

Spectrophotometry

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. ***The basic principle is that each compound absorbs or transmits light over a certain range of wavelength.*** This measurement can also be used to measure the amount of a known chemical substance. Spectrophotometry is one of the most useful methods of quantitative analysis in various fields such as chemistry, physics, biochemistry, material and chemical engineering and clinical applications.

Introduction

Every chemical compound absorbs, transmits, or reflects light (electromagnetic radiation) over a certain range of wavelength. Spectrophotometry is a measurement of how much a chemical substance absorbs or transmits. Spectrophotometry is widely used for quantitative analysis in various areas (e.g., chemistry, physics, biology, biochemistry, material and chemical engineering, clinical applications, industrial applications, etc.). Any application that deals with chemical substances or materials can use this technique. In biochemistry, for example, it is used to determine enzyme-catalysed reactions. In clinical applications, it is used to examine blood or tissues for clinical diagnosis. There are also several variations of the spectrophotometry such as atomic absorption spectrophotometry and atomic emission spectrophotometry.

A **spectrophotometer** is an instrument that measures the amount of photons (the intensity of light) absorbed after it passes through sample solution. With the spectrophotometer, the amount of a known chemical substance (concentrations) can also be determined by measuring the intensity of light detected. 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 infrared range (700 - 15000 nm) of electromagnetic radiation spectrum.

In visible spectrophotometry, the absorption or the transmission of a certain substance can be determined by the observed colour. 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 colour 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 particular beam of light is passed through a solution sample.

Devices and mechanism

Figure 1 below illustrates the basic structure of spectrophotometers. It consists of a light source, a collimator, a monochromator, a wavelength selector, a cuvette for sample solution, a photoelectric detector, and a digital display or a meter. Detailed mechanism is described below.

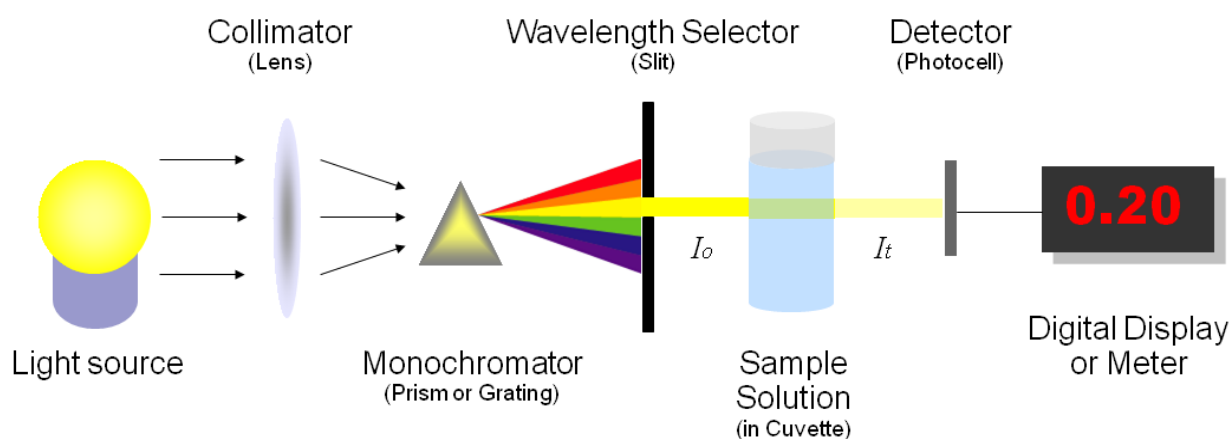


Figure 1: Basic structure of spectrophotometers.

A spectrophotometer, in general, consists of two devices; a spectrometer and a photometer. A spectrometer is a device that produces, typically disperses and measures light. A photometer indicates the photoelectric detector that measures the intensity of light.

- **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, as shown in Figure 1.
- **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 absorbed and then sends a signal to a galvanometer or a digital display, as illustrated in Figure 1.



Figure 2: A single wavelength spectrophotometer.

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 3.

