13. Biochemistry-3

Chemical Analysis

(instrumental analysis or dry chemical analysis)

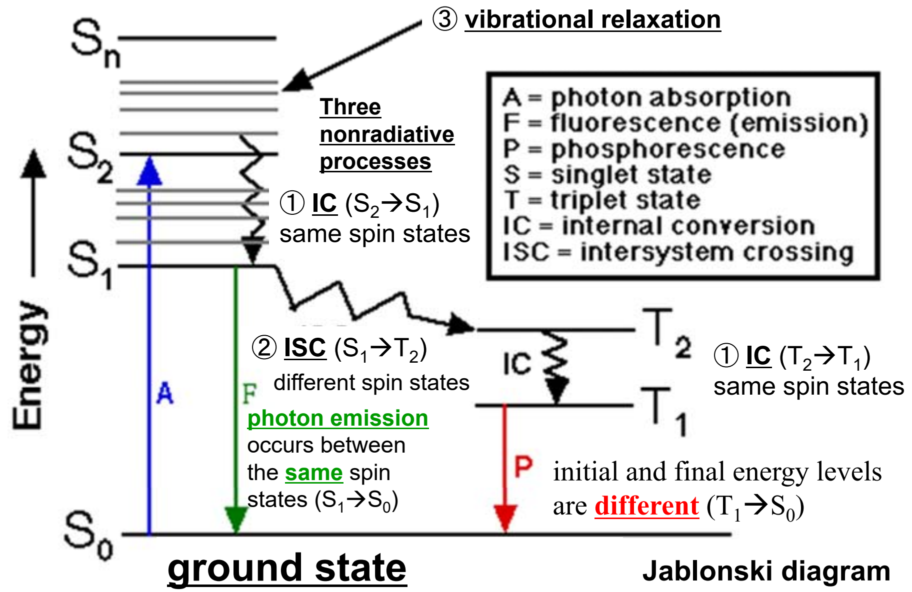
- Spectrophotometry
- Mass spectrometry
- Chromatography
- Electrophoresis
- Electron spin resonance
- Nuclear magnetic resonance

Chemical Analysis

- **Physical properties** or **chemical composition** of the assays
- **Gravimetric** and **volumetric** analyses
- **Classical analysis** (wet chemical analysis) uses a balance. It relies on **chemical reactions** between the analyte and reagent, and depends on the formation of a product of the chemical reaction, e.g., the **color** or a **precipitated solid** in a solution
- **Instrumental analysis** (dry chemical analysis) uses **instruments** to characterize chemical reactions between the analyte and reagent, or to measure a **property of the analyte**

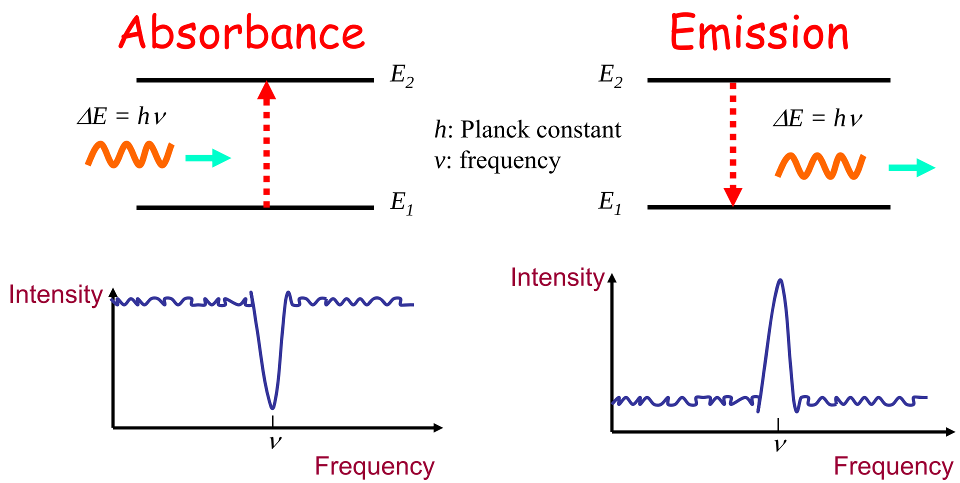
Energy Levels

Once a molecule absorbs energy **A** from electromagnetic radiation, it can return to **ground state** by several routes.



Jablonski diagram

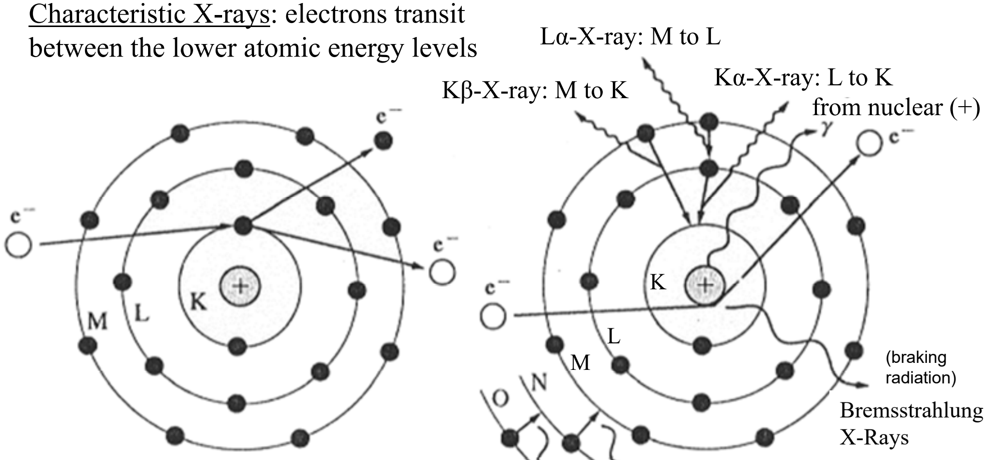
Energy Level Transition



1. detect the frequency or wavelength of the emitted or absorbed photons
2. provide information on the energy levels and electronic structure of materials by analyzing the spectrum

Atom Shell and Emission

Characteristic X-rays: electrons transit between the lower atomic energy levels

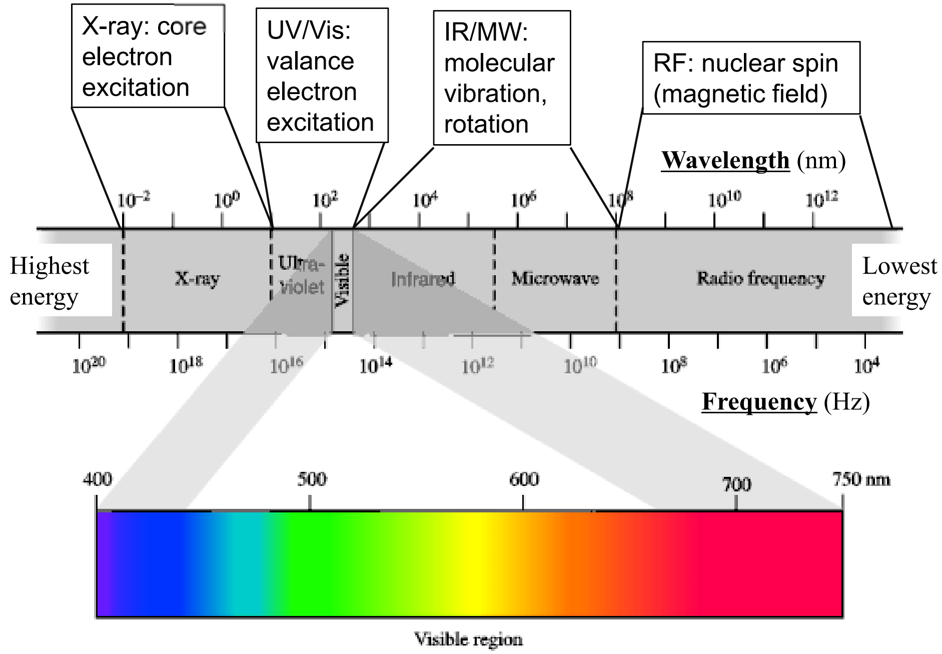


Bremsstrahlung X-ray: when accelerated electrons (○e⁻) approach metal atom nuclear, they are braked and dramatically decelerated by the Coulomb force

UV (Ultraviolet): higher shell than X ray

VR (Visible Ray): higher shell than X and UV, visible light (380 ~ 780nm).

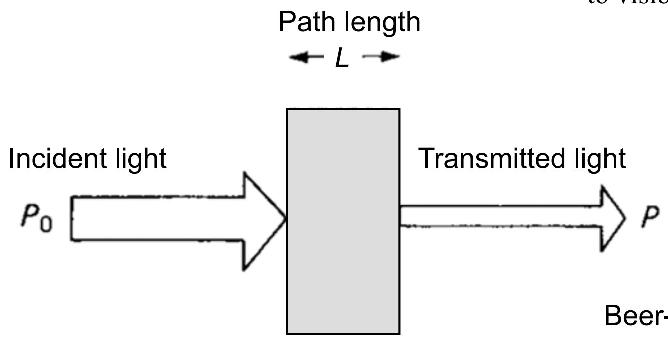
Excitation Methods



Light Absorption

Material composition and properties can be analyzed through investigating which wavelengths of a light are absorbed.

