Department of Rural Technology and Social Development, GGU, Bilaspur (C.G.)

Examination 2013: M.Sc. Ist Semester (AU 6292)

RT-704 (Instrumentation and Techniques)

Time: 3 hours **Max Marks: 60**

Section A

Multiple Choice Questions (02 Mark each) **20 Marks**

1. Centrifugal force is defined by

   (a) $G = \omega^2 r$
2. Hematoxylin is used in histology for

   (a) Staining
3. Filters are used to isolate desired wavelength of radiation in

   (c) Both
4. Centrifugation technique is based on behaviour of particle in

   (a) Centrifugal field
5. Maximum speed of bench top centrifuge is

   (d) None
6. In colorimeter if we have to quantify blue color compound then which color filter we should use-

   (b) Red
7. Primary excitation filter in fluorescence microscope is placed between

   (a) UV source and specimen
8. In phase contrast microscope, phase plate decreases phase change by

   (a) ¼ λ
9. SDS modify the protein in which way-

   (c) Both a and b
10. An atom excites (goes lower energy level to higher) by–

    (a) Absorbing energy

Section B

Short answer type questions: (Attempt any four: 5 Marks each) **20 Marks**

1. Write short note on relative centrifugal force.

   The centrifugal field is generally expressed in multiples of the gravitational field, g ($981 cms^{-2}$). The relative centrifugal field (g), RCF, which is the ratio of the centrifugal acceleration at a specified radius and the speed to the standard acceleration of gravity, can be calculated from the following equation:

$$RCF = \frac{4\pi^2(\text{rev min}^{-1})^2 r}{3600 \times 981} = \frac{G}{g}$$

RCF units are therefore dimensionless (denoting multiples of g) and revolutions per minute are usually abbreviated as r.p.m.: RCF 1.12 X $^{-5}$ r.p.m.^{2}r.

For example:

Question A fixed-angle rotor exhibits a minimum radius, r_{min}, at the top of the centrifuge tube of 3.5 cm, and a maximum radius, r_{max}, at the bottom of the tube of 7.0 cm. See Fig. 3.2a for a cross-sectional diagram of a fixed-angle rotor illustrating the position of the minimum and maximum radius. If the rotor is operated at a speed of 20 000 r.p.m., what is the relative centrifugal field, RCF, at the top and bottom of the centrifuge tube?

Answer The relative centrifugal field may be calculated using the equation:

$RCF = 1{,}12 \times 10^{-5}$ r.p.m.$^2 r$

Top of centrifuge tube:

$RCF = 1{,}12 \times 10^{-5} \times (20\,000)^2 \times 3.5 = 15\,680$

Bottom of centrifuge tube:

$RCF = 1{,}12 \times 10^{-5} \times (20\,000)^2 \times 7.0 = 31\,360$

This calculation illustrates that with fixed-angle rotors the centrifugal field at the top and bottom of the centrifuge tube might differ considerably, in this case exactly two-fold.

2. **Write short note on significance of fixation.**

Fixation

For the various histological and cytological study of a living cell, it is necessary to isolate or to cut, the sections of the tissue from its organ. In such a case the isolated cell or tissue would not remain alive or retain its form which may cause to misresult after examination. For overcoming this problem, the sections or cells require to be fixed more or less in their living form before sectioning.

If a piece of tissue is cut out of a living or recently dead organism and no special case is taken to keep it alive or maintain its structure, it will soon undergo marked its structure, it will soon undergo marked changes. When it left in the air or in fluid it may be shrink or swell or it may attacked by bacteria and moulds. Besides these, the tissue will gradually fall to piece by self digestion or autolysis. Cell contains enzymes collectively known as cathepsin, capable of dissolving their own protein constituents

when they die. For these reason it is usual to treat a piece of a tissue with a fluid called as "Fixative", to embed it after fixation in some solid medium such as paraffin wax, that will hold its constituents parts in the right relation to one another during sectioning, then to section, it next to stain the section and finally to mount the stained section in a medium that senders them transparent.

