NATIONAL UNIVERSITY OF PHARMACY
Department of Educational and Information Technologies

BIOPHYSICS, PHYSICAL METHODS OF ANALYSIS

Lecture 10

Physical methods of analysis.

Plan of the Lecture

1. Classification of the methods of analysis.
2. Optical methods.
3. Microscopy.
4. Refractometry.
5. Polarimetry.
6. Colorimetry.
7. Spectral analysis.
8. Other methods.

Purpose of the lecture is

- to review the common methods of analysis of drugs.

Physical methods of analysis: optical methods

1. Classification of the methods of analysis.
2. Microscopy.
3. Refractometry.
4. Polarimetry.
5. Colorimetry.

Methods of analysis are divisible into:

1.Chemical methods:

- gravimetry
- titrimetry

2. Physical and chemical methods:

- electrochemical
- photometric
- kinetic
- chromatographic

3. Physical methods:

- spectral
- optical
- diffraction
- other

4. Biological methods

Physical and physical- chemical methods of analysis

are based on the using of the relationship between measurable physical properties of substances and qualitative and quantitative composition.

Physical properties of materials are measured with different instruments (tools), so physical and chemical methods are called **instrumental**.

Advantages of PhMA

1. High sensitivity.
2. High selectivity.
3. Ability of rapid testing.
4. Ability of remote testing.
5. Ability of non-destructive testing.
6. Ability of local testing.

Disadvantages of PhMA

1. Measurement errors are higher reproducibility of measurements is lower than for classical chemical methods.
2. High complexity and high cost of equipment.

Three branches of Microscopy

- Optical
- Electron
- Scanning Probe
- Optical and Electron microscopy measure refraction, diffraction, and reflection of the source radiation
  - Optical uses white light, fluorescent light, or lasers
  - Electron uses electromagnetic radiation/electron beams
- Scanning uses a physical probe to interact with the surface of the specimen

Imaging Techniques

Technique	Image Formed By	Lowest Resolvable Unit	Approx Lower Limit
Optical Microscopy	Light Rays	Microns (μm)	1 μm (monochromatic light)
Confocal Microscopy	Coherent Light Source (Laser)	Microns (μm)	.1 μm (X-Y Direction)
Transmission Electron Microscopy (TEM)	Electrons	Angstroms (Å)	2 Å (high resolution TEM)
Scanning Electron Microscopy (SEM)	Electrons	Nanometers (nm) to Angstroms (Å)	10 nm (100 Å)
Atomic Force & Scanning Tunneling Microscopies (AFM/STM)	Molecular Mechanical Probes	Angstroms (Å)	40 Å (theoretical)

Optical Microscopy

Properties of light

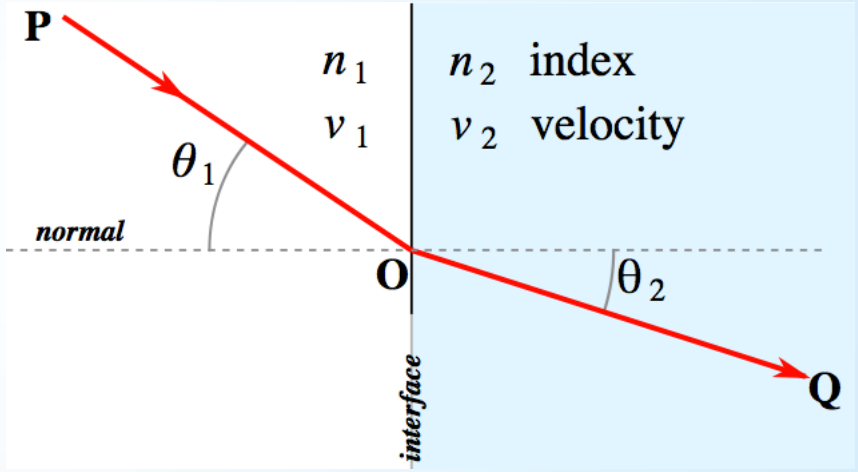
- Reflection
- Refraction
- Numerical Aperture

Refraction

- Change in the direction of a wave (light) due to a change in speed
- The straw in the picture looks bent because the light is bending as it moves from the water to the air

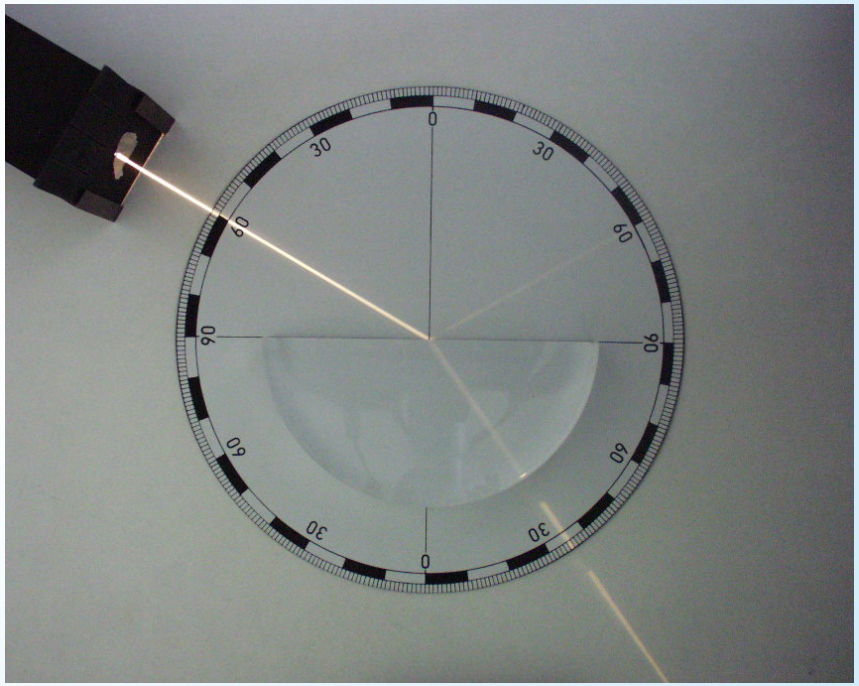
Refractive Index (RI)

- RI of a material a measure of the speed of light in material
- RI is the ratio of the velocity of light in a vacuum to the speed of light in the specified material
- Incident angle (θ_1) is related to the refraction angle (θ_2) by Snell's Law
- $n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$
- Used in calculating focusing power of lenses and dispersion properties of prisms



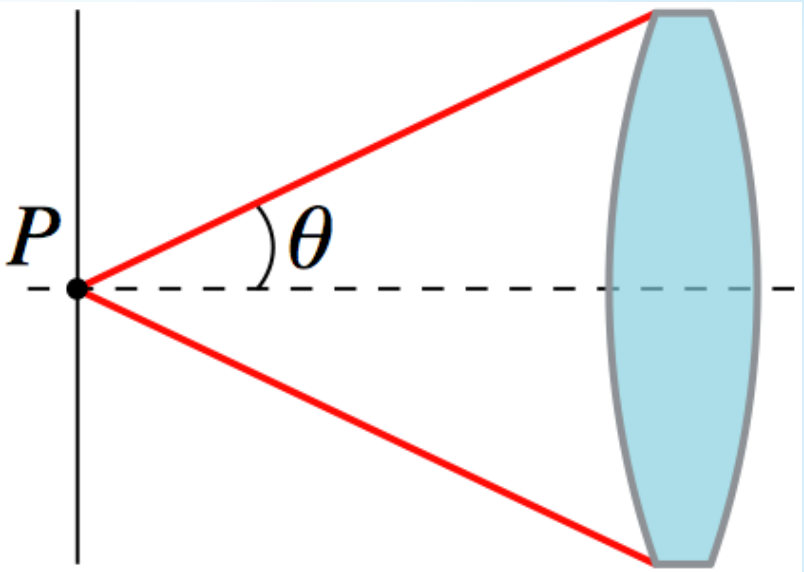
Reflection

- Reflection is defined as a change in direction of a wave at an interface between 2 different media so that the waveform returns to the media from which it came
- Used in focusing light waves to increase transmitted light



