II. THE SPECTROPHOTOMETER

1. Definition of spectrophotometer

The **spectrophotometer** is a vital instrument used to measure the intensity of light absorbed by a sample. This measurement helps determine the concentration of solutes in a solution.

2. **Biotechnology Applications:** Used in DNA, RNA, and protein quantification.
3. **Spectrophotometer Instrumentation**

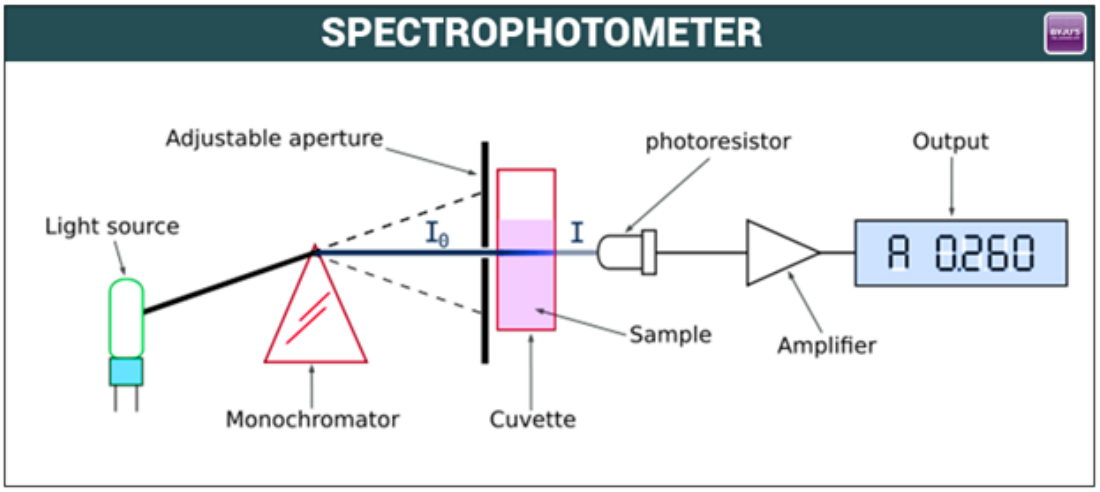

Fig 1: Spectrophotometer Instrumentation. https://cdn1.byjus.com/wp-content/uploads/2018/11/chemistry/2017/01/20060501/Spectrophotometer-Principle.png

A spectrophotometer is made up of two instruments:

- Spectrometer: is to produce light of any wavelength
- Photometer: is to measure the intensity of light

The spectrophotometer is designed in a way that the liquid or a sample is placed between spectrometer and photometer. The photometer measures the amount of light that passes through the sample and delivers a voltage signal to the display. If the absorbing of light changes, the voltage signal also changes. Spectrophotometers come in a variety of shapes and sizes and have multipurpose uses to them. The different types of spectrophotometers available are all different from one another, based on their application and desired functionality. The most popular spectrophotometers are 45 degrees, sphere and multi-angle

spectrophotometers. Another closely related concept is Spectroscopy that simply measures the absorption of light from its source and the intensity of light as well.

The basic spectrophotometer instrument consists of a light source, a digital display, a monochromator, a wavelength sector to transmit a selected wavelength, a collimator for straight light beam transmission, photoelectric detector and a cuvette to place a sample.

The intensity of light is symbolized as l_0 measure the number of photons per second. When the light is passed through the blank solution, it does not absorb light and is symbolized as (l). Other important factors are Absorbance (A) and Transmittance (T).

- When a monochromatic wavelength I_0 passes through an absorbent solution of concentration C_1 contained in a vessel of thickness L_1. Note that part of this radiation is absorbed by the sample and part is transmitted (I_1).
- When a monochromatic wavelength I0 passes through an absorbent solution of high concentration C_2 ($C_2 > C_1$) contained in a vessel of thickness L1. Note that part of this radiation is absorbed by the sample and part is transmitted (I_2) or ($I_1 > I_2$).
- When a monochromatic wavelength I0 passes through an absorbent solution of high concentration C_2 contained in a cuvette of thickness L_2 (or $L_2 > L_1$). Note that part of this radiation is absorbed by the sample and part is transmitted (I_3) or ($I_2 > I_3$).