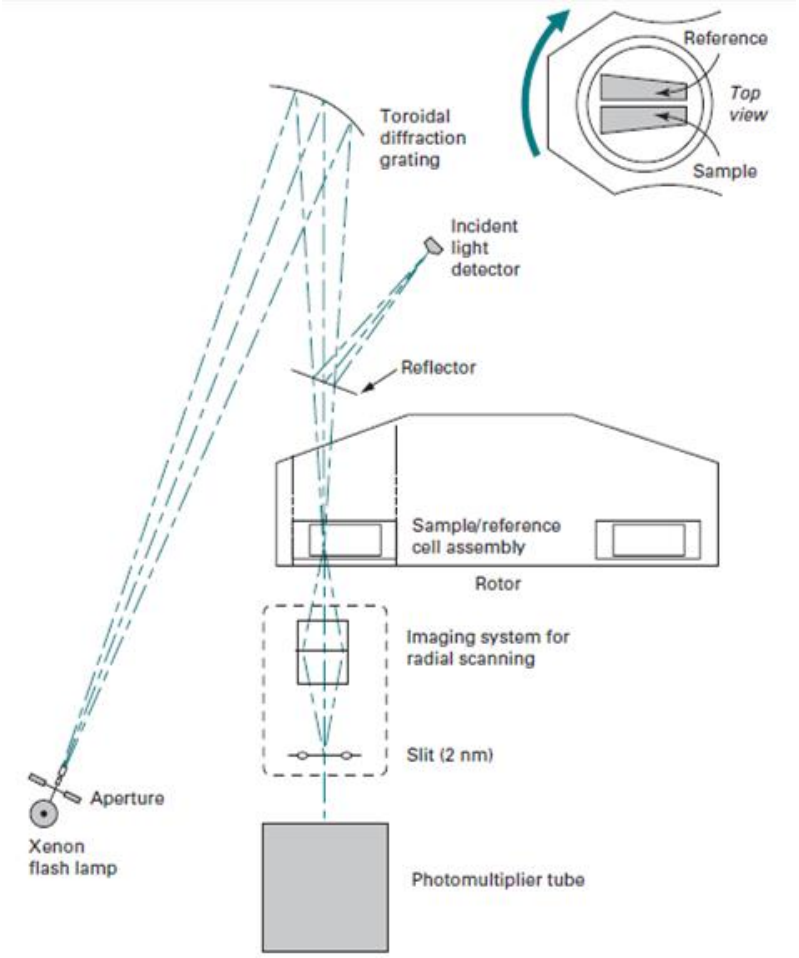
To presence to piece of tissue one requires a fluid that will not shrink or swell or dissolve or distort, will kill bacteria and moulds and will render the autolytic enzyme inactive, such fluid is a preservative. A fixative must do everything that a preservative does, but it addition it must modify the tissue in such a way that they become capable of resisting subsequent treatment of various kinds. Of these treatments the ones that are most likely to cause damage are embedding, sectioning and mounting. Fixation usually makes many tissue constituents (especially chromatin) readily colourable by the suitable dye. The essential function of fixation is the stabilization of protein part of the framework of the cell. The concentration of fixative at or near which it is only used in fixation is termed the "standard concentration".

3. **Briefly describe analytical centrifuge.**

Analytical ultracentrifuges: These instruments are capable of operating at speeds of 70,000 rpm (500000g) and consist of a motor, a rotor contained in a protective armoured chamber that is refrigerated and evacuated and an optical system to observe sedimenting material throughout the duration of centrifugation to determine concentration distribution in the sample at any time during centrifugation. Three types of optical system are available in the analytical centrifuge-a light absorption system, and the alternative schlieren system or Rayleigh system, both of which detect changes in the refractive index of the solution.

The rotor is solid with holes to hold the cells that contain the samples. The tip of the rotor contains a thermistor for measuring temperature. The simplest type of rotor incorporates two cells-the analytical cell and the counterpoise cell and analytical cell. The analytical cells have upper and lower plane windows of optical grade quartz or synthetic sapphire, the latter being used with interference optics, as they have less tendency to distort under a high gravitational field. The rotor chamber contains anupper condensing lens and a lower collimating lens. The former together with a camera lens, focuses light on photographic plate, whereas the latter collimates the light so that the sample cell is illuminated by parallel light. In more advanced instruments, the photographic detector system is replaced by an electronic scanning system.

