Unit – 4 Spectroscopy

Spectroscopy is that branch of physics which deals with interaction light with materials. In other words it is an analytical method for qualitative and quantitative estimation by use of light.

Light is as we all know is an electromagnetic radiation which has wave and frequency as measurable characters.

Spectroscopy finds wide spread application in daily life. It is used in analytical chemistry, phyto-chemistry (plant chemistry), biological analysis, health care and medicine. Further is became a part of other means of estimation and analysis like in chromatography, elemental analysis and also identification tools.

The different types of spectroscopy include:

A). Based on the level of study.

Here the classification is done based on the study made at atomic or molecular level. When light is allowed to pass through the substance, changes in atomic level or molecular level is observed.

1) Atomic spectroscopy:

Here energy changes takes place at atomic levels. The measurement is done to study the atoms and their quantity. This is of two types as atomic absorption spectroscopy and atomic emission spectroscopy. These are referred as atomic absorption spectroscopy and flame photometry respectively.

Here the sample mostly metals and their formulations are converted to atomic forms and then measured. Ex: Sodium, calcium, magnesium related formulations.

This finds less application as the characters of most substance are depended on the molecular nature and not just atoms in it.

Also the methods are expensive, time taking and even quite tedious to perform.

2) Molecular spectroscopy:

Here the energy changes occurring at the level of molecule are studied. The characters like molecular absorption. emission and vibration are studied.

The examples include, colorimetry, UV-spectroscopy, infra red, FTIR, fluorimetry etc.

This method of spectroscopy is widely used due to many applications. The methods are quick, easy and accurate in determination. Further they are easily integrated into other analytical techniques like chromatography.

B) Based on the property of either absorption or emission.

Here the principle of abortion or emission of electromagnetic radiation is taken into consideration.

1) Absorption spectroscopy:

As the name suggests, here there is absorption of light by the sample. The extent of absorption and the wavelength of the absorbed light is considered. The wavelength of light absorbed tells the nature of the compound while the intensity of absorbed light tells the concentration.

The examples of the spectroscopic methods coming under this method are colorimetry, UV-spectroscopy, infra red spectroscopy, Nuclear magnetic resonance (NMR), atomic absorption spectroscopy.

2) Emission spectroscopy:

Contrary to the above method, here the emitted light is measured. Once the light impinges on the sample, some of it is absorbed. This absorption of light leads to transition of electrons from ground state to excited state. These excited electrons return back to ground state by release of electromagnetic radiation (light) of specific wavelength. The intensity gives the concentration while the wavelength tells the nature of the compound.

Examples include fluorimetry, flame photometry.

C) Based on the level of study i.e. electronic or magnetic levels.

Here the study is done based on electronic or magnetic properties of the compound. Light is an electromagnetic radiation. That is it has both electronic and magnetic properties.

1. **Electronic spectroscopy**:

When a compound is estimated without the magnetic field we call it electronic spectroscopy. In this method the substance under test is exposed to light without the influence of magnetic field.

Examples of this method are again colorimetry, UV-spectroscopy, IR, fluorimetry etc.

2. Magnetic spectroscopy:

Here the substance is exposed to electromagnetic radiation in presence of external magnetic field.

Examples include Nuclear magnetic resonance spectroscopy (NMR), Electron spin resonance spectroscopy (ESR).

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Electromagnetic radiation

Electromagnetic radiation light is a form of energy whose behavior is described by the proper ties of both waves and particles. Some properties of electromagnetic radiation, such as its refraction when it passes from one medium to another (Figure), are explained best by describing light as a wave. Other properties, such as absorption and emission, are better described by treating light as a particle.

Electromagnetic radiation consists of oscillating electric and magnetic fields that propagate through space along a linear path and with a constant velocity. In a vacuum electromagnetic radiation travels at the speed of light, c, which is $2.997\ 92 \times 10^8$ m/s. When electromagnetic radiation moves through a medium other than a vacuum its velocity, v, is less than the speed of light in a vacuum.

An electromagnetic wave is characterized by several fundamental properties, including its velocity, amplitude, frequency, phase angle, polariza tion, and direction of propagation. The wavelength, λ, is defined as the distance between successive maxima (Figure). For ultraviolet and visible electromagnetic radiation the wavelength is usually expressed in nanometers (1 nm = 10^{-9}m), and for infrared radiation it is given in microns (1 μm = 10^{-6}m).

Particle properties of electromagnetic radiation

When matter absorbs electromagnetic radiation it undergoes a change in energy. The interaction between matter and electromagnetic radiation is easiest to understand if we assume that radiation consists of a beam of energetic particles called photons. When a photon is absorbed by a sample it is "destroyed," and its energy acquired by the sample.

The energy of a photon, in joules, is related to its frequency, wavelength, and wavenumber.

The electromagnetic spectrum

The frequency and wavelength of electromagnetic radiation vary over many orders of magnitude. For convenience, we divide electromagnetic radiation into different regions—the electromagnetic spectrum —based on the type of atomic or molecular transition that gives rise to the absorption or emission of photons (Figure). The boundaries between the regions of the electromagnetic spectrum are not rigid, and overlap between spectral regions is possible.

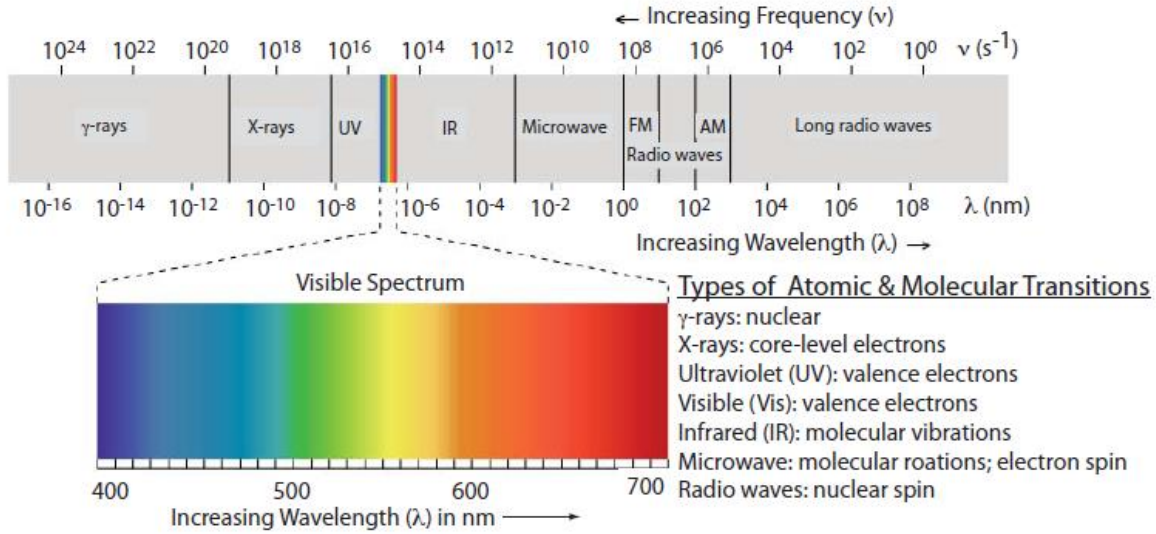

Figure. The electromagnetic spectrum showing the boundaries between different regions and the type of atomic or molecular transition responsible for the change in energy. The colored inset shows the visible spectrum.

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❖ Spectrophotometry

Spectrophotometry is a method to measure how much a chemical substance absorbs light by measuring the intensity of light as a beam of light passes through sample solution. The basic principle is that each compound absorbs or transmits light over a certain range of wavelength. This measurement can also be used to measure the amount of a known chemical substance. Spectrophotometry is one of the most useful methods of quantitative analysis in various fields such as chemistry, physics, biochemistry, material and chemical engineering and clinical applications.

Introduction

Every chemical compound absorbs, transmits, or reflects light (electromagnetic radiation) over a certain range of wavelength. Spectrophotometry is a measurement of how much a chemical substance absorbs or transmits. Spectrophotometry is widely used for quantitative analysis in various areas (e.g., chemistry, physics, biology, biochemistry, material and chemical engineering, clinical applications, industrial applications, etc). Any application that deals with chemical substances or materials can use this technique. In biochemistry, for example, it is used to determine enzyme-catalyzed reactions. In clinical applications, it is used to examine blood or tissues for clinical diagnosis. There are also several variations of the spectrophotometry such as atomic absorption spectrophotometry and atomic emission spectrophotometry.

A spectrophotometer is an instrument that measures the amount of photons (the intensity of light) absorbed after it passes through sample solution. With the spectrophotometer, the amount of a known chemical substance (concentrations) can also be determined by measuring the intensity of light detected. Depending on the range of wavelength of light source, it can be classified into two different types:

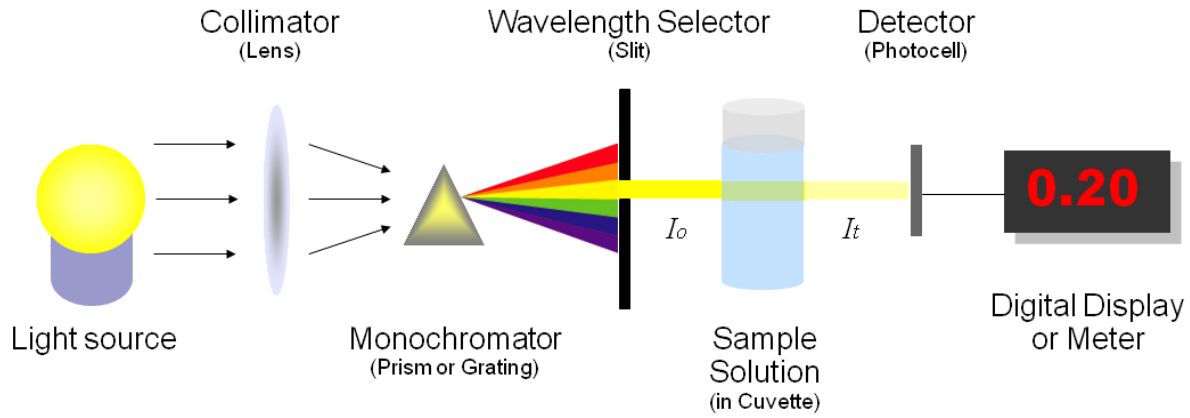
- **UV-visible spectrophotometer**: uses light over the ultraviolet range (185 - 400 nm) and visible range (400 - 700 nm) of electromagnetic radiation spectrum.
- **IR spectrophotometer**: uses light over the infrared range (700 - 15000 nm) of electromagnetic radiation spectrum.

In visible spectrophotometry, the absorption or the transmission of a certain substance can be determined by the observed color. For instance, a solution sample that absorbs light over all visible ranges (i.e., transmits none of visible wavelengths) appears black in theory. On the other hand, if all visible wavelengths are transmitted (i.e., absorbs nothing), the solution sample appears

white. If a solution sample absorbs red light (~700 nm), it appears green because green is the complementary color of red. Visible spectrophotometers, in practice, use a prism to narrow down a certain range of wavelength (to filter out other wavelengths) so that the particular beam of light is passed through a solution sample.

Devices and mechanism

Figure illustrates the basic structure of spectrophotometers. It consists of a light source, a collimator, a monochromator, a wavelength selector, a cuvette for sample solution, a photoelectric detector, and a digital display or a meter. Detailed mechanism is described below



A spectrophotometer, in general, consists of two devices; a spectrometer and a photometer. A spectrometer is a device that produces, typically disperses and measures light. A photometer indicates the photoelectric detector that measures the intensity of light.

- **Spectrometer**: It produces a desired range of wavelength of light. First a collimator (lens) transmits a straight beam of light (photons) that passes through a monochromator (prism) to split it into several component wavelengths (spectrum). Then a wavelength selector (slit) transmits only the desired wavelengths, as shown in Figure 1.
- **Photometer**: After the desired range of wavelength of light passes through the solution of a sample in cuvette, the photometer detects the amount of photons that is absorbed and then sends a signal to a galvanometer or a digital display.

Beer-Lamberts Law:

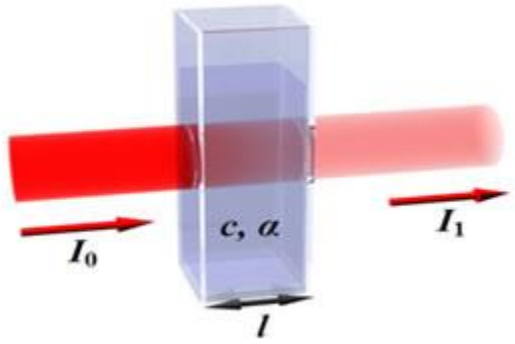


Diagram of Beer-Lambert absorption of a beam of light as it travels through a cuvette of width .

Beer-Lambert's law is the linear relationship between the absorbance and concentration of the absorbing sample, i.e. a logarithmic relation exist between the transmission of light through a substance (T) and the product of absorption coefficient of a substance (α) and distance travelled by the light through the material(path length (l) The absorption coefficient is the product of molar absorptivity, the concentration the material, or an absorption cross section, σ and the (number) density,

Where,
Ɛ is the molar absorptivity of the absorber, C is the concentration of the absorbing species in the material and N is the density (number) of adsorbers.

For liquids, these relations are usually written as:

$$T = \frac{I}{I_0} = 10^{-\alpha l} = 10^{-\epsilon l c}$$

Whereas for gases, these relations are written as:

$$T = \frac{I}{I_0} = e^{-\alpha' l} = e^{-\sigma l N}$$

where,

I_o=intensity of the incident light
I =intensity of the transmitted light

σ =cross section of light absorption by a single particle
N=density of absorbing particles

The transmission (or transmissivity) for liquids in terms of absorbance, is defined as:

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

The relationship between absorbance (A) and percent transmittance (%T) is also quantitative and this can be written as:

$$A = -\log\left(\%T / 100\right)$$

Percent transmittance is $\frac{I}{I_0} \times 100$

Whereas, for gases, it is usually defined as:

$$A' = -\ln\left(\frac{I}{I_0}\right)$$

The above equation shows that the absorbance becomes linear relationship with the concentration according to:

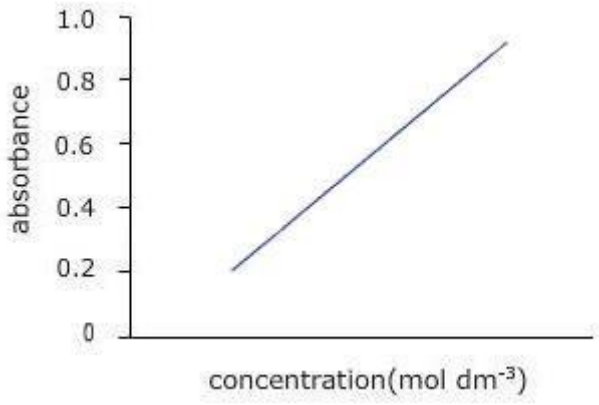
$$A = \epsilon l c = \alpha l$$

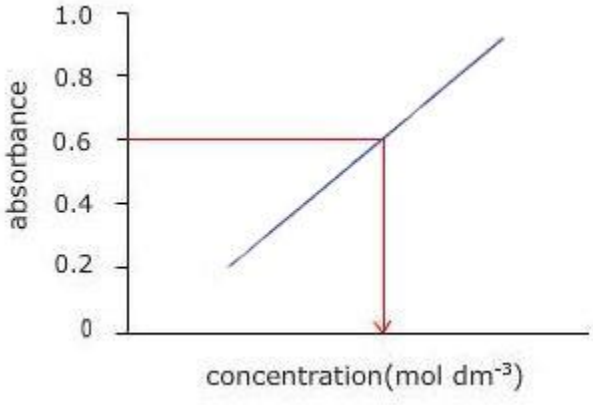
and

$$A' = \sigma l N = \alpha' l$$

Thus, the absorbance is measured, if the path length and the molar absorptivity are known and the concentration of the substance can be deduced.

