TEJASVI NAVADHITAMASTU

"Let our (the teacher and the taught) learning be radiant"

Let our efforts at learning be luminous and filled with joy, and endowed with the force of purpose

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PHOTOMETRY

COLORIMETER & SPECTROPHOTOMETER

ULTRAVIOLET AND VISIBLE LIGHT SPECTROSCOPY

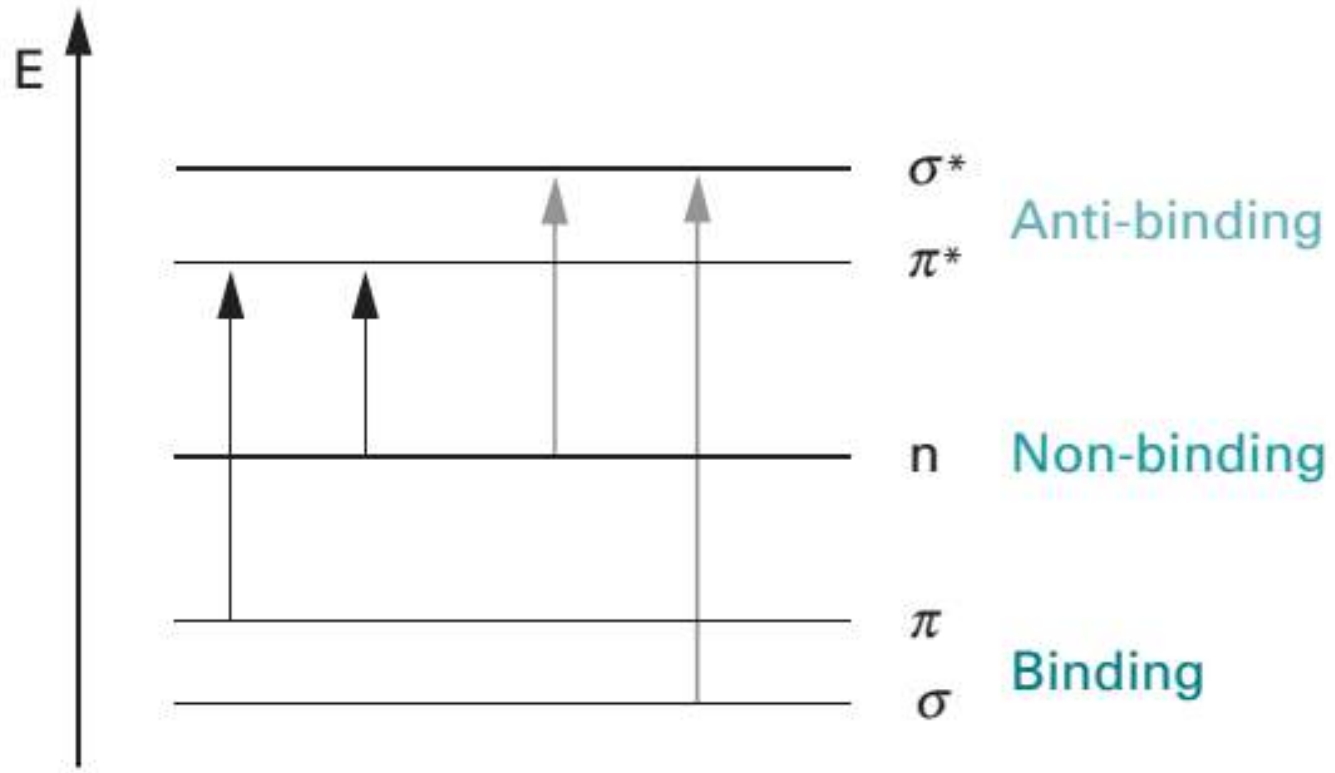
The electronic transitions in molecules can be classified according to the participating molecular orbitals.

From the four possible transitions $(n\rightarrow\pi^*,\ \pi\rightarrow\pi^*,\ n\rightarrow\sigma^*,\ \sigma\rightarrow\sigma^*)$ only two can be elicited with light from the UV/Vis spectrum for some biological molecules i.e. $n\rightarrow\pi^*$ and $\pi\rightarrow\pi^*$.

Then $n\rightarrow\sigma^*$ and $\sigma\rightarrow\sigma^*$ transitions are energetically not within the range of UV/Vis spectroscopy and require higher energies.

