# Introduction to Spectrophotometric Techniques

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**Abstract**

Spectrometric techniques are used to measure the interaction of different frequency components of electromagnetic radiations (EMR) with that of matter. After interaction with matter, these radiations are absorbed by the matter. It is not possible that we look at the matter instead, we observe the interaction of light with different degrees of freedom of matter and/or substance. This chapter describes the basics of spectrophotometry and various types of spectrophotometric techniques. Spectrophotometry is a key method that is frequently used for identification and quantification of raw materials and pharmaceutical products.

**Keywords**

Electromagnetic radiations · Frequency · Electronic energy level · Spectrophotometer · Types of spectrophotometric techniques

## 2.1 Introduction

Spectrometric techniques are used to measure the interaction of different frequency components of electromagnetic radiations (EMR) with that of matter. Electromagnetic radiations interact with matter at specific energy levels. After interaction with matter, these radiations are absorbed then atoms/molecules of analyte present in sample move from one energy state (usually ground state having low energy level) to another energy state (usually excited stated having high energy level). It is not possible that we look at the matter and/or substance but its ghost when the matter and/or substance interacts with light. Instead, we observe the interaction of light with different degrees of freedom of matter and/or substance. In spectrophotometry, we either measure the absorbance or emission of EMR after its interaction with matter. Interaction of EMR with matter is directly dependent on the energy of radiation. Spectrophotometry is used to understand how different frequency components of EMR interact with matter and how we can use this information to understand



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the quantity of analyte present in sample. Broadly speaking, spectrophotometry is basically the set of tools that can be used together in different ways to understand the chemical properties and/or nature of the analyte present in sample.

### 2.1.1 Photometry

It is the branch of science that deals with the measurement of light. Photometer is a tool that is used for the measurement of intensity of light. It is the measurement of the amount of luminous light (either absorbed or emitted) falling on the surface of analyte present in sample from a source of radiation.

### 2.1.2 Spectrophotometry

It is the phenomenon of measurement of the intensity of light at selected wavelengths. This method depends on the light absorbing and/or emission property of either the analyte or a derivative of analyte being analyzed. Spectrophotometry is used in pharmaceutical analysis for the identification of the analyte through the spectrum that is absorbed and emitted by an analyte present in the sample solution.

## 2.2 Spectrum

It is defined as the measurement of response as a function of wavelength or more precisely the frequency.

## 2.3 Electromagnetic Radiations

Electromagnetic radiations (EMR) contain discrete energy packets. These discrete energy packets are known as photons. A photon contains an oscillating magnetic field and an oscillating electric field (Fig. 2.1). Both of these fields fall perpendicular to one another.

### 2.3.1 Frequency ($\nu$)

Frequency is defined as the number of oscillations produced by an electrical field radiation per second. Hertz (Hz) is the unit used for frequency (1 Hz = 1 cycle per second).

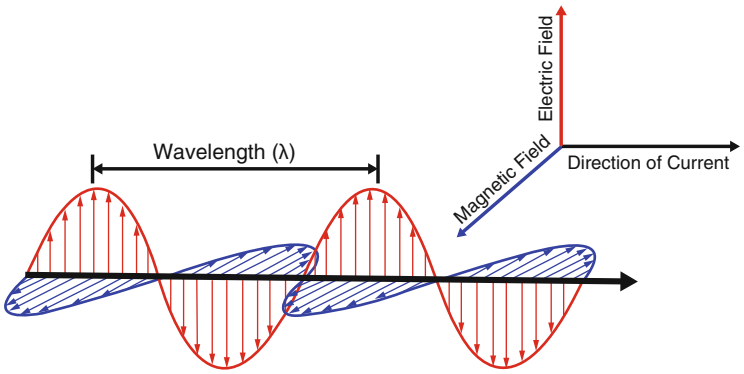


**Fig. 2.1** Schematic representation of levels of electromagnetic radiations

### 2.3.2 Wavelength (λ)

It is the measurement of distance that exists between two adjacent parts of a wave present in the same phase or the distance present between two adjacent troughs or crest.

### 2.3.3 Levels of Electromagnetic Radiations

In EMR, three levels exist which are as follows:

#### 2.3.3.1 Electronic Energy Level

Molecules exist in the lowest energy level ($E_0$) at room temperature that is known as ground state. Whenever, the EMR are absorbed by the molecules, valence electrons are promoted to next higher energy state abbreviated as $E_1$ that is also known as excited state. This shifting of electrons from lower energy level (ground state) to higher energy level (excited state) by absorbing the energy in the form of EMR is known as electronic transition and the difference is calculated as: $DE = E_1 - E_o$.

#### 2.3.3.2 Vibrational Energy Level

These are the less energy levels than electronic energy levels. The difference between these energy levels is comparatively small such as 0.01–10 kcal/mole. For example, when infrared (IR) radiations are absorbed by the molecules, they are excited from lower vibrational energy level to higher vibrational energy level and start vibrating with relatively higher amplitude.

