Chapter

Fundamentals of Colorimetry

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Abstract

There are several kinds of analytical techniques following the principle of photometry in which colorimetry comes under absorption photometry. The colorimetry is commonly used analytical technique involved in quantitative estimation of color i.e. it is utilized to find out concentration of the colored substance in the sample solution e.g. water, biological samples at visible spectrum of light (380–780 nm). The colorimeter is an instrument in which this technique is used. It is also called absorptiometer.
A substance must be colorful or should have property of forming chromogens through the addition of reagents which will absorb light according to their color intensity to be measured. The intensity of the color is in proportion to the concentration of colored compound. Most of the analytical techniques used in our clinical laboratory presently are based on this photometric principles in which absorbed, transmitted or emitted light are measured. When intensity at different wavelength on the whole range of electromagnetic spectrum is measured, it is called spectrophotometry. Smartphone accessories have been evolved to allow the simple, quick reproducible values of the molecules.

Keywords: Beer's and Lambert's law, chromogen, colorimetry, photometry, standard curve

1. Introduction

Colorimetry is a type of photometry which is basically considered as the techniques in which light is detected and also detects changes in its intensity. The root word "photo," means light. A photometer is a machine measuring the energy of electromagnetic wave in the range of infrared radiation to ultra-violet radiation, including the visible part of the electromagnetic spectrum (**Figure 1**). It changes light into electric current by using a photocell. Colorimetry is referred if the measured light is in the visible range of the electromagnetic radiation. In this method a beam of light from a light source is allowed to pass via sample holder containing the analyte in the solution, the intensity of light transmitted will be less than the light passing through sample in the cuvette. The absorbed light is in proportion to the concentration of the analyte. The color of the sample is an intrinsic characteristic of the solution or it can be evolved by the addition of suitable reagents. The absorption of the sample is compared to that of standards from which the concentration of test sample can be calculated [1–3].

Photometric methods can be divided into two catagories, visual and physical. The physical photometry is commonly practiced.

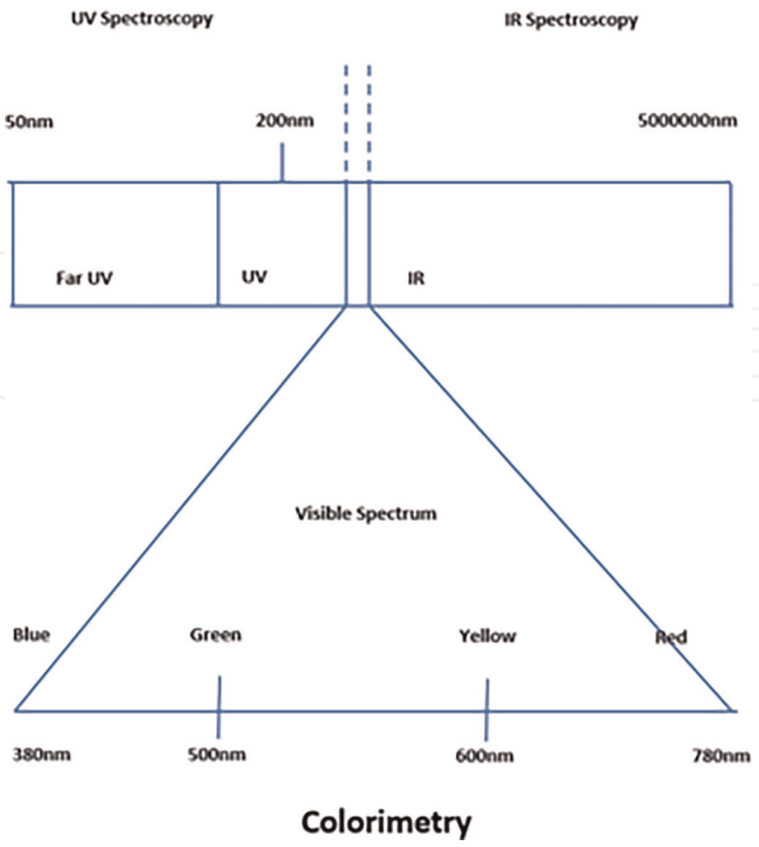

Figure 1.
Visible electromagnetic spectrum.

There are various kinds of analytical techniques that are based upon photometric principles. In Spectrophotometry, atomic absorption, turbidometry etc. absorbed or transmitted light is measured while in flame emission photometry emitted light is measured.

2. History

Scientist James Clerk Maxwell from Scotland, invented colorbox in 1860 which was the primitive colorimeter featuring a prism that assisted in control of red, green, and blue light beams separately to match the color in the sample. Later Louis Jules Duboscq (1817–86), a French optical instrument maker, designed Duboscq colorimeter in 1854 (**Figure 2**). It was one of the early colorimeters to be one of the popular. It allowed for the coexistent color comparison of two liquids. Duboscq explained an improvised variety to the French Academy of Sciences in 1868. A Duboscq colorimeter calculates the concentration of a substance through a visual comparison of the color intensity of the compound against that of a standard solution. This method of identification was revolutionary when first introduced, but the colorimeter was replaced by the emergence of the more precise spectrophotometer in the early 1940s. Bausch & Lomb then remodeled the first Duboscq colorimeter (**Figure 3**).

Later photoelectric colorimetry became widely known in the mid-1930s, and in 1938 William Henry Summerson brought up colorimeter containing a photocell. There is fascinating background on use of photocells which were mainly used by Germans. After the world war was stopped, an English pupil Arthur Evans was acquainted to a

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Figure 2.
Duboscq colorimeter, by F. Hellige and Co., Freiburg, Germany, 1901–1914.

leading medical researcher Dr. Rose of the Hammersmith Hospital, (UK) was looking for inventing a device which would enable him calculating the intensity of a color in a solution thereby calculating the concentration of an element in it. Colorimetry was already in use widely as analytical technique in medical, scientific and industrial laboratories which was performed by matching the solution under direct visual examination to known standards. Evan's EEL company started to produce first selenium photocell colorimeters (the model 2) commercially and were distributed all over the world. It gave both accurate and reliable results. Later the company was bought by Corning which was the pioneer company to produce pH sensitive glass for pH electrodes which are still popular in medical and industrial laboratories. However, new colorimeters do not use selenium photocells. This pioneer company is still gaining it's momentum in this field.

