

Spectroscopic Techniques

Lecturer: Komalharini Tiwari
(M.Sc. Bioinformatics, PG Dipl. Data Science)

Academic Year: 2024- 2025

Outline:

1. X-ray Spectroscopy
2. Infrared Spectroscopy
3. Raman Spectroscopy
4. NMR Spectroscopy (Refer Notes shared)

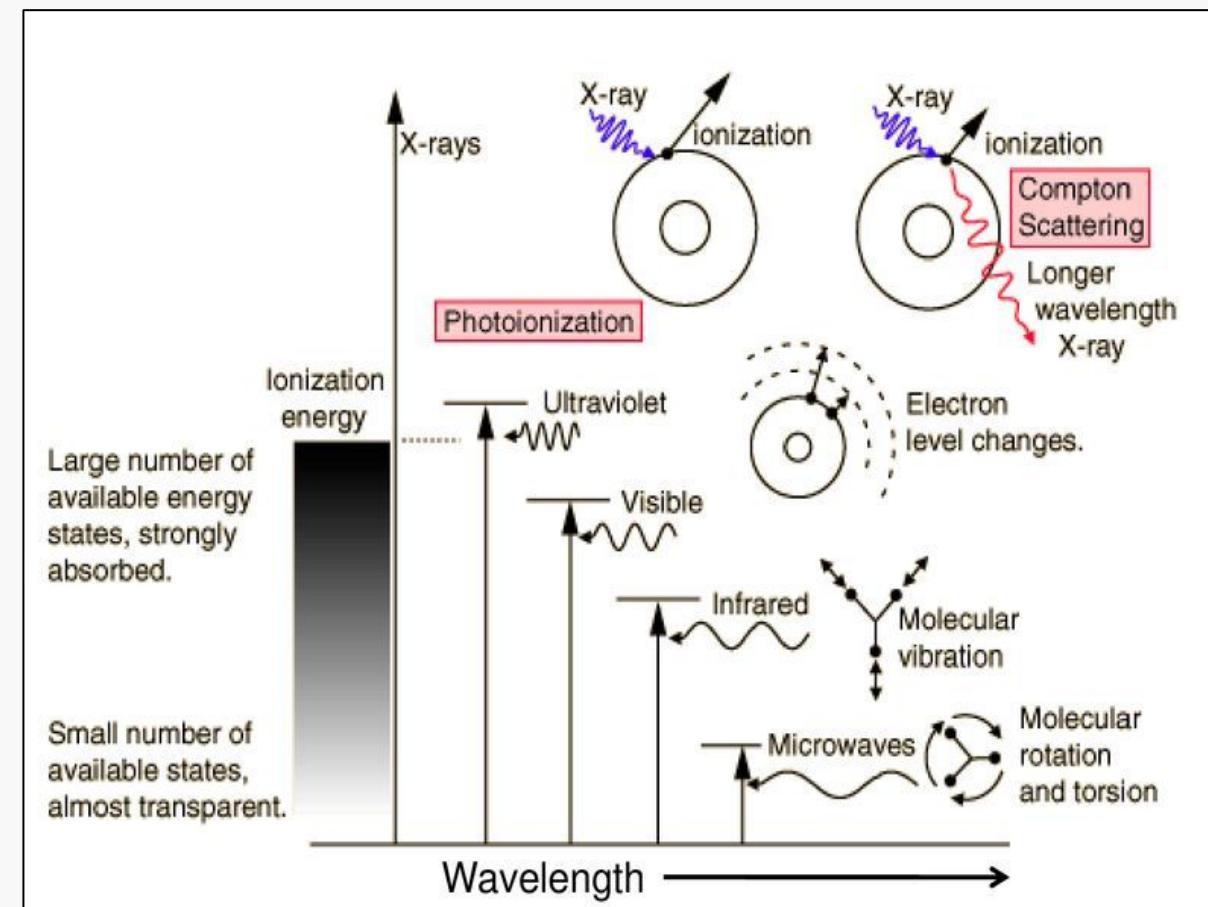


Figure 1: Electromagnetic Spectrum

Outline:

- **X-ray Spectroscopy** is invaluable in determining the detailed 3D structure of biomolecules, particularly proteins and nucleic acids, but requires crystallization and can be destructive to samples.
- **IR Spectroscopy** excels in identifying functional groups and analyzing secondary structures in proteins and lipids, with the major drawback being interference from water in biological samples.
- **Raman Spectroscopy** complements IR by providing detailed vibrational information with minimal water interference, making it ideal for studying live cells and aqueous samples, though its weak signal can be a limitation.

These techniques, when used in bioinformatics, offer powerful tools for understanding the structure, function, and interactions of biomolecules, thereby aiding in drug discovery, protein engineering, and other biological research areas.

Types of Spectra:

1. **Microwave spectra:** Pure rotational (Far IR or Microwave region)
2. **Infrared spectra:** Rotational and vibrational (Near IR region)
3. **Raman spectra:** Rotational and vibrational (Visible region) Based on scattering not absorption.
4. **Band spectra:** Rotational, vibrational and transitional (UV and Visible region)
5. **NMR spectra:** Transition between nuclear spin energy levels of the molecule when external field is applied. Energy is very high in these transitions. (Radio frequency regions)
6. **EMR or Electron Spin Resonance (ESR) spectra:** Transition between electron spin energy levels of the molecule when external magnetic field is applied. (Microwave regions with a specific frequency range, typically in the range of 10^4 to 10^6 MHz). In EMR spectroscopy, we measure the absorption of microwaves by paramagnetic centers with one or more unpaired electrons

Types of Spectra and movements:

- **Rotational** (Microwave spectra): *Far IR and Microwave region.* Low energy to change the rotational level on the same vibrational and transitional level, but not enough to cause translation spectra.
- **Vibrational & Rotational** (Infrared spectra): *Near IR region.* Same electronic level, and the same transitional level but a higher vibrational level
- **Transitional** (Band spectra): *Visible and UV region.* Exciting energy is large enough to cause a transition from one electronic level to another electronic level (E_0 to E_+).

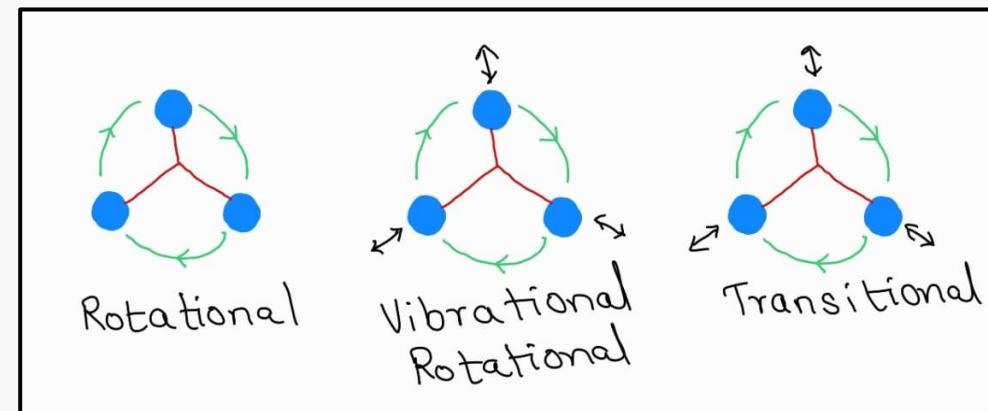


Figure 2: Molecular representation

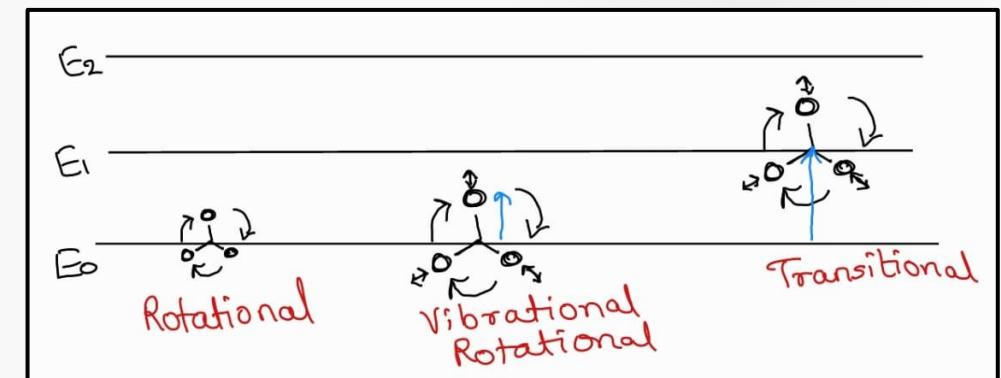


Figure 3: Energy level representation

Actual movement:

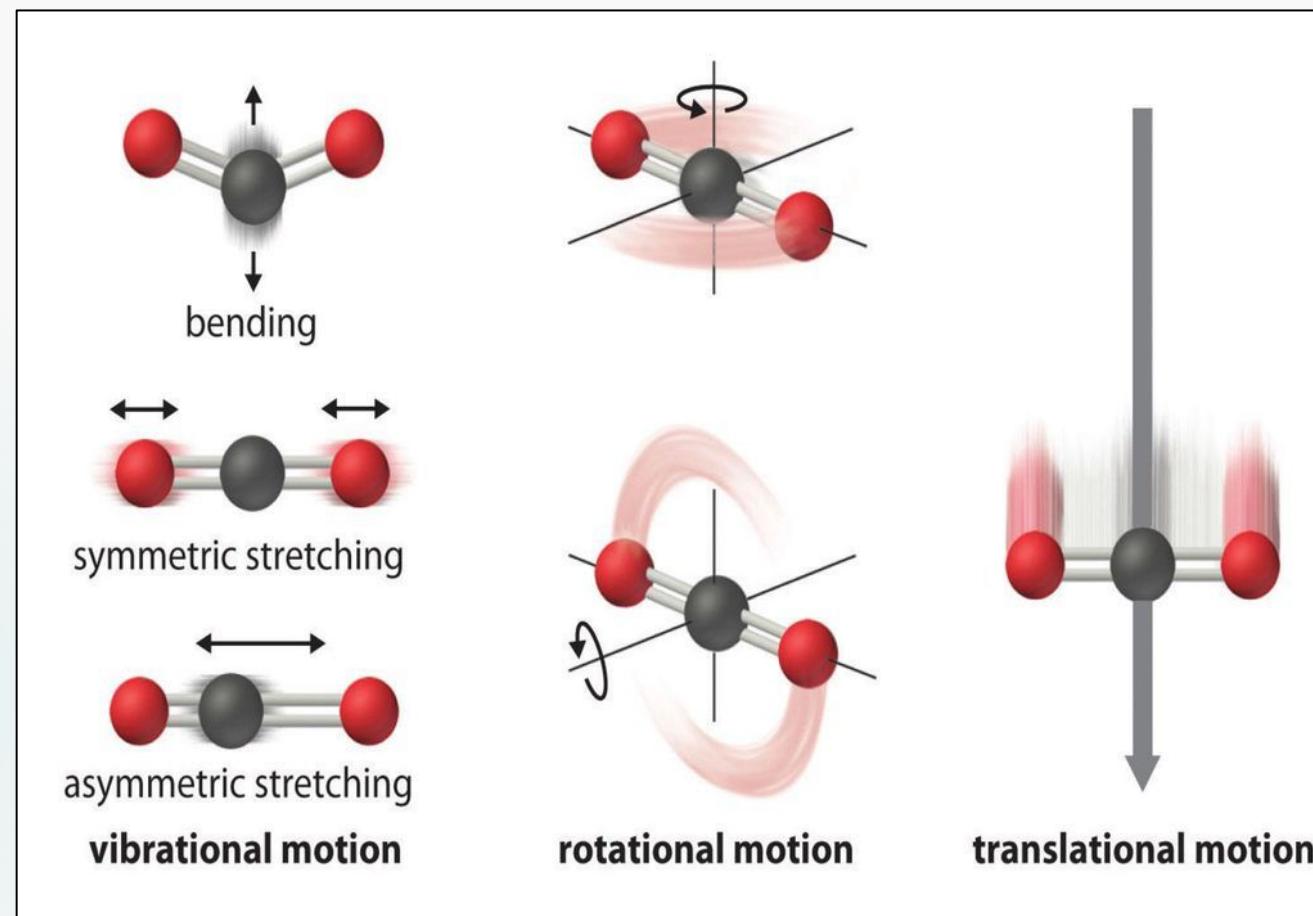


Figure 4: Electron movements

Questions

X-Ray Spectroscopy

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Introduction to X-Ray Spectroscopy:

What are X-Rays:

- ▶ X-rays make up X-radiation, which is a form of electromagnetic radiation.
- ▶ **Wavelength:** 0.01 to 10 nanometers.
- ▶ **Frequencies:** 30 petahertz to 30 exahertz (3×10^{16} Hz to 3×10^{19} Hz).
- ▶ **Energies:** 100 eV to 100 keV, produced by the deceleration of high-energy electrons.
- ▶ “*X-Ray Spectroscopy*” is a general term for several spectroscopic techniques for characterization of materials by using X-Ray excitation.

Note- Analytical purpose: 0.07 to 0.2 nm or 0.7 to 2 Å*

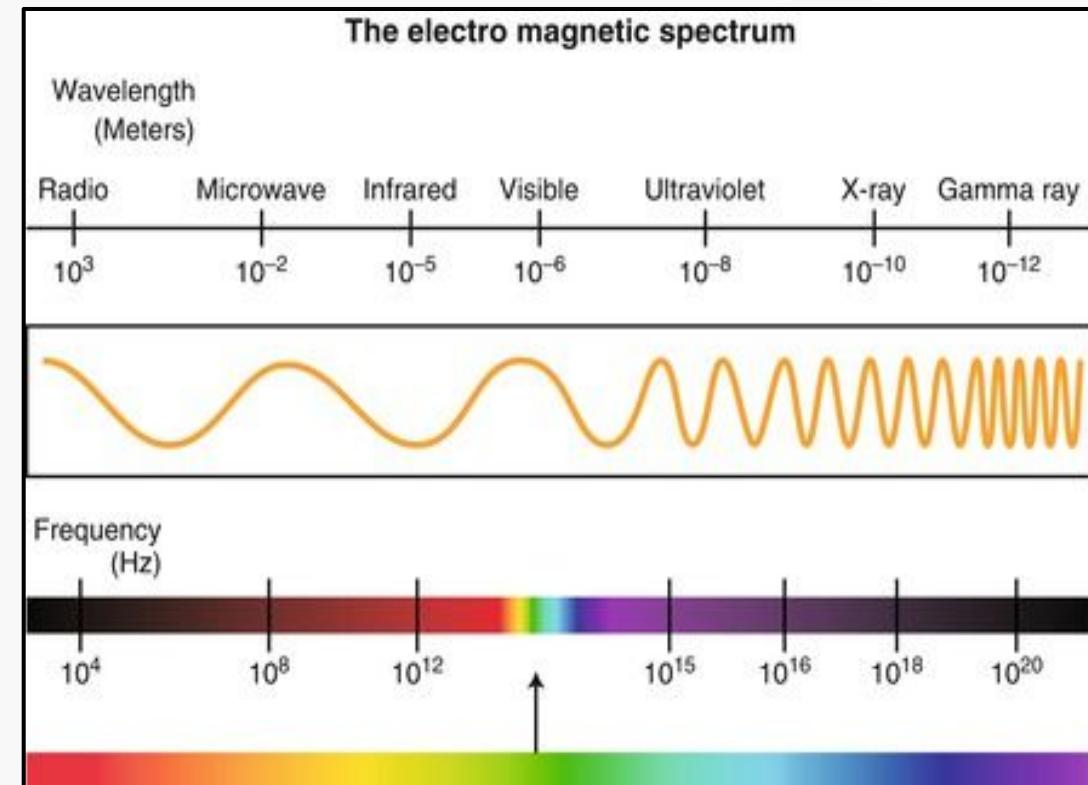


Figure 1: Electromagnetic Spectrum

Notes on types of X-Ray Spectroscopy:

Principle:

X-ray spectroscopy involves the study of the interaction between X-rays and matter. When X-rays are incident on a material, they can be absorbed or scattered. The absorption leads to the excitation of inner-shell electrons to higher energy levels or even ejects them from the atom. The resulting electronic transitions (when an electron drops back to fill the vacancy) produce characteristic X-rays.

Types:

1. X-ray Diffraction (XRD):

- Used to study the crystalline structure of materials by analyzing the diffraction patterns.

2. X-ray Absorption Spectroscopy (XAS):

- Focuses on the absorption of X-rays as a function of energy.

3. X-ray Fluorescence (XRF):

- Analyzes the secondary X-rays emitted by a material when it is excited by X-rays.

Types of X-Ray Spectroscopy:

➤ X-Ray Diffraction (XRD):

- ▶ The most popularly used.
- ▶ Use: Structural Determination of any crystalline substance.
- ▶ Based on scattering of X-Rays by atoms present in the sample.

➤ X-Ray Absorption:

- ▶ Number of photons absorbed is directly proportional to concentration of Sample.
- ▶ Intensity of absorption (I_{abs}) = $I_0 - I_t$

Where, $I_0 \rightarrow$ Incident Radiation, $I_t \rightarrow$ Transmitted Radiation

- ▶ Uses: Determining true oxidation states, structural changes during processes, chemical composition in common material.

Continued...