Molecular (sub-)structures responsible for interaction with electromagnetic radiation are called chromophores. In proteins, there are three types of chromophores relevant for UV/Vis spectroscopy-

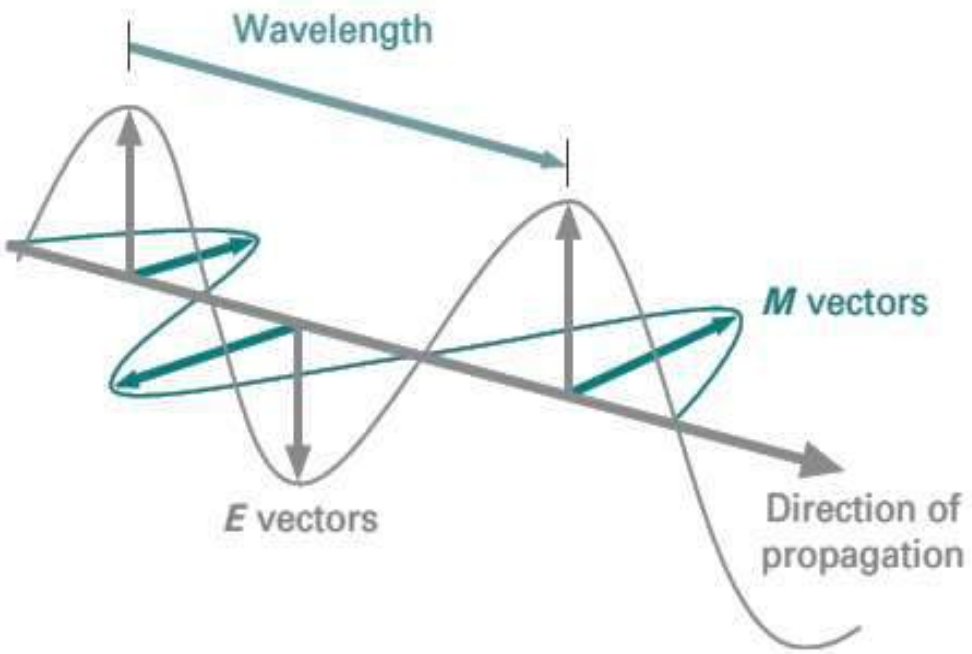
- peptide bonds (amide bond);
- certain amino acid side chains (mainly tryptophan and tyrosine)
- certain prosthetic groups and coenzymes (e.g. porphyrine groups such as in haem)



Energy scheme for molecular orbitals (not to scale). Arrows indicate possible electronic transitions. The length of the arrows indicates the energy required to be put into the system in order to enable the transition. Black arrows depict transitions possible with energies from the UV/Vis spectrum for some biological molecules. The transitions shown by grey arrows require higher energies (e.g. X-rays)

