UNIT V

Instrumental Methods and Applications

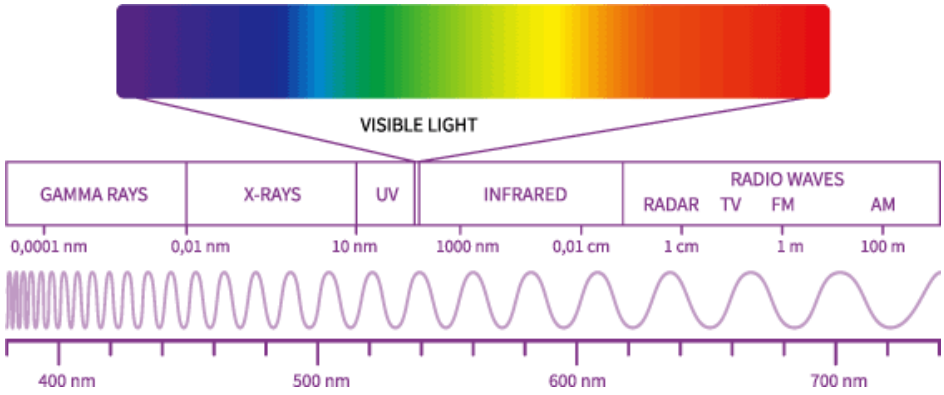
Electromagnetic spectrum-Absorption of radiation: Beer-Lambert's law. UV-Visible Spectroscopy, electronic transition, Instrumentation, IR spectroscopies, fundamental modes and selection rules, Instrumentation. Chromatography-Basic Principle, lassification-HPLC: Principle, Instrumentation and Applications.

ELECTROMAGNETIC SPECTRUM

The electromagnetic spectrum is a range of frequencies, wavelengths and photon energies covering frequencies from below 1 hertz to above 10^{25} Hz, corresponding to wavelengths which are a few kilometers to a fraction of the size of an atomic nucleus in the spectrum of electromagnetic waves. Generally, in a vacuum, electromagnetic waves tend to travel at speeds which are similar to that of light. However, they do so at a wide range of wavelengths, frequencies and photon energies.

The electromagnetic spectrum consists of a span of all electromagnetic radiation which further contains many sub ranges, which are commonly referred to as portions. These can be further classified as infrared radiation, visible light or ultraviolet radiation.

The entire range (electromagnetic spectrum) is given by radio waves, microwaves, infrared radiation, visible light, ultra-violet radiation, X-rays, gamma rays and cosmic rays in the increasing order of frequency and decreasing order of wavelength. The type of radiation andtheir frequency and wavelength ranges are as follows:



Beer-Lambert Law Statement

For a given material sample path length and concentration of the sample are directly proportional to the absorbance of the light.

$$A = \varepsilon Lc$$

Where,

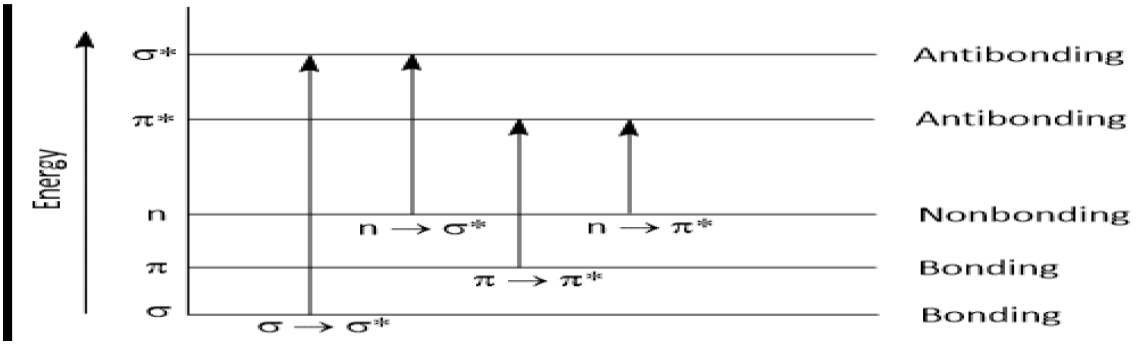
- A is the amount of light absorbed for a particular wavelength by the sample
- ε is the molar extinction coefficient
- L is the distance covered by the light through the solution
- c is the concentration of the absorbing species

UV-VISIBLE SPECTROSCOPY:

UV-visible spectroscopy is a technique that measures the amount of light absorbed by a chemical substance. It is absorption spectroscopy or reflectance spectroscopy technique within the ultraviolet and visible regions of the electromagnetic spectrum. When continuous radiation is passed through a compound a portion of that compound is absorbed by the compound. The residual radiation after passing through the compound yields a spectrum with gaps in it due to absorption by the compound, this spectrum is called the absorption spectrum. Absorption of UV-Visible radiation results in the electronic transition of the compound, i.e., an electron in the ground state (occupied orbital) is promoted to the excited state (unoccupied orbital), and the amount of radiation absorbed corresponds to the energy difference between the ground state and the excited state.