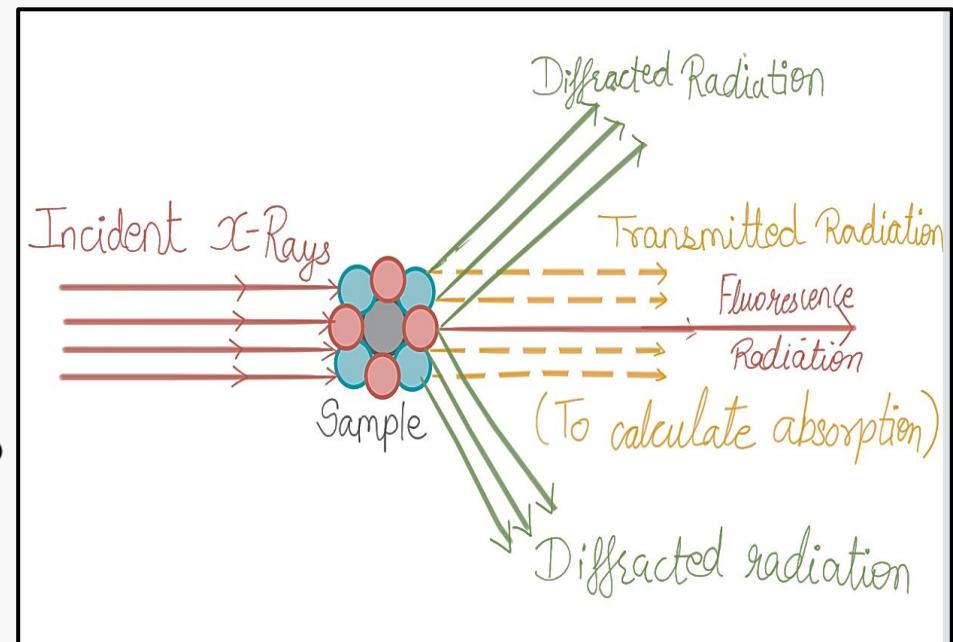


Figure 2A: Types of X-Rays generated

Types of X-Ray Spectroscopy:

...Continued

➤ X-Ray Fluorescence (XRF):

- ▶ Some of the incident radiation is absorbed by atoms in the sample (I_{abs}) which supplies the energy
- ▶ Electrons in the ground state jump to excited state causing instability.
- ▶ Release of some energy in the form of X-Rays with longer wavelength, thus resulting in the fluorescence.
- ▶ X-Rays are generated within the sample.
- ▶ Uses: in Qualitative as well as quantitative analysis, to accurately measure both, the major constituents of a material, and its trace elements.

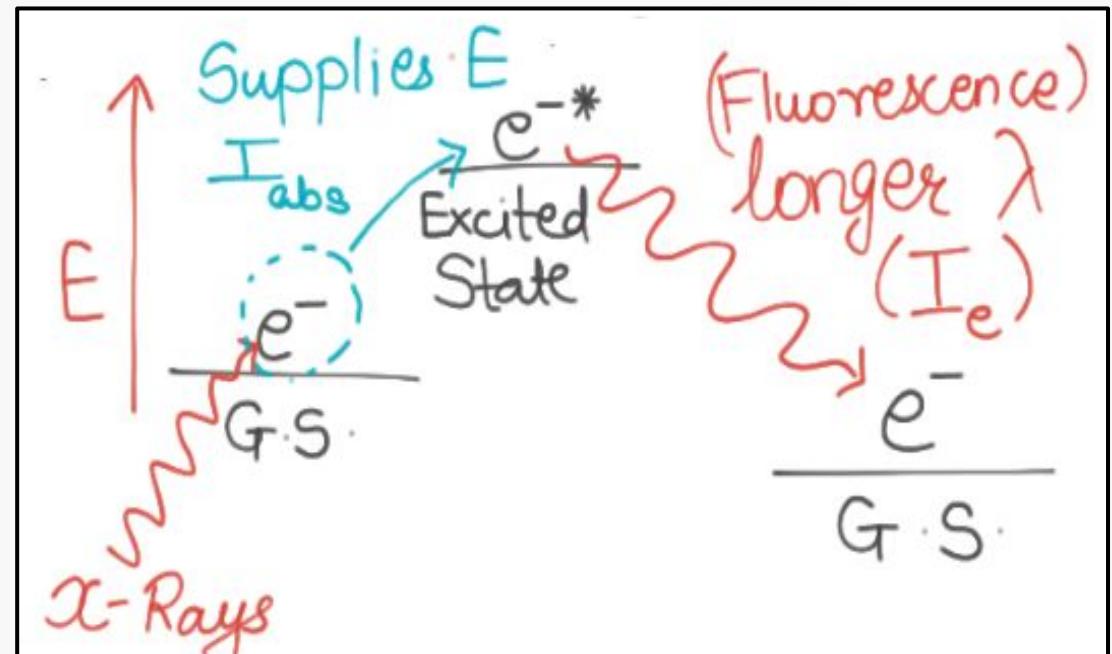


Figure 2B: X-Ray Fluorescence Spectroscopy within the sample

Instrumentation of X-Ray Diffraction Spectroscopy:

- **X-Ray Tube** (produce X-Rays):
 - Large vacuum tube with a heated **cathode** of tungsten filament and a target metal (Cu or Mo) **anode**.
- **Collimator** (narrows X-Ray beam):
 - 2 closely packed metal plates, separated by small **gap**.
- **Monochromator:** (Filter & Crystal-(Plate & Curved))
 - Absorbs undesirable radiations, filters the required radiations.
- **Detector:** (Photographic Method & Counter Methods)
 - A film is developed, when X-rays pass through the sample and hit the film.
 - Counter methods – GM counter, Proportional Counter, Scintillation Detector, SS-SC detector, SC detector.

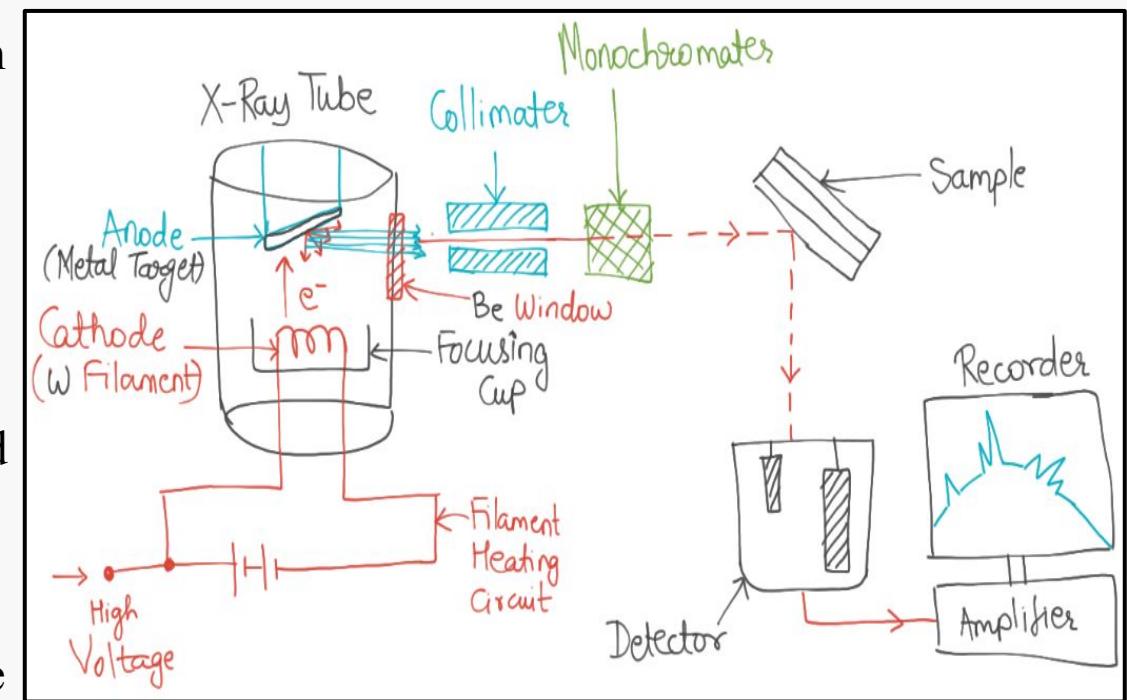


Figure 10A: Instrumentation of XRD

Instrumentation of X-Ray Diffraction Spectroscopy:

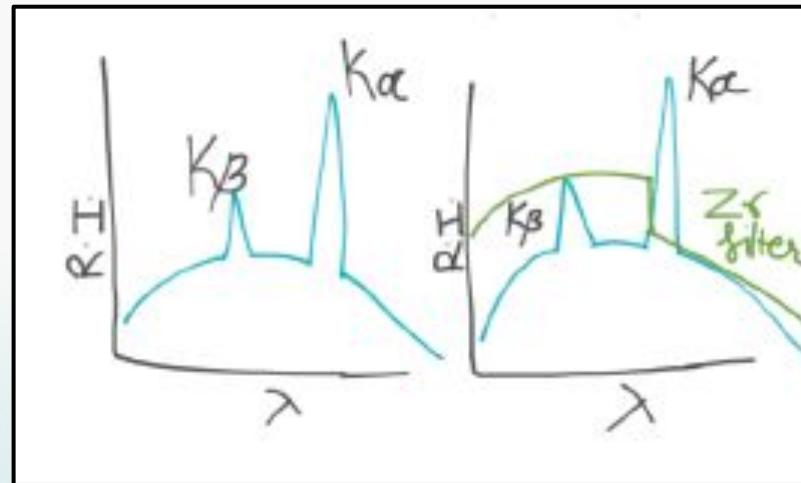


Figure 10C: Filter monochromator
for Mo radiation

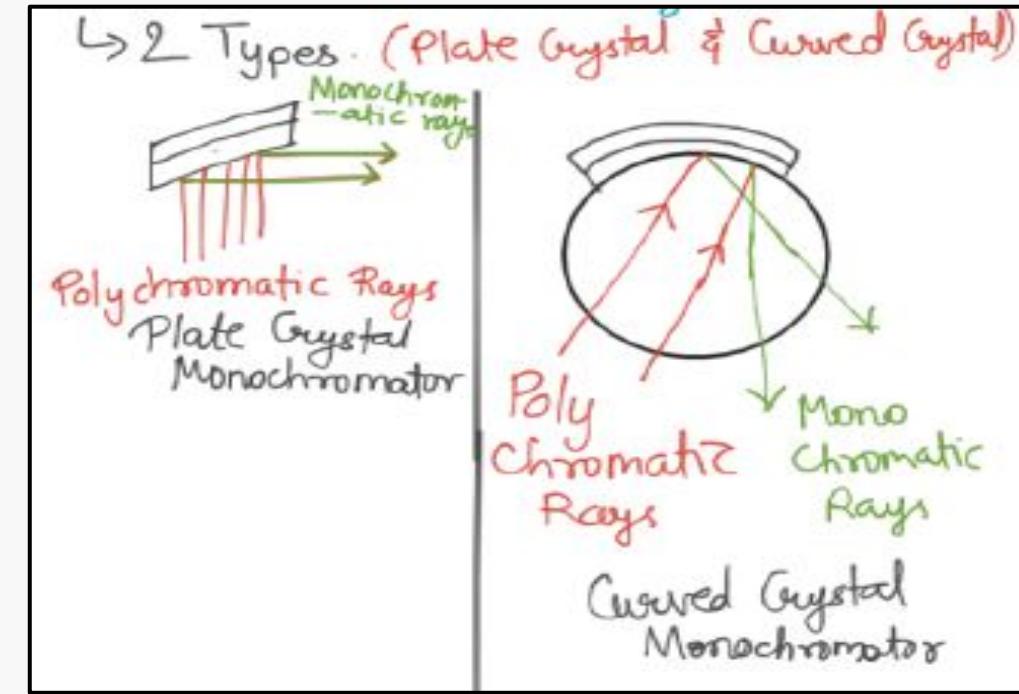


Figure 10D: Two types of Crystal monochromators-
Plate & Curved

Principle of XRD Method: (Bragg's Law)

Bragg's Law Derivation:

Suppose, **beams** of X-Rays fall on a crystal sample, at a **glancing angle Θ** , some of these rays will be **reflected** (**diffracted**) from the upper plate at the same **angle Θ** .

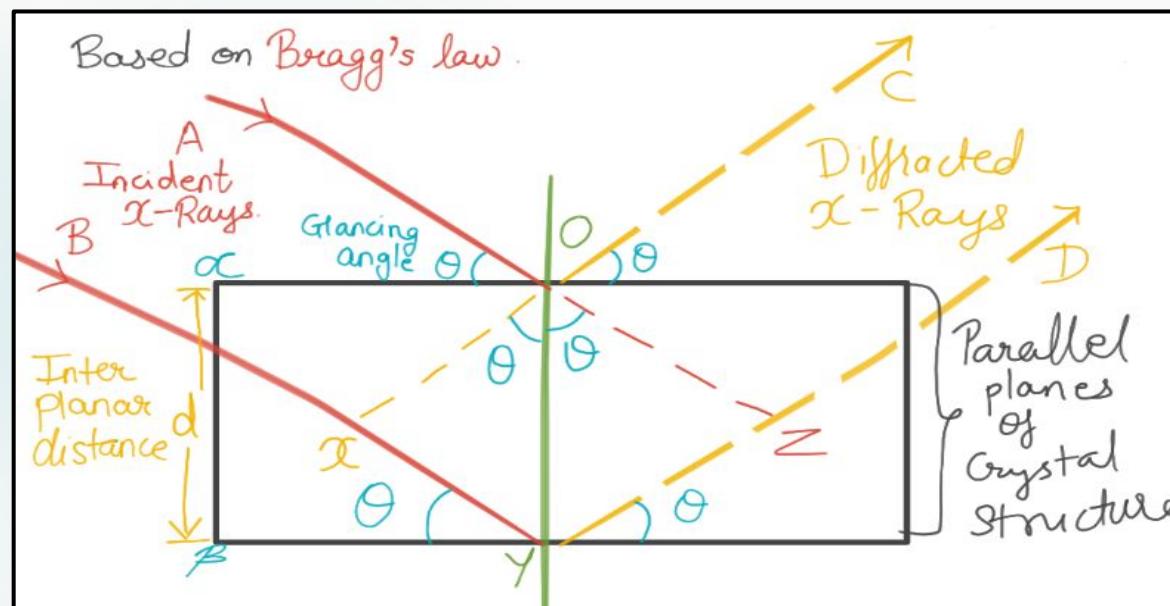


Figure 8: Bragg's law representation

Angles:
 $\angle COY = \theta$ & $\angle ZOY = \theta$

Distance:
 $CO = BX$ & $CO = DZ$

Path difference:
 ↳ an integral multiple of wavelength
 $= n\lambda$

↳ Path difference = $XY + YZ$

↳ $n\lambda = XY + YZ$

↳ $\sin \theta = \frac{\text{Length of } 1^{\text{er}} \text{ arm}}{\text{Hypotenuse}}$

1) $\triangle COY$, $\sin \theta = \frac{XY}{CY}$ $\therefore XY = CY \cdot \sin \theta$
 $\therefore XY = d \times \sin \theta$

2) $\triangle ZOY$, $\sin \theta = \frac{ZY}{CY}$ $\therefore ZY = CY \cdot \sin \theta$
 $\therefore ZY = d \times \sin \theta$

↳ $\therefore n\lambda = 2d \times \sin \theta$ (Bragg's equation)

$n \rightarrow$ Order of diffraction
 $d \rightarrow$ Interplanar distance

Figure 9: Bragg's law derivation

Principle of XRD Method: (Bragg's Law)

- Bragg's Law is fundamental in X-ray diffraction (XRD) and describes the condition for constructive interference of X-rays reflected from crystal planes.
- Bragg's Law explains how X-rays interact with the periodic structure of a crystal.
- For constructive interference (which produces a detectable signal), the path difference between rays reflected from successive planes must be an integral multiple of the wavelength.
- This relationship is crucial for determining the crystal structure.
- $n\lambda = 2d \sin \theta$
- n is the order of reflection (an integer).
- λ is the wavelength of the incident X-rays.
- d is the interplanar spacing in the crystal.
- θ is the angle between the incident X-ray and the crystal plane (and the angle of reflection)

$$n\lambda = 2d \sin \theta$$

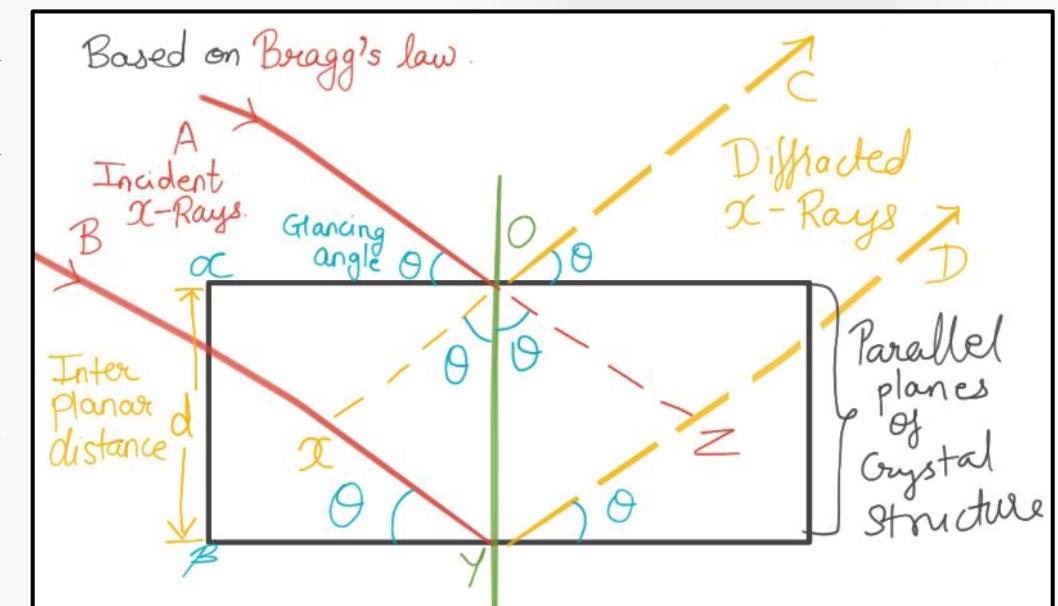


Figure 8: Bragg's law representation

Instrumentation of X-Ray Diffraction Spectroscopy:

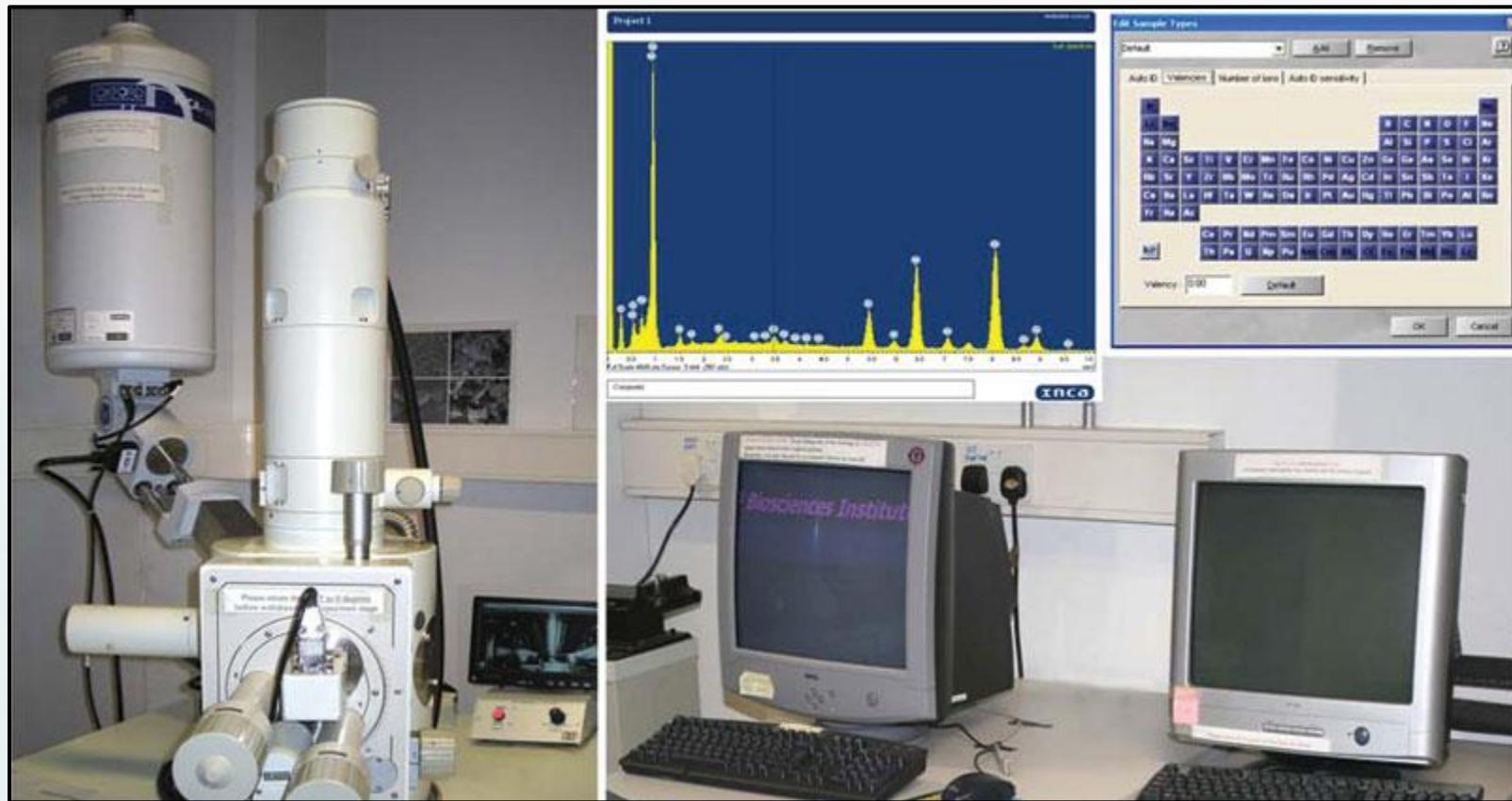


Figure 10B: Instrumentation of X-Ray Spectroscopy.

Working of X-Ray Spectroscopy

High voltage (up to 60kV)

Tungsten filament is heated

Emits an electron (Striking electron)

Hits the metal target (Anode)

X-Rays produced in random directions

Directed by and pass through Beryllium Window

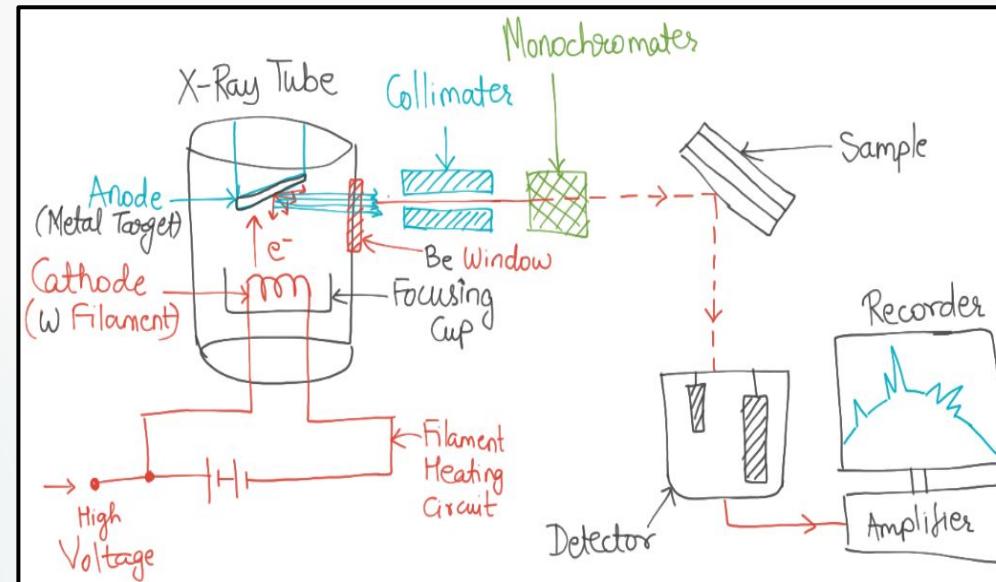


Figure 10E: Instrumentation of XRD for Working.

Collimator – Narrows the beam

Monochromator – Monochromatic radiation

Hit the sample and diffract

Detector detects the signal

Amplifier amplifies the signal

Recorder reads the signal

Advantages & Disadvantages:

ADVANTAGES

- ▶ X-ray spectroscopy is an excellent method to determine the structure of a compound.
- ▶ In the event when other spectral methods fail to reveal a compound's identity, X-ray spectroscopy is the method of choice.
- ▶ Structural determination where the other parameters such as bond lengths and bond angles are also determined.
- ▶ Suitable for a broad range of crystalline materials, including large biomolecules like proteins and nucleic acids.

DISADVANTAGES

- ▶ Crystallization Requirement: The need to crystallize the sample can be a significant bottleneck, especially for large or flexible biomolecules.
- ▶ Radiation Damage: X-rays can damage biological samples, potentially altering the structure being studied.
- ▶ Most chemists find this process very tedious, time consuming and it requires a skillful hand.

Applications:

X-Ray spectrometry is used in a wide range of applications, including-

- Research in igneous, sedimentary, and metamorphic petrology
- Soil surveys
- Mining (e.g., measuring the grade of ore)
- Cement production
- Ceramic and glass manufacturing
- Metallurgy (e.g., quality control)
- Environmental studies (e.g., analyses of particulate matter on air filters)
- Petroleum industry (e.g., sulfur content of crude oils and petroleum products)
- Field analysis in geological and environmental studies (using portable, handheld XRF spectrometers)

Applications:

□ Protein Crystallography:

- XRD is widely used to determine the 3D structure of proteins at atomic resolution.
- By analyzing the diffraction pattern of X-rays passing through protein crystals, researchers can model the electron density and infer the protein's structure, which is crucial for understanding function, interactions, and drug design.

□ Nucleic Acid Structure:

- It has been instrumental in elucidating the structures of DNA and RNA, helping to understand the molecular basis of genetic information storage and expression.