Later Klett Bio Colorimeters were made for clinical laboratory work. This Klett colorimeter includes standard color disks made of glass, which can be used in place of standard solutions in color comparisons [4–6].

2.1 Terminologies

Light: It is an electromagnetic radiation (EMR). It is composed of γ rays, X rays, ultra violet rays, visible rays, infrared rays, radio waves and micro waves. Of the components only those visible to eye is known as white light [7–10].

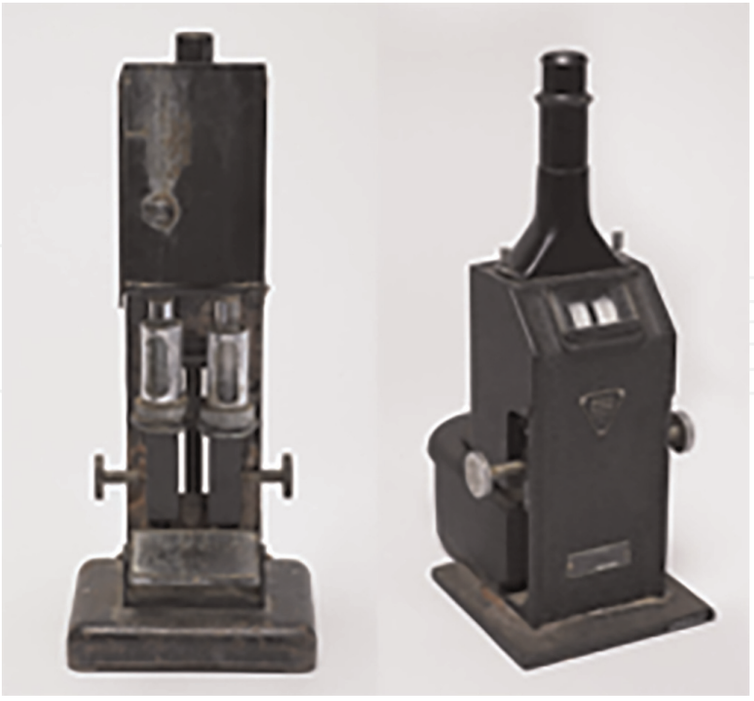

Figure 3.
Bausch & Lomb Duboscq Colorimeter.

Photons: Light is energy which exists in the form of bundles or as discrete packets of quanta called photons. The energy (E) of EMR is directly proportional to its frequency (u) and velocity (c) but inversely proportional to its wavelength (λ).

Thus, $E \alpha uBut, u = c/\lambda So, E \alpha c/\lambda E = hu = h\, c/\lambda$

where, h = plank's constant, c is the velocity of light in vacuum, 3×10^8 m/sec.

EMR is produced by events at the molecular, atomic, or nuclear level. Some of the events which give rise to EMR are oscillations of nuclei and electrons in electrical or magnetic fields, molecular bending and vibrations, excitation of orbital electrons, ejection of an inner orbital electron and rearrangement of the other electrons, nuclear break-up etc. The radiation they emit will have different wavelengths as each of these events differ in terms of the energy involved (**Table 1**). Thus, a full spectrum of EMR will be produced. Their magnitude of electrical vector and magnetic vector are denoted by the symbol **E and H** respectively. A beam of light from a light source consists of many randomly oriented plane polarized components being propagated in the same direction (**Figure 4**).

Polychromatic light: A beam of waves from a light source consists of several wavelength and is known as polychromatic.

Monochromatic light: A beam of waves in which all the rays have the same wavelength is known as monochromatic.

Spectrum: When EMR allowed to pass through a prism, all the 7 rays are dispersed into an orderly pattern called spectrum and can be measured by a spectrometer. The components have different wavelength and frequency. It can be classified as emission spectrum & absorption spectrum. When radiation is directly examined by a spectrometer the resultant pattern of wavelength is called emission spectrum. If a radiation is examined after interacting with an absorbing medium it is called absorption spectrum. Emission and absorption spectrum may be either line or band type. Spectrum produced by atoms in gaseous state appears as lines and it is called line or atomic spectrum. Spectrum produced by molecules in solution or crystal appears as bands

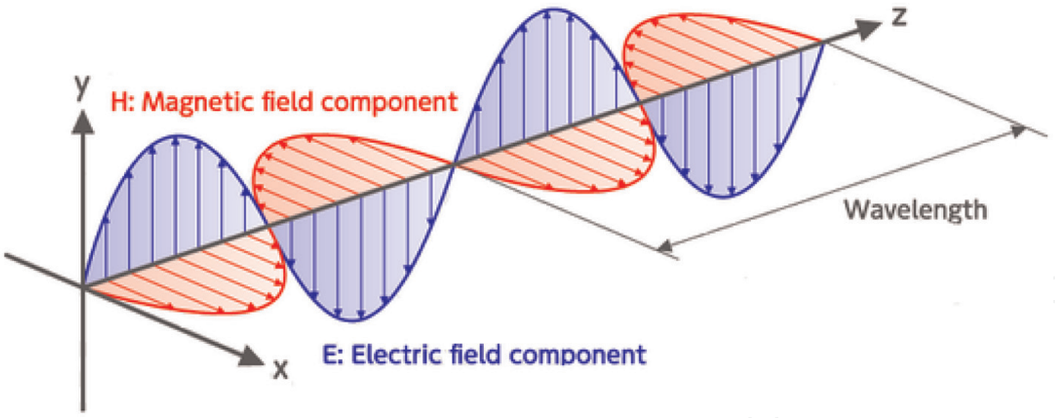

Figure 4.
Electromagnetic radiation wave of which is propagating transversally in space and time consists of the electric (E) and magnetic (H) field vectors directed perpendicular to each other.