Wood is opaque to visible light. Some materials are opaque to some wavelengths of light, but transparent to others. Glass and water are opaque to ultraviolet light, but transparent to visible light.



Transmittance

$$T = \frac{P}{P_0} = e^{-\alpha(\lambda)Lc}$$

Beer-Lambert's law

$P = P_0 e^{-\alpha(\lambda)Lc}$

α: extinction coefficient

λ: wavelength

An object becomes dark or opaque or color due to absorption of some wavelengths of the incoming light.

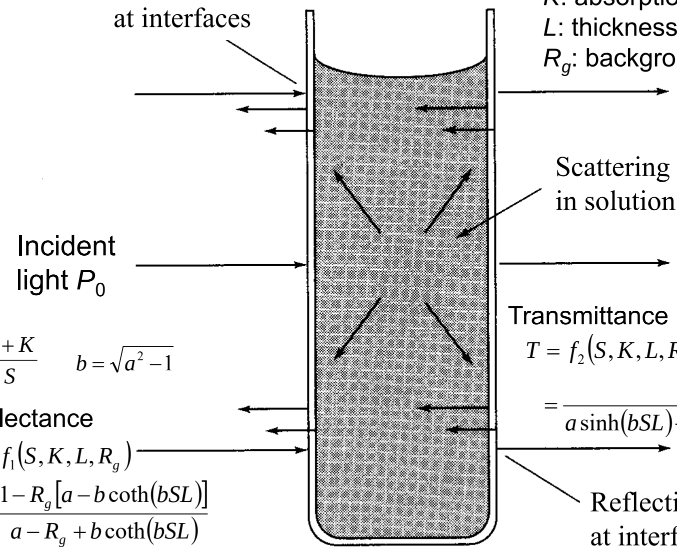
Light Reflection and Scattering

S: scattering coefficient

K: absorption coefficient

L: thickness of the layer

R_g: background reflectance



$$a = \frac{S+K}{S} \qquad b = \sqrt{a^2 - 1}$$

Transmittance

$$T = f_2(S, K, L, R_g) = \frac{b}{a\sinh(bSL) + b\cosh(bSL)}$$

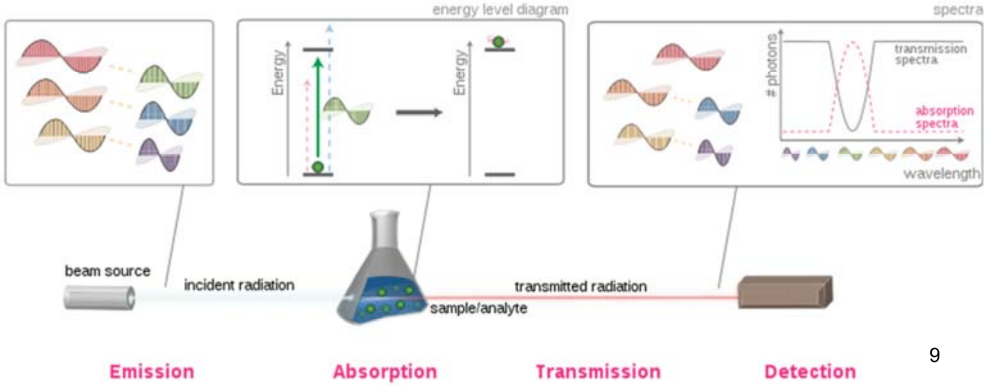
Reflectance

$$R = f_1(S, K, L, R_g) = \frac{1 - R_g[a - b\coth(bSL)]}{a - R_g + b\coth(bSL)}$$

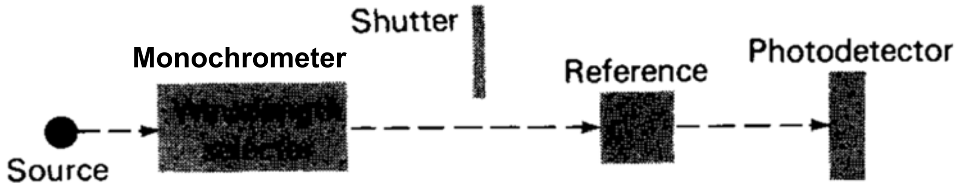
Spectroscopy

study of the absorption and emission of light and other radiation by matter, as related to the dependence of these processes on the wavelength of the radiation.

Radio-frequency spectroscopy → magnetic resonance imaging
Microwave spectroscopy → big bang of the universe
Optical spectroscopy → chemical composition and physical structure
X-ray spectroscopy → computerized tomography

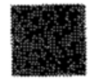


Single-beam Light Absorption Measurement



quantitative measurement of the reflection or transmission properties of a material as a function of wavelength

Sample



relative intensity of the light between reference and test sample is measured separately.

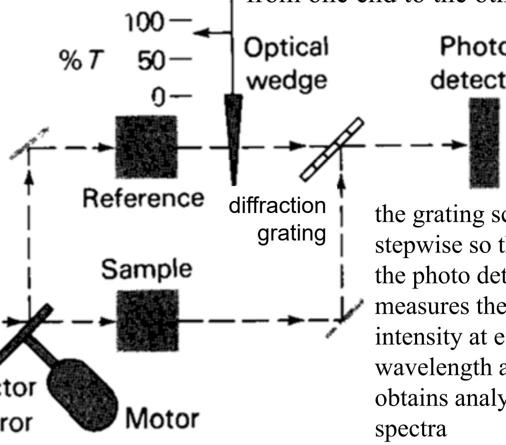
- A certain chemical reaction within a solution may occur in forward and reverse direction where **reactants** form **products** and products break down into reactants. This chemical reaction will reach an **equilibrium point**.
- To determine the **respective concentrations** of **reactants** and **products** at the equilibrium point, the light transmittance is detected using photo detector.
- The transmitted light density is indicative of the concentration of certain chemicals that barricade light.

Double-beam Light Absorption Measurement

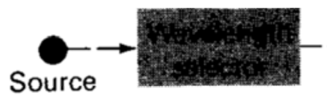
two light paths = one for a **reference** sample and the other for **test** sample

Source light passes through a monochrometer. Discrete frequencies are transmitted through the test sample. The photon flux density of the transmitted light is measured with a photo detector. The transmittance value for each wavelength of the test sample is compared with the transmission values from the reference sample

transmittance decreases from one end to the other



Monochrometer



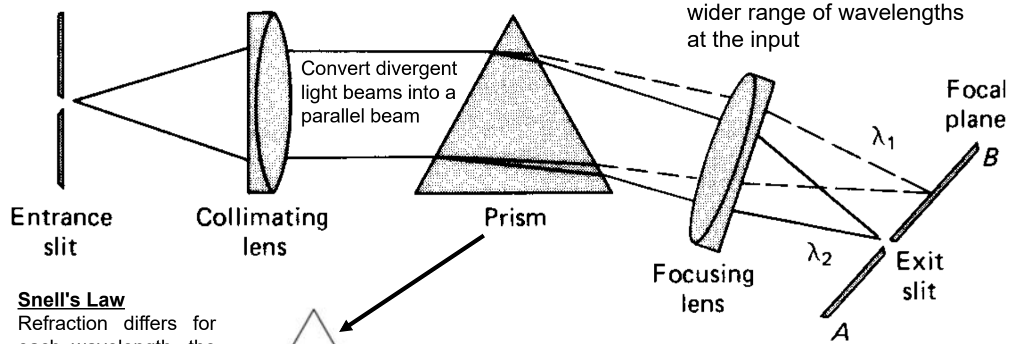
diffract the light into a "rainbow" of wavelengths and output a narrow bandwidth of this diffracted spectrum

the grating scans stepwise so that the photo detector measures the light intensity at each wavelength and obtains analytical spectra

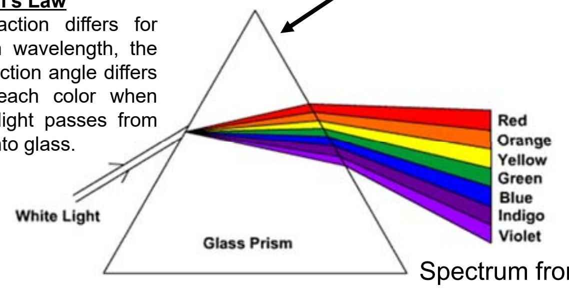
Prism Monochrometer

Spatially separate the wavelengths of light by using the phenomena of optical dispersion in a prism

Transmit a narrow band of wavelengths of light from a wider range of wavelengths at the input



Snell's Law
Refraction differs for each wavelength, the refraction angle differs for each color when the light passes from air into glass.