Numeric Problems (Bragg's Law):

Q1) The used reflecting plane of LiF analyzing crystal has an interplanar distance of 2.5 \AA . Calculate λ of 2nd order diffracted line which has a glancing angle of 60°

$$\begin{aligned} n\lambda &= 2d \sin \theta \\ 2\lambda &= 2 \times 2.5 \times \sin 60^\circ \\ &= \frac{2 \times 2.5 \times \sqrt{3}/2}{2} \\ &= \frac{2.5 \times 1.732}{2} = \frac{4.33}{2} \text{ \AA} \\ \lambda &= 2.165 \text{ \AA} \end{aligned}$$

Bragg's law

↳ gives relationship between:

- λ (wavelength of X-Rays)
- d (Interplanar distance in crystals)
- θ (Angle of Reflection).

$\sin \theta$	0°	30°	45°	60°	90°
Values	0	$\frac{1}{2}$	$\frac{1}{\sqrt{2}}$	$\frac{\sqrt{3}}{2}$	1

Numeric Problems (Bragg's Law):

Q2) Calculate the angle at which

a) 1st order reflection

b) 2nd order reflection

will occur, when x-ray of $\lambda = 1.54 \text{ \AA}$ ^{*}
are diffracted by the atoms of a
crystal, given $d = 4.04 \text{ \AA}$

a) First order reflection ($n=1$)

$$n\lambda = 2d \sin \theta$$

$$1 \times 1.54 \text{ \AA} = 2 \times 4.04 \text{ \AA} \times \sin \theta$$

$$\sin \theta = 1.54 / 8.08$$

$$= 0.191 \checkmark$$

$$\theta = \sin^{-1}(0.191)$$

$$\boxed{\theta = 11^\circ}$$

b) Second Order reflection ($n=2$)

$$n\lambda = 2d \sin \theta$$

$$2 \times 1.54 \text{ \AA} = 2 \times 4.04 \text{ \AA} \times \sin \theta$$

$$\sin \theta = \frac{2 \times 1.54}{2 \times 4.04}$$

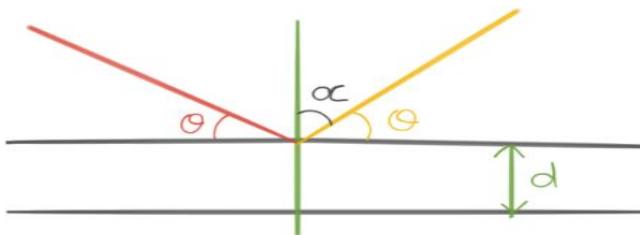
$$= 0.381 \checkmark$$

$$\theta = \sin^{-1}(0.381)$$

$$\boxed{\theta = 22.4^\circ}$$

Numeric Problems (Bragg's Law):

Q3) X-ray of $\lambda = 1.5 \text{ \AA}$ are diffracted by a set of atomic plane in a crystal in the following manner:



Find the angle (α) for first order diffraction.

Given: $n=1$ (First order)
 $\lambda = 1.5 \text{ \AA}$
 $d = 2.9 \text{ \AA}$

$$1 \times 1.5 = 2 \times 2.9 \times \sin \theta$$

$$\sin \theta = 1.5 / 5.8 \\ = 0.259 \quad \checkmark$$

$$\theta = \sin^{-1}(0.259) \\ = 15^\circ \quad \checkmark$$

$$\alpha = (90^\circ - 15^\circ) \\ \alpha = 75^\circ$$

Q4) For first order diffraction by a crystal plane having $d = 2.3 \text{ \AA}$ in a solid observed at the angle of 30° . Using the same radiation & first order diffraction, $\theta = 60^\circ$ for another solid; calculate the d value for 2nd solid.

I (First Solid)

Given: $n=1$
 $d = 2.3 \text{ \AA}$
 $\theta = 30^\circ$

$$n\lambda = 2d \sin \theta \\ 1 \times \lambda = 2 \times 2.3 \text{ \AA} \times \sin 30^\circ \\ \lambda = 2 \times 2.3 \text{ \AA} \times \frac{1}{2}$$

$$\lambda = 2.3 \text{ \AA}$$

II (Second Solid)

Given: $n=1$
 $\lambda = 2.3 \text{ \AA}$
 $\theta = 60^\circ$

$$n\lambda = 2d \sin \theta \\ 1 \times 2.3 \text{ \AA} = 2d \sin 60^\circ \\ 2.3 \text{ \AA} = 2d \times \frac{\sqrt{3}}{2}$$

$$d = \frac{2.3 \text{ \AA}}{\sqrt{3}} \\ = \frac{2.3 \text{ \AA}}{1.732}$$

$$d = 1.33 \text{ \AA}$$

IR Spectroscopy

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Principle of IR Spectroscopy:

- Infrared spectroscopy is based on the absorption of IR radiation by molecules, causing vibrational transitions.
- Molecules absorb IR radiation at specific frequencies that correspond to the vibrational modes of their chemical bonds.
- Different bonds in a molecule absorb IR radiation at characteristic frequencies, allowing for the identification of functional groups and molecular structure.
- The Beer-Lambert Law is used in IR spectroscopy to quantify the concentration of analytes.
- It is based on the fact that the amount of light absorbed by a sample is directly proportional to the concentration of absorbing species in the path of the light.

- $A = \epsilon cl$
- A is the absorbance.
- ϵ is the molar absorptivity (a constant specific to the substance).
- c is the concentration of the solution.
- l is the path length through which the light passes.

Applications in Bioinformatics:

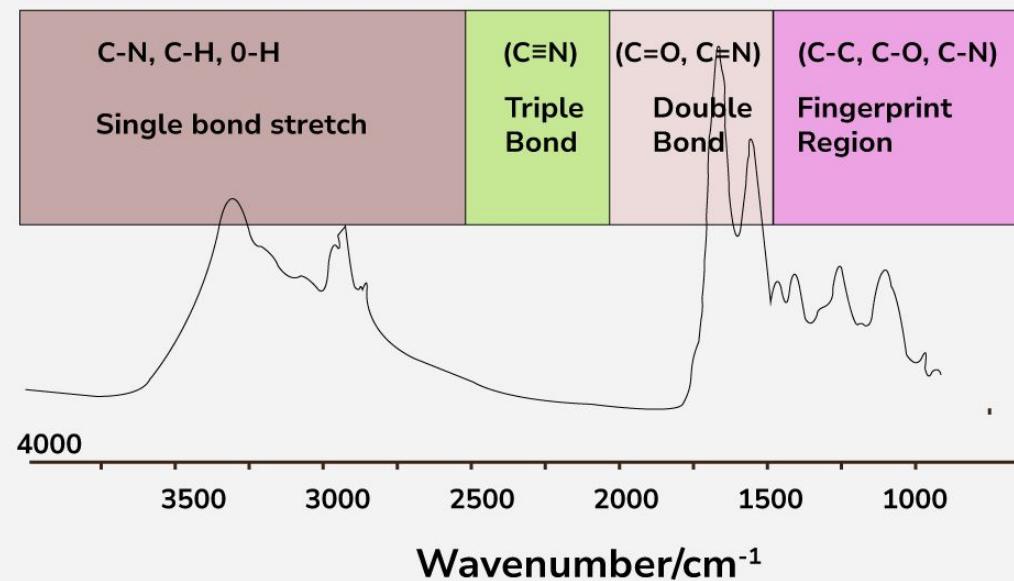
- Infrared Spectroscopy is widely used in organic and inorganic chemistry, environmental science, pharmaceuticals, forensics, and food quality analysis for identifying substances and understanding their compositions.
- Protein Secondary Structure Analysis:
 - IR spectroscopy, especially using Fourier Transform IR (FTIR), can be used to determine the secondary structure of proteins (e.g., α -helices, β -sheets) by analyzing the amide bands.
- Lipid Analysis:
 - IR spectroscopy is useful in studying lipid compositions and conformational changes in biological membranes.
- Biomolecular Interaction Studies:
 - It can monitor changes in the molecular structure of proteins, nucleic acids, and other biomolecules upon interaction with ligands or drugs.
- Purity control:
 - In quality control, it can quickly identify impurities and measure concentrations of components in a mixture, ensuring products meet specified standards without destructive testing.

Types of IR Spectroscopy:

- **Fourier Transform Infrared Spectroscopy (FTIR):** Uses an interferometer to collect high-resolution spectral data.
- **Dispersive IR Spectroscopy:** Uses a monochromator to disperse IR radiation into its component wavelengths.