TYPES OF ELECTRONIC TRANSITIONS

- $\sigma - \sigma$ * **Transition:** transition of an electron from bonding sigma orbital (σ) to anti-bonding sigma orbital (σ*), is represented by $\sigma - \sigma$ * transition. For example, alkanes because in alkane all the atoms are held together with sigma bond.
- $n - \sigma$ * **&** $n - \pi$* **Transition:** Transitions from non-bonding molecular (n) orbital to anti- bonding sigma orbital or anti-bonding pi orbital (π*), are represented by $n - \sigma$* or $n - \pi$* transition respectively. These transition required less energy than $\sigma - \sigma$* transition. For example, alkyl halide, aldehydes, ketones etc.



- $\pi - \pi$* **Transition:** This type of transition generally show in unsaturated molecules like alkenes, alkynes, aromatics, carbonyl compounds etc. This transition required less energy as compare to $n - \sigma$* transition.

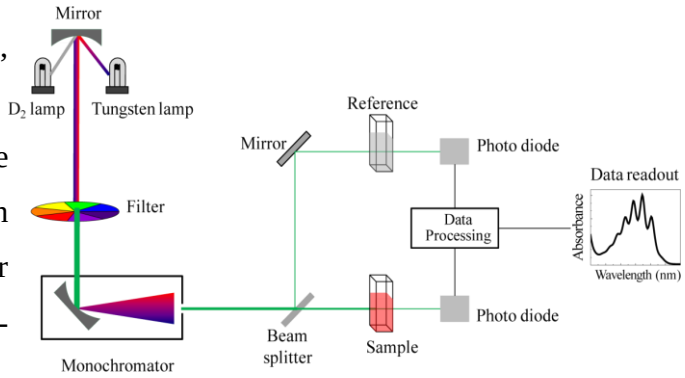
CHROMOPHORES AND AUXOCHROMES

Chromophores: It is a covalently unsaturated group absorbed electromagnetic radiation in UV-visible region and impart color to the compound. For example, C=C, C≡C, benzene, NO2 etc.

Auxochromes: It is a saturated group (containing lone pair of electron), when auxochrome attach to the Chromophore absorption shift towards longer wavelength. For example: -OH, -SH, -NH etc.

INSTRUMENTATION OF UV-VISIBLE SPECTROSCOPY

Principle: According to Beer-Lambert's Law, the absorbance of a solution (containing the compound) is directly proportional to the concentration of the absorbing species (the compound) and the path length. This translates to, as the number of molecules (concentration) capable of absorbing the radiation at a given wavelength increases, the extent of absorption is increased. Also, the efficiency of the molecule (recorded by its molar absorptivity) in absorbing the radiation contributes to greater absorption. The formulation for Beer-Lambert's law is given by A= εcl.



Light Source: A UV spectrophotometer has two light sources, a tungsten lamp for visible light and a hydrogen or deuterium lamp, and the former gives more extensive and intense light than the latter. The monochromator disperses the light, and the desired wavelength is focused on the exit slit.

Monochromator: Monochromators generally composed of prisms and slits. The most of the spectrophotometers are double beam spectrophotometers. The radiation emitted from the primarysource is dispersed with the help of rotating prisms. The various wavelengths of the light source which are separated by the prism are then selected by the slits such the rotation of the prism results in a series of continuously increasing wavelength to pass through the slits for recording purpose. The beam selected by the slit is monochromatic and further divided into two beams with the help of another prism.

Sample and reference cells: One of the two divided beams is passed through the sample solution and second beam is passé through the reference solution. Both sample and reference solution are contained in the cells. These cells are made of either silica or quartz.

Detector: Generally two photocells serve the purpose of detector in UV spectroscopy. One of the photocell receives the beam from sample cell and second detector receives the beam from thereference. The intensity of the radiation from the reference cell is stronger than the beam of sample cell. This results in the generation of pulsating or alternating

currents in the photocells.