Region	X-rays	Ultraviolet	Visible	Infrared	Microwave
Wavelength	0.1–100 nm	100–380 nm	380–750 nm	750 nm to 100 μm	100 μm to 30 cm
Effect on molecules	Excitation of sub-valence electrons	Excitation of valence electrons	Excitation of valence electrons	Molecular vibrations	Molecular rotations

Table 1.
Effect of absorption of electromagnetic radiation on the molecules.

and it is referred as band or molecular spectrum. Emission spectrum are of line & band emission spectrum while Absorption spectrum are of line & band absorption spectrum.

Spectroscopy: It is a technique which is used for quantification, characterization and structural analysis of a substance based on interaction of light with that substance. Interaction of light with a substance may result in different phenomena and generate a specific analytical signal. The signal is detected by a detector and recorded by a digital recorder in the form of a spectrum. The spectrum can be correlated with the amount, structure, property and functional group of the sample.

a. Emission spectrum

It is characteristic of the emitting atom or molecule in excited state. Line emission spectrum appears as fine bright lines on a dark background. e.g. line emission spectrum of sodium atoms excited by flame. Band emission spectrum appears as broad bright bands. e.g. Band emission spectrum of Ca salts heated in a flame.

b. Absorption spectrum

It is characteristic of the emitting atom or molecule in ground state.
Line absorption spectrum appears as dark lines on a bright background e.g. line absorption spectrum of sodium atoms irradiated by white light. Band absorption spectrum appears as broad dark bands e.g. Band absorption spectrum of iodine vapor irradiated with white light (**Figure 5**).

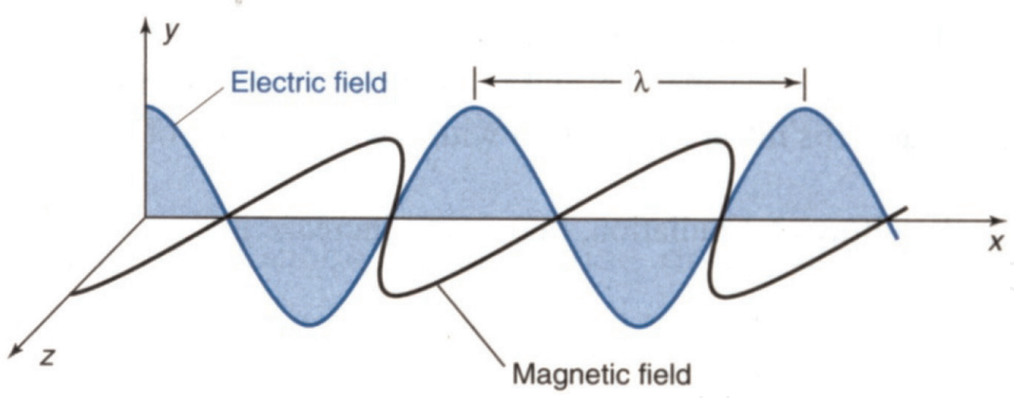

Figure 5.
EMR spectrum.

Frequency: When radiation is propagated into a medium, it oscillates. Number of oscillations occurring per second is called as the frequency (**u**) of the radiation. It is expressed as Hertz (**Hz**).

Wave number: Number of waves propagating per centimeter of the medium is called its wave number (**σ**) or **ū**.

Wavelength: The distance between two successive peaks of a wave is known as wavelength (λ) (**Figure 5**). The wavelength of a light wave determine its color or visibility. For e.g. light wave with λ between 380 and 780 nm is visible to human eye and therefore called visible light or white light. Visible light has a range of colors which are abbreviated in mnemonic VIBGYOR.

Velocity: Distance traveled by the light wave per second is called its velocity (**c**). It is expressed as cm/sec.

3. Parts of colorimeter

The parts of the colorimetry are as follows [3, 7, 9–11]:

1. **Light Source/Radiation source:** An electric lamp is used as radiation source mostly. Commonly used lamp is tungsten filament lamp (for wavelength 320–700 nm). Any lamp source which gives adequate intensity of radiation over the entire wavelength region can be used. The source of light for colorimetry should provide continuous radiation from 320 to 700 nm with adequate intensity which should be stable and fluctuations free. As the tungsten filament lamp fulfills the above criterias, this lamp makes its place in the most of colorimeters. The major drawback of the tungsten lamp is that it mostly emits its radiant energy in the near-infrared region of the spectrum. But at higher temperatures the life of the lamp is much reduced. In order to remove the unwanted infrared radiation a filter that absorbs heat is placed between the lamp and sample which absorbs most of the infrared radiation without affecting radiation energy at other wavelengths. Only about 15% of radiant energy emitted lies in the visible region.

2. **Slit:** It allows only a beam of light within visible spectrum excluding unwanted light.

3. **Condensing lens**: A beam of light after passing through slit falls on condenser lens which turns into parallel beam of light.

4. **Filters or monochromator:** Only light of required wavelength is passed through filter or monochromator while light of other wavelengths are absorbed.

   Filter: It permits only monochromatic light to pass through while absorbs unwanted ones. It is usually made up of colored glass or dyed gelatin. This is a means of selecting light of narrow wavelength λ (50 nm or more). A green filter allows green color to pass through while rest are absorbed. The color of filter used is always complementary to color of solution.

   I. Absorption filter: It may be of glass absorbance or gelatin absorbance type. Filter is solid sheet of glass being colored by a pigment (dyed gelatin or similar materials) which is dissolved or dispersed in the glass.

   II. Interference filter: It may be of constructive interference or destructive interference (Fabry-Perot). It has dielectric spacer film made up of CaF2, MgF2 or SiO, between two parallel reflecting silver films. The thickness of dielectric spacer film can be 1/2λ (1st order), 2 λ/2 (2nd order), 3 λ/2 (3rd order) etc.

   Monochromators: These are better and more efficient than filters in converting a polychromatic light into monochromatic light. It is usually found in spectrophotometer. Monochromators are of various types:

   I. Prism: It is either of refractive type prism or reflective type prism.

   II. Grating: It may be diffraction grating or transmission grating.

5. **Cuvette/sample cell/sample holder:** They are specially designed tubes made up of optical glass (borosilicate or quartz) to hold the colored sample for measurement in colorimeter for accurate reading. There are square, rectangular or round shaped cuvettes. It is used to hold samples of fixed optical pathlength which is usually 1 cm. Its capacity is 3–4 ml or even lesser in case of micro cuvettes. The colored solution in cuvette absorbs the complementary color (**Table 2**). It is made of material that does not absorb light of wavelength range of the interest.