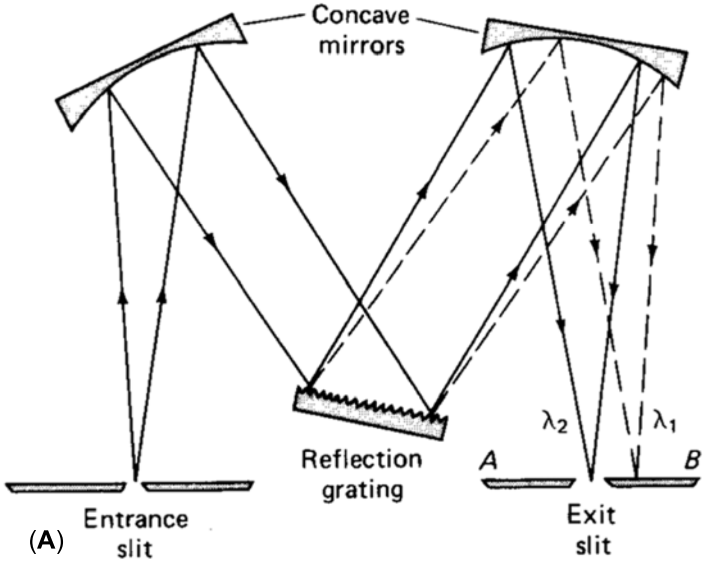


Spectrum from prism

If the material is shaped like a prism, the angles for each color will be exaggerated, and the colors will be displayed as a spectrum of light.

Grating Monochrometer

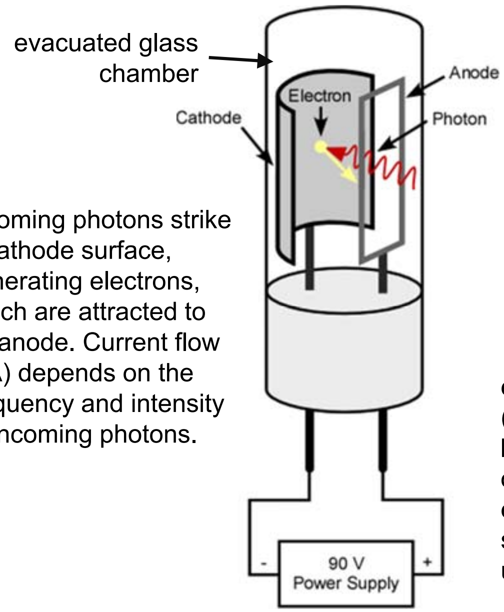
Spatially separate the wavelengths of light by using the phenomena of optical diffraction in a grating



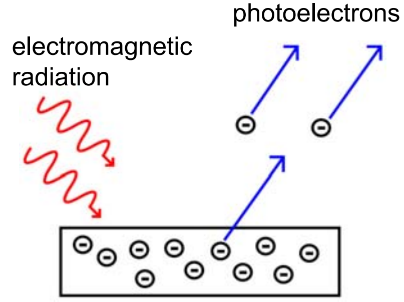
- A wide band light aims at an entrance slit which is placed at the focus of a curved mirror (collimator)
- The reflected light from the mirror is collimated (focused at infinity)
- The collimated light is diffracted by the grating and collected by another mirror which refocuses the light on the exit slit.

Focal plane

Phototube (PT)



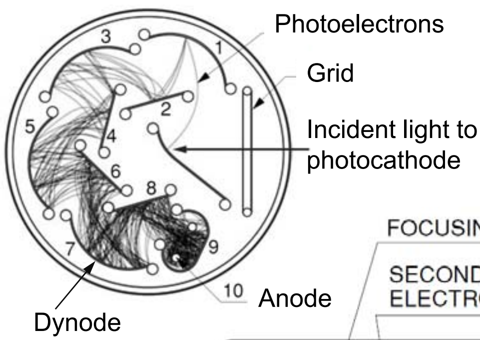
Incoming photons strike a cathode surface, generating electrons, which are attracted to an anode. Current flow (μA) depends on the frequency and intensity of incoming photons.

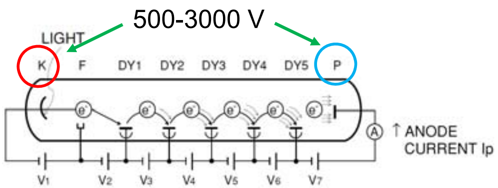


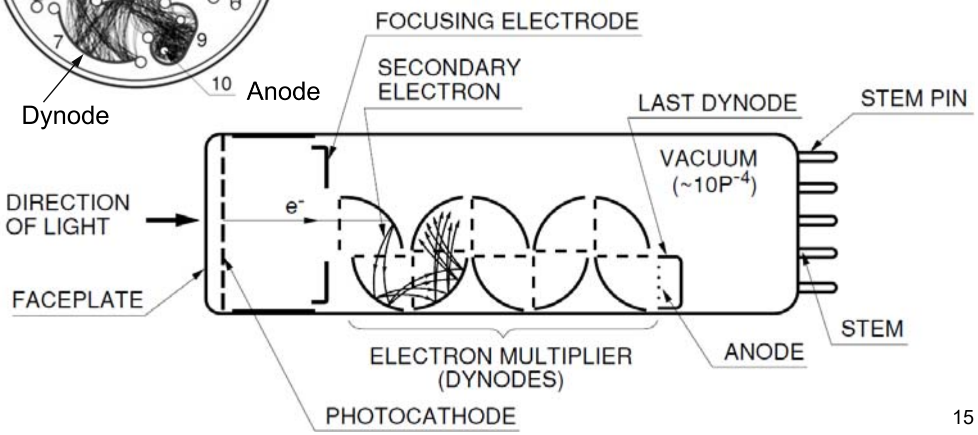
photoelectric effect

electrons are emitted from matter (metals and non-metallic solids, liquids or gases) as a consequence of their absorption of energy from electromagnetic radiation of very short wavelength, such as visible or ultraviolet radiation.

Photomultiplier Tube (PMT)







Photodiode (PD)

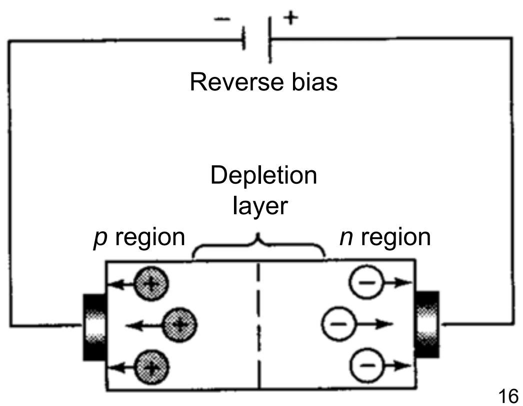
Inner photoelectric effect:

- when a **photon** of sufficient energy strikes the diode, it creates negatively charged electrons and positively charged holes.

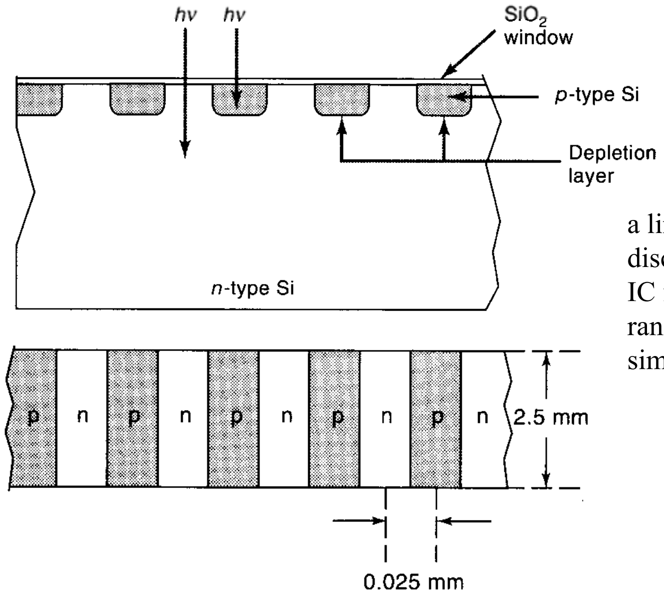
Electrons and holes may

- remain free
- other electrons may combine with holes to form complete atoms again
- be pulled away from the **depletion region** by an **external electric field**

If light absorption occurs in the depletion region,
->> **holes** move toward the **cathode**, and **electrons** toward the **anode**
->>A photocurrent is produced



Photodiode Array (PDA)

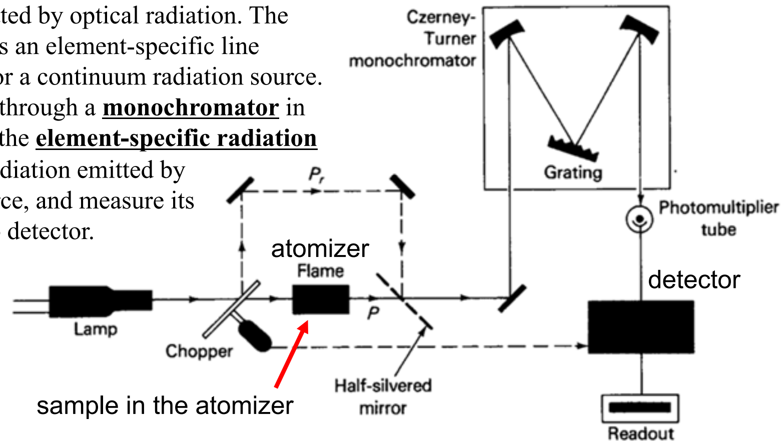


a linear array of discrete PDs on an IC for detecting a range of wavelengths simultaneously

Quantitative determination of chemical elements employing the **absorption** of light by free atoms in the **gaseous state**.