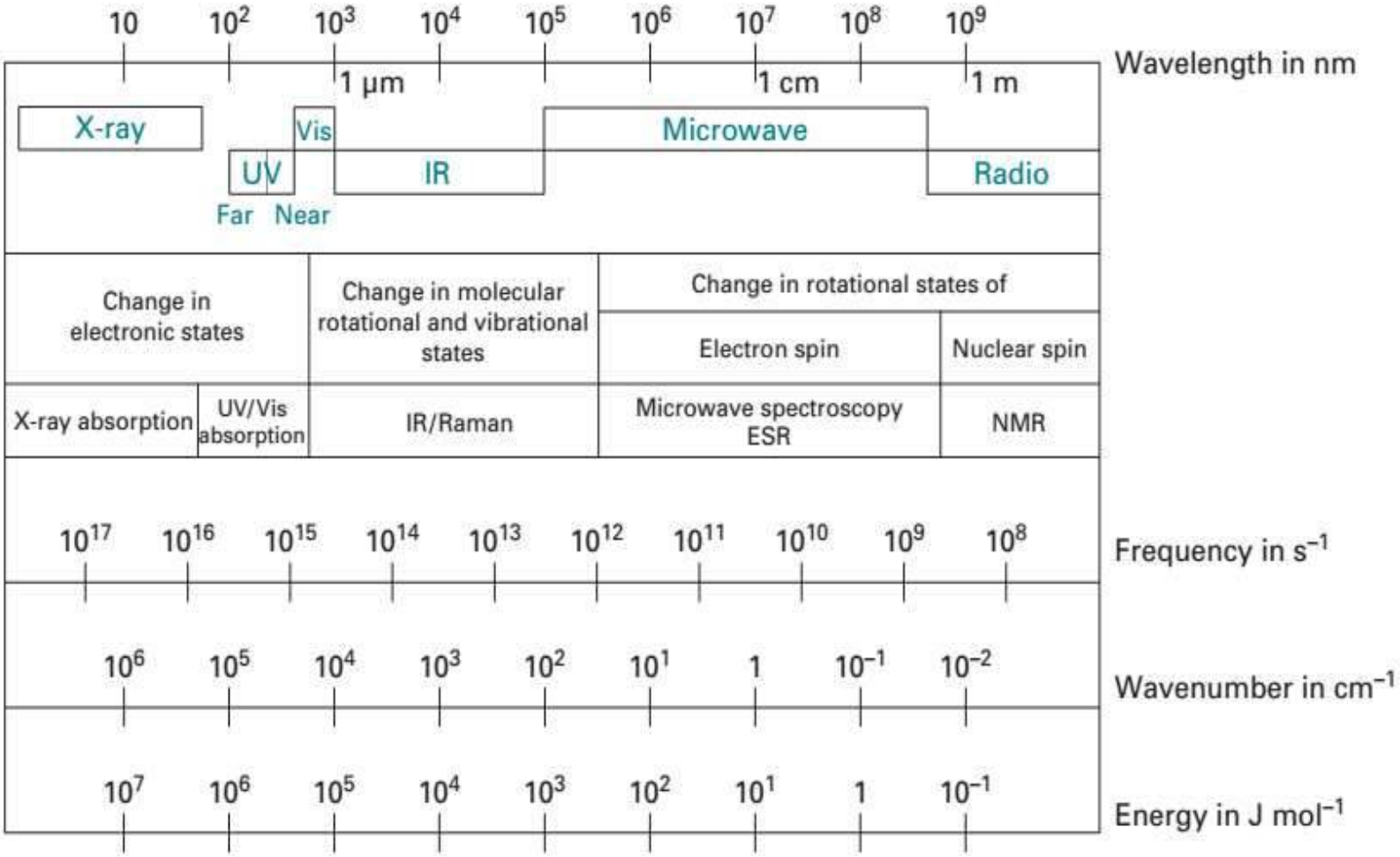
ELECTROMAGNETIC RADIATION

Electromagnetic radiation is composed of an electric and a perpendicular magnetic vector, each one oscillating in plane at right angles to the direction of propagation. The wavelength is the spatial distance between two consecutive peaks (one cycle) in the sinusoidal waveform and is measured in submultiples of metre, usually in nanometres (nm). The maximum length of the vector is called the amplitude



Light is electromagnetic radiation and can be described as a wave propagating transversally in space and time. The electric (E) and magnetic (M) field vectors are directed perpendicular to each other. For UV/Vis, circular dichroism and fluorescence spectroscopy, the electric field vector is of most importance. For electron paramagnetic and nuclear magnetic resonance, the emphasis is on the magnetic field vector.

ELECTROMAGNETIC SPECTRUM AND THEIR PROPERTIES



Various spectrometer and their working range in electromagnetic radiations

COLORIMETER & SPECTROPHOTOMETER

Photometry broadly deals with the study of the phenomenon of light absorption by molecules in solution. The specificity of a compound to absorb light at a particular wavelength (monochromatic light) is exploited in the laboratory for quantitative measurements. Colorimeter and spectrophotometer are the laboratory instruments used for this purpose. When a light at a particular wavelength is passed through a solution (incident light), some amount of it is absorbed and, therefore, the light that comes out (transmitted light) is diminished. The nature of light absorption in a solution is governed by Beer-Lambert law.

BEER-LAMBERT LAW

Beer's law states that the amount of transmitted light decreases exponentially with an increase in the concentration of absorbing material (i.e. the amount of light absorbed depends on the concentration of the absorbing molecules).

Lambert's law states that the amount of transmitted light decreases exponentially with increase in the thickness of the absorbing molecules (i.e. the amount of light absorbed is dependent on the thickness of the medium).

By combining the two laws (Beer-Lambert law), the following derivation can be obtaine-

$$I = I_0^{\varepsilon cd}$$

where I= Intensity of the transmitted light

I_0 = Intensity of the incident light

H= Molar extinction coefficient

c = Concentration of the absorbing substance (moles/l or g/dl)

d = Thickness of medium through which light passes.

Transmittance (T)

When the thickness of the absorbing medium is kept constant (i.e. Lambert's law), the intensity of the transmitted light depends only on concentration of the absorbing material. In other words, the Beer's law is operative. The ratio of transmitted light (I) to that of incident light (I_0) is referred to as Transmittance (T).

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

Absorbance (A) or optical density (OD)

Absorbance (A)or optical density (OD) is very commonly used in laboratories. The relation between absorbance and transmittance is expressed by the following equation.

$$\lg\frac{I_0}{I} = \lg\frac{1}{T} = \varepsilon \times c \times d = A$$

where $[d] = 1\ \text{cm}$, $[c] = 1\ \text{mol dm}^{-3}$, and $[\varepsilon] = 1\ \text{dm}^3\ \text{mol}^{-1}\ \text{cm}^{-1}$. ε is the **molar absorption coefficient** (also **molar extinction coefficient**) ($\alpha = 2.303 \times c \times \varepsilon$). A is the **absorbance** of the sample, which is displayed on the spectrophotometer.

Deviations from the Beer–Lambert law

According to the Beer–Lambert law, absorbance is linearly proportional to the concentration of chromophores/ Produced colour. This might not be the case any more in samples with high absorbance. Every spectrophotometer has a certain amount of stray light, which is light received at the detector but not anticipated in the spectral band isolated by the monochromator.

In order to obtain reasonable signal-to-noise ratios, the intensity of light at the chosen wavelength should be 10 times higher than the intensity of the stray light (I_{stray}). If the stray light gains in intensity, the effects measured at the detector have nothing or little to do with chromophore concentration.