According to the Beer-Lambert Law, absorbance is proportional to concentration, so that at dilute solutions a plot of concentration vs. absorbance would be straight line, but the Law breaks down for solutions of higher concentration, and so you might get a curve under those circumstances.





Applications of a Spectrophotometer:

1. It is directly used to measure light intensity at different wavelengths.

2. It is used to determine the unknown concentration of solution.

3. Spectrometers can be used to determine the equilibrium constant of a reaction involving ions.

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- **Turbidometry**

Suspended solids obstruct the transmittance of light through fluids. The turbidity of a sample may be due to a single chemical substance or a combination of several. A turbidimeter measures this obstruction to determine the haziness, or intensity of light, in a sample, in nephelometric turbidity units (NTU). A cloudy solution has a high haziness value, while a relatively clear solution has a low value.

Note that while tubidimeters measure the effect of particles on the clarity of a fluid, they cannot be used to determine particle size.

A turbidimeter is composed of a light source , a focusing lens to direct a beam of light through a sample, a photoelectric detector positioned at a 90° angle from the beam to measure the amount of light scattered, and a light trap, to prevent any light already past the sample from being detected and giving faulty readings.

Turbidimeters send beams of light into a sample (blue) that contains particles. The particles absorb this light energy and radiate it in all directions. The manner in which the two interact depend on the size, shape, and composition of the particle, the wavelength of the light beam, and the refractive index of the sample. The refractive index of the particle must be different than the suspending fluid. As this difference increases, the scattering becomes more intense.

The term “turbidity” is used to describe the cloudy or milky appearance of liquid or solid media such as water (drinking, mineral, bathing or waste water), beverages (beer, wine or soft drinks) or window glass (translucent glass).

In physical terms, turbidity is due to particles of varying sizes scattering or absorbing light, giving the medium in question a cloudy appearance. This turbidity is caused by suspended particles such as sludge, limestone, yeast or microorganisms.

When particles are suspended in a solution in a cuvette, they make the solution unclear (turbid).

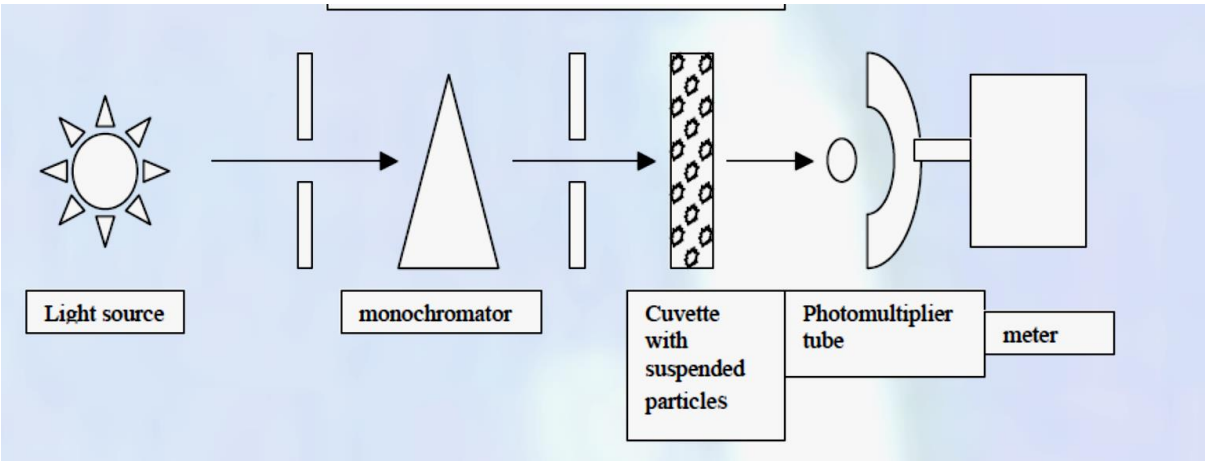
Incident light entering the cuvette will be subjected to three reactions;

1- some of the light will be absorbed (blocked) by the particles
2- some will be transmitted through the cuvette
3- some will be scattered in various directions.

Considerations in turbidimetry and nephelometry

- The reaction in turbidimetry & nephelometry does not follow Beer's Law
- Therefore, standard curves must be plotted and the concentration of the unknown is determined from the standard curve.
- Because the absorbance is dependent on both number and size of particles, the standard solution which is used for the standard curve must have similar size in suspension as unknown.

- Because some precipitation and settlement of particles may occur with time, in order to obtain good accuracy it is important to ; a) mix the sample well prior to placing the cuvette in the instrument, and, b) keep the same time for measurement of every sample throughout the measurement.
- Kinetic reactions (measurement of the progress of reaction with time) provides higher degree of accuracy, sensitivity, precision and less time than end-point reactions (measuring the reaction at the start and finish of the reaction).
- Additionally in kinetic reactions there is no need for reagent blank since the previous reading is taken as the base-line for the next reading.
- Kinetic reaction may be taken in 60, 90 or 120 seconds (taking readings at 10 seconds intervals), whereas endpoint reactions may take much longer time e.g. 15 -120 minutes.
- Selection of a wavelength.
- If both solution and suspended particles are colorless, then use any wave length in the visible range.
- If the solution is coloured but the particles are not coloured, then use a wave length that gives minimum absorption for the solution.
- If the particles are coloured and the solution is colorless then use a wavelength that gives maximum absorption with the particles.
- If both solution and particles are coloured then use two wavelengths; one that gives minimum absorbance for the solution and the other one maximum absorbance for the particles. Subtract the solution absorbance from the particles absorbance.



- A turbidimeter is used in many process applications in the industrial, municipal, pharmaceutical , and pulp/paper industries.
- Turbidimetry is involved with measuring the amount of transmitted light (and calculating the absorbed light) by particles in suspension to

determine the concentration of the substance in question. Amount of absorbed light, and therefore, concentration is dependent on; a) number of particles, and b) size of particles.

- Measurements are made using light spectrophotometers Clinical Applications.
- Determination of the concentration of total protein in biological fluids such as urine and CSF which contain small quantities of protein (mg/L quantities) using trichloroacetic acid.
- Determination of amylase activity using starch as substrate. The decrease in turbidity is directly proportional to amylase activity.
- Determination of lipase activity using triglycerides as substrate. The decrease in turbidity is directly proportional to lipase activity.

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- **Nephelometry.**

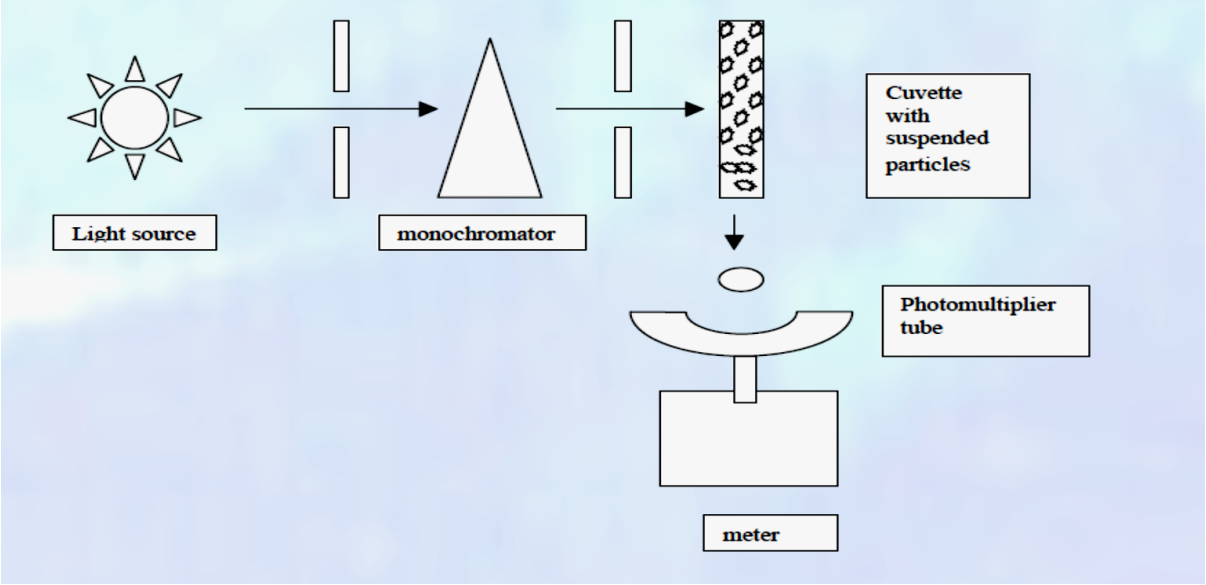
Principle

- Nephelometry is concerned with measurement of scattered light from a cuvette containing suspended particles in a solution.
- The components of a nephelometer are the same as a light spectrophotometer except that the detector is placed at a specific angle from the incident light.
- The detector is a photomultiplier tube placed at a position to detect forward scattered light. Detectors may be placed at 90^o, 70^o or 37^o depending on the angle at which most scattered light are found.
- Since the amount of scattered light is far greater than the transmitted light in a turbid suspension, nephelometry offers higher sensitivity than turbidimetry.
- The amount of scattered light depends on the size and number of particles in suspension.
- For most clinical applications, the light source is a tungsten lamp giving light in the visible region.
- For higher sensitivity and for applications that determine the size and number of particles in suspension, laser light nephelometers is used.

Clinical applications of nephelometry.

Widely used to determine concentrations of unknowns where there is antigen-antibody reactions such as -

- Determination of immunoglobulins (total, IgG, IgE, IgM, IgA) in serum and other biological fluids.
- Determination of the concentrations of individual serum proteins; hemoglobin, haptoglobin, transferring, c-reactive protein, 1-antitrypsin, albumin (using antibodies specific for each protein).
- Determination of the size and number of particles (laser-nephelometer}



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❖ **Luminometry**

As the name suggests, this type of spectrophotometry is associated with the phenomenon of luminescence.

Luminescence can be described as the emission of light by certain materials which do NOT result from heating (that is, the emission of light is when the temperature is below that of incandescence). Luminescence is the basic principle behind the working of luminometers. This phenomenon is usually ascribed to oxidative reactions which take place in solution producing molecules in an excited state. Some of these reactions release energy in the form of heat while others release in the form of photons. Examples of luminescent compounds are luciferin (light emitting compound found in organisms), luminol (chemical exhibiting luminescence).

There are two major categories of luminescence as **chemiluminescence** and **bioluminescence**.

Chemiluminescence is the luminescence produced by some chemical means. For example, luminol when oxidized with hydrogen peroxide (H_2O_2) in

the presence of a catalyst produces luminescence which is called the chemiluminescence.

Bioluminescence is the luminescence which is produced by the interference of an enzyme is referred to as *bioluminescence.*

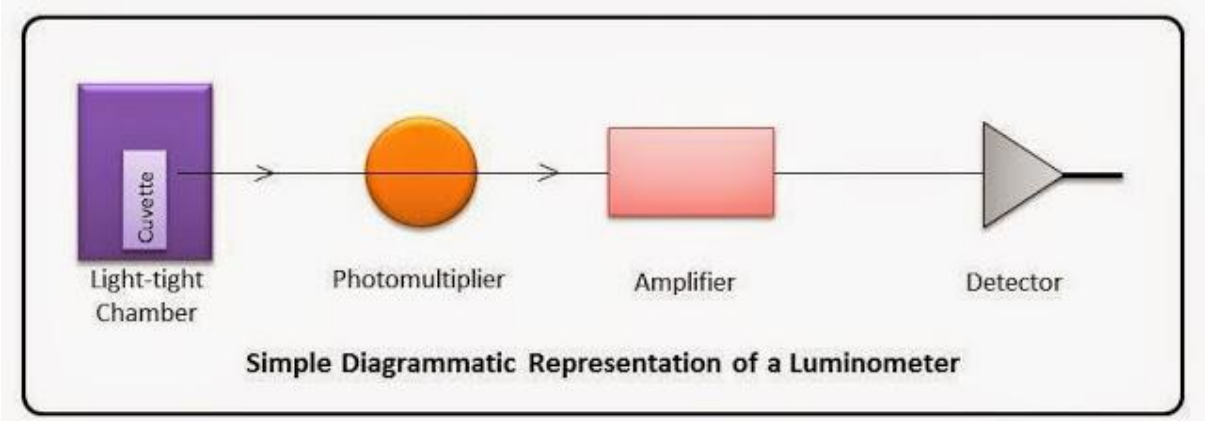
Measurement of light from a chemical reaction is highly useful because the concentration of an unknown can be inferred from the rate at which light is emitted. The rate of light output is directly related to the amount of light emitted and accordingly, proportional to the concentration of the luminescent material present. Therefore, light measurement is a relative indicator of the amount of luminescent material present in the sample of interest.

Advantages of luminometry

i. There are various advantages of luminometry over spectrophotometry. Firstly, luminometry is *more sensitive* as around femtomole quantities can be measured. Next advantage is that of a simple instrumentation. In luminometers, wavelength selectors are not required. This is so because the luminescent light is monochromatic as a result of its emission from a specific reaction.
ii. Superior sensitivity and low background distinguish luminometry from other analytical methods. Luminometry is up to 100,000 times more sensitive than absorption spectroscopy and is at least 1,000 times more sensitive than fluorometry. A well-designed luminometer can detect as little as 0.6 picograms of adenosine triphosphate (ATP) or 0.1 femtograms of luciferase, two common luminescent analytes.
iii. Chemi- and bioluminescence measurements have become extremely popular in recent years. They are often used to determine the amount of a specific unknown present in a sample, and in the last decade, have become extremely important in the study of gene expression and gene regulation.

Instrumentation

The basic components of luminometers are:
a. A light-tight chamber in which the cuvette containing the sample can be kept
b. A facility for the addition of luminescent reagents in light-tight fashion
c. A detector (which is generally a photomultiplier)
d. An amplifier
e. A recorder



Simple Diagrammatic Representation of a Luminometer

Applications:

We will discuss here three main systems which are of frequent use as firefly, bacterial luminescence and luminol chemiluminescence. The principle and applications of each of these are described below:

a. Firefly luminescence and ATP measurement:

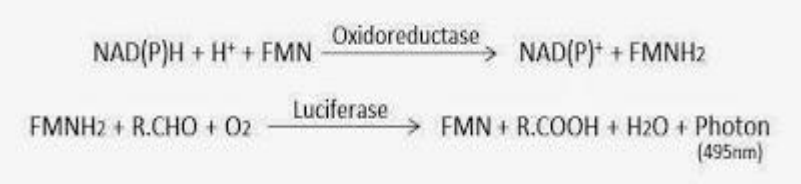
Luciferase enzyme catalyses the following reaction in the presence of magnesium:

$$\text{Luciferin} + \text{ATP} + O_2 \xrightarrow[Mg]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + CO_2 + \underset{(562nm)}{\text{Photon}}$$

Here, for each molecule of ATP reacting, one photon of intensity of 562nm is produced. This system is highly specific for ATP if all the reagents are pure. By linking this reaction with various other reactions, it can be used to assay a number of ATP-specific enzymes and their substrates such as creatine kinase, creatine phosphate etc.

b. Bacterial luminescence and coenzymes measurement:

The coenzymes that can be measured by this method are the NADH and NADPH. This system utilizes a purified oxidoreductase obtained from the bacterium *Benecka harveyi*. The reaction can be then coupled to bacterial luciferase as follows:



Here, bacterial luciferase catalyzes the oxidation of aldehyde by oxygen in the presence of FMNH2 during which a photon of maximum intensity at 495nm is produced.

c. Luminol based chemiluminescent assays:

Luminol is oxidized by hydrogen peroxide at pH 10-11 if chromium, copper or iron compounds are used as catalysts. Photons with maximum intensity at 430nm are produced.

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❖ **UV-Visible spectrophotometry**

UV spectroscopy is an important tool in analytical chemistry. The other name of UV (Ultra-Violet) spectroscopy is Electronic spectroscopy as it involves the promotion of the electrons from the ground state to the higher energy or excited state.

