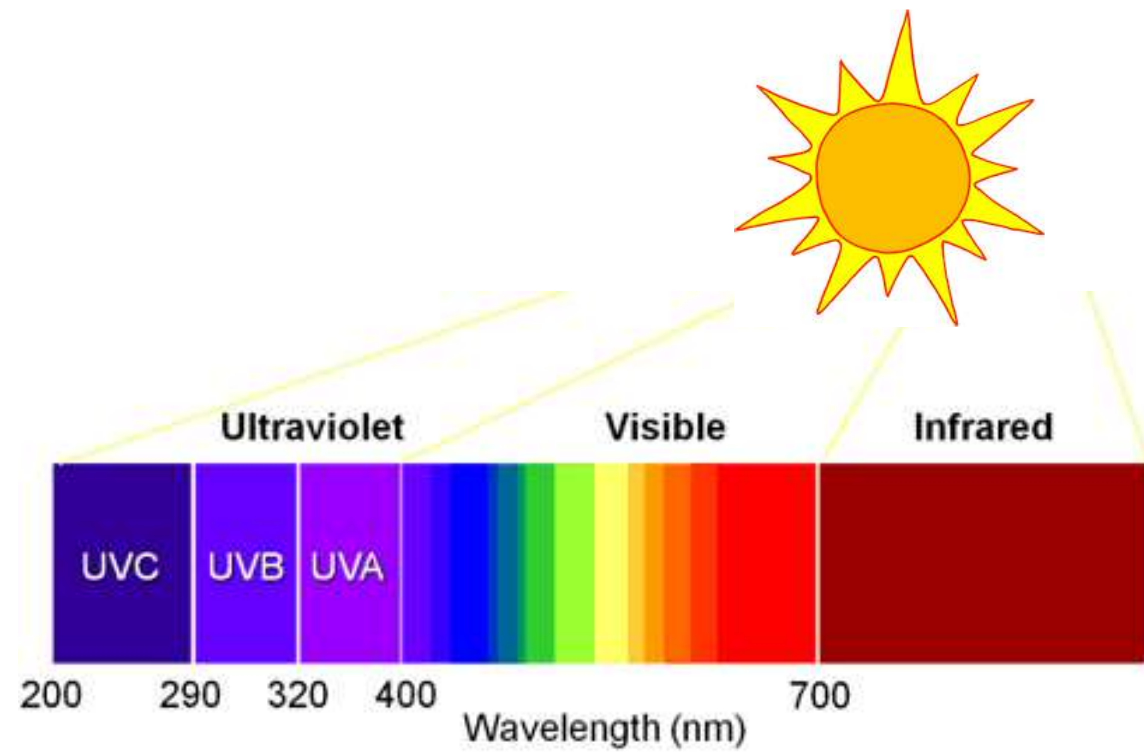
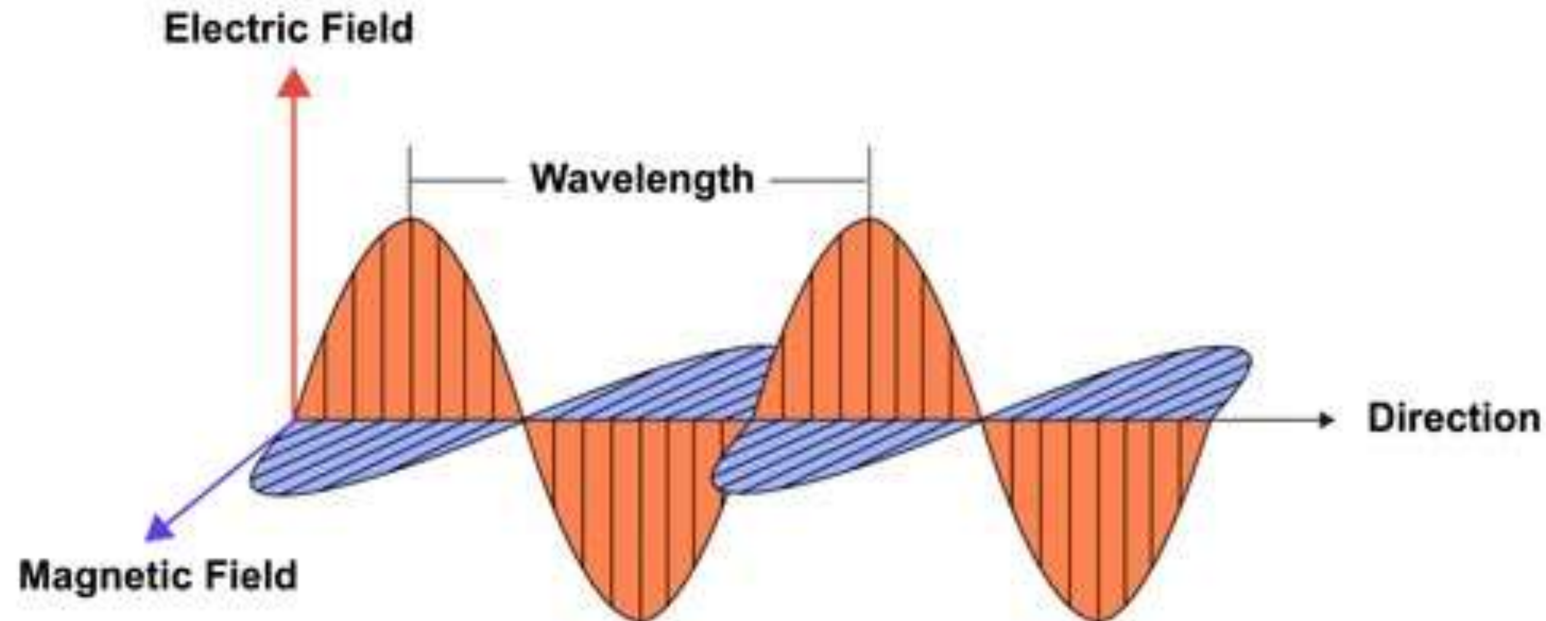


UV/VIS Spectroscopy

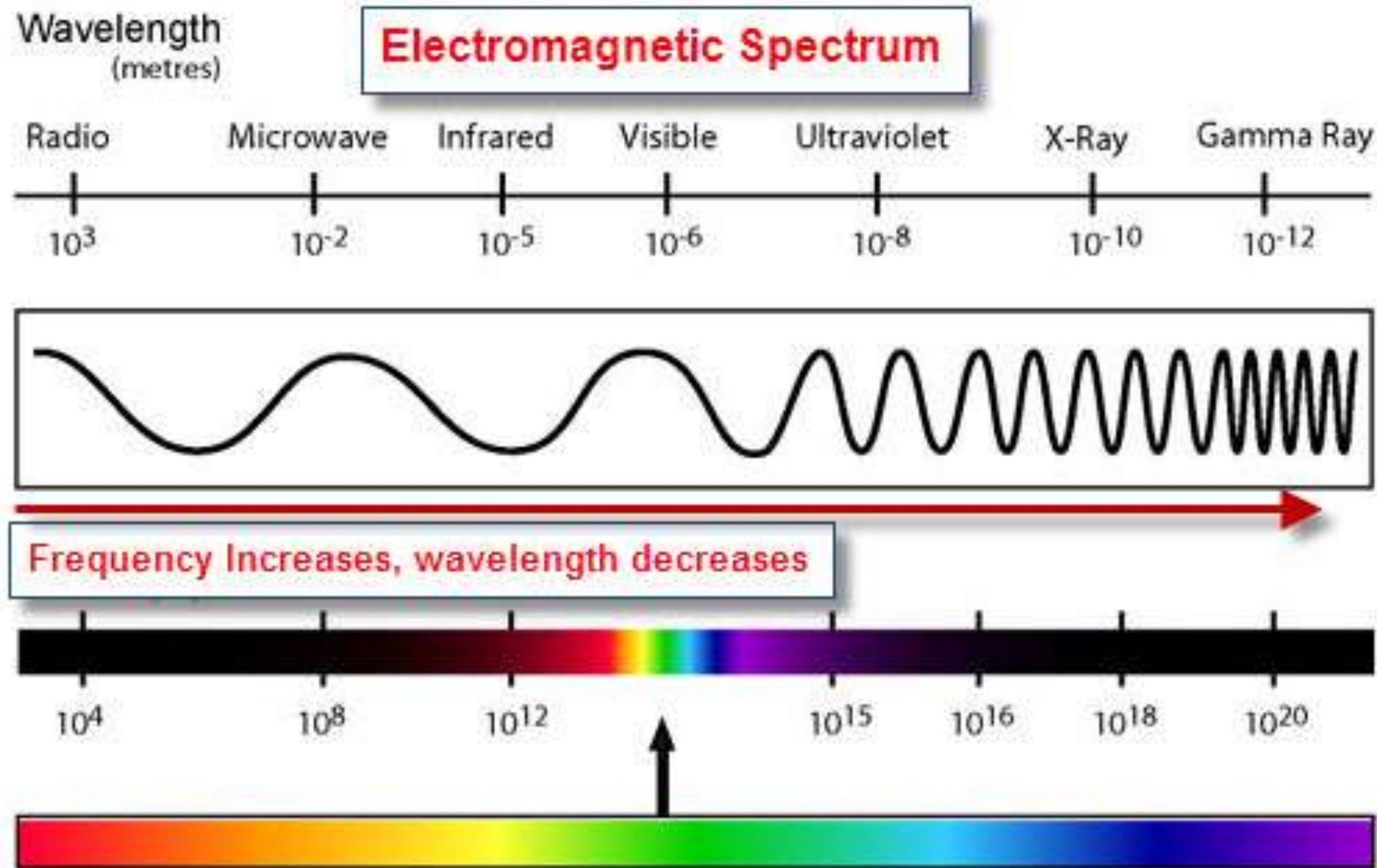
Spectroscopy

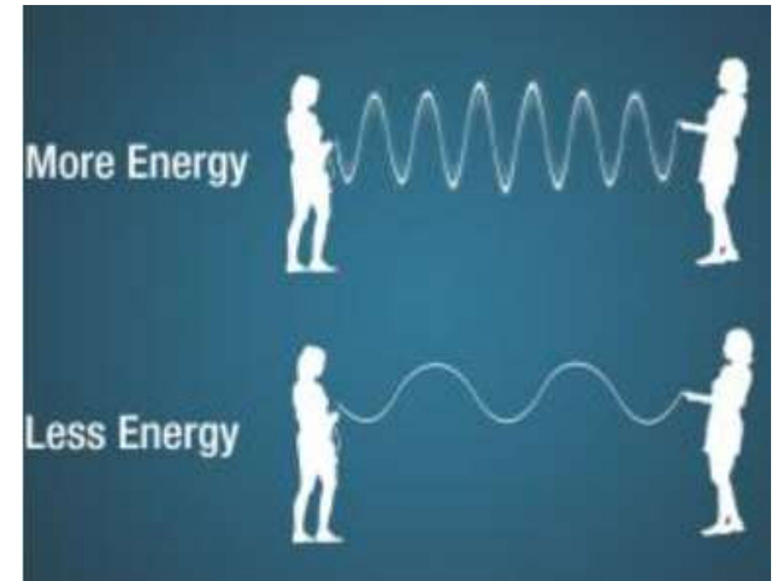
- Spectroscopy is the study of the interaction between electromagnetic radiation and matter
- It is the absorption, emission or scattering of radiation by matter that is used to quantitatively or qualitatively study the matter or a physical process
- Identity→ Qualitative spectroscopy
- Measurement of the total amount→ Quantitative spectroscopy

Electromagnetic Radiation?



Electromagnetic Radiation?





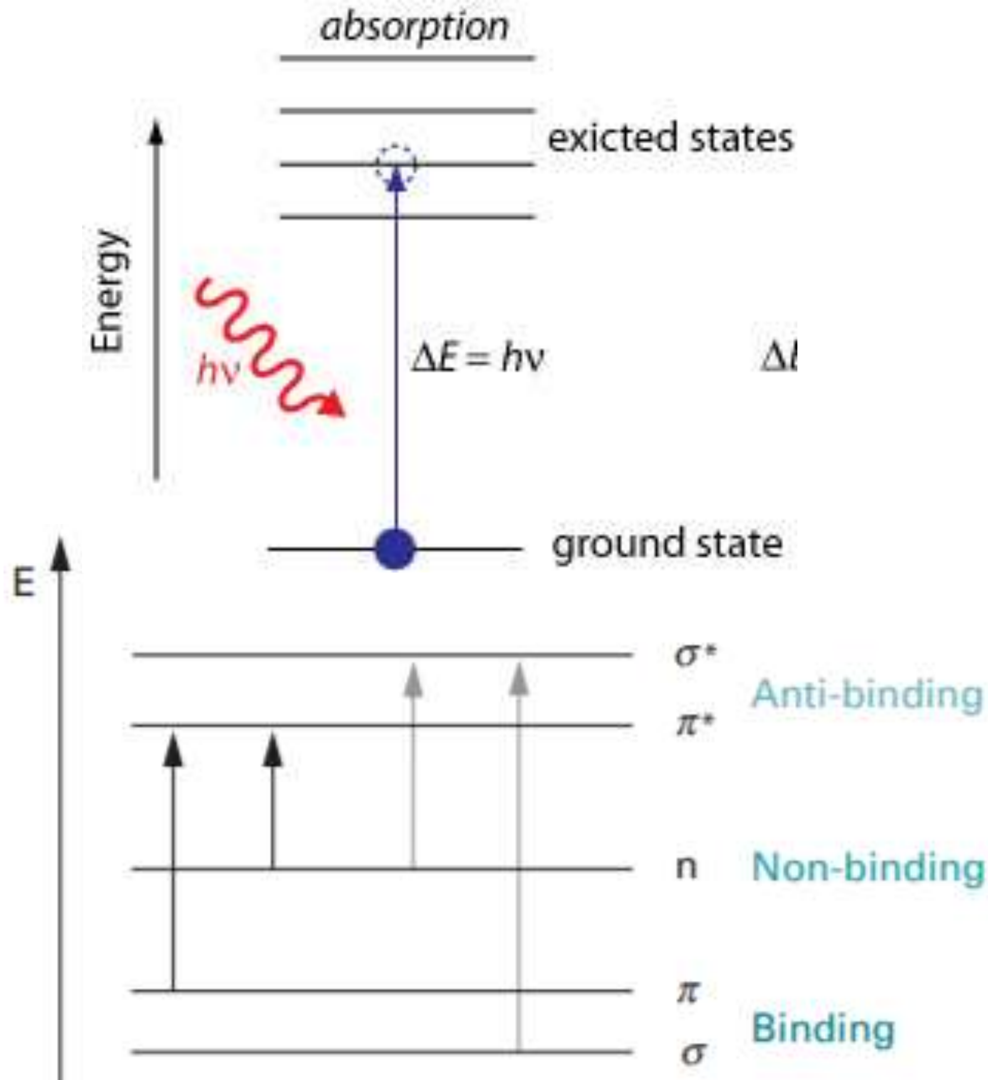
UV-Visible Spectroscopy

The UV radiation region extends from 10 nm to 400 nm and the visible radiation region extends from 400 nm to 700 nm.