Atomic Absorption Spectroscopy

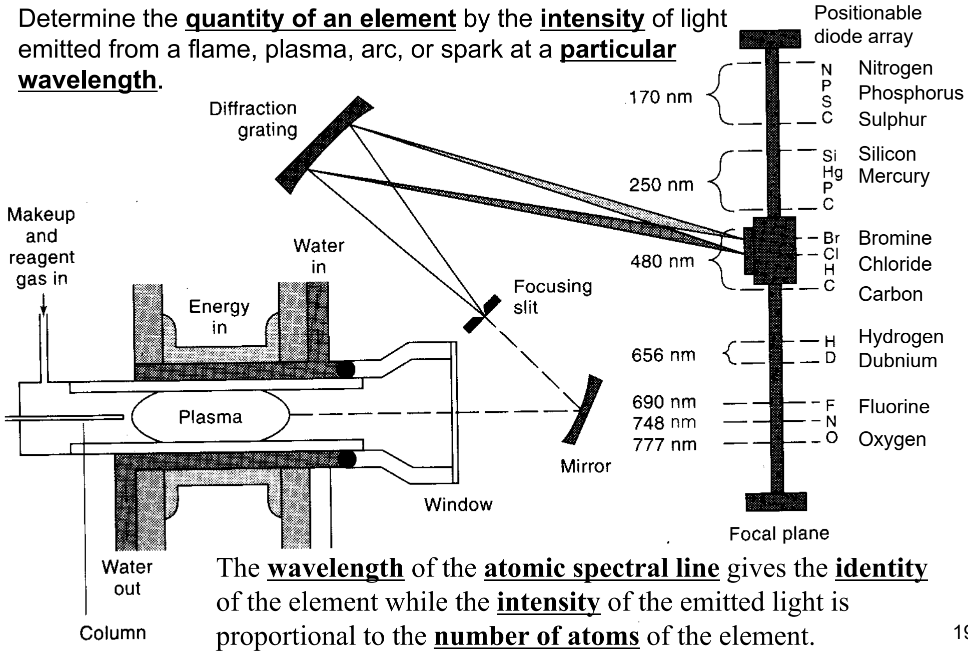
Atoms are irradiated by optical radiation. The radiation source is an element-specific line radiation source or a continuum radiation source. Radiation passes through a **monochromator** in order to separate the **element-specific radiation** from any other radiation emitted by the radiation source, and measure its output by a photo detector.



The electrons of the atoms in the **atomizer** are promoted to higher orbitals (excited state) for a short period of time (nanoseconds) by absorbing a **defined quantity of energy** (radiation of a given wavelength). This amount of energy, i.e., **wavelength**, is specific to a particular electron transition in a particular element. Each **wavelength** corresponds to **one element**.
The radiation flux without/with a sample in the atomizer is measured using a detector, and the **ratio** between the two **absorbance** is converted to analyte **concentration** or **mass** using Beer-Lambert Law.

Atomic Emission Spectroscopy

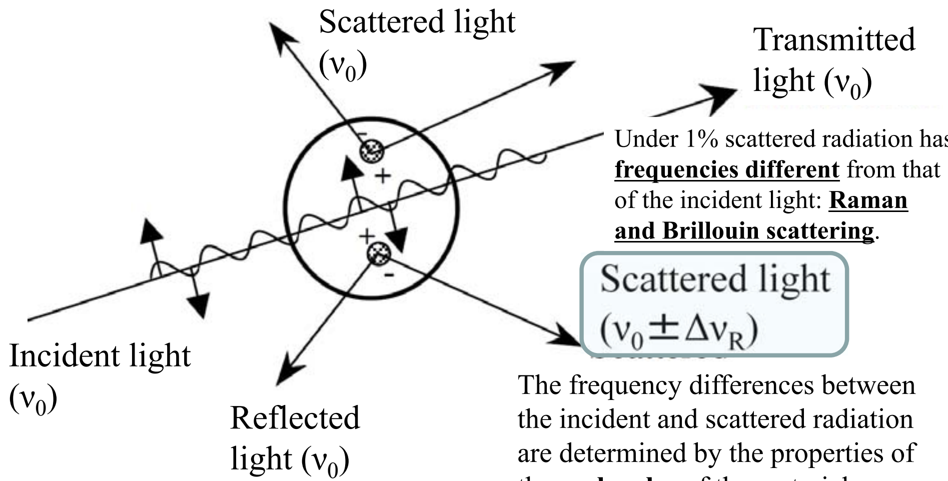
Determine the **quantity of an element** by the **intensity** of light emitted from a flame, plasma, arc, or spark at a **particular wavelength**.



The **wavelength** of the **atomic spectral line** gives the **identity** of the element while the **intensity** of the emitted light is proportional to the **number of atoms** of the element.

Raman Scattering

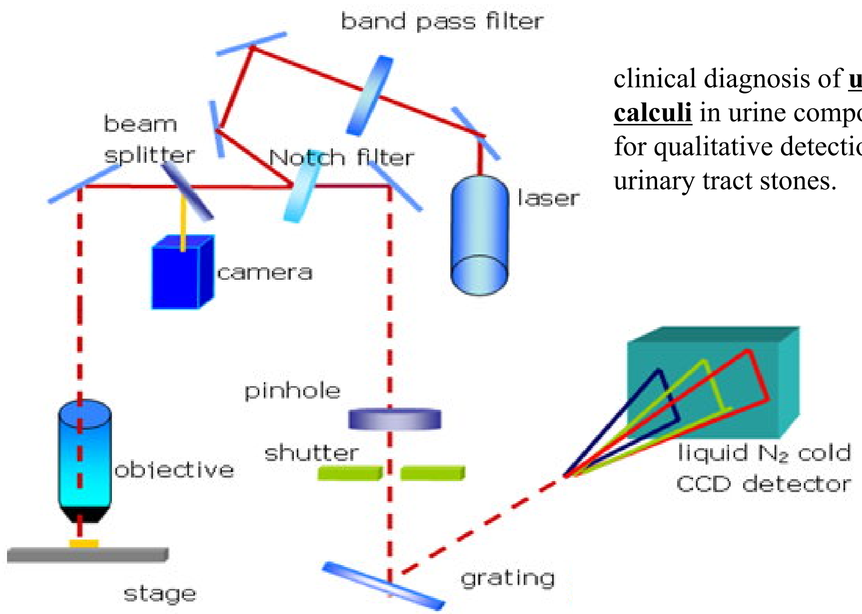
When an incident light interacts with a material, part of it is **transmitted**, part **reflected**, and part **scattered**.
Over 99% of the **scattered** radiation has the **same frequency** as the incident light: **Mie** and **Rayleigh scattering**.



Under 1% scattered radiation has **frequencies different** from that of the incident light: **Raman and Brillouin scattering**.

The frequency differences between the incident and scattered radiation are determined by the properties of the **molecules** of the material.

Raman Spectroscopy



clinical diagnosis of **urinary calculi** in urine composition for qualitative detection of urinary tract stones.