Amplifier: The alternating current generated in the photocells is transferred to the amplifier. Theamplifier is coupled to a small servo meter. Generally current generated in the photocells is of very low intensity, the main purpose of amplifier is to amplify the signals many times so we can get clear and recordable signals.

Recording devices: Most of the time amplifier is coupled to a pen recorder which is connected to the computer. Computer stores all the data generated and produces the spectrum of the desiredcompound.

APPLICATIONS

- UV-Visible spectroscopes are unmistakably progressively refined instruments and givepreferable goals and exactness over a colorimeter.
- It is utilized to evaluate the concentration of colored and colorless solutions, which couldretain light.
- Because of its higher susceptibility, it is used to determine even minimal quantities in thematter.
- It, for the most part, does not corrupt or change the substance under examination and henceforth can be recuperated or reused.
- It is utilized to discover the absorption maxima of compounds with a broad scope of wavelengths.
- It additionally empowers to pursue the subtleties of reactions and dynamic enzyme energy.
- It is additionally used to decide the development of bacteria and yeast and decide the numberof cells in culture.

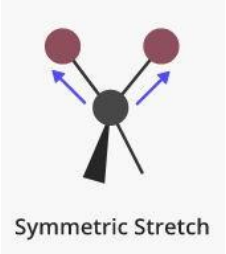
INFRARED (IR) SPECTROSCOPY

Infrared (IR) spectroscopy is an analytical technique used to determine the bonds and functional groups in the structure of chemical compounds. It destroys no sample during analysis. Infrared spectroscopy is a type of spectroscopic technique that involves the interaction of infrared radiation with matter. It is based on the absorption, emission, or scattering of infrared radiation by a sample, and is used to identify and analyze the chemical structure of a sample. The principle of infrared spectroscopy is based on the vibrations of atoms and the dipole moment of the compounds. When infrared radiation passes through the sample, a fraction of incident radiation of particular energy is absorbed by the vibrating atoms. The energy of the vibratory bonds corresponds to the energy of absorption. This way infrared spectrum is obtained.

MOLECULAR VIBRATIONS TO INFRARED RADIATIONS

Molecular vibrations fall into two main categories: stretching or bending. The stretching vibration entails a change in the inter-atomic distance along the bond axis, whereas in bending vibrations the bond lengths remain constant, but the bond angles change.

Stretching Vibration: In this type of vibrations the distance between the two atoms changes i.e. the distance between the atoms either increases or decreases but the atoms remains in the same bond axis. There are two types of stretching vibrations



Symmetric Stretching: In this type of stretching vibrations, the movement of the atoms with respect to a particular central atom takes place in the same direction.

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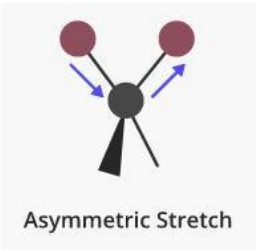
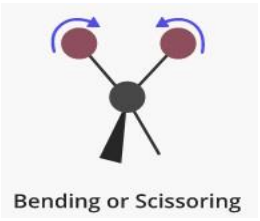
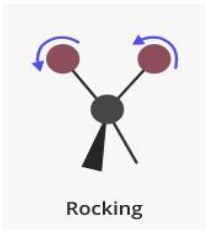


Asymmetric Stretching: In this type of stretching vibrations, the movement of the atoms with respect to a particular central atom takes place in opposite direction i.e. one atom approaches towards the central atom while the other atom moves away from it.

Bending Vibrations: In this type of vibrations, the angle between the bonded atoms changes i.e., the positions of the atoms changes with respect to the original bond axis. Bending vibrations are of four types.

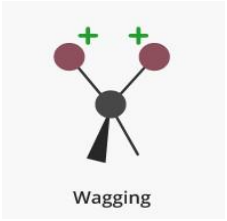


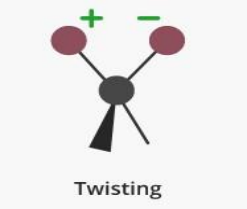
Scissoring: In this type of bending vibrations, two atoms either move away from each other or move towards each other with respect to a central atom i.e. they operate like a scissor. In this type of bending vibration, the operation takes place in plane.



Rocking: In this type of bending vibrations, two atoms move in the same direction with respect to a central atom. In this type of bending vibration, the operation takes place in plane.

Wagging: In this type of bending vibrations, two atoms either move up of the plane or move down of the plane with respect to the plane containing the central atom. In this type of bending vibration, the operation takes place out of-plane.