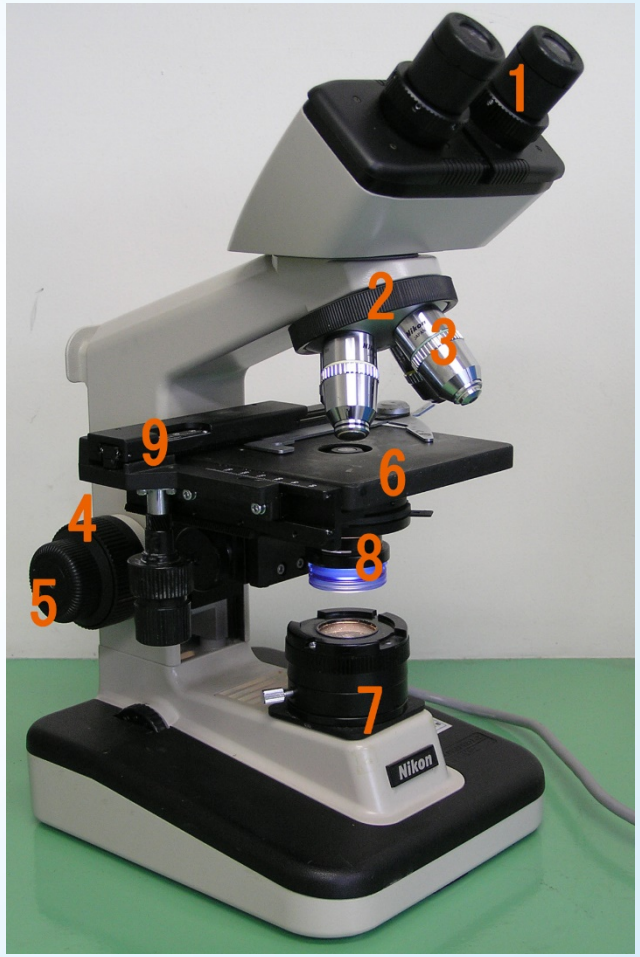
Numerical Aperture

- NA of a microscope objective is a measure of its ability to gather light
- The more light (higher NA) the better the resolving power of the lens
  - Better resolution
- NA = (n)sin(θ)
  - n = Refractive Index
  - θ = ½ the maximum cone of light than can enter the lens
- Usually the NA of an objective increases with its magnifying power.
- The smallest detail that can be resolved is proportional to:
  - λ/NA



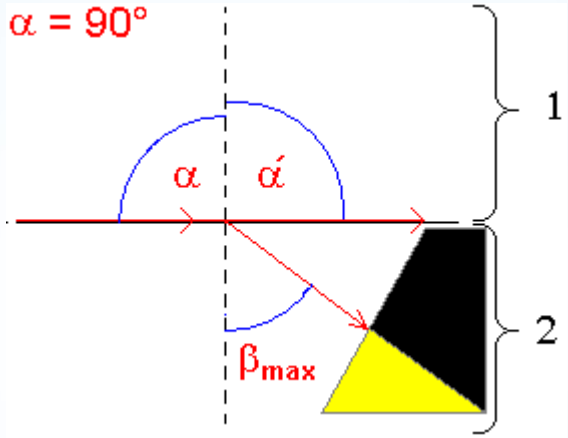
Optical Microscope

1. Ocular lens
2. Objective turret
3. Objective
4. Coarse Adjustment
5. Fine Adjustment
6. Stage
7. Light source
8. Condenser
9. X-Y Control



Refractometry

- principal - detection of limiting angle



- applications - verifying purity of liquid materials

The speed of light in a vacuum is always the same, but when light moves through any other medium it travels more slowly since it is constantly being absorbed and reemitted by the atoms in the material. The ratio of the speed of light in a vacuum to the speed of light in another substance is defined as the **index of refraction** (aka **refractive index** or ***n***) for the substance.

$$\text{refractive index of substance } (n) = \frac{\text{speed of light in a vacuum}}{\text{speed of light in substance}} \quad \text{(Eqn 1)}$$

Whenever light changes speed as it crosses a boundary from one medium into another its direction of travel also changes, i.e., it is refracted (Figure). (In the special case of the light traveling perpendicular to the boundary there is no change in direction upon entering the new medium.) The relationship between light's speed in the two mediums (v_A and v_B), the angles of incidence (q_A) and refraction (q_B) and the refractive indexes of the two mediums (n_A and n_B) is shown below:

$$\frac{v_A}{v_B} = \frac{\sin\theta_A}{\sin\theta_B} = \frac{n_B}{n_A} \quad \text{(Eqn 2)}$$

Thus, it is not necessary to measure the speed of light in a sample in order to determine its index of refraction. Instead, by measuring the angle of refraction, and knowing the index of refraction of the layer that is in contact with the sample, it is possible to determine the refractive index of the sample quite accurately. Nearly all refractometers utilize this principle, but may differ in their optical design.

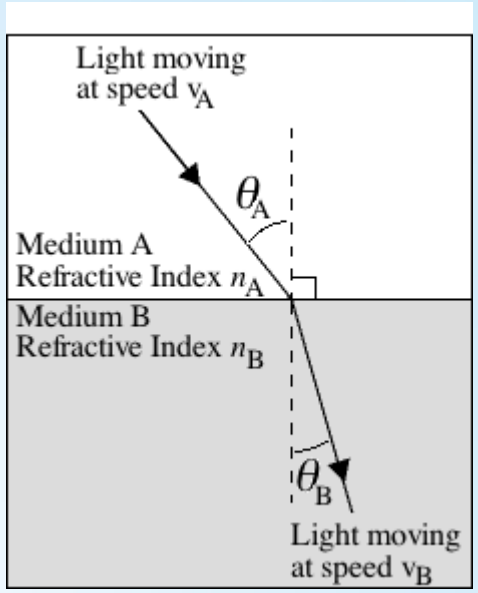


Figure. Light crossing from any transparent medium into another in which it has a different speed, is refracted, i.e., bent from its original path (except when the direction of travel is perpendicular to the boundary). In the case shown, the speed of light in medium A is greater than the speed of light in medium B.

In the Abbe' refractometer the liquid sample is sandwiched into a thin layer between an illuminating prism and a refracting prism (Figure 2). The refracting prism is made of a glass with a high refractive index (e.g., 1.75) and the refractometer is designed to be used with samples having a refractive index smaller than that of the refracting prism. A light source is projected through the illuminating prism, the bottom surface of which is ground (i.e., roughened like a ground-glass joint), so each point on this surface can be thought of as generating light rays traveling in all directions. Inspection of Figure 2 shows that light traveling from point A to point B will have the largest angle of incidence (q_i) and hence the largest possible angle of refraction (q_r) for that sample. All other rays of light entering the refracting prism will have smaller q_r and hence lie to the left of point C. Thus, a detector placed on the back side of the refracting prism would show a light region to the left and a dark region to the right.

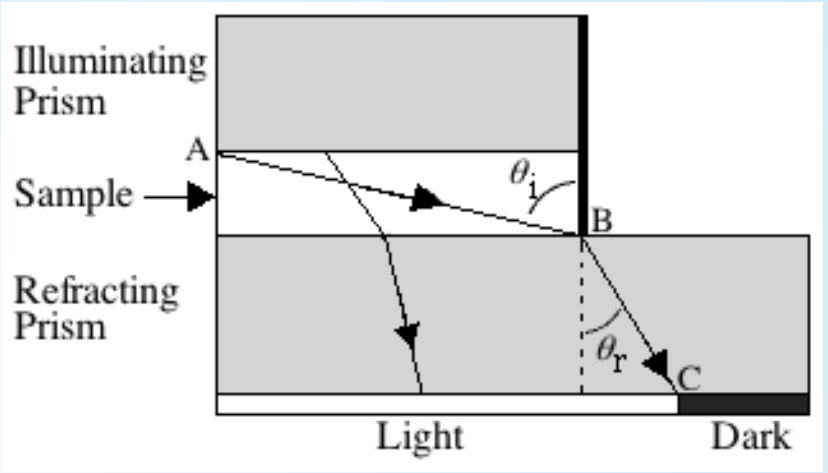


Figure. Cross section of part of the optical path of an Abbe refractometer. The sample thickness has been exaggerated for clarity.

