Unit VIII: Spectroscopy

BB/BM/BZ 406
Biophysics and Bioethics

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Introduction

Definition of Spectroscopy

It is a branch of science that deals with interaction of matter with light or electromagnetic radiation (EMR).

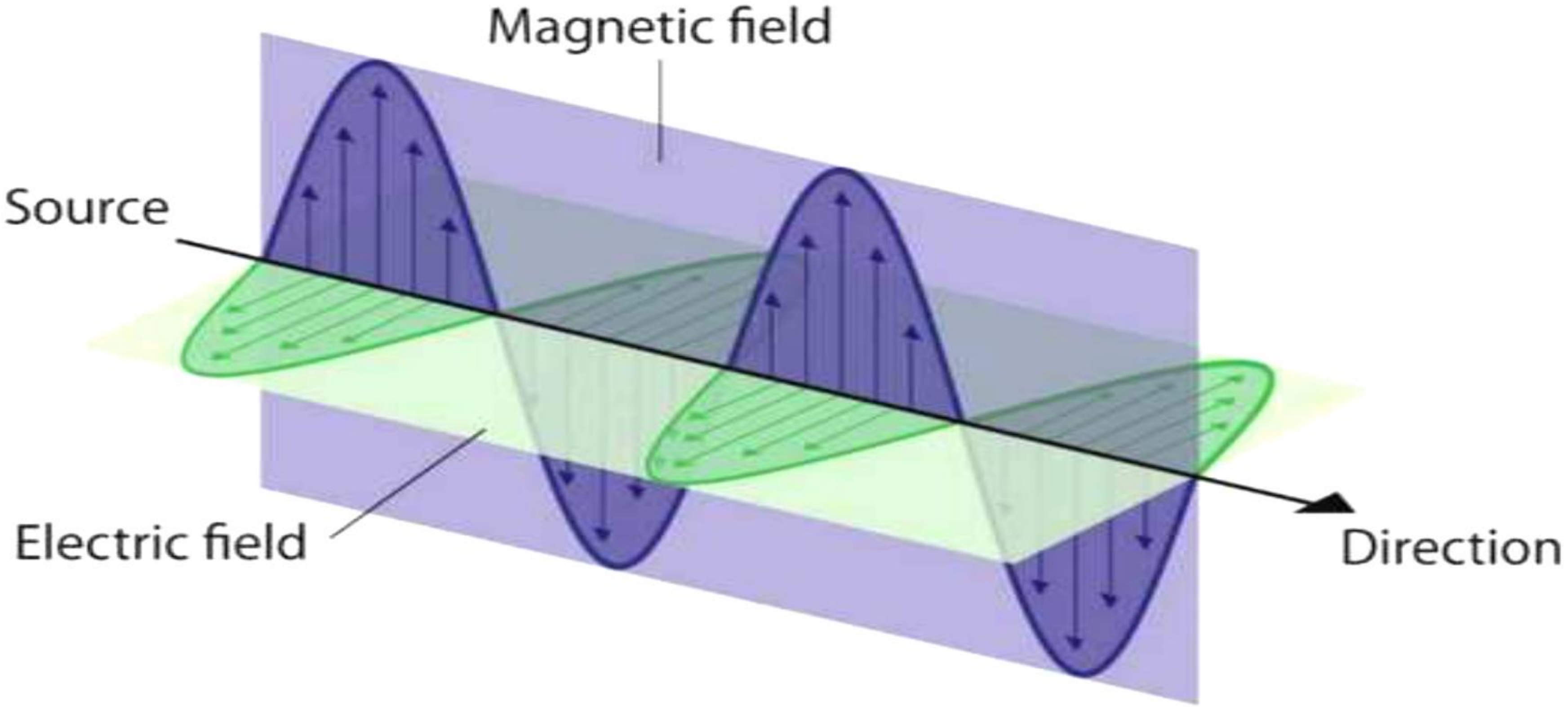
OR

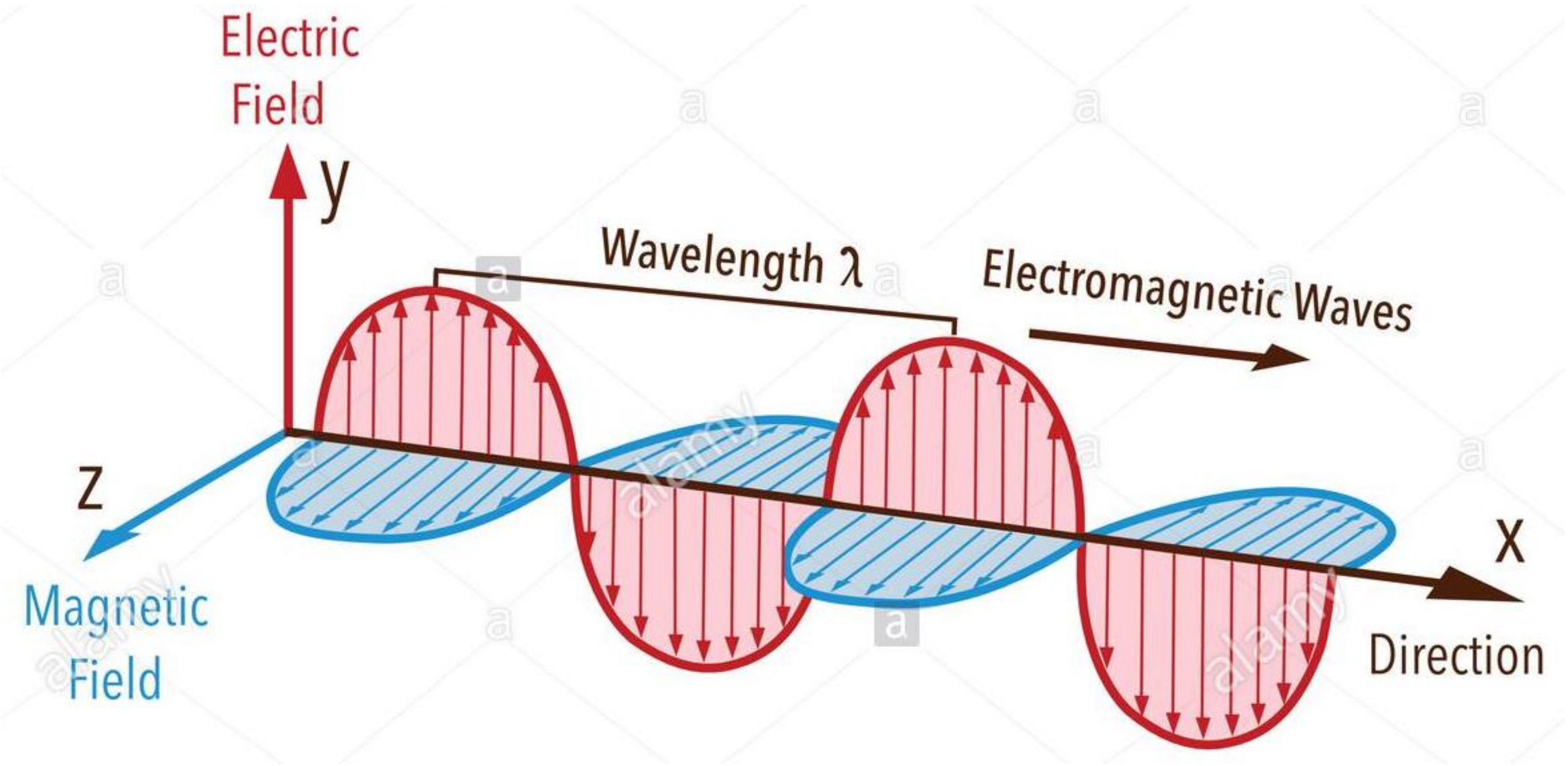
It is a branch of science in which EMR of particular wavelength or range of wavelength is used for qualitative and quantitative analysis of matter.

Matter- anything which occupies space in universe and having mass is called matter.

Electromagnetic Radiation

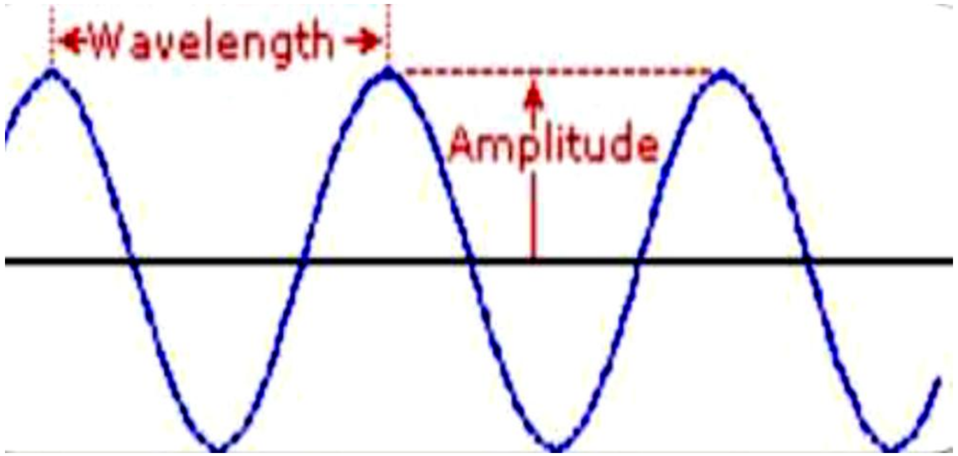
- Wave produced by motion of electrically charged particles (Photon)
- Consists of two components – Electric and Magnetic




Electric
Field
y
Wavelength λ
Electromagnetic Waves
z
x
Magnetic
Field
Direction

Properties of Waves

- Wavelength (λ) – Distance between two nearest crest or troughs
- Frequency – Number of wave cycle in a given time, measured in Hertz (Hz)
- Amplitude – Wave's height or length