In the U.V. light absorption system, light of a suitable wavelength is passed through the moving analytical cell containing solution under analysis for example, protein and nucleic acid, and the intensity of the transmitted light is recorded either on a photographic plate or by an automatic single or split beam photoelectric scanning system. The scanner system, unlike the u.v. photographic method has the advantage of allowing direct visualization of the results during the course of experiment and can provide a plot of the concentration of the sample at all points in the analytical cell at any particular time.



Schematic diagram of Analytical Centrifuge.

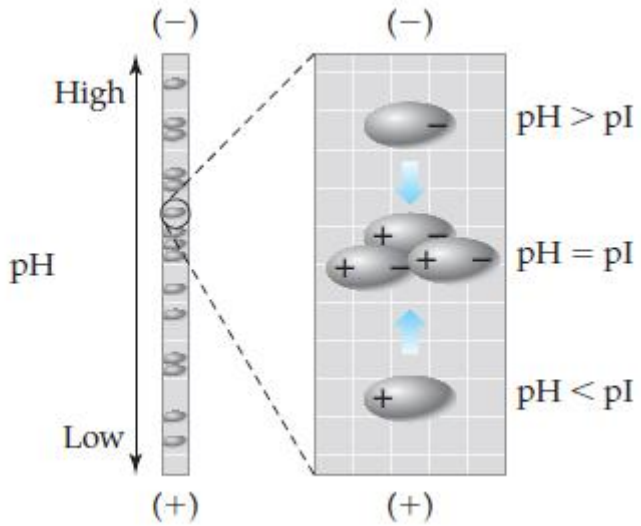
4. **Write in detail about *iso* electric focusing?**

***iso* electric focusing (**IEF) is a method used to separate zwitterions (substances with both acidic and basic groups. Zwitterions are separated in IEF based on their isoelectric points by having these compounds migrate in an electric field across a pH gradient.

In this pH gradient, each zwitterion will migrate until it reaches a region where the pH is equal to its isoelectric point. At this point, the zwitterion will no longer have any net charge and its electrophoretic mobility will become zero, causing the analyte to stop migrating.

The result is a series of tight bands, where each band appears at the point where pH = pI for a given zwitterion. The reason isoelectric focusing produces tight bands for these analytes is that even if zwitterions momentarily diffuses out of the region where the pH is equal to its pI, the system will tend to "focus" the zwitterions back into this region.
This focusing occurs because of the way the pH gradient is aligned with the electric field. High pH occur toward the negative electrode, so as solutes diffuse out of their band and toward this region they will take on a more negative charge and be attracted back to the positive electrode. At the same time, zwitterions that move toward the positive electrode and region of lower pH will acquire a more positive charge and be attracted back toward the negative electrode. It is this focusing property that makes it possible for IEF to separate zwitterions with only very small differences in their pI values.

To obtain a separation in IEF, it is necessary to have a stable pH gradient. This pH gradient is produced by placing in the electric field a mixture of small reagent zwitterions known as *ampholytes*. These are usually polyprotic amino carboxylic acids with a range of p*K*a values.When these ampholytes are placed in an electric field, they will travel through the system and align in the order of their p*K*a values. The result is a *p*H gradient that can be used directly or by cross-linking the ampholytes to a support to keep them stationary in the system.



Iso Electric Focusing

Application of IEF

1. IEF is a valuable tool for separating proteins or other compounds that contain both positive and negative charges. These include some drugs, as well as bacteria, viruses, and cells.

2. Applications of this method range from biotechnology and biochemistry to forensic analysis and paternity testing.

3. IEF is particularly useful in providing high-resolution separations between different forms of enzymes or cell products. For example, it is possible with this method to separate proteins with differences in pI values as small as 0.02 pH units.