Secondly, molecular events might lead to deviations from the Beer–Lambert law. For instance, chromophores might dimerise at high concentrations and, as a result, might possess different spectroscopic parameters.

The absorption spectrum of a chromophore is only partly determined by its chemical structure. The environment also affects the observed spectrum, which mainly can be described by three parameters:

- protonation/deprotonation (pH, RedOx);
- solvent polarity (dielectric constant of the solvent); and
- orientation effects.

Protonation/deprotonationarises either from changes in pH oroxidation/reduction reactions, which makes chromophores pH- and RedOx-sensitive reporters. As a rule of thumb the sample will displays a batho- and hyperchromic shift, if a titratable group becomes charged.

Solvent polarity affects the difference between the ground and excited states. Generally, when shifting to a less polar environment one observes a batho- and hyperchromic effect. Conversely, a solvent with higher polarity elicits a hypso- and hypochromic effect.

Orientation effects, such as an increase in order of nucleic acids from single stranded to double-stranded DNA, lead to different absorption behavior.

- A wavelength shift to higher values is called **Red Shift or Bathochromic Effect.**
- A shift to lower wavelengths is called **Blue Shift or Hypsochromic Effect.**
- An increase in absorption is called **Hyperchromicity (more colour).**
- A decrease in absorption is called **Hypochromicity (less colour)**

COLORIMETER

Colorimeter (or photoelectric colorimeter) is the instrument used for the measurement of coloured substances. This instrument is operative in the visible range (400-800 nm) of the electromagnetic spectrum of light.

The working of colorimeter is based on the principle of Beer-Lambert law.

The colorimeter, in general consists of light source, filter sample holder and detector with display (meter or digital). A filament lamp usually serves as a light source. The filters allow the passage of a small range of wave length as incident light. The sample holder is a special glass cuvette with a fixed thickness.

The photoelectric selenium cells are the most common detectors used in colorimeter.

Diagrammatic representation of the components in a colorimeter.

SPECTROPHOTOMETER/ UV-Visible Spectrophotometer

The spectrophotometer primarily differs from colorimeter by covering the ultraviolet region (200-400 nm) of the electromagnetic spectrum.

Further, the spectrophotometer is more sophisticated with several additional devices that ultimately increase the sensitivity of its operation several fold when compared to a colorimeter.

A precisely selected wavelength (say 234 nm or 610 nm) in both ultraviolet and visible range can be used for measurements. In place of glass cuvettes (in colorimeter), quartz cells are used in a spectrophotometer.

The spectrophotometer operation is also based on the Beer-Lambert law.