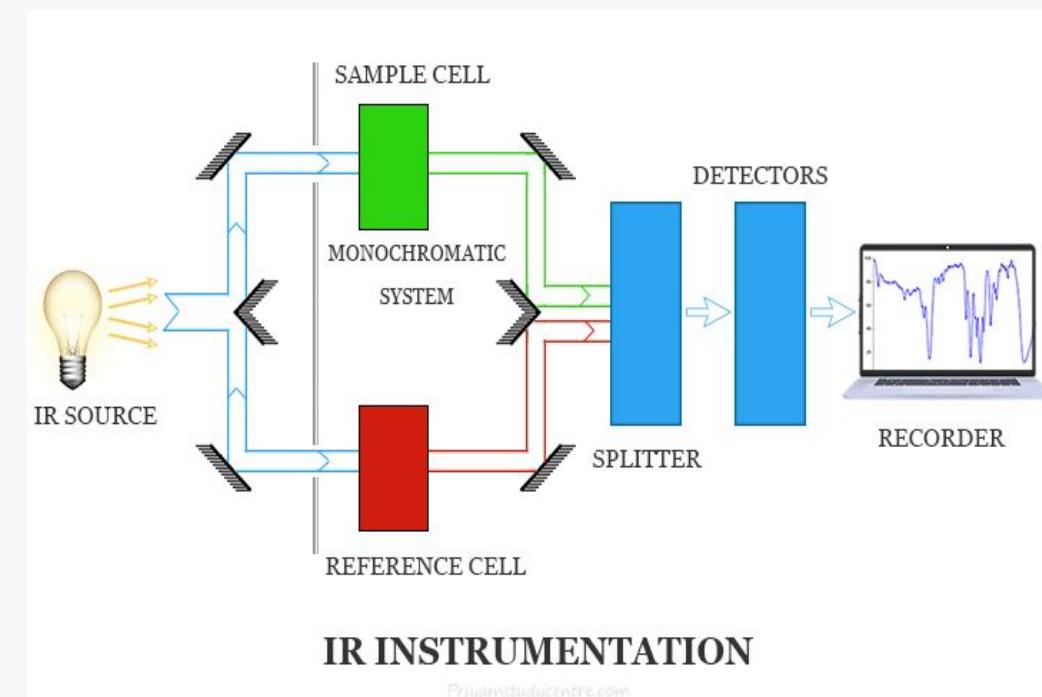


IR Spectrum Graph



Instrumentation of IR Spectroscopy:

- **IR Source:** A thermal emitter, like a Globar, that produces a broad spectrum of IR radiation.
- **Sample Holder:** Can be a cell for liquids or a solid sample holder.
- **Interferometer (FTIR):** Combines light from different paths to produce an interference pattern.
- **Detector:** Converts the IR signal into a measurable electronic signal.



Instrumentation of IR Spectroscopy:

Component	Description
Source of Infrared Radiation	Emits infrared radiation over a wide range of wavelengths using nichrome wire, Nernst glower, or heated filament.
Sample Compartment	Holds the sample being analyzed, accommodating solids, liquids, and gases. Equipped with windows made of transparent materials.
Monochromator or Interferometer	Selects specific wavelengths of infrared light for analysis. Monochromators disperse radiation using a prism or grating. Interferometers modulate light to produce interferograms.
Detector	Captures the intensity of infrared radiation transmitted through or reflected from the sample. Converts infrared signal into an electrical signal for analysis.
Data Processing and Analysis	Software for processing and analyzing data obtained from the detector. Performs tasks like baseline correction, peak identification, and spectral interpretation.

Advantages and Limitations:

□ Advantages:

- Non-Destructive: IR spectroscopy is typically non-invasive, making it suitable for studying biological samples without destroying them.
- Fast and Efficient: IR spectra can be obtained quickly, allowing for rapid analysis.

□ Limitations

- Water Interference: Water strongly absorbs in the IR region, which can interfere with the analysis of biological samples.
- Complex Spectra: The overlapping of vibrational bands can complicate the interpretation of spectra, especially for complex mixtures.

Raman Spectroscopy

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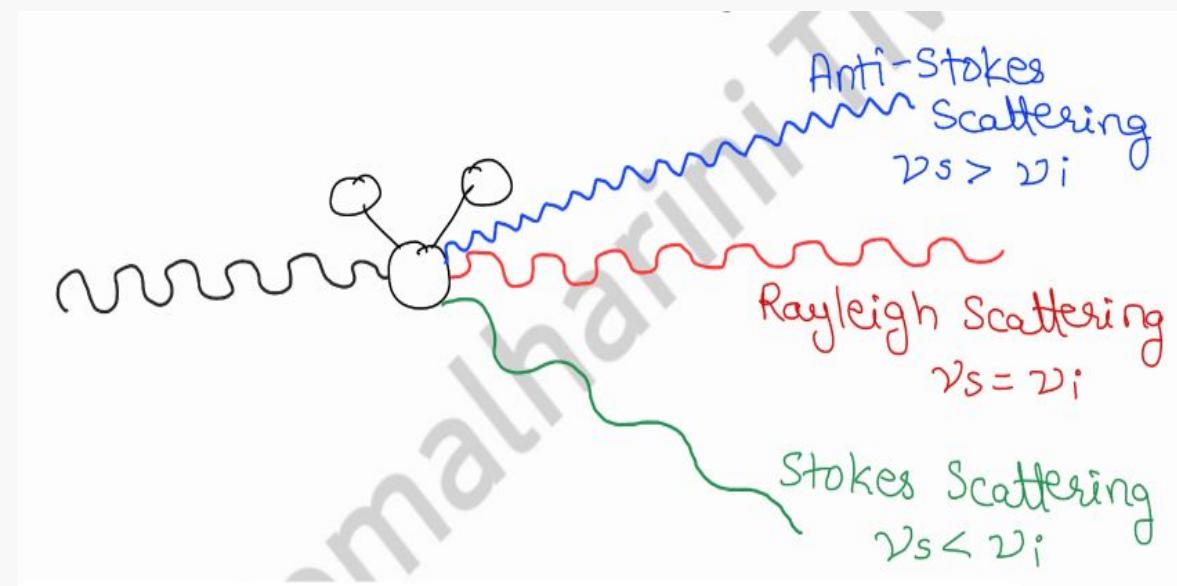
Academic Year: 2024 - 2025

Principle & Instrumentation:

- Raman spectroscopy is based on the inelastic scattering of light (Raman scattering). When light interacts with molecular vibrations, some photons are scattered at different energies, corresponding to the vibrational modes of the molecules.
- **Instrumentation**
 - ▶ Laser Source: Provides monochromatic light, typically in the visible or near-infrared range.
 - ▶ Sample Holder: Holds the sample under investigation.
 - ▶ Monochromator: Filters the scattered light to isolate the Raman-shifted light.
 - ▶ Detector: Captures and measures the intensity of Raman-scattered light.

Raman Effect:

- When light strikes sample, some light is absorbed, some is transmitted and a minute part is scattered at the right angle to the incident beam.
- Raman spectroscopy employs and measures this scattered radiation.
- As incident radiation has frequency (ν_i), scattered radiation also has frequency (ν_s).
- For $\sim 1\%$ of total scattered intensity occurs at frequencies different than scattered frequency. This is Raman scattering. It is inelastic in nature i.e. ν_s is not equal to ν_i
- Raman spectroscopy exploits stokes and anti-stokes scattering.



Raman Effect:

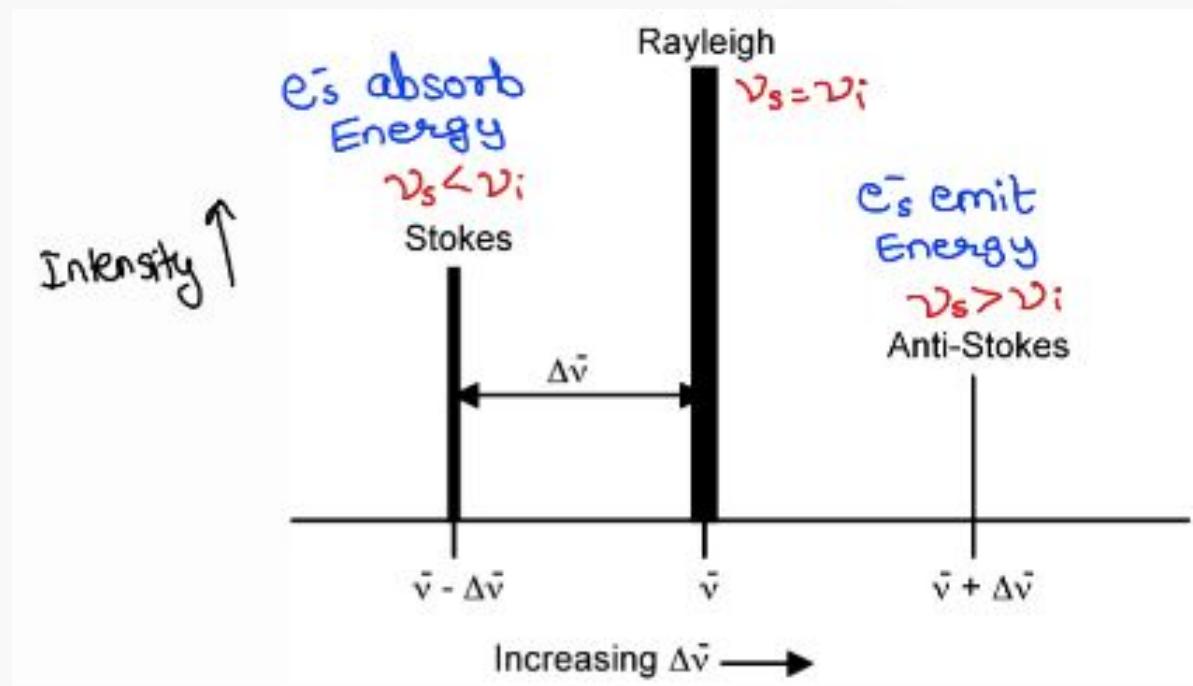
- Raman spectra gives molecular fingerprint.
- Different molecules have different Raman spectra.