5. **Write the principle and application of fluorimetry?**

Fluorescence is a emission phenomenon, the energy transition from a higher to lower state within the molecule concerned being measured by the detection of this emitted radiation rather than the absorption. The excitation event caused by absorption of the electromagnetic radiation the wavelength of absorbed radiation must be at lower values (Higher energy) than the emitted (fluoresced) wavelength. The difference between these two wavelengths is known as stokes shift.The best results are obtained from compounds involving large shifts. It is possible for a compound to absorb in ultraviolet region and emit or fluoresce in the visible.

The ratio-

Q= Quanta fluoresced / Quanta absorbed -------- 1

Gives Q as the quantum efficiency and is usually independent of the exciting wavelength.

At low concentrations, the intensity of fluorescence (I_f) is related to the intensity of the incident radiation (I_0)by-

$I_f = 2.3\ I_0\ \epsilon_\lambda cdQ$

i.e. $I_f\ \alpha c$

where-

c is the concentration of the fluorescence solution (molar),

d is the light path in fluorescing solution (cm), and

ϵ_λ is the molar extinction coefficient coefficient for the absorbing material at at wavelength λ ($dm^3mol^{-1}cm^{-1}$).

The technique of fluorimetry is most accurate at very low concentrations, whereas absorption spectrophotometry is least accurate at these concentrations. For example , 100 pg NADH may be measured fluorimetrically, whereas absorption spectrophotometry requires 100µg NADH. This is due to increased sensitivity.

6. **Shortly describe construction of phase contrast microscope.**

Construction of phase contrast microscope: The phase contrast microscope is a specially designed light microscope with the annular diaphragm and annular phase plate fitted into it. The annular diaphragm is fitted below the condenser. It consists of a circular disc with a curricular annular groove of the annular diaphragm. An image of this illuminated opening is formed at the back focal plane of the objective. At this plane is placed the annular phase plate which may be either a negative phase plate with an elevated (thick) circular area corresponding to the annular groove, or a positive phase plate with a thin circular groove corresponding to the annular groove. The thicker or thinner area in the annular phase plate is termed the conjugate area.

The phase contrast is obtained with the help of phase plate and the annular diaphragm by separating the central or direct rays from the diffracted rays. In an ordinary light microscope, light rays are focused on the object by a condenser lens. If there is no object, there will be no diffracted rays and the light rays will be of one type i.e. central rays (direct rays) alone. If an annular diaphragm is placed below the condenser its image is produced at the rear focal plane of the objective. If the eye piece is removed, the image of the diaphragm is seen at the back of the objective. If an object is placed under the objective, some light ray will be diffracted by it. The annular diaphragm has a slit or annulus, the direct light will form an image of this annulus but diffracted light will be evenly spread over the whole of the back lens of the objective. The phase plate inserted at the rear focal plane of the objective alters the phase by ¼ λ. The direct or central rays came from the annulus of diaphragm pass through the groove of phase plate, whereas the diffracted light rays pass through the region out side the groove. In its passage through phase plate the light is retarded by ¼ of the wave length. Finally the diffracted light ray which is also retarded by ¼ λ by object combines with the retarded direct rays and forms the image. The intensity of the direct light can be reduced by depositing a thin metal film on the groove. In phase contrast microscope, the diaphragm has to be matched to the phase plate in each particular objective. Usually a number of annular diaphragm of different size are arranged on a rotating turret attached to the condenser.

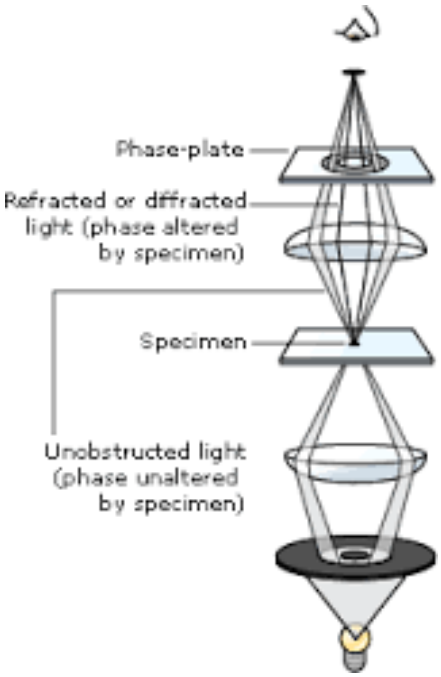


Figure: A part of the phase contrast microscope showing the light rays passing through the annular diaphragm and the annular phase plate. While the direct or central rays pass through the annular groove (conjugate area) of the phase plate the diffracted rays pass through the entire phase plate.