Near UV Region: 200 nm to 400 nm

Far UV Region: below 200 nm

Interaction with matter



Types of transitions in organic compounds

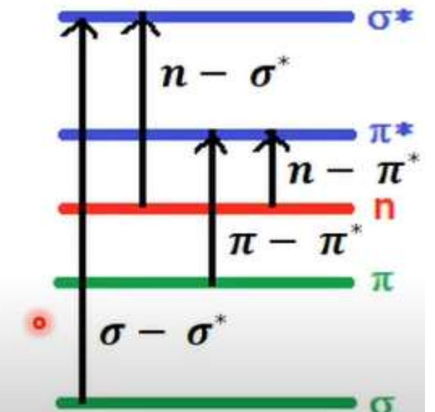
$\sigma \rightarrow \sigma^*$ $C-H, C-C$

$n \rightarrow \sigma^*$ $C-O, C-S, C-N$

$\pi \rightarrow \pi^*$ $C=C, C \equiv C$

$n \rightarrow \pi^*$ $C=O, C=S, N=N$

$$\sigma \rightarrow \sigma^* > n \rightarrow \sigma^* > \pi \rightarrow \pi^* > n \rightarrow \pi^*$$



From the four possible transitions (n->π*, π->π*, σ->σ*, n->σ*), only two can be elicited with light from the UV/Vis spectrum for some biological molecules: n->π* and π->π*

Interaction with matter

In proteins, there are three types of chromophores relevant for UV/Vis spectroscopy:

- peptide bonds (amide bond) **[180-230 nm, far-UV range];**
- certain amino acid side chains (mainly tryptophan and tyrosine) **[260-280 nm, near-UV range];**
- certain prosthetic groups and coenzymes (e.g. porphyrine groups such as in haem)

Substance	Wavelength (nm)
Phenylalanine	257
Tyrosine	274
Tryptophan	280
Cystine (Cys2)	250
Porphyrins	~400
NAD+	260
NADH	340
FAD	438
Purines and Pyrimidines	260-275

Porphyrins are the prosthetic groups of haemoglobin, myoglobin, catalase and cytochromes

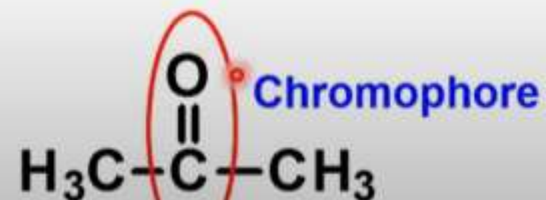
Interaction with matter

Chromophore – Covalently bonded group present in a molecule which is capable of absorbing UV or Visible light and exhibit a characteristic absorption band. eg. ethylenic, acetylenic, carboxylic acids, carbonyl, esters etc.

Chromogen – Compound possessing the chromophore.

$\pi \rightarrow \pi^{*}$ – $-C=C,\ -C\equiv C$, etc.

$n \rightarrow \pi^{*}$ – $-COOH,\ -C\equiv N$,

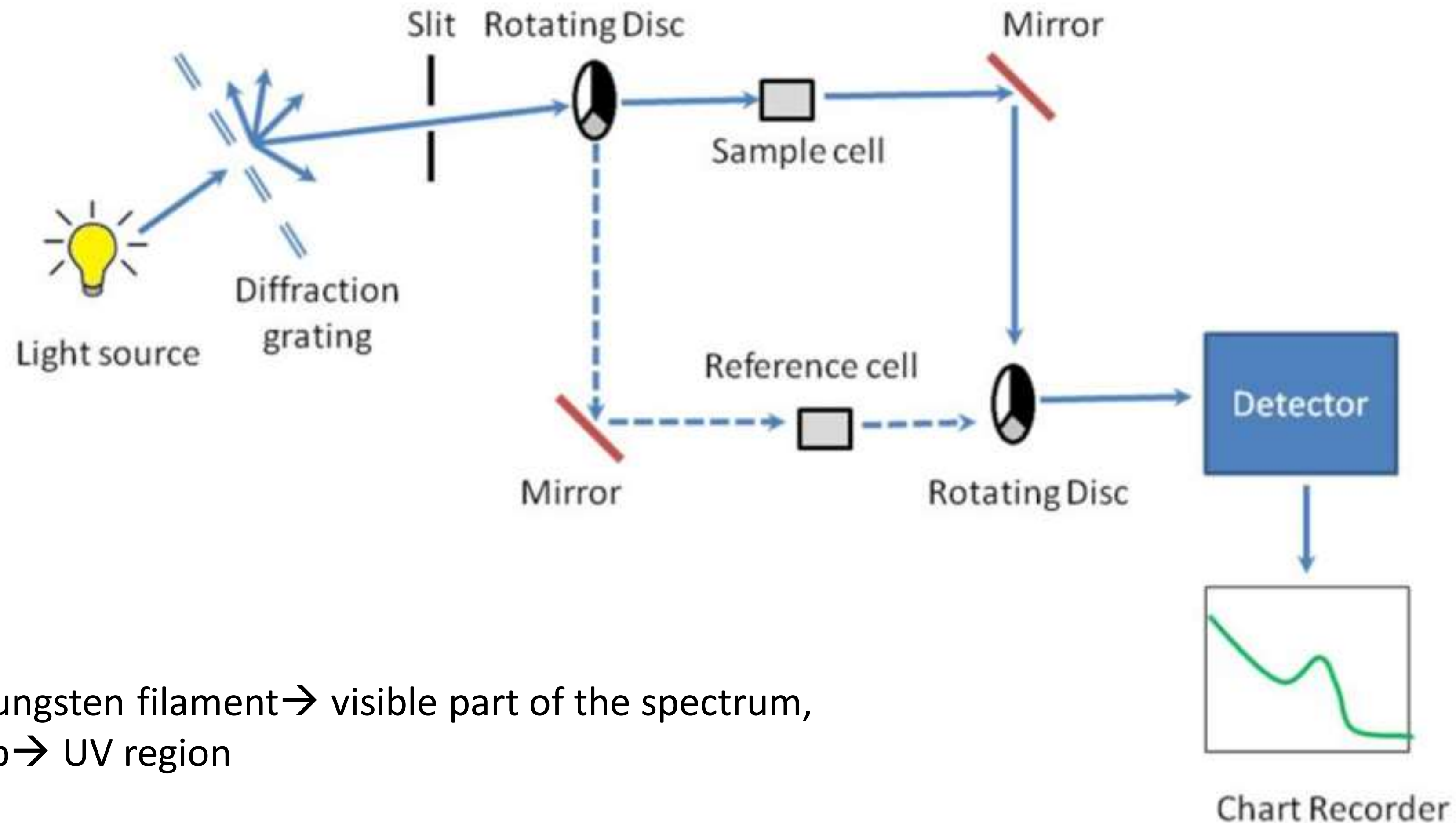


Auxochrome –

- A functional group attached to the chromophore
- It does not absorb light, but alters the absorption wavelength and/or intensity of the absorption band of the chromophore.

$-OH, -SH, -NH_2, -OR, -NR_2, -Cl$

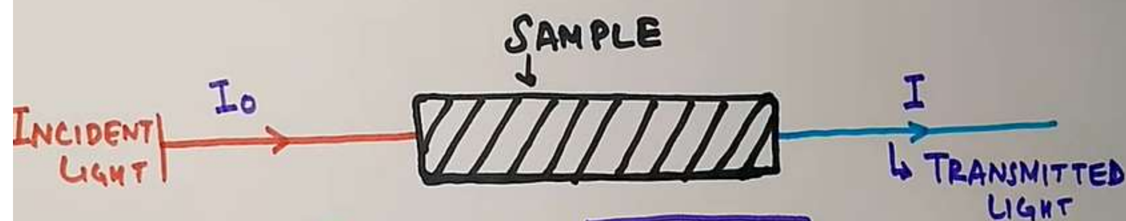
ULTRAVIOLET AND VISIBLE LIGHT SPECTROSCOPY



Light source: tungsten filament→ visible part of the spectrum, deuterium bulb→ UV region

Quantification of light absorption

→ MEASURING THE ABSORBANCE OF SOLUTION



ABSORBANCE : $A = \frac{I_0}{I}$

TRANSMISSION : $T = \frac{I}{I_0}$

ABSORBANCE A: $A = \log_{10}\left(\frac{1}{T}\right)$

$$\Rightarrow A = \log\left(\frac{I_0}{I}\right)$$

LAMBERT'S LAW

STATEMENT : ABSORBANCE OF LIGHT IN HOMOGENOUS SOL. IS DIRECTLY PROPORTIONAL TO THE LENGTH OF SAMPLE IN WHICH THE LIGHT PASSES.

$$A = \log_{10}\left(\frac{I_0}{I}\right) \propto l$$

$$\boxed{A = \varepsilon l}$$

ε = MOLAL EXTINCTION COEFFICIENT

BEER'S LAW

STATEMENT : ABSORBANCE OF LIGHT IN SAMPLE/SOL IS DIRECTLY PROPORTIONAL TO THE CONC. OF SOL IN WHICH LIGHT TRAVELS.

$$A = \log_{10}\left(\frac{I_0}{I}\right) \propto C$$

$$\boxed{A = \varepsilon C}$$

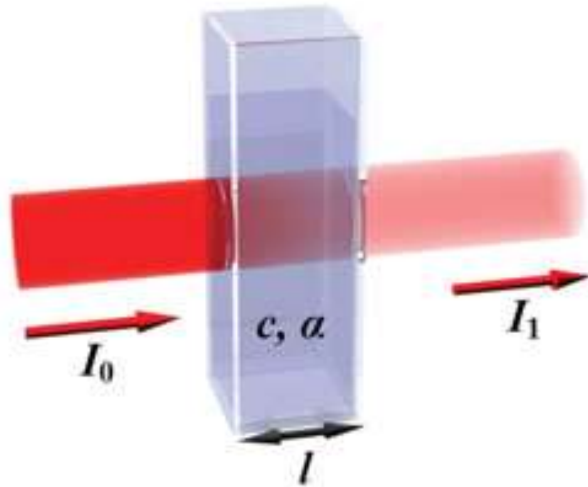
COMBINING BOTH LAWS

FOR ABSORBANCE:

$$A = \log_{10}\left(\frac{I_0}{I}\right) = \varepsilon c l$$

$$\boxed{A = \varepsilon c l}$$

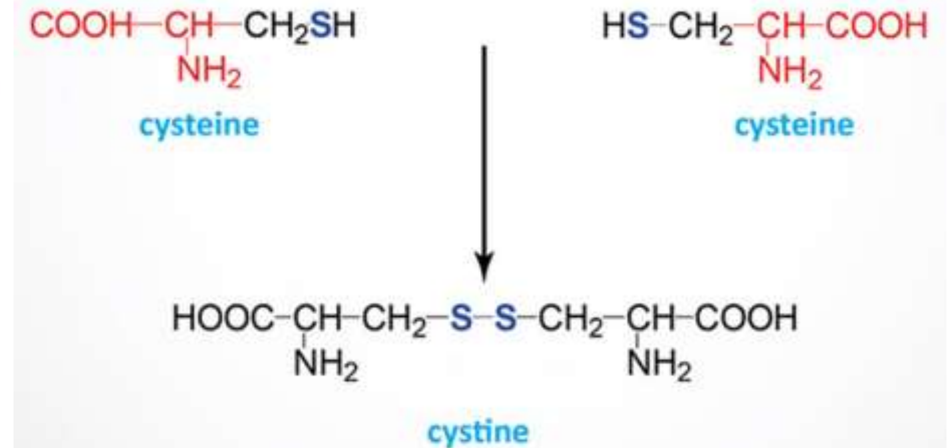
Beer-Lambert's law



where,
- A = Absorbance
- c = Concentration ($1\ mol/dm^3$)
- l = path length ($1\ cm$)
- ϵ = extinction coefficient ($1\ dm^3\ mol^{-1}\ cm^{-1}$) (molar extinction coefficient)

Molar extinction coefficient (ε)

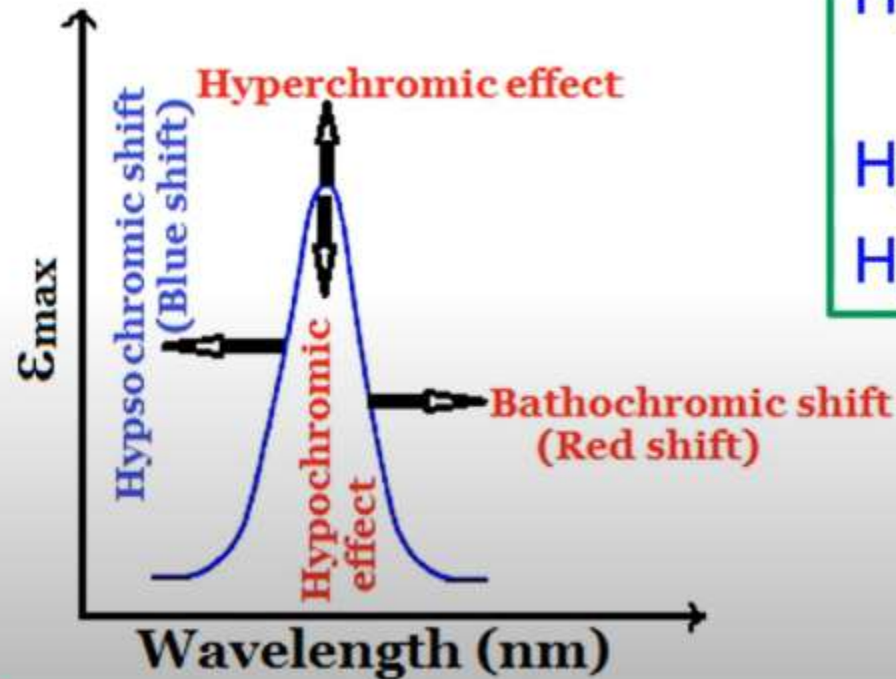
Residue	Gill and von Hippel ε_i (280 nm) in $dm^3\ mol^{-1}\ cm^{-1}$
Cys_2	120
Trp	5690
Tyr	1280



MAMVSEFLKQ AWFIENEEQE YVQTVKSSKG GPGSAVSPYP TFNPSS

For the peptide above, one obtains $\varepsilon = (1 \times 5690 + 2 \times 1280)\ dm^3\ mol^{-1}\ cm^{-1} = 8250\ dm^3\ mol^{-1}\ cm^{-1}$

Chromic shifts/Effect of substituents on absorption spectra



Bathochromic shift – **shifts to longer** wavelength (red shift)

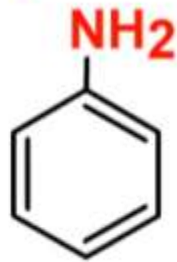
Hypsochromic shift – **shifts to shorter** wavelength (blue shift)

