Biochemistry
Quantitative analysis
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BIOCHEMISTRY

- Is the study of the chemical make-up and functional behaviour of the living matter at the molecular level
- Term was introduced by ***Carl Neuberg*** in 1903
- **Scope** :

Helps to understand

- The physiological reactions and transformations taking place in living systems
- Study of intermediary metabolism
- Physical chemistry of biomolecules
- Organic chemistry of enzyme – catalysed reactions
- Biosynthesis of molecules
- Bioenergetics
- Cellular structure & control mechanisms

1: COLOURIMETRY

- Is the quantitative analysis by which the concentration of a coloured solute in solution is determined by ***comparing the colour intensity of the test solution against the colour intensity of a standard solution of known concentration of the same solute***

- **Principle** : Works based on ***Beer – Lambtert law*** which holds that *"the intensity of a beam of monochromatic light passing through a solution decreases inversely with solute concentration and decreases exponentially with an increase in the thickness of a solution"*
- **Procedure:** A light beam of the same intensity

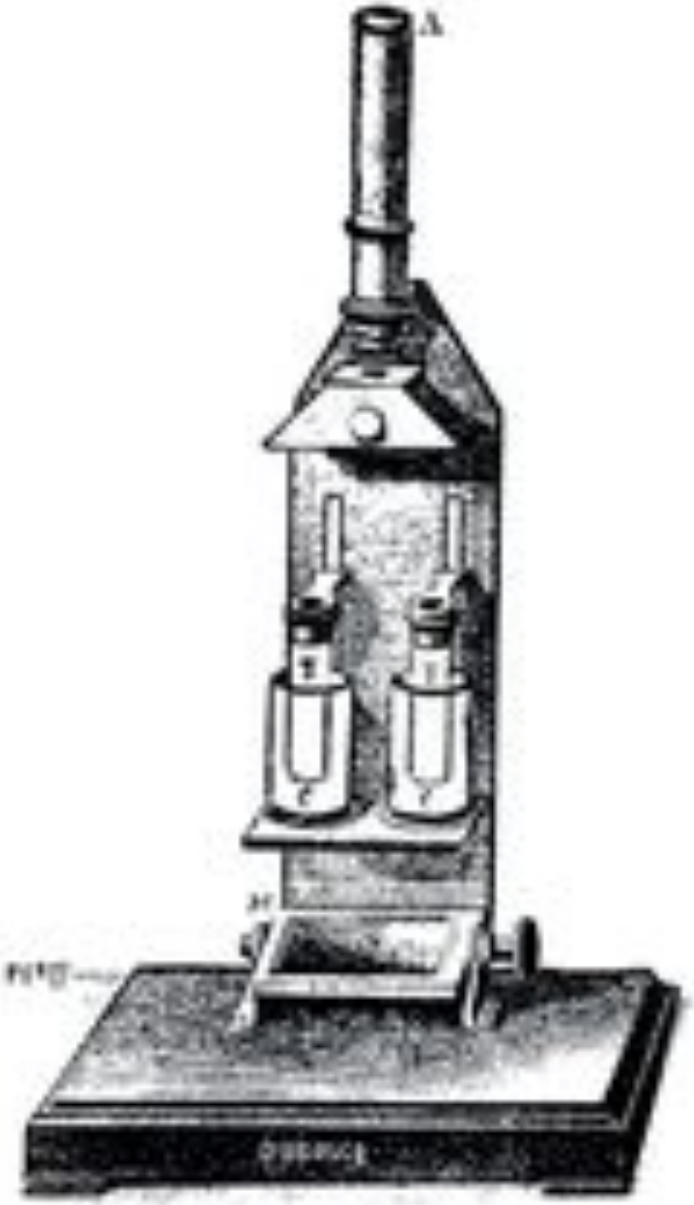
- The instrument used for measuring the color intensities is known as **CLOURIMETER**

Visual Colourimeter

- Used in visual colourimetry where the intensities of the colours of the standard and the test solutions are exactly matched by adjusting the thickness of the test solutions and keeping the thickness of the standard solution constant

- Visible light form incandescent electric lamp ------> Passes through a glass filter ------> Passes through the cups (one containing standard and other containing test solutions with a glass rod immersed in it) ------> Enters to the optical system composed of prisms and lenses ------> Converges on the circular plate

- Here, one half of the glass plate get illuminated by



- $S_1C_1 = S_2C_2$

$$C_1 = \frac{S_2C_2}{S_1}$$

S_1= Colour intensity of the test solution

S_2= Colour intensity of the standard solution

C_1= Concentration of the test solution

C_2= Concentration of the standard solution

Photoelectric Colourimeter

- Used in Photoelectric colourimetry where the intensities of the colours of the standard and the test solutions are measured by keeping the wavelength and intensity of the incident light and thickness of both the solutions constant and the distance traversed by light through the two solutions equal