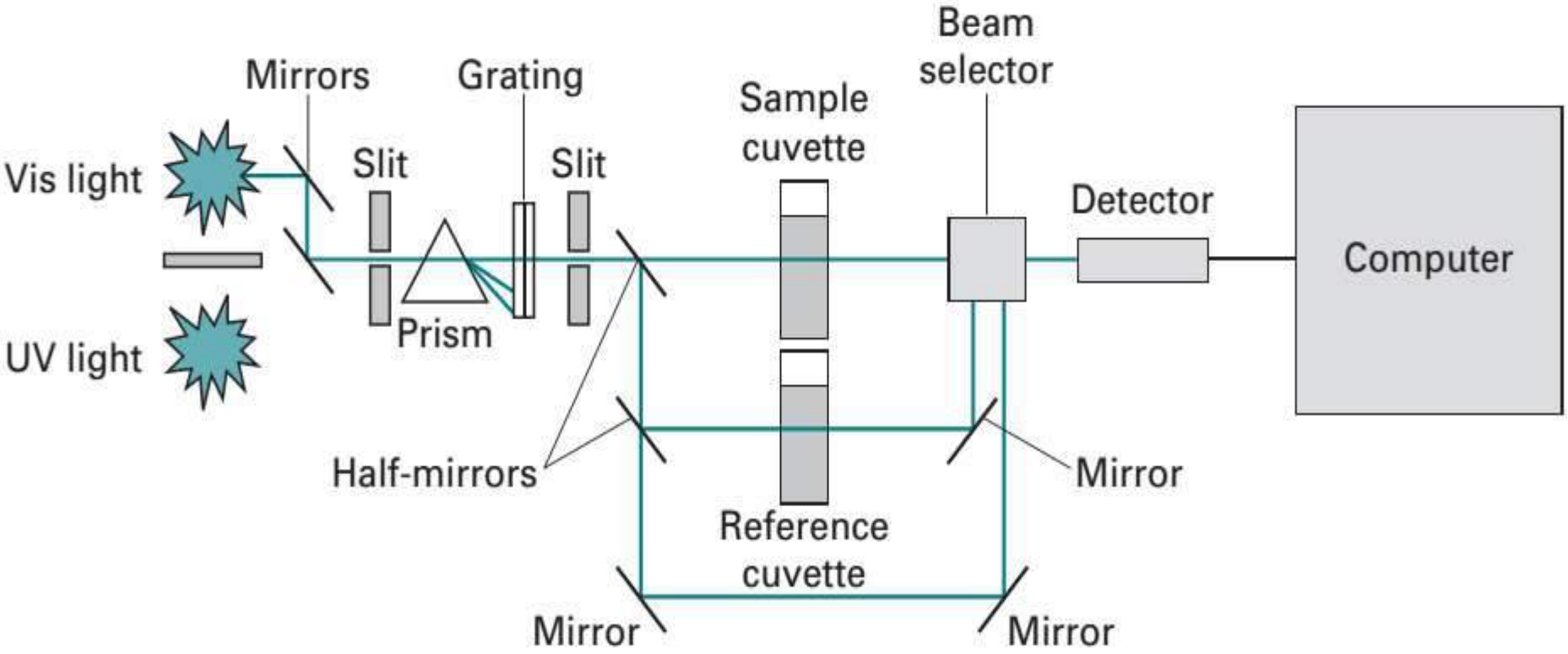
Instrumentation

- UV/Vis spectrophotometers are usually dual-beam spectrometers where the first channel contains the sample and the second channel holds the control.

- The light source is a tungsten filament bulb for the visible part of the spectrum, and a deuterium bulb for the UV region. Since the emitted light consists of many different wavelengths, a monochromator, consisting of either a prism or a rotating metal grid of high precision called **grating**, is placed between the light source and the sample.

- In a dual-beam instrument, the incoming light beam is split into two parts by a half mirror. One beam passes through the sample, the other through a control (blank, reference). This approach obviates any problems of variation in light intensity, as both reference and sample would be affected equally.

- Wavelength selection can also be achieved by using coloured filters as monochromators that absorb all but a certain limited range of wavelengths. This limited range is called the bandwidth of the filter. Filter-based wavelength selection is used in colorimetry, a method with moderate accuracy, but best suited for specific colorimetric assays where only certain wavelengths are of interest. If wavelengths are selected by prisms or gratings, the technique is called spectrophotometry



Optical arrangements in a dual-beam spectrophotometer. Either a prism or a grating constitutes the monochromator of the instrument. Optical paths are shown as green lines.

Applications

The usual procedure for (colorimetric) assays is to prepare a set of standards and produce a plot of concentration versus absorbance called **calibration curve**. This should be linear as long as the Beer–Lambert law applies. Absorbances of unknowns are then measured and their concentration interpolated from the linear region of the plot.

Qualitative and quantitative analysis

1. Qualitative analysis may be performed in the UV/Vis regions to identify certain classes of compounds both in the pure state and in biological mixtures (e.g. protein-bound).

2. The application of UV/Vis spectroscopy to further analytical purposes is used for quantification of biological samples either directly or via colorimetric assays. In many cases, proteins can be quantified directly using their intrinsic chromophores, tyrosine and tryptophan.

3. The region from 500 to 300 nm provides valuable information about the presence of any prosthetic groups or coenzymes.

4. Protein quantification can also be done by single wavelength measurements at 280 and 260 nm.

INFRARED AND RAMAN SPECTROSCOPY

Within the electromagnetic spectrum the energy range below the UV/Vis is the infrared region, encompassing the wavelength range of about 700 nm to 25mm, and thus reaching from the red end of the visible to the microwave region.

The absorption of infrared light by a molecule results in transition to higher levels of vibration.

Bonds between atoms can be considered as flexible springs, with constant vibrational motion within a molecule. Bond vibrations can be stretching or bending (deformation).

Theory predicts that a molecule with n atoms will have a total of 3n - 6 fundamental vibrations (3n - 5, if the molecule is linear): 2n - 5 bending, and n – 1 stretching modes