UV-Vis Spectroscopy is used to determine analyte concentration either at one time or often over a desired time period**.** The technique measures the absorption of light across the ultraviolet and visible light wavelengths through a liquid sample. Samples are dispensed into cuvette and placed in the path between a UV-Vis light and a detector. According to Beer-Lambert's law, with a constant light path length and known absorption coefficient (dependent upon wavelength), the concentration of a compound in question can be determined from the light absorbed by the sample at that wavelength.

UV-Vis is often called a general technique because most molecules will absorb in the UV-Vis wavelength range. The UV extends from 100–400 nm and the visible spectrum from 400–700 nm. The 100–200 nm range is called the deep UV. Light sources are more difficult to find for this range, so it is not routinely used for UV-Vis measurements. Typical UV-Vis spectrometers use a deuterium lamp for the UV that produces light from 170–375 nm and a tungsten filament lamp for visible, which produces light from 350–2,500 nm.

When a photon hits a molecule and is absorbed, the molecule is promoted into a more excited energetic state. UV-visible light has enough energy to promote electrons to a higher electronic state, from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO). The energy difference between the HOMO and the LUMO is called the band gap. Typically, these orbitals are called bonding and anti-bonding. The energy of the photon must exactly match the band gap for the photon to be absorbed. Thus, molecules with different chemical structures have different energy band gaps and different absorption spectra.

The appearance of broad bands or shoulders on the UV-Vis structure is due to the numerous vibrational and rotational states of a molecule, which lead to separate energy band gaps of slightly different energies.

For molecules with absorption in the visible region, the compounds will often appear colored. However, a common misconception is that the wavelength of peak absorption (λ_{max}) for a compound is the color it appears. A compound that appears red does not have much absorption in the red region of the spectrum. Instead, the λ_{max} for a compound that looks red is green. The color of a compound arises because those wavelengths of light are selectively transmitted through the sample, and thus they are not absorbed. A color wheel is helpful in determining what color a compound will absorb and what range the λ_{max} will be, as the color directly across the wheel from the observed color is the color that is most absorbed.

Principle of UV spectroscopy

UV spectroscopy obeys the Beer-Lambert law, which states that: *when a beam of monochromatic light is passed through a solution of an absorbing substance, the rate of decrease of intensity of radiation with thickness of the absorbing solution is proportional to the incident radiation as well as the concentration of the solution.*

The expression of Beer-Lambert law is-
$A = \log (I_0/I) = Ecl$
Where, A = absorbance
I_0 = intensity of light incident upon sample cell
I = intensity of light leaving sample cell
C = molar concentration of solute

L = length of sample cell (cm.)
E = molar absorptivity

From the Beer-Lambert law it is clear that greater the number of molecules capable of absorbing light of a given wavelength, the greater the extent of light absorption. This is the basic principle of UV spectroscopy.

Instrumentation and working of UV spectroscopy

Instrumentation and working of the UV spectrometers can be studied simultaneously. Most of the modern UV spectrometers consist of the following parts-

Light Source- Tungsten filament lamps and Hydrogen-Deuterium lamps are most widely used and suitable light source as they cover the whole UV region. Tungsten filament lamps are rich in red radiations; more specifically they emit the radiations of 375 nm, while the intensity of Hydrogen-Deuterium lamps falls below 375 nm.

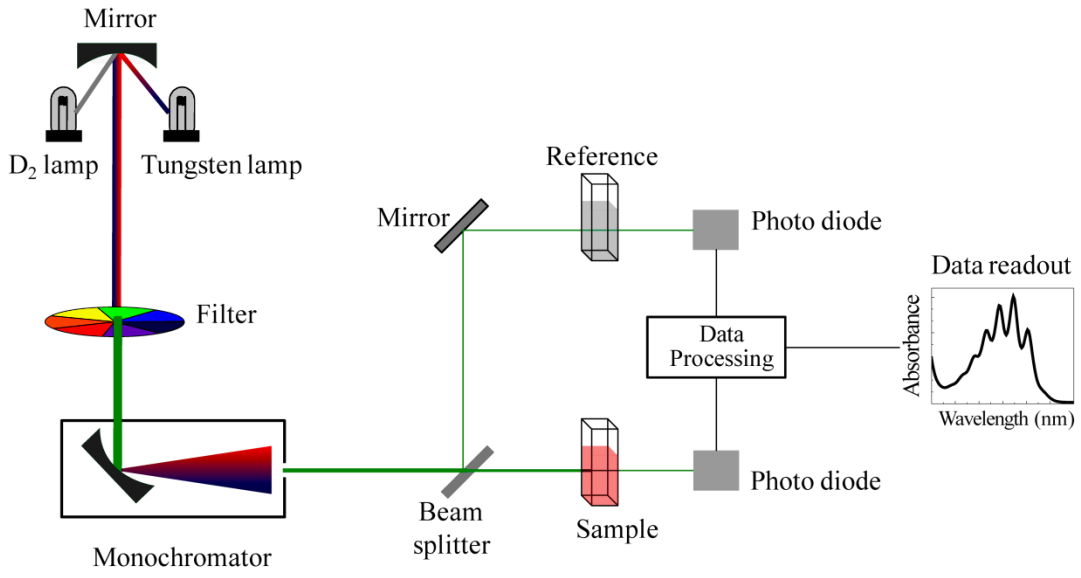
Monochromator- Monochromators generally composed of prisms and slits. The most of the spectrophotometers are **double beam spectrophotometers**. The radiation emitted from the primary source is dispersed with the help of rotating prisms. The various wavelengths of the light source which are separated 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. Glass can't be used for the cells as it also absorbs light in the UV region.

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 the reference. 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. The amplifier is coupled to a small servometer. 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 desired compound.



Procedure

A. Calibrate the Spectrometer

1. Turn on the UV-Vis spectrometer and allow the lamps to warm up for an appropriate period of time (around 20 min) to stabilize them.
2. Fill a cuvette with the solvent for the sample and make sure the outside is clean. This will serve as a blank and help account for light losses due to scattering or absorption by the solvent.
3. Place the cuvette in the spectrometer. Make sure to align the cuvette properly, as often the cuvette has two sides, which are meant for handling (may be grooved) and are not meant to shine light through.
4. Take a reading for the blank. The absorbance should be minimal, but any absorbance should be subtracted out from future samples. Some instruments might store the blank data and perform the subtraction automatically.

B. Perform an Absorbance Spectrum

1. Fill the cuvette with the sample. To make sure the transfer is quantitative, rinse the cuvette twice with the sample and then fill it about ¾ full. Make sure the outside is clean of any fingerprints, etc.
2. Place the cuvette in the spectrometer in the correct direction.
3. Cover the cuvette to prevent any ambient light.
4. Collect an absorbance spectrum by allowing the instrument to scan through different wavelengths and collect the absorbance. The wavelength range can be set with information about the specific sample, but a range of 200–800 nm is standard. A diode-array instrument is able to collect an entire absorbance spectrum in one run.
5. From the collected absorbance spectrum, determine the absorbance maximum (λ_{max}). Repeat the collection of spectra to get an estimate of error in λ_{max}.
6. To make a calibration curve, collect the UV-Vis spectrum of a variety of different concentration samples. Spectrometers are often limited in linear range and will not be able to measure an absorbance value greater than 1.5. If the absorbance values for the sample are outside the instrument's linear range, dilute the sample to get the values within the linear range.

C. Kinetics Experiments with UV-Vis Spectroscopy

1. UV-Vis can be used for kinetics experiments by examining the change in absorbance over time. For a kinetics experiment, take an initial reading of the sample.
2. Quickly add the reagent to start the chemical reaction.
3. Stir it well to mix with the sample. If a small amount is added, this could be done in a cuvette. Alternatively, mix the reagent with sample and quickly pour some in a cuvette for a measurement.
4. Measure the absorbance at the λ_{max} for the analyte of interest over time. If using up the reagent being measuring (*i.e.* absorbance is going up because there is less reagent to absorb), then the decay will indicate the order of the reaction.
5. Using a calibration curve, make a plot of analyte concentration vs time, converting the absorbance value into concentration. From there, this graph can be fit with appropriate equations to determine the reaction rate constants.

Applications of UV spectroscopy

1. <u>Detection of functional groups</u>- UV spectroscopy is used to detect the presence or absence of chromophore in the compound. This is technique is not useful for the detection of chromophore in complex compounds. The absence of a band at a particular band can be seen as an evidence for the absence of a particular group. If the spectrum of a compound comes out to be transparent above 200 nm than it confirms the absence of –

a) Conjugation b) A carbonyl group c) Benzene or aromatic compound d) Bromo or iodo atoms.

2. <u>Detection of extent of conjugation</u>- The extent of conjugation in the polyenes can be detected with the help of UV spectroscopy. With the increase in double bonds the absorption shifts towards the longer wavelength. If the double bond is increased by 8 in the polyenes then that polyene appears visible to the human eye as the absorption comes in the visible region.

3. <u>Identification of an unknown compound</u>- An unknown compound can be identified with the help of UV spectroscopy. The spectrum of unknown compound is compared with the spectrum of a reference compound and if both the spectrums coincide then it confirms the identification of the unknown substance.

4. <u>Determination of configurations of geometrical isomers</u>- It is observed that cis-alkenes absorb at different wavelength than the trans-alkenes. The two isomers can be distinguished with each other when one of the isomers has non-coplanar structure due to steric hindrances. The cis-isomer suffers distortion and absorbs at lower wavelength as compared to trans-isomer.

5. <u>Determination of the purity of a substance</u>- Purity of a substance can also be determined with the help of UV spectroscopy. The absorption of the sample solution is compared with the absorption of the reference solution. The intensity of the absorption can be used for the relative calculation of the purity of the sample substance.

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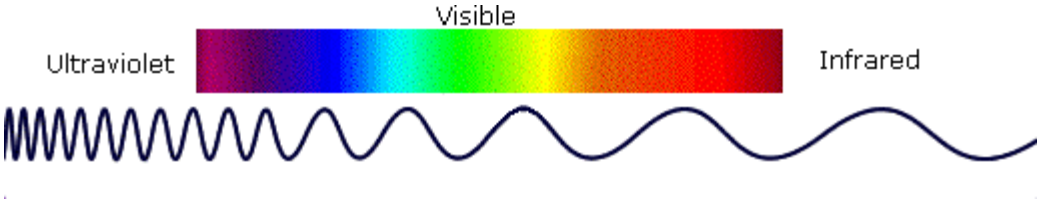
❖ **Infrared spectroscopy** (**IR spectroscopy** or **vibrational spectroscopy**)

Infrared spectroscopy (**IR spectroscopy** or **vibrational spectroscopy**) involves the interaction of infrared radiation with matter. It covers a range of techniques, mostly based on absorption spectroscopy. As with all spectroscopic techniques, it can be used to identify and study chemicals. Samples may be solid, liquid, or gas. The method or technique of infrared spectroscopy is conducted with an instrument called an **infrared spectrometer** (or spectrophotometer) to produce an **infrared spectrum**. An IR spectrum is essentially a graph of infrared light absorbance (or transmittance) on the vertical axis vs. frequency or wavelength on the horizontal axis. Typical units of frequency used in IR spectra are reciprocal centimeters (sometimes called wave numbers), with the symbol cm^{-1}. Units of IR wavelength are commonly given in micrometers (formerly called "microns"), symbol μm, which are related to wave numbers in a reciprocal way. A common laboratory instrument that uses this technique is a Fourier transform infrared (FTIR) spectrometer. Two-dimensional IR is also possible as discussed below.

The infrared portion of the electromagnetic spectrum is usually divided into three regions; the near-, mid- and far- infrared, named for their relation to the visible spectrum. The higher-energy near-IR, approximately 14000–4000 cm^{-1} (0.8–2.5 μm wavelength) can excite overtone or harmonic vibrations. The mid-infrared, approximately 4000–400 cm^{-1} (2.5–25 μm) may be used to study the fundamental vibrations and associated rotational-vibrational structure. The far-infrared, approximately 400–10 cm^{-1} (25–1000 μm), lying adjacent to the microwave region, has low energy and may be used for rotational spectroscopy. The names and classifications of these subregions are conventions, and are only loosely based on the relative molecular or electromagnetic properties.

On the immediate high energy side of the visible spectrum lies the ultraviolet, and on the low energy side is the infrared. The portion of the infrared region most useful for analysis of organic compounds is not immediately adjacent to the visible spectrum, but is that having a wavelength range from 2,500 to 16,000 nm, with a corresponding frequency range from $1.9*10^{13}$ to $1.2*10^{14}$ Hz.



Photon energies associated with this part of the infrared (from 1 to 15 kcal/mole) are not large enough to excite electrons, but may induce vibrational excitation of covalently bonded atoms and groups. The covalent bonds in molecules are not rigid sticks or rods, such as found in molecular model kits, but are more like stiff springs that can be stretched and bent. The mobile nature of organic molecules was noted in the chapter concerning conformational isomers. We must now recognize that, in addition to the facile rotation of groups about single bonds, molecules experience a wide variety of vibrational motions, characteristic of their component atoms. Consequently, virtually all organic compounds will absorb infrared radiation that corresponds in energy to these vibrations. Infrared spectrometers, similar in principle to the UV-Visible spectrometer described elsewhere, permit chemists to obtain absorption spectra of compounds that are a unique reflection of their molecular structure

Theory

Infrared spectroscopy exploits the fact that molecules absorb frequencies that are characteristic of their structure. These absorptions occur at resonant frequencies, i.e. the frequency of the absorbed radiation matches the vibrational frequency. The energies are affected by the shape of the molecular potential energy surfaces, the masses of the atoms, and the associated vibronic coupling.

In particular, in the Born–Oppenheimer and harmonic approximations, i.e. when the molecular Hamiltonian corresponding to the electronic ground state can be approximated by a harmonic oscillator in the neighborhood of the equilibrium molecular geometry, the resonant frequencies are associated with the normal modes corresponding to the molecular electronic ground state potential energy surface. The resonant frequencies are also related to the strength of the bond and the mass of the atoms at either end of it. Thus, the frequency of the vibrations are associated with a particular normal mode of motion and a particular bond type.

Infrared Spectroscopy

- Spectroscopy is based upon the idea of measuring the energy needed to produce a change from one energy level to another.
- The energy possessed by chemical particles is quantised; there can only be a number of definite energy values, rather than a whole range of energy values.

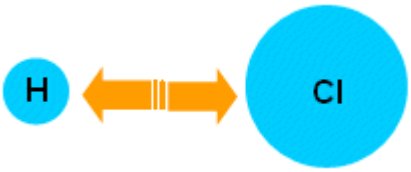
- Infrared spectroscopy takes advantage of the **vibrational energy changes** that occur in molecules when they are exposed to infrared radiation (10^{14} – 10^{13} Hz).
- Frequency and Wavelength are related to each other:

  C = λv
  (C = Speed of Light)

- The **Wavenumber** is the reciprocal of the wavelength (**1/** λ), and thus is a direct measure of the frequency.

Interaction between Infrared radiation and molecules

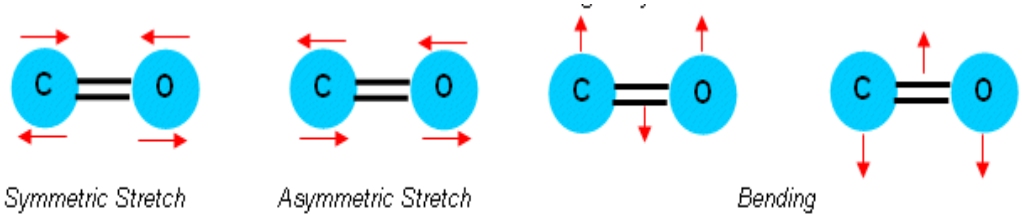
- The bonds in simple diatomic molecules (HCl, HI, HBr…) can only vibrate by stretching:



- For these molecules there is only one infrared absorption. The energy of this corresponds to the molecules changing from their lowest vibrational energy level, to the next higher one.
- The energy needed to excite a vibration is different for each molecule; this is because this energy is related to the bond strength (weaker bonds require less energy to vibrate than stronger ones).
- Thus the frequencies of infrared absorption differ for each molecule.