- Tungsten filamemt electric lamp as light source ------> Coloured optic filters composed of a prism to select the most desirable wavelength of light which the solute would absorb at the maximum rate ------> Light transparent Cuvettes ------> Photocell to convertlight enegy to electrical energy ------> Measuring device

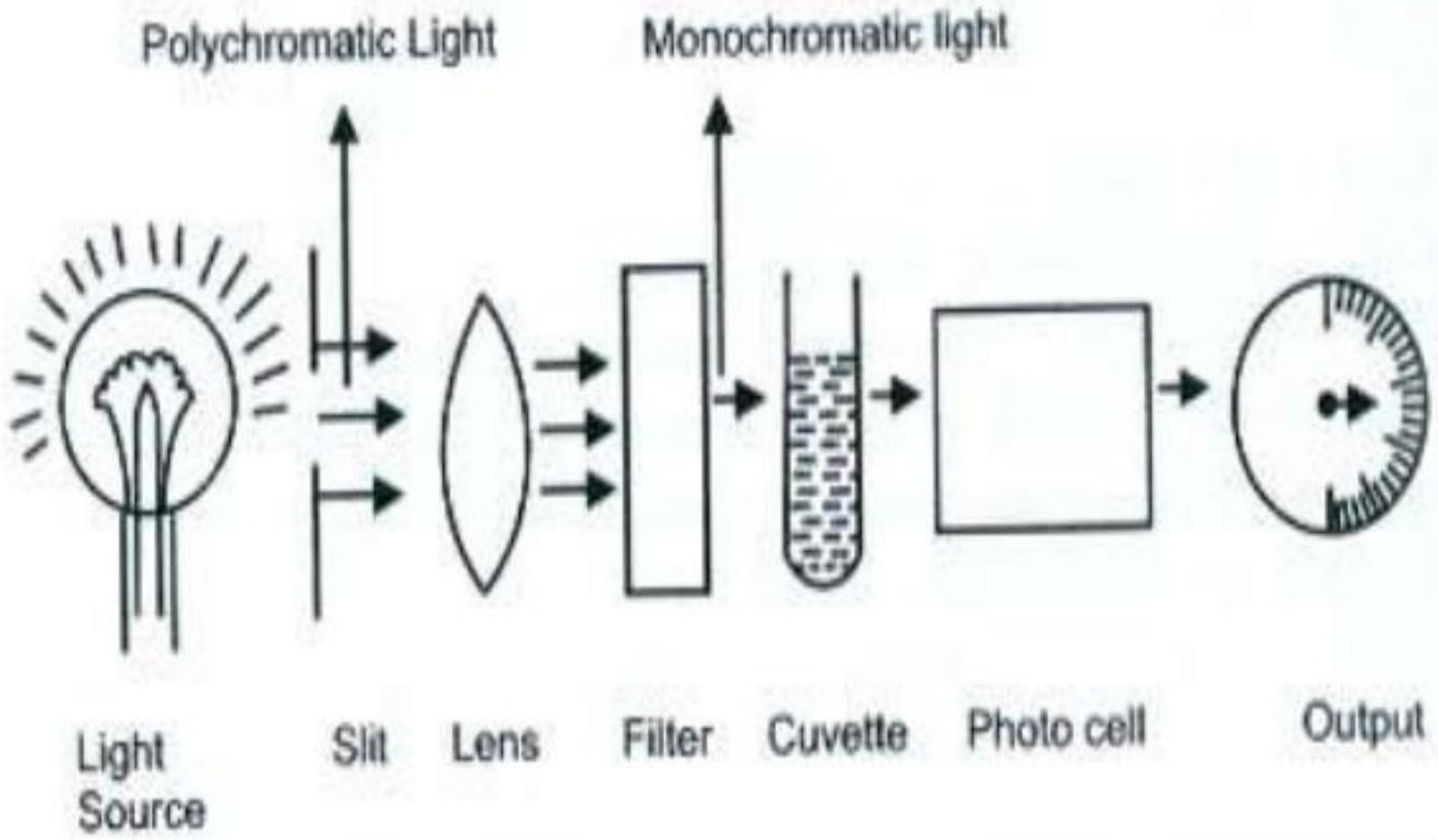
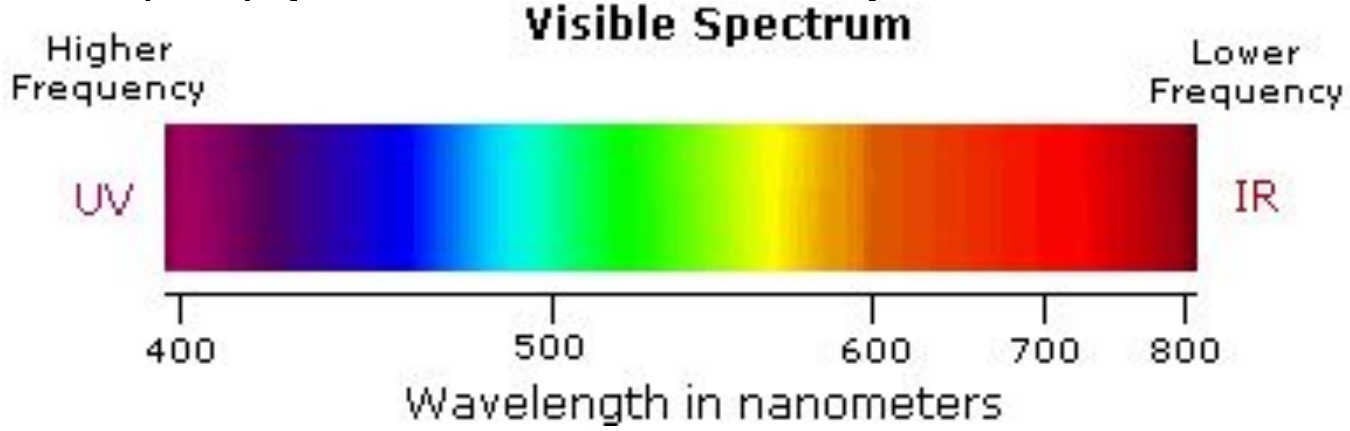