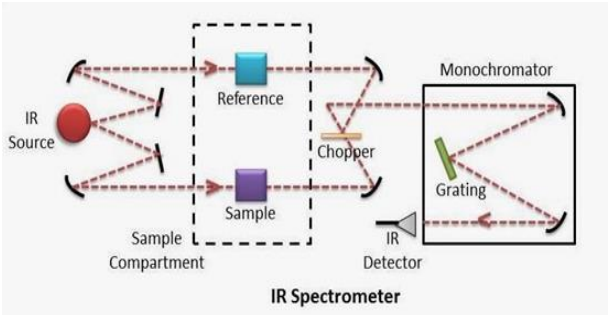


Twisting: In this type of bending vibrations, one atom move up of the plane while other atom move down of the plane with respect to the plane containing the central atom. In this type of bending vibration, the operation takes place out of-plane.

INSTRUMENTATION OF IR SPECTROSCOPY

The main parts of an IR spectrometer are:

- IR radiation source.
- Monochromators
- Sample cell and sampling of substance
- Detector.



IR Radiation Sources: Infrared instruments need a source of radiant energy which provides a means for isolating narrow frequency band. The radiation source must emit IR radiation which should be intense enough for detection, steady and extend over the desired wavelengths. The various popular source of IR radiation are as follows

- Incandescent lamp
- Nernest glower
- Globar Source
- Mercury Arc

Monochromators: The radiation source emits radiation of various frequency as the sample in IR spectroscopy absorb only at certain frequency, it thus becomes essential to select desired frequencies from the radiation source and reject the radiation of other frequencies. Thus, selection has been achieved by means of Monochromators which are mainly of two types

- Prism Monochromators
- Grating Monochromators.

Chopper: The two beams are reflected to a chopper which is rotating at a speed of 10 rotations per second. This chopper makes the reference and the sample beam to fall on the monochromator grating alternately.

Detector: At the wavelength where the sample has absorbed, the detector will receive a weak beam from the sample while the reference beam will retain full intensity. This leads to apulsating or alternating current to flow from detector to amplifier. On the other hand, at the frequencies where the sample doesn't absorb, both the beams will have equal

intensities and the current flowing from the detector to the amplifier will be direct and not alternating. The amplifieris designed to amplify only the alternating current.

APPLICATIONS

- Identification of functional group and structure elucidation
- Identification of substances
- Studying the progress of the reaction
- Detection of impurities
- Quantitative analysis
- Study the presence of impurities in water sample

CHROMATOGRAPHY

Chromatography is based on the principle where molecules in mixture applied onto the surfaceor into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase. The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights. Because of these differences, some components ofthe mixture stay longer in the stationary phase, and they move slowly in the chromatography system, while others pass rapidly into mobile phase, and leave the system faster. Based on this approach three components form the basis of the chromatography technique

Stationary phase: This phase is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface a solid support".

Mobile phase: This phase is always composed of "liquid" or a "gaseous component."

Retention Volume: Retention volume (Vr) is the volume of mobile phase required to elute a compound from the chromatographic column. It is the total volume of the mobile phase that passes through the column during the retention time of the compound.

Retention Time: Retention time (tR) is the time taken for a compound to travel from the injection point to the detector in a chromatographic system. It is a measure of the time the compound spends within the column, including both the time spent in the mobile phase and any time spent interacting with the stationary phase.

CLASSIFICATION OF CHROMATOGRAPHY

Based on the bed Shape

- **Column Chromatography:** In this type of chromatography, the stationary phase of the setup is placed inside a tube. Then, the particles of the stationary phase (which is in the solid state) are made to fill the inside with the tube. An unrestricted, open path is then prepared for the mobile phase (somewhere along the middle of the tube).

Planar Chromatography: In this type of chromatography, the stationary phase of the apparatus usually has a planar shape. Different subcategories of planar chromatography include paper chromatography (where the stationary phase is a special type of paper) and thin layer chromatography (usually abbreviated as TLC).

Based on the Physical State of the Mobile Phase

- **Gas Chromatography:** In this type of chromatography, the mobile phase is a substance that exists in the gaseous state. It can be noted that gas chromatography is also known as gas- liquid chromatography, and is often abbreviated to GLC. This type of chromatography almost always involves the use of a packed column.
- **Liquid Chromatography:** This type of chromatography involves the use of a mobile phase that exists in the liquid state. Liquid chromatography, often abbreviated to LC, can be carried out either on a plane or in a column. It can be noted that there exist many subcategories under liquid chromatography such as high-performance liquid chromatography and reversed phase liquid chromatography.