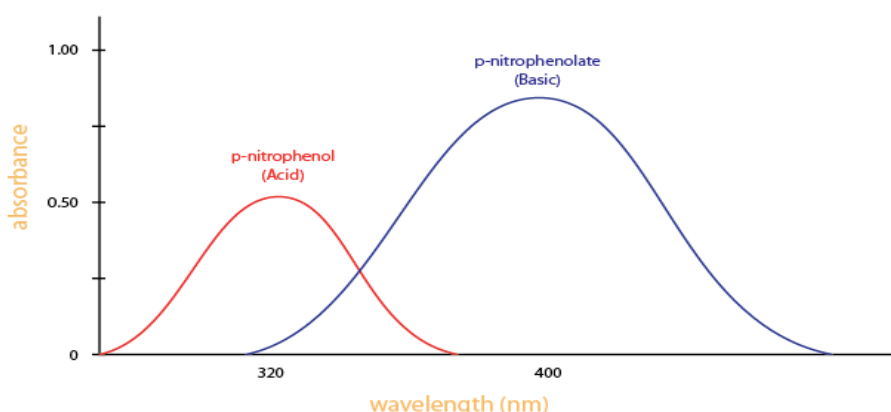


Figure 3: Absorbance of two different compounds

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 4 shows an example of an isosbestic point.

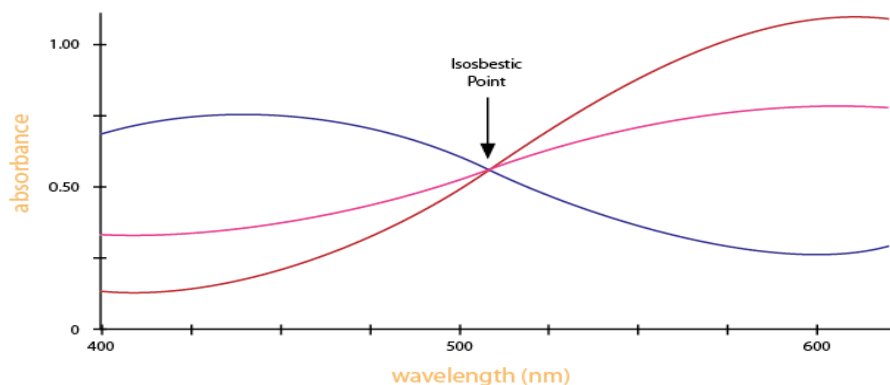


Figure 4: An example of isosbestic point

Referring back to Figure 1 (and Figure 5), the amount of photons that goes through the cuvette and into the detector is dependent on the length of the cuvette and the concentration of the sample. Once you know the intensity of light after it passes through the cuvette, you can relate it to transmittance (T). Transmittance is the fraction of light that passes through the sample. This can be calculated using the equation:

$$\text{Transmittance (T)} = L_t/L_o$$

Where L_t is the light intensity after the beam of light passes through the cuvette and L_o is the light intensity before the beam of light passes through the cuvette. Transmittance is related to absorbance by the expression:

$$\text{Absorbance (A)} = -\log(T) = -\log(L_t/L_o)$$

Where absorbance stands for the amount of photons that is absorbed. With the amount of absorbance known from the above equation, you can determine the unknown concentration of the sample by using Beer-Lambert Law. Figure 5 illustrates transmittance of light through a sample. The length l is used for Beer-Lambert Law described below.

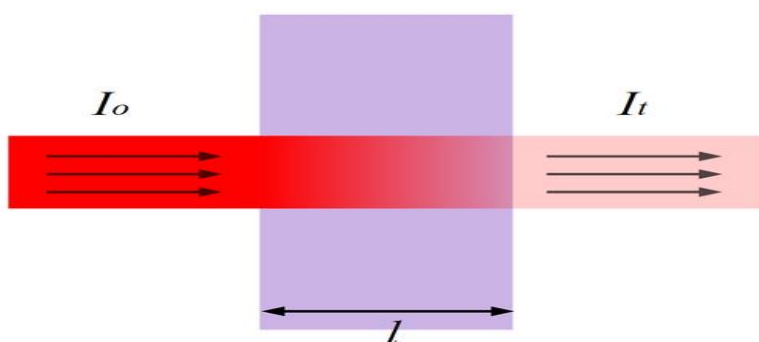


Figure 5: Transmittance

Beer-Lambert Law

[Beer-Lambert Law](#) (also known as Beer's Law) states that there is a linear relationship between the absorbance and the concentration of a sample. For this reason, Beer's Law can *only* be applied when there is a linear relationship. Beer's Law is written as:

$$A = \epsilon Lc$$

where

- A is the measure of absorbance (no units),
- ϵ is the molar extinction coefficient or molar absorptivity (or absorption coefficient),
- L is the path length, and
- c is the concentration.

The molar extinction coefficient is given as a constant and varies for each molecule. Since absorbance does not carry any units, the units for ϵ must cancel out the units of length and concentration. As a result, ϵ has the units: $\text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. The path length is measured in centimetres. Because a standard spectrometer uses a cuvette that is 1 cm in width, L is always assumed to equal 1 cm.

Types of Spectrophotometers

There are some common types of spectrophotometers include: UV-vis spectrophotometer: Measures light absorption in UV and visible ranges (200-800 nm). Used for quantification of many inorganic and organic compounds. 1. Infrared spectrophotometer: Measures infrared light absorption, allowing identification of chemical bonds and functional groups. 2. Atomic absorption spectrophotometer (AAS): Uses absorption of light by vaporized analyte atoms to determine concentrations of metals and metalloids. 3. Fluorescence spectrophotometer: Measures intensity of fluorescent light emitted from samples after excitation. Allows highly sensitive analysis of samples with native or induced fluorescence. 4. Colorimeter: Simple spectrophotometers used to measure light absorption for colorimetric assays and tests.

Applications in Biochemistry

Spectrophotometry is an important technique used in many biochemical experiments that involve DNA, RNA, and protein isolation, enzyme kinetics and biochemical analyses. Since samples in these applications are not readily available in large quantities, they are especially suited to be analyzed in this non-destructive technique. In addition, precious sample can be saved by utilizing a micro-volume platform where as little as 1 μL of sample is required for complete analyses. A brief explanation of the procedure of spectrophotometry includes comparing the absorbency of a blank sample that does not contain a colored compound to a sample that contains a colored

compound. This coloring can be accomplished by either a dye such as Coomassie Brilliant Blue G-250 dye measured at 595 nm or by an enzymatic reaction as seen between β -galactosidase and ONPG (turns sample yellow) measured at 420 nm. The spectrophotometer is used to measure colored compounds in the visible region of light (between 350 nm and 800 nm), thus it can be used to find more information about the substance being studied. In biochemical experiments, a chemical and/or physical property is chosen and the procedure that is used is specific to that property to derive more information about the sample, such as the quantity, purity, enzyme activity, etc. Spectrophotometry can be used for a number of techniques such as determining optimal wavelength absorbance of samples, determining optimal pH for absorbance of samples, determining concentrations of unknown samples, and determining the pKa of various samples. Spectrophotometry is also a helpful process for protein purification and can also be used as a method to create optical assays of a compound. Spectrophotometric data can also be used in conjunction with the Beer–Lambert.

Because a spectrophotometer measures the wavelength of a compound through its colour, a dye-binding substance can be added so that it can undergo a colour change and be measured. It is possible to know the concentrations of a two-component mixture using the absorption spectra of the standard solutions of each component. To do this, it is necessary to know the extinction coefficient of this mixture at two wavelengths and the extinction coefficients of solutions that contain the known weights of the two components. In addition to the traditional Beer-Lamberts law model, cuvette based label free spectroscopy can be used, which add an optical filter in the pathways of the light, enabling the spectrophotometer to quantify concentration, size and refractive index of samples following the hands law. Spectrophotometers have been developed and improved over decades and have been widely used among chemists. Additionally, Spectrophotometers are specialized to measure either UV or Visible light wavelength absorbance values. It is considered to be a highly accurate instrument that is also very sensitive and therefore extremely precise, especially in determining colour change. This method is also convenient for use in laboratory experiments because it is an inexpensive and relatively simple process.