Compound	Bond energy KJ mol^{-1}	I.R. Absorption cm^{-1}
HCl	432	2886
HBr	366	2559
HI	298	2230

- In more complex molecules, there are more ways in which bond deformations are possible. Most of these more complex molecules involve more than two atoms.
- Carbon dioxide can vibrate in the following ways:



- Below 1500cm^{-1} there is a very detailed "fingerprint" region which can be used with a database of infrared spectra for identification by comparison.
- Even more complex molecules have more vibrational modes. These are sometimes labelled by the descriptions (rocking, scissoring, twisting and wagging).
- All these vibrational modes and the fact that different bonds have different energies lead to infrared spectrums being very complicated.
- Not all of the spectrum has to be identified however, only one or two peaks may need to be identified.
- The peaks are characteristic of specific bonds.
- Below is the infrared spectrum of ethanol with some identified fingerprints:

Sample preparation

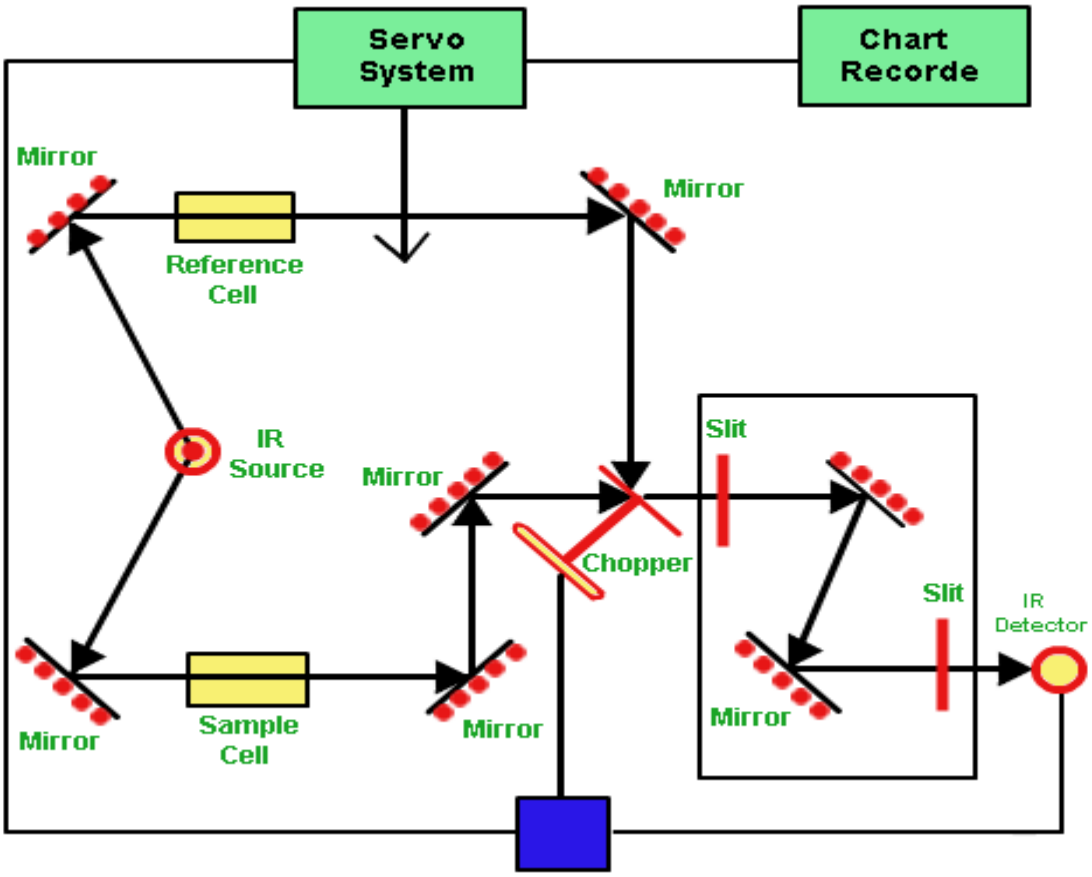
Gaseous samples require a sample cell with a long pathlength to compensate for the diluteness. The pathlength of the sample cell depends on the concentration of the compound of interest. A simple glass tube with length of 5 to 10 cm equipped with infrared-transparent windows at the both ends of the tube can be used for concentrations down to several hundred ppm. Sample gas concentrations well below ppm can be measured with a White's cell in which the infrared light is guided with mirrors to travel through the gas. White's cells are available with optical pathlength starting from 0.5 m up to hundred meters.

Liquid samples can be sandwiched between two plates of a salt (commonly sodium chloride, or common salt, although a number of other salts such as potassium bromide or calcium fluoride are also used).[2] The plates are transparent to the infrared light and do not introduce any lines onto the spectra.

Solid samples can be prepared in a variety of ways. One common method is to crush the sample with an oily mulling agent (usually mineral oil Nujol). A thin film of the mull is applied onto salt plates and measured. The second method is to grind a quantity of the sample with a specially purified salt (usually potassium bromide) finely (to remove scattering effects from large crystals). This powder mixture is then pressed in a mechanical press to form a translucent pellet through which the beam of the spectrometer can pass.[2] A third technique

is the "cast film" technique, which is used mainly for polymeric materials. The sample is first dissolved in a suitable, non hygroscopic solvent. A drop of this solution is deposited on surface of KBr or NaCl cell. The solution is then evaporated to dryness and the film formed on the cell is analysed directly. Care is important to ensure that the film is not too thick otherwise light cannot pass through. This technique is suitable for qualitative analysis. The final method is to use microtomy to cut a thin (20–100 μm) film from a solid sample. This is one of the most important ways of analysing failed plastic products for example because the integrity of the solid is preserved.

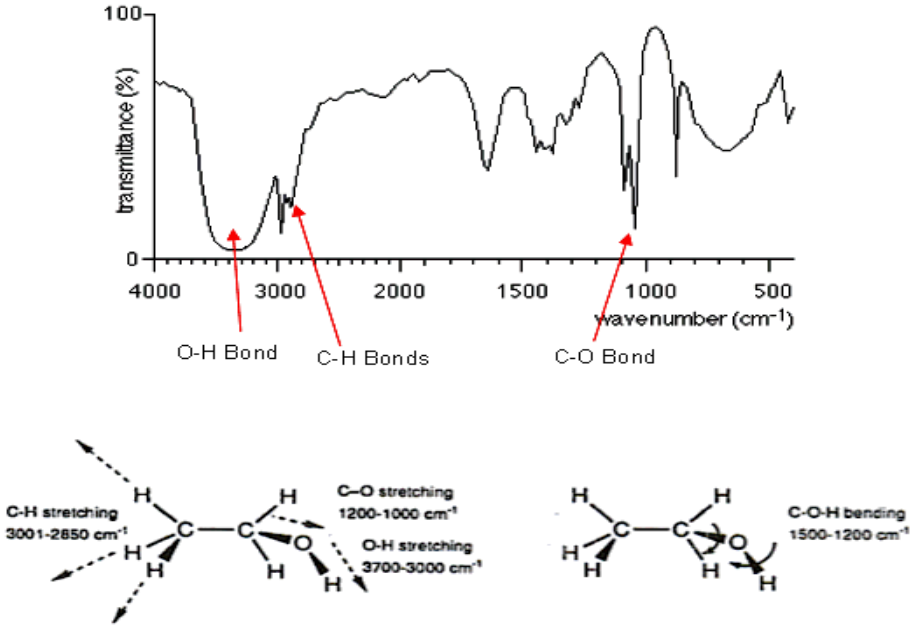
Comparing to a reference



Schematics of a two-beam absorption spectrometer.

A beam of infrared light is produced, passed through an interferometer (not shown), and then split into two separate beams. One is passed through the sample, the other passed through a reference. The beams are both reflected back towards a detector, however first they pass through a splitter, which quickly alternates which of the two beams enters the detector. The two signals are then

compared and a printout is obtained. This "two-beam" setup gives accurate spectra even if the intensity of the light source drifts over time.

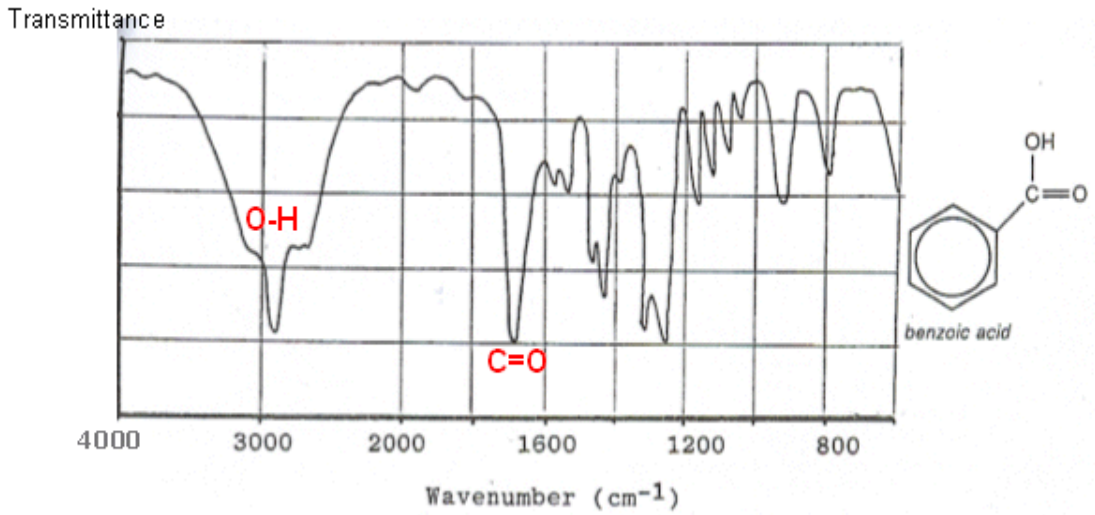


How the Infrared Spectrometer works

- Infrared radiation from a heat source is split into two beams.
- One of the beams goes through the sample, and the other goes through a reference chamber, ensuring that absorptions from water and carbon dioxide in the air are cancelled out.
- The beams are then sent along the same path, in separate alternating pulses, achieved using a beam chopper (rotating disc with segment cut out of it).
- The beams are then analysed by passing them through a sample of sodium chloride or potassium chloride (both invisible to IR radiation) or through a diffraction grating.
- Only light of one particular frequency is allowed to focus onto the detector at one time.
- The spectrum is achieved by scanning the frequencies and recording the associated light intensities.
- When the sample is not absorbing, there will be no difference between the alternating pulses reaching the detector, so no signal is recorded.
- When a vibration is being excited, the sample beam intensity will be reduced and a signal generated.
- The record of an IR spectrum seems to be upside down (the baseline is at the top); however it is transmittance that is being recorded and this is at a maximum when no light is being absorbed.

Example analysis of a compound

The infrared spectrum of benzoic acid is shown below:



1. An O-H group is present in the molecule (identified by the region between 2500-3300 cm^{-1})
2. Absorption between 1680-1750 shows that a C=O is present.
3. Absorption around 1300 cm^{-1} again shows the presence of an OH group.
4. Absorption around 900-1100 cm^{-1} could be due to the benzene ring or to the carbon-oxygen bond of the acid grouping.

APPLICATIONS OF IR SPECTROSCOPY

Infrared spectroscopy is widely used in industry as well as in research. It is a simple and reliable technique for measurement, quality control and dynamic measurement. It is also employed in forensic analysis in civil and criminal analysis.

Some of the major applications of IR spectroscopy are as follows:

1. Identification of functional group and structure elucidation

Entire IR region is divided into group frequency region and fingerprint region. Range of group frequency is 4000-1500 cm^{-1} while that of finger print region is 1500-400 cm^{-1}.

In group frequency region, the peaks corresponding to different functional groups can be observed. According to corresponding peaks, functional group can be determined.

Each atom of the molecule is connected by bond and each bond requires different IR region so characteristic peaks are observed. This region of IR spectrum is called as finger print region of the molecule. It can be determined by characteristic peaks.

2. Identification of substances

IR spectroscopy is used to establish whether a given sample of an organic substance is identical with another or not. This is because large number of absorption bands is observed in the IR spectra of organic molecules and the probability that any two compounds will produce identical spectra is almost zero. So if two compounds have identical IR spectra then both of them must be samples of the same substances.

IR spectra of two enatiomeric compound are identical. So IR spectroscopy fails to distinguish between enantiomers.

For example, an IR spectrum of benzaldehyde is observed as follows.

C-H stretching of aromatic ring- 3080 cm^{-1}

C-H stretching of aldehyde- 2860 cm^{-1} and 2775 cm^{-1}

C=O stretching of an aromatic aldehyde- 1700 cm^{-1}

C=C stretching of an aromatic ring- 1595 cm^{-1}

C-H bending- 745 cm^{-1} and 685 cm^{-1}

No other compound then benzaldehyde produces same IR spectra as shown above.

3. Studying the progress of the reaction

Progress of chemical reaction can be determined by examining the small portion of the reaction mixure withdrawn from time to time. The rate of disappearance

of a characteristic absorption band of the reactant group and/or the rate of appearance of the characteristic absorption band of the product group due to formation of product is observed.

4. Detection of impurities

IR spectrum of the test sample to be determined is compared with the standard compound. If any additional peaks are observed in the IR spectrum, then it is due to impurities present in the compound.

5. Quantitative analysis

The quantity of the substance can be determined either in pure form or as a mixure of two or more compounds. In this, characteristic peak corresponding to the drug substance is chosen and log I0/It of peaks for standard and test sample is compared. This is called base line technique to determine the quantity of the substance.

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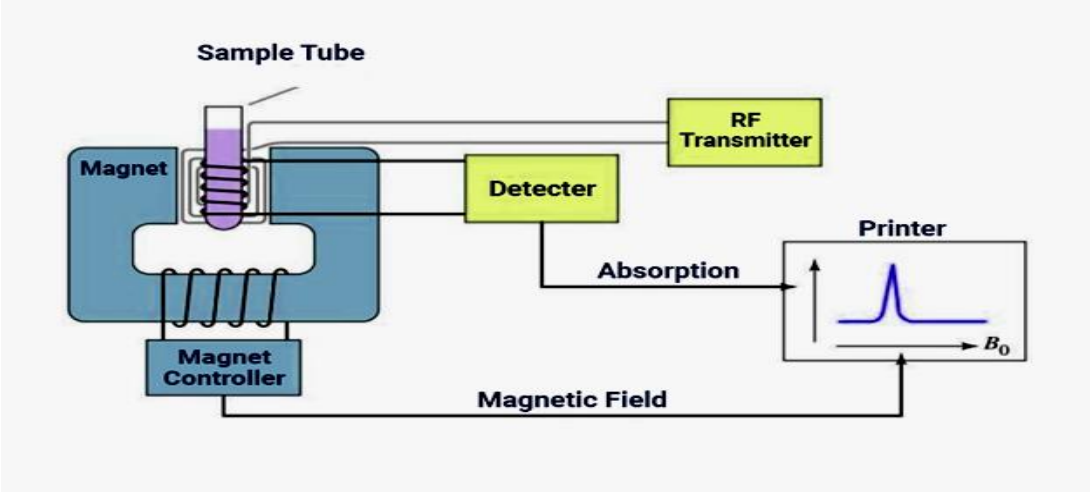
❖ **Nuclear magnetic resonance spectroscopy**

Nuclear magnetic resonance spectroscopy, most commonly known as **NMR spectroscopy** or **magnetic resonance spectroscopy** (**MRS**), is a research technique that exploits the magnetic properties of certain atomic nuclei. This type of spectroscopy determines the physical and chemical properties of atoms or the molecules in which they are contained. It relies on the phenomenon of nuclear magnetic resonance and can provide detailed information about the structure, dynamics, reaction state, and chemical environment of molecules. The intramolecular magnetic field around an atom in a molecule changes the resonance frequency, thus giving access to details of the electronic structure of a molecule and its individual functional groups.