Fig. 27.1: Parts of the colorimeter

i. Light rays from the lamp pass through a prism system to produce a spectrum.
ii. From this spectrum, the filters allow only specific wavelength to pass across them.
iii. The selected wavelength is transmitted to the test solution in the cuvette
iv. The light emerging from the test solution is focused by a lens system to the photoelectric cell
v. The potential difference generated by the photoelectric cell is measured by the potentiometer

- Although we see sunlight (or white light) as uniform or homogeneous in color, it is actually composed of a broad range of radiation wavelengths in the ultraviolet (UV), visible and infrared (IR) portions of the spectrum.

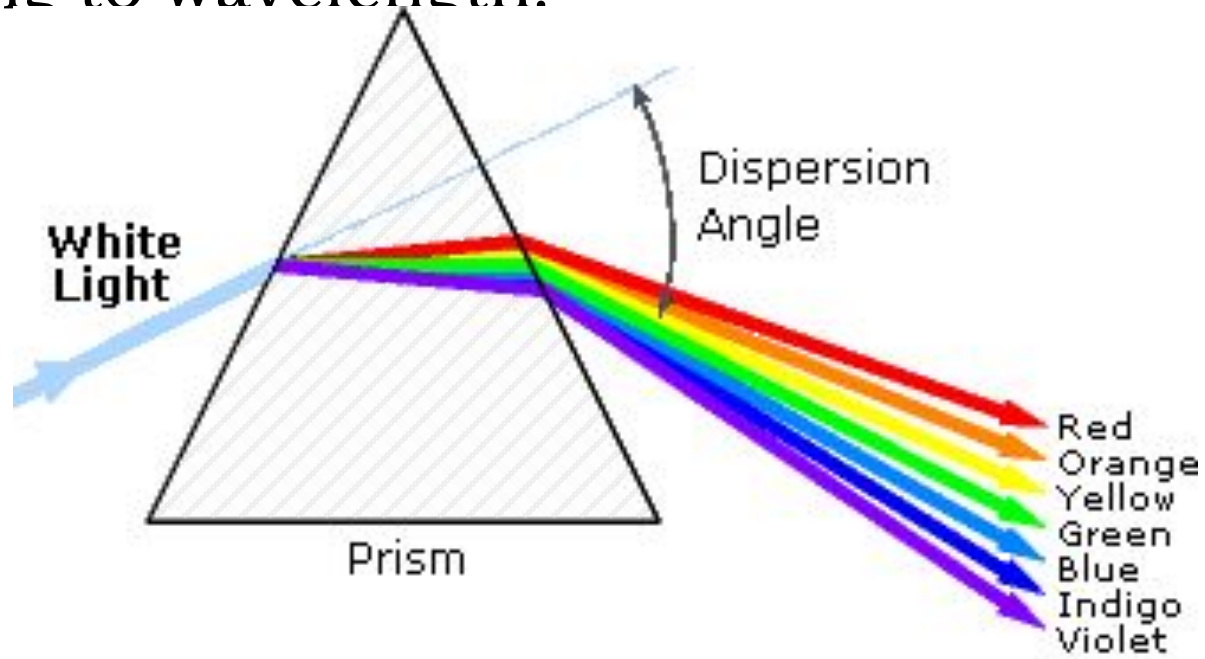


Violet: 400 - 420 nm
Indigo: 420 - 440 nm
Blue: 440 - 490 nm
Green: 490 - 570 nm
Yellow: 570 - 585

Light spectrum and their wavelengths

Wavelength (nm)	Region name	Observed
<380	Ultraviolet	Invisible
380-440	Visible	Violet
440-500	Visible	Blue
500-580	Visible	Green
580-600	Visible	Yellow
600-620	Visible	Orange
620-750	Visible	Red
800-2500	Near-infrared	Not visible

- The component colors of the visible portion can be separated by passing sunlight through a prism, which acts to bend the light in differing degrees according to wavelength.