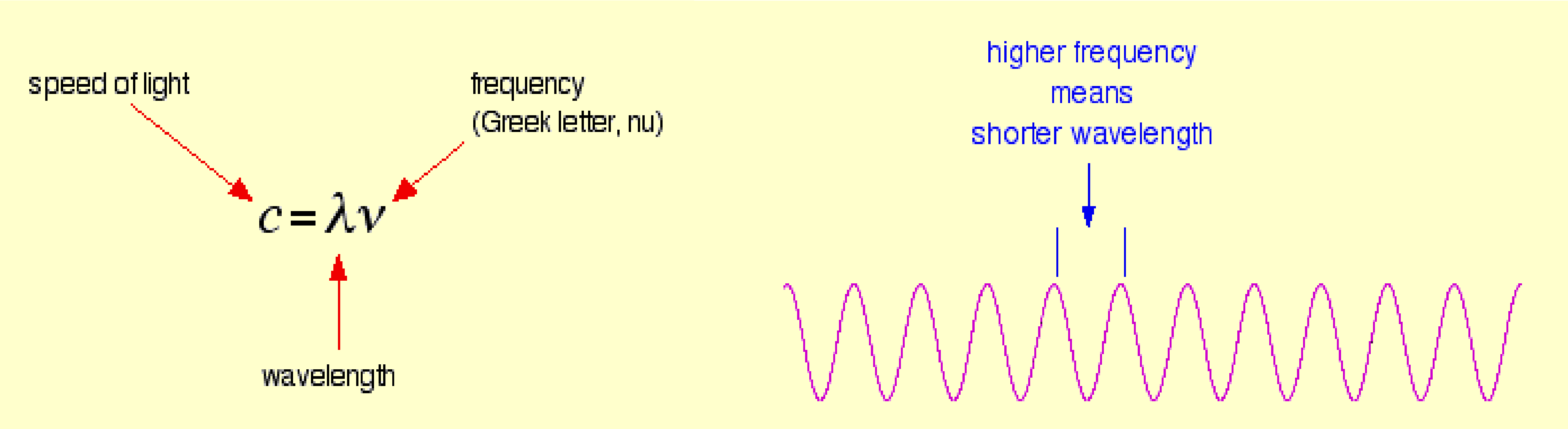


Relation between frequency and wavelength – velocity (speed) of propagation

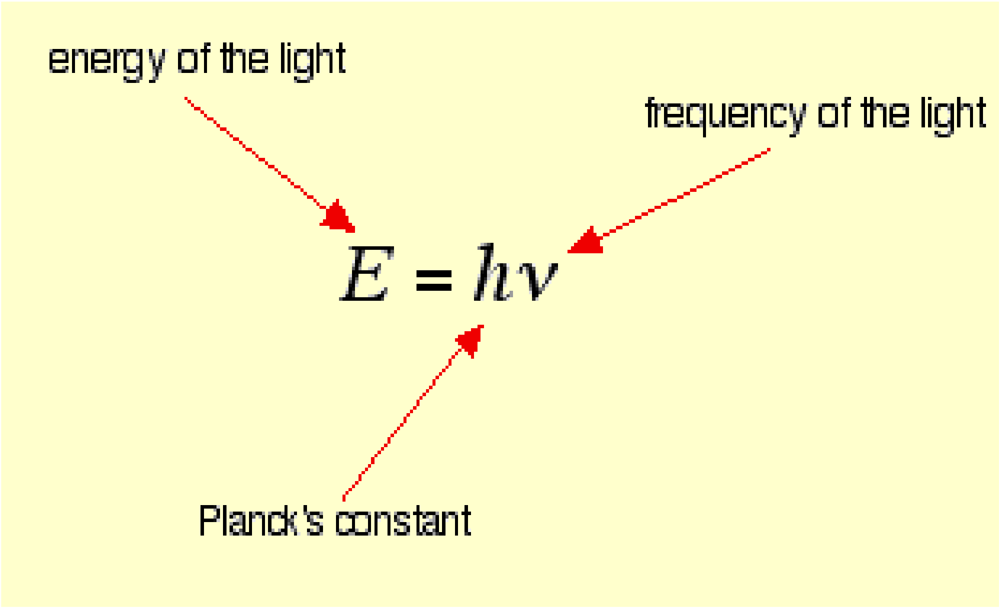
$$C = \nu \lambda$$

Where,

$$C = 2.99792 \times 10^8 \text{ m/s}$$



Relation between frequency of light and energy

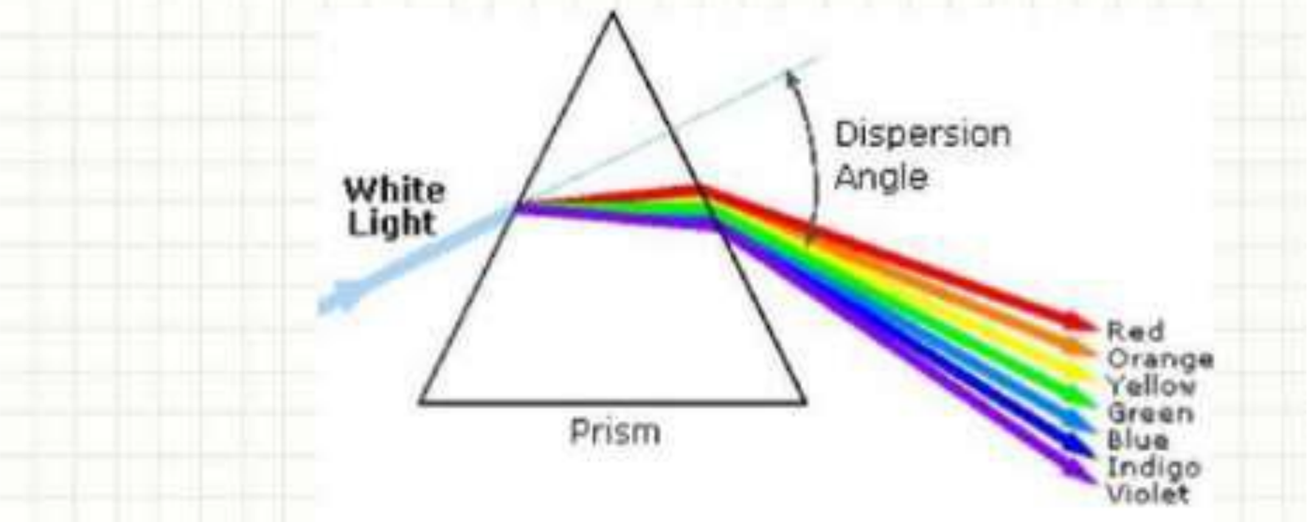


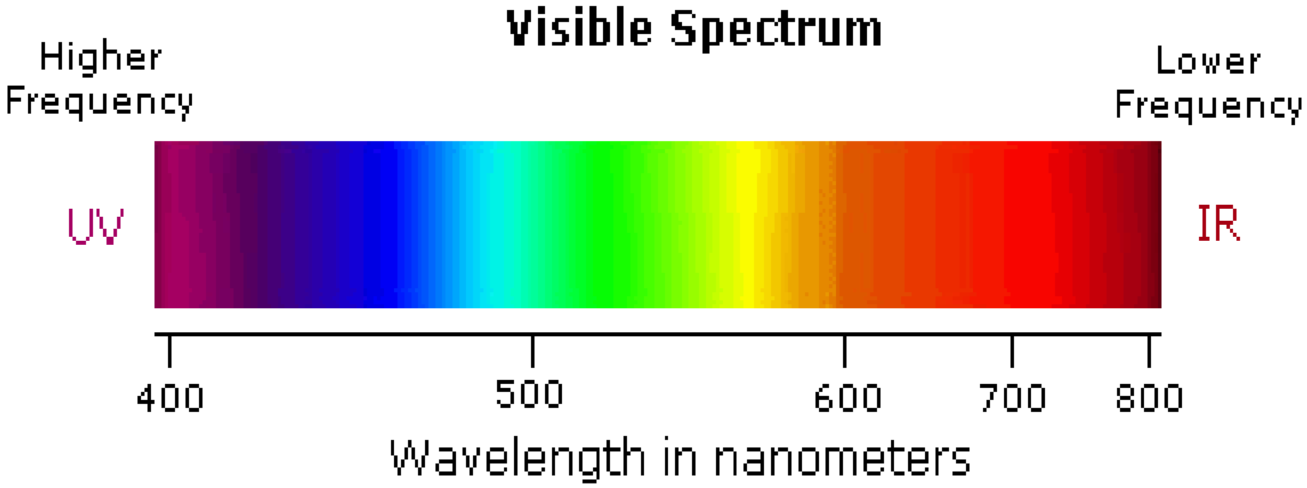
$E = h\,C / \lambda$ $\qquad\qquad\qquad\qquad$ $(C = \upsilon\,\lambda)$

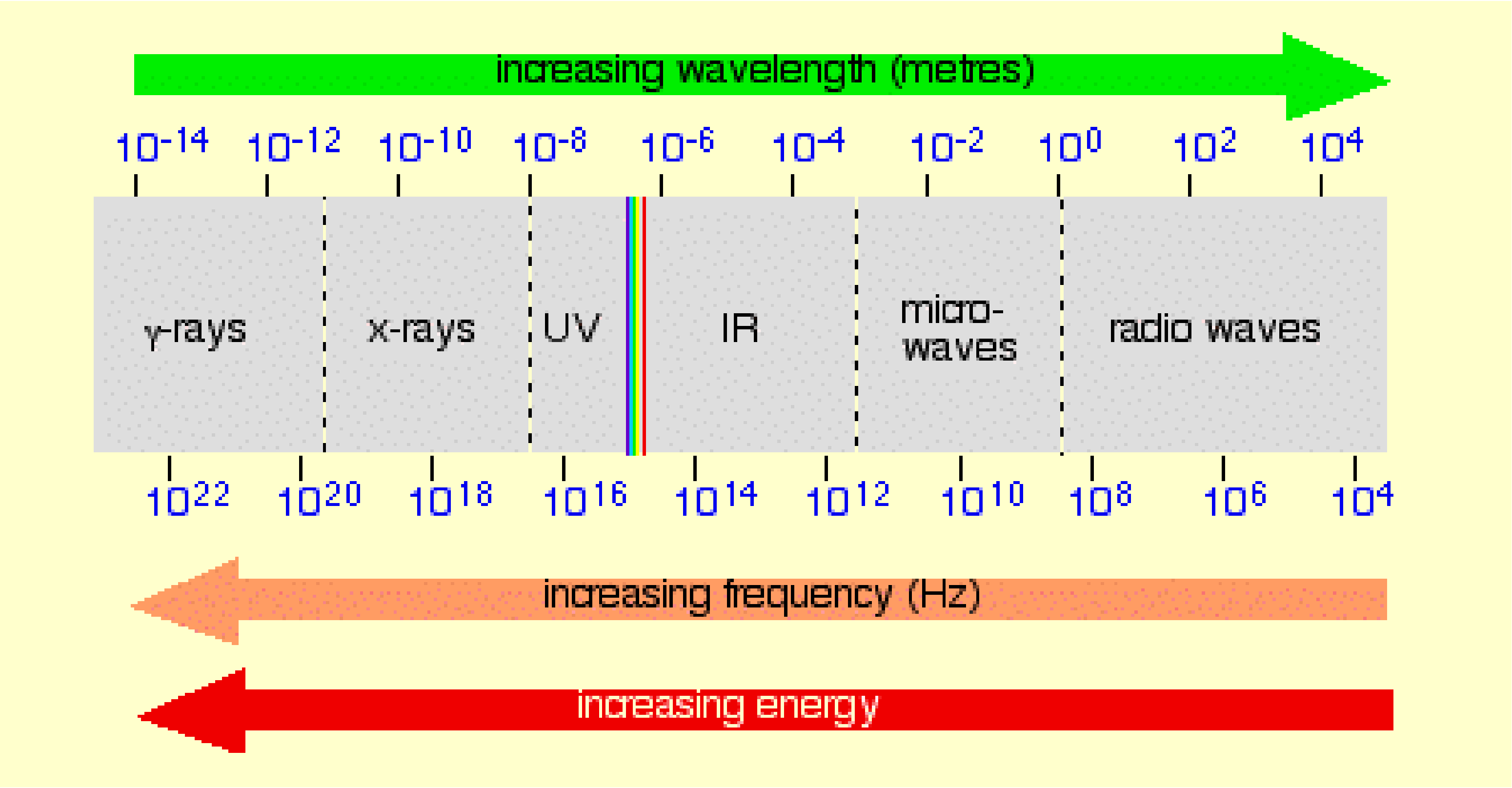
Where,

h = Planck’s constant = 6.6261 x 10-34 J.s

Electromagnetic Radiation







Violet	400 - 420 nm	Yellow	570 - 585 nm
Indigo	420 - 440 nm	Orange	585 - 620 nm
Blue	440 - 490 nm	Red	620 - 780 nm
Green	490 - 570 nm		

Interaction of EMR with matter

Molecules undergo processes like rotation, vibration, electronic transitions, and nuclear transitions.