We can see that the amount of absorber is related to the concentration of the solution and the thickness of the cell.

- Transmittance $T = I/I_0$
- transmittance = T%
- Absorbance A = -log T

$$A = \log (I/I_0) = \log (I_0/I)$$

- We then obtain the relationship known as **Beer-Lambert's** law:

$$A = \varepsilon l C$$

ε = the molar extinction coefficient (L.mol-1.cm-1) or molar absorptivity (or absorption coefficient); this is a characteristic of the substance studied at a given wavelength.

C = concentration and **l** = cuvette length.

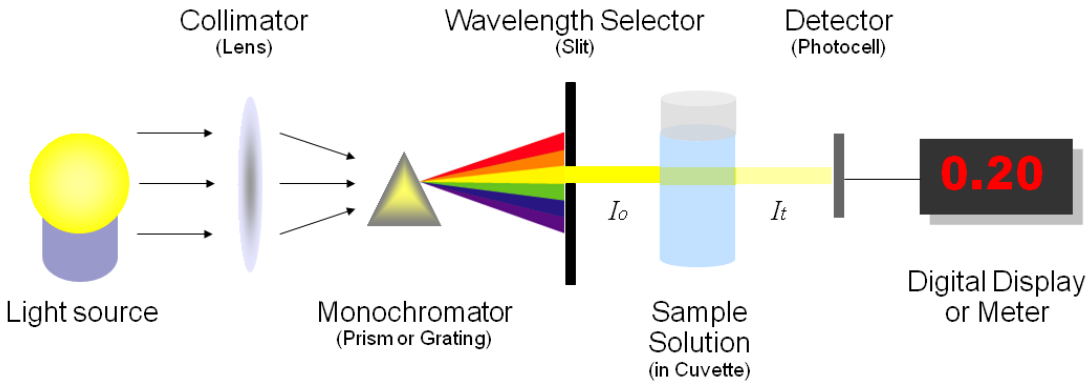

Fig 2: Basic structure of spectrophotometers (CC BY-4.0; Heesung Shim via LibreTexts)
https://chem.libretexts.org/@api/deki/files/8475/spectrophotometer_structure.png?revision=1&size=bestfit&width=893&height=324

3. Excitation of electrons

When a wavelength is applied to a solution, electrons absorb energy and go from the ground state to the excited state. To return to the ground state they emit a wavelength, which is detected and gives a spectrum called the absorption spectrum. It shows the absorbance in relation to the wavelength.

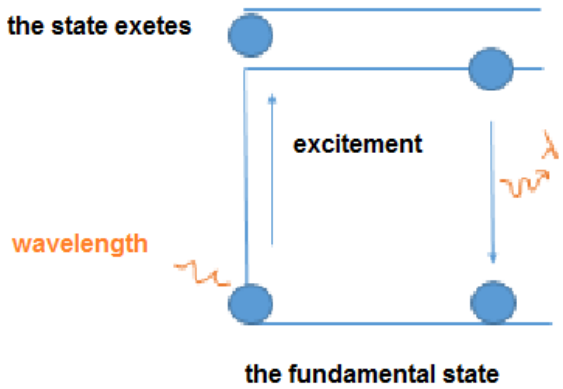

Fig 3: excitation of electrons @belfethi.l

You need a spectrometer to produce a variety of wavelengths because different compounds absorb best at different wavelengths. For example, p-nitrophenol (acid form) has the maximum absorbance at approximately 320 nm and p-nitrophenolate (basic form) absorb best at 400nm, as shown in Figure 4.