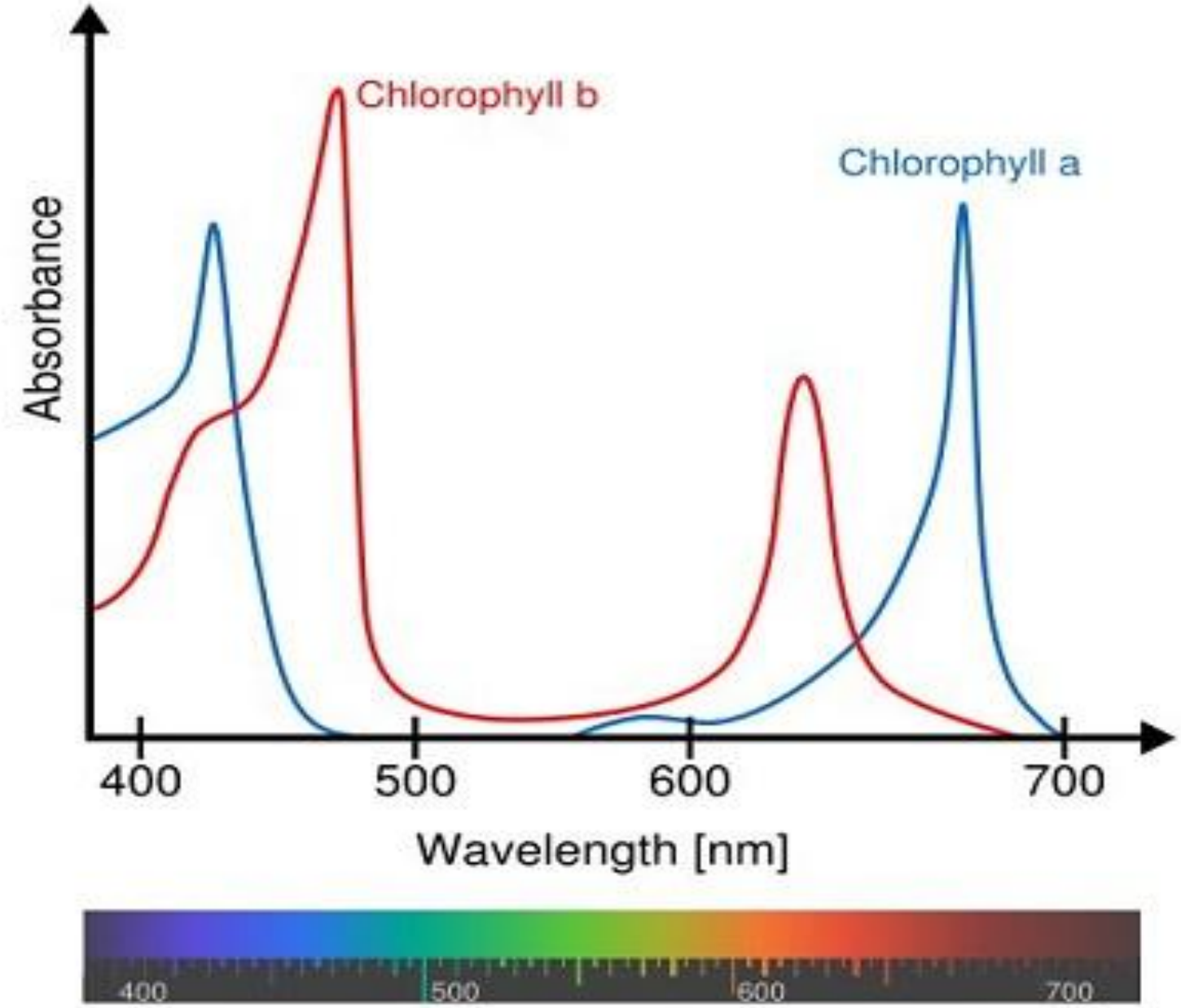
- When white light passes through or is reflected by a colored substance, a characteristic portion of the mixed wavelengths is absorbed. The remaining light will then assume the complementary color to the wavelength(s) absorbed.

- The absorption or the transmission of a certain substance can be determined by the observed color. For instance, a solution sample that absorbs light over all visible ranges (i.e., transmits none of visible wavelengths) appears black in theory. On the other hand, if all visible wavelengths are transmitted (i.e., absorbs nothing), the solution sample appears white.