The energies underlying these processes correspond to different regions in the electromagnetic spectrum

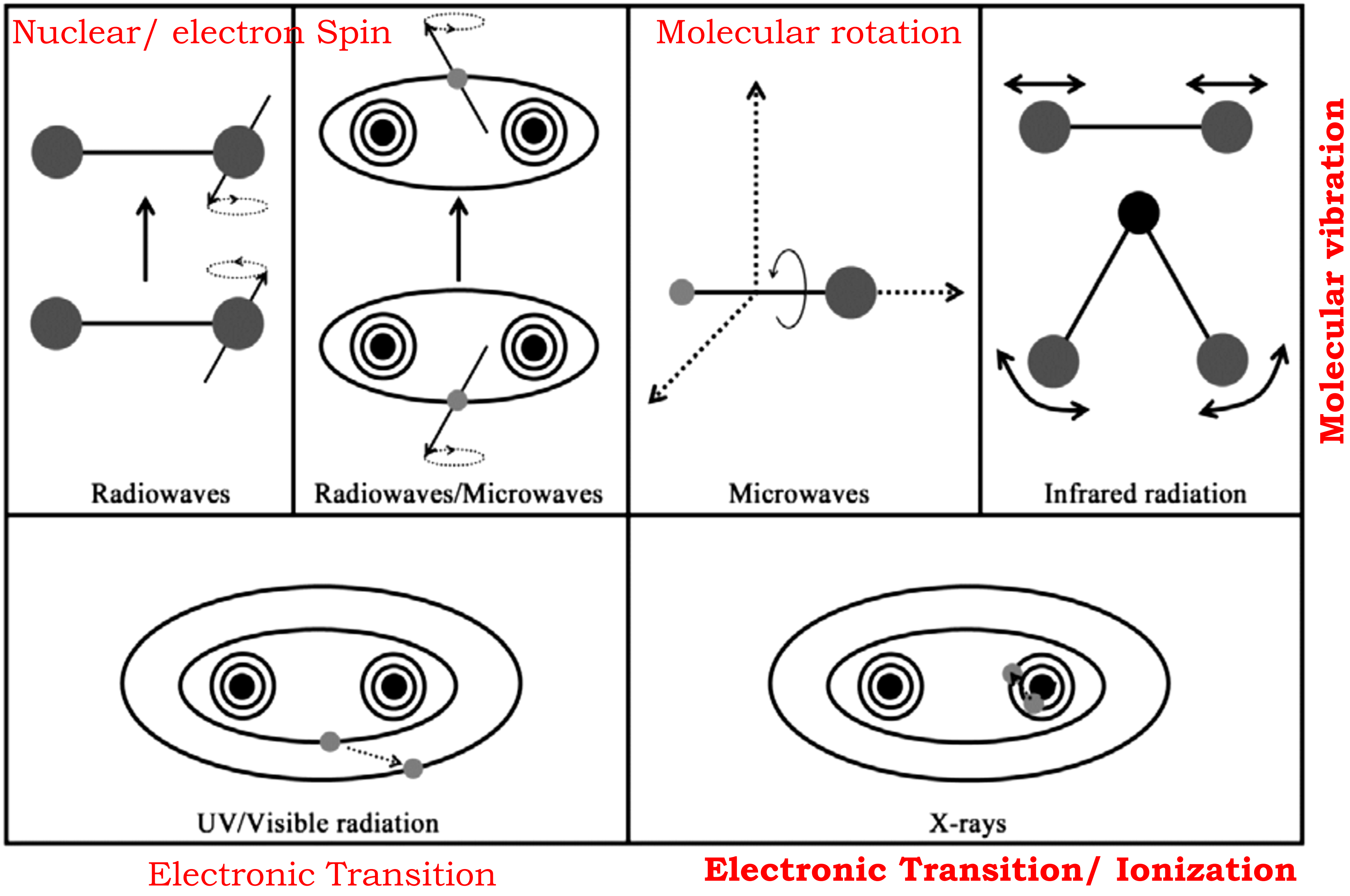
i. Radiofrequency waves: Radiofrequency region has very low energies that correspond to the energy differences in the nuclear and electron spin states. These frequencies, therefore, find applications in nuclear magnetic resonance and electron paramagnetic resonance spectroscopy.

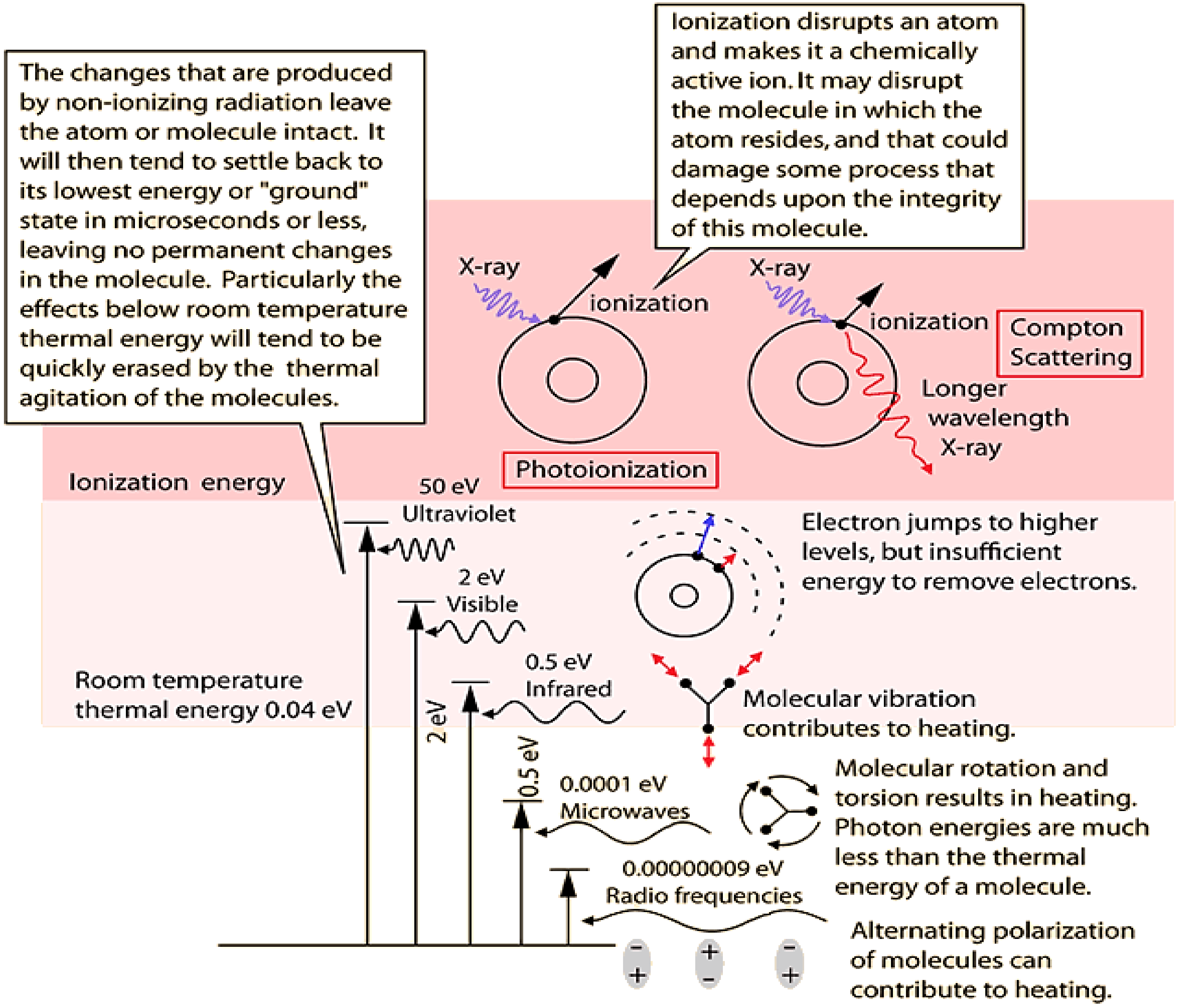
ii. Microwaves: Microwaves have energies between those of radiofrequency waves and infrared waves and find applications in rotational spectroscopy and electron paramagnetic resonance spectroscopy.

iii. Infrared radiation: The energies associated with molecular vibrations fall in the infrared region of electromagnetic spectrum. Infrared spectroscopy is therefore also known as vibrational spectroscopy and is a very useful technique for functional group identification in organic compounds.

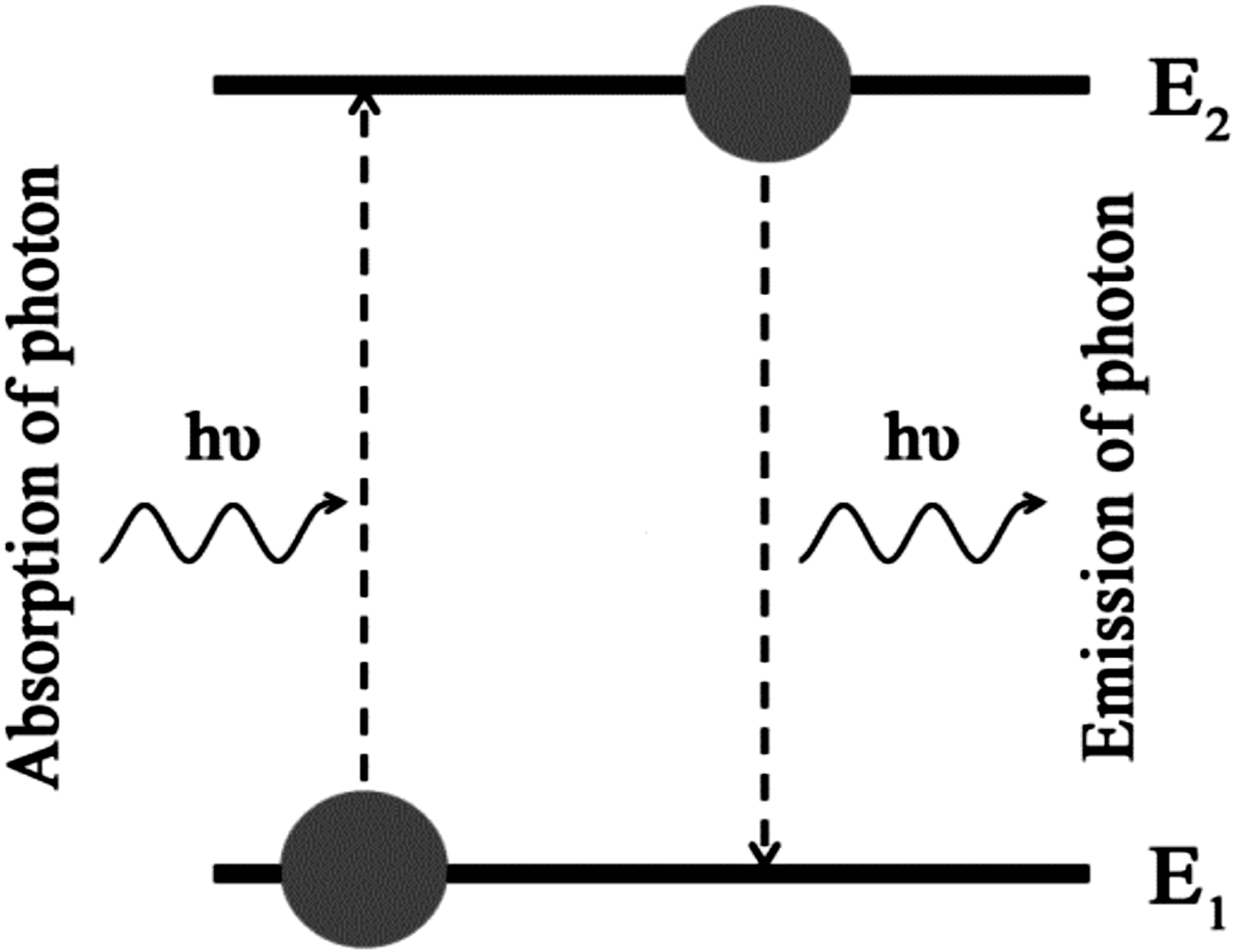
iv. UV/Visible region: UV and visible regions are involved in the electronic transitions in the molecules. The spectroscopic methods using UV or visible light therefore come under 'Electronic spectroscopy'.

v. X-ray radiation: X-rays are high energy electromagnetic radiation and causes transitions in the internal electrons of the molecules.