Section C

Long answer type questions: (Attempt any two: 10 Marks each) **20 Marks**

1. Explain the process of fixation and staining and their significance.

Fixation → Small pieces of tissues are best fixed by direct immersion, since this brings the fixative most rapidly into contact with the cells throughout the piece. When it is necessary to fix a piece of tissue a centimeter or more thick, it is best to inject the fixative through a blood vessel in order to send it quickly to all depths. This method of fixation is called Perfusion, it has the disadvantage that the total amount of fixative that can be contained in the blood vessel is usually small, and nothing outside the vessels is fixed until the fixative substance has passed through their walls into the intercellular spaces and thus reached the cells.

PREPERETION OF SLIDE MOUNTING and STAINING

In XYLENE Overnight

↓

100% for 10 min

↓

90% For 10 min

↓

70 % For 10 min

↓

50% For 10 min

↓

30% For 10 min

↓

DH_20 for 10 min

↓

Heamatoxylene (For 2 – 2 ½ min)

↓

Under tap water Running water for 10 min

↓

In 30% For 10 min

↓

In 50% For 10 min

↓

In 70% For 10 min

↓

In EOSIN Dye For 10 min

↓

In 90% For 1 min (only single Dip)

↓

In 100% For 1 min (only single Dip)

↓

Than Again XYLENE (Fresh) for Over night

↓

Than Mount With DPX and Dipped Coverslip in XYLENE (For Removal of Air Bubble) and Put on the Slide of DPX

Significance of Fixation

The purpose of fixation is usually to stabilize the tissue so that they retain as nearly as possible in the form that they had in life, but clearly it is not the purpose to leave their chemical composition unchanged. This would indeed by the negative of fixation, for the cells would be still alive or like recently dead ones. The purpose is to change the chemical composition in such a way as to confer structural stability.

2. **Explain construction, function and applications of transmission electron microscope.**

Construction of EM: The construction of the electron microscope is based on the same principle as that of light microscope. It consists of a central column, electron gun, electromagnetic lenses and a fluorescent screen.

Microscope column: The microscope column consists of an evacuated metal tube. It is housed at the top of electron gun, a number of electromagnetic lenses, viewing screen and photographic plate. These components are aligned one above the other. The microscope column provides a shielding to the operator from X rays that are generated when electrons strike the metal surfaces.

1. Electron gun: the source of electrons is the electron gun and is located at the top of the microscope body. It consists of a hot tungsten filament. It is the source of electrons forming the beam. The tungsten filament is surrounded by a negatively biased shield with an aperture through which the electron beam is drawn off to a grounded and positive anode below.

2. Electromagnetic lenses: The electromagnetic coil corresponds to the condenser, objective and ocular lens of the light microscope. These coils are called condenser, objective and projector coils. Each coils have "coils of electric wires" wounded on hollow metal cylinder, designed in such a way that an electric current passing through the magnetic coil produces an axially symmetrical magnetic field in the centre of the lens. The magnetic field forces the electrons to spiral around a central axis. The electron beam passes through the microscope column and gets deflected by a variable degree depending on the current flowing through the coil of the lens. The microscope is fitted with a variable resistance with a rotary switch control. It controls focusing and image formation in the electron microscope. The magnetic field functions as magnifying lens. Focusing is also performed by adjusting the voltage of current.

3. Fluorescent screen: As electrons are harmful to our eyes, the magnified image is observed on a fluorescent screen. The screen is coated with a chemical which by its excitation forms the image as on the T.V. screen.

4. Transformers: The electron microscope requires high voltage current for the electron gun and electromagnetic coils. This is possible with the aid of high voltage transformers

which can boost the voltage from 220 volts to 50-100 kilo volts. This requires elaborate arrangements and contributes to the massive size of the microscope.