- If a solution sample absorbs red light (~700 nm), it appears green because green is the complementary color of red. Visible spectrophotometers, in practice, use a prism to narrow down a certain range of wavelength (to filter out other wavelengths) so that the

Colors and complimentary colors of visible spectrum

Color of the solution/ solution color transmitted	Filter used/ color absorbed	Wavelength (nm)
Yellow blue	Violet	380 – 430
Yellow	Blue	430 – 475
Orange	Green blue	475 – 495
Red	Blue green	495 – 505
Purple	Green	505 – 555
Violet	Yellow green	555 – 575
Blue	yellow	575 – 600
Green blue	Orange	600 – 650
Blue green	Red	650 - 750


Chlorophyll b
Chlorophyll a
Absorbance
400
500
600
700
Wavelength [nm]
400
500
600
700

2: SPECTROPHOTOMETRY

- Every chemical compound absorbs, transmits, or reflects light (electromagnetic radiation) over a certain range of wavelength. Spectrophotometry is a method to measure how much a chemical substance absorbs light by measuring the intensity of light as a beam of light passes through sample solution.

OR

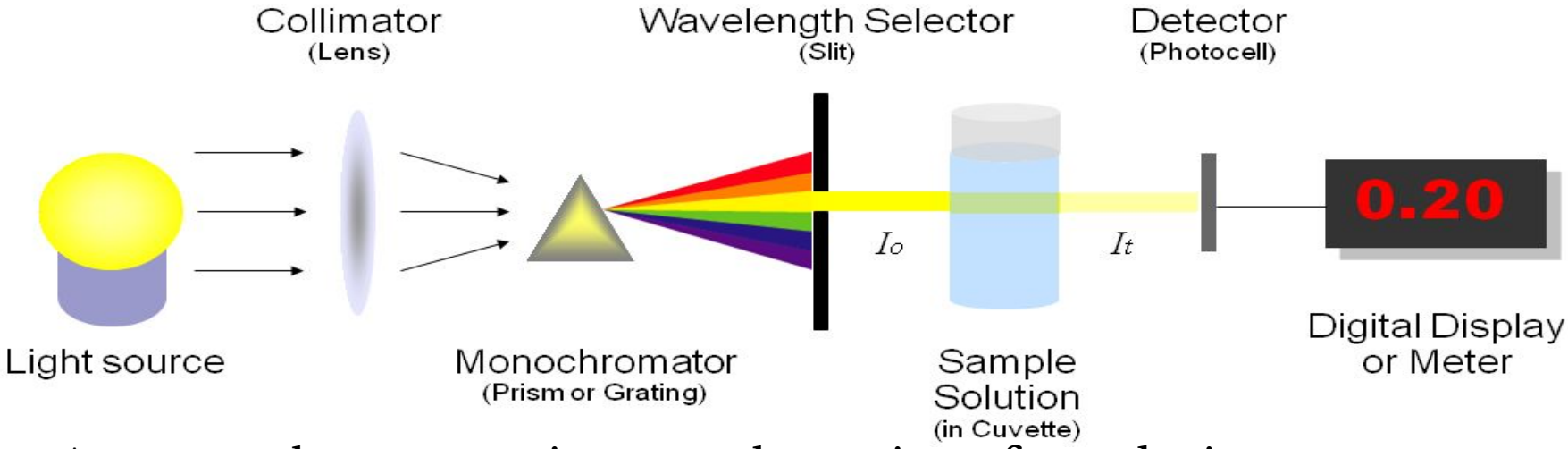
- **Is a technique used for the characterization, identification and quantitative estimation of biomolecules in a solution by measuring the intensity of the light transmitted**

- **Principle** : Works based on ***Beer – Lambtert law*** which holds that *"the intensity of a beam of monochromatic light passing through a solution decreases inversely with solute concentration and decreases*

SPECTROPHOTOMETER

- A spectrophotometer is an instrument that measures the concentrations of a chemical substance by measuring the amount of photons (the intensity of light) absorbed after it passes through sample solution.
- Depending on the range of wavelength of light source, it can be classified into two different types:
  - **UV-visible spectrophotometer**: uses light over the ultraviolet range (185 - 400 nm) and visible range (400 - 700 nm) of electromagnetic radiation spectrum.
  - **IR spectrophotometer**: uses light over the

spectrophotometers



A spectrophotometer, in general, consists of two devices; a spectrometer and a photometer.

Spectrometer: It produces a desired range of wavelength of light. First a collimator (lens) transmits a straight beam of light (photons) that passes through a monochromator (prism) to split it into several component wavelengths (spectrum). Then a wavelength selector (slit) transmits only the desired wavelengths.
Photometer: After the desired range of wavelength of light passes through the solution of a sample in cuvette, the photometer detects the amount of photons that is

Differences between Colorimetry and Spectrophotometry

- A colorimeter quantifies color by measuring three primary color components of light (red, green, blue), whereas spectrophotometer measures the precise color in the human-visible light wavelengths.
- Colorimetry uses fixed wavelengths, which are in the visible range only, but spectrophotometry can use wavelengths in a wider range (UV and IR also)
- Colorimeter measures the absorbance of

3: CHROMATOGRAPHY

- Is an analytical technique used for the separation, purification and identification of the different chemical constituents of complex mixtures or solutions
- First introdused by Mikhail Tsewett in 1906 for separation of chlorophylls and other pigments from plant extract. He packed a vertical column with calcium carbonate and mixtures to be separated was poured on it after dissolving it in petroleum ether. He observed different coloured zones on the adsorbent column by the separation of different pigments