Nuclear/ electron Spin
Molecular rotation
Molecular vibration
Radiowaves
Radiowaves/Microwaves
Microwaves
Infrared radiation
UV/Visible radiation
X-rays
Electronic Transition
Electronic Transition/ Ionization



The changes that are produced by non-ionizing radiation leave the atom or molecule intact. It will then tend to settle back to its lowest energy or "ground" state in microseconds or less, leaving no permanent changes in the molecule. Particularly the effects below room temperature thermal energy will tend to be quickly erased by the thermal agitation of the molecules.
Ionization disrupts an atom and makes it a chemically active ion. It may disrupt the molecule in which the atom resides, and that could damage some process that depends upon the integrity of this molecule.
X-ray
ionization
X-ray
ionization
Compton Scattering
Longer wavelength X-ray
Photoionization
Ionization energy
50 eV
Ultraviolet
Electron jumps to higher levels, but insufficient energy to remove electrons.
2 eV
Visible
0.5 eV
Infrared
Room temperature thermal energy 0.04 eV
Molecular vibration contributes to heating.
2 eV
0.5 eV
0.0001 eV
Microwaves
Molecular rotation and torsion results in heating. Photon energies are much less than the thermal energy of a molecule.
0.00000009 eV
Radio frequencies
Alternating polarization of molecules can contribute to heating.



Transitions of a molecule between energy levels, E_1 and E_2 by absorbing/emitting the EMR

Interaction of EMR with matter

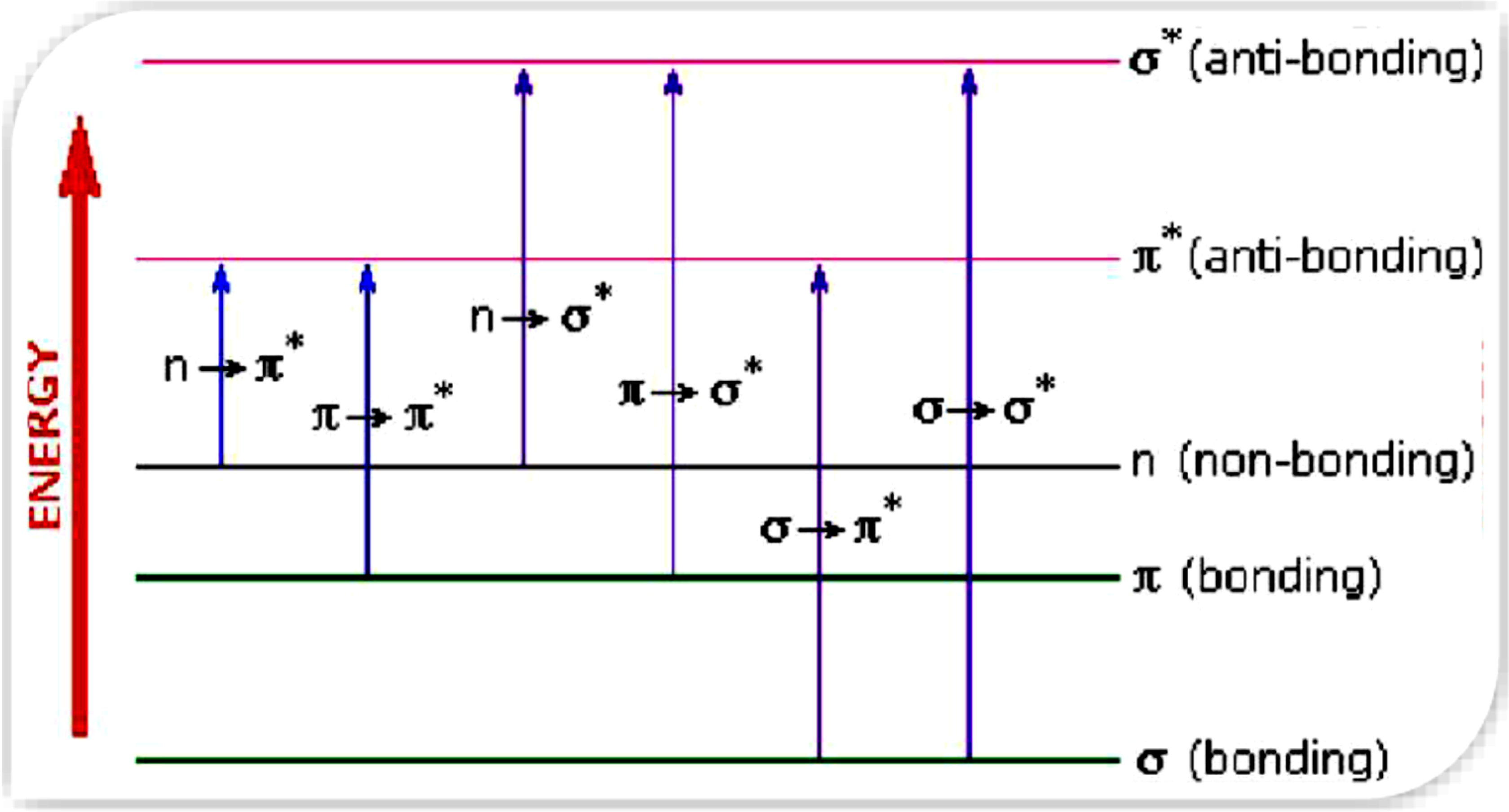
- **Absorption** : Light is absorbed
- **Emission**: Light is emitted or released
- **Transmission**: light is allowed to pass through
- **Reflection**: light is reflected or bounced away
- **Diffraction**: shows wave nature
- **Refraction**: shows particle nature
- **Interference**: light is disturbed
- **Scattering**: light is dispersed
- **Polarization**: light vibration is restricted to one direction

UV/Visible Absorption Spectroscopy

- If light in the UV/visible part of the electromagnetic spectrum is passed through a sample in solution, some light energy may be **absorbed**.
- Absorption of UV and visible radiation is one of the most routinely used analytical tools in life sciences research.
- **UV region of EMR λ** : ~10 nm – ~400 nm
    - Near UV: λ ~ 250 nm – 400 nm
    - Far UV: λ ~ 190 nm – 250 nm
    - Vacuum UV: λ< 190 nm

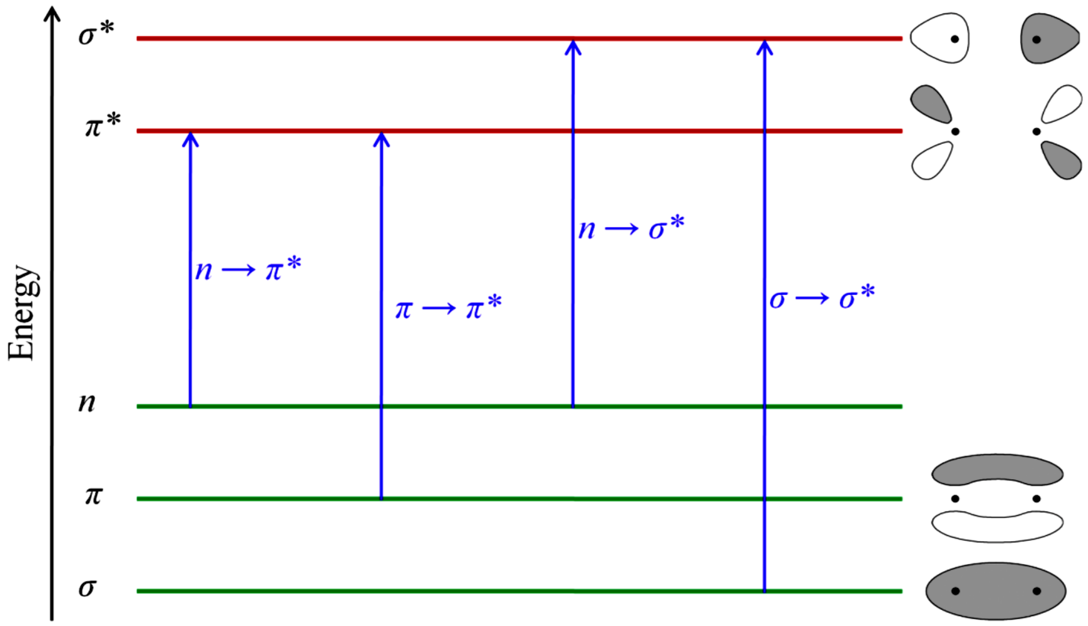
  * These ranges are very loosely defined. Slightly different ranges are also used.
- **Visible region λ** : ~400 nm – ~780 nm

Electronic Transitions



The possible electronic transitions are:

- σ → σ* transition
- π → π* transition
- n → σ* transition
- n → π* transition
- σ → π* transition
- π → σ* transition



σ → σ* transition

- σ → σ* transition is a high energy process and therefore lies in the vacuum UV region (λ< 190 nm).
- σ electron from orbital is excited to corresponding anti-bonding orbital σ*.
- Alkanes, wherein only σ → σ* transition is possible show absorption bands ~150 nm.

e.g. Methane (CH4) has C-H bond only and can undergo σ → σ* transition and shows absorbance maxima at 125 nm.

$\pi \rightarrow \pi^*$ transition

- π electron in a bonding orbital is excited to corresponding anti-bonding orbital π^*.
- Compounds containing multiple bonds like alkenes, alkynes, carbonyl, nitriles, aromatic compounds, etc undergo $\pi \rightarrow \pi^*$ transitions.

e.g. Alkenes generally absorb in the region 170 to 205 nm.

$n \rightarrow \sigma^*$ transition

- The presence of nonbonding electrons in a molecule further expands the number of possible transitions. The entire molecule, however, is not generally involved in the absorption of the radiation in a given wavelength range.
- Saturated compounds containing atoms with lone pair of electrons like O, N, S and halogens are capable of $n \rightarrow \sigma^*$ transition.
- These transitions usually requires less energy than $\sigma \rightarrow \sigma^*$ transitions.
- The number of organic functional groups with $n \rightarrow \sigma^*$ peaks in UV region is small (150 – 250 nm).

n → π* transition

- An electron from non-bonding orbital (n) is promoted to anti-bonding π* orbital.
- Compounds containing double bond involving hetero atoms (C=O, C≡N, N=O) undergo such transitions.
- n → π* transitions require minimum energy and show absorption at longer wavelength around 300 nm.