□ Raman Shift Calculation:

- The Raman shift is calculated as the difference between the wavenumber of the incident light ($\tilde{\nu}_i$) or ($\tilde{\nu}_o$) and the wavenumber of the scattered light ($\tilde{\nu}_s$).
- $\Delta\tilde{\nu} = \tilde{\nu}_i - \tilde{\nu}_s$

□ Stokes vs. Anti-Stokes Scattering:

- Stokes lines appear at lower energy (longer wavelength) than the incident light, while anti-Stokes lines appear at higher energy (shorter wavelength).



Raman Shift Calculation:

The Raman shift ($\Delta\tilde{\nu}$) is calculated as the difference between the wavenumber of the incident light ($\tilde{\nu}_i$) or ($\tilde{\nu}_\theta$) and the wavenumber of the scattered light ($\tilde{\nu}_s$).

□ **Raman Shift ($\Delta\tilde{\nu}$) = $\tilde{\nu}_\theta - \tilde{\nu}_s$**

Wavenumber Calculation: The wavenumber ($\tilde{\nu}$) in cm^{-1} is:

$$\tilde{\nu} = \frac{1}{\lambda} \times 10^7$$

Raman Shift: Calculated as:

$$\Delta\tilde{\nu} = \left(\frac{1}{\lambda_0} - \frac{1}{\lambda_s} \right) \times 10^7 \text{ cm}^{-1}$$

Where λ_0 is the wavelength of incident light, and λ_s is the wavelength of scattered light.

Advantages and Limitations:

□ Advantages

- Non-Destructive: Like IR, Raman is non-destructive, and samples can be analyzed in their natural state, including in aqueous solutions.
- Minimal Water Interference: Unlike IR, water has a weak Raman signal, making Raman spectroscopy suitable for studying aqueous biological samples.

□ Limitations

- Weak Signal: Raman scattering is inherently weak, requiring sensitive detectors and sometimes enhancement techniques.
- Fluorescence Interference: Some samples can fluoresce under the laser light used in Raman spectroscopy, which can overwhelm the Raman signal.

Applications in Bioinformatics:

□ Structural Analysis of Biomolecules:

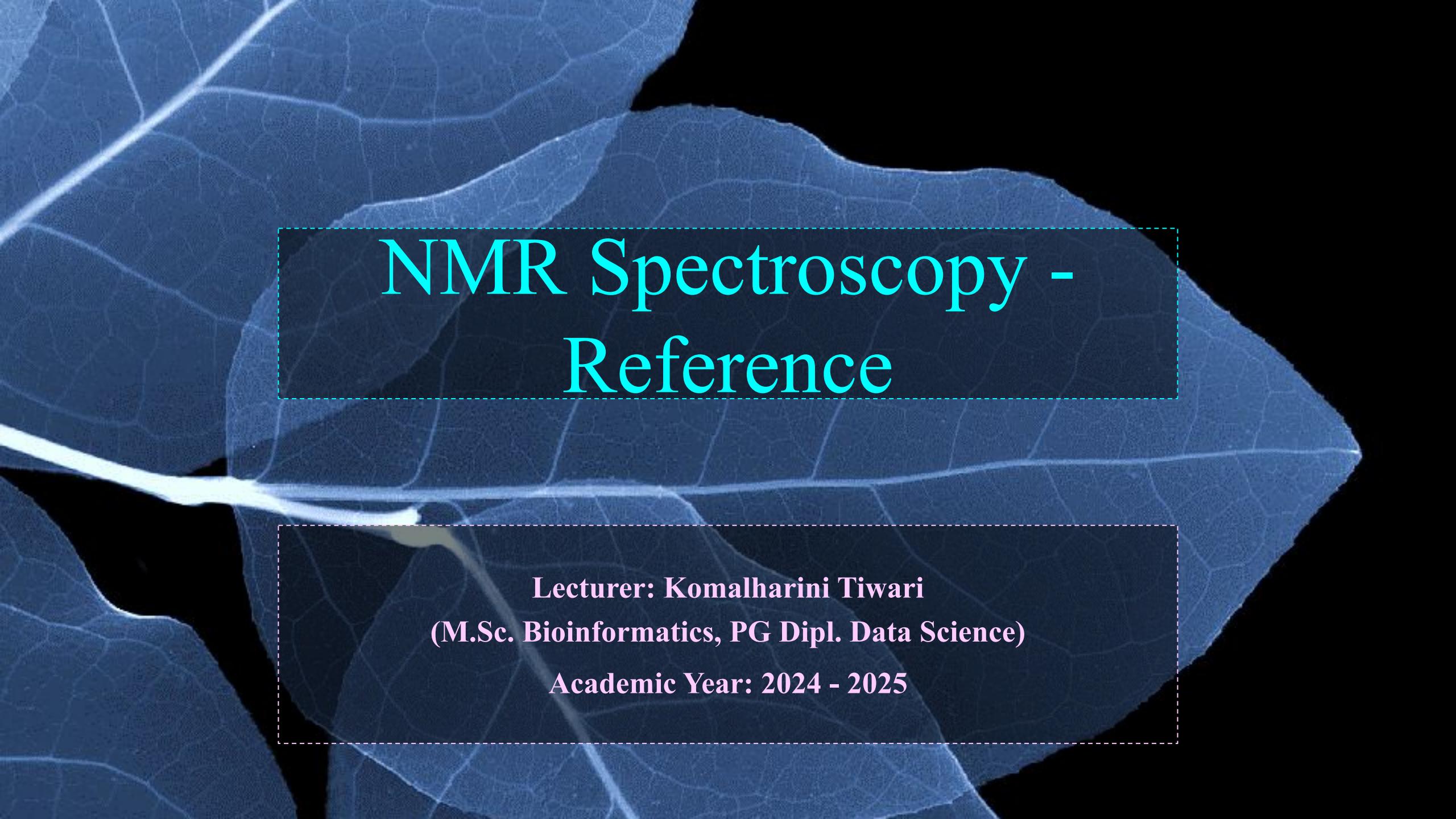
- Raman spectroscopy can provide information about the secondary and tertiary structures of proteins, nucleic acids, and other biomolecules.

□ Live Cell Imaging:

- Raman spectroscopy allows for the non-invasive study of living cells, providing insights into cellular processes in real-time.

□ Drug-Biomolecule Interactions:

- Raman can be used to study the binding of drugs to biomolecules, aiding in drug design and development.



NMR Spectroscopy - Reference

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Principle of NMR:

- **Magnetic Properties of Nuclei:** NMR-active nuclei have a non-zero nuclear spin (I). For example, 1H and ^{13}C both have a nuclear spin $I = \frac{1}{2}$. These nuclei possess a magnetic moment that interacts with an external magnetic field.
- **Zeeman Effect:** In the presence of an external magnetic field (B_0), the magnetic moments of nuclei align either with or against the field, creating two energy levels for $I = \frac{1}{2}$ nuclei. This difference in energy (ΔE) between the two states is proportional to the strength of the magnetic field.
- **Resonance Condition:** When RF radiation of a specific frequency (ν) is applied, nuclei can transition between the two energy levels if the energy of the radiation matches ΔE . The resonance frequency ν_0 for a nucleus in a magnetic field B_0 is given by:

$$\nu_0 = \frac{\gamma B_0}{2\pi}$$

where γ is the gyromagnetic ratio, a constant specific to each type of nucleus.

Principle of NMR:

- **Chemical Shift (δ):** The chemical shift is the resonance frequency of a nucleus relative to a standard (usually TMS for 1H and ^{13}C NMR). It is expressed in parts per million (ppm):

$$\delta = \frac{\nu_{\text{sample}} - \nu_{\text{reference}}}{\nu_{\text{spectrometer}}} \times 10^6$$

- **Spin-Spin Coupling (J-Coupling):** Neighboring nuclei interact with each other through bonds, causing splitting of NMR signals into multiplets. The splitting pattern provides information about the number and type of neighboring nuclei.

Thank-You!