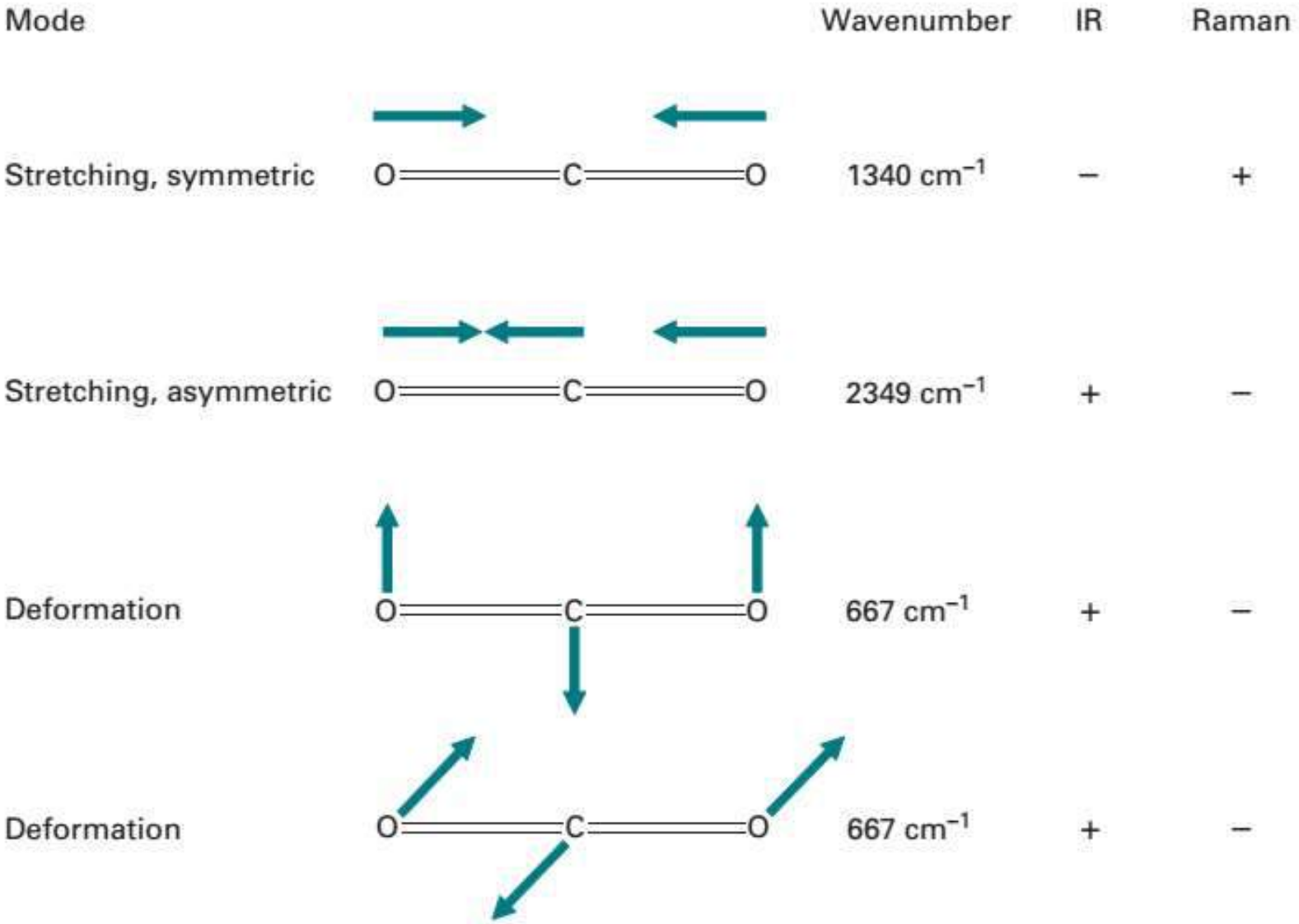
Infrared and Raman spectroscopy give similar information about a molecule, but the criteria for the phenomena to occur are different for each type.

For asymmetric molecules, incident infrared light will give rise to an absorption band in the infrared spectrum, as well as a peak in the Raman spectrum.

However, symmetric molecules, such as for example CO2, that possess a center of symmetry show a selective behavior: bands that appear in the infrared spectrum do not appear in the Raman spectrum, and vice versa.

An **infrared spectrum** arises from the fact that a molecule absorbs incident light of a certain wavelength which will then be 'missing' from the transmitted light. The recorded spectrum will show an absorption band

A **Raman spectrum** arises from the analysis of scattered light. The largest part of an incident light beam passes through the sample (transmission). A small part is scattered isotropically, i.e. uniformly in all directions (**Rayleigh scatter**), and possesses the same wavelength as the incident beam. The Raman spectrum arises from the fact that a very small proportion of light scattered by the sample will have a different frequency than the incident light.



Normal vibrational modes for CO2 . For symmetric molecules that possess a centre of symmetry, bands that appear in the IR do not appear in the Raman spectrum.

As different vibrational states are excited, energy portions will be missing, thus giving rise to peaks at lower frequencies than the incident light (**Stokes lines**). Notably, higher frequencies are also observed (**anti-Stokes lines**); these arise from excited molecules returning to ground state.

The emitted energy is dumped onto the incident light which results in scattered light of higher energy than the incident light. The criterion for a band to appear in the infrared spectrum is that the transition to the excited state is accompanied by a change in **dipole moment**, i.e. a change in charge displacement. Conversely, the criterion for a peak to appear in the Raman spectrum is a change in **polarisability** of the molecule during the transition.

Infrared spectroscopy

Infrared spectroscopy (**IR spectroscopy** or **Vibrational Spectroscopy**) is the spectroscopy that deals with the infrared region of the electromagnetic spectrum, that is light with a longer wavelength and lower frequency than visible light.

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. For a given sample which may be solid, liquid, or gaseous, the method or technique of infrared spectroscopy uses an instrument called an **infrared spectrometer** (or spectrophotometer) to produce an **infrared spectrum**.