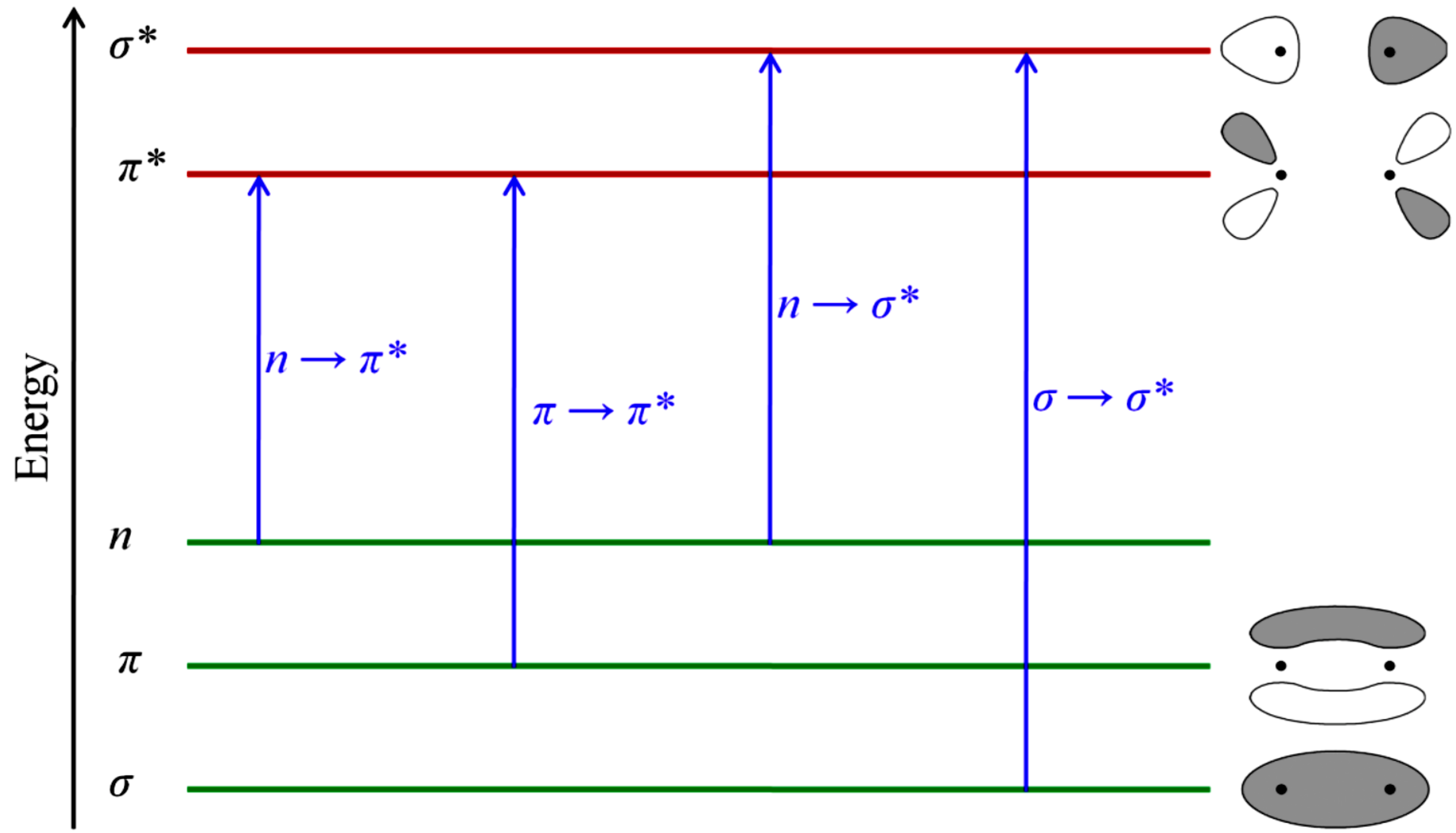
σ → π* transition & π → σ* transition

- These electronic transitions are **forbidden transitions & are only theoretically possible**.

Thus, n → π & π → π* electronic transitions show absorption in region above 200 nm which is accessible to UV-visible spectrophotometer.*

The UV spectrum is of only a few broad of absorption.

The possible electronic transitions can graphically shown as



Schematic diagram showing energy levels of different orbitals and **possible absorption transitions**

Chromophore

- Molecules or parts of molecules that are capable of absorbing light are called **chromophores.**
- Atoms that comprise the molecular orbitals involved in the **electronic transitions** constitute the molecular moiety that is directly involved in the transition. Such a group of atoms is called a **chromophore**.
- A structural modification in a chromophore is generally accompanied by changes in the absorption properties.
- Chromophores that form part of the structure of biomacromolecules are called **intrinsic chromophores**. e.g. aromatic amino acids (in Protein) and nucleotides (in nucleic acids).
- **Extrinsic chromophores** (chemically attached artificial groups) are used as reporter groups.
- Ideally, reporter groups should have a single site of attachment to the target macromolecule and should not affect its normal structure and function.
- e.g. pH indicators, lycopene, β-carotene etc.

Principle of absorption Spectroscopy

The phenomenon of absorption can be described by two laws- Beers Law and Lamberts Law, together called as **Beer-Lambert Law,** which states-

$$\mathbf{A = logI_0/I = \Sigma.c.}\boldsymbol{l}$$

(Note: Detail of the Law and its application has been discussed in previous class)

The equality showing linear relationship between absorbance and the concentration of the chromophore is known as the Beer-Lambert law (or Beer's law).

Deviations from Beer-Lambert law:

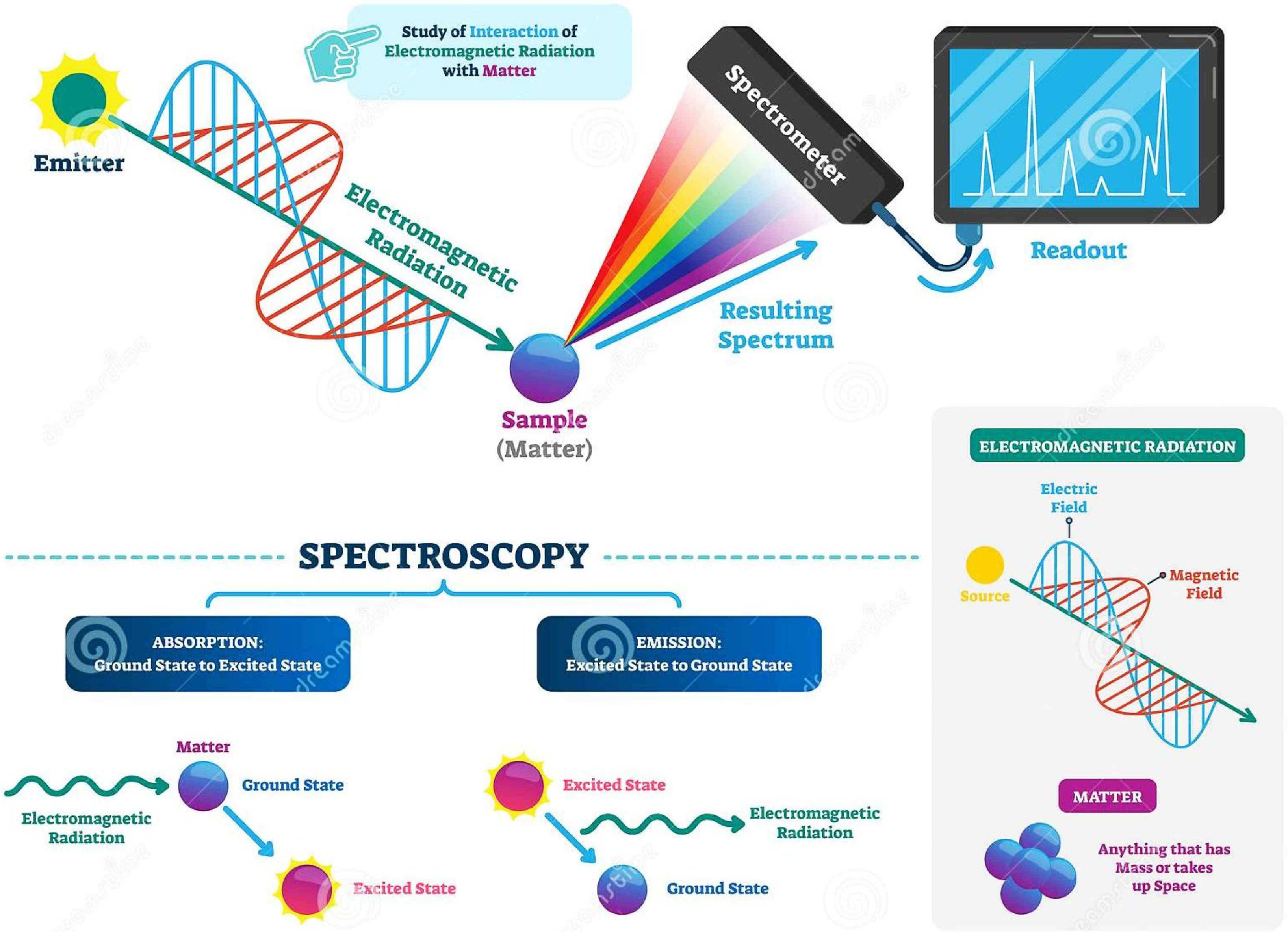
Three major reasons that are responsible for the breakdown of linear relationship between absorbance and the concentration of the absorbing molecule are-

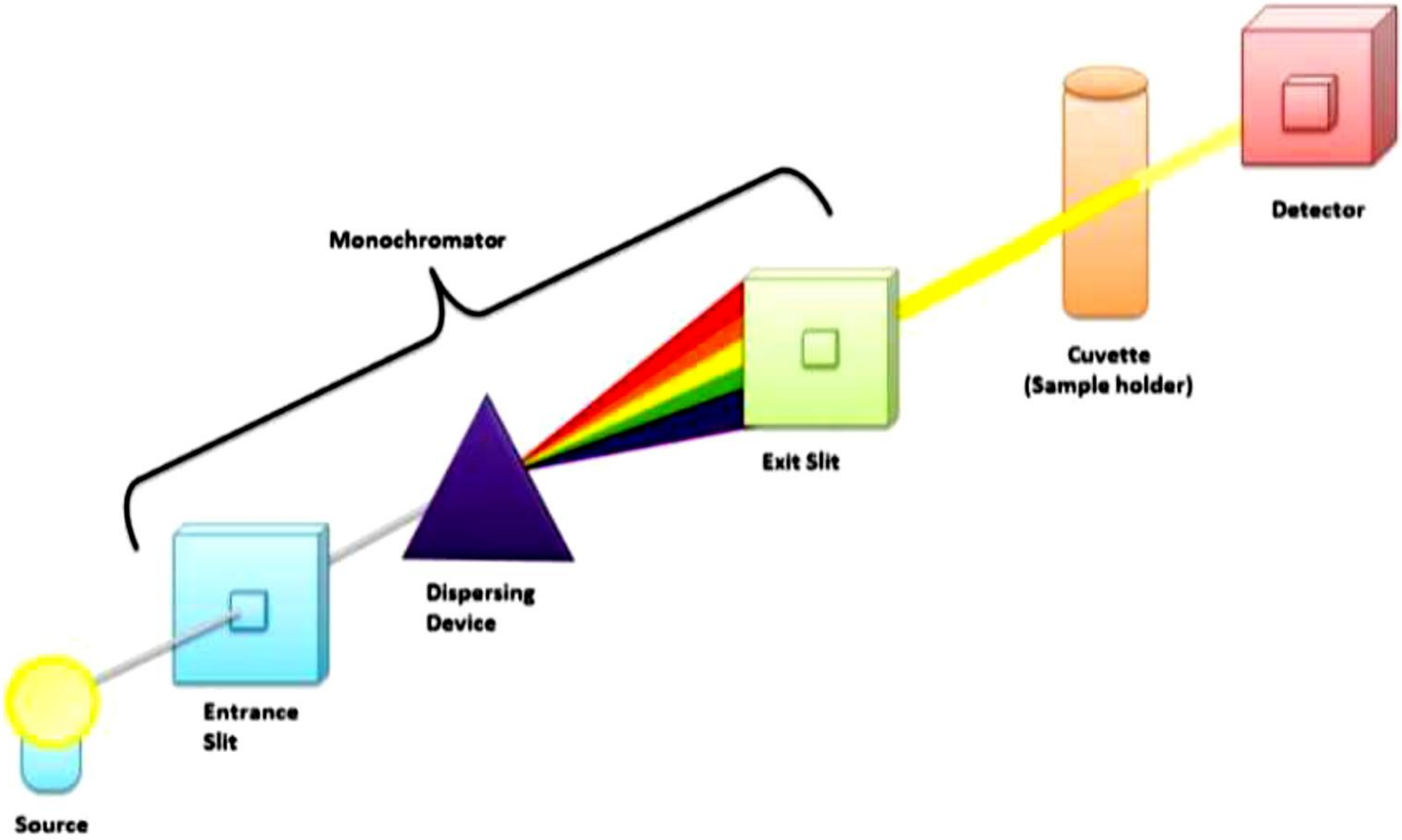
i. High sample concentration:

ii. Chemical reactions:

iii. Instrumental factors:

Instrumentation





Representative Diagram of a Simple UV-Visible Spectrophotometer

Five Basic Optical Instrument Components

1. **Source** – A stable source of radiant energy at the desired wavelength (range).
2. **Wavelength Selector** – A device that isolates a restricted region of the EM spectrum used for measurement (**Monochromators**, prisms & filters).
3. **Sample Container** – A transparent container used to hold the sample (cells, cuvettes, etc).
4. **Detector/Photoelectric Transducer** – Converts the radiant energy into a useable signal (usually electrical).
5. **Signal Processor & Readout** – Amplifies or attenuates the transduced signal and sends it to a readout device as a meter, digital readout, chart recorder, computer, etc.

1. Light Sources

Various UV radiation sources are as follows

a. Deuterium lamp

b. Hydrogen lamp

c. Tungsten lamp

d. Xenon discharge lamp

e. Mercury arc lamp

Various Visible radiation sources are as follow

a. Tungsten lamp

b. Mercury vapour lamp

c. Carbonone lamp

2. Wavelength Selectors

Wavelength selectors output a limited, narrow, continuous group of wavelengths called a *band.*

Two types of wavelength selectors: A) **Filters** B) **Monochromators**

A. Filters – Two types of filters:

a) Interference Filters b) Absorption Filters

B. Monochromators

- Wavelength selector that can continuously scan a broad range of wavelengths.
- Used in most scanning spectrometers including UV, visible, and IR instruments.

PRISM TYPE

– Refractive type

– Reflective type

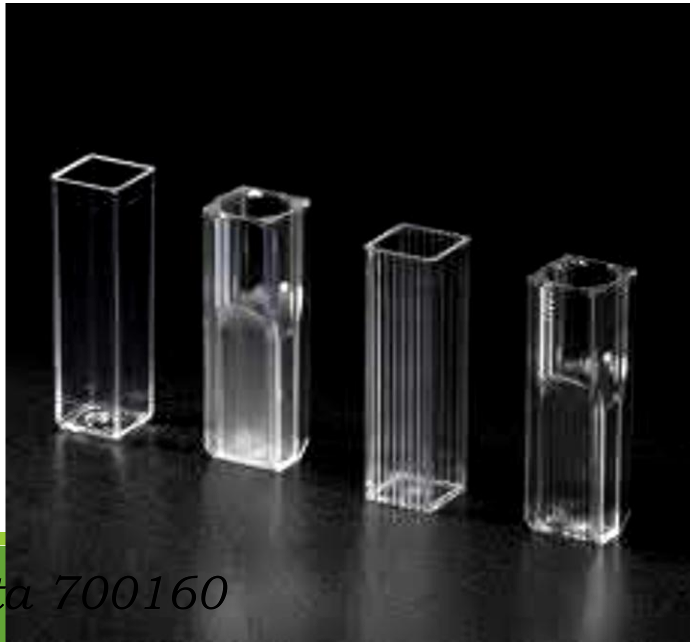
GRATING TYPE

– Diffraction type

– Transmission Type

3. Sample Compartment

- Sample is contained in tubes called **cuvettes** which may be constructed from a number of materials.
- Glass or plastic cuvettes are useful in the visible part of the spectrum but have the disadvantage that they may absorb ultraviolet radiation.
- **Quartz** cuvettes do not absorb in the ultraviolet but are fragile and expensive.
- Disposable plastic cuvettes which do not absorb at wavelengths greater than 280 nm are now commercially available.
- Plastic cuvettes may be damaged by exposure to organic solvents which can limit their usefulness in certain types of experiments
- Some typical materials are:
    - Optical Glass - 335 - 2500 nm
    - Special Optical Glass – 320 - 2500 nm
    - Quartz (Infrared) – 220 - 3800 nm
    - Quartz (Far-UV) – 170 - 2700 nm

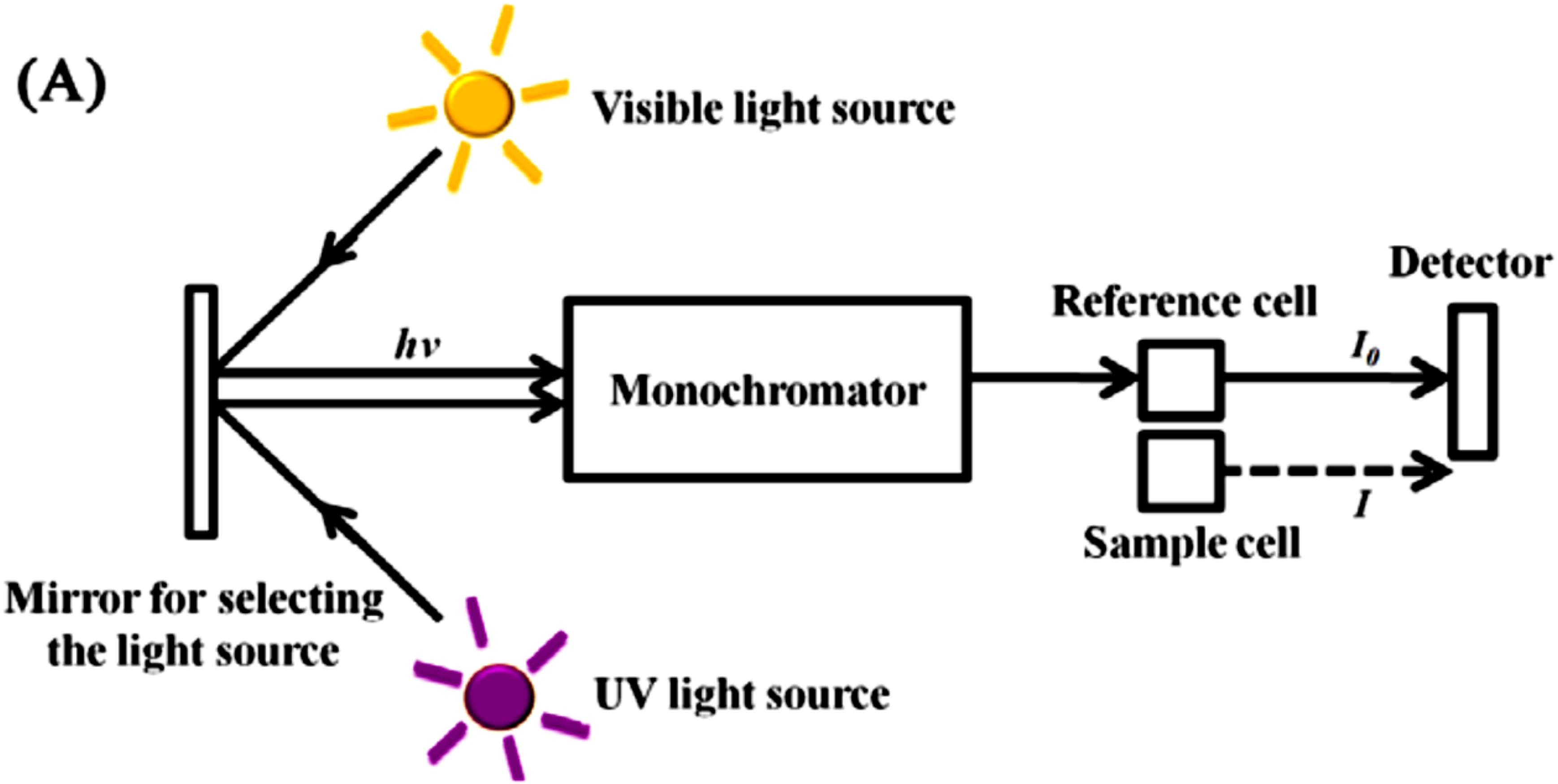


4. Detectors

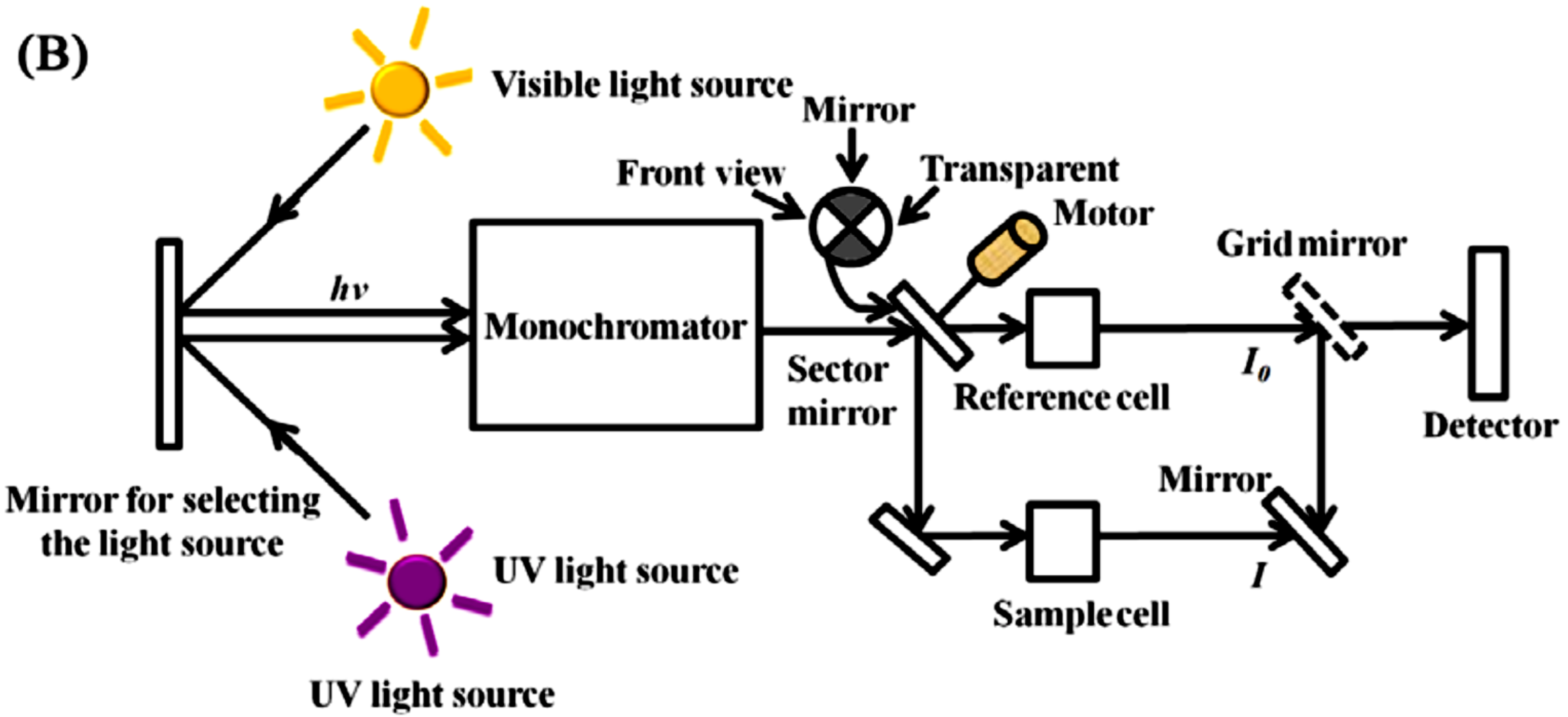
- After the light has passed through the sample, we want to be able to detect and measure the resulting light.
- These types of detectors come in the form of transducers that are able to take energy from light and convert it into an electrical signal that can be recorded, and if necessary, amplified.