Hyperchromic effect – increase in intensity

Hypochromic effect – decrease in intensity

Bathochromic shift (Red shift) – Absorption band shifts to longer wavelength due to

- presence of an auxochrome
- change in the polarity of solvent
- presence of conjugation



Benzene
(λ_{max}= 256 nm)
(ε_{max}= 200)

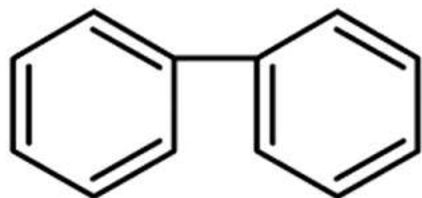
Aniline
(λ_{max}= 280 nm)
(ε_{max}= 1430)

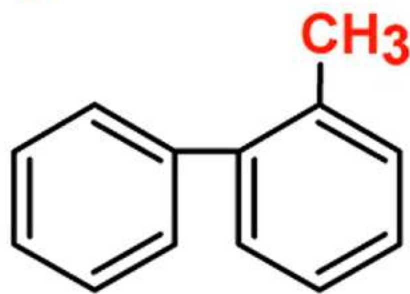
Aniline

- **lone pair on nitrogen delocalizes the π system of the aromatic ring**
- **Conjugation increases**
- **Low energy gap**
- **Bathochromic shift**

Hypsochromic shift- Absorption band shifts to shorter wavelength due to

- presence of an auxochrome
- change in the polarity of solvent
- removal of conjugation



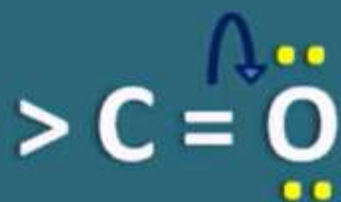


Biphenyl	2-Methyl biphenyl
(λ_{max}= 250 nm)	(λ_{max}= 237 nm)
(ε_{max}= 19000)	(ε_{max}= 10250)

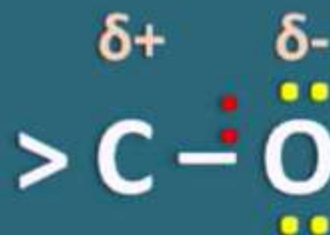
- Biphenyl rings are not completely planar (rings at an angle of 450)
- 2-methyl biphenyl is pushed further out of coplanarity
- Weak orbital overlap

$\pi \rightarrow \pi^*$ transition

Polar solvent



$\pi \rightarrow \pi^*$



Less polar

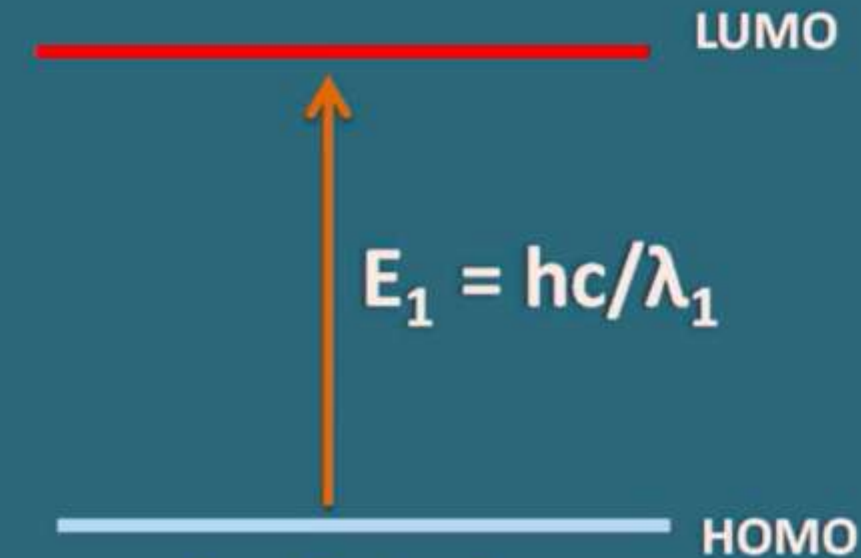
π Bonding molecular orbital

More polar

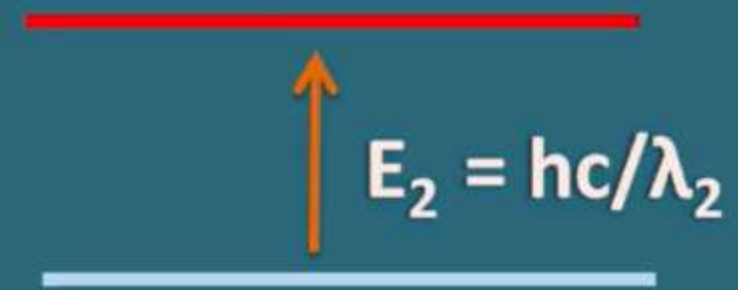
π^* anti-bonding molecular orbital

$\pi \rightarrow \pi^*$ transition

Bathochromic shift or red shift



Analyte in Non polar solvent



Analyte in polar solvent

$E_1 > E_2$ or $\lambda_1 < \lambda_2$

n → π* transition

Polar solvent

n → π*

More H-Bonding

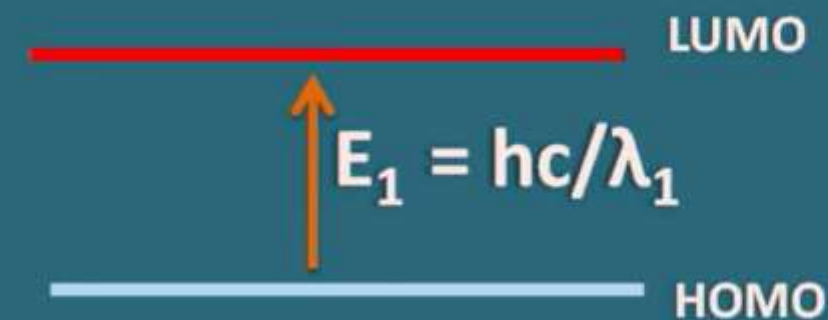
Less H-bonding

HOMO

LUMO

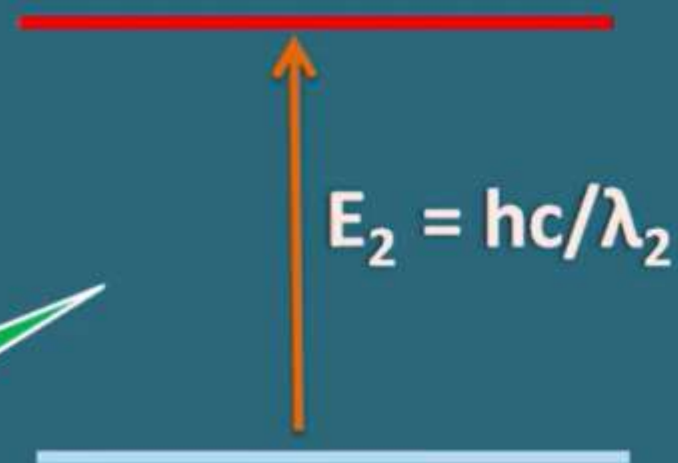
$n \rightarrow \pi^*$ transition

$E_1 < E_2$ or $\lambda_1 > \lambda_2$



Analyte in
Non polar solvent

Hypsochromic shift or
Blue shift



Analyte in
polar solvent

Effect of polarity of solvent on λ_{max}

Positive solvatochromism

POLAR solvent

$\pi \rightarrow \pi^*$ transition λ_{max} Bathochromic or red shift

Negative solvatochromism

$n \rightarrow \pi^*$ transition λ_{max} Hypsochromic or blue shift

Limitations of UV/Vis Spectroscopy

- Stray light
- Light Scattering
- Interference from multiple absorbing species
- Molecular events- ex: chromophore dimerization
- Geometrical considerations

Applications of UV/Vis Spectroscopy

- DNA and RNA Analysis
- Pharmaceutical analysis
- Bacterial Culture
- Beverage Analysis
- Hemoglobin concentrations->cancer research
- Wastewater treatments

Wavelength used in absorbance analysis in nanometers	What does UV absorbance at this wavelength indicate the presence of?	What causes UV absorbance at this wavelength?
230	Protein	Protein Shape
260	DNA and RNA	Adenine, guanine, cytosine, thymine, uracil
280	Protein	Mostly tryptophan and tyrosine

Absorbance ratio	Typical values
260/280	1.8 absorbance ratio typical for pure DNA 2.0 absorbance ratio typical for pure RNA
260/230	Absorbance ratio varies; 2.15 to 2.50 typical for RNA and DNA

Instruments specifically designed to measure turbid samples are **nephelometers** or **Klett meters**

Question 1

A solution contains NADH and NAD+, both at 0.1mM concentration. If NADH has a molar extinction coefficient of 6220 and that of NAD+ is negligible, the optical density measured in a cuvette of 5mm path length will be?

A. 0.62
B. 0.062
C. 0.31
D. 0.031

Solution:

Given: $C = 0.1\ mM$ ($1\ mol/dm^3$)

$l = 5\ mm$ ($1\ cm$)

$\epsilon = 6220$

$A = ?$

Sol^n: $C = 10^{-4}\ mol/dm^3$

$l = 0.5\ cm$

$\epsilon = 6220$

$\therefore A = \epsilon c l$ (Beer-lambert's law)

$= 6220 \times 10^{-4} \times 0.5$

$= 3110 \times 10^{-4}$

$A = 0.311$

$1\ mM = 10^{-3}\ mol/dm^3$

$0.1\ mM = x\ mol/dm^3$

$\therefore x = 0.1 \times 10^{-3}$

$\therefore x = 10^{-4}\ mol/dm^3$

$1\ cm = 10\ mm$

$x\ cm = 5\ mm$

$\therefore x = \frac{5}{10} = 0.5$

$\therefore x = 0.5\ cm$

Question 2

What percentage of NAD^+ is reduced in a solution that had an absorbance of 0.9 at 260 nm before reduction, and an absorbance of 0.11 at 340 nm after the reaction? (The molar extinction coefficients of NAD^+ are E_{260}=18000, E_{340} = negligible. The molar extinction coefficient of NADH are E_{260} = 18000, E_{340} = 6200.)

Soln.:

260 nm:

$c = A/\varepsilon = 0.9/18000 = 5 \cdot 10^{-5}$ M (total amount of NAD^+)

340 nm:

$c = A/\varepsilon = 0.11/6200 = 1.77 \cdot 10^{-5}$ M (the amount of NAD^+ that became NADH)

$(1.77 \cdot 10^{-5}/5 \cdot 10^{-5}) \cdot 100 = 35.5\%$

Question 3

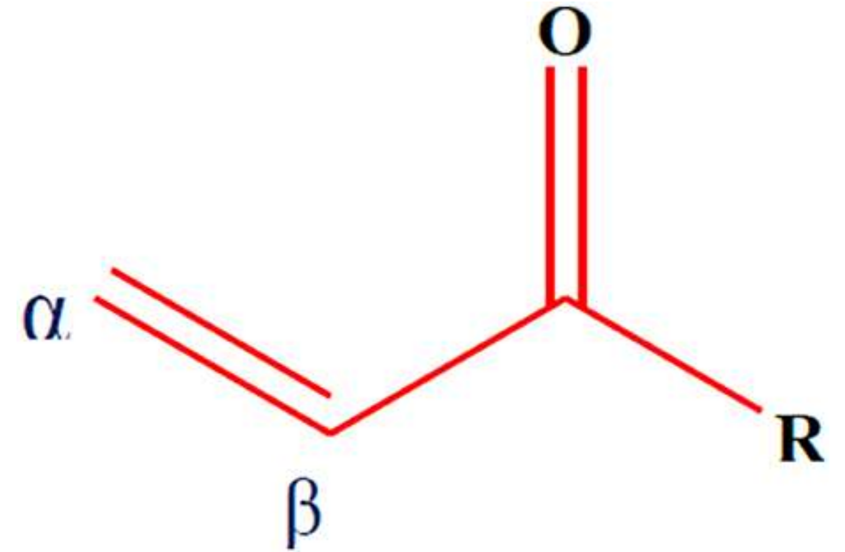
In the UV-visible absorption spectrum of an α,β-unsaturated carbonyl compound, with increasing solvent polarity,

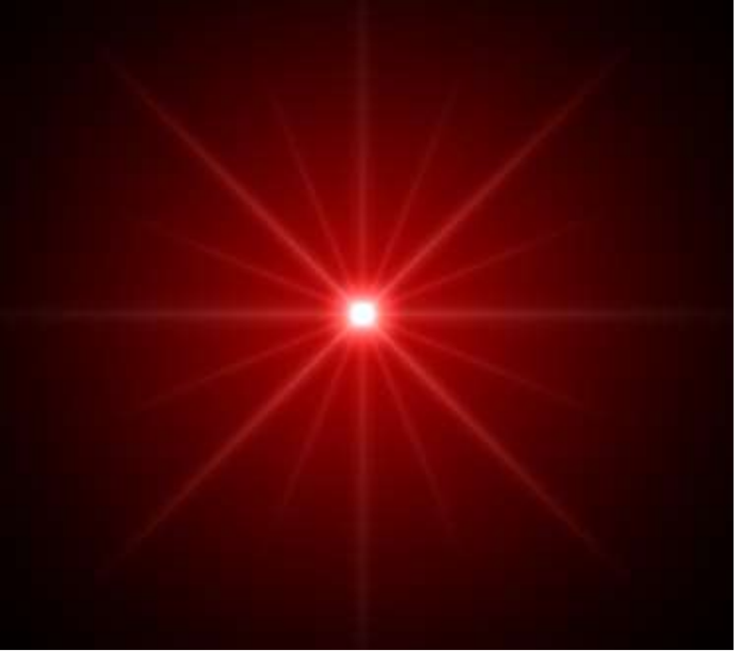
A. n-π* transitions undergo hypsochromic shift, π-π* undergo bathochromic shift