A basic 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.

The infrared portion of the electromagnetic spectrum is usually divided into three regions as named for their relation to the visible spectrum are-

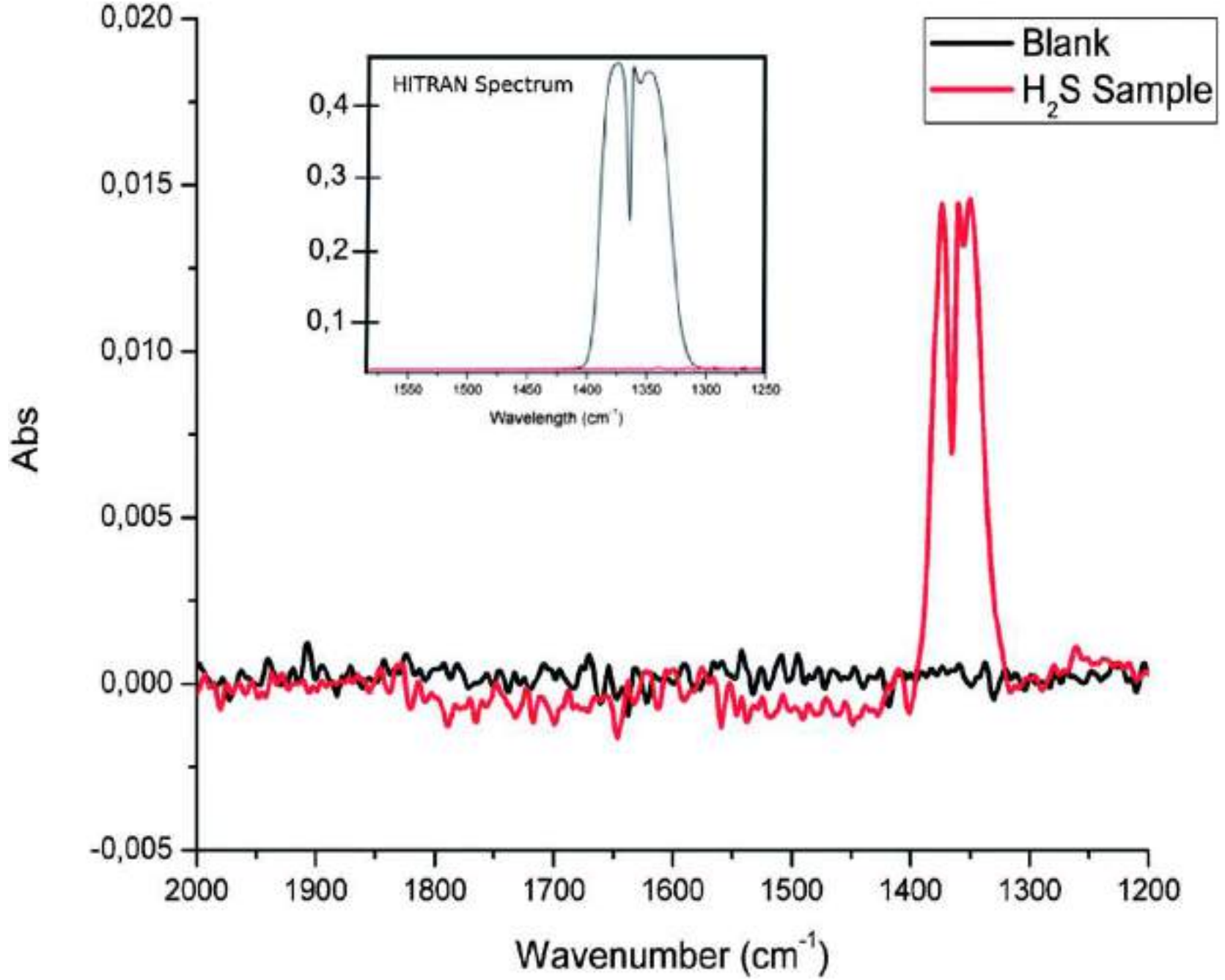
1. Near infrared spectrum
2. Mid- infrared spectrum
3. Far- infrared spectrum

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


Blank
H2S Sample
HITRAN Spectrum
Wavelength (cm-1)
Abs
Wavenumber (cm-1)
0,020
0,015
0,010
0,005
0,000
-0,005
2000
1900
1800
1700
1600
1500
1400
1300
1200
0,4
0,3
0,2
0,1
1550
1500
1450
1400
1350
1300
1250

Practical IR spectroscopy

The infrared spectrum of a sample is recorded by passing a beam of infrared light through the sample.

When the frequency of the IR is the same as the vibrational frequency of a bond, absorption occurs. Examination of the transmitted light reveals how much energy was absorbed at each frequency (or wavelength). This can be achieved by scanning the wavelength range using a monochromator.