Color of solution	Filter	Wavelength (nm)	Range (nm)
Blue green	Red	620	650–700
Blue	Yellow	590	570–600
Purple	Green	550	505–555
Red	Blue green	500	495–505
Orange	Green blue	490	475–495
Yellow	Blue	445	420–475
Yellowish green	Violet	410	400–420

Table 2.
Colors of solutions and their complementary filters.

Color wheel: The Color of Light *absorbed* and *observed* passing through the compound are complementary. Complementary colors lie across the diameter on the color wheel and combine to form "white light", so the color of a compound seen by the eye is the complement of the color of light absorbed by a colored compound.

6. **Detector (photocell)**: A device used to convert the light to electrical energy, comprising metal plate coated with photosensitive element such as selenium or cadmium (+ve electrode). The light sensitive material is covered with a transparent thin layer of gold or copper (−ve electrode). When the light strikes the selenium layer, electrons are liberated which pass into the transparent layer making it electronegative. A potential difference is created between the transparent layer and metal plate. The electric current thus generated is directly proportional to the intensity of light striking the photocell.

7. **Readout device**: The potential difference or electrical signal in photocell is detected by readout device. The device also can be called galvanometer or ammeter or digital readout, which shows absorbance & transmittance or both.

4. Principle of colorimetry

Principle of colorimetry: When a beam of monochromatic light passes through a colored solution, the coloring substances absorbs a portion of the light & the rest is transmitted. Absorption of light is related to the color intensity. The color intensity will be proportional to the concentration of the chemical (analyte) responsible for producing the color [7–11].

Absorbance (A): When light passes through a medium, the ratio of log of intensity of incident light to the intensity of transmitted light is called as absorbance or optical density (OD). Previously it was called extinction.

Transmittance (%T): When light passes through a medium, the ratio of intensity of the transmitted light to the intensity of incident light is called as transmittance.

Path length (l): The internal cross length of the cuvette through which light passes is called as path length. Usually it is 1 cm.

Absorbance maxima: Wavelength of maximum absorbance is known as absorbance maxima. It is denoted by λ_{max}. The wavelength at which a substance shows maximum absorbance is called absorption maximum or λ_{max} (**Figure 6**).

The amount of light is absorbed or transmitted by a colored solution in accordance with 2 laws.

1. **Beer's Law:** When monochromatic light passes through a colored solution, amount of light transmitted decreases exponentially with increase in concentration of colored substance. i.e. the amount of light absorbed by a colored solution is directly proportional to the concentration of substance in the colored solution **Aαc** (**Figure 7**).

2. **Lambert's law:** The amount of the light transmitted decreases, exponentially with increase in pathlength (l) of the cuvette or thickness of colored solution through which light passes. i.e. the amount of the light absorbed by a colored

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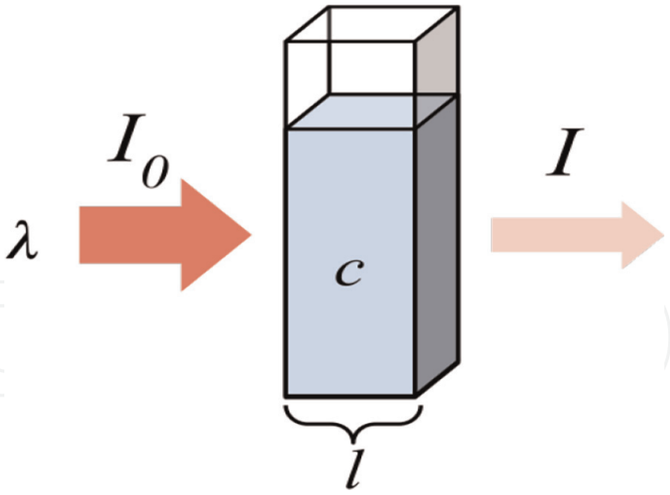

Figure 6.
Showing relationship between absorbance, absorptivity, pathlength and concentration where, io = incident light, I = transmitted light, a = absorbance, c = concentration of absorbing compound, l = pathlength, ε = molar absorptivity.

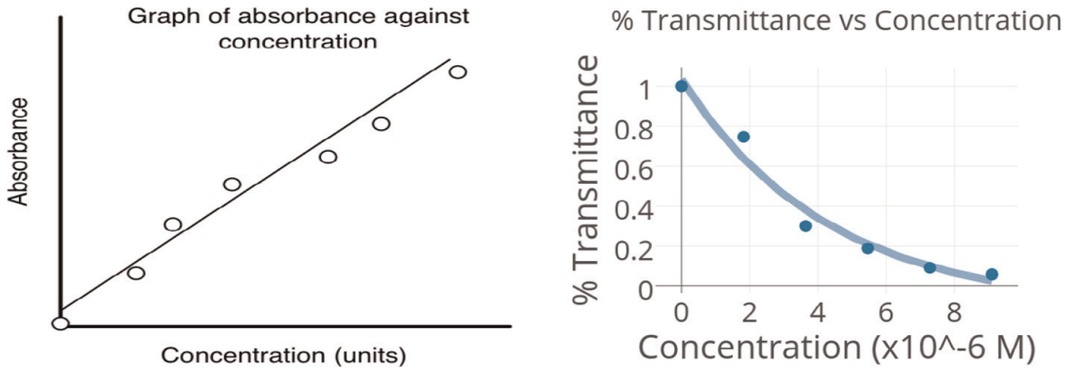

Figure 7.
Beer's law.

solution depends on pathlength of cuvette or thickness or depth of colored solution **Aαl** (**Figures 8** and **9**).

Relationship between absorbance (A) and transmittance (T): Relationship between (A) & (T) i.e.

Io = A + I A = Io–I.

By definition, A = log Io/I.

A = − log I/Io.

A = − log T (As T = I/Io).

A = log 1/T.

A = log 100/(%T) to take into percentage multiply by 100.

A = log 100– log%T (As log a/b = Log a–Log b).