PRINCIPLE

- Relies on the differential distribution of the constituents of a mixture or solution between two immiscible phases due to their selective adsorption and differential migration
  - The two immiscible phases is represented by a ***mobile phase*** (Gas/ Liquid) and a ***stationary phase*** (Solid/ Liqid)
  - The sample to be analysed is called ***solute***. The stationary phase is contained in a region called ***sorbent***. The mobile phase moves the solute through the sorbent.
- Thus the propelling force for the movement of the solute is provided by the carrier mobile phase and the selective impedence for slowing down the movement of the selected molecules

PROCEDURE

- As the mobile phase carrying the solute passes through the stationary phase, the molecular components of the solute get distributed between the two phases. Molecules which have high affinity for the stationary phase, preferentially interact and bind with it. Hence they get retarded in their movement throgh the chromatographic system.
- The other components, having only low affinity for the stationary phase spent more time with the mobile phase. So they are rapidly removed from the chromatographic system. This is called ***elusion***

Based on phase there are two types of chromatography

1: **LIQUID CHROMATOGRAPHY** – where mobile phase is always a liquid and stationary phase is a liquid/solid

- Liquid – Liquid ***partition*** chromatography

  Eg: Paper chromatography & Thin layer chromatography

- Liquid – solid ***adsorption*** chromatography

  Eg: Column chromatography (affinity,

Based on phase there are two types of chromatography

2: **GAS CHROMATOGRAPHY** – where mobile phase is always a gas and stationary phase is a liquid/solid

- Gas – Liquid ***partition*** chromatography
- Gas – Solid ***adsorption*** chromatography

PAPER CHROMATOGRAPHY

- Is a type of liquid-liquid partition chromatography
- Stationary phase is a film of immobilized water adsorbed on a special type of paper mat or filter paper usually made of highly purified cellulose
- The mobile phase is an organic solvent such as butanol, acetic acid

APPARATUS

- Support for chromatographic paper, Solvent trough and an air tight glass cylinder

PROCEDURE

- Sample is spotted at the lower end of the paper & dried

- The solvent ascends through the paper by capillary action, carrying the various components of the sample along with it
- Components move at different rates on account of their differential distribution between the two immiscible solvents
- After some time paper is removed and dried. Coloured compounds form a number of coloured spots while the others can be detected by spraying a reagent

CALCULATION

- In partition chromatography the distribution can be quantified by using Partition coefficient, K_D

$$K_D = \frac{\text{Concentration of solute in stationary phase}}{}$$

COLUMN CHROMATOGRAPHY

- Is a type of liquid-solid adsorption chromatography
- The stationary state is formed by a vertical glass/steel tube filled with inert adsorbent such as Calcium carbonate, Aluminium oxide, Cellulose etc.
- The mobile phase may be petroleum, ether, benzene, acetone etc in which the molecular mixture to be separated is dissolved

PROCEDURE

- The mobile phase along with solute is poured down and continuously washed through the column (Elusion)
- Different components of the mixture adsorb

4: ELECTROPHORESIS

- Is the analytical technique applied for the separation of charged molecules based on their movement in an electric field. It is widely used to analyse and separate biomolecules like peptides, proteins, nucleotides, nucleic acid etc.

PRINCIPLE

- Every biological molecules in a solution carry a net electric charge except at isoelectric point. When two electrodes are placed in such a solution and an electric field is applied, the positively charged molecules will move to the cathode and negative ones to anode.
- Separation usually occurs at pH values above or below to the iso electric point (pH at which the net charge of the particle is zero)

[The greater the charge and lesser the molecular weight, the greater would be the electrophoretic mobility of the particle ie, a molecule with a double charge will move twice the speed of a molecule with single charge]

Velocity of the movement of the particle V = Eq/f

E = Electric field in volts, q= net charge of the molecule, f = frictional coefficient due to shapeand mass of the molecule

APPARATUS

1: Inert supporting media for the movement of charged particles – Wetted filter paper or a Polymerised gel like matrix (starch, cellulose-acetate, acrylamide, agarose, silica etc.)

Types of Electrophoresis

- Paper electrophoresis
- Poly Acrylamide Gel Elecrophoresis [PAGE]
- Agarose Gel Electrophoresis [AGE]
- Sodium Dodecyl Sulphate Poly Acrylamide Gel Elecrophoresis [SDS -PAGE]
- Pulse Field Gel Electrophoresis [PFGE]
- Capillary Elecrophoresis [CE]

Poly Acrylamide Gel Elecrophoresis [PAGE]

- A type of electrophoresis where poly acrylamide is used as the supporting medium
- Prepared by the free radical polymerisation of acrylamide and the cross linking agent methylene-bis-acrylamide
- Produced either as column or slab gels. Slab gels are more advantageous since several samples can be analysed at a time
- The gel forms a spongy network through which the macromolecules can only squeeze through it
- Movment is based on size and molecular weight of the migrating molecules