Samples with different refractive indexes will produce different angles of refraction (see Equation 2 above and recall that the angle of incidence and the refractive index of the prism are fixed) and this will be reflected in a change in the position of the borderline between the light and dark regions. By appropriately calibrating the scale, the position of the borderline can be used to determine the refractive index of any sample. In an actual Abbe' refractometer there is not a detector on the back of the refracting prism, and there are additional optics, but this is the essential principle.

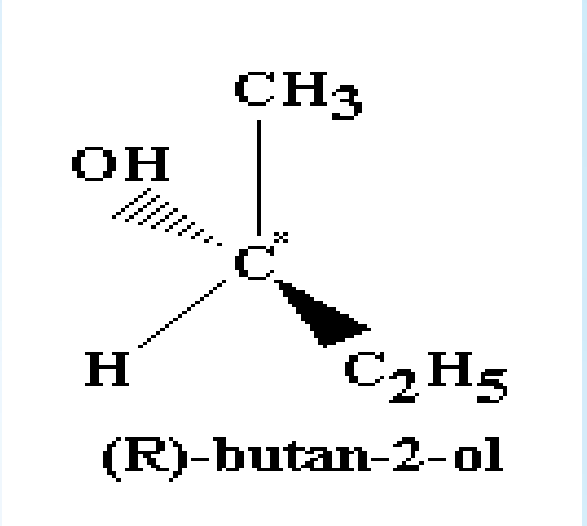
In most liquids and solids the speed of light, and hence the index of refraction, varies significantly with wavelength. (This variation is referred to as **dispersion**, and it is what causes white light moving through a prism to be refracted into a rainbow. Shorter wavelengths are normally refracted more than longer ones.) Thus, for the most accurate measurements it is necessary to use monochromatic light. The most widely used wavelength of light for refractometry is the sodium D line at 589 nm.

If white light were used in the simple Abbe' refractometer optics shown in Figure 2, dispersion would result in the light and dark borderline being in different places for different wavelengths of light. The resulting "fuzziness" of the borderline would make precise work impossible. However, many Abbe' refractometers are able to operate satisfactorily with white light by introducing a set of "compensating prisms" into the optical path after the refracting prism. These compensating prisms are designed so that they can be adjusted to correct (i.e., compensate for) the dispersion of the sample in such a way that they reproduce the refractive index that would be obtained with monochromatic light of 589 nm, the sodium D line.

As mentioned earlier, the speed of light in a substance is slower than in a vacuum since the light is being absorbed and reemitted by the atoms in the sample. Since the density of a liquid usually decreases with temperature, it is not surprising that the speed of light in a liquid will normally increase as the temperature increases. Thus, **the index of refraction normally decreases as the temperature increases** for a liquid. For many organic liquids the index of refraction decreases by approximately 0.0005 for every 1 °C increase in temperature. However for water the variation is only about -0.0001/°C.

Many refractometers are equipped with a thermometer and a means of circulating water through the refractometer to maintain a given temperature. Most of the refractive index measurements reported in the literature are determined at 20 or 25 °C.

Polarimetry



(R)-butan-2-ol

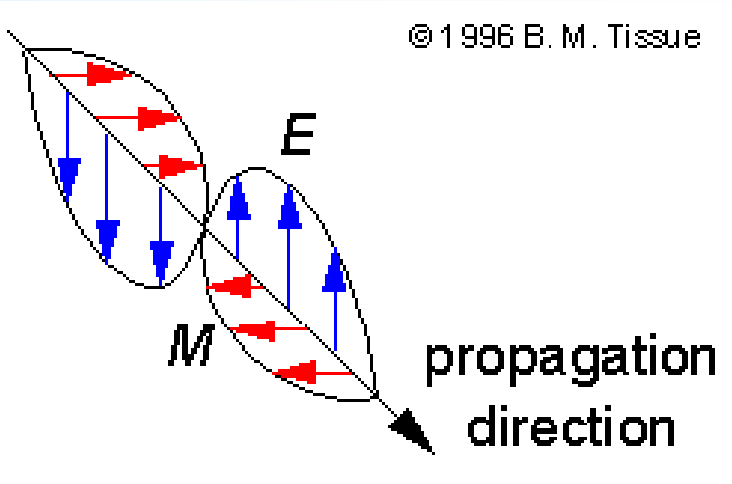
- It’s a type of qualitative and quantitative technique,used mostly for **optically active** compounds

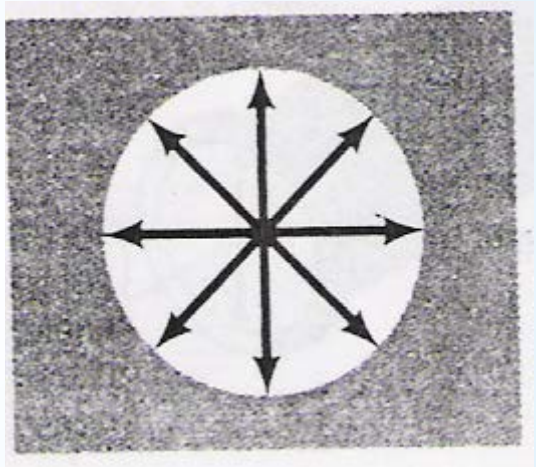
- the tendency of the molecules to **rotate** the plane of plane polarized light (clockwise or anticlockwise) and the **extent of rotation** is measured

- these properties are unique for a molecule, thus polarimetry can be used to **identify** and **estimate** the compounds

Plane polarized light:

A beam of light consists of two mutually perpendicular oscillating fields:electric field and magnetic field





In a beam of ordinary light (ex from bulb) the oscillation of electric field are occurring in <u>all possible planes</u> perpendicular to the direction of propagation, c/a **Unpolarized light**

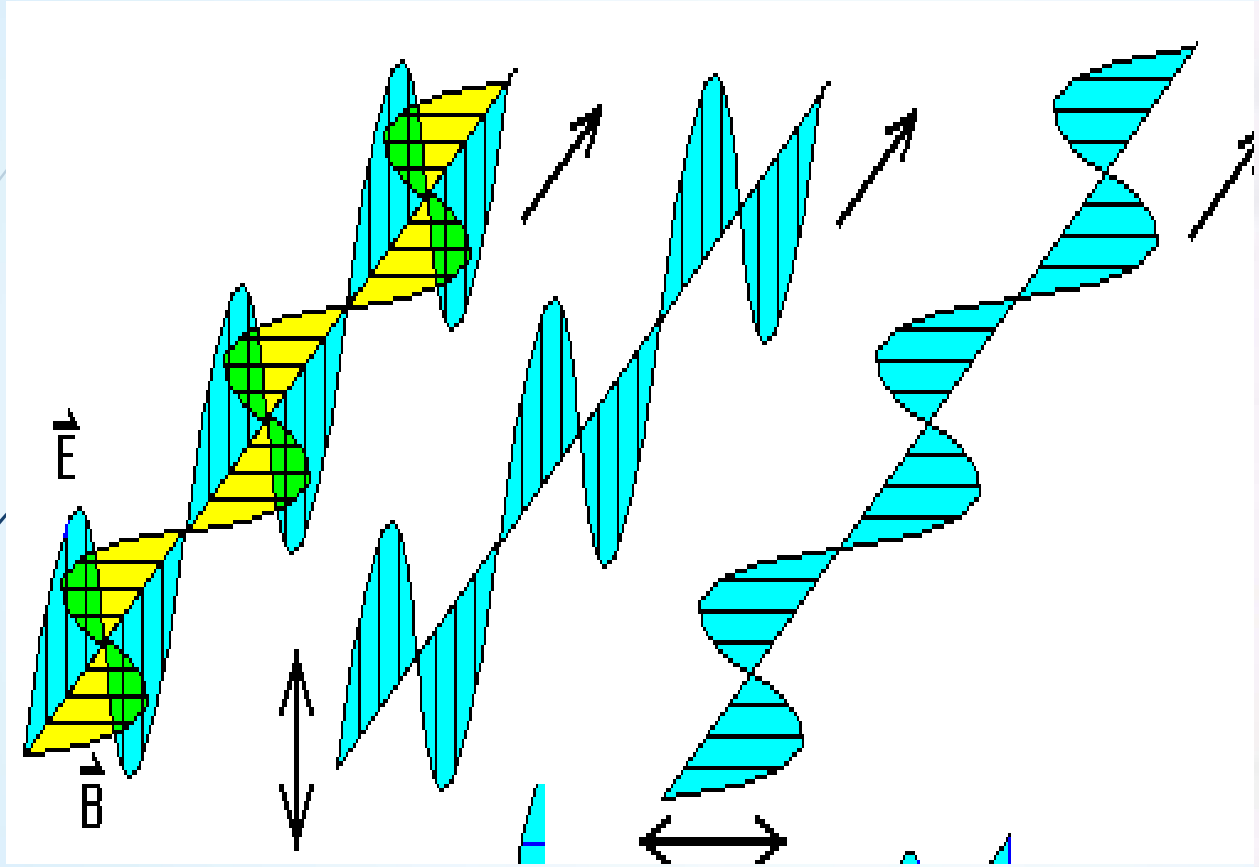
When an unpolarized light is passed through a **polarizer**, the polarizer interacts with the electrical field