A = 2 − log%T (As log 100 = 2).

Combined Beer's – Lambert's law: It is denoted as, amount of light transmitted through a colored solution decreases exponentially with increase in concentration of colored solution & increase in the pathlength of cuvette or thickness of the colored solution.

$$\mathbf{A \alpha c} \text{ or } \mathbf{A = k\,c} \qquad (1)$$

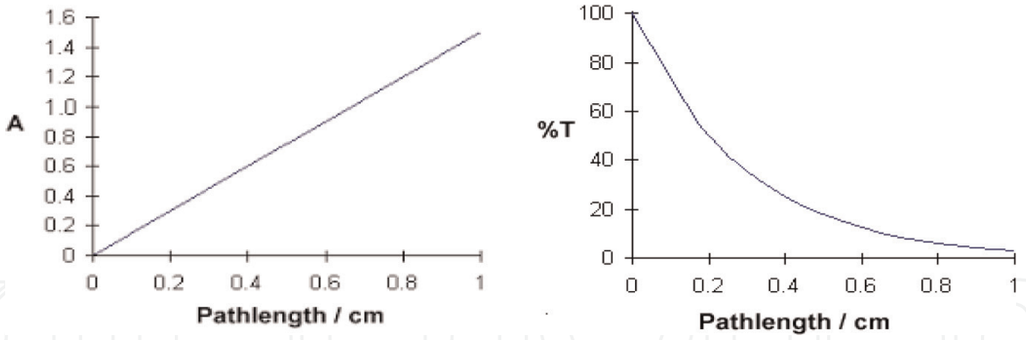

Figure 8.
Lambert's law.

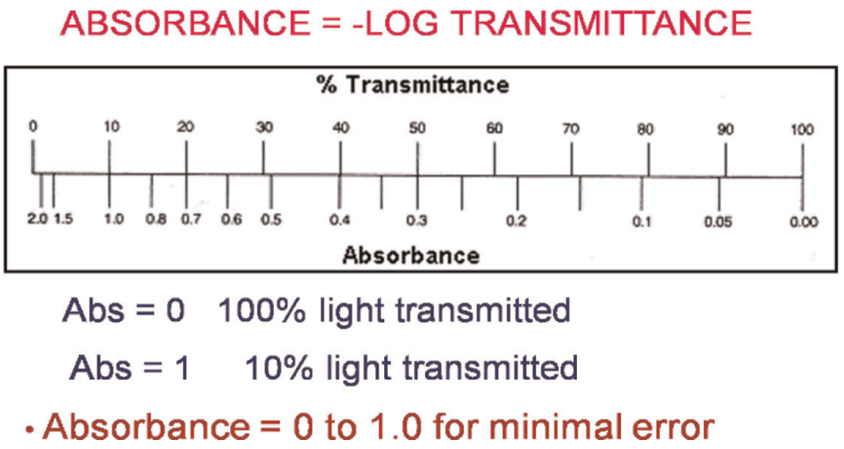

Figure 9.
Relationship between absorbance (a) and transmittance (T).

Where, k = linear absorption coefficient of the absorbing material.

$$\mathbf{A\alpha l\ A = k' l} \tag{2}$$

Where, k′ = absorptivity constant.

Combinely from Beer-Lambert law both k & k′ can be merged to form a single constant a & hence, combined equation can be expressed as **A** = **acl** (**Figure 6**)

Where,

A = absorbance.

a = proportionality constant defined as absorptivity.

c = concentration of the absorbing compound sample in solution usually expressed in grams per liter.

l = pathlength of the sample (cm).

A = **acl**, this equation forms the basis of quantitative analysis by absorption photometry. **A** values have no units, hence, the units for **a** are the reciprocal of those for **l**, when **l** is **1 cm** and **c** is expressed in moles per liter the symbol **ε** (epsilon) is substituted for the constand **a**.

A = **εcl**,

where,

ε = molar absorptivity/ molar absorption coefficient/molar extinction coefficient ($Lmol^{-1}\ cm^{-1}$).

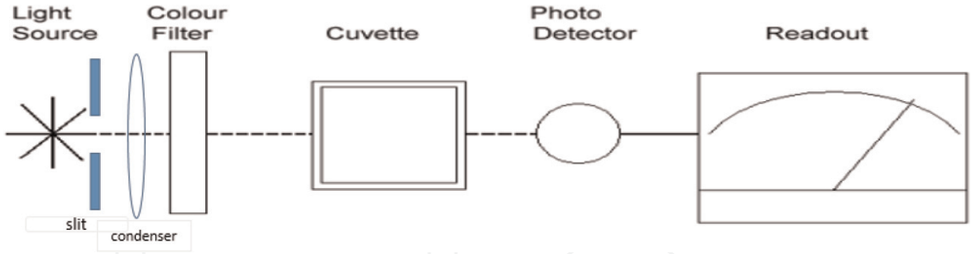

Figure 10.
Working principle of colorimetry.

The value for molar absorptivity ε is constant for a given compound at a given wavelength under prescribed conditions of solvent, temperature, pH, etc. It is a measurement of how strongly a chemical substance absorbs light at a given wavelength. For any particular molecular type, absorptivity changes as wavelength of radiation changes.

Let,

A_T = Absorbance of test A_S = Absorbance of standard.

C_S = Concentration of standard C_T = concentration of test.

$A_T = k \times C_T \times L$ and $A_S = k' \times C_S \times L$.

$A_T/A_S = (k \times C_T \times L)/(k' \times C_S \times L)$.

$A_T/A_S = C_T/C_S)$.

Therefore,

$$\mathbf{C_T = A_T/A_{S\times}\ C_S}$$

i.e. Concentration of test = Absorbance of the test X concentration of a standard/ Absorbance of the standard.

Therefore, above equation states that optical density (OD) is proportional to the sample concentration if the pathlength is constant. It follows then, that if the OD of a standard solution is known, then an unknown sample concentration can be calculated from its OD by applying the above formula. The standard chosen should have an optical density nearer to the OD of the unknown. The most accurate way of measuring the concentration in the unknown sample is through preparation of a calibrated curve or standard graph from a number of standards (**Figure 10**).