5. Vacuum pumps: The electron microscope requires vacuum inside the micro scope column. This is maintained with the help of high vacuum pumping by diffusion pumps requiring a lot of space. A high vacuum is maintained by keeping every sections of the microscope and every moving control sealed with the help of rubber or plastic rings between adjoining metal surfaces.

6. Water cooling system: The water cooling system is required to prevent overheating of the parts, which is performed by the water pumps and cooling coils.

However, modern electron microscopes have a circulating pump, refrigeration plant and filter system in the form of a closed circuit included in the design of microscope itself.

Working:

The working of the electron microscope is based on the same plan as that of the light microscope. The electrons are used for magnification and image formation. Since, electrons have a much shorter wavelength; they can be used for much better resolution.

Image formation: Image formation occurs by electron scattering. Electrons strike the atomic nuclei and get dispersed and the dispersed electrons form the image. The electron image is converted into a visible form by projecting on a fluorescent screen.

Electrons in the form of collimated beam pass through the condenser coil and fall on the object. They get scattered and transmitted through the object and pass through the objective coil, which magnifies the image of the object. The projector coil further magnifies the image and projects it on the fluorescent screen or photographic film. The image formation occurs when the energy of the electrons is transformed into visible light through excitation of the chemical coating of the screen. Those electrons which reach to fluorescent screen form the bright spots while the areas where the electrons do not reach the screen forms the dark spots. The area which scatter electrons are term electron dense. The varying degree of intensity of electrons forms the image with varying degree of grey. Electron dispersion, however, due to the atomic nuclei which consist of protons and neutrons. The higher the atomic number greater the dispersion. Since, biological materials generally have a low atomic number; the dispersion of electrons is poor. Very poor dispersion means very poor contrast in image formation. In order to increase contrast, a number of salt with high atomic number are used. Such salts can be used during the process of fixation or staining.

3. **Write in detail about principle, functioning and application of atomic absorption spectrophotometry.**

PRINCIPLE of atomic absorption spectrophotometry

Molecules give rise to band spectra and atom clearly defined line spectra. Atomic absorption spectra give black lines against a bright back ground. Some elements

particularly metals, have an important role to play in biological systems, weather as simple cofactors as enzymes, the central atom in biological macromolecules such as iron in haemoglobin or magnesium in chlorophyll, or as toxic substances that affect metabolism. Use of atomic spectrometry will enable data to be obtained that are important in understanding the biological roles of these elements.

In a spectrum of an element, the wavelength at which absorption are absorbed are associated with transitions where the minimal energy change occurs. For example transition of electrons from s to p orbit or D- lline transition of sodium atom that gives rise to emission of orange light. When electron transition occur in an atom they are limited by the availability of an empty orbital or level. An orbital or level could not be overfilled without contravening the Pauli Exclusion Principle. In order for energy changes to be minimal, transition tend to occur between levels close together in energy terms these limitations means that emissions and absorption lines are absolutely characteristics of the element concerned. The wavelength emitted from excited atoms may be identified using a spectrometer. In atomic absorption spectrophotometer atom concentrations are not measured directly in solution. The atoms have to be volatilised either in a flame or electrothermally in an oven. In this state the elements will readily emit or absorb monochromatic radiation at the appropriate wavelength. Usually nebulisers will be used to spray the standard or test solution into the flame through which the light is passed. Alternatively the light beam is passed, in an oven, through a cavity containing the vaporised material.