B. n-π* transitions undergo bathochromic shift, π-π* undergo hypsochromic shift

C. both n-π* and π-π* transitions undergo bathochromic shift

D. both n-π* and π-π* transitions undergo hypsochromic shift



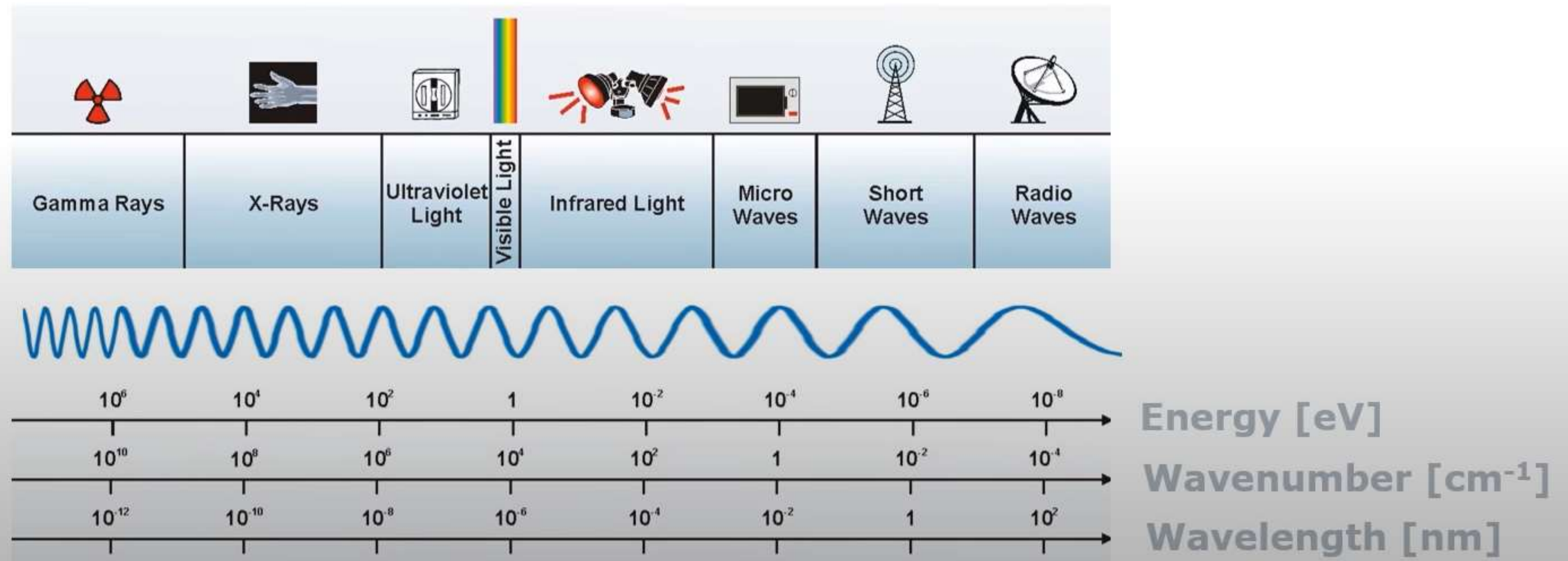


Infrared (IR) Spectroscopy

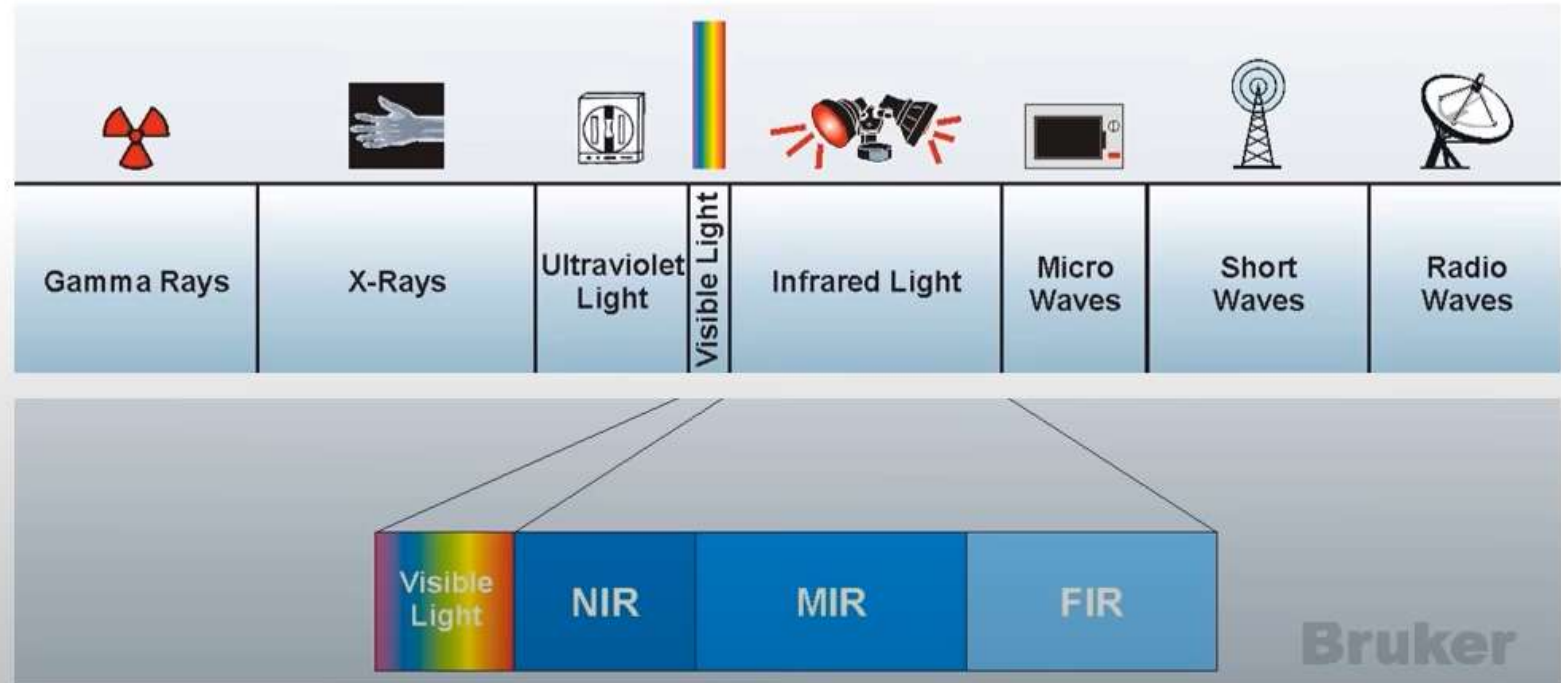
Infrared Spectroscopy?

What is infrared (IR) spectroscopy?

- IR spectroscopy studies the interaction between matter and infrared radiation
- Infrared radiation is electromagnetic radiation (light) with a longer wavelength than visible light

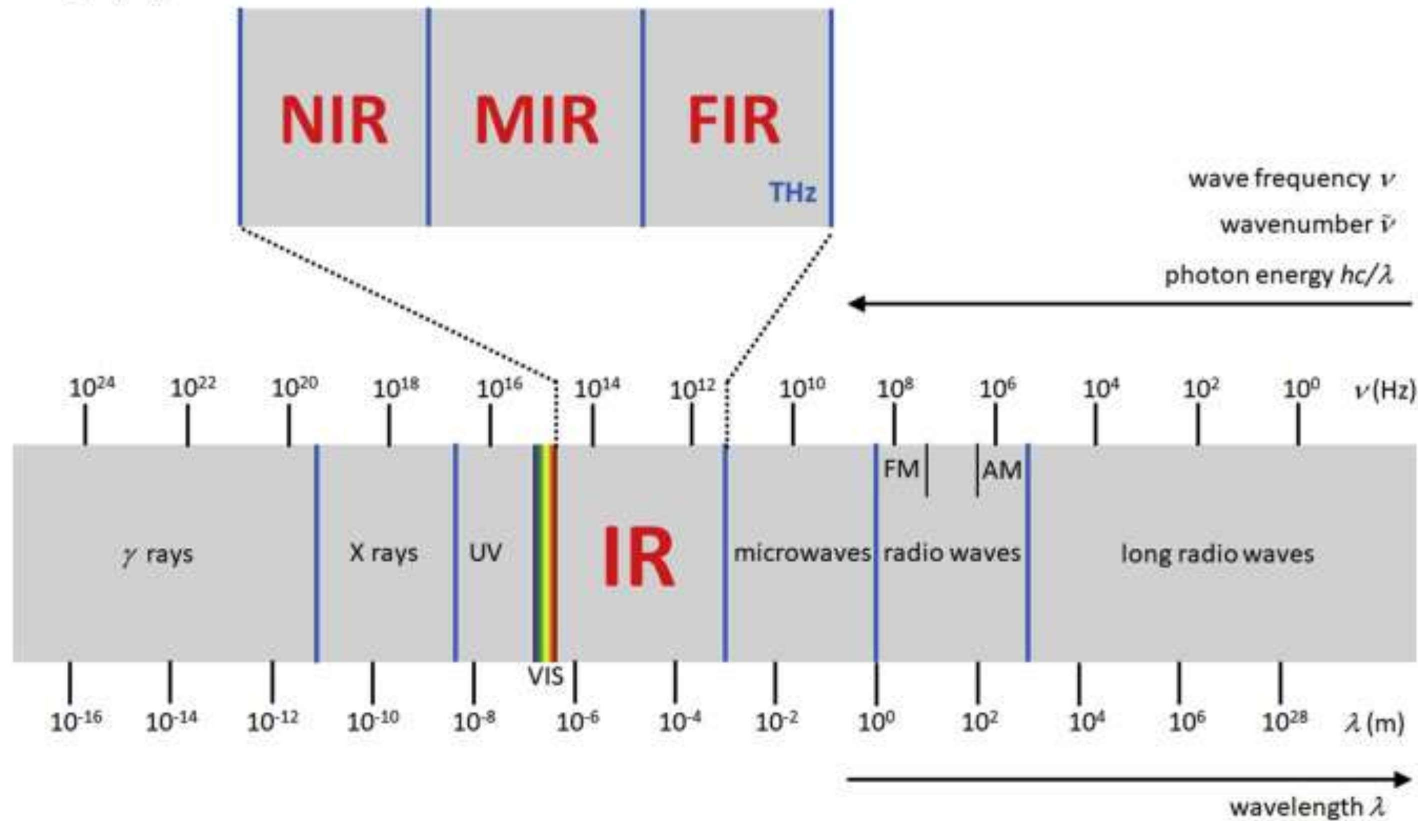


Infrared Spectroscopy

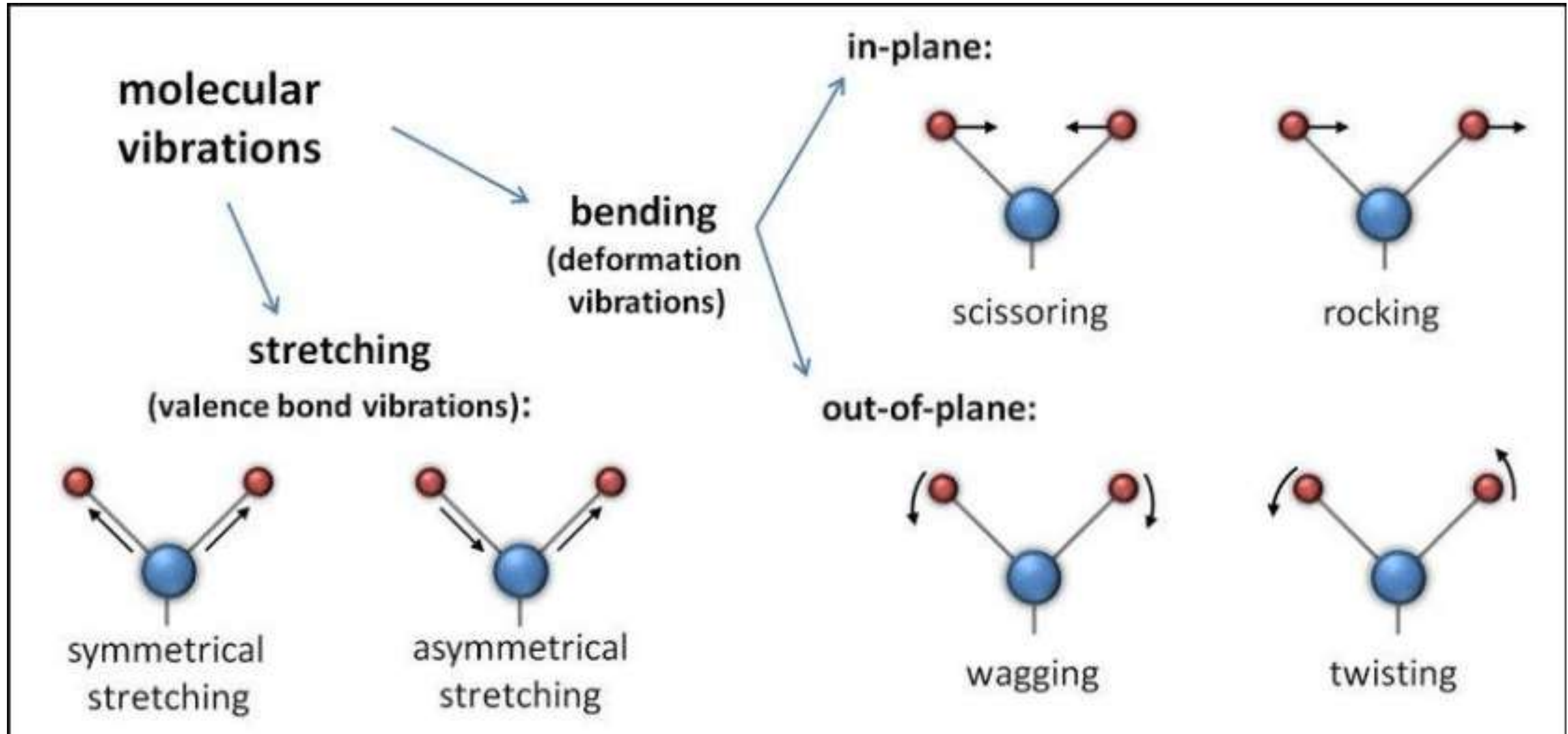


- Depending on the wavelength, the IR can be subdivided into near, mid and far infrared

Infrared Radiation

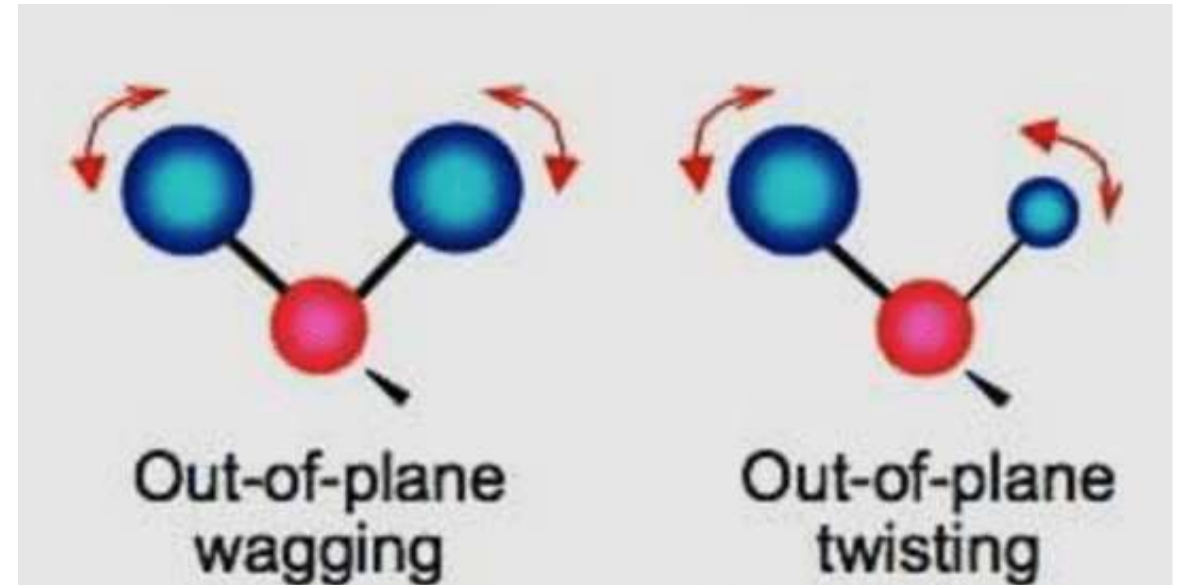


How does infrared light interacts with matter?