Three common types of detectors are used

- Barrier layer cells
- Photo emissive cell detector
- **Photomultiplier**



Schematic diagram showing a **single beam** spectrophotometer



Schematic diagram showing a **double beam** spectrophotometer

Applications of UV/Visible Spectroscopy

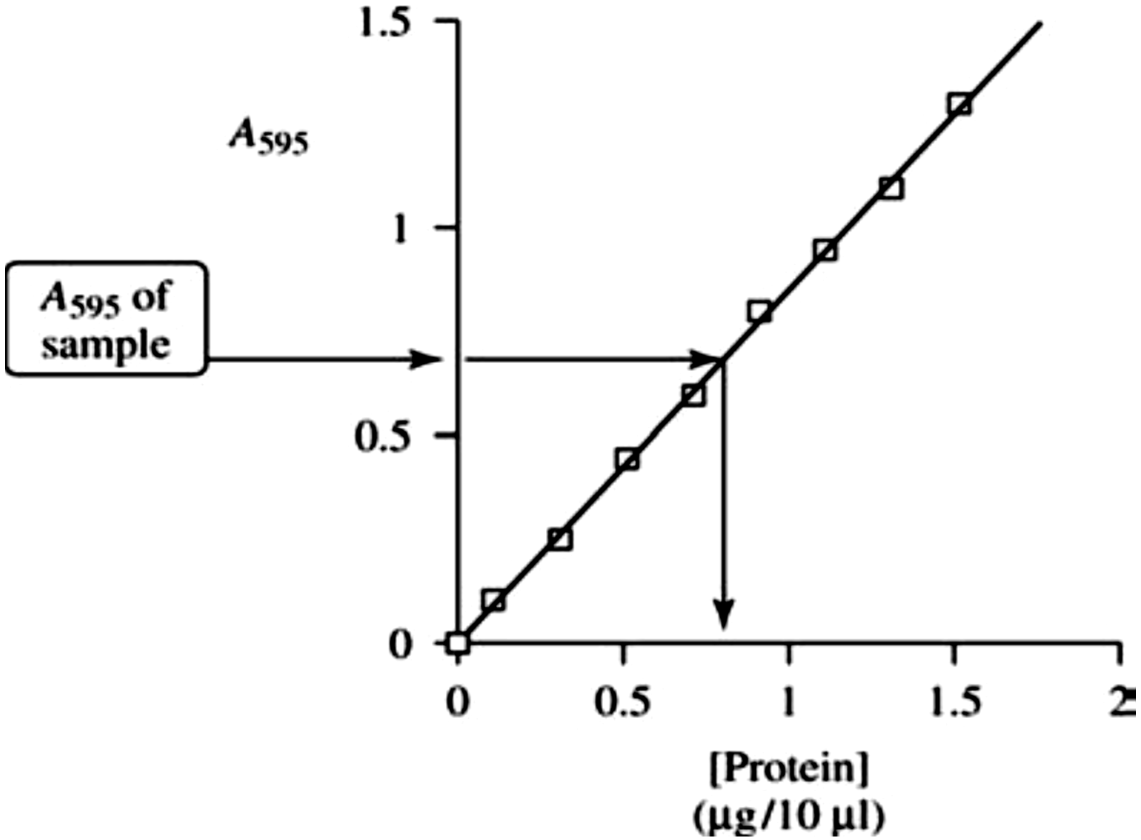
***1. Determination of molar absorption coefficient*:**

Beer-Lambert law states $A = \varepsilon cl$. Therefore it is straightforward to calculate the molar absorption coefficient (ε) of a compound if the concentration of compound is accurately determined.

2. Quantification of compounds

If the molar absorption coefficient at a wavelength is known for the compound, the concentration can easily be estimated using Beer-Lambert law.

However, the compounds can still be quantified if their molar absorption coefficients are not known. For example, determination of protein concentration by using protein binding dyes that form colored complex (Bradfords method for instance).



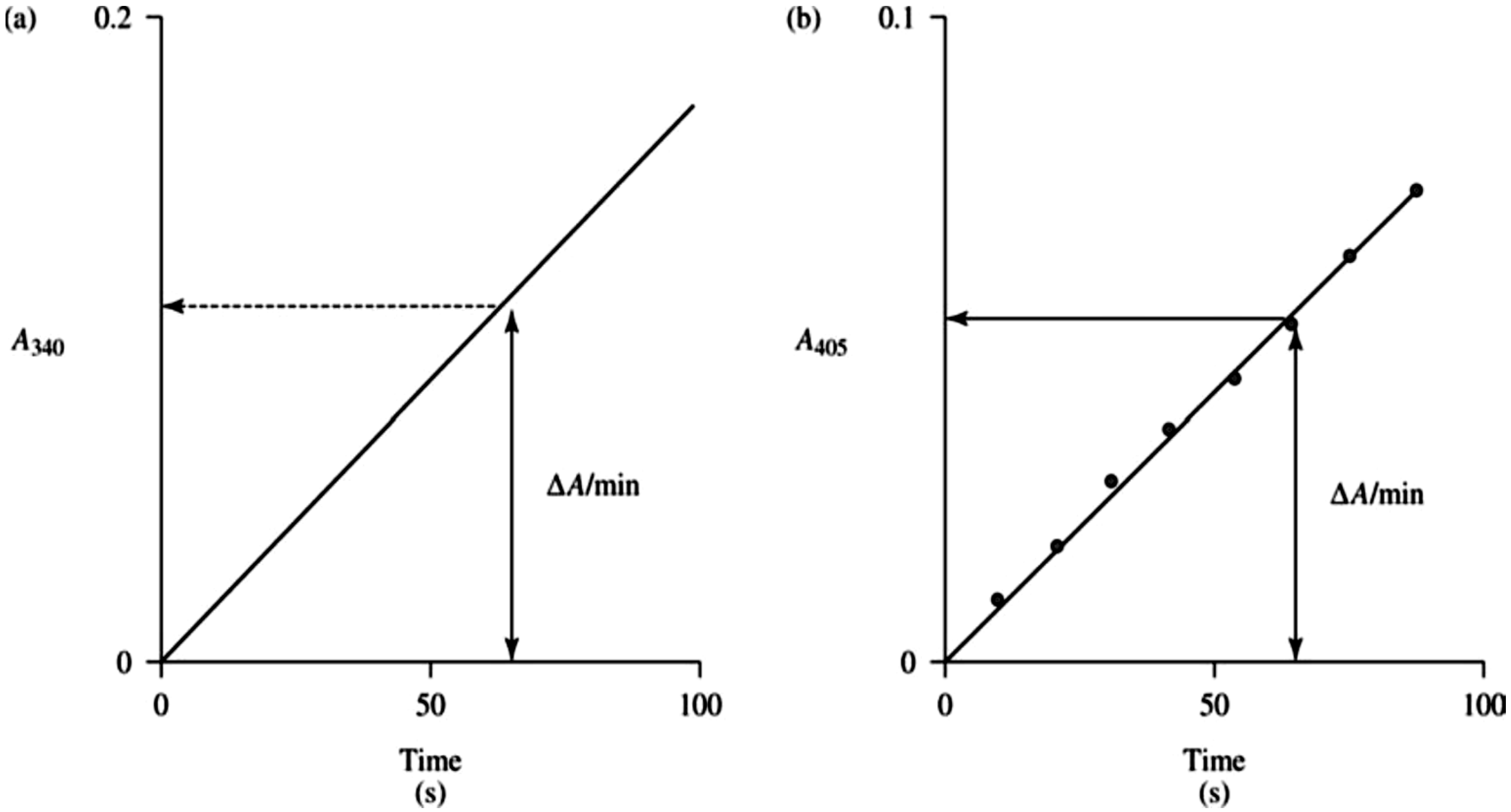
Determination of protein concentration using a standard curve – the Bradford assay. Coomassie brilliant blue has a λ_{max} at 596 nm when it complexes with protein. A standard curve is constructed with various known concentrations of bovine serum albumin. The concentration of an unknown sample may be estimated by comparison with this curve (arrows). The use of a standard curve for the relationship between A_λ and concentration depends on the Beer–Lambert law.

3. Quality control

- A given organic compound such as a drug can be studied for its purity.
- Comparison of spectrum with the standard drug will detect the impurities, if any.
- UV/Visible absorption is often used to check the purity of nucleic acid as well as protein.
- A typical nucleic acid containing all four bases shows an absorption band centered ~260 nm while a protein having aromatic amino acids shows absorption band centered ~280 nm.
- A ratio of the absorbance at 260 nm to that at 280 nm i.e. A_{260}/A_{280} is a measure of the purity.

4. Chemical kinetics:

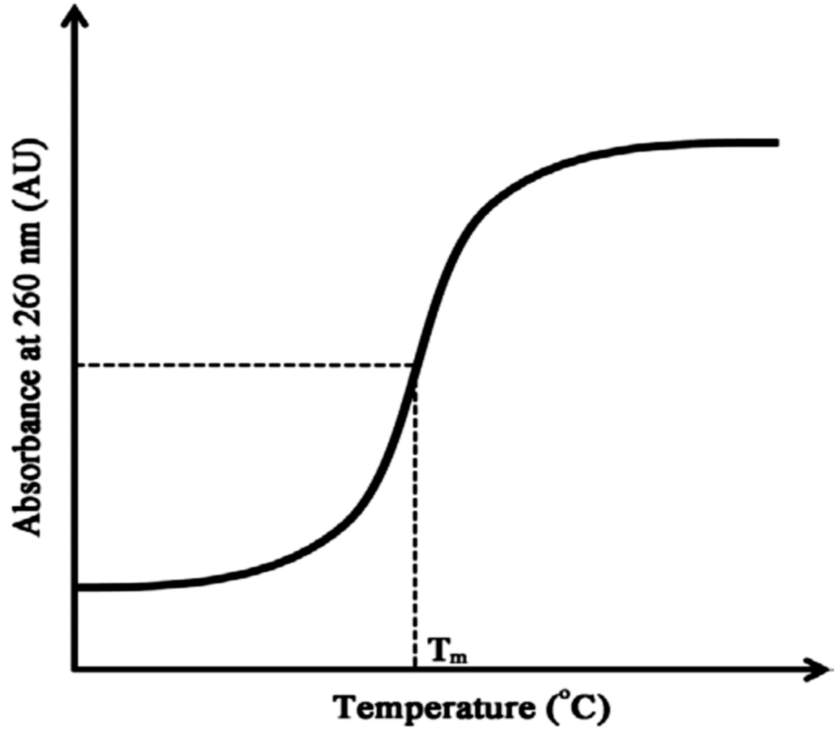
- UV/visible spectroscopy can be used to monitor the rate of chemical reactions if one of the reactants or products absorbs in a region where no other reactant or product absorbs significantly.
- In **enzyme assays** the concentration of either substrate or product may be measured to allow calculation of rates of enzyme-catalyzed reactions.
- For example, reduction of NAD to NADH + H+ by **oxidoreductase** enzymes is conveniently followed by continuous measurement of A_{340}.