Most frequently, NMR spectroscopy is used by chemists and biochemists to investigate the properties of organic molecules, although it is applicable to any kind of sample that contains nuclei possessing spin. Suitable samples range from small compounds analyzed with 1-dimensional proton or carbon-13 NMR spectroscopy to large proteins or nucleic acids using 3 or 4-dimensional techniques. The impact of NMR spectroscopy on the sciences has been substantial because of the range of information and the diversity of samples, including solutions and solids.



Resonant frequency

When placed in a magnetic field, NMR active nuclei (such as ^{1}H or ^{13}C) absorb electromagnetic radiation at a frequency characteristic of the isotope.[3] The resonant frequency, energy of the absorption, and the intensity of the signal are proportional to the strength of the magnetic field. For example, in a 21 Tesla magnetic field, protons resonate at 900 MHz. It is common to refer to a 21 T magnet as a 900 MHz magnet, although different nuclei resonate at a different frequency at this field strength in proportion to their nuclear magnetic moments.

Sample handling

An NMR spectrometer typically consists of a spinning sample-holder inside a very strong magnet, a radio-frequency emitter and a receiver with a probe (an antenna assembly) that goes inside the magnet to surround the sample, optionally gradient coils for diffusion measurements and electronics to control the system. Spinning the sample is necessary to average out diffusional motion. Whereas, measurements of diffusion constants (*diffusion ordered spectroscopy* or DOSY) are done the sample stationary and spinning off, and flow cells can be used for online analysis of process flows.

NMR Spectroscopy Techniques

Resonant frequency: It refers to the energy of the absorption, and the intensity of the signal that is proportional to the strength of the magnetic field. NMR active nuclei absorb electromagnetic radiation at a frequency characteristic of the isotope when placed in a magnetic field.

Acquisition of spectra: Upon excitation of the sample with radio frequency pulse, a nuclear magnetic resonance response is obtained. It is a very weak signal and requires sensitive radio receivers to pick up.

Chemical shift: A spinning charge generates a magnetic field that results in a magnetic moment proportional to the spin. In the presence of an external magnetic field, two spin states exist; one spin up and one spin down, where one aligns with the magnetic field and the other opposes it.

Deuterated solvents

The vast majority of nuclei in a solution would belong to the solvent, and most regular solvents are hydrocarbons and would contain NMR-reactive protons. Thus, deuterium (hydrogen-2) is substituted (99+%). The most used deuterated solvent is deuterochloroform ($CDCl_3$), although deuterium oxide (D_2O) and deuterated DMSO (DMSO-d_6) are used for hydrophilic analytes and deuterated benzene is also common. The chemical shifts are slightly different in different solvents, depending on electronic solvation effects. NMR spectra are often calibrated against the known solvent residual proton peak instead of added tetramethylsilane.

Shim and lock

To detect the very small frequency shifts due to nuclear magnetic resonance, the applied magnetic field must be constant throughout the sample volume. High resolution NMR spectrometers use shims to adjust the homogeneity of the magnetic field to parts per billion (ppb) in a volume of a few cubic centimeters. In order to detect and compensate for inhomogeneity and drift in the magnetic field, the spectrometer maintains a "lock" on the solvent deuterium frequency with a separate lock unit. In modern NMR spectrometers shimming is adjusted automatically, though in some cases the operator has to optimize the shim parameters manually to obtain the best possible resolution.[6][7]

Acquisition of spectra

Upon excitation of the sample with a radio frequency (60–1000 MHz) pulse, a nuclear magnetic resonance response - a free induction decay (FID) - is obtained. It is a very weak signal, and requires sensitive radio receivers to pick up. A Fourier transform is carried out to extract the frequency-domain spectrum from the raw time-domain FID. A spectrum from a single FID has a low signal-to-noise ratio, but fortunately it improves readily with averaging of repeated acquisitions. Good ^{1}H NMR spectra can be acquired with 16 repeats, which takes only minutes. However, for elements heavier than hydrogen, the

relaxation time is rather long, e.g. around 8 seconds for ^{13}C. Thus, acquisition of quantitative heavy-element spectra can be time-consuming, taking tens of minutes to hours.

If the second excitation pulse is sent prematurely before the relaxation is complete, the average magnetization vector still points in a nonparallel direction, giving suboptimal absorption and emission of the pulse. In practice, the peak areas are then not proportional to the stoichiometry; only the presence, but not the amount of functional groups is possible to discern. An inversion recovery experiment can be done to determine the relaxation time and thus the required delay between pulses. A 180° pulse, an adjustable delay, and a 90° pulse is transmitted. When the 90° pulse exactly cancels out the signal, the delay corresponds to the time needed for 90° of relaxation.[8] Inversion recovery is worthwhile for quantitive ^{13}C, 2D and other time-consuming experiments.

Chemical shift

A spinning charge generates a magnetic field that results in a magnetic moment proportional to the spin. In the presence of an external magnetic field, two spin states exist (for a spin 1/2 nucleus): one spin up and one spin down, where one aligns with the magnetic field and the other opposes it. The difference in energy (ΔE) between the two spin states increases as the strength of the field increases, but this difference is usually very small, leading to the requirement for strong NMR magnets (1-20 T for modern NMR instruments). Irradiation of the sample with energy corresponding to the exact spin state separation of a specific set of nuclei will cause excitation of those set of nuclei in the lower energy state to the higher energy state.

For spin 1/2 nuclei, the energy difference between the two spin states at a given magnetic field strength is proportional to their magnetic moment. However, even if all protons have the same magnetic moments, they do not give resonant signals at the same frequency values. This difference arises from the differing electronic environments of the nucleus of interest. Upon application of an external magnetic field, these electrons move in response to the field and generate local magnetic fields that oppose the much stronger applied field. This local field thus "shields" the proton from the applied magnetic field, which must therefore be increased in order to achieve resonance (absorption of rf energy). Such increments are very small, usually in parts per million (ppm). For instance, the proton peak from an aldehyde is shifted ca. 10 ppm compared to a hydrocarbon peak, since as an electron-withdrawing group, the carbonyl deshields the proton by reducing the local electron density. The difference between 2.3487 T and 2.3488 T is therefore about 42 ppm. However a frequency scale is commonly used to designate the NMR signals, even though

the spectrometer may operate by sweeping the magnetic field, and thus the 42 ppm is 4200 Hz for a 100 MHz reference frequency (rf).

However given that the location of different NMR signals is dependent on the external magnetic field strength and the reference frequency, the signals are usually reported relative to a reference signal, usually that of TMS (tetramethylsilane). Additionally, since the distribution of NMR signals is field dependent, these frequencies are divided by the spectrometer frequency. However, since we are dividing Hz by MHz, the resulting number would be too small, and thus it is multiplied by a million. This operation therefore gives a locator number called the "chemical shift" with units of parts per million.[9] In general, chemical shifts for protons are highly predictable since the shifts are primarily determined by simpler shielding effects (electron density), but the chemical shifts for many heavier nuclei are more strongly influenced by other factors including excited states ("paramagnetic" contribution to shielding tensor).

The chemical shift provides information about the structure of the molecule. The conversion of the raw data to this information is called *assigning* the spectrum. For example, for the ^{1}H-NMR spectrum for ethanol (CH_3CH_2OH), one would expect signals at each of three specific chemical shifts: one for the C*H*$_3$ group, one for the C*H*$_2$ group and one for the O*H* group. A typical CH_3 group has a shift around 1 ppm, a CH_2 attached to an OH has a shift of around 4 ppm and an OH has a shift anywhere from 2–6 ppm depending on the solvent used and the amount of hydrogen bonding. While the O atom does draw electron density away from the attached H through their mutual sigma bond, the electron lone pairs on the O bathe the H in their shielding effect.

NMR Spectroscopy Uses

NMR spectroscopy is used by chemists and biochemists to investigate the properties of organic molecules, although it is applicable to any kind of sample that contains nuclei possessing spin. For example, the NMR can quantitatively analyze mixtures containing known compounds. NMR can either be used to match against spectral libraries or to infer the basic structure directly for unknown compounds.

Once the basic structure is known, NMR can be used to determine molecular conformation in solutions as well as in studying physical properties at the molecular level such as conformational exchange, phase changes, solubility, and diffusion.

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❖ Fluorescence spectroscopy

Fluorescence spectroscopy (also known as **fluorometry** or **spectrofluorometry**) is a type of electromagnetic spectroscopy that analyzes fluorescence from a sample. It involves using a beam of light, usually ultraviolet light, that excites the electrons in molecules of certain compounds and causes them to emit light; typically, but not necessarily, visible light. A complementary technique is absorption spectroscopy. In the special case of single molecule fluorescence spectroscopy, intensity fluctuations from the emitted light are measured from either single fluorophores, or pairs of fluorophores.

Devices that measure fluorescence are called fluorometers.

Theory

Molecules have various states referred to as energy levels. Fluorescence spectroscopy is primarily concerned with electronic and vibrational states. Generally, the species being examined has a ground electronic state (a low energy state) of interest, and an excited electronic state of higher energy. Within each of these electronic states there are various vibrational states.[1]

In fluorescence, the species is first excited, by absorbing a photon, from its ground electronic state to one of the various vibrational states in the excited electronic state. Collisions with other molecules cause the excited molecule to lose vibrational energy until it reaches the lowest vibrational state of the excited electronic state.

The molecule then drops down to one of the various vibrational levels of the ground electronic state again, emitting a photon in the process.[1] As molecules may drop down into any of several vibrational levels in the ground state, the emitted photons will have different energies, and thus frequencies. Therefore, by analysing the different frequencies of light emitted in fluorescent spectroscopy, along with their relative intensities, the structure of the different vibrational levels can be determined.

For atomic species, the process is similar; however, since atomic species do not have vibrational energy levels, the emitted photons are often at the same wavelength as the incident radiation. This process of re-emitting the absorbed photon is "resonance fluorescence" and while it is characteristic of atomic fluorescence, is seen in molecular fluorescence as well.[2]

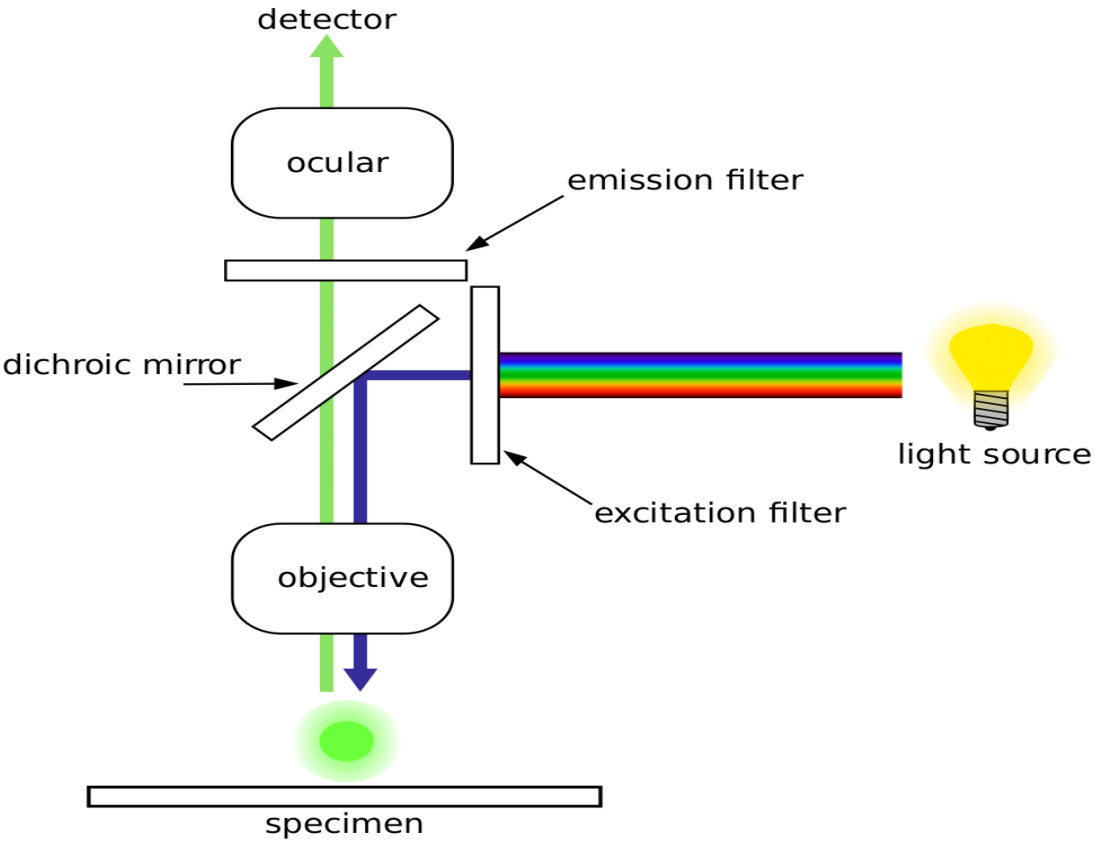
In a typical fluorescence (emission) measurement, the excitation wavelength is fixed and the detection wavelength varies, while in a fluorescence excitation measurement the detection wavelength is fixed and the excitation

wavelength is varied across a region of interest. An **emission map** is measured by recording the emission spectra resulting from a range of excitation wavelengths and combining them all together. This is a three dimensional surface data set: emission intensity as a function of excitation and emission wavelengths, and is typically depicted as a contour map.

Instrumentation

Two general types of instruments exist: filter fluorometers that use filters to isolate the incident light and fluorescent light and spectrofluorometers that use a diffraction grating monochromators to isolate the incident light and fluorescent light.

Both types use the following scheme: the light from an excitation source passes through a filter or monochromator, and strikes the sample. A proportion of the incident light is absorbed by the sample, and some of the molecules in the sample fluoresce. The fluorescent light is emitted in all directions. Some of this fluorescent light passes through a second filter or monochromator and reaches a detector, which is usually placed at 90° to the incident light beam to minimize the risk of transmitted or reflected incident light reaching the detector.



A simplistic design of the components of a fluorimeter

Various light sources may be used as excitation sources, including lasers, LED, and lamps; xenon arcs and mercury-vapor lamps in particular. A laser only emits light of high irradiance at a very narrow wavelength interval, typically under 0.01 nm, which makes an excitation monochromator or filter unnecessary. The disadvantage of this method is that the wavelength of a laser cannot be changed by much. A mercury vapor lamp is a line lamp, meaning it emits light near peak wavelengths. By contrast, a xenon arc has a continuous emission spectrum with nearly constant intensity in the range from 300-800 nm and a sufficient irradiance for measurements down to just above 200 nm.

Filters and/or monochromators may be used in fluorimeters. A monochromator transmits light of an adjustable wavelength with an adjustable tolerance. The most common type of monochromator utilizes a diffraction grating, that is, collimated light illuminates a grating and exits with a different angle depending on the wavelength. The monochromator can then be adjusted to select which wavelengths to transmit. For allowing anisotropy measurements, the addition of two polarization filters is necessary: One after the excitation monochromator or filter, and one before the emission monochromator or filter.

As mentioned before, the fluorescence is most often measured at a 90° angle relative to the excitation light. This geometry is used instead of placing the sensor at the line of the excitation light at a 180° angle in order to avoid interference of the transmitted excitation light. No monochromator is perfect and it will transmit some stray light, that is, light with other wavelengths than the targeted. An ideal monochromator would only transmit light in the specified range and have a high wavelength-independent transmission. When measuring at a 90° angle, only the light scattered by the sample causes stray light. This results in a better signal-to-noise ratio, and lowers the detection limit by approximately a factor 10000, when compared to the 180° geometry. Furthermore, the fluorescence can also be measured from the front, which is often done for turbid or opaque samples .

The detector can either be single-channeled or multichanneled. The single-channeled detector can only detect the intensity of one wavelength at a time, while the multichanneled detects the intensity of all wavelengths simultaneously, making the emission monochromator or filter unnecessary. The different types of detectors have both advantages and disadvantages.