Functioning of atomic absorption spectrophotometer

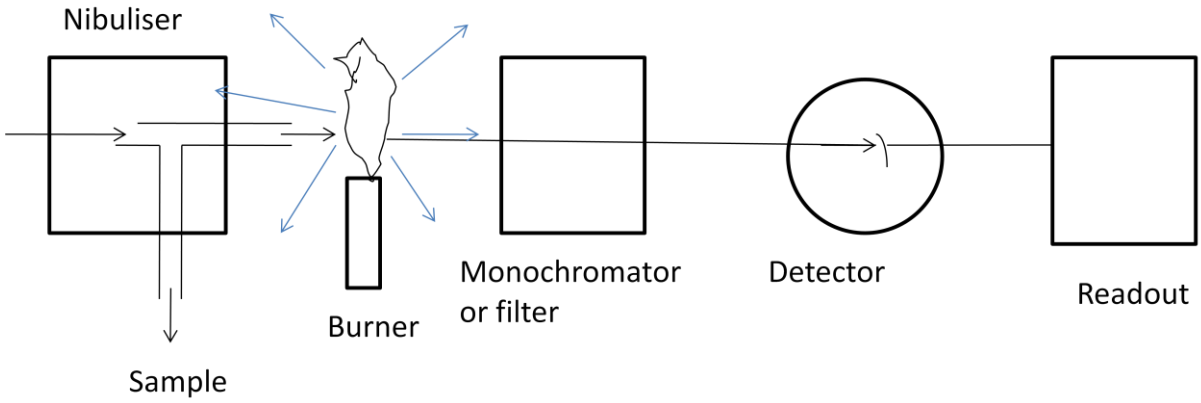
In this instrument either a double monochromator with a source of white light or a hollow cathode discharge lamp is used to produce radiation in a very narrow band width. Discharge lamps emit radiation at a wavelength specific for the element being assayed. This specificity can be obtained only from a pure sample of the element that is excited electrically to produce an arc spectrum of that element.

Nebulisers and burners are similar to the emission devices but 10cm flames are often used to obtain an increased optical length. Both single and double beam instruments are available. Double beam instrument incorporating a chopper to give intermittent pulses and prevent stray light from the frame reaching the detector. Most useful wavelength range is 190 to 850nm.

Flameless instrument

A flameless atomic absorption spectrophotometer incorporates a graphite tube as an oven. Which may be heated electrically to 3000ºC monochromatic light specific to the element being assayed is produced either by a hollow cathode discharge lamp or an

electrodeless discharge lamp. The graphite tube forms an optical cavity, in which sample resides and through which monochromatic radiation is passed.