4.1 Preparation of standard graph and evaluation of unknown

A calibration curve is prepared by plotting known concentrations of a given substance against absorbance at a particular wavelength (usually the λ_{max}). The relationship between absorbance and concentration is linear if the Beer's law is obeyed (**Figure 11**).

$y = mx$.

Where, y = A, x = concentration and m = ab.

Unknown concentration of a substance is measured by comparing the absorbance of unknown to the absorbance of known concentrations of the same substance measured under exactly identical conditions (same pathlength l usually 1 cm, same wavelength or the same band of wavelength, same temperature, same solvent and same instrument etc).

A plot of absorbance values against known concentrations of a substance is known as a standard graph or a calibration curve and standard curve should always be linear. The linearity of the curve represents that it follows Beer's-Lambert law.

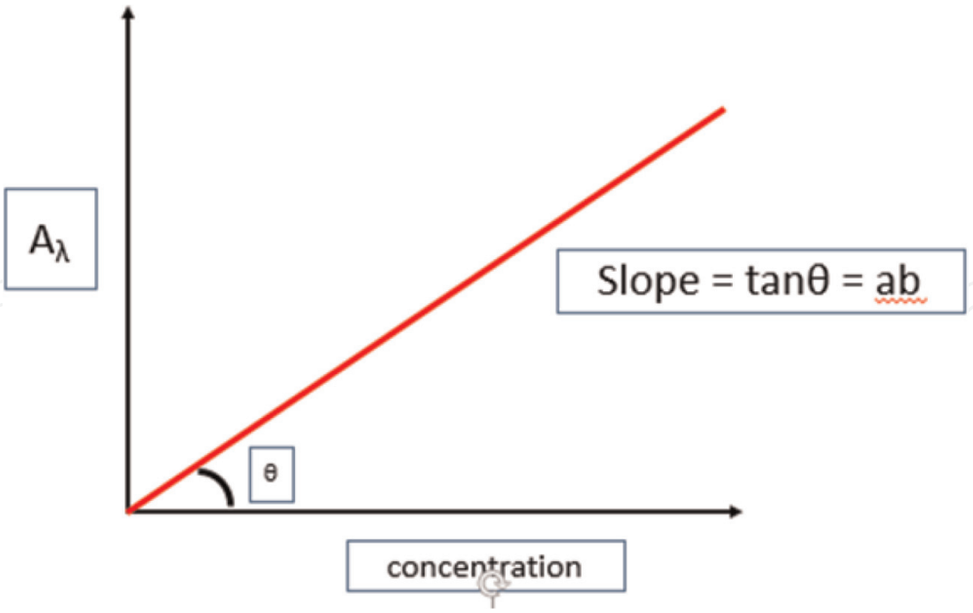

Figure 11.
Preparation of standard graph and evaluation of unknown.

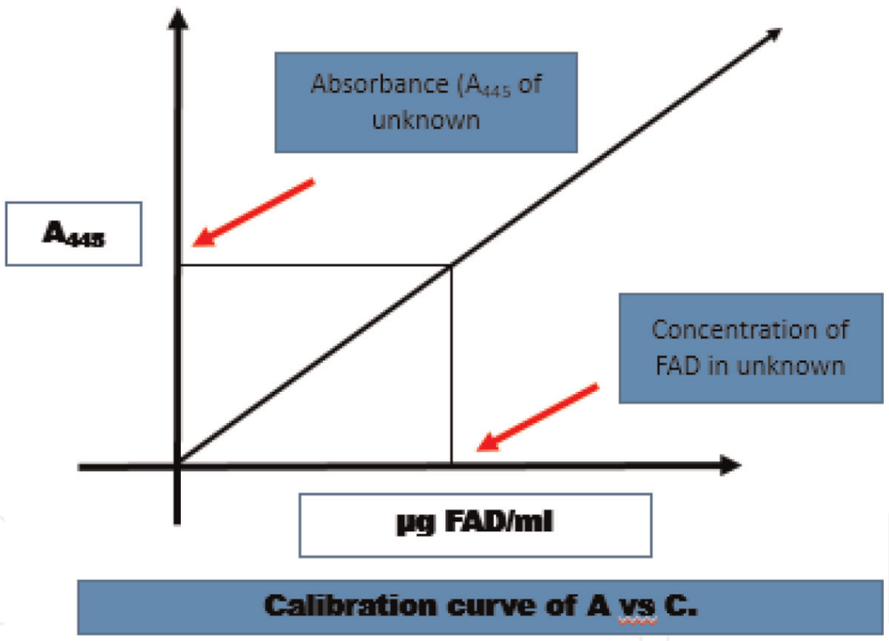

Figure 12.
Evaluation of unknown concentration of FAD from standard graph.

From this graph the unknown concentration of flavin adenine dinucleotide (FAD) can be read off (**Figure 12**).

5. Linearity limit

It is the range of concentrations of an analyte which the instrument can measure linearly beyond which the instrument does not response definetly.

Importance of knowing linearity limit: In biochemistry and other allied sciences, the linearity limit of each test is to be known. It is different for different

kits of same parameter. Reading beyond this range is not reliable. In such condition, the sample is diluted and retested and multiplied by the dilution factor. In conclusion calibration curve or standard graph can be used to detect limit of linearity of a test.

6. How to draw standard curve?

Standard curves are defined as a graphs with absorbance or % transmittance plotted on the y axis, and increasing concentrations of standard along the X axis. If the Beer's law is followed, the resulting line representing absorbance vs. concentration will be straight.

A standard curve is constructed after obtaining the (%T) or (A) readings from a number of solutions of known concentration used in a reaction or procedure.

For example, Let us draw standard curve of glucose:

The glucose can be estimated even by plotting graph. This method is helpful to learn more about pipetting and plotting a graph with different concentration of glucose ranging from 50 to 400 mg.

1. Standard glucose (stock) 500 mg of glucose is dissolved in 100 ml of water. It contains 5.0 mg of glucose/ml

2. Working standards: series of working standards of various concentrations are prepared which ranges from 50 to 400 mg/dl.