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❖ Atomic absorption spectroscopy

Atomic absorption spectroscopy (**AAS**) is a spectroanalytical procedure for the quantitative determination of chemical elements using the absorption of optical radiation (light) by free atoms in the gaseous state.

In analytical chemistry the technique is used for determining the concentration of a particular element (the analyte) in a sample to be analyzed. AAS can be used to determine over 70 different elements in solution or directly in solid samples used in pharmacology, biophysics and toxicology research.

Atomic absorption spectrometry has many uses in different areas of chemistry such as clinical analysis of metals in biological fluids and tissues such as whole blood, plasma, urine, saliva, brain tissue, liver, muscle tissue, semen, in some pharmaceutical manufacturing processes, minute quantities of a catalyst that remain in the final drug product, and analyzing water for its metal content.

Atomic absorption spectrometry has many uses in different areas of chemistry.

1. **Clinical analysis.** Analysing metals in biological fluids such as blood and urine.
2. **Environmental analysis.** Monitoring our environment – *eg* finding out the levels of various elements in rivers, seawater, drinking water, air, petrol and drinks such as wine, beer and fruit drinks.
3. **Pharmaceuticals.** In some pharmaceutical manufacturing processes, minute quantities of a catalyst used in the process (usually a metal) are sometimes present in the final product. By using AAS the amount of catalyst present can be determined.
4. **Industry.** Many raw materials are examined and AAS is widely used to check that the major elements are present and that toxic impurities are lower than specified – *eg* in concrete, where calcium is a major constituent, the lead level should be low because it is toxic.
5. **Mining.** By using AAS the amount of metals such as gold in rocks can be determined to see whether it is worth mining the rocks to extract the gold.

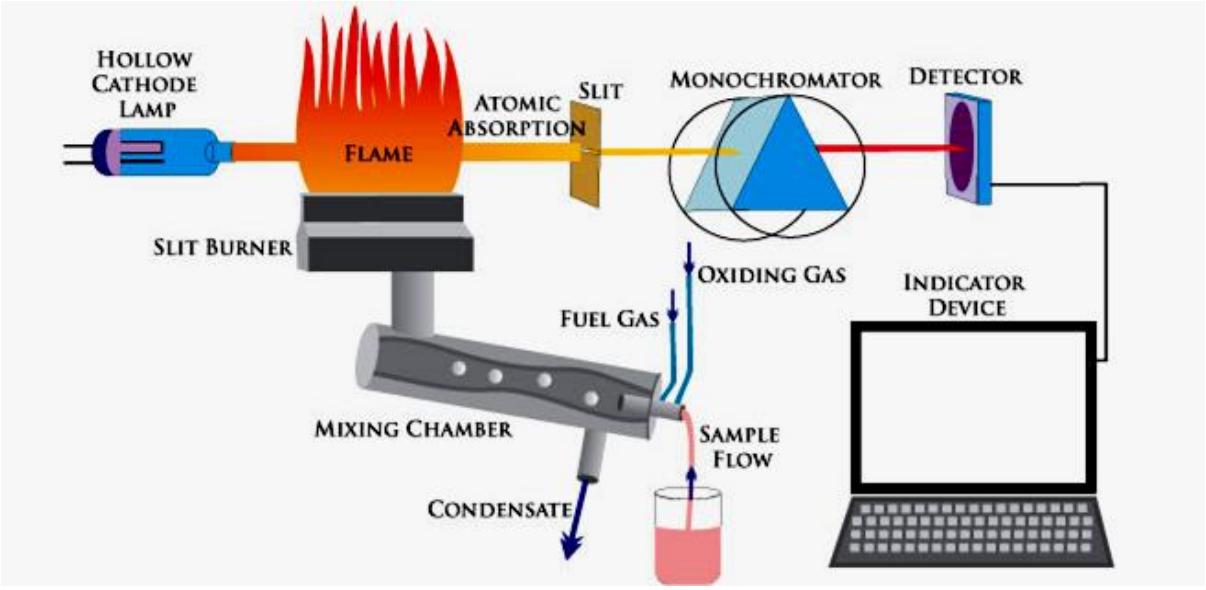
Principle

Atomic absorption methods measure the amount of energy (in the form of photons of light, and thus a change in the wavelength) absorbed by the sample. Specifically, a detector measures the wavelengths of light transmitted by the sample (the "after" wavelengths), and compares them to the wavelengths, which originally passed through the sample (the "before" wavelengths). A signal

processor then integrates the changes in wavelength, which appear in the readout as peaks of energy absorption at discrete wavelengths. Any atom has its own distinct pattern of wavelengths at which it will absorb energy, due to the unique configuration of electrons in its outer shell. This allows for the qualitative analysis of a pure sample.

In order to tell how much of a known element is present in a sample, one must first establish a basis for comparison using known quantities. It can be done producing a calibration curve**.** For this process, a known wavelength is selected, and the detector will measure only the energy emitted at that wavelength. However, as the concentration of the target atom in the sample increases, absorption will also increase proportionally. Thus, one runs a series of known concentrations of some compound, and records the corresponding degree of absorbance, which is an inverse percentage of light transmitted. A straight line can then be drawn between all of the known points. From this line, one can then extrapolate the concentration of the substance under investigation from its absorbance. The use of special light sources and specific wavelength selection allows the quantitative determination of individual components of a multielement mixture.

Atomic absorption analysis involves measuring the absorption of light by vaporized ground state atoms and relating the absorption to concentration. The incident light beam is attenuated by atomic vapor absorption according to Beer's law.



The process of atomic absorption spectroscopy (AAS) involves two steps:

1. Atomization of the sample
2. The absorption of radiation from a light source by the free atoms

The sample, either a liquid or a solid, is atomized in either a flame or a graphite furnace. Upon the absorption of ultraviolet or visible light, the free atoms undergo electronic transitions from the ground state to excited electronic states.

To obtain the best results in AA, the instrumental and chemical parameters of the system must be geared toward the production of neutral ground state atoms of the element of interest. A common method is to introduce a liquid sample into a flame. Upon introduction, the sample solution is dispersed into a fine spray, the spray is then desolvated into salt particles in the flame and the particles are subsequently vaporized into neutral atoms, ionic species and molecular species. All of these conversion processes occur in geometrically definable regions in the flame. It is therefore important to set the instrument parameters such that the light from the source (typically a hollow-cathode lamp) is directed through the region of the flame that contains the maximum number of neutral atoms. The light produced by the hollow-cathode lamp is emitted from excited atoms of the same element which is to be determined. Therefore the radiant energy corresponds directly to the wavelength which is absorbable by the atomized sample. This method provides both sensitivity and selectivity since other elements in the sample will not generally absorb the chosen wavelength and thus, will not interfere with the measurement. To reduce background interference, the wavelength of interest is isolated by a monochromator placed between the sample and the detector.

In atomic absorption there are two methods of adding thermal energy to a sample. A graphite furnace AAS uses a graphite tube with a strong electric current to heat the sample. In flame AAS (see photo above), we aspirate a sample into a flame using a nebulizer. The flame is lined up in a beam of light of the appropriate wavelength. The flame (thermal energy) causes the atom to undergo a transition from the ground state to the first excited state. When the atoms make their transition, they absorb some of the light from the beam. **The more concentrated the solution, the more light energy is absorbed!**

The light beam is generated by lamp that is specific for a target metal. The lamp must be perfectly aligned so the beam crosses the hottest part of the flame. The light passed through the flame is received by the monochromator, which is set to accept and transmit radiation at the specified wavelength and travels into the detector. The detector measures the intensity of the beam of light. When some of the light is absorbed by metal, the beam's intensity is reduced. The detector records that reduction as absorption. That absorption is shown on output device by the data system.

We can find the concentrations of metals in a sample running a series of calibration standards through the instrument. The instrument will record the absorption generated by a given concentration. By plotting the absorption versus the concentrations of the standards, a calibration curve can be plotted. We can then look at the absorption for a sample solution and use the calibration curves to determine the concentration in that

A) Sample preparation

Depending on the information required, total recoverable metals, dissolved metals, suspended metals, and total metals could be obtained from a certain environmental matrix.

B) Calibration and standard curves

As with other analytical techniques, atomic absorption spectrometry requires careful calibration. EPA QA/QC demands calibration through several steps including interference check sample, calibration verification, calibration standards, bland control, and linear dynamic range.

The idealized calibration or standard curve is stated by Beer's law that the absorbance of an absorbing analyte is proportional to its concentration.

Unfortunately, deviations from linearity usually occur, especially as the concentration of metallic analytes increases due to various reasons, such as unabsorbed radiation, stray light, or disproportionate decomposition of molecules at high concentrations.

If the sample concentration is too high to permit accurate analysis in linearity response range, there are three alternatives that may help bring the absorbance into the optimum working range:

1) sample dilution

2) using an alternative wavelength having a lower absorptivity

3) reducing the path length by rotating the burner hand.

C) EPA method for metal analysis

Flame atomic absorption methods are referred to as direct aspiration determinations. They are normally completed as single element analyses and are relatively free of interelement spectral interferences. For some elements, the temperature or type of flame used is critical. If flame and analytical conditions are not properly used, chemical and ionization interferences can occur.

Graphite furnace atomic absorption spectrometry replaces the flame with an electrically heated graphite furnace. The major advantage of this technique is that the detection limit can be extremely low. It is applicable for relatively clean

samples, however, interferences could be a real problem. It is important for the analyst to establish a set of analytical protocol which is appropriate for the sample to be analyzed and for the information required.

D) Interferences

Since the concentration of the analyte element is considered to be proportional to the ground state atom population in the flame, any factor that affects the ground state population of the analyte element can be classified as interference. Factors that may affect the ability of the instrument to read this parameter can also be classified as interference.

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❖ Circular Dichroism (CD) spectroscopy

Circular Dichroism (CD) is an absorption spectroscopy method based on the differential absorption of left and right circularly polarized light. Optically active chiral molecules will preferentially absorb one direction of the circularly polarized light. The difference in absorption of the left and right circularly polarized light can be measured and quantified. UV CD is used to determine aspects of protein secondary structure. Vibrational CD, IR CD, is used to study the structure of small organic molecules, proteins and DNA. UV/Vis CD investigates charge transfer transitions in metal-protein complexes.

Circular Dichroism, an absorption spectroscopy, uses circularly polarized light to investigate structural aspects of optically active chiral media. It is mostly used to study biological molecules, their structure, and interactions with metals and other molecules.

Circular Polarization of Light

Electromagnetic radiation consists of oscillating electric and magnetic fields perpendicular to each other and the direction of propagation. Most light sources emit waves where these fields oscillate in all directions perpendicular to the propagation vector. Linear polarized light occurs when the electric field vector oscillates in only one plane. In circularly polarized light, the electric field vector rotates around the propagation axis maintaining a constant magnitude. When looked at down the axis of propagation the vector appears to trace a circle over the period of one wave frequency (one full rotation occurs in the distance equal to the wavelength). In linear polarized light the direction of the vector stays constant and the magnitude oscillates. In circularly polarized light the magnitude stays constant while the direction oscillates.

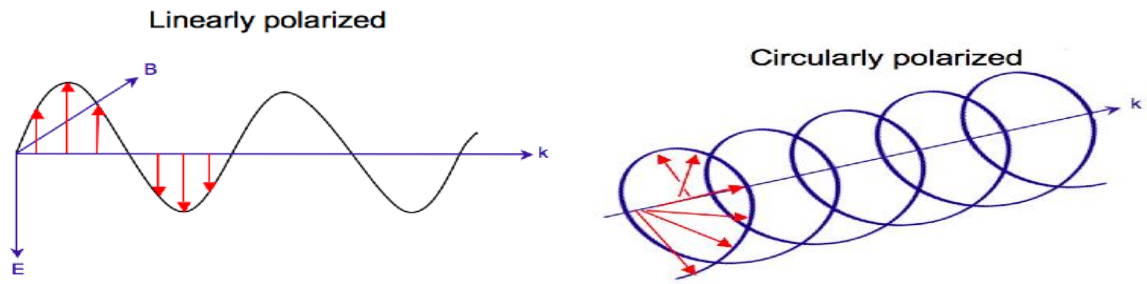


Diagram of linearly polarized and circularly polarized light

As the radiation propagates the electric field vector traces out a helix. The magnetic field vector is out of phase with the electric field vector by a quarter turn. When traced together the vectors form a double helix.

Light can be circularly polarized in two directions: left and right. If the vector rotates counterclockwise when the observer looks down the axis of propagation, the light is left circularly polarized (LCP). If it rotates clockwise, it is right circularly polarized (RCP). If LCP and RCP of the same amplitude, they are superimposed on one another and the resulting wave will be linearly polarized.

As with linear polarized light, circularly polarized light can be absorbed by a medium. An optically active chiral compound will absorb the two directions of circularly polarized light by different amounts.

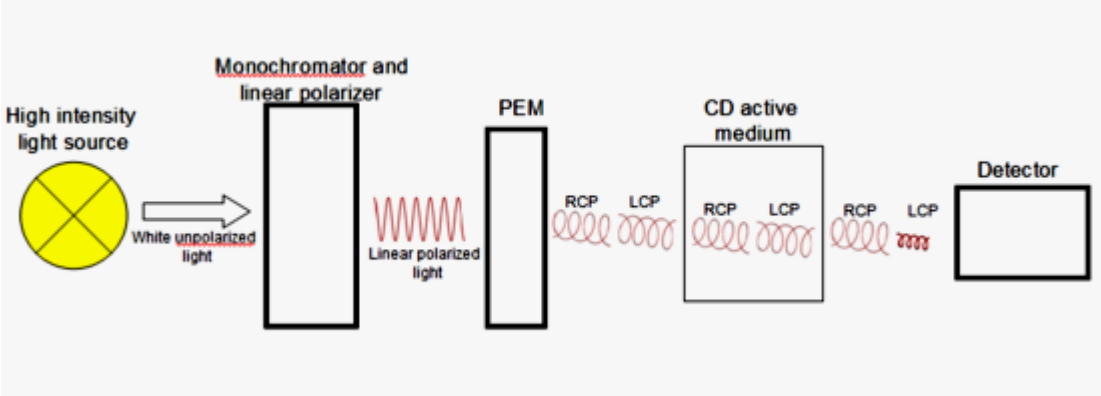
This can be extended to the Beer-Lambert Law. The molar absorpitivty of a medium will be different for LCP and RCP.

The difference in molar absorptivity is also known as the molar circular dichroism. The molar circular dichroism is not only wavelength dependent but also depends on the absorbing molecules conformation, which can make it a function of concentration, temperature, and chemical environment.

Any absorption of light results in a change in amplitude of the incident wave; absorption changes the intensity of the light and intensity of the square of the amplitude. In a chiral medium the molar absorptivities of LCP and RCP light are different so they will be absorbed by the medium in different amounts. This differential absorption results in the LCP and RCP having different amplitudes which means the superimposed light is no longer linearly polarized. The resulting wave is elliptically polarized.

Instrumentation

Most commercial CD instruments are based on the modulation techniques introduced by Grosjean and Legrand. Light is linearly polarized and passed through a monochromator. The single wavelength light is then passed through a modulating device, usually a photoelastic modulator (PEM), which transforms the linear light to circular polarized light. The incident light on the sample switches between LCP and RCP light. As the incident light swtches direction of polarization the absorption changes and the differention molar absorptivity can be calculated.



Applications of CD

1. The most widely used application of CD spectroscopy is identifying structural aspects of proteins and DNA. The peptide bonds in proteins are optically active and the ellipticity they exhibit changes based on the local conformation of the molecule.
2. Secondary structures of proteins can be analyzed using the far-UV (190-250 nm) region of light. The ordered alpha-helices, beta-sheets, beta-turn, and random coil conformations all have characteristic spectra. These unique spectra form the basis for protein secondary structure analysis.
3. The real value in CD comes from the ability to show conformational changes in molecules. It can be used to determine how similar a wild type protein is to mutant or show the extent of denaturation with a change in temperature or chemical environment.
4. It can also provide information about structural changes upon ligand binding. In order to interpret any of this information the spectrum of the native conformation must be determined.
5. Some information about the tertiary structure of proteins can be determined using near-UV spectroscopy. Absorptions between 250-300 nm are due to the dipole orientation and surrounding environment of the aromatic amino acids, phenylalanine, tyrosine, and tryptophan, and cysteine residues which can form disulfide bonds.