Mass Spectrometer

$$r = \frac{mv}{qB}$$

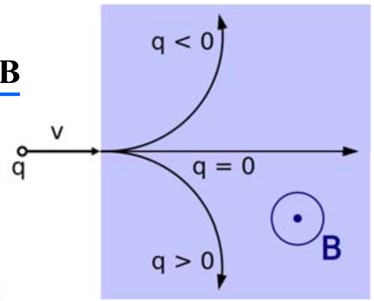
r: radius of the circular motion of the particle
B: magnetic field strength
q: charge of the particle
m: mass of the particle
v: velocity of the particle

Lorentz force

$$\mathbf{F}_L = q\mathbf{E} + q\mathbf{v}\times\mathbf{B}$$

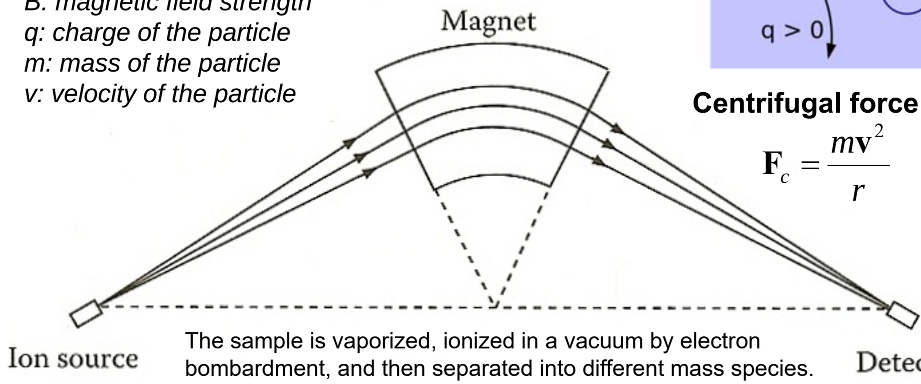
electric force

magnetic force



Centrifugal force

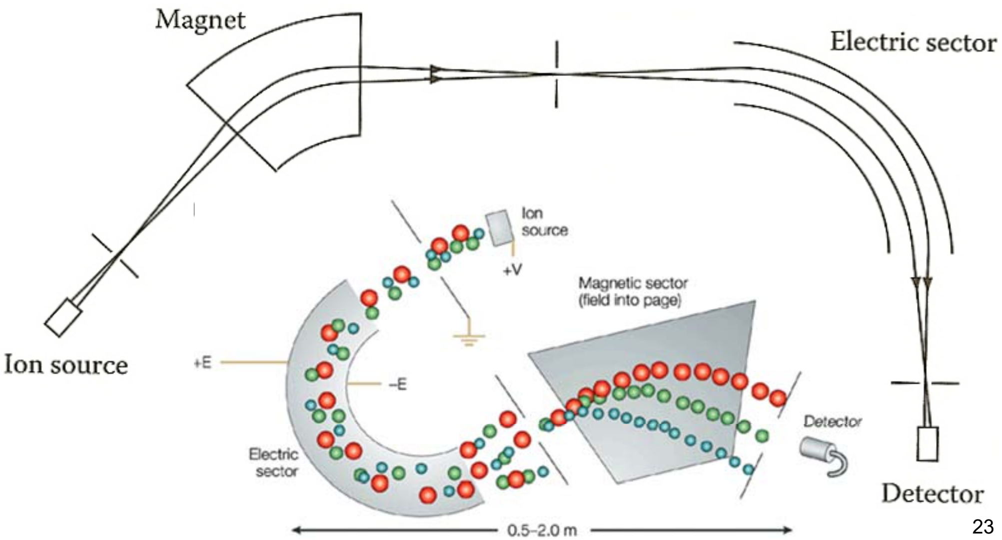
$$\mathbf{F}_c = \frac{m\mathbf{v}^2}{r}$$



The sample is vaporized, ionized in a vacuum by electron bombardment, and then separated into different mass species.

By combining it with an electrostatic sector, focusing characteristics is improved.

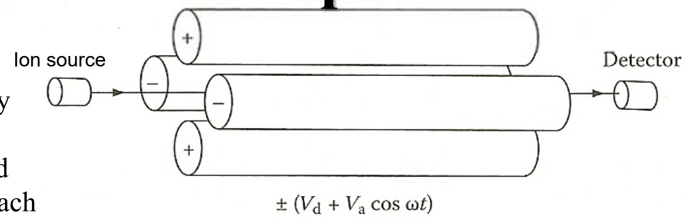
Double-focus Mass Spectrometer

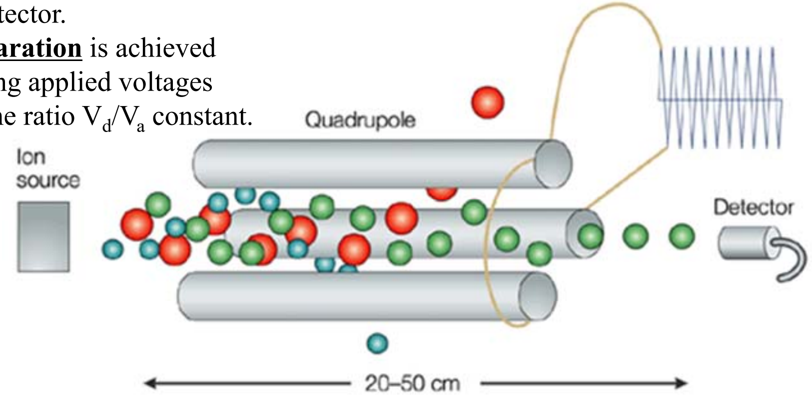


Quadrupole Mass Spectrometer

A voltage ($V_d + V_a\cos\omega t$) is applied between adjacent rods. The ion injected into the center of the rods travels along the axis and oscillates due to $V_a\cos\omega t$ electric field. The oscillation is stable only when a certain condition is fulfilled between V_d, V_a, and the mass, so that the ions reach the ion detector.

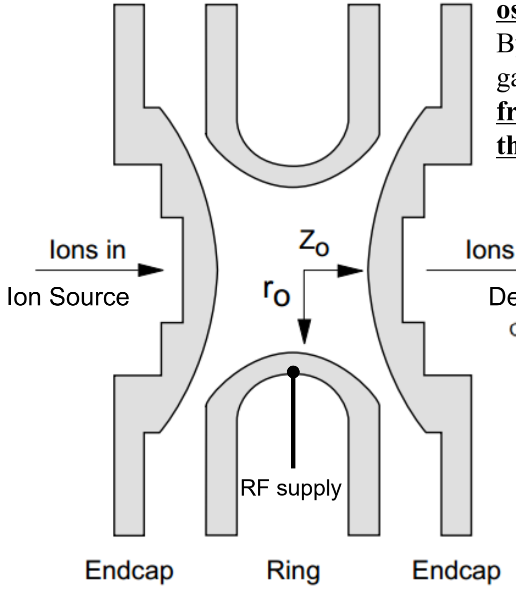
Mass separation is achieved by scanning applied voltages keeping the ratio V_d/V_a constant.



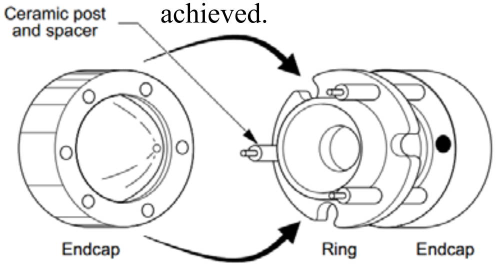


Ion Trap Mass Spectrometer

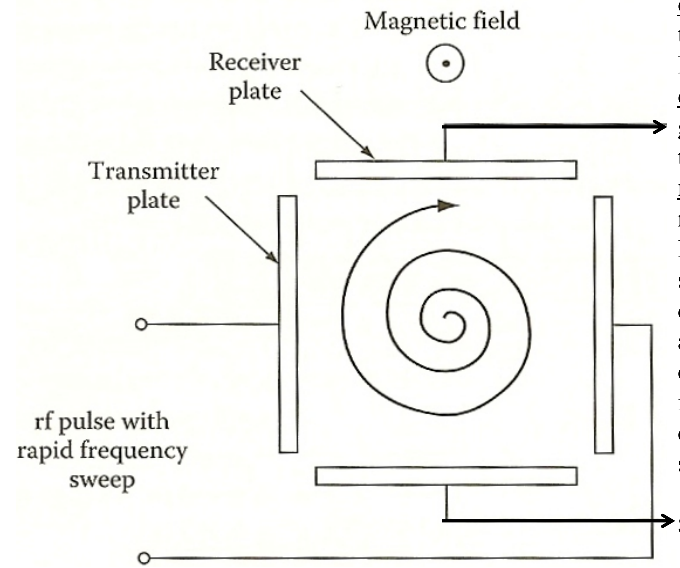
<u>RF voltage</u> is applied to the **<u>ring electrode</u>** relative to the **<u>endcap electrodes</u>**. The ions injected into the cell are trapped in it, being constrained in a **<u>stable orbit oscillating both axially and radially</u>**. By increasing the RF voltage, each ion gains kinetic energy so that it is **<u>ejected from stable orbit</u>** and some ions **<u>strike the detector</u>**.



By a gradual increase of the RF field, instability of ionic motion occurs in order to increase the mass-to-charge ratio, and the mass scan is achieved.