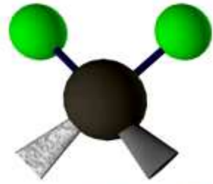


How does infrared light interact with matter?

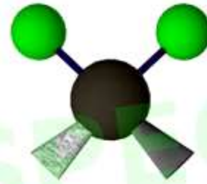
- Stretching Vibration: change in bond length
- Bending Vibration: change in bond angle



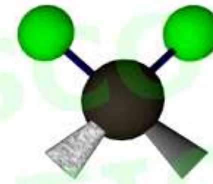
INFRARED SPECTROSCOPY VIBRATION ANIMATION
DOWNLOAD AT CAPTAINCORROSION.COM



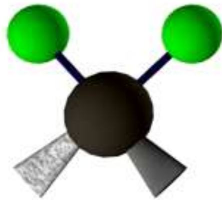
SYMMETRIC STRECHING



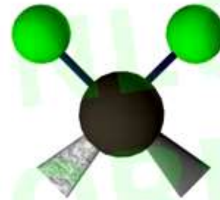
ANTISYMMETRIC STRECHING



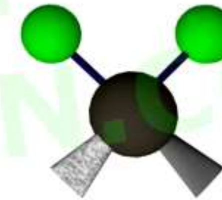
ROCKING



WAGGING



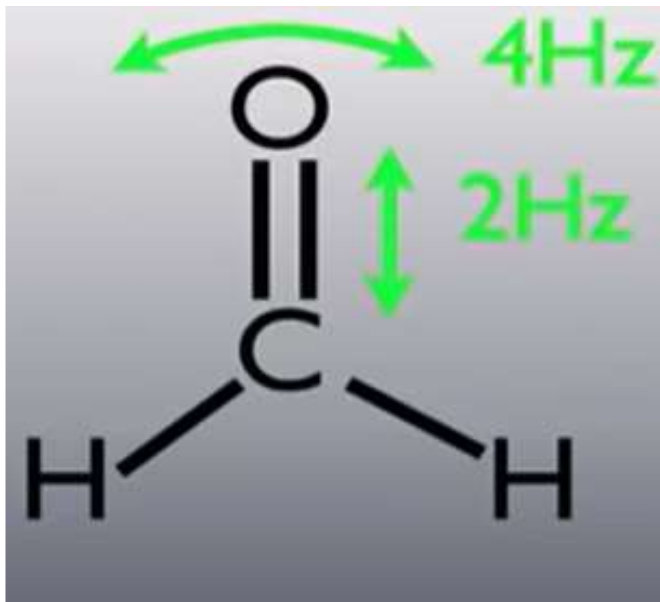
TWISTING

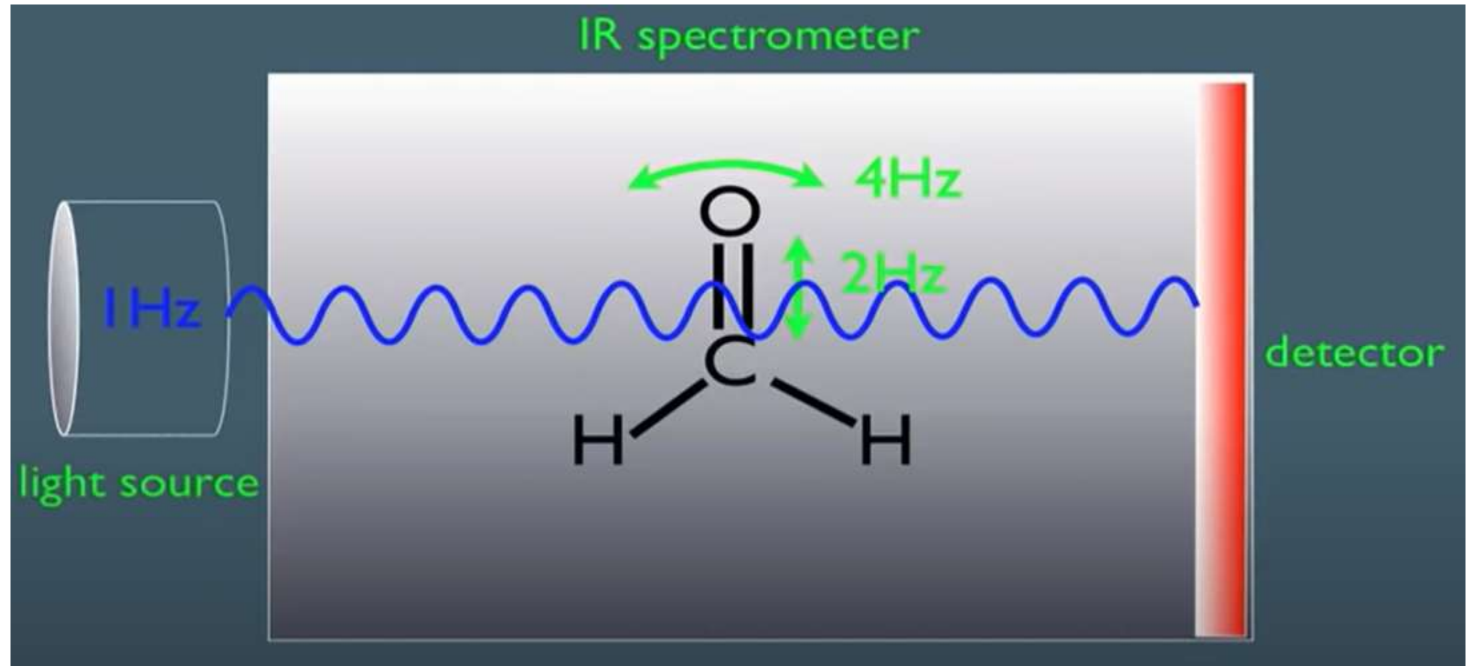


SCISSORING

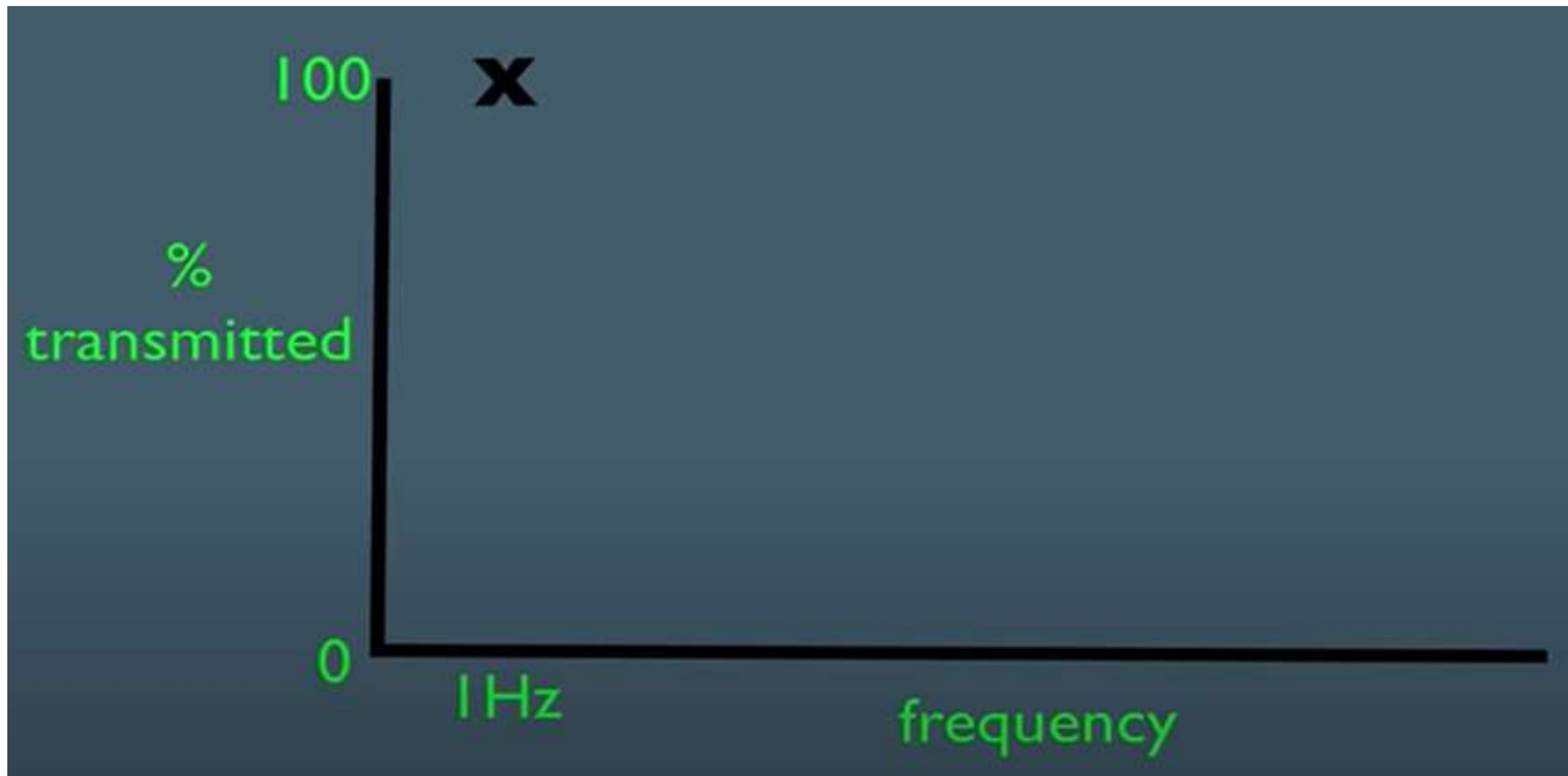
Reference: https://www.youtube.com/watch?v=1PQqDfJKXvA&ab_channel=CaptainCorrosion

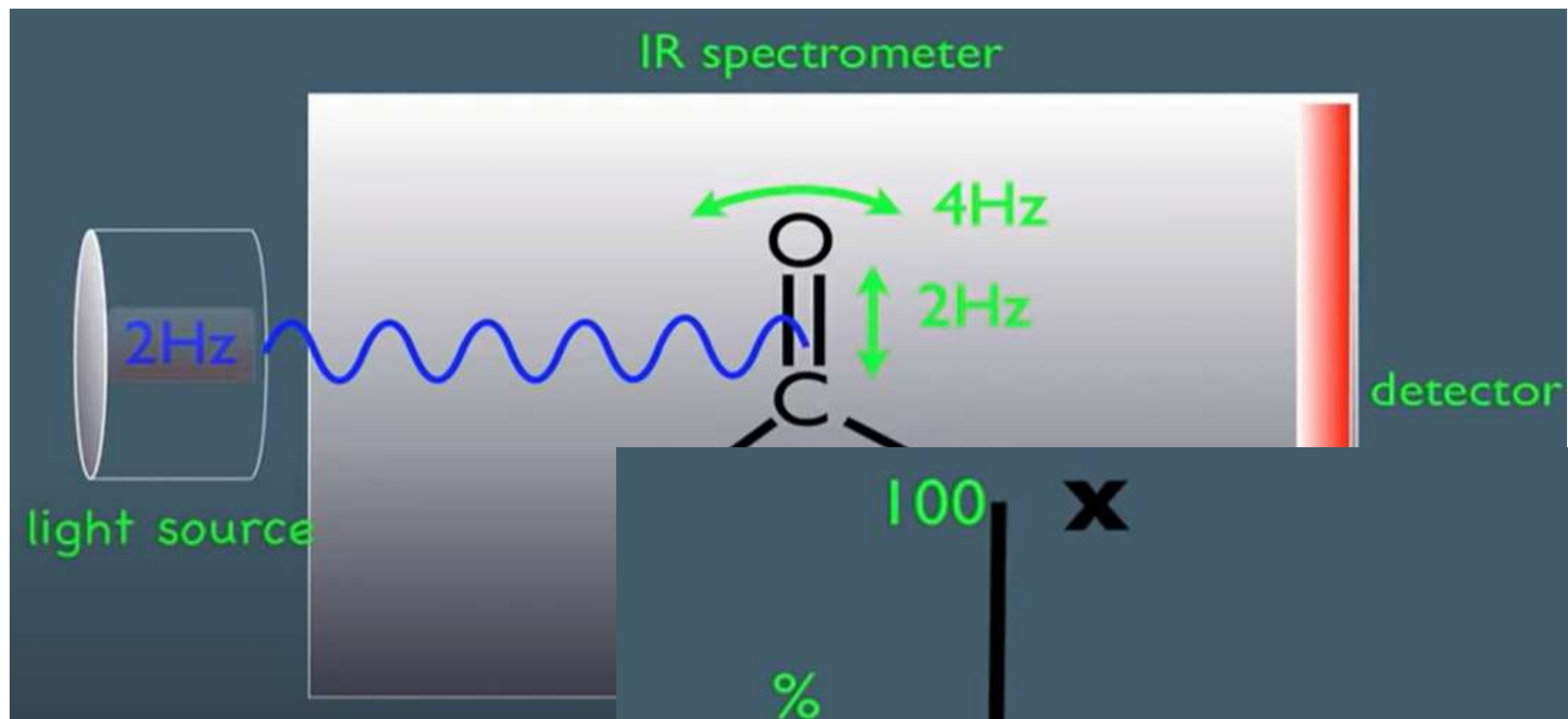
How is an infrared spectrum measured?