3. 50 mg/dl – 1.0 ml stock +9.0 ml water

4. 100 mg/dl – 2.0 ml stock +8.0 ml water

5. 150 mg/dl – 3.0 ml stock +7.0 ml water

6. 200 mg/dl – 4.0 ml stock +6.0 ml water

7. 250 mg/dl – 5.0 ml stock +5.0 ml water

8. 300 mg/dl – 6.0 ml stock +4.0 ml water

9. 350 mg/dl – 7.0 ml stock +3.0 ml water

10. 400 mg/dl – 8.0 ml stock +2.0 ml water

Followed by measurement of the absorbance of those standards in colorimeter.

After the readings are obtained each is plotted on semi-log graph paper (% transmittance) or linear graph paper (absorbance) against the corresponding concentration. If the procedure follows Beer's Law, the points plotted will generally lie such that a straight line can be drawn through them. The concentration of controls and other unknowns (patient samples) can be determined by locating their %T or A reading on the line, then dropping an imaginary line down from that point to intersect the concentration axis.

Once a standard curve is developed for a particular test method on a particular spectrophotometer, it should be checked periodically to determine that it is still good.

A new curve should be constructed when there is a change in reagent lot numbers, methodology or procedure, an instrument parameter (change bulb, optics cleaned, etc.),

Once the curve is drawn, a number of things must be considered to determine its acceptability. The majority of the curve's points should be on or close to the line. There could be many reasons for a point not being on the line. Whether or not the curve passes through the point of origin (the "0"), varies with the procedure. If Beer's law is followed and the procedure is linear at the lower concentrations, the curve's line generally goes through the zero.

Always a standard curve should be prepared to confirm that Beer's law is applied to the analysis being carried out. The curve should be linear expectantly. There are several factors that may cause deviations from linearity which refers to deviations from Beer's law. Following factors may cause deviations from Beer's law:

Imperfect monochromacy or polychromatic radiation: The most common factor for deviation from linearity in most colorimeters is the use of a band of wavelengths to measure absorbance. Beer-lambert's law is applicable for monochromatic radiation only.

Other factors which includes followings:

High concentration of chromophores: According to the Beer-Lambert law absorbance (A) is linearly proportional to the concentration of chromophores. There may be deviation in samples with high sample concentrations (>10 mMol of solute) & high absorbance. The intensity of light at choosen wavelength (I_λ) should be 10 times higher than the intensity of the stray light (I_{stray}) to reduce deviation from linearity [2]. In addition, the chromophore molecules may dimerize at high concentration and the absorption spectra of the dimers are not the same as that of the monomers. If the spectra differ, the absorption coefficient will differ leading to a positive or negative deviation (**Figure 13**).

Polymerization: It results into the spectral shift. This wavelength at which molar absorption coefficient is not changed is called the isobestic point.

Aggregation: Moreover, high concentration may also lead to aggregation. The intensity of the radiation reaching the detector is decreased because large aggregates scatter light. In such a case a positive deviation will be seen. Aggregation can also lead to electronic interactions that can either decrease or increase the absorption coefficient. At high concentrations, chemical events (association, polymerization,

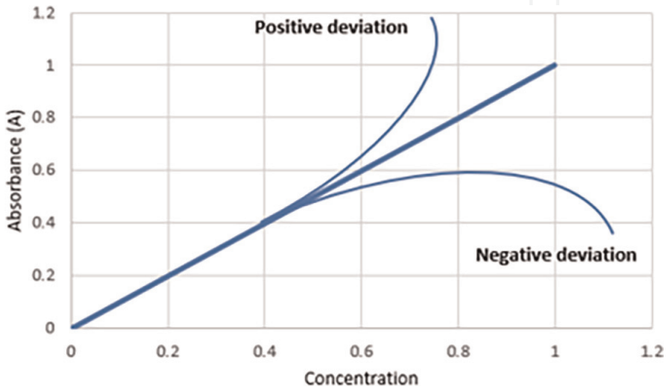

Figure 13.
Deviation from Beer-Lambert law.

dissociation, pH change, interaction with the solvent to produce a product with different absorption characteristics) which may lead to a change in the chemical composition of the solution. Consequently, a deviation from linearity will result.

Low concentrations of chromophores: Deviation may also occur at low concentrations. Proteins are known to denature at low concentrations and, the denatured product has an absorption spectrum that is different from the native protein.

Apart from imperfect monochromacy, other instrumental drawbacks may also result in deviations from Beer's law. Stray radiation reaching the detector changes sensitivity of the detector and power fluctuations of the radiation source and detector amplification system also cause deviation.

Besides above factors, the following factors can also cause deviations from the Beer's law:

1. **Temperature:** There are changes in the degree of solubility, dissociation/association properties of the solute, and several other factors with the changes in temperature. The absorbance is also changed.

2. **Sample Instability:** Some colored compounds are unstable and undergo changes within short duration. In such cases the color might increase, or decrease.

3. **Fluorescence:** Those substances which fluoresce, there might be deviations because fluorescent intensity also reaches the detector apart from the transmitted intensity.

4. **Turbidity:** Turbid solutions give higher absorbance.

5. **Mismatched cell:** If length of sample and blank cell are not equal there may be deviation.

6. **Spectral slit width (SSW):** The deviation occurs if the width of the instrument is not proper. Spectral slit width (SSW) should be as narrow as possible. As SSW increases resolution decreases.

7. Unstable radiation source

8. **Miscellaneous factors**: Impurities, Solvent, pH, suspension, coagulation and so on also deviate from Beer's Lambert law.

7. Variety of applications

- Quantitative estimation of hemoglobin (Hb) and biochemical concentration of analyte in serum sample (glucose, protein, albumin, triglyceride, cholesterol, bilirubin, urea, creatinine, uric acids, calcium etc).

- Quantitative estimation of enzymes such as alkaline phosphatase, alanine transaminase, alpha amylase.

- Testing the water quality for chemicals such as chloride, fluoride, zinc, iron etc.