UV-visible spectrophotometry

Most spectrophotometers are used in the UV and visible regions of the spectrum, and some of these instruments also operate into the near-infrared region as well. The concentration of a protein can be estimated by measuring the OD at 280 nm due to the presence of tryptophan, tyrosine and phenylalanine. This method is not very accurate since the composition of proteins varies greatly and proteins with none of these amino acids do not have maximum absorption at 280 nm. Nucleic acid contamination can also interfere. This method requires a spectrophotometer capable of measuring in the UV region with quartz cuvettes.

Ultraviolet-visible (UV-vis) spectroscopy involves energy levels that excite electronic transitions. Absorption of UV-vis light excites molecules that are in ground-states to their excited-states.

Visible region 400–700 nm spectrophotometry is used extensively in colorimetry science. It is a known fact that it operates best at the range of 0.2–0.8 O.D. Ink manufacturers, printing companies, textiles vendors, and many more, need the data provided through colorimetry. They take readings in the region of every 5–20 nanometers along the visible region, and produce a spectral reflectance curve or a data stream for alternative presentations. These curves can be used to test a new batch of colorant to check if it makes a match to specifications, e.g., ISO printing standards.

Traditional visible region spectrophotometers cannot detect if a colorant or the base material has fluorescence. This can make it difficult to manage color issues if for example one or more of the printing inks is fluorescent. Where a colorant contains fluorescence, a bi-spectral fluorescent spectrophotometer is used. There are two major setups for visual spectrum spectrophotometers, d/8 (spherical) and 0/45. The names are due to the geometry of the light source, observer and interior of the measurement chamber. Scientists use this instrument to measure the amount of compounds in a sample. If the compound is more concentrated more light will be absorbed by the sample; within small ranges, the Beer–Lambert law holds and the absorbance between samples vary with concentration linearly. In the case of printing measurements two alternative settings are commonly used- without/with uv filter to control better the effect of uv brighteners within the paper stock.

Samples are usually prepared in cuvettes; depending on the region of interest, they may be constructed of glass, plastic (visible spectrum region of interest), or quartz (Far UV spectrum region of interest). Some applications require small volume measurements which can be performed with micro-volume platforms.

Applications

- Estimating dissolved organic carbon concentration
- Specific ultraviolet absorbance for metric of aromaticity
- Bial's test for concentration of pentoses

Experimental application

As described in the applications section, spectrophotometry can be used in both qualitative and quantitative analysis of DNA, RNA, and proteins. Qualitative analysis can be used and spectrophotometers are used to record spectra of compounds by scanning broad wavelength regions to determine the absorbance properties (the intensity of the color) of the compound at each wavelength. One experiment that can demonstrate the various uses that visible spectrophotometry can have is the separation of β -galactosidase from a mixture of various proteins. Largely,

spectrophotometry is best used to help quantify the amount of purification your sample has undergone relative to total protein concentration. By running an affinity chromatography, B-Galactosidase can be isolated and tested by reacting collected samples with Ortho-Nitrophenyl- β -galactoside (ONPG) and determining if the sample turns yellow. Following this testing the sample at 420 nm for specific interaction with ONPG and at 595 for a Bradford Assay the amount of purification can be assessed quantitatively. In addition to this spectrophotometry can be used in tandem with other techniques such as SDS-Page electrophoresis in order to purify and isolate various protein samples.

IR spectrophotometry

Spectrophotometers designed for the infrared region are quite different because of the technical requirements of measurement in that region. One major factor is the type of photosensors that are available for different spectral regions, but infrared measurement is also challenging because virtually everything emits IR as thermal radiation, especially at wavelengths beyond about 5 μm .

Another complication is that quite a few materials such as glass and plastic absorb infrared, making it incompatible as an optical medium. Ideal optical materials are salts, which do not absorb strongly. Samples for IR spectrophotometry may be smeared between two discs of potassium bromide or ground with potassium bromide and pressed into a pellet. Where aqueous solutions are to be measured, insoluble silver chloride is used to construct the cell.