Cyclotron Resonance Mass Spectrometer

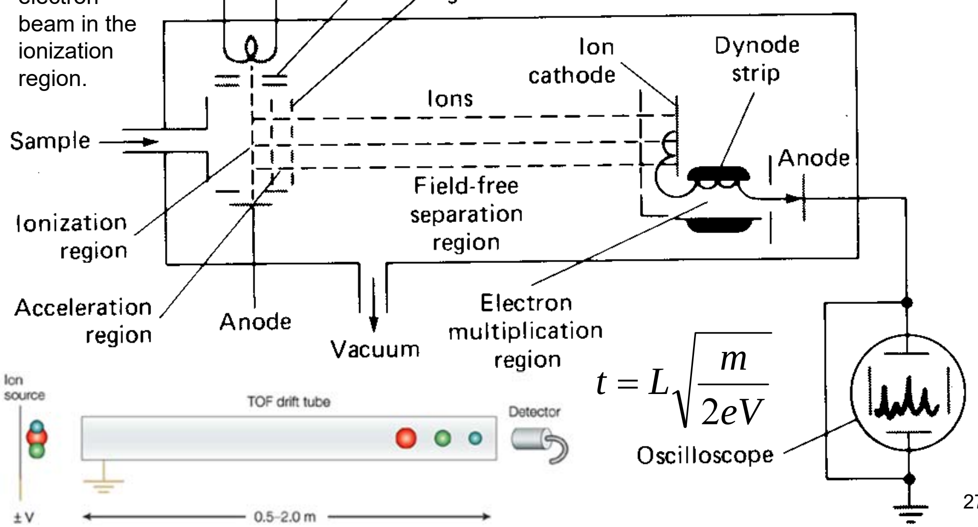


AC voltage is applied to the **<u>transmitter plates</u>**.
An ion generated in the electric field moves in a **<u>circular path</u>** perpendicular to the magnetic field direction.
If the **<u>cyclotron resonance condition</u>** is fulfilled, the ion gains energy from the field so that the **<u>radius of circular motion increases</u>**, and finally reaches the **<u>receiver plate</u>**.
If the applied frequency is swept rapidly, all ions having different masses are excited and this causes a signal containing many different frequency components corresponding to ionic species of different masses.

Time-of-flight Mass Spectrometer

<u>Ionization</u> is achieved by the pulsed electron beam in the ionization region.

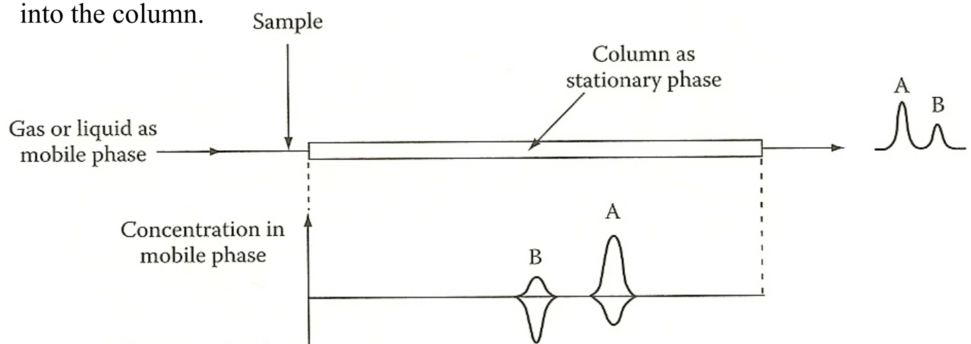
The mass of an ion is determined from the time an ion takes to travel a constant distance when it is accelerated by a constant voltage.



$$t = L\sqrt{\frac{m}{2eV}}$$

Principle of Chromatography

The components in a mixture are separated based on their differences in **<u>speeds</u>** of **<u>moving</u>** through a column when a **<u>gas</u>** or **<u>liquid</u>** is forced to flow through it, due to the **<u>differential affinities</u>** of substances for a gas or liquid mobile medium and for a stationary adsorbing medium through which they pass, such as paper, gelatin, or magnesia.
The chromatographic method consists of a **<u>stationary phase</u>**, which is a **<u>solid</u>** or **<u>liquid</u>** held in the column, and a **<u>mobile phase</u>**. which is a **<u>gas</u>** or a **<u>liquid</u>** forced into the column.

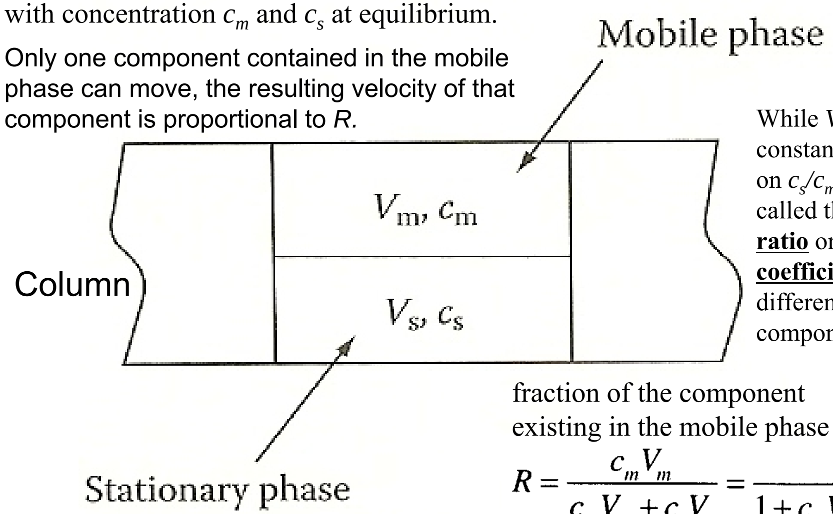


Different affinity between mobile phase and stationary phase ->> different moving speed

Equilibrium Between Two Phases

A small section of the column with the volume of the mobile phase V_m and that of the stationary phase V_s.
A component is dissolved in the mobile phase and the stationary phase with concentration c_m and c_s at equilibrium.

Only one component contained in the mobile phase can move, the resulting velocity of that component is proportional to *R*.



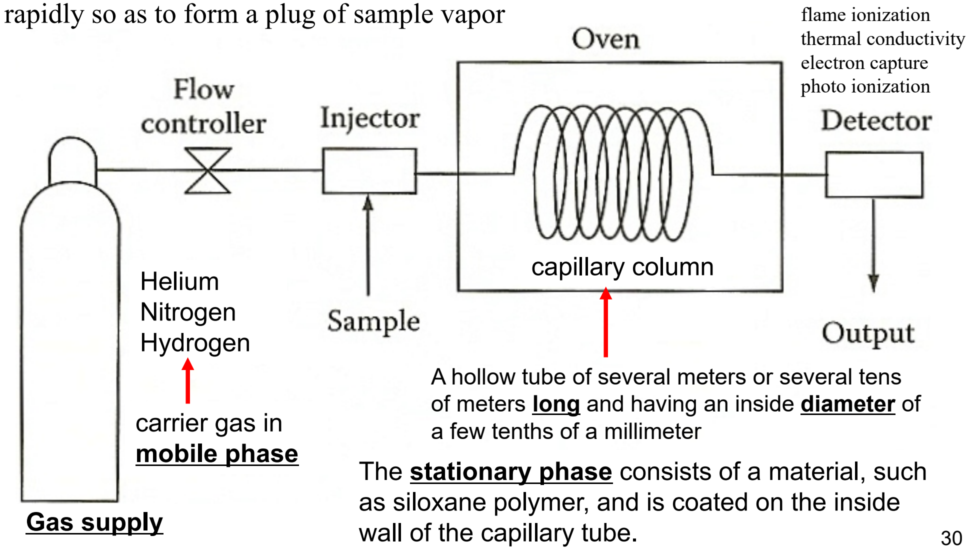
While *Vs/Vm* is constant, *R* depends on c_s/c_m which is called the **partition ratio** or the **partition coefficient**, and is different for different components.

fraction of the component existing in the mobile phase

$$R = \frac{c_m V_m}{c_m V_m + c_s V_s} = \frac{1}{1 + c_s V_s / c_m V_m}$$

Gas Chromatography

The sample is **vaporized** and introduced rapidly so as to form a plug of sample vapor

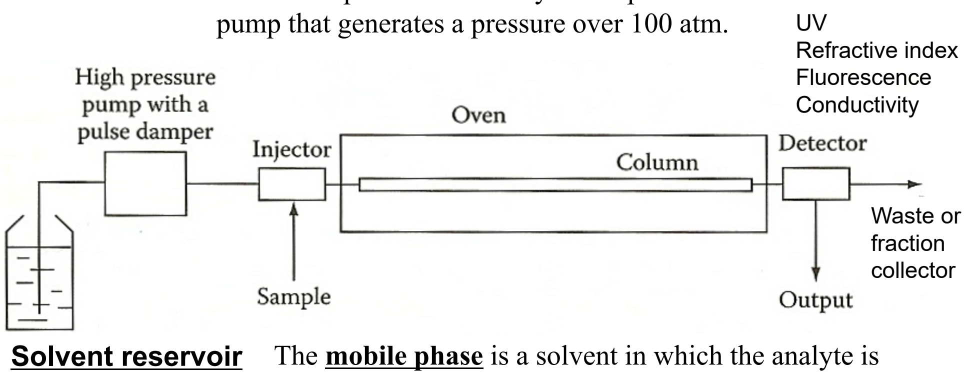


A hollow tube of several meters or several tens of meters **long** and having an inside **diameter** of a few tenths of a millimeter

The **stationary phase** consists of a material, such as siloxane polymer, and is coated on the inside wall of the capillary tube.