PROCEDURE

- The slab is inserted vertically between two buffer reservoirs. The upper reservoir usually contain an anode and lower one a cathode.
- The sample to be analysed is placed on the slab as a spot or as a thin band and a voltage is applied
- The charged molecules of the sample move to their respective poles

Application

- Mainly used to separate large molecules such as proteins

Agarose Gel Elecrophoresis [AGE]

- Is an ideal technique for the analysis of the DNA fragments and also for the identification, assaying and characterisation of enzymes like topoisomerase. The technique is simole, rapid and relatively less expensive

PRINCIPLE

- When an electric current is applied through the agarose gel containing charged molecules like DNA or RNA, The positively charged cations move towards the cathode and negatively charged anions move to the anode

PROCEDURE

- Here agarose is used as the standard supporting medium

  [Agarose is a polysaccharide extracted from sea weed. It is a purified form of agar and is a linear polymer of galactose derivatives]
- Gels are prepared by dissolving agarose in powder in a warm electrophoretic buffer. It is then cooled to 50°C and poured between glass plates to form gel slabs
- The sample to be analysed is placed in a sample well and voltage is applied until separation is completed
- After separation nucleic acids are visualised by soaking the gel in

  solution of ethidium bromide. It displays enhanced fluorescence when intercalated between stacked

QUALITATIVE TESTS

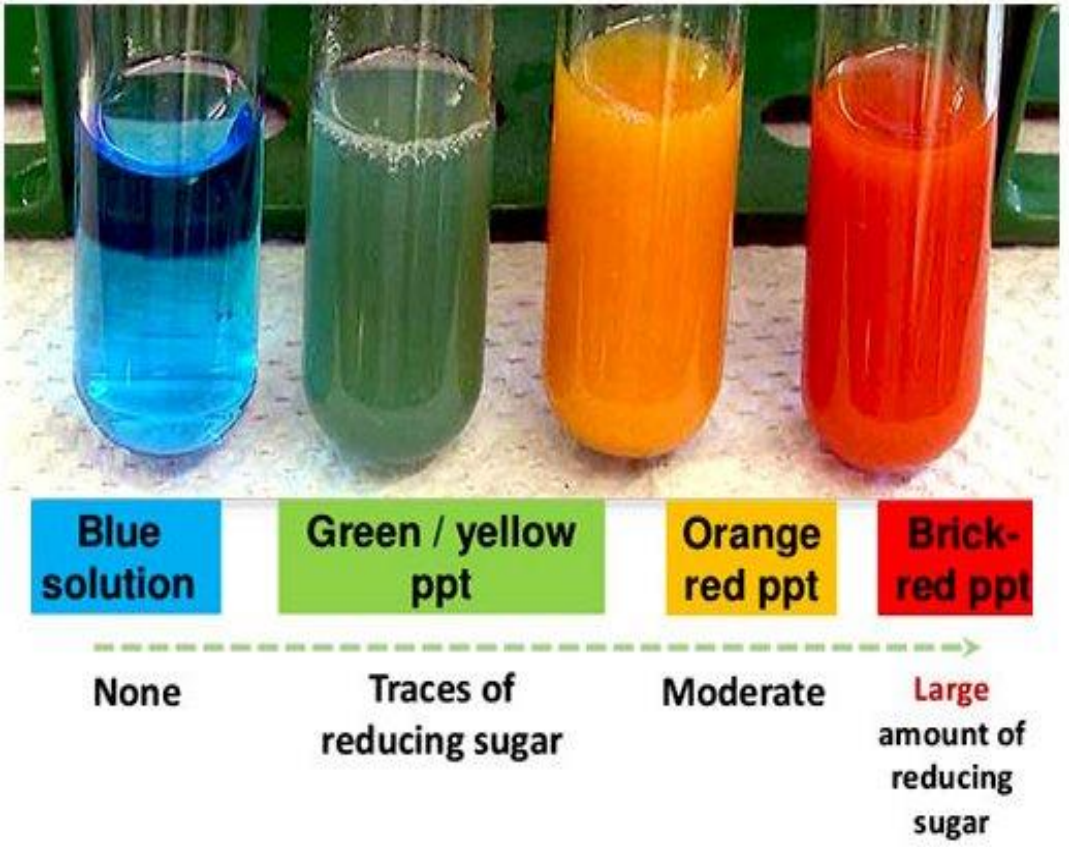
BENEDICT'S TEST

Principle:

- Carbohydrates with free aldehyde or ketone groups have the ability to reduce solutions of various metallic ions.
- Reducing sugars under alkaline conditions tautomerise and form enediols.
- Enediols are powerful reducing agents.
- They reduce cupric ions to cuprous form and are themselves converted to sugar acids.
- The cuprous ions combine with OH- ions to form yellow cuprous hydroxide which upon heating is converted to red cuprous oxide.