6. Near-UV techniques can also be used to provide structural information about the binding of prosthetic groups in proteins.
7. Metal containing proteins can be studied by visible CD spectroscopy. Visible CD light excites the d-d transitions of metals in chiral environments. Free ions in solution will not absorb CD light so the pH dependence of the metal binding and the stoichiometry can be determined.
8. Vibrational CD (VCD) spectroscopy uses IR light to determine 3D structures of short peptides, nucleic acids, and carbohydrates.
9. VCD has been used to show the shape and number of helices in A-, B-, and Z-DNA. VCD is still a relatively new technique and has the potential to be a very powerful tool.

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❖ Optical Rotatory Dispersion (ORD) spectroscopy

Optical rotatory dispersion is the variation in the optical rotation of a substance with a change in the wavelength of light. Optical rotatory dispersion can be used to find the absolute configuration of metal complexes. For example, when plane-polarized white light from an overhead projector is passed through a cylinder of sucrose solution, a spiral rainbow is observed perpendicular to the cylinder.

Optical rotatory dispersion (ORD) is the phenomenon by which the amount of polarisation rotation by a material depends on the wavelength.

These phenomena allow us to study chiral structures. ORD spectroscopy allows us to study protein structure, for example. An instrument which allows the measurement of both transmitted intensity and optical rotation will allow you to measure ORD.

Principles of operation

When white light passes through a polarizer, the extent of rotation of light depends on its wavelength. Short wavelengths are rotated more than longer wavelengths, per unit of distance. Because the wavelength of light determines its color, the variation of color with distance through the tube is observed. This dependence of specific rotation on wavelength is called optical rotatory dispersion. In all materials the rotation varies with wavelength. The variation is caused by two quite different phenomena. The first accounts in most cases for the majority of the variation in rotation and should not strictly be termed rotatory dispersion. It depends on the fact that optical activity is actually circular birefringence. In other words, a substance which is optically active transmits

right circularly polarized light with a different velocity from left circularly polarized light.

In addition to this pseudodispersion which depends on the material thickness, there is a true rotatory dispersion which depends on the variation with wavelength of the indices of refraction for right and left circularly polarized light.

For wavelengths that are absorbed by the optically active sample, the two circularly polarized components will be absorbed to differing extents. This unequal absorption is known as circular dichroism. Circular dichroism causes incident linearly polarized light to become elliptically polarized. The two phenomena are closely related, just as are ordinary absorption and dispersion. If the entire optical rotatory dispersion spectrum is known, the circular dichroism spectrum can be calculated, and vice versa.

Chiral molecules exhibit circular birefringence, which means that a solution of a chiral Substance presents an anisotropic medium through which left circularly polarised (L-CPL) &Right circularly polarised (RCPL) propagate at different speeds. • This leads to optical rotation which is measured by using a polarimeter. • Measuring optical rotation as a function of wavelength is termed Optical rotatory dispersion (ORD) spectroscopy Introduction.

Plane/linearly polarized light • Light from ordinary lamp consists of waves vibrating in many different planes. When it is passed through polaroid lense it is found to vibrate in one plane is said to be plane polarised light or polarised light.

The compounds which are having the ability to rotate the plane of polarised light are called Optically active compounds. • This property of compound is called optical activity. It is measured by Polarimeter.

Compound which rotates plane of polarised light to right(clock wise) is called DEXTROROTATORY & is denoted by (+) sign.If the compound rotates plane of polarised light towards left side (anti clock wise) is called LEVOROTATORY & is denoted by (-) sign.

Enantiomers are optically active compounds. Optically active molecules have different refractive indices, and different extinction coefficients for L and R circularly polarised light. For a compound to be optically active it must be devoid of the following properties – 1. Plane of symmetry (σ) 2. Center of symmetry (i) 3. Alternating rotation – reflection axis of symmetry or an improper axis.

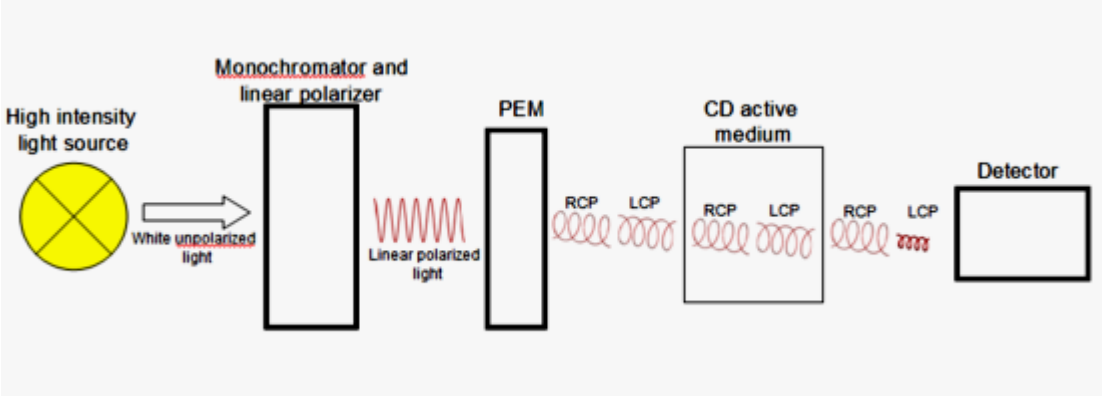
Plane polarized light may be considered as the combination of two circularly polarized light of which one is right circularly polarized light (RCPL) & other is left circularly polarized light (LCPL) which are in equal & opposite in nature. RCPL+LCPL = Plane Polarized light (PPL).

If Refractive index is same for two circularly polarized light then it vibrate in opposite direction with the same angular velocity. • In the below image we can observe the change of E.

Specific rotation: It is defined as the observed angle of optical rotation α when plane-polarized light is passed through a sample with a path length of 1 decimeter and a sample concentration of 1 gram per 1 milliliter. Where, α – Angle of Rotation in degrees l –path length is in decimeters d – Density of liquid is in g 100/ml 1 T– Temperature.

It depends on various factors like: • Nature of substance. • Length of the column. • Concentration. of the solution • Temperature of the sol. • Nature of the solvent. • Wavelength of the light used.

The rotation is the result of an increment in the relative phase of the left and right circularly polarized components in which the plane polarized light can be decomposed. • The increment (which can be either positive or negative) is caused by differences in the interactions of left and right circularly polarized light with a medium consisting of chiral molecules.



APPLICATIONS OF ORD

i. Determination of optically active substance such as amino acids, polypeptides, proteins, steroids, antibiotics, terpenes.
ii. Stereochemistry of Aliphatic amino acids: Aliphatic amino acids show a unique cotton effect. α -amino acids of levo configuration show positive effect around 215nm while dextro enantiomers show negative effect.

iii. Stereochemistry of Steroids: In one form the specific rotation increases with decreasing wave length(positive curve) and in the other form the specific rotation decreases with increasing wave length(negative curve).
iv. Quantitative Analysis: specific rotation is a good measure of concentration. Determination of Absolute configuration. Conformational studies.

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❖ Mass spectroscopy

Mass spectrometry is a powerful analytical technique used to quantify known materials, to identify unknown compounds within a sample, and to elucidate the structure and chemical properties of different molecules. The complete process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances.

This technique basically studies the effect of ionizing energy on molecules. It depends upon chemical reactions in the gas phase in which sample molecules are consumed during the formation of ionic and neutral species.

Basic Principle

A mass spectrometer generates multiple ions from the sample under investigation, it then separates them according to their specific mass-to-charge ratio (m/z), and then records the relative abundance of each ion type.

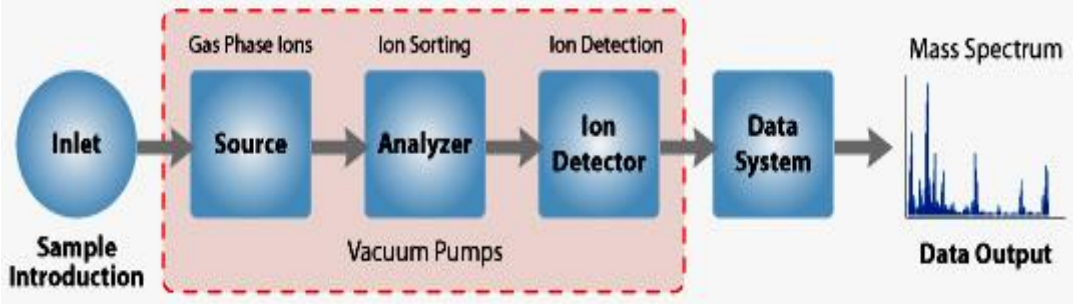
The first step in the mass spectrometric analysis of compounds is the production of gas phase ions of the compound, basically by electron ionization. This molecular ion undergoes fragmentation. Each primary product ion derived from the molecular ion, in turn, undergoes fragmentation, and so on. The ions are separated in the mass spectrometer according to their mass-to-charge ratio, and are detected in proportion to their abundance. A mass spectrum of the molecule is thus produced. It displays the result in the form of a plot of ion abundance versus mass-to-charge ratio. Ions provide information concerning the nature and the structure of their precursor molecule. In the spectrum of a pure compound, the molecular ion, if present, appears at the highest value of m/z (followed by ions containing heavier isotopes) and gives the molecular mass of the compound.

Instrumentation

The instrument consists of three major components:

1. **Ion Source:** For producing gaseous ions from the substance being studied.
2. **Analyzer:** For resolving the ions into their characteristics mass components according to their mass-to-charge ratio.
3. **Detector System:** For detecting the ions and recording the relative abundance of each of the resolved ionic species.

In addition, a sample introduction system is necessary to admit the samples to be studied to the ion source while maintaining the high vacuum requirements (~10-6 to 10-8 mm of mercury) of the technique; and a computer is required to control the instrument, acquire and manipulate data, and compare spectra to reference libraries.

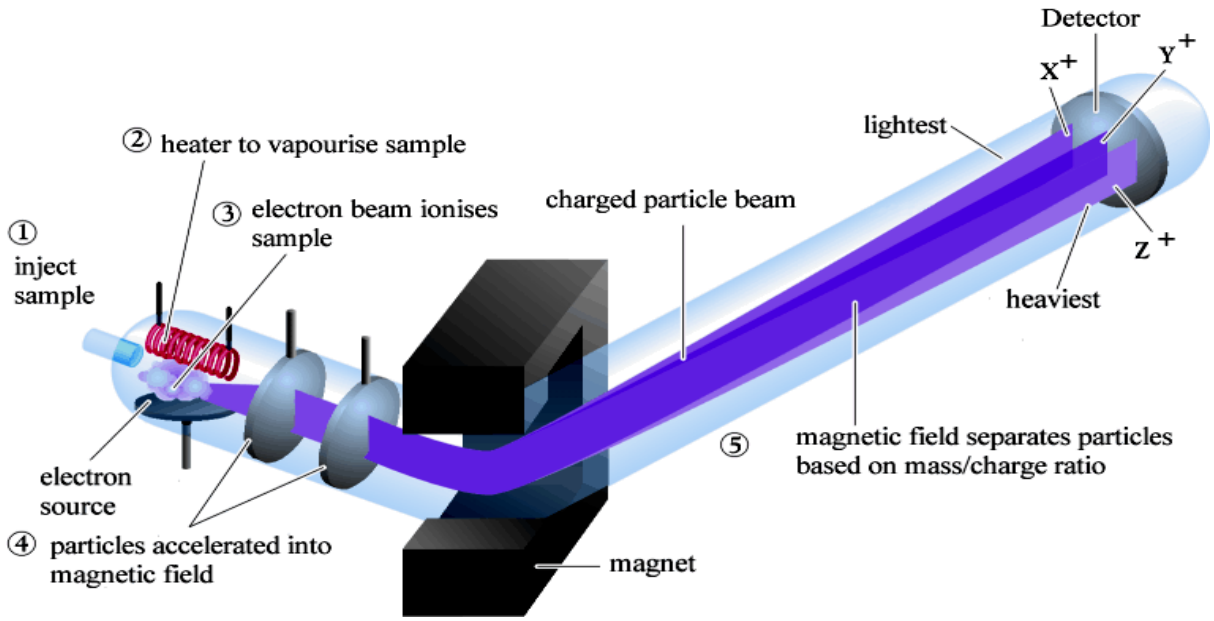


With all the above components, a mass spectrometer should always perform the following processes:

1. Produce ions from the sample in the ionization source.
2. Separate these ions according to their mass-to-charge ratio in the mass analyzer.
3. Eventually, fragment the selected ions and analyze the fragments in a second analyzer.
4. Detect the ions emerging from the last analyzer and measure their abundance with the detector that converts the ions into electrical signals.
5. Process the signals from the detector that are transmitted to the computer and control the instrument using feedback.

- There are four key stages in the process for Mass Spectrometry.

1. Ionization
2. Acceleration
3. Deflection
4. Detection



1. Ionzsation

- Electro Ionisation is the most common type of ionisation.
- The sample is bombarded by electrons which come from a heated filament.
- The electrons run in a stream between the cathode and anode.
- When the sample passes through the electron stream, the high energy electrons in the stream knock electrons out of the sample to form ions.

2. Acceleration

- Acceleration is a simple step where the ions are placed between a set of charges parallel plates.
- The ions will then be repelled by one plate and attracted to the other.
- There is a slit cut in the plate which the ions are attracted to. the force of attraction and repulsion forces the ions through the slit at an accelerated rate.
- The speed of acceleration can be adjusted by changing the charge on the plates.

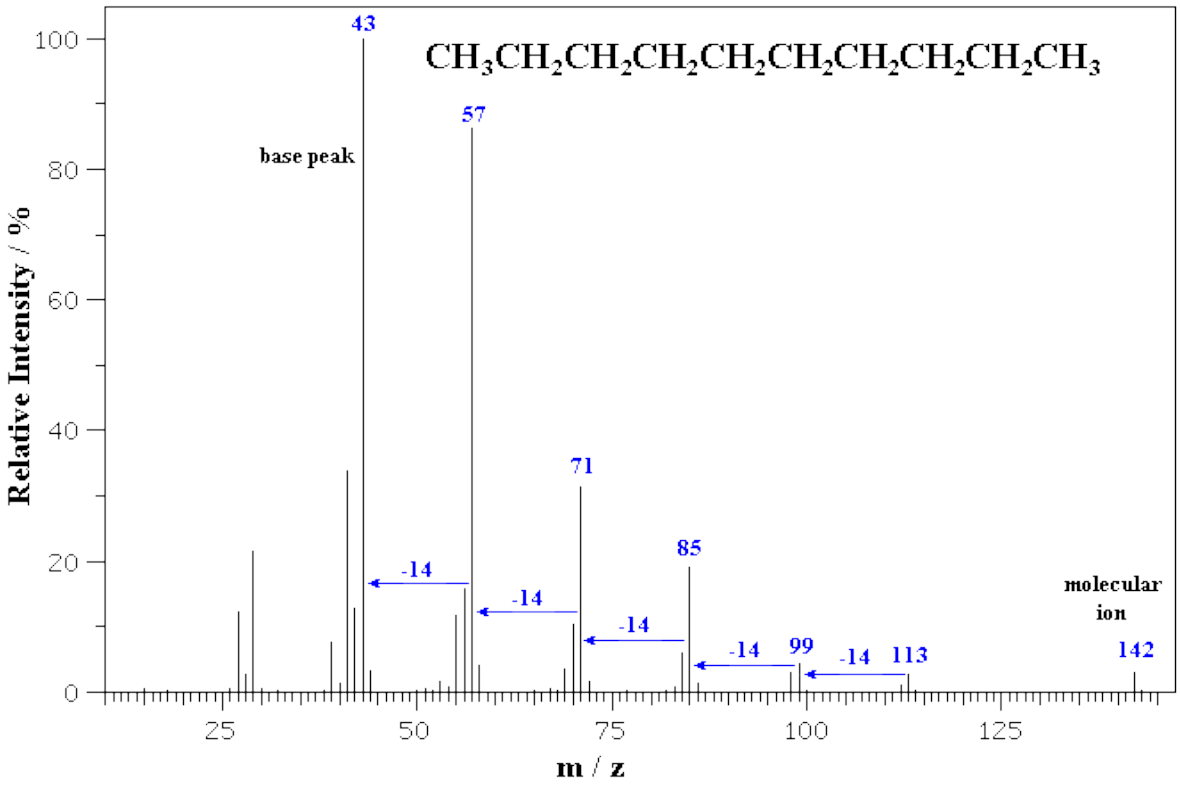
3. Deflection

- Ions are deflected by the magnetic field surrounding the instrument.
- The amount of deflection depends on the mass and charge of the ions.
- The heavier ions and ions with a positive charge of 2 or more, are deflected the least (Ion stream C)
- The lightest ions and ions with 1 positive charge are deflected the most (Ion Stream A)

- The ions at the correct mass and charge travel to the detector. (Ion Stream B)
- The mass to charge ratio (m/z) is determined from the ion that hits the detector.