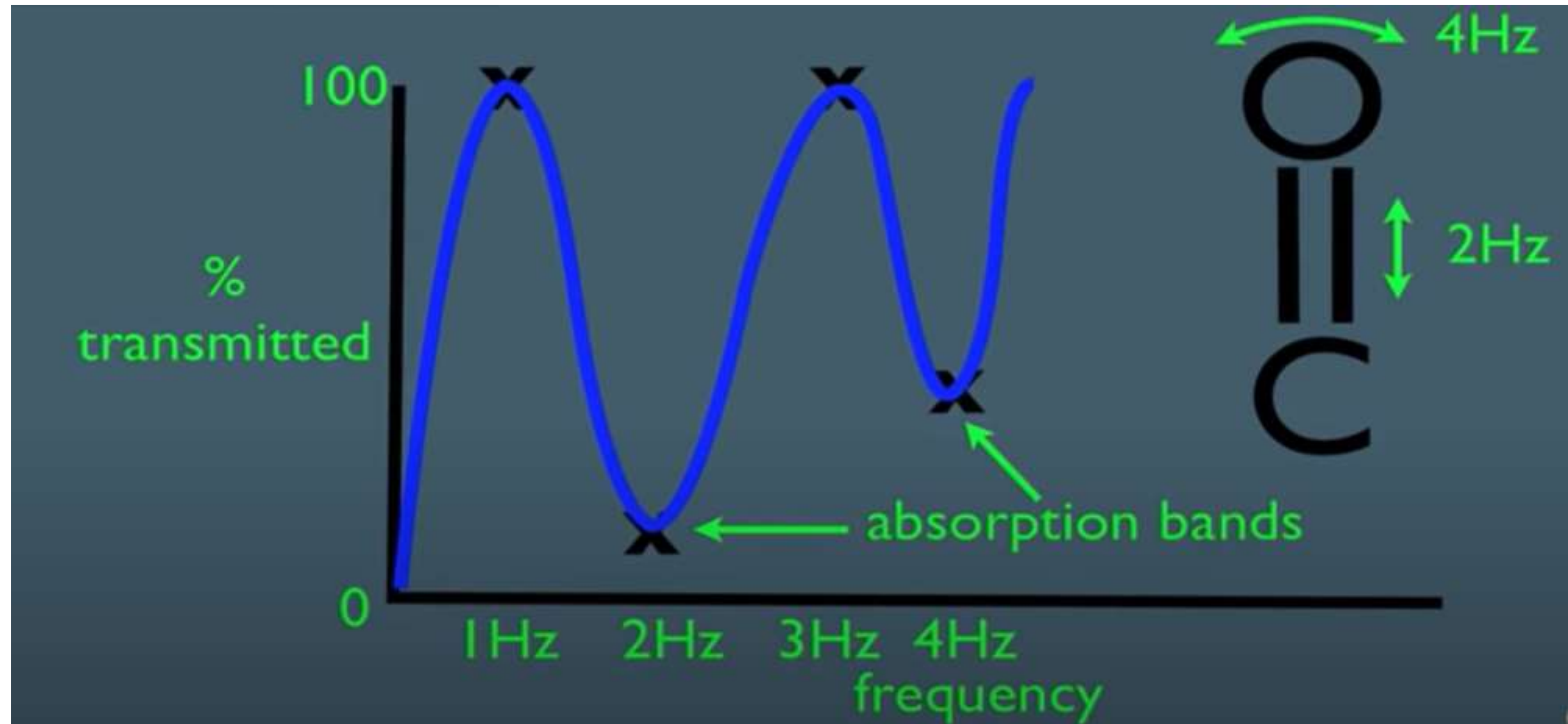


How is an infrared spectrum measured?



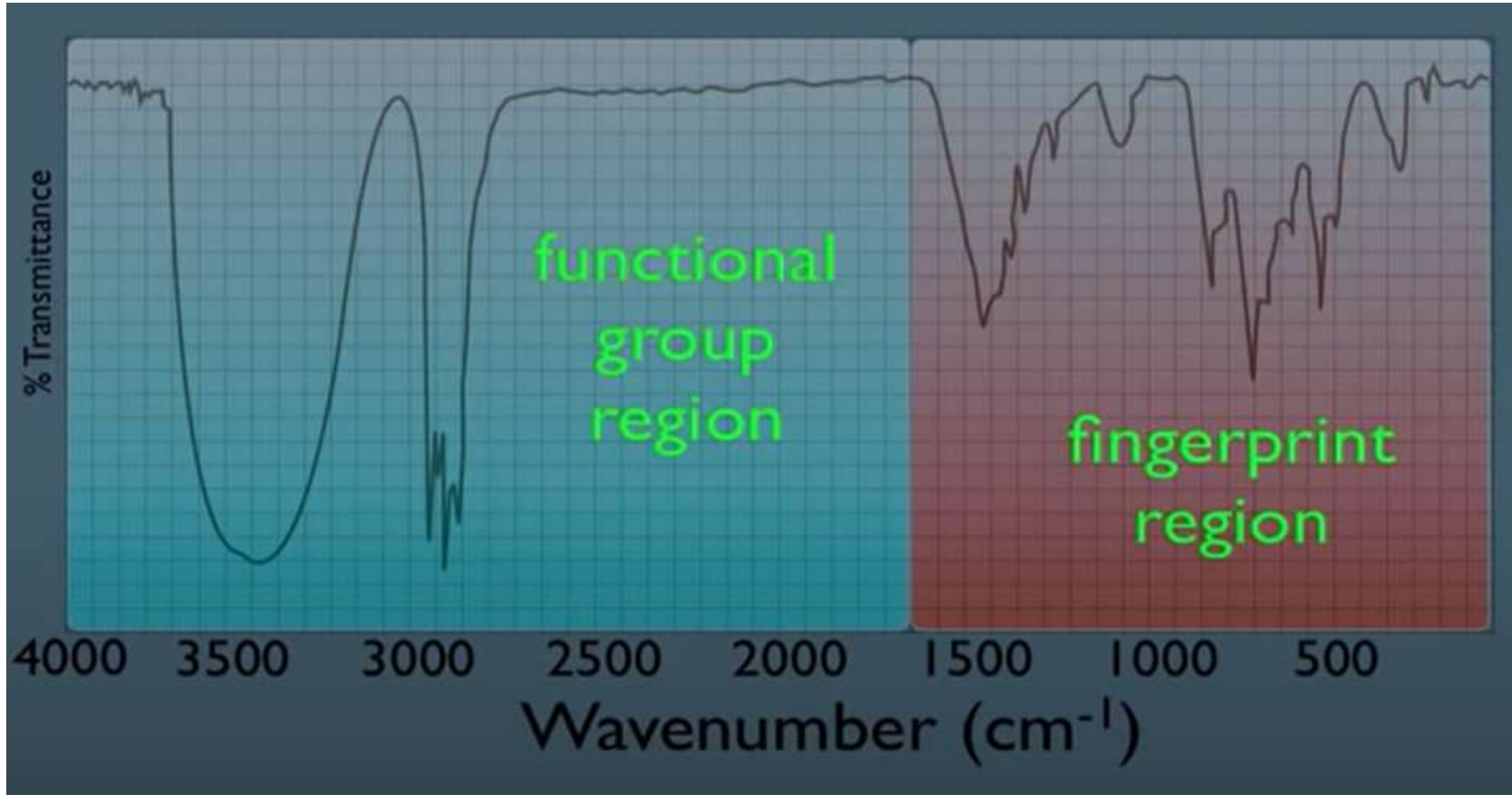


100
% transmitted
0
1Hz
2Hz
frequency



100
%
transmitted
0
1Hz
2Hz
3Hz
4Hz
frequency
absorption bands
4Hz
2Hz
O
C

Infrared Spectrum



Functional Groups and Wavenumbers

functional group wavenumbers		
bond	wave#	intensity
O—H	3650-3200	strong, broad
C—H	3300-2700	medium
N—H	3500-3300	medium, broad
C≡N	2260-2220	medium
C≡C	2260-2100	weak-medium
C=C	1680-1600	medium
C=N	1650-1550	medium
C=O	1780-1650	strong
C—O	1250-1050	strong

Protein- Secondary structure determination

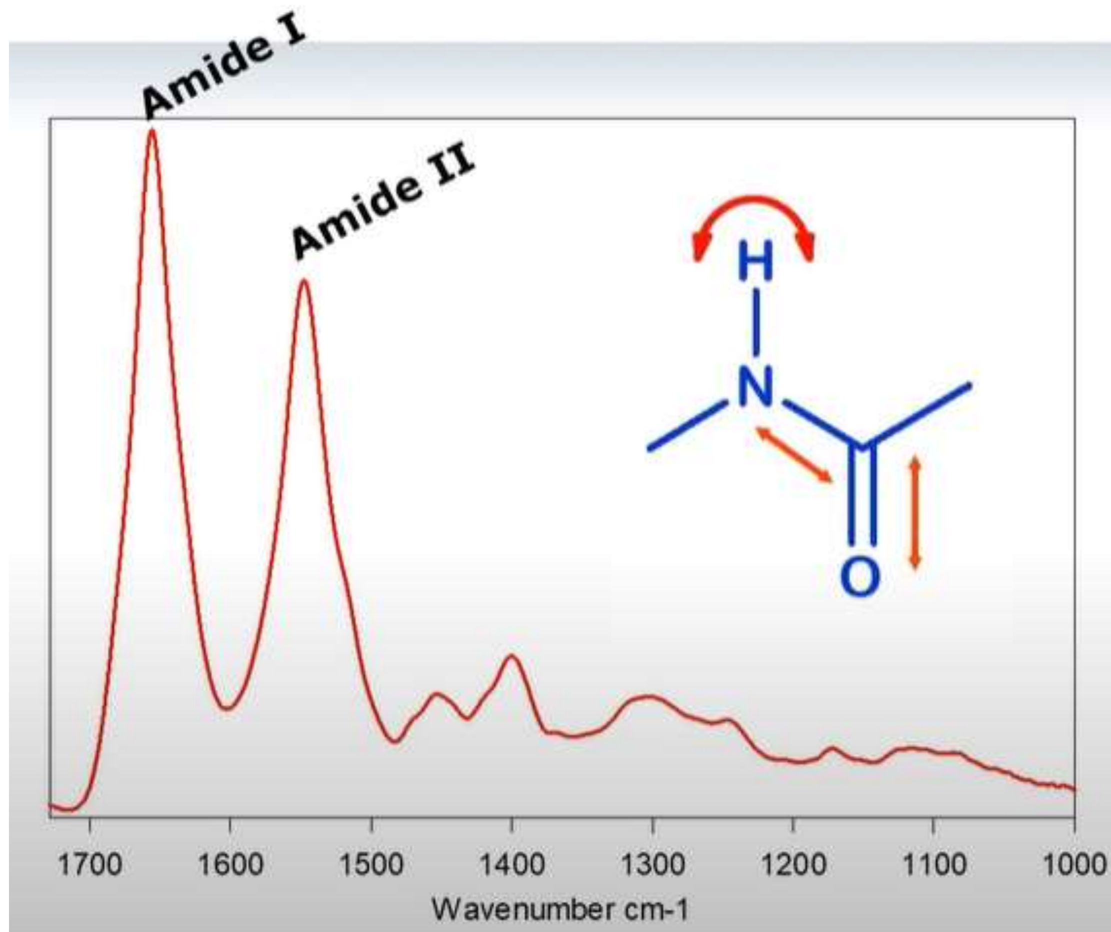
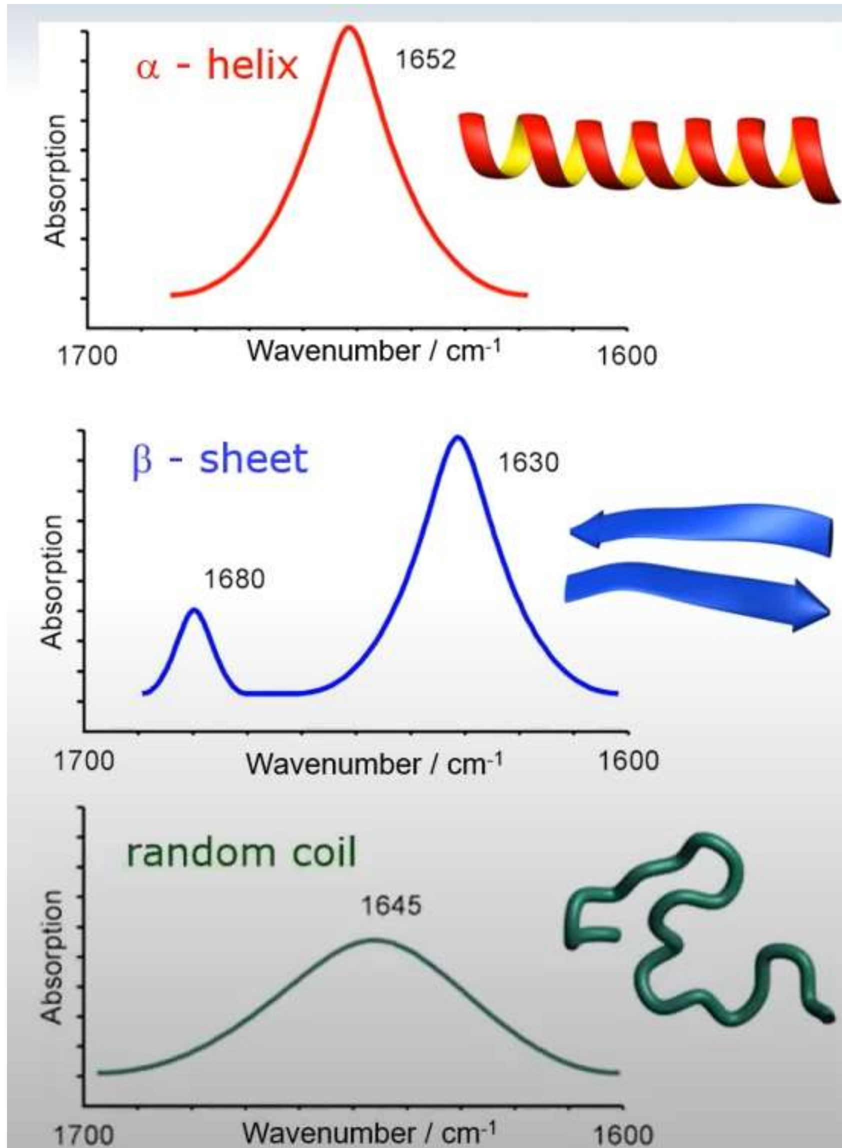


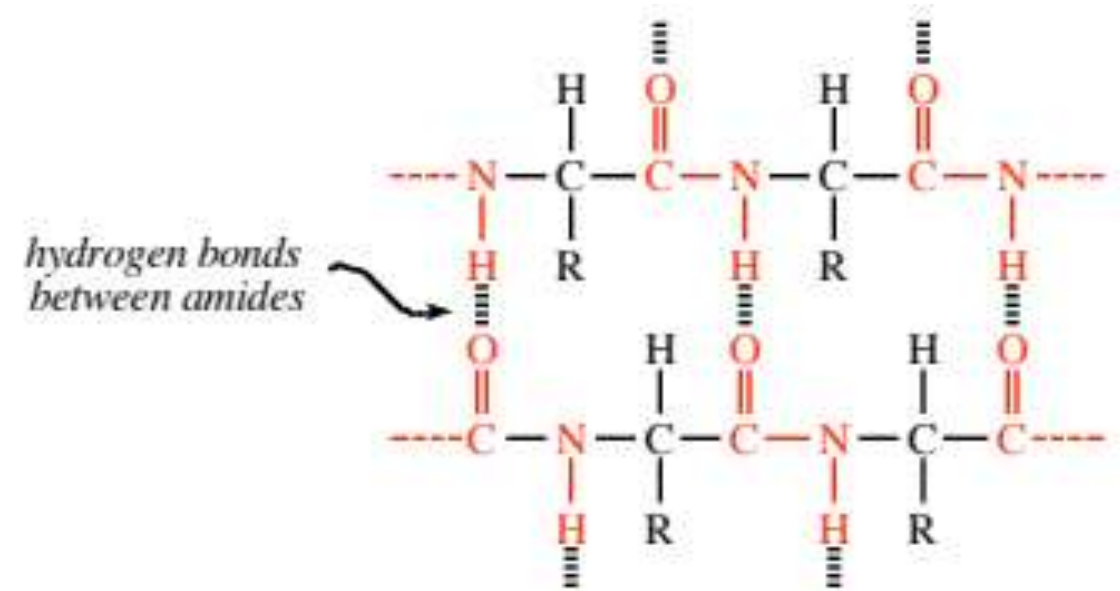
Table 1 Characteristic infrared bands of peptide linkage