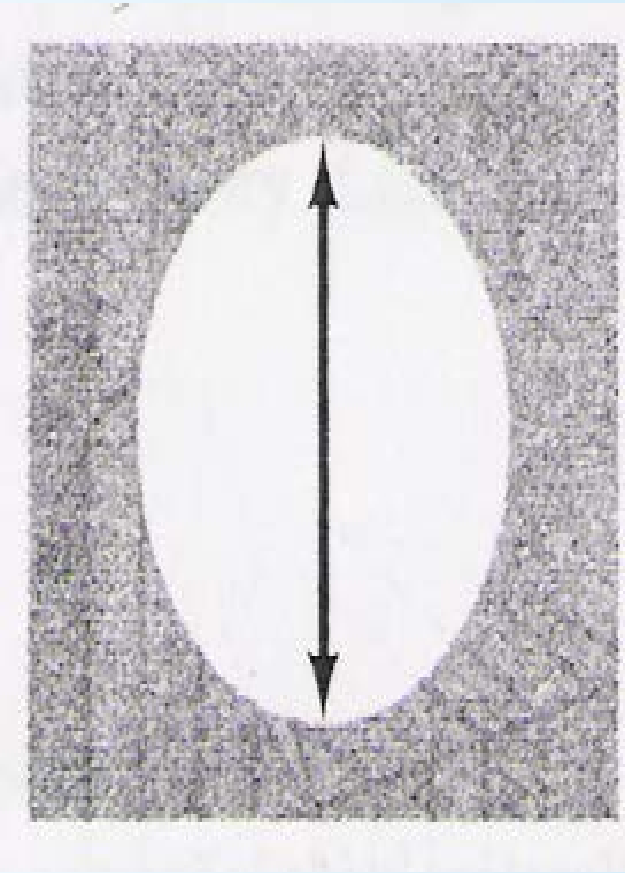
•The resultant light which emerge from the polarizer has their electric field vector **oscillating in only one direction**