#### 2.3.3.3 Rotational Energy Level

Rotational energy levels are quantized and discrete. The difference between these levels is relatively smaller as compared to that of vibrational energy levels. Overall, order of different energy level is represented as follows:

$$\Delta E_{rotational} < \Delta E_{vibrational} < \Delta E_{electronic}.$$

## 2.4 Principle of Spectroscopy

The principle of spectroscopy is based upon the measurement of spectrum of given analyte present in sample having either atoms or molecules. Spectrum consists of the graph of intensity radiations either absorbed or emitted by the sample versus wavelength ($\lambda$) or frequency ($\nu$). Spectrometer is a device which is used to measure the spectrum of analyte. It is more advanced instrument than colorimeter. It is because there are filters present in colorimeter which allow wide range of wavelengths of radiation to pass through, whereas in spectrophotometer, grating and/or prism is utilized in order to split the light beam into multiple wavelengths. With the help of selective mechanism, specific wavelength of EMR may interact with sample solution.

## 2.5 Spectrophotometer

This is an optical instrument that is used to measure the intensity of EMR relative to the specific wavelength. It is useful for the measurement of analyte concentration present in sample solution by measuring the amount of EMR absorbed and/or emitted by the sample solution. The sample to be analyzed by spectrophotometer must be in solution form and the solvent that contains analyte must be optically transparent in specific wavelength region.

### 2.5.1 Components of Spectrophotometer

Following are the four major components of spectrophotometer:

1. *Light source*: It provides the polychromatic light to the monochromator which diffracts the light. The sources of light depend on the type of spectrophotometric technique. For UV-radiation, the most common sources of light are hydrogen lamp and the deuterium lamp. For visible radiation, the most common sources are tungsten filament and carbon arc. For IR-radiation, the most common sources are Nernst Glower and Global.
2. *Monochromator*: It splits polychromatic light into individual wavelengths and separates these individual wavelengths into narrow bands.
3. *Cuvette and/or sample holder*: It holds the sample and allows the specific wavelength to pass through itself. Cuvettes are transparent in nature and are made of ordinary glass or quartz material.
4. *Photosensitive detector*: Detector must be highly sensitive and long-term stable that have the ability to detect the low level of the radiant energy.


**Fig. 2.2** Schematic representation of single-beam spectrophotometer

5. *Read-out device*: It has the ability to interpret the signals received from the detector.

According to the number of beams used, spectrophotometer has the following two types:

## 2.5.2 Single-Beam Spectrophotometer

Single beam passes through sample present in cuvette (Fig. 2.2). At first, the spectrophotometer is standardized by putting a reference solution in the cuvette, and the resulting absorbance is subtracted from absorbance of sample solution in order to remove solvent effect. Single-beam spectrophotometer follows Beer–Lambert law to determine the unknown concentration of the analyte.

### 2.5.2.1 Advantages

1. Less expensive.
2. High sensitivity due to non-splitting of the light source.

### 2.5.2.2 Disadvantages

Single-beam spectrophotometer is not suitable due to the lack of the compensation of the following disturbances:

1. Analysis is time consuming as it has single cell. At first, the reference solution is loaded to record the reading, then cuvette is washed and sample solution is loaded to record the recording.
2. Electronic circuit fluctuations.
3. Voltage fluctuations.
4. Mechanical component's instability.
5. Drift in energy of light sources.

## 2.5.3 Double-Beam Spectrophotometer

A double-beam spectrophotometer is used for the comparison of the light intensity between two light paths, one beam passes through a reference and the other from the test sample (Fig. 2.3).

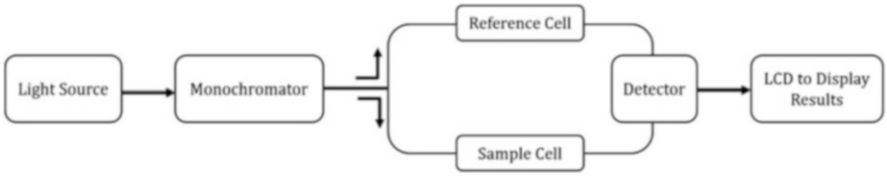


**Fig. 2.3** Schematic representation of double-beam spectrophotometer

#### 2.5.3.1 Advantages

1. It offers better detection than single-beam spectrophotometer.
2. Instability factors, lamp drift, voltage fluctuations, and stray light do not affect the measurement of light as these factors influence the measurement of results in single-beam spectrophotometer.

According to the range and type of EMR used, it has the following types:

1. UV-VIS spectrophotometer.
2. IR spectrophotometer.
3. Atomic absorption spectrophotometer (AAS).
4. Atomic fluorescence spectrophotometer (AFS).
5. X-ray fluorescence spectrophotometer (XFS).
6. Mass spectrophotometer (MS).