Designation	Approximate frequency (cm^{-1})	Description
Amide A	3300	NH stretching
Amide B	3100	NH stretching
Amide I	1600–1690	C=O stretching
Amide II	1480–1575	CN stretching, NH bending
Amide III	1229–1301	CN stretching, NH bending
Amide IV	625–767	OCN bending
Amide V	640–800	Out-of-plane NH bending
Amide VI	537–606	Out-of-plane C=O bending
Amide VII	200	Skeletal torsion

- Amide I: C=O stretching (80%)
- Amide II: CN stretching (18-40%), NH bending (40-60%)

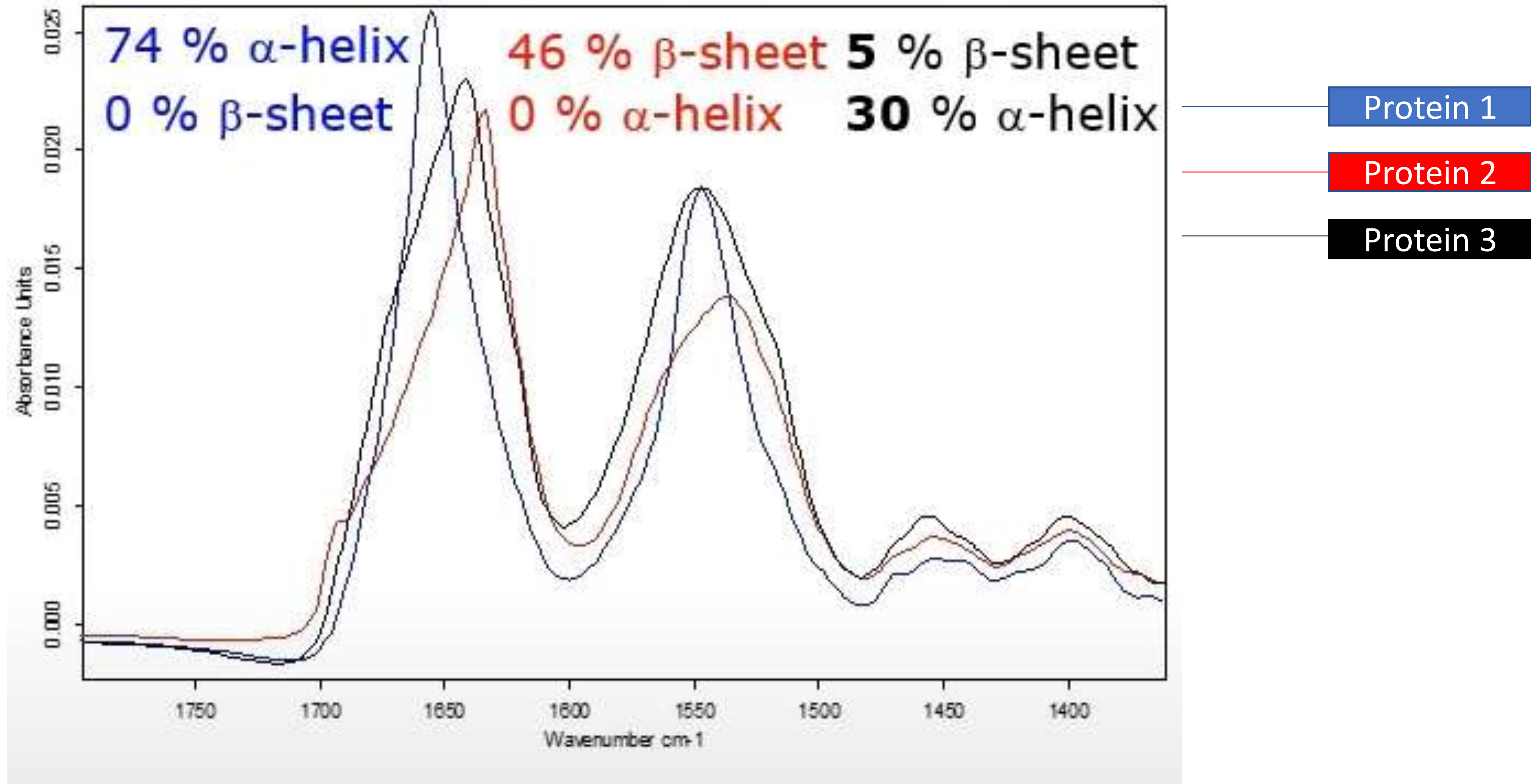
Protein- Secondary structure determination



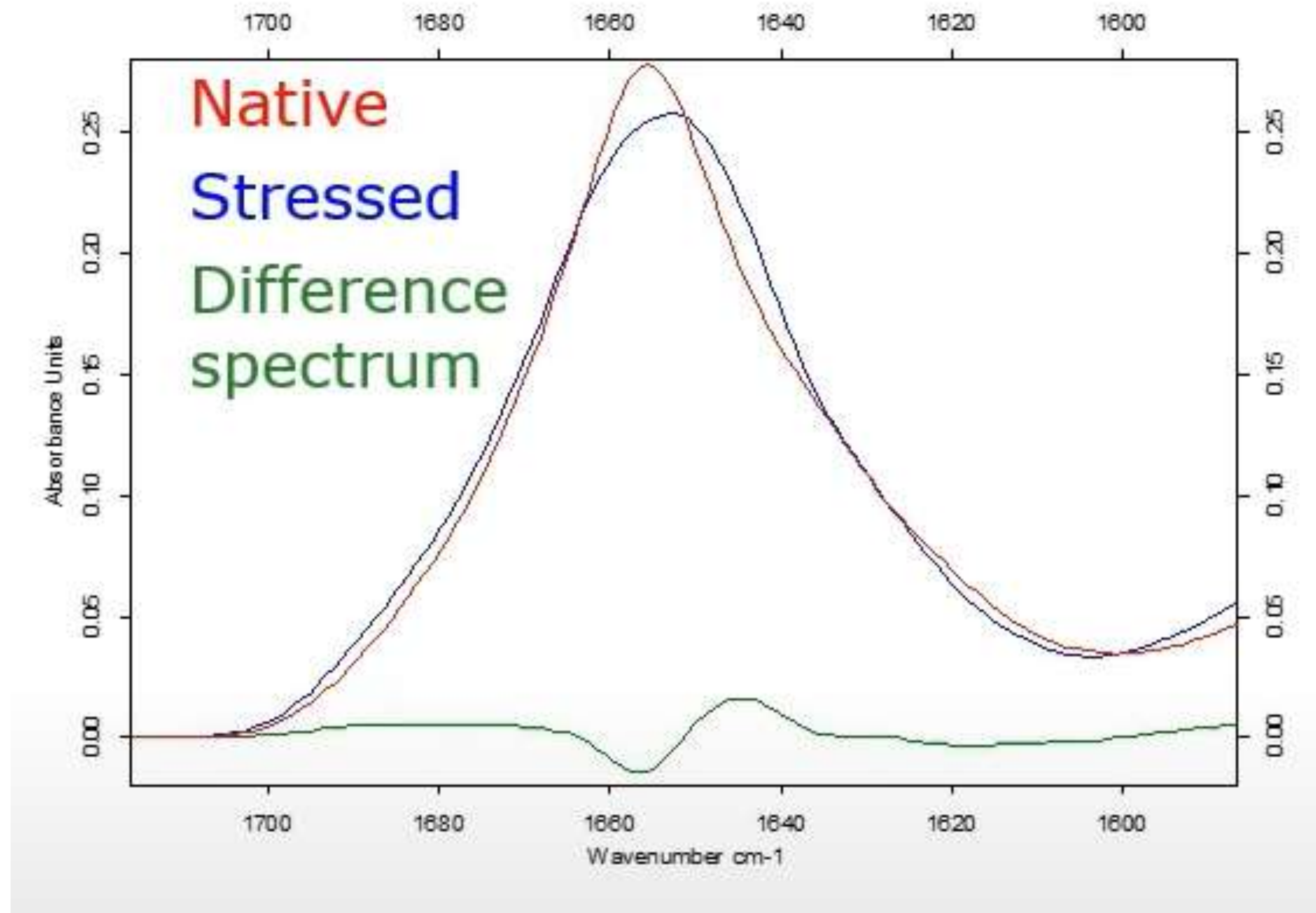


- 1658 and 1650 cm^{-1} → α-helix
- 1640−1620 cm^{-1} and 1695−1690 cm^{-1} → β-sheet
- 1640 and 1648 cm^{-1} → random coils
- 1670, 1683, 1688 and 1694 cm^{-1} → β-turns

Characterize Secondary Structure

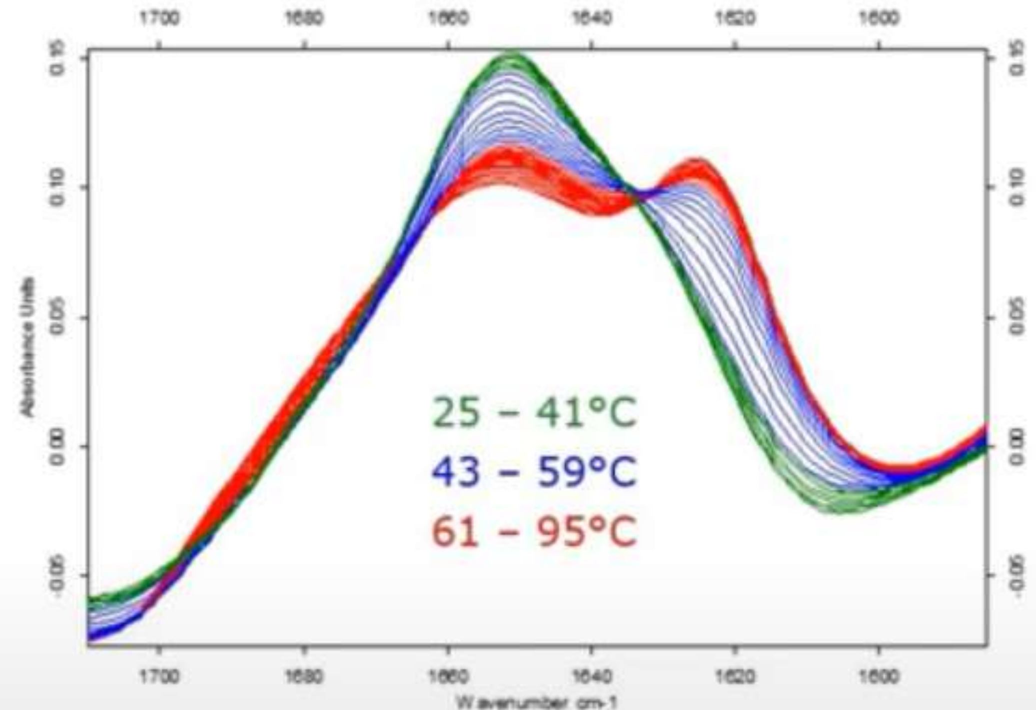


Detection of Conformational Changes



Following structural transitions

Fibrillation is the process by which misfolded proteins form large aggregates, for example a amyloid fibrils (associated with diseases like Alzheimer’s and Parkinson’s)



✓ Follow Structural Transitions

Temperature ramps

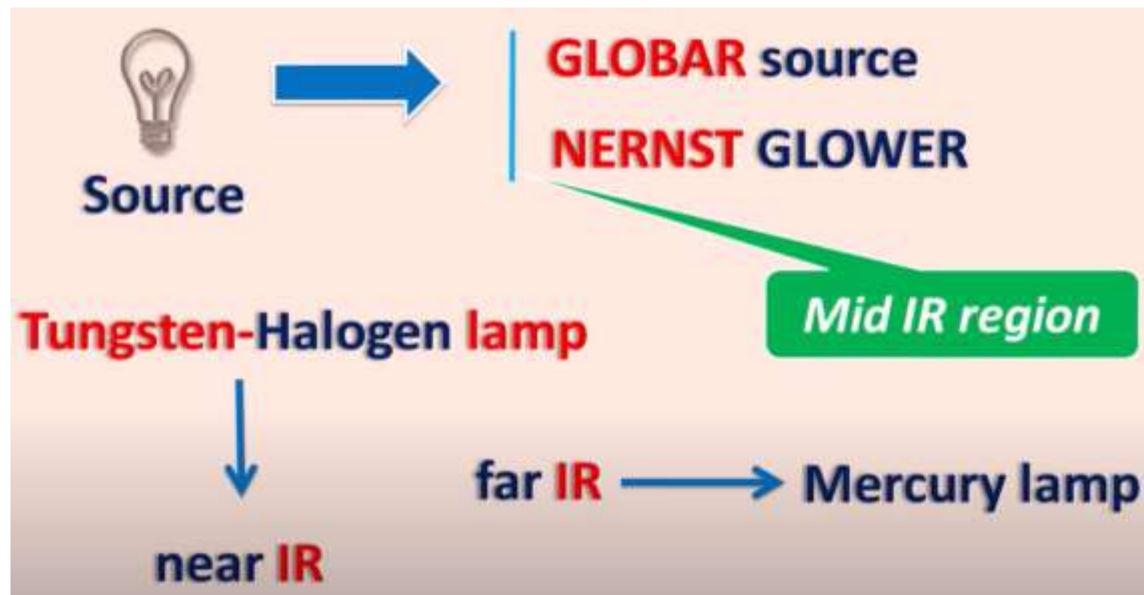
Aggregation

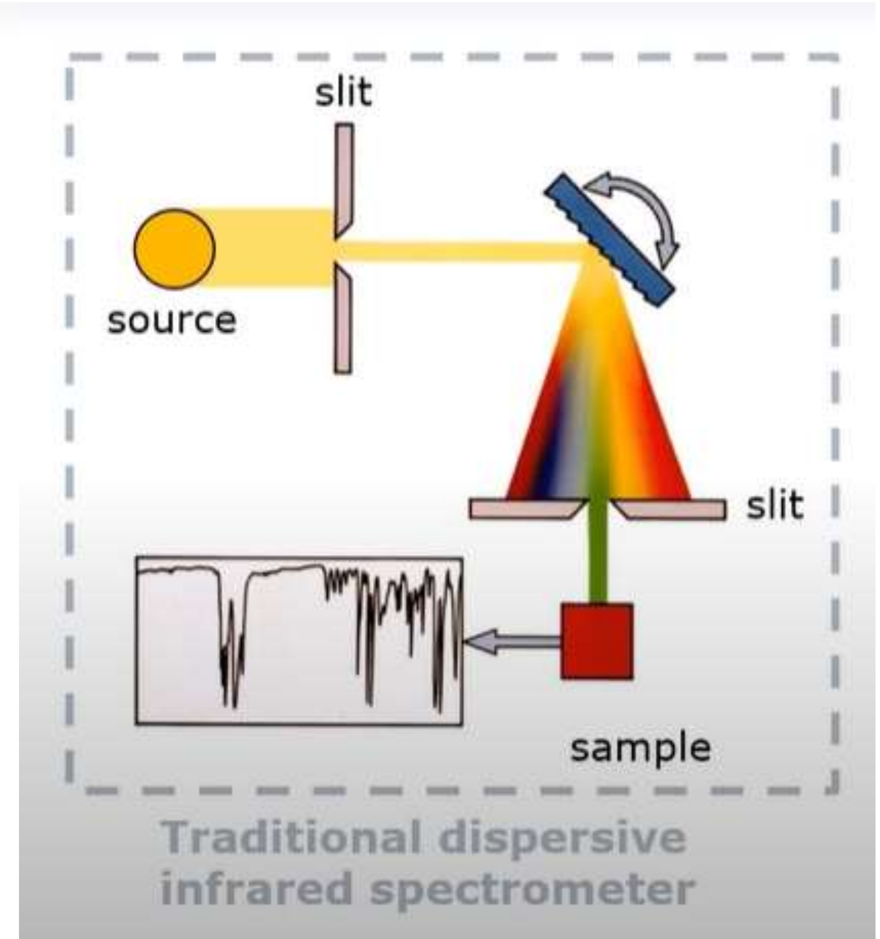
Fibrillation

Instrumentation

How is IR spectroscopy practically applied?

