	At 100000 g _____ separated	Cystol	DNA	Mitochondria	plasmids	cystol
18	_____ used in Lipoprotein fractionations	Sodium bromide	alcohol	Methanol	mercury	Sodium bromide
19	RCF is an abbreviation of _____	relative centrifugal field	relative computer field	relative centrifuge field	relative common field	relative centrifugal field
20	Cesium salt gradients are used in the separation of _____	Nucleic acid	cell debris	Mitochondria	plasmids	Nucleic acid
21	Sodium iodide is used for separating of _____	DNA and RNA	plasmids	protein	carbohydrate	DNA and RNA
22	Analytical cells are made up of	quartz	steel	glass	plastic	quartz
23	The best rotor used in centrifuge is	Steel	aluminum	titanium alloy	brass	titanium alloy
24	At 1000 g_____separated	Cell debris	DNA	Mitochondria	plasmids	Cell debris
25	Zonal rotors are used in	industry	Clinical labs	Laboratory	Medical field	industry
26	Sedimentation pattern can be obtained in ultracentrifugation is _____	UV system	Louis system	Schlieren system	Lamberts system	Schlieren system
27	Centrifuge consists of _____	rotor, motor	rotor and soft	rotor, motor	motor and soft	rotor, motor

				and soft		and soft
28	Sucrose concentration upto _____ % can be used in centrifugation	100	60	90	80	60
29	Small volume can be separated by_____	Effendorf tube	Fixed angle	swinging bucket	Zonal	Effendorf tube
30	Sedimentation is based on _____	Speed	medium	centrifugal field	moderate	centrifugal field
31	The rate of sedimentation is dependent upon the centrifugal field (G) is determined by _____	G – Wr^2	Gwr^3	G w^2r	Gwr	G w^2r
32	High weight rotor will affect the _____	Separation process	high speed centrifugation	speed	low density	separation process
33	_____ lamp is used in ultracentrifuge	UV	mercury	xenon	tungsten	xenon
34	Swinging rotor is most useful for _____	gradient separation	Pelleting of molecules	isopynic separation	normal sedimentation	gradient separation
35	Sedimentation coefficient may be calculated by using _____	Ultra centrifuge	Low volume centrifuge	low speed centrifuge	high speed centrifuge	Ultra centrifuge
36	Relative molecular mass determination can be done by using _____	Ultra centrifuge	Low volume centrifuge	Low speed centrifuge	High speed centrifuge	Ultra centrifuge
37	Fixed angle rotor is useful for _____	separation of molecules	pelting of molecules	pelting of molecules	all the above	all the above
38	Analytical centrifuge developed by	Johnson	Marry	Louis	Svedberg	Svedberg
39	Vertical rotor is useful for _____	isopynic separation	none of the above	gradient separation	normal separation	isopynic separation
40	Centrifuge mainly used for _____	pelleting and sedimentation		sedimentation	separation of macromolecules	all the above
41	rmax is more in _____	vertical rotor	zonal rotor	swinging	fixed angle	swinging

				bucket rotor	rotor	bucket rotor
42	Sodium bromide is used for separating of _____	DNA	Nucleic acids	lipoprotein	RNA	lipoprotein
43	High speed centrifuge may be _____	refrigerated	none of the above	room temperature	above room temperature	refrigerated
44	Rotor must be _____	corrosive resistant	glass	high weight	plastic	corrosive resistant
45	_____ % of sucrose solution is used density gradients	10-20.0	50-100	20-30	10-60.0	10-60.0
46	Ultra centrifuge must be enclosed with _____	Steel	glass	heavy armour plating	plastic	heavy armour plating
47	High speed centrifuge must be enclosed with _____	Steel	glass	heavy armour plating	plastic	heavy armour plating
48	Sub cellular fraction can be done with _____	Analytical centrifuge	Medium speed centrifuge	Preparative centrifuge	High speed centrifuge	Preparative centrifuge
49	RCF value is _____	1.12 x $10^5 rpm^2 r$	4.12 x $10^5 rpm^2 r$	2.12 x $10^5 rpm^2 r$	3.12 x $10^5 rpm^2 r$	1.12 x $10^5 rpm^2 r$
50	Differential centrifugation is useful for _____	biochemical research	physical and chemical	chemical research	physical research	biochemical research
51	High sedimentation rate may be achieved by _____	Motor	flexible soft	rotor	centrifugal field	centrifugal field
52	Analytical centrifuge is used for _____	Ligand-binding studies	Pelleting of molecules	Separation of molecules	Attachment of molecules	Separation of molecules
53	Rotor may be washed with_____	Distilled water	Salt water	Hot water	Tap water	Distilled water
54	Isopynic solution is_____	Same concentration	None of the above	Different concentration	Gradient	Same concentration
55	Motor and rotor is connected by a_____	Flexible rod	needle	wire	Flexible soft	Flexible soft

56	Cystol may be separated at _____ rpm	10000	4000	20000	100000	100000
57	Caesium chloride is used for separation of _____	DNA	enzymes	Protein	mitochondria	DNA
58	Rotor must be kept in a _____ condition	Dry	liquid	hot	Moisture	dry
59	Gravitational field is _____	9.81cm/s^2	981cm/s^2	98.1cm/s^2	0.981cm/s^2	981cm/s^2
60	_____ will determine the sedimentation rate	Speed	soft	motor	rotor	speed
61	Fixed angle rotors are ideal tool for_____	pelleting	sedimentation	quantification	qualitative	pelleting
62	Analytical centrifuge is useful for _____	Separation of molecules	sedimentation	Determination of purity	Pelleting of molecules	Determination of purity
63	Analytical centrifuge is useful for _____	Determination of relative mass	sedimentation	Separation of molecules	Pelleting of molecules	Determination of relative mass