•Such light is c/a **plane-polarized light**

•Plane polarized light can be polarized in different directions

Plane polarize light



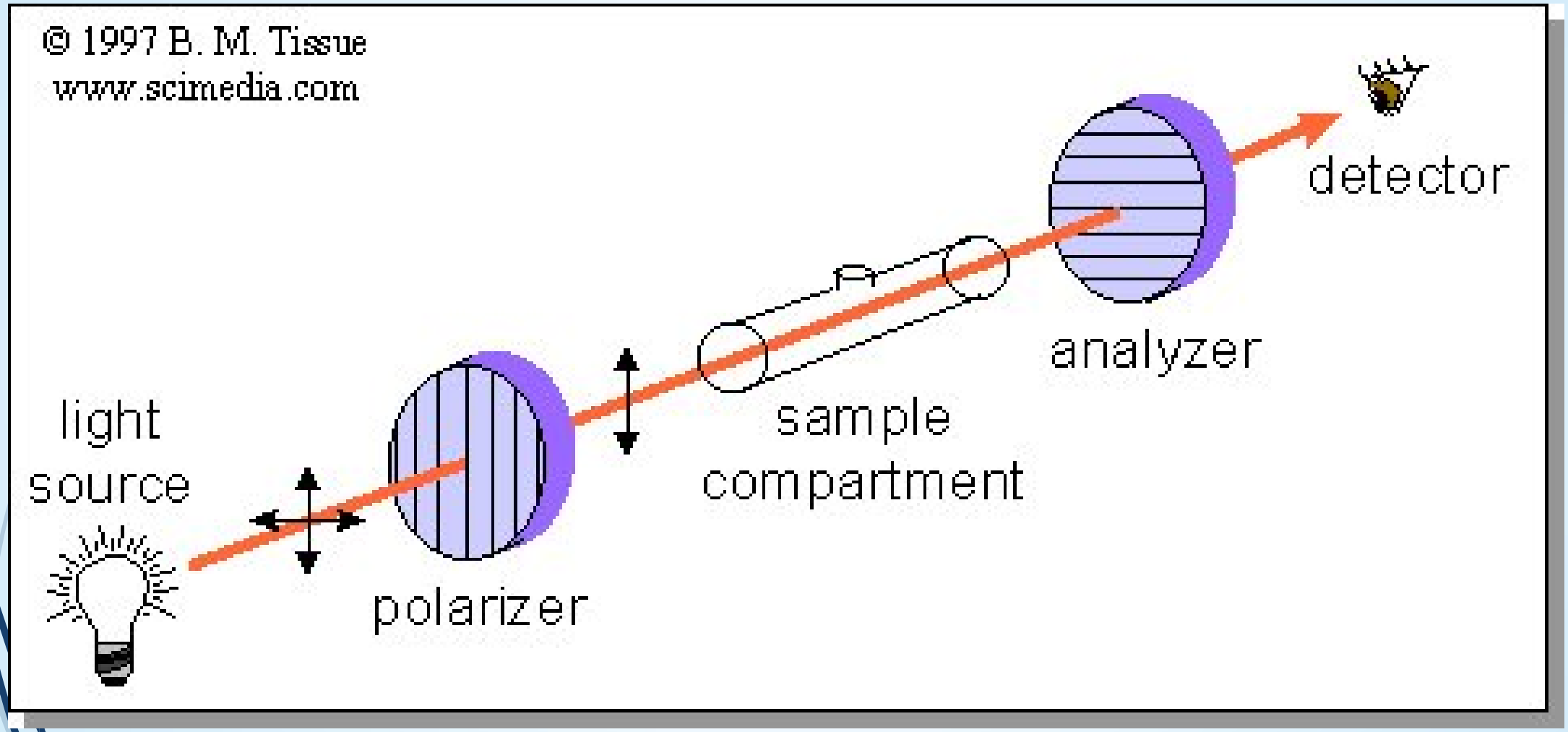


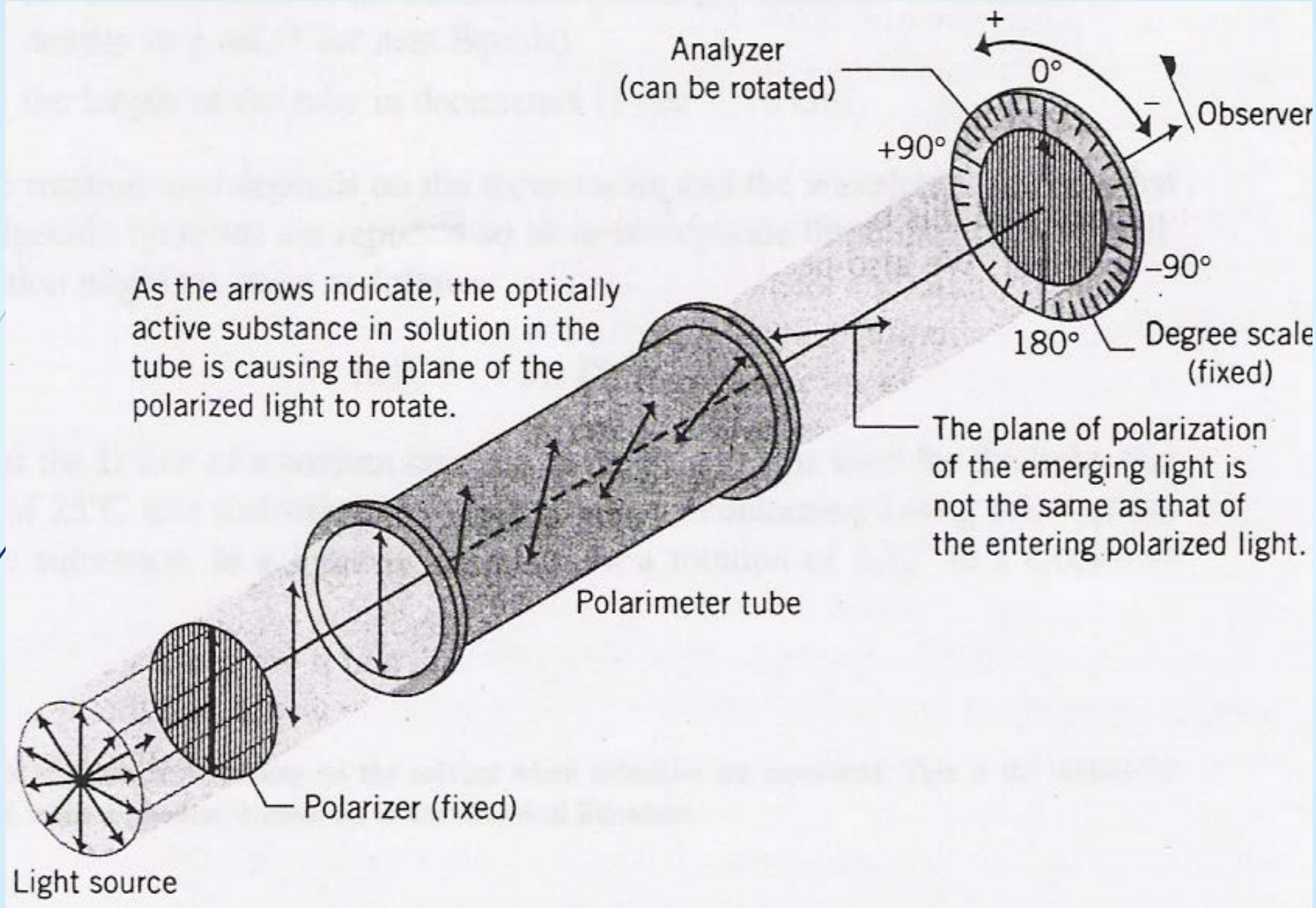
The Polarimeter: a device used to measure the effect of plane-polarized light on optically active compounds

The components of polarimeter are:

- a light source - (usually a sodium lamp)
- a polarizer
- a tube for holding sample in the light beam- a sample cell
- an analyzer- second polarizer, and
- a scale- to measure the rotation of plane polarized light

Schematic of a polarimeter



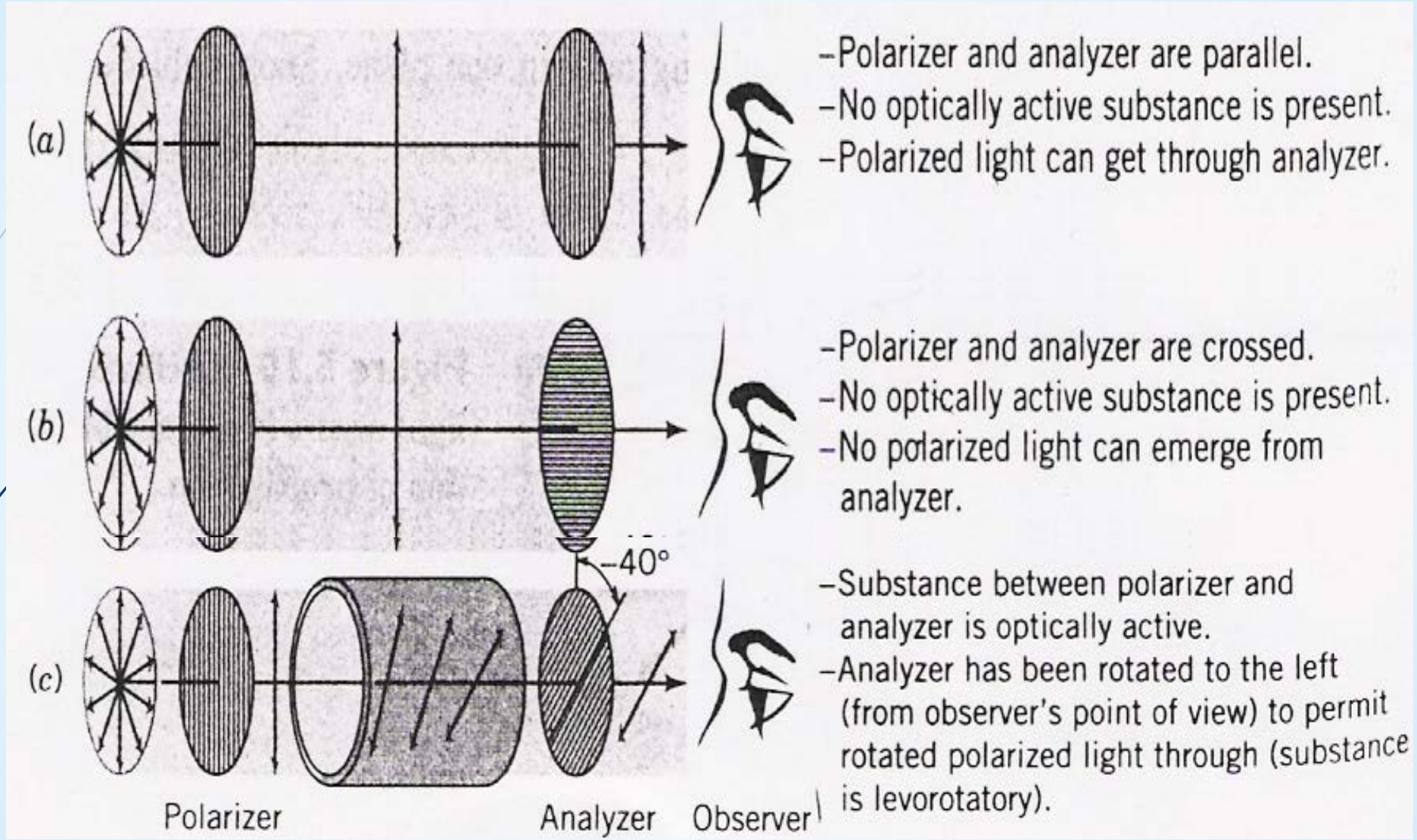

Analyzer
(can be rotated)
+
0°
–
Observer
+90°
–90°
180°
Degree scale
(fixed)
As the arrows indicate, the optically active substance in solution in the tube is causing the plane of the polarized light to rotate.
The plane of polarization of the emerging light is not the same as that of the entering polarized light.
Polarimeter tube
Polarizer (fixed)
Light source

- if no or optically inactive sample is present in the tube and the instrument is reading zero (0^o), the axes of plane polarized light and the analyzer is exactly **parallel**
- the observer will detect **maximum amount** (100 % transmittance) of light passing through.
- if the sample is optically active the plane of PPL will be **rotated** as it pass through the tube

• in order to detect the maximum brightness of the light (ie. 100% transmittance) observer will have to rotate the axis of the analyzer in either clockwise or counterclockwise direction

• if the analyzer is rotated in a **clockwise direction**, the rotation (**α** in degree) is said to be positive (+), and such substance are c/a **dextrorotatory**

• if the rotation is **counterclockwise**, the α is –ve, and such substances are c/a **levorotatory**


(a)
(b)
(c)
-40°
Polarizer
Analyzer
Observer
-Polarizer and analyzer are parallel.
-No optically active substance is present.
-Polarized light can get through analyzer.
-Polarizer and analyzer are crossed.
-No optically active substance is present.
-No polarized light can emerge from analyzer.
-Substance between polarizer and analyzer is optically active.
-Analyzer has been rotated to the left (from observer's point of view) to permit rotated polarized light through (substance is levorotatory).