- Originally, light was first directed to a diffraction grating, splitting into several beams travelling in different directions.
- These beams were then mechanically directed at the sample and each wavelength examined individually.



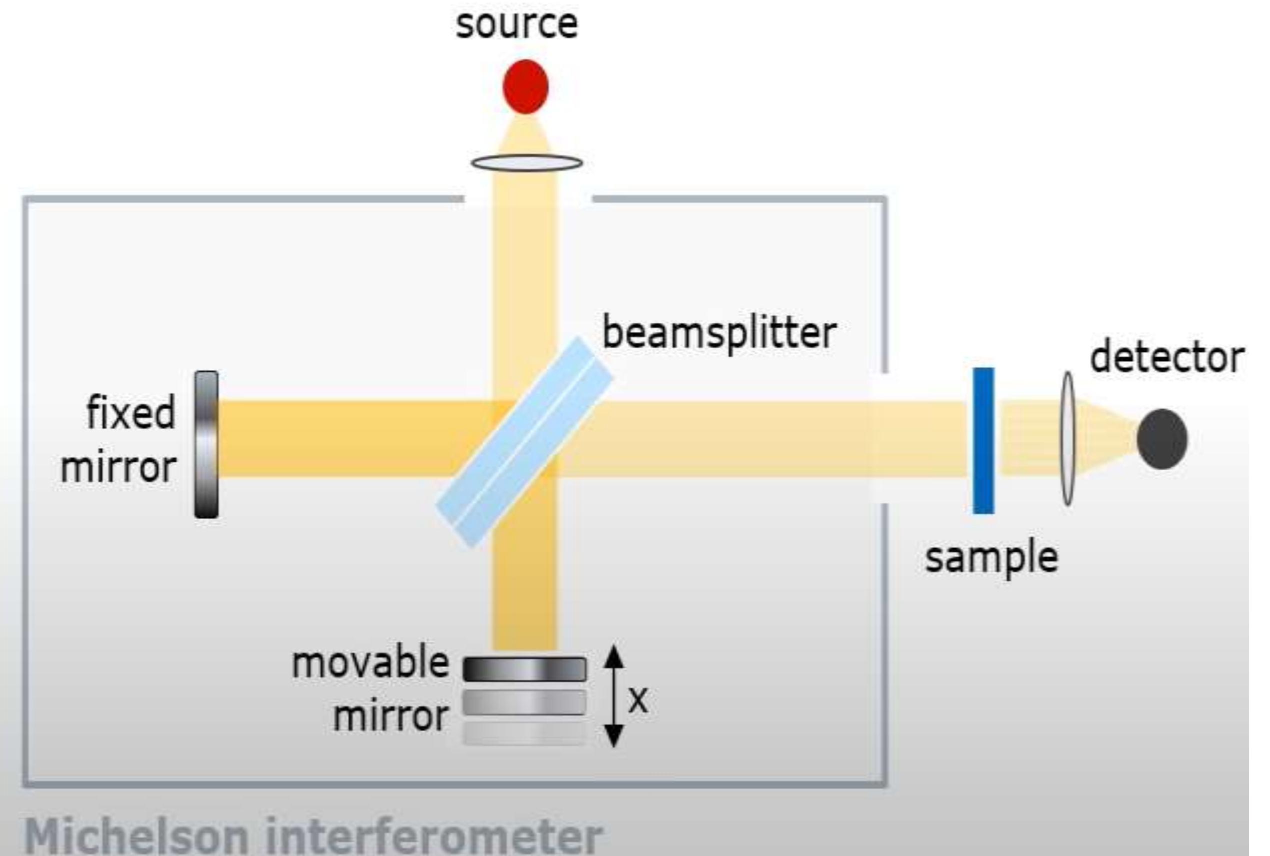


Traditional dispersive infrared spectrometer

Instrumentation

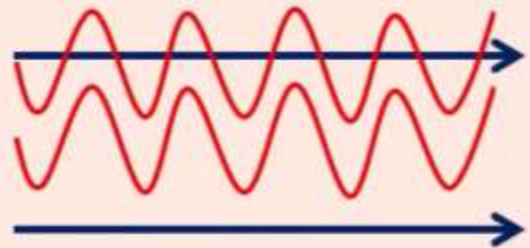
What is a Fourier-transform infrared (FTIR) spectrometer?

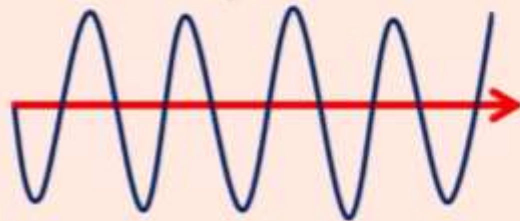
- The center of an FTIR spectrometer is the (e.g. Michelson) interferometer
- During the measurement the IR-beam enters the interferometer and is directed at a beamsplitter
- The beam is then split, and directed at a fixed and moving mirror respectively
- The beam is recombined (causing interference) and finally directed at the sample material
- The spectral information of all wavelengths is acquired simultaneously saving a lot of time.



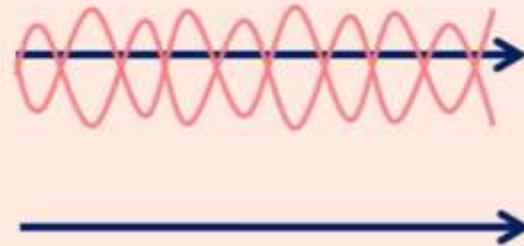
Optical path difference **Interference**

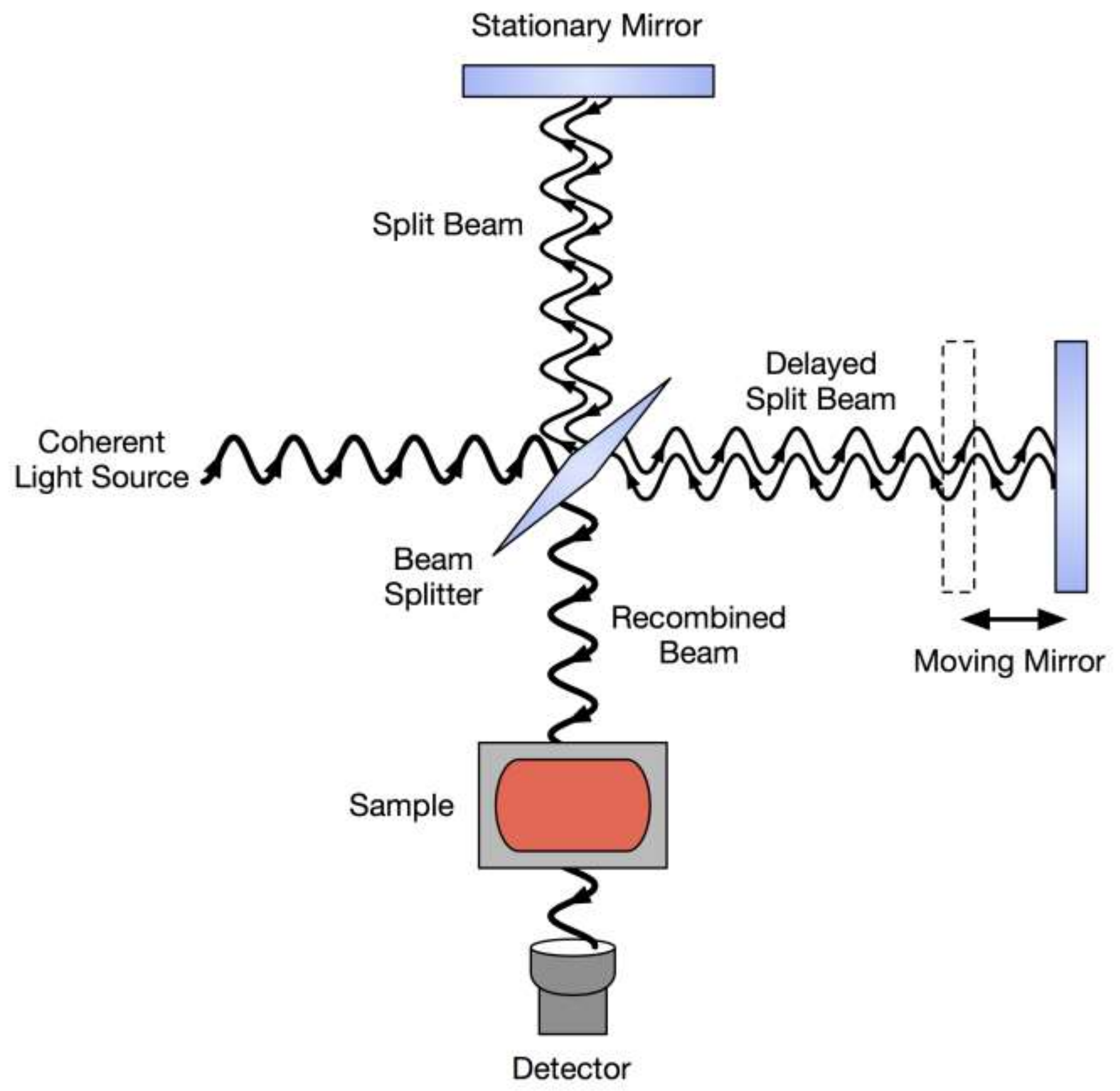
Constructive



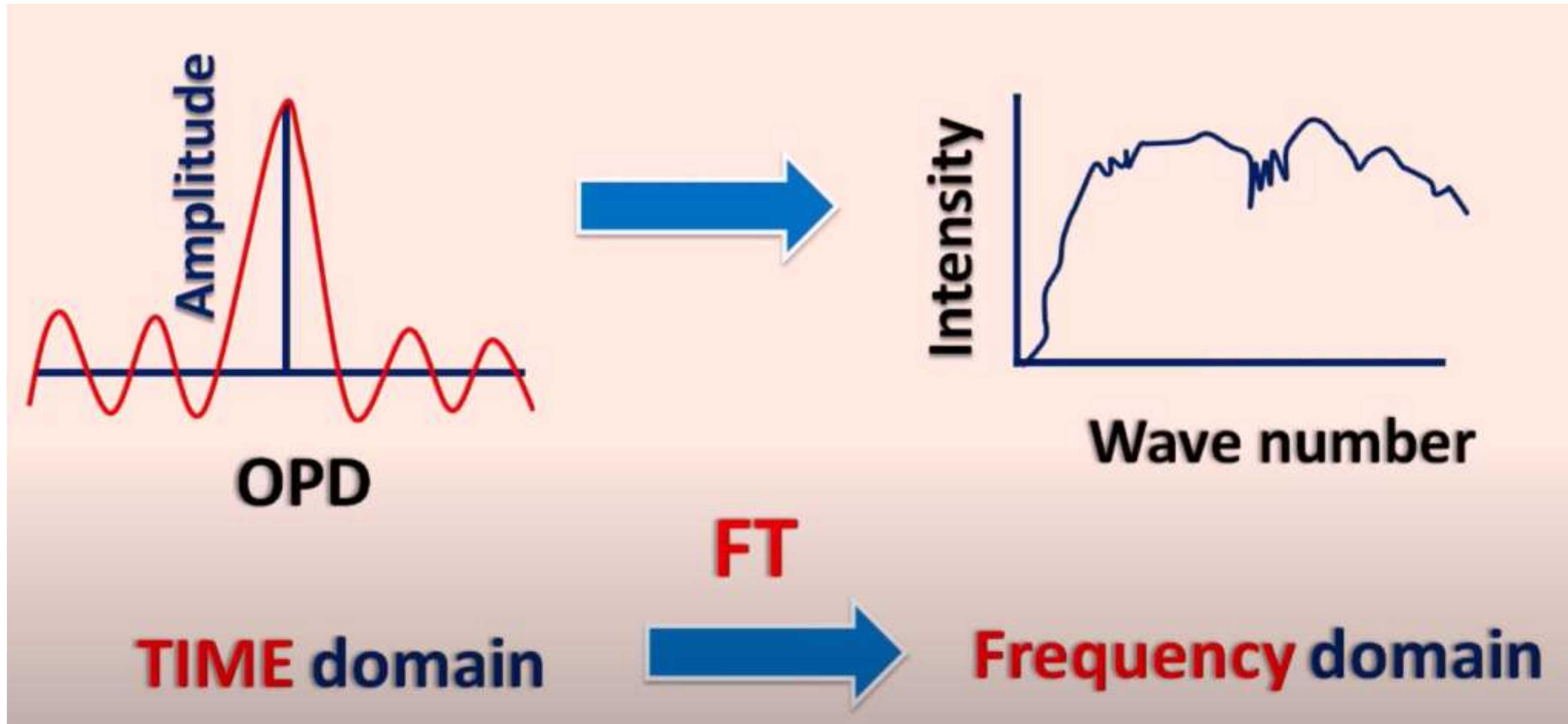


Destructive




Stationary Mirror
Split Beam
Delayed
Split Beam
Coherent
Light Source
Beam
Splitter
Recombined
Beam
Moving Mirror
Sample
Detector

Fourier Transform



Logic behind using Interferometer?