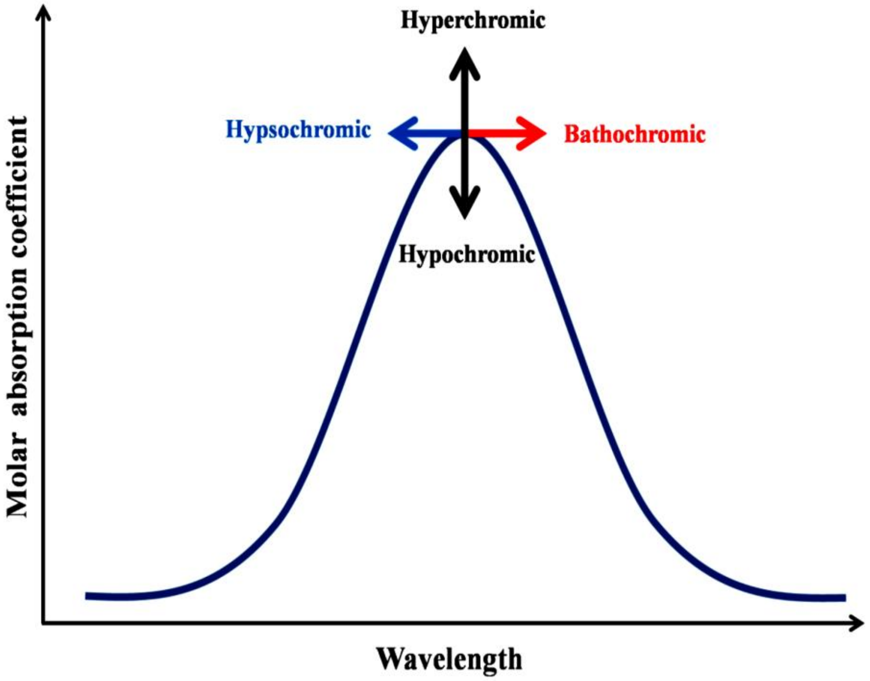


Use of spectroscopy in enzyme asays. (a) Continuous assay of a dehydrogenase producing NADH+H^+. (b) Stopped assay of alkaline phosphatase.

5. *Determination of melting temperature (Tm) of DNA*:

- A double stranded DNA molecule can be denatured into the single strands by heating it.
- Melting temperature, Tm is the temperature at which 50% of the DNA gets denatured into single strands.
- Denaturation of DNA is accompanied by hyperchromic shift in the absorption spectra in the near UV region





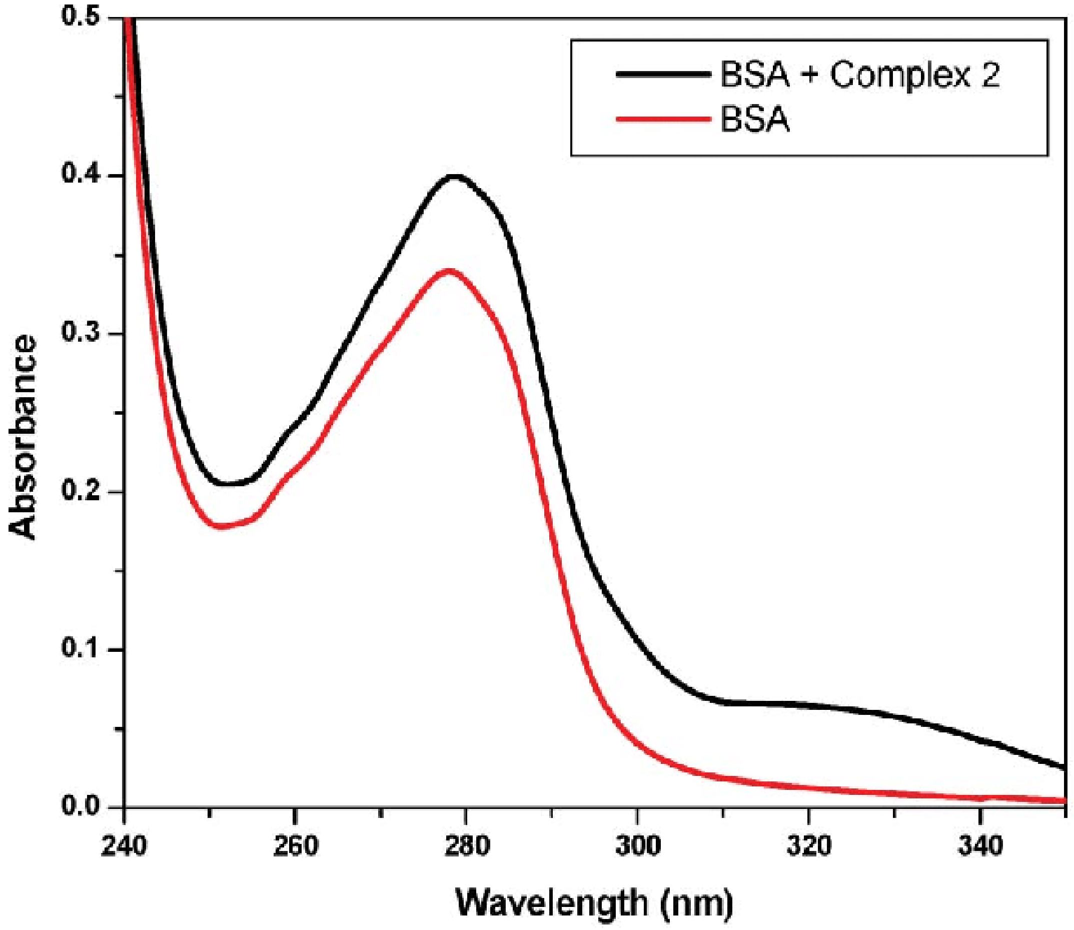
***6. Detectors in liquid chromatography instruments*:**

- UV/visible detectors are perhaps the most common detectors present in liquid chromatography systems.
- Modern instruments use photodiode array detectors that can detect the molecules absorbing in different spectral regions

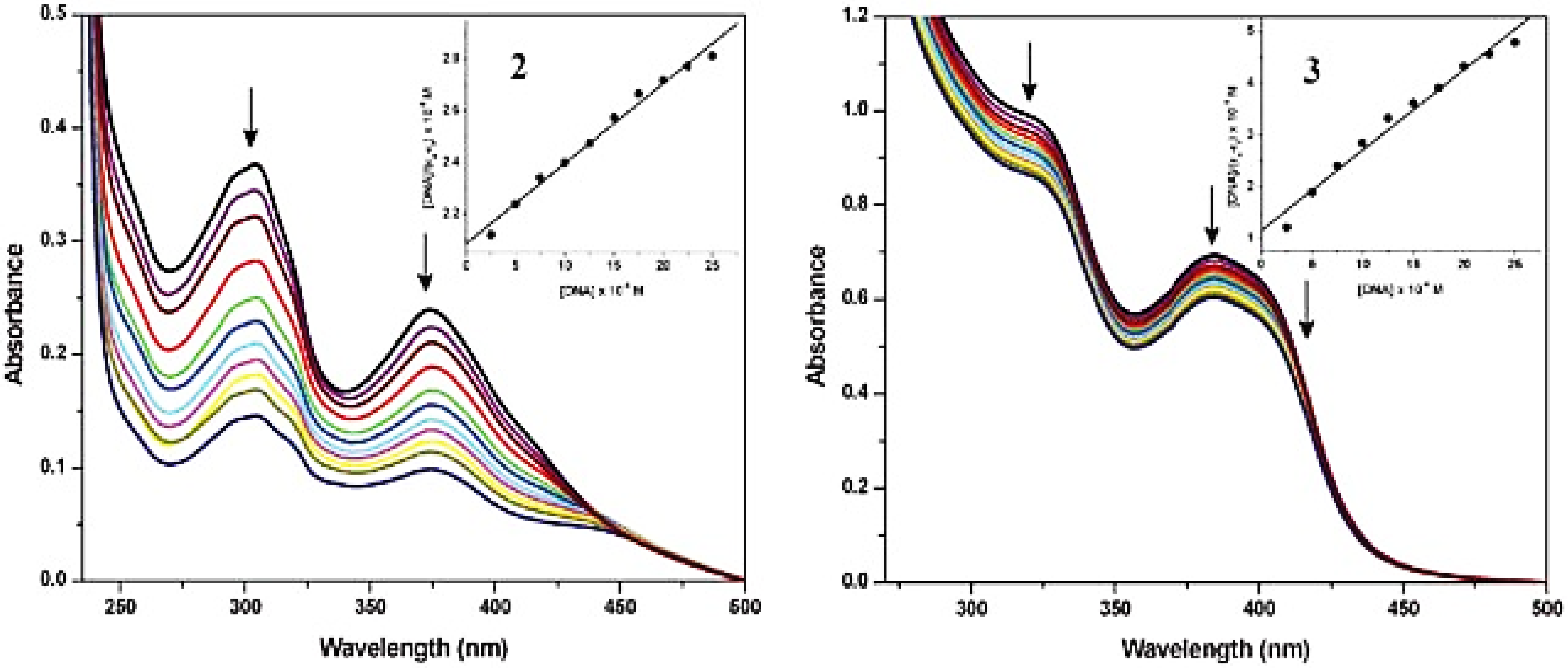
7. Microbial growth kinetics:

- A UV/visible spectrophotometer is routinely used to monitor the growth of microorganisms.
- **The underlying principle behind this, however, is not absorbance but scattering**.
- As the number of microbial cells increase in a culture, they cause more scattering in light.
- The detector therefore receives less amount of radiation, recording this as absorbance.
- To distinguish this from actual absorbance, the observed value is referred to as the optical density.

8. Drug-DNA / Drug- protein interaction



Representative figure to show the change in protein spectrum after interaction with a Drug



Representative figure to show the change in DNA spectra after interaction with a various concentrations of drug

Relationship between light absorption and color

Color absorbed	Color observed	Absorbed radiation(nm)
Violet	Yellow-green	400-435
Blue	Yellow	435-480
Green-blue	Orange	480-490
Blue-green	Red	490-500
Green	Purple	500-560
Yellow-green	Violet	560-580
Yellow	Blue	580-595
Orange	Green-blue	595-605
Red	Blue-green	605-750

Suggested Readings:

- Physical Biochemistry: Principles and Applications, 2nd edition (2009), David Sheehan, JohnWiley. ISBN13: 978-0470856031.
- Principles and Techniques of Biochemistry and Molecular Biology. 7th Edition (2010), Keith Wilson and John Walker. Cambridge University Press, ISBN 978-0-521-73167-6

Thank You

(To be continued…)