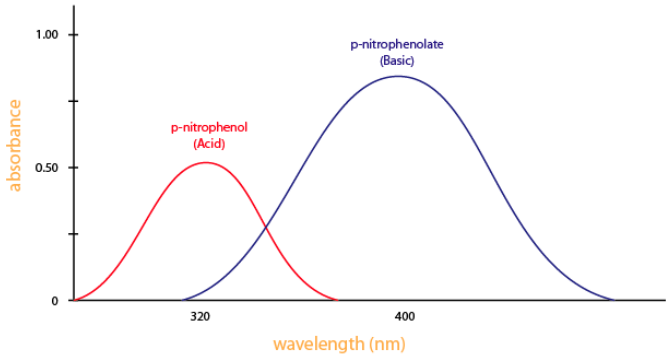

Fig 4: Absorbance of two different compounds.
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Looking at the graph that measures absorbance and wavelength, an isosbestic point can also be observed. An **isosbestic point** is the wavelength in which the absorbance of two or more species are the same. The appearance of an isosbestic point in a reaction demonstrates that an intermediate is NOT required to form a product from a reactant. Figure 5 shows an example of an isosbestic point.

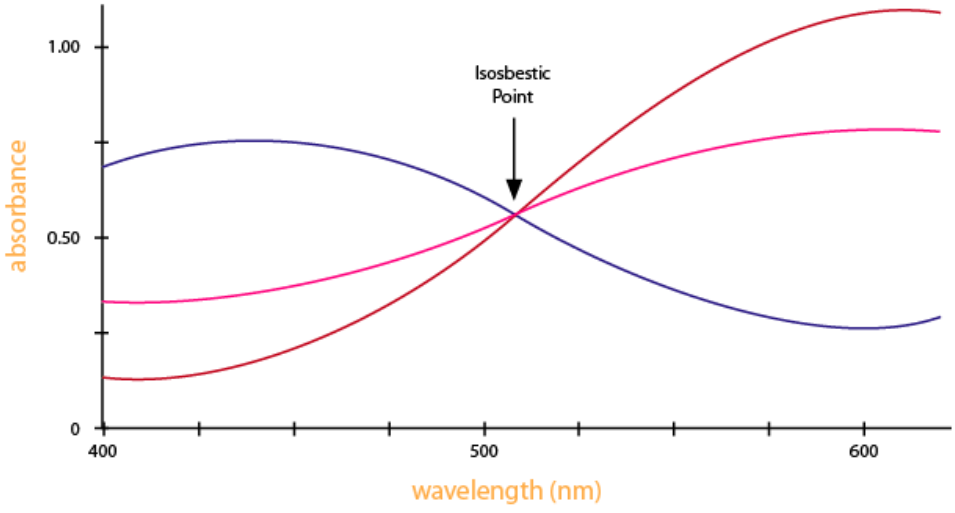

Fig 5 : An example of isosbestic point (CC BY-4.0; Heesung Shim via LibreTexts).
https://chem.libretexts.org/@api/deki/files/8471/isopoint.png?revision=1

4. Use a spectrophotometer

- To determine the absorbance of the coloured species, it is necessary to first perform a "blank", i.e. record the intensity of the light the intensity of the light passing through a cuvette containing solvent (without the chemical species whose absorbance is to be measured).
- Quartz cells are required for UV measurements as plastic and glass absorb this radiation. because plastic and glass absorb this radiation. Water also absorbs UV radiation, so a UV-transparent solvent such as cyclohexane or dichloromethane

4.1.Installation of tanks

- Fill one cuvette with the solution to be analysed; fill another identical cuvette with the solvent of the solution to be analysed: this is the "blank" cuvette.
- Do not touch the entrance and exit surfaces of the light beam with your fingers; they must be perfectly clean.
- Insert the cuvette with the clean side perpendicular to the light beam.