- Determination of concentration of plant nutrients such as nitrates, NH3.
- Phosphate determination.
- Chrominium in aluminum alloys

In serum sample: colorimetry may be applied for

1. Estimation of serum glucose by glucose oxidase-peroxidase (GOD-POD) method.
2. Estimation of total protein in the given serum sample by Biuret method.
3. Estimation of serum albumin in the given sample by Bromocresol Green Method.
4. Estimation of triacylglycerol in given serum sample by enzymatic method.
5. Estimation of cholesterol in given serum sample by CHOD-4AP method.
6. Estimation of creatinine in given serum sample by Jaffe's alkaline picrate method
7. Estimation of serum bilirubin by modified Jendrassik/Grof method.
8. Estimation of serum calcium based on colorimetric method using (O-cresolpthalein complexone)
9. Estimation of serum urea by enzymatic (urease) method using Modified Berthelot reaction.
10. Estimation of serum uric acid by enzyme urease method (4-AP method).
11. Estimation of alkaline phosphatase in the given serum sample by kinetic method.
12. Estimation of alpha-amylase in the given serum sample by the CNPG3 method.
13. Estimation of alanine transaminase in the given serum sample by the kinetic method.

In whole blood:

1. Hemoglobin estimation by Drabkin method.

In urine:

1. Determination of primaquine metabolites in urine.

2. Determination of urinary chloride.

3. Determination of urinary cystine

4. Quantitative determination of urinary p-aminophenol

In CSF: colorometry is applied for

1. protein determination in CSF.

2. chloride determination in CSF.

3. glucose determination in CSF.

In water:

1. Iron

2. Chloride

Plant material:

1. Phosphorus estimation

8. Pre-requisitive for good colorimetry

1. Steady power supply with voltage stabilizer

2. Centrifuge should be away from colorimeter

3. No bubbles

4. No contamination with thumb prints, scratches

9. Advantages & disadvantages of colorimetry

Advantages: Colorimetry is cheap and useful for quantitative determination of colored substances. It is portable. Since it has limited manual operations it is easy to handle.

Disadvantages: It can not be used for those substances which do not possess color. Errors in inferences can be seen from alike color of interfering compounds. Moreover, very elevated concentrations cannot be measured hence is less sensitive. The filters available are of only narrow range. Unstable light source has possibility of errors since there is change in the light intensities.

10. Case study

- Under aseptic condition total 2 ml of blood from every participant was drawn which was placed in Ethylenediaminetetraacetic acid (EDTA) test tube at school

and transpoted to Biochemistry laboratory of Nepal Medical College and Teaching Hospital (NMCTH) in cold blood box and hemoglobin was estimated on the same day [12].

- Hemoglobin (Hb) estimation by Drabkin method was performed at Biochemistry laboratory.

Principle: Hemoglobin (Hb) + Ferriccyanide → Methhaemoglobin → Cyanomethhaemoglobin (stable red compound) which is measured colorimetrically, it's intensity is directly in proportion to amount of Hb.

- **Drabkin method sample:** whole blood

- At biochemistry lab, out of 2 ml whole blood in EDTA test tube, 20 μL (i.e. 0.02 ml) was mixed with 2.5 ml of Drabkin reagent i.e. (tests) in glass tubes with the help of micro pipette then was rinsed for several times for proper mixing. The blank tube was used to adjust the colorimeter to zero optical density at 546 nm wavelength. Finally readings of optical density of the tests was taken which is directly proportional to amount of Hb in the tests as stated by Beer's law of absorbance.

- **Results:**

Hemoglobin (gm/dl)	Range	Mean ± SD	Median
	6–22.8	12.63 ± 2.91	12.60

Table 3.
Mean, Range and Median of haemoglobin of the participants enrolled in the study.

Discussion: Hb was found to be ranged from 6.0 to 22.80 g/dl, mean ± SD of Hb being 12.63 ± 2.91 g/dl.

11. Advances in colorimetry

The accessibility of smartphones and the speedy development of different mobile applications is having a great effect in our daily life [13]. Nevertheless, till now, smartphones have been rarely applied to other sciences and their applications in this research field may provide an immense leap in terms of routine sample analysis with an accessible, fast, simple and low-cost strategy. Smartphone cameras produces images using a red, green, and blue (RGB) color code. Based on this code, simple analysis of these digital pictures to quantify color changes is done with the help of different applications. These differences in color have been used for the quantification of various analytes in liquid suspensions, both in simple solutions and direct sampling matrices [14]. In many cases, a reagent is used to produce a colored compound through the reaction with a particular analyte, and the color of the newly formed analyte-reagent complex is measured [15].

Smartphones are embedded with an optical chemical or biosensing base via the use of an attached lighting control enclosure that is fitted to the device and the assay

platform that may be strip, cassette, cuvette etc. In this configuration, the smartphone can function as an illumination source (Light Emitting Diode), a signal detector, and a signal processor. Based on the conditions of the assay, various lenses, filters, diffraction gratings, and alternative light or power sources may be incorporated within the attached enclosure to intensify signal detection from colorimetric, fluorescence-based, chemi/bioluminescence-based, and scattering-based assays [16, 17].

Pharmaceutical compounds can be determined in dosage forms and biological matrices by using various colorimetric reagents with the help of spectrophotometric and chromatographic methods. The colorimetric methods have more sensitivity than that of ultraviolet spectroscopic methods. Moreover colorimetric methods measure light of longer wavelengths which decreases effects of interfering excipients. Most of the procedures are inexpensive and reagents easily available. There is a great scope for development of new reagents and new colorimetric methods [18].

There has been a focus on newly developed chemosensors on systems that permit for portable, on-site testing of the target analytes without requiring high yield, costly laboratory instrumentation [19, 20]. It is challenging to design a portable chemosensors to maintain high selectivity, sensitivity, and wide applicability in the efficient measurement of various analytes especially that are similar in structure and size. The Levine group has developed sensitive and selective fluorescence-based systems for analyte detection by using the property of cyclodextrin to act as a supramolecular scaffold that aids proximity-induced, highly analyte-specific interactions between an analyte of interest and a high-quantum yield fluorophore [21–25].

Recently there has been developments in the the field of optofluidics making use of light matter interactions in integrated devices with significantly improved features [26]. The newly developed microfluidic device integrated in optofluidic system has been introduced. The sensitivity of measuring concentration of sample by optofluidic system is better than that of other methods [27].

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