Procedure

- Take 5 ml of Benedict's reagent.
- Add 8 drops of carbohydrate solution.
- Boil over a flame or in a boiling water bath for 2 minutes.
- Let the solution cool down.



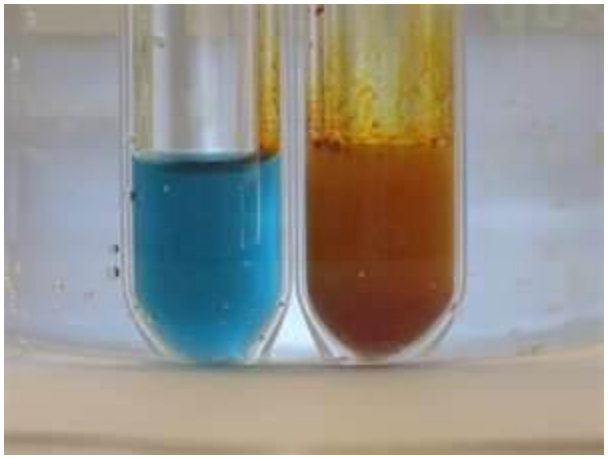
Interpretation:

- Benedict's test is a semi quantitative test. The color of the precipitate gives a rough estimate of a reducing sugar present in the sample.
- **Green color - Up to 0.5 G% (+)**
- **Green precipitate - 0.5-1.0 G% (++)**
- **Yellow precipitate -1.0-1.5 G% (+++)**
- **Orange precipitate- 1.5-2.0 G% (++++)**
- **Brick red precipitate- > 2.0 G %(+++++)**

- This is a very simple and effective method of ascertaining the presence or the amount of glucose in the urine and can be done by the diabetic himself

- It is essential that the above test be performed two hours after a meal. In the initial stages of the disease, a diabetic does not lose sugar in his urine, when on empty stomach. Hence if the Benedict's test is performed in the fasting state, it is possible to miss the diagnosis of the disease

2) FEHLING'S TEST



3)**BARFOED'S TEST**

Principle: Aldoses and ketoses can reduce cupric ions even in acidic conditions. This test is used to distinguish reducing mono saccharides from disaccharides by controlling pH and time of heating. Mono saccharides react very fast whereas disaccharides react very slowly.

BARFOED'S TEST

Procedure:

- To 2 ml of Barfoed's reagent, add 2 ml of carbohydrate solution.
- Keep the test tubes in the boiling water bath for 3 minutes.
- Cool under running water.
- Over-heating should be avoided.

A scanty brick red precipitate is observed in a positive reaction.

4. SELIWANOFF'S TEST

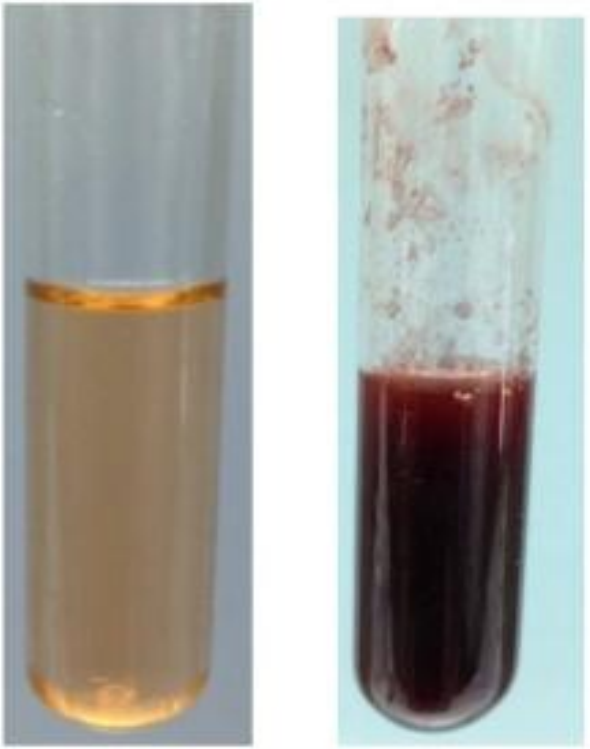
Principle:

Keto hexoses on treatment with hydrochloric acid form 5-hydroxy methyl furfural which on condensation with resorcinol gives a cherry red colored complex.

SELIWANOFF'S TEST

Procedure:

- To 3 ml of Seliwanoff reagent add 1ml of fructose.
- Boil for 30 seconds only.
- Cool the solution.



A cherry red color is observed in a positive reaction.

SELIWANOFF'S TEST

Interpretation:

- This test is given positive by ketohexoses so it is answered by fructose, sucrose and other fructose containing carbohydrates.
- This test distinguishes between glucose and fructose.
- Overheating of the solution should be avoided.
- Upon continuous boiling, aldoses get converted to ketoses and give a positive reaction with Seliwanoff reagent.

BIURET TEST

XANTHOPROTEIN TEST

NINHYDRIN TEST