Main components of Atomic Absorption spectrophotometer

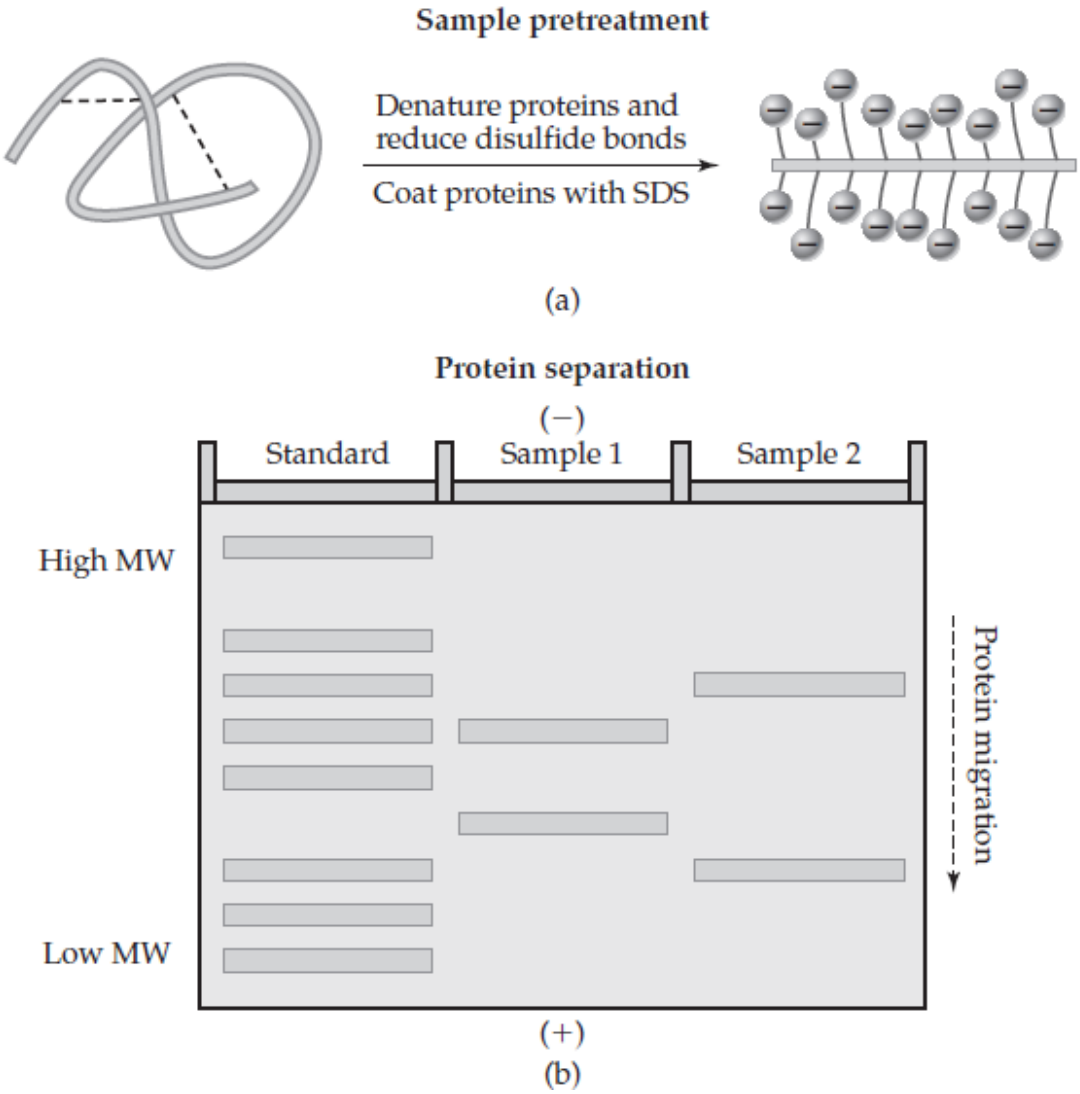
Application

1. This technique is widely used in clinical laboratories, for determination of metals in body fluid.
2. In physiological and pharmacological research sodium, potassium, calcium, magnesium, cadmium and zinc may be measured directly, but copper, lead, iron and mercury require prior extraction from the biological source.
3. The methods are also widely used in determination of elements in soil and plant materials and after suitable ashing procedures, may be used for metals in macromolecules, organells, cells and tissues.

4. **Explain SDS-PAGE. Write difference between native and SDS-PAGE.**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis, or **SDS-PAGE.** In SDS-PAGE, the proteins in a sample are first denatured and their disulfide bonds broken through the use of a reducing agent. This pretreatment converts the proteins into a set of single-stranded polypeptides. These polypeptides are then treated with *sodium dodecyl sulfate* (*SDS*), a surfactant with a nonpolar tail and a negatively charged sulfate group. The nonpolar end of this surfactant coats each protein, forming roughly linear rods that have an exterior layer of negative charge. The result for a mixture of proteins is a series of rods with different lengths but similar charge-to-mass ratios. Then these protein rods are passed through a porous polyacrylamide gel in the presence of an electric field. The negative charges on these rods (from the SDS coating) cause them to all move toward the positive electrode, while the pores of the gel allow small rods to travel more quickly to this electrode than large rods. At the end of an SDS-PAGE run, the positions of protein bands from a sample are compared to those obtained for known protein standards applied

to the same gel. This comparison is made either qualitatively or by preparing a calibration curve.



Diagrammatic representation of SDS PAGE

Difference between Native PAGE and SDS PAGE

S.No.	Particulars	Native PAGE	SDS PAGE
1.	Basis of separation	The molecules separated in an electric field on the basis of their net charge and size of the protein.	As all the proteins have similar charge to mass ratio they are separated on the basis of their mass.
2.	Treatment of samples	Proteins are not treated with any compound prior to separation	Proteins are treated with reducing agent such as mercapto-ethanol and SDS (detergent).

3.	Application	Used for separation of proteins	Widely used for determine purity of protein, molecular mass of unknown sample, and the number of polypeptide units with a protein.