Specific Rotation:

• extent of optical rotation depends on both the **sample path length** and the **analyte concentration**.

Specific rotation, [α], provides a normalize quantity to correct for this dependence, and is defined as: $[\alpha_0] = \alpha / c.l$ where,

$[\alpha_0]$= the specific rotation ; α = observed rotation

c = conc. of sample in gm/ml

l = length of the tube in decimeter (1dm = 10cm)

• [α] depends on the temperature and the wavelength of the light used

• these quantities are also incorporated while reporting [α]

$$[\alpha]_D^{25} = +3.12^o$$

• means D line of a sodium lamp (λ=589.6nm) is used for the light at a temperature of 25°C, and that a sample containing 1.00g/ml of the optically active substance, in a 1-dm tube, produces a rotation of 3.12° in a clockwise direction

Application

• polarimetric method is a simple and accurate means for determination of structure in micro analysis of expensive and non-duplicable samples.

• it is employed in quality control, process control and research in the pharmaceutical, chemical, essential oil, flavor and food industries.

• it is so well established that the United States Pharmacopoeia and the Food & Drug Administration include **polarimetric specifications** for numerous substances.

Pharmaceutical Applications

Determines **product purity** by measuring specific rotation and optical rotation of: Amino acids, Amino sugars, Analgesics, Antibiotics Cocaine, Dextrose Diuretics Serums Steroids Tranquilizers Vitamins

Utilizes polarimetry for incoming **raw materials inspection** of: Camphors, Citric acid, Glyceric acid Gums Lavender oil, Lemon oil Orange oil Spearmint oil

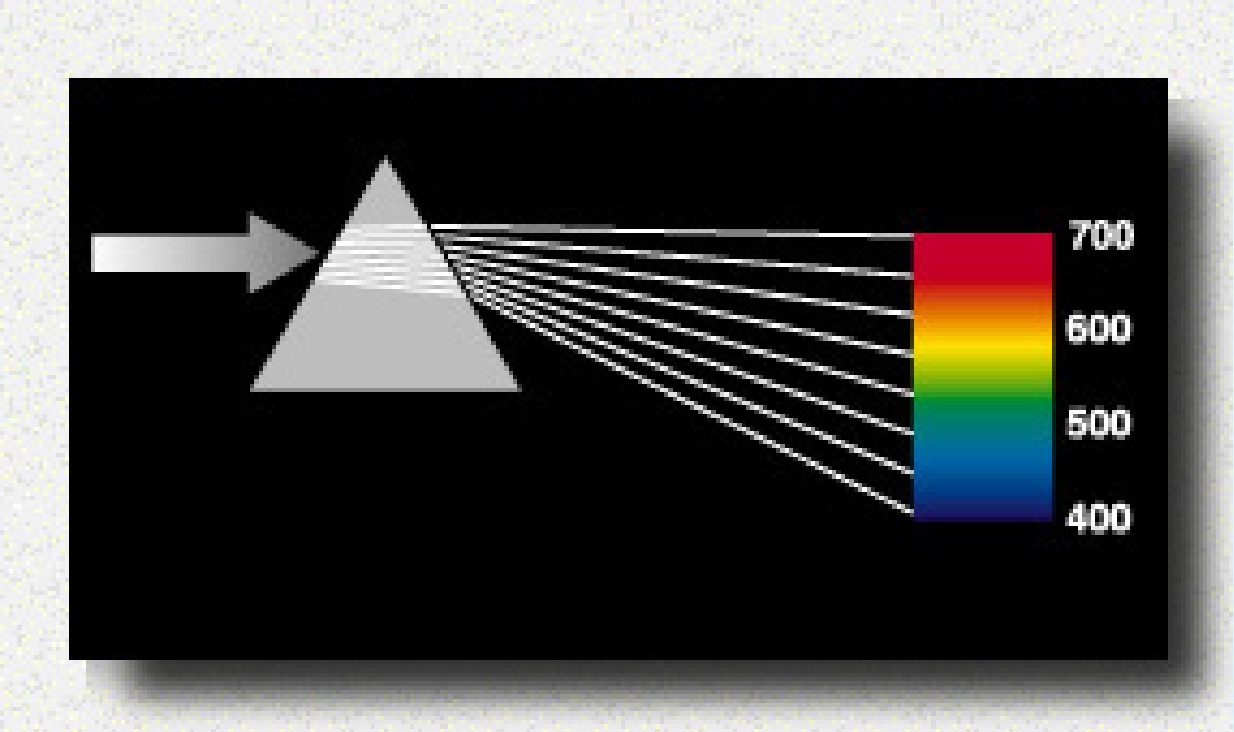
Colorimetry

- **Colorimetry** is the use of the human eye to determine the concentration of colored species.

- **Spectrophotometry** is the use of instruments to make the same measurements. It extends the range of possible measurements beyond those that can be determined by the eye alone.

Note: This experiment will demonstrate **both techniques** on the **same set of dyes**.

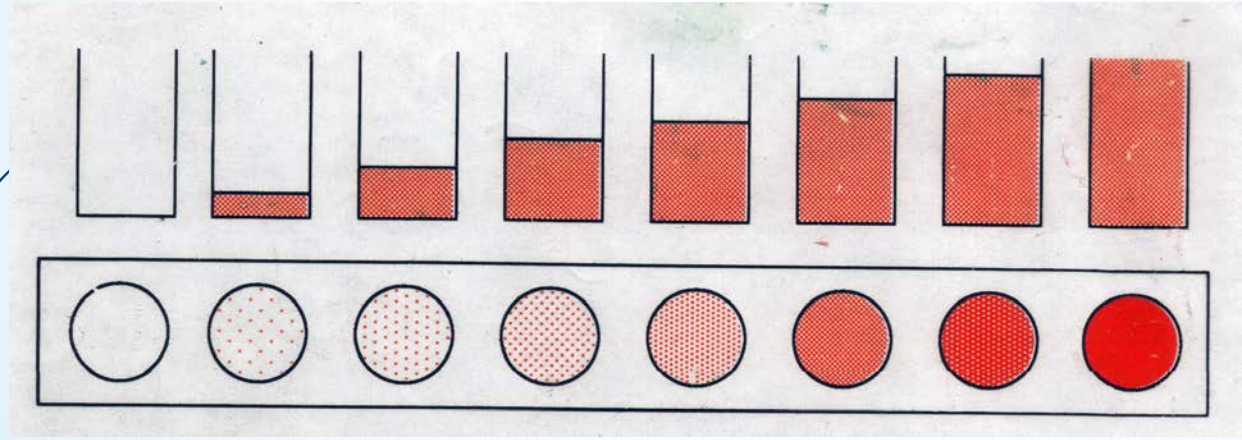
- **Visual Observations** – Because colorimetry is based on inspection of materials with the human eye, it is necessary to review aspects of visible light.
- **Visible light** is the narrow range of electromagnetic waves with the wavelength of **400-700 nm**.



ROY G. BIV the mnemonic used to remember the colors of the visible spectrum.