4.2.Computer-interfaced spectrophotometer

4.2.1. Recording an absorption spectrum Go to “spectrum” mode and select absorbance display.

❶ set the acquisition parameters:

- the wavelength range to be scanned; if no information is available, choose from 400 nm to 800 nm ;
- the “step”; in general, choose 1 nm.

❷ **Start acquisition;** the software asks you to insert the blank” cell. Insert the blank cell and click on OK.

The computer records the intensity of transmitted light for each wavelength in the selected range.

❸ Replace the “white” cuvette with the cuvette containing the solution studied. Click on OK.

The computer records the intensity of transmitted light for each wavelength, calculates the absorbance and then displays the absorption spectrum.

2.2 Recording absorbance at a given wavelength

Set to “absorbance measurement” mode.

❶ set the measurement wavelength.

❷ Repeat steps ❷ and ❸ of 4.2.1.

4.3.Spectrophotometer not interfaced to computer

❶ set the measurement wavelength.

❷ Insert “blank” cell. Press “zero absorbance” button.

❸ Insert the cell containing the solution to be studied. Read the absorbance displayed.

❹ To plot a spectrum, repeat the above three steps for each wavelength studied.

5. Good practices of use of the spectrophotometer

- Perform the calibration of the spectrophotometer, each time the analysis of a group of samples is carried out.
- Keep the lid of the sample holder closed during the measurement process, to ensure an adequate reading.
- Avoid reusing disposable cuvettes.
- Use only quartz cuvettes, to perform analyzes below 310 nm.
- Avoid the use of plastic buckets, if organic solvents are used.
- Use high quality silicate borosilicate glassware to prepare the standards. Avoid the use of sodium-sodium-glassware whenever possible, because prolonged contact with standards can permeate it and, consequently, produce erroneous results.
- Carefully clean the glass cuvettes after use. Discard those that present scratches on the polished surface.
- Use high quality reagents as much as possible. Low quality reagents can cause contamination even at very low concentrations. The diluents used – water or solvents – must be free of impurities.
- Verify that the samples or standards have not been degassed inside the cuvettes.
- Take into account, when trying to use new procedures, that not all substances comply with Beer’s law. Carry out linearity tests on the range of concentrations to be used.

6. the maintenance of a spectrophotometer

Spectrophotometers, in general, are very specialized and expensive equipment. Its conservation depends to a great extent on the way of installation and use. The

environment that surrounds them and the quality of the electricity services are factors of paramount importance, so that the equipment can provide services in accordance with the specifications for which they were manufactured.

Maintenance routines that may require vary in complexity, ranging from the careful cleaning of its components to specialized procedures, which should only be performed by technicians or engineers who have received the corresponding training and have the technical information developed by the manufacturers.

Note that preventive maintenance of a spectrophotometer must respond to the routines and frequencies recommended by the manufacturer. we present a group of basic routines that can be performed in the laboratory:

- Externally clean the spectrophotometer, including controls, screens or measuring meters. This can be done with a piece of fine fabric – similar to the texture of handkerchiefs – moistened with distilled water.
- Inspect and clean the power cord.
- Verify that the lamp is clean and in good condition. If it does not work, install a new one, with the same specifications as the original one. In modern spectrophotometers, lamp status is automatically detected by software that controls the status and operation of the instrument, making it easy to determine when the lamp needs to be changed. Replace the bulb and adjust the bulb afterwards following the procedure recommended by the manufacturer.
- Check the protection fuse. Before opening the fuse housing, check that the spectrophotometer is off and that its contacts are clean and in good condition.
- Place the computer in the operational configuration.
- Operate the ignition switch to allow operation for five (5) minutes.
- Perform a leakage current test in the ON and OFF positions.
- Calibrate the front panel of the spectrophotometer according to the manufacturer’s instructions.
- Measure the sensitivity of the equipment.
- Conduct a test following Beer’s law.

- Return the spectrophotometer to the initial setting, if calibration has been successfully performed.