4. Detection

- When the ion stream reached the detector the hit a wire. On hitting the wire they become neutralised by an electron jumping from the metal wire to the ion.
- The amplifier picks up on this current being created between the wire and the ion and amplifies the signal being detected.
- The computer picks up on this and converts it to mass/charge ratio and a spectrum is produced.
- **Mass spectra**
- A mas spectrum is really just a frequency bar graph. The x axis is the mass to charge ratio (m / z), where z is usually 1. The y axis is the relative intensity (how often is the ion observed compared to the most common ion (the base peak)).
- The MS of a typical hydrocarbon, n-decane is shown below.



Applications

Mass spectrometry is an analytical technique that involves the study in the gas phase of ionized molecules with the aim of one or more of the following:

- Molecular weight determination
- Structural characterization
- Gas phase reactivity study
- Qualitative and quantitative analysis of components in a mixture.

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❖ Raman spectroscopy

Raman spectroscopy (named after Indian physicist Sir C. V. Raman) is a spectroscopic technique used to observe vibrational, rotational, and other low-frequency modes in a system. Raman spectroscopy is commonly used in chemistry to provide a structural fingerprint by which molecules can be identified.

Theoretical basis

It relies on inelastic scattering, or Raman scattering, of monochromatic light, usually from a laser in the visible, near infrared, or near ultraviolet range. The laser light interacts with molecular vibrations, phonons, or other excitations in the system, resulting in the energy of the laser photons being shifted up or down. The shift in energy gives information about the vibrational modes in the system. Infrared spectroscopy yields similar, but complementary, information.

Typically, a sample is illuminated with a laser beam. Electromagnetic radiation from the illuminated spot is collected with a lens and sent through a monochromator. Elastic scattered radiation at the wavelength corresponding to the laser line (Rayleigh scattering) is filtered out by either a notch filter, edge pass filter, or a band pass filter, while the rest of the collected light is dispersed onto a detector.

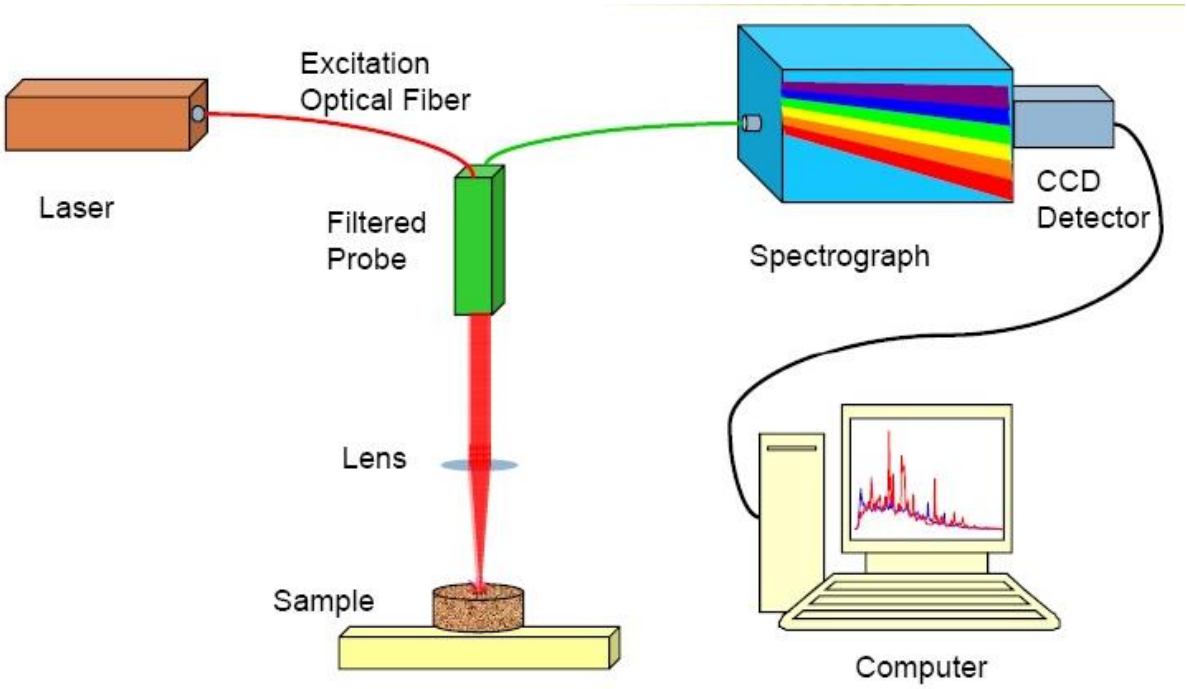
Spontaneous Raman scattering is typically very weak, and as a result the main difficulty of Raman spectroscopy is separating the weak inelastically scattered light from the intense Rayleigh scattered laser light. Historically, Raman spectrometers used holographic gratings and multiple dispersion stages to achieve a high degree of laser rejection. In the past, photomultipliers were the detectors of choice for dispersive Raman setups, which resulted in long

acquisition times. However, modern instrumentation almost universally employs notch or edge filters for laser rejection and spectrographs either axial transmissive (AT), Czerny–Turner (CT) monochromator, or FT (Fourier transform spectroscopy based), and CCD detectors.

Raman spectroscopy is a type of molecular spectroscopy that involves the dispersion of electromagnetic radiation by molecules or atoms. It measures the rotational, vibrational, and other low-frequency modes of molecules.

Different types of information can be obtained from a Raman spectrum. Every molecule has its own unique Raman spectrum, which can be correlated to a molecular fingerprint. This helps in developing databases of known standards that can be subsequently utilized for verification of unknown standards.

A BWID qualitative software package is utilized to develop databases or spectral libraries. With the help of BWID, users can preprocess some of the information using sample smoothing, baseline corrections, derivatives, etc. Later, methods or models can be developed using partial-least-squares regression or other similar algorithms and employ these models to predict unknowns.



Instrumentation

A Raman spectrometer includes three main components such as the laser, the sampling interface, and the spectrometer itself.

i. A typical Raman laser will consists of different characteristics, such as a small form factor, low power consumption, narrow linewidth, a stable power output, and a stable wavelength output. In case Raman measurements are conducted using a 785nm source, it is important to ensure that the source is only emitting 785nm.
ii. The second component includes the sampling interface. In many Raman spectrometers, fiber-optic probe is typically used which offers an extremely flexible sampling interface. These fiber-optic probes can be easily adapted to a range of optical microscopes, gas flow cells, liquid flow cells, and other sampling chambers. One critical aspect of a fiber-optic probe is a high-optical-density Raman cutoff. This means, when users are looking at the Raman spectrum, they need to ensure that the laser wavelength is blocked as much as possible so that the Raman shift can be observed. It is extremely important that the Raman shift is observed very close to the laser line since many materials have vital spectral features very near to the line.
iii. The third component is the spectrometer. Here, important performance factors are small form factor, high resolution, low power consumption, and low noise. An appropriate detector is very important and must be utilized depending on which excitation laser is being used. For visible excitation, a standard CCD is selected; for UV excitation, a photomultiplier tube (PMT) or CCD is typically chosen; and for NIR excitation, an indium gallium arsenide (InGaAs) array is normally employed.

Applications of Raman spectroscopy

1. It is used in chemistry to identify molecules and study chemical bonding. Because vibrational frequencies are specific to a molecule's chemical bonds and symmetry (the fingerprint region of organic molecules is in the wavenumber range 500–1500 cm^{-1}, Raman provides a fingerprint to identify molecules. For instance, Raman and IR spectra were used to determine the vibrational frequencies of SiO, Si_2O_2, and Si_3O_3 on the basis of normal coordinate analyses.[6] Raman is also used to study the addition of a substrate to an enzyme.
2. In solid-state physics, it is used to characterize materials, measure temperature, and find the crystallographic orientation of a sample. As

with single molecules, a solid material can be identified by characteristic phonon modes.

3. In solid state chemistry and the bio-pharmaceutical industry, it can be used to not only identify active pharmaceutical ingredients (APIs), but to identify their polymorphic forms.
4. It has a wide variety of applications in biology and medicine. It has helped confirm the existence of low-frequency phonons in proteins and DNA, promoting studies of low-frequency collective motion in proteins and DNA and their biological functions.
5. It also has a wide usage for studying biominerals. Lastly, Raman gas analyzers have many practical applications, including real-time monitoring of anesthetic and respiratory gas mixtures during surgery.
6. It has been used in several research projects as a means to detect explosives from a safe distance using laser beams.

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❖ **Atomic force microscopy (AFM)**

An **atomic force microscope** is a type of high resolution scanning probe microscope that has a resolution that you can measure in fractions of a nanometer.

It was pioneered in 1986 by Nobel Prize Winner Gerd Binnig along with Calvin Quate and Christoph Gerber.

One of the most important tools for imaging on the nanometer scale, **Atomic Force Microscopy** uses a cantilever with a sharp probe that scans the surface of the specimen.

When the tip of the probe travels near to a surface, the forces between the tip and sample deflect the cantilever according to Hooke's law.

Atomic force microscopy will measure a number of different forces depending on the situation and the sample that you want to measure.

As well as the forces, other microscopes can include a probe that performs more specialized measurements, such as temperature.

The force deflects the cantilever, and this changes the reflection of a laser beam that shines on the top surface of the cantilever onto an array of photodiodes. The variation of the laser beam is a measure of the applied forces.

Contact and Non-Contact Modes

There are two primary modes of operation for an atomic force microscope, namely contact mode and non-contact mode depending on whether the cantilever vibrates during the operation.

In **contact mode**, the cantilever drags across the sample surface and it uses the deflection of the cantilever to measure the contours of the surface.

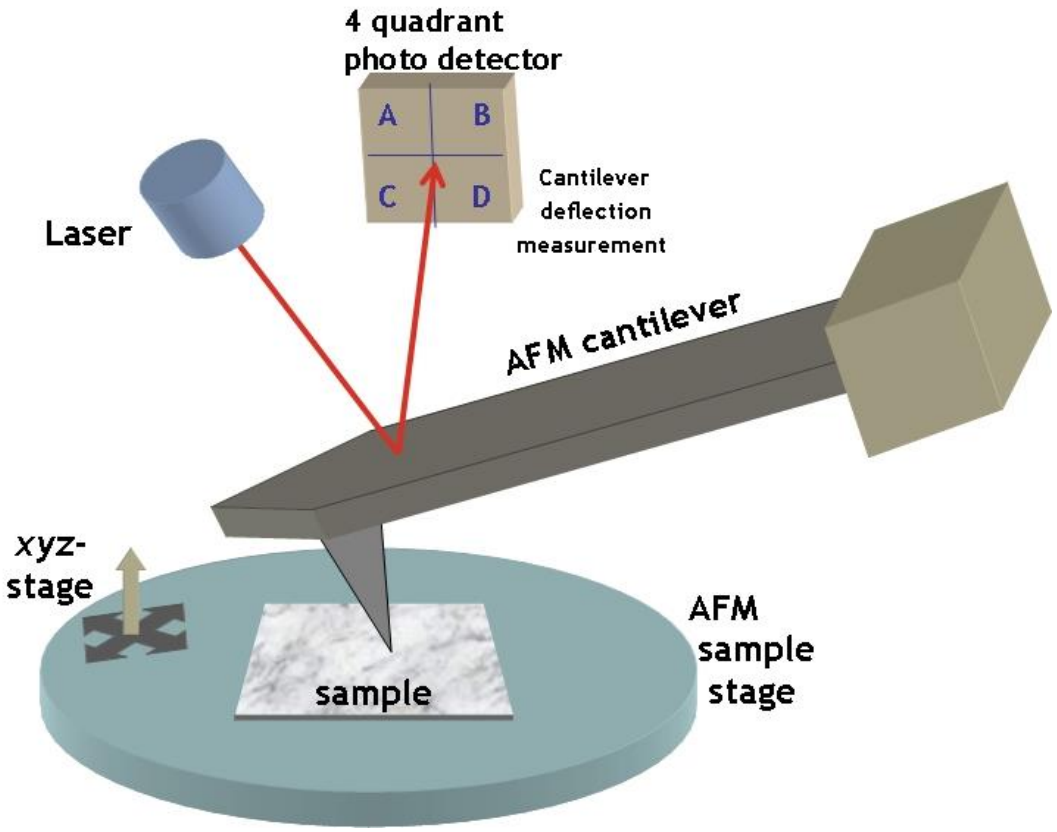
To eliminate the noise and drift that can affect a static signal, low stiffness cantilevers are used, but this allows strong attractive forces to pull the tip to the surface. To eliminate this attraction, the tip is in contact with the surface where the overall force is repulsive.

In **non-contact mode**, the tip vibrates slightly above its resonance frequency and does not contact the surface of the sample. Any long range forces, like van der Waals forces, decreases the resonant frequency of the cantilever.

A feedback loop system helps to maintain the oscillation amplitude constant by changing the distance from the tip to the sample. Recording the distance between the tip and sample at each point allows the software to construct a topographic image of the sample surface.

Most samples will form a layer of moisture on the surface if stored at ambient conditions, and this can make it difficult to measure the sample accurately.

If the probe tip is close enough to detect the short-range forces then it is close enough to stick to the moisture. One way around this is tapping, or dynamic contact mode



Tapping Mode

In tapping mode, the cantilever uses a piezoelectric element mounted on the top to oscillate it at near to its resonance frequency with an amplitude of up to 200nm.

The forces cause the amplitude to decrease as the tip gets close to the surface, and the height of the cantilever adjusts to keep the amplitude constant.

This tapping results in less damage to the sample than contact mode and is more accurate than non-contact mode when moisture is present on a sample.

The atomic force microscope is a powerful tool that is invaluable if you want to measure incredibly small samples with a great degree of accuracy.

Unlike rival technologies it does not require either a vacuum or the sample to undergo treatment that might damage it.

At the limits of operation however, researchers have demonstrated atomic resolution in high vacuum and even liquid environments.

Applications

1. The AFM has been applied to problems in a wide range of disciplines of the natural sciences, including solid-state physics, semiconductor science and technology, molecular engineering, polymer chemistry and physics, surface chemistry, molecular biology, cell biology, and medicine.
2. Applications in the field of solid state physics include (a) the identification of atoms at a surface, (b) the evaluation of interactions between a specific atom and its neighboring atoms, and (c) the study of changes in physical properties arising from changes in an atomic arrangement through atomic manipulation.
3. In molecular biology, AFM can be used to study the structure and mechanical properties of protein complexes and assemblies. For example, AFM has been used to image microtubules and measure their stiffness.
4. In cellular biology, AFM can be used to attempt to distinguish cancer cells and normal cells based on a hardness of cells, and to evaluate interactions between a specific cell and its neighboring cells in a competitive culture system.
5. AFM can also be used to indent cells, to study how they regulate the stiffness or shape of the cell membrane or wall.

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