Liquid Chromatography

Due to a very high flow resistance of the column, the mobile phase is driven by a non-pulsatile pump that generates a pressure over 100 atm.

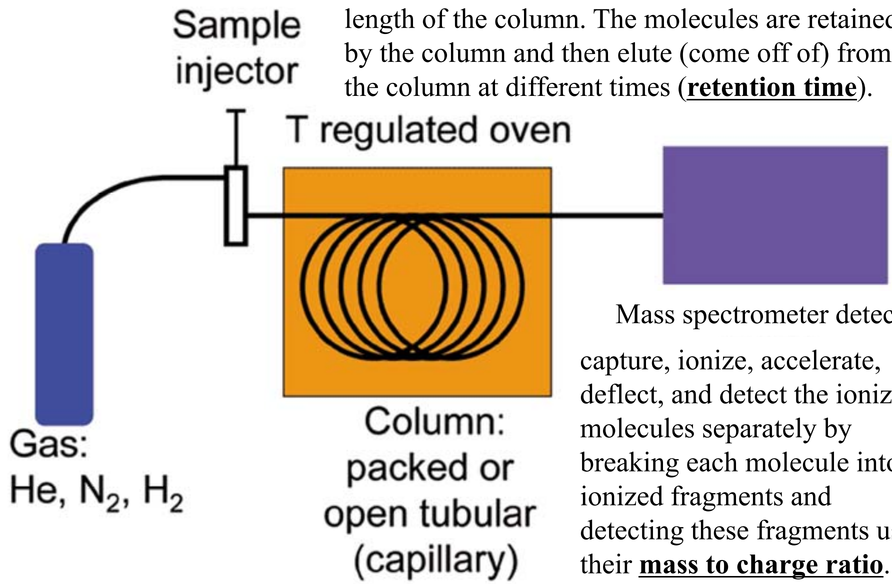


The **mobile phase** is a solvent in which the analyte is soluble. For water-soluble substances, a polar solvent such as water is used and for fat-soluble substances, a nonpolar solvent such as hydrocarbons is used as the mobile phase. The **stationary phase** consists of fine particles, typically 5-10um in diameter, packed in a column.

Gas Chromatography+Mass Spectrometry to identify different substances in a sample

GC-MS

The difference in the **chemical properties** between different molecules in a mixture will separate the molecules as the sample travels the length of the column. The molecules are retained by the column and then elute (come off of) from the column at different times (**retention time**).

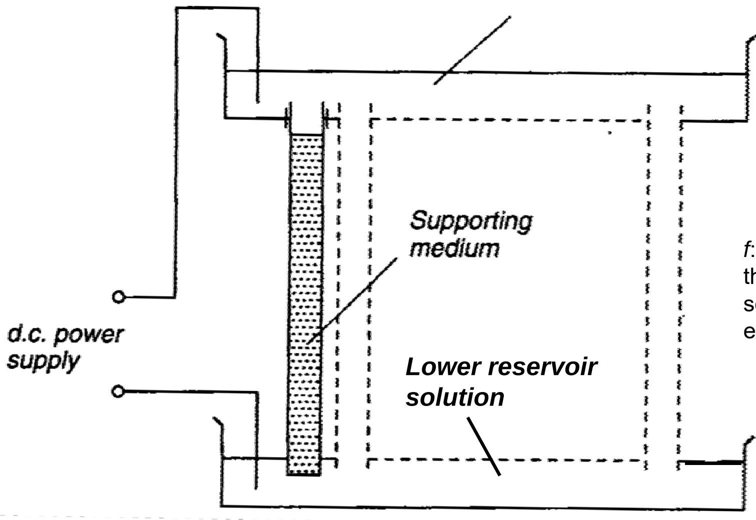


capture, ionize, accelerate, deflect, and detect the ionized molecules separately by breaking each molecule into ionized fragments and detecting these fragments using their **mass to charge ratio**.

migration of ions in an electric field causes the separation of ions exposed to the field.

Electrophoresis

If an ion with a charge q is exposed to an electric field E, a force qE arises and drives the ion.



In a viscous medium, a frictional force arises that reaches an equilibrium at which the driving and the frictional forces are in balance so that the ion moves at a constant velocity v given by

$$v = qEf$$

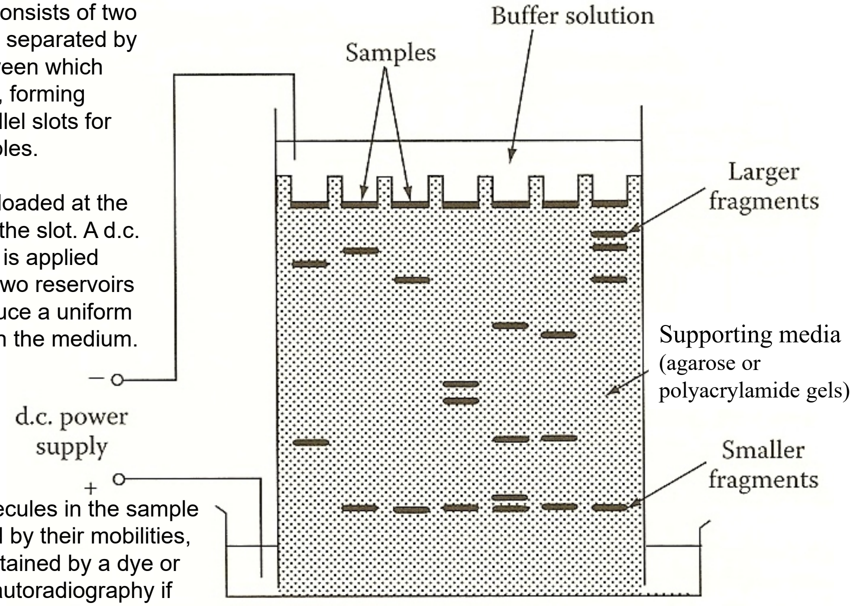
f: frictional coefficient that is proportional to the solvent viscosity and the effective radius of the ion.

a smaller molecule moves faster and a larger one moves slower if the charges are identical.

Electrophoresis Unit

The column consists of two **glass plates**, separated by 1-2 mm, between which gels are filled, forming identical parallel slots for multiple samples.

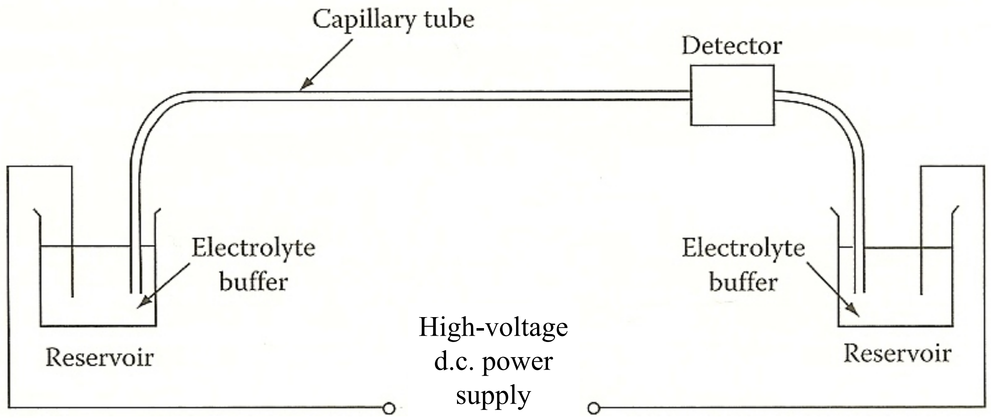
Samples are loaded at the upper part of the slot. A d.c. power supply is applied between the two reservoirs so as to produce a uniform electric field in the medium.



After the molecules in the sample are separated by their mobilities, the gels are stained by a dye or analyzed by autoradiography if the sample is radiolabeled.

Capillary Electrophoresis

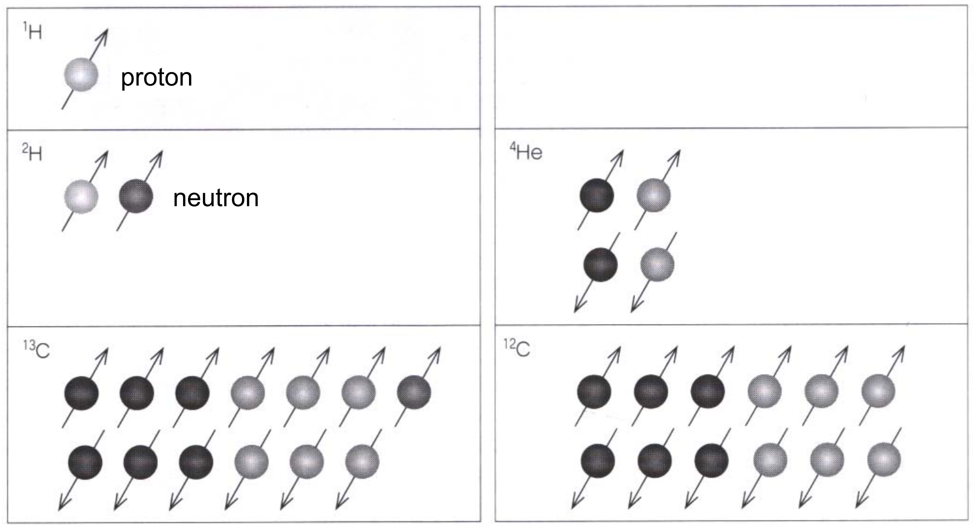
A narrow-bore tube (50 um in diameter and 50-100 cm long) is filled with a supporting medium. The molecules close to the end of the capillary are detected by an UV detector.