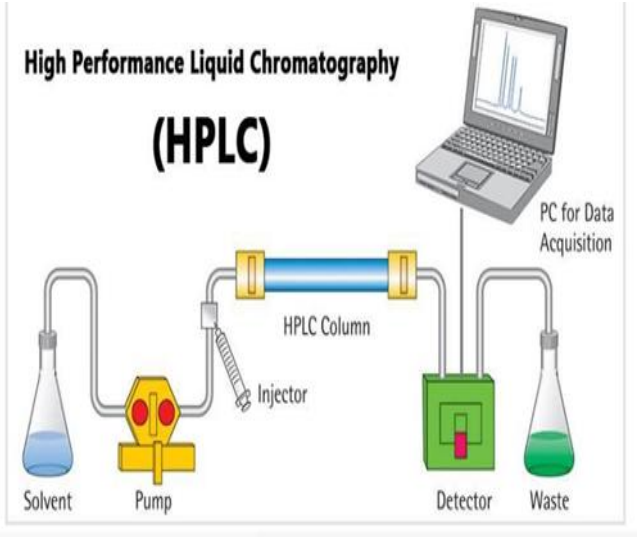
Based on the Mechanism of the Separation

- **Ion Exchange Chromatography:** This type of chromatography is also known as ion chromatography. Ion exchange chromatography involves the separation of the components of the mixture via an ion exchange mechanism. Differently charged components of the mixture are separated with the help of different ions in this separation technique.
- **Size Exclusion Chromatography:** This type of chromatography involves the separation of different components of the mixture based on their sizes. In size exclusion chromatography, components of the mixture are filtered based on their hydrodynamic volume or hydrodynamicdiameters. It can be noted that size exclusion chromatography is also known as gel permeationchromatography or gel filtration chromatography.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

High-performance liquid chromatography or commonly known as HPLC, is an analytical technique used to separate, identify or quantify each component in a mixture. The mixture is separated using the basic principle of column chromatography and then identified and quantified by spectroscopy. HPLC is thus basically a highly improved form of column liquidchromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres.

The Pump



- The development of HPLC led to the development of the pump system.
- The pump is positioned in the most upper stream of the liquid chromatography system and generates a flow of eluent from the solvent reservoir into the system.
- High-pressure generation is a "standard" requirement of pumps besides which, it should also to be able to provide a consistent pressure at any condition and a controllable and reproducible flow rate.
- Most pumps used in current LC systems generate the flow by back-and-forth motion of a motor-driven piston (reciprocating pumps). Because of this piston motion, it produces "pulses".

Injector

- An injector is placed next to the pump.
- The simplest method is to use a syringe, and the sample is introduced to the flow of eluent.
- The most widely used injection method is based on sampling loops.

Column

- The separation is performed inside the column.
- The recent columns are often prepared in stainless steel housing, instead of glass columns.
- The packing material generally used is silica or polymer gels compared to calcium carbonate.The eluent used for LC varies from acidic to basic solvents.
- Most column housing is made of stainless steel since stainless is tolerant towards a largevariety of solvents.

Detector

- Separation of analytes is performed inside the column, whereas a detector is used to observethe obtained separation.
- The composition of the eluent is consistent when no analyte is present. While the presence of analyte changes the composition of the eluent. What detector does is to measure these differences.
- This difference is monitored as a form of an electronic signal. There are different types of detectors available.

Recorder

- The change in eluent detected by a detector is in the form of an electronic signal, and thus it isstill not visible to our eyes.
- In older days, the pen (paper)-chart recorder was popularly used. Nowadays, a computer- based data processor (integrator) is more common.
- There are various types of data processors; from a simple system consisting of the in-built printer and word processor while those with software that are specifically designed for an LC system which not only data acquisition but features like peak-fitting, baseline correction, automatic concentration calculation, molecular weight determination, etc.

APPLICATIONS

- Analysis of drugs
- Analysis of synthetic polymers
- Analysis of pollutants in environmental analytics
- Determination of drugs in biological matrices
- Isolation of valuable products
- Product purity and quality control of industrial products and fine chemicals
- Separation and purification of biopolymers such as enzymes or nucleic acids
- Water purification
- Pre-concentration of trace components
- Ligand-exchange chromatography
- Ion-exchange chromatography of proteins
- High-pH anion-exchange chromatography of carbo hydrates and oligosaccharides.