Visual Colorimetry

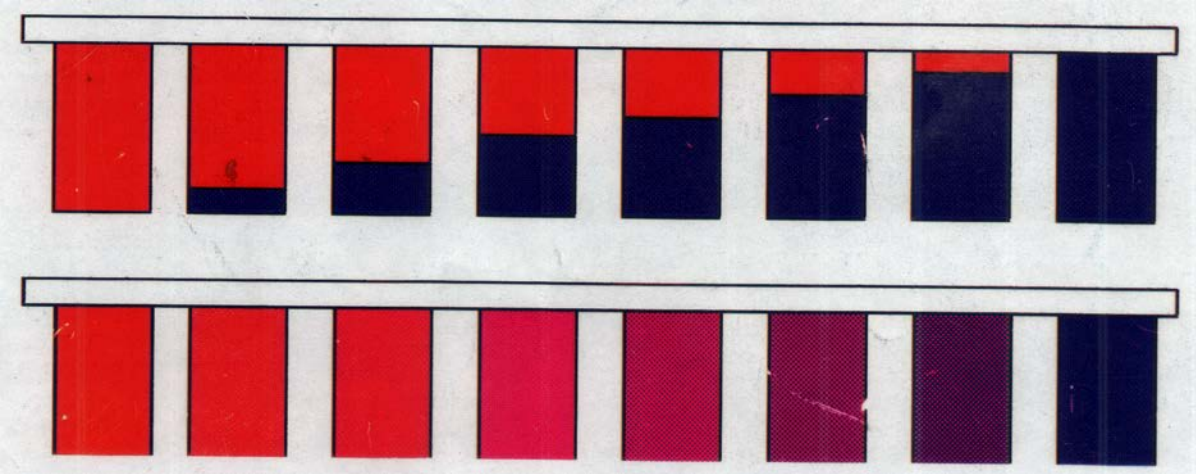
Intensity: For light shining through a colored solution,the observed intensity of the color is found to be **dependent on both the thickness of the absorbing layer (pathlength) and the concentration of the colored species.**



←**Side view**

←**Top view**
(a.k.a. Bird's eye view)

For One Color: A series of solutions of a single color demonstrates the **effect of either concentration or pathlength**, depending on how it is viewed.



←Ratio used

←Purple produced

For more than one color: the ratio of an unknown mixture can also be determined by matching the shade of the color to those produced from known ratios.

In this example, the ratio of a mixture of **red** and **blue** can be determined visibly by comparing the mixture to **purples** produced from known ratios of red and blue.

Dilution Factor (constant pathlength)

Recall: $C_1V_1 = C_2V_2$

Then for the dilution,

$$C_{diluted} \times V_{diluted} = C_{std} \times V_{std}$$

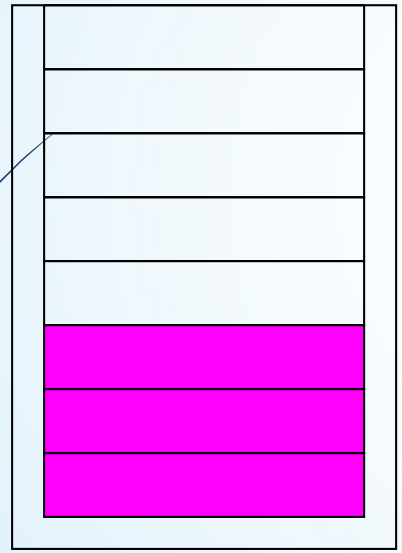
$$C_{diluted} = C_{std} \times (V_{std} / V_{diluted})$$

Since $V_{diluted} = V_{total}$

$$C_{diluted} = C_{std} \times (V_{std} / V_{total})$$

Substituting the volumes:

$$C_{diluted} = C_{std} \times (3 \text{ drops} / 8 \text{ drops})$$



3 drops of dye std
+ 5 drops water
8 drops total volume

If the **original concentration is 5.88 ppm**, then:

$$C_{diluted} = 5.88 \text{ ppm} \times (3 / 8)$$

$$C_{diluted} = 2.21 \text{ ppm}$$

Intensity: When the product of the concentration and the pathlength of any two solutions of a colored compound are the same, the same intensity or darkness of color is observed.

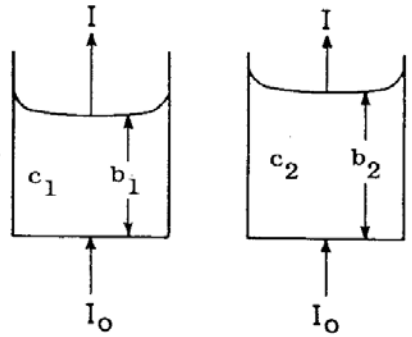


Fig. I-1. Transmission of Light Through Solutions

$$\log \frac{I_o}{I} = a_s c_1 b_1 = a_s c_2 b_2 \qquad (5)$$

$$c_1 b_1 = c_2 b_2 \qquad (6)$$

$$\frac{c_1}{c_2} = \frac{b_2}{b_1} \qquad (7)$$

This is the fundamental relationship used in color comparators.

Duboscq visual colorimeter

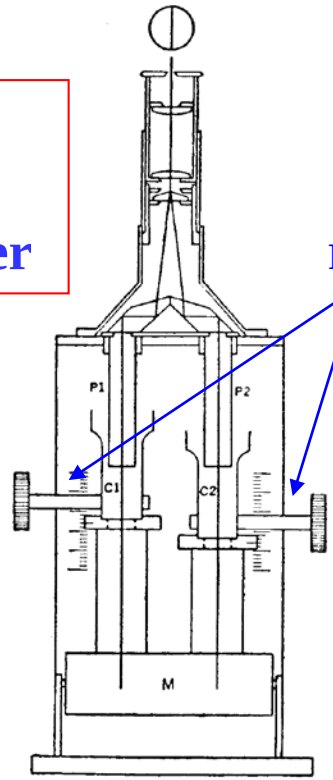


Fig. I-6. Optical Path in a Colorimeter of the Duboscq Type. P_1, P_2, plungers; C_1, C_2, cups to hold the solutions; M, mirror. The two halves of the field viewed through the ocular appear equally bright when a match has been obtained.

Spectrophotometry

- **Spectrophotometer** - an instrument that measures the amount of light absorbed, or the intensity of color at a given wavelength.

- The **intensity of color** can be given a numerical value by comparing the amount of light prior to passing it through the sample and after passing through the sample.

- These **quantitative measurements** of light absorbed are the **Transmittance** and the **Absorbance**.

Absorbance

Beer-Lambert Law (a.k.a. Beer's law) - the linear relationship between absorbance and concentration of an absorbing species.

$$A = abc$$

A is the absorbance

"a" is molar absorptivity in L/[(mole)(cm)]

Also called "**extinction coefficient**" or "**ε**";
it is dependent on the material being studied.

"b" is the path length in cm

The **diameter of the cuvette** or sample holder which is the distance the light travels through the absorbing sample. "b" is a **constant** when the **same size cuvette** is used for all samples.

"c" is the concentration of the sample in (mol/L)

Main use of Beer's Law is to **determine the concentration** of various solutions.

Transmittance is Related to Absorbance

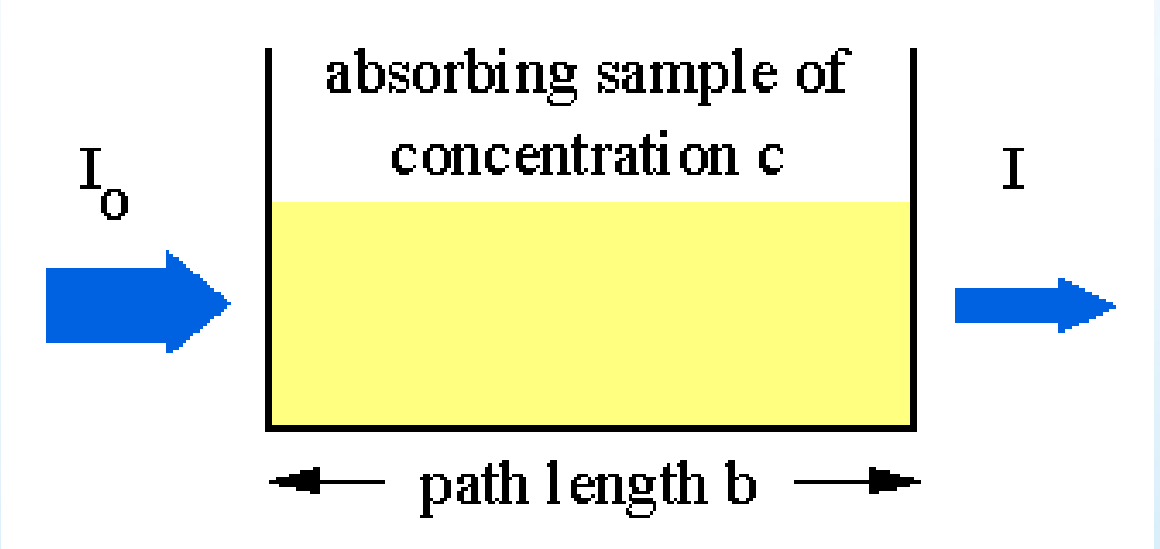
Transmittance is given by the equation:

$$T = I/I_o$$

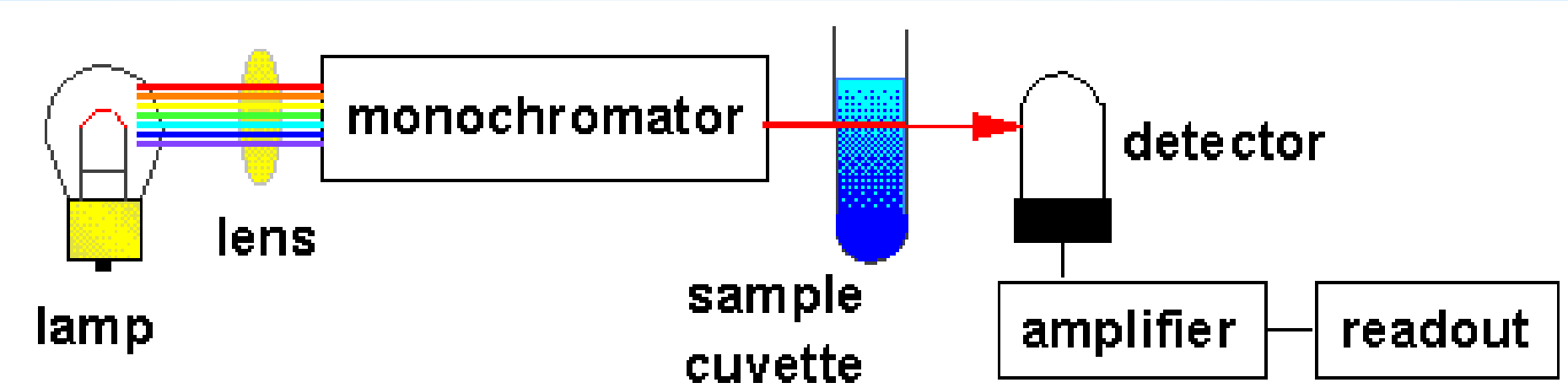
where I is the intensity of the light after it has gone through the sample & I_o is the initial light intensity.

Absorbance is related to the %T:

$$A = -\log T = -\log(I/I_o)$$



Simple Spectrophotometer Schematic

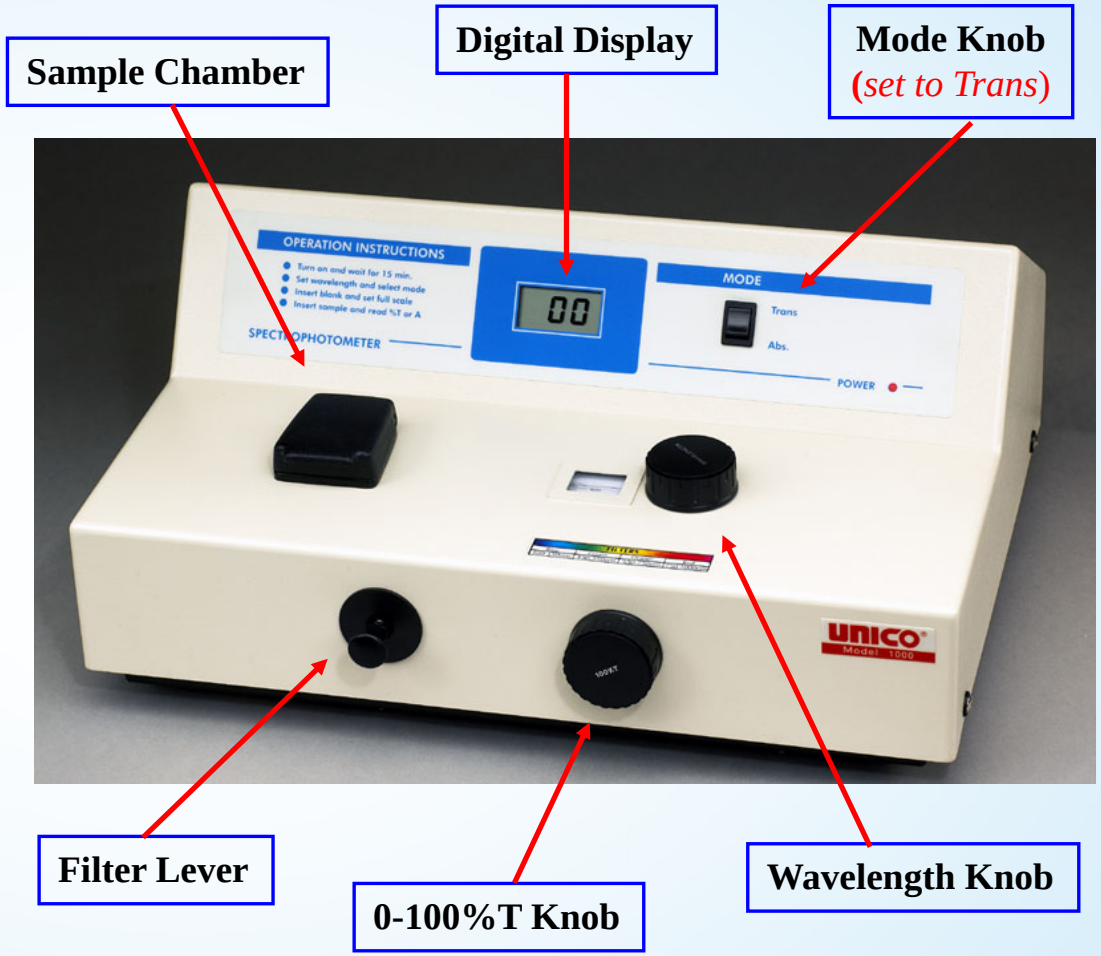


- **The lamp** emits all colors of light (i.e., white light).
- **The monochromator** selects **one wavelength** and that wavelength is sent through the sample.
- **The detector** detects the wavelength of light that has passed through the sample.
- **The amplifier** increases the signal so that it is easier to read against the background noise.

Spectronic 20 Instructions

(Directions below will be available next to each instrument)

1. With sample **chamber empty**, set desired wavelength then adjust to **0%T** with right knob on front panel.
2. **Insert blank solution**, close lid and adjust **100%T** with right knob on front panel.
3. **Insert dye solutions**, read and record **%T** values.
4. **Change wavelength***, **repeat steps 2-4.**



***NOTE:** The **filter** must be **changed** periodically **to coordinate with the wavelength** range studied: **blue (400-449), green (450-549)** and **orange (550-749).**

Control Questions

1. Microscopy.
2. Refractometry.
3. Polarimetry.
4. Colorimetry.
5. Spectral analysis.

Recommended literature:

Basic:

1. Vladimir Timanyuk, Elena Zhivotova, Igor Storozhenko. Biophysics: Textbook for students of higher schools / Kh.: NUPh, Golden Pages, 2011.- 576p.
2. Vladimir Timaniuk, Marina Kaydash, Ella Romodanova. Physical methods of analysis / Manual for students of higher schools/– Kharkiv: NUPh: Golden Pages, 2012. – 192 p.
3. Philip Nelson. Biological Physics. – W. H. Freeman, 1st Edition, 2007. – 600 p.
4. Biophysics, physical methods of analysis. Workbook: Study guide for the students of higher pharmaceutical educational institutions / Pogorelov S. V., Krasovskyi I. V., Kaydash M. V., Sheykina N. V., Frolova N. O., Timaniuk V. O., Romodanova E.O., Kokodii M.H. – Kharkiv., – 2018. – 130 p.
5. Center for distance learning technologies of NPhaU. Access mode: http://nuph.edu.ua/centr-distancijjnih-tehnologijj-navcha/

Support:

1. Eduard Lychkovsky. Physical methods of analysis and metrology: tutorial / Eduard Lychkovsky, Zoryana Fedorovych. – Lviv, 2012. – 107 p.
2. Daniel Goldfarb. Biophysics DeMYSTiFied. – McGraw-Hill Professional, 1st Edition, 2010. – 400 p.


Thanks for
your attention