### 2.5.4 Types of Spectrophotometric Techniques

Types of the spectrophotometric technique depend on the quantitative pharmaceutical analysis of analyte which is measured by the interaction of different frequency components of EMR with analyte. Mostly, the spectrophotometric techniques can be classified into the following major types:

### 2.5.5 Absorption Spectroscopy

In this spectrophotometric technique, we measure the amount of EMR that is being absorbed by the sample (Fig. 2.4). The examples are UV-VIS spectroscopy, atomic absorption spectroscopy, infra-red spectroscopy, and nuclear magnetic resonance spectroscopy.

### 2.5.6 Emission Spectroscopy

In this spectrophotometric technique, when EMR interacts with sample, the sample emits the radiations of specific wavelength which are then detected to predict the

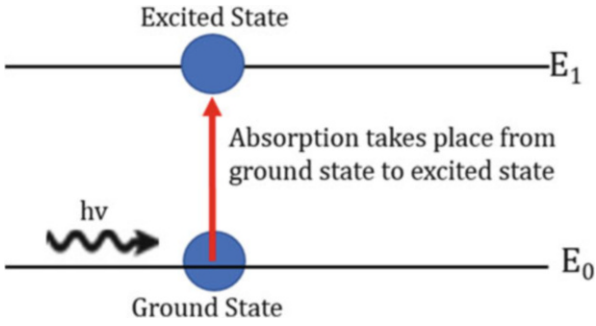


**Fig. 2.4** Schematic representation of absorption of light in atomic absorption spectroscopy

amount of the sample. In emission spectrophotometric technique, the sample first absorbs the EMR and then emits the light of specific wavelength. The examples are atomic emission spectroscopy, fluorescence emission spectroscopy.

### 2.5.7 Scattering Spectroscopy

In this technique, the substance scatters the EMR at specific wavelength and polarization angles. Scattering of light gives the information regarding the molecular structure. The scattering phenomenon is much faster than the absorption and/or emission phenomenon. One of the most important examples of light scattering spectroscopy is Raman spectroscopy. It is used for qualitative purpose. It is helpful for the identification of molecules and chemical bonds.

## 2.6 Applications

Spectroscopic techniques have wide range of applications in the field of pharmaceutical analysis. The most important are as follows:

### 2.6.1 Quantitative Analysis

Spectrophotometer is widely used in the quantitative pharmaceutical analysis. In quantitative pharmaceutical analysis, the unknown concentration of analyte may be determined with the help of absorption spectrophotometry because most of the biological organic compounds have the ability to absorb EMR in UV-VIS region. For example, nucleic acids and proteins absorb at 254 nm and 280 nm, respectively.

### 2.6.2 Qualitative Analysis

UV-VIS spectrophotometer is used to identify the compounds both not only in pure form but also in biological preparations. Identification of compounds can be done by plotting the absorption spectrum of the analyte because the absorption at specific wavelength gives some hints to the structure of the compound. For example, absorption at 220–280 nm indicates that the analyte may be aliphatic, alicyclic hydrocarbons or their derivatives. Absorption at 250–330 nm indicates that the analyte may contain more than two conjugated double bonds.

### 2.6.3 Enzyme Assay

Enzyme assay is basically performed with the help of spectrophotometer. In this assay, the substrate absorbs light in UV region. For example, lactate dehydrogenase (LDH) is an enzyme that converts lactate to pyruvate with the help of co-enzyme $NAD^+$. In this reaction, LDH transfer the electrons from lactate to $NAD^+$ as shown in the following equation.

$$\text{Lactate} + \text{NAD}^+ \leftrightarrow \text{Pyruvate} + \text{NADH} + \text{H}^+.$$

In spectrophotometer, NADH absorbs radiations at the wavelength of 340 nm in UV range, while $NAD^+$ does not absorb radiation at this wavelength.

Another example of enzyme assay is pyruvate kinase that converts phosphoenol-pyruvate to pyruvate and produces one molecule of ATP. Then pyruvate is converted into lactate.

$$\text{Phosphoenolpyruvate} + \text{ADP} \leftrightarrow \text{Pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \leftrightarrow \text{Lactate} + \text{NAD}^+.$$

In this reaction, NADH is oxidized to $NAD^+$ by converting pyruvate into lactate. As $NAD^+$ does not absorb radiations at 340 nm in UV range, the absorbance decreases which indicate the conversion of pyruvate to lactate.

### 2.6.4 Molecular Weight Determination

Spectrophotometer can help to determine the molecular weight of the analyte. Spectrophotometer can only determine the molecular weight of the small compounds.

### 2.6.5 Physicochemical Properties

Spectrophotometer can be used to determine the following physicochemical properties of the analyte:

1. Heats of formation for molecular addition in compounds and/or complexes.
2. Determination of the empirical formula of the substrates.
3. Dissociation constants of acids and bases.
4. Determination of the rate of reactions of the substrates.
5. Quantitative and qualitative determination of the complexes.
6. Hydration equilibration of carbonyl compounds.

## Further Reading

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