The advantage of using a capillary is the enhancement of heat dissipation due to a large surface-to-volume ratio that permits the use of a high voltage, which decreases the analysis time. By applying 10-50 kV, the typical analysis time can be reduced to 10-30 min from the many hours required in the original apparatus.

Nucleus and Magnetic Moment

Nucleus are composed of protons and neutrons
If at least either proton or neutron is odd, the nucleus will have a magnetic moment
If both protons and neutrons are even, nuclear magnetic moment is 0 and would not be the subject of NMR

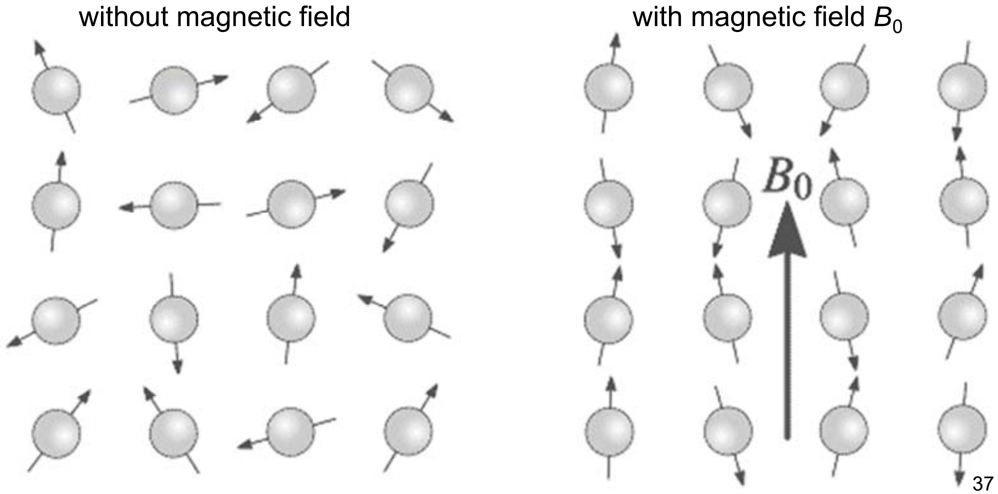


Nucleus with magnetic moment
Nucleus without magnetic moment

Magnetic resonance based technique measures absorption of electromagnetic radiation by either electrons or nuclei in the presence of an external magnetic field.

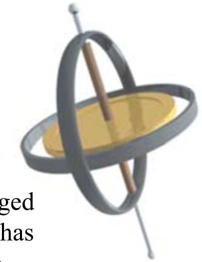
Nuclear Moment μ

As long as ^{1}H nucleus are not exposed to the magnetic field, their magnetic moments are in arbitrary and random direction. No magnetism is exposed to the outside.



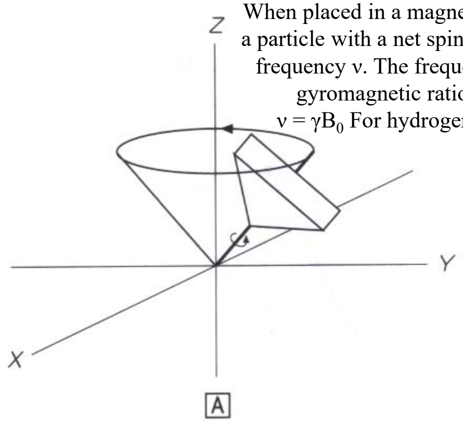
Spin and Precession

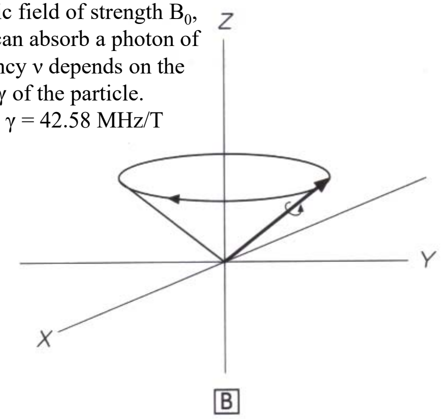
An electron or a nucleus can be regarded as a spinning charged particle. Due to the spinning motion, the particle has an angular momentum. At the same time, it causes a magnetic moment induced by the effective ring current due to the rotating charge.



Because angular momentum is quantized in atomic scale, the emerged magnetic moment is also quantized, and consequently, the particle has a magnetic moment at discrete levels only, which is proportional to the quantized angular momentum.

When placed in a magnetic field of strength B_0, a particle with a net spin can absorb a photon of frequency ν. The frequency ν depends on the gyromagnetic ratio γ of the particle.
$\nu = \gamma B_0$ For hydrogen, γ = 42.58 MHz/T





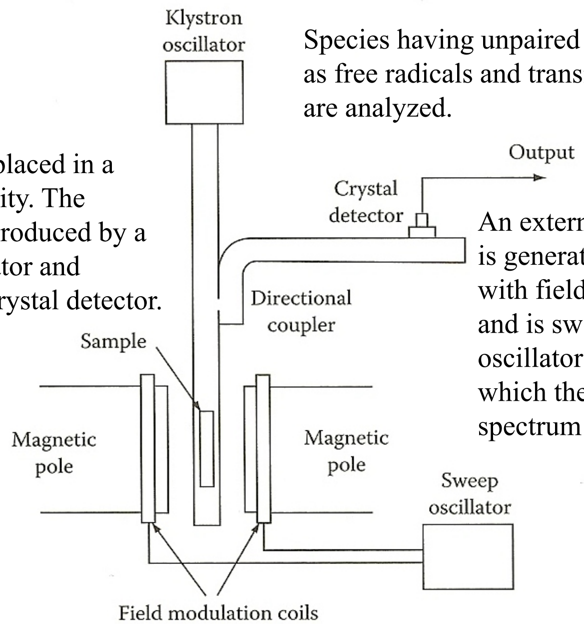
measurement of the absorption of electrons resonant frequency = microwave range

Electron Spin Resonance (ESR)

Species having unpaired electrons, such as free radicals and transition metal ions, are analyzed.

The sample is placed in a microwave cavity. The microwave is produced by a klystron oscillator and detected by a crystal detector.

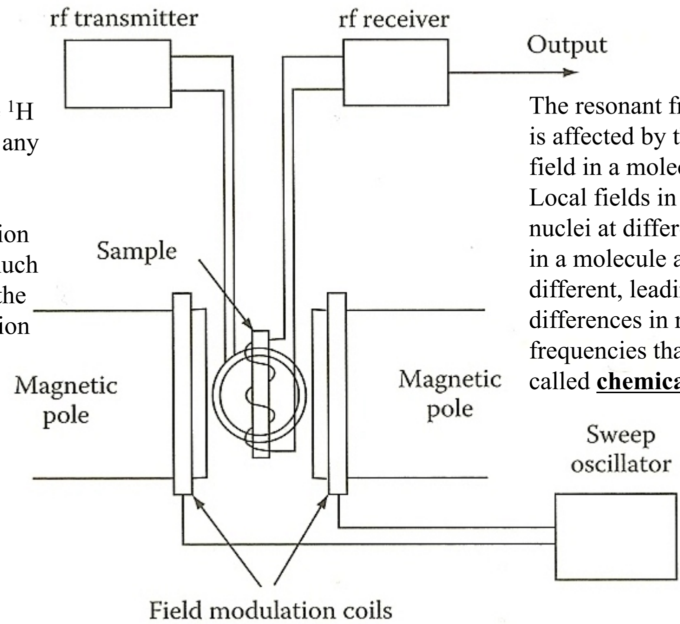
An external magnetic field is generated by magnets with field modulation coils, and is swept by sweep oscillator in a range in which the resonant spectrum can be observed.



measurement of the absorption of nuclei resonant frequency = radio frequency range

Nuclear Magnetic Resonance

The resonance of protons (^{1}H) is measured. Because ^{1}H exists in water and any organic substance, observation of the strength of absorption does not provide much information about the chemical composition of a sample.



The resonant frequency is affected by the local field in a molecule. Local fields in hydrogen nuclei at different sites in a molecule are different, leading to differences in resonant frequencies that are called **chemical shifts**.