Alternatively, the whole wavelength range is measured at once using a Fourier transform instrument and then a transmittance or absorbance spectrum is generated using a dedicated procedure. **Analysis of the position, shape and intensity of peaks in this spectrum reveals details about the molecular structure of the sample.**

This technique works almost exclusively on samples with covalent bonds. Simple spectra are obtained from samples with few IR active bonds and high levels of purity.

More complex molecular structures lead to more absorption bands and more complex spectra. The technique has been used for the characterization of very complex mixtures. Spectra issues with infrared fluorescence are rare.

Raman spectroscopy

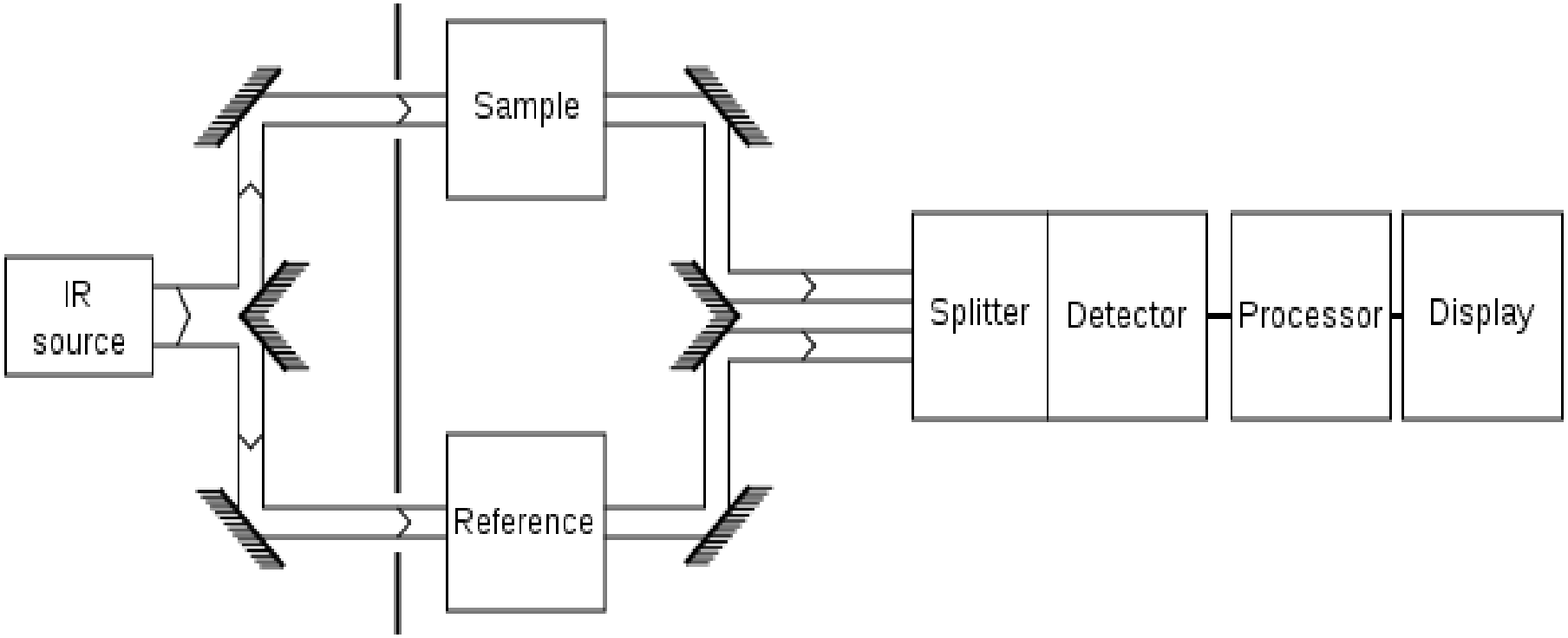
A **Raman spectrum** arises from the analysis of scattered light. The largest part of an incident light beam passes through the sample (transmission). A small part is scattered isotropically, i.e. uniformly in all directions (**Rayleigh scatter**), and possesses the same wavelength as the incident beam. The Raman spectrum arises from the fact that a very small proportion of light scattered by the sample will have a different frequency than the incident light.

The assignment of peaks in Raman spectra usually requires consideration of peak position, intensity and form, as well as depolarisation. This allows identification of the type of symmetry of individual vibrations, but not the determination of structural elements of a molecule.

The depolarisation is calculated as the ratio of two intensities with perpendicular and parallel polarisation with respect to the incident beam.

The use of lasers as light source for Raman spectroscopy easily facilitates the use of linearly polarised light. Practically, the Raman spectrum is measured twice. In the second measurement, the polarisation plane of the incident beam is rotated by 90^{o}.

INSTRUMENTATION



The most common source for infrared light is white-glowing zircon oxide or the so-called globar made of silicium carbide with a glowing temperature of 1500 K. The beam of infrared light passes a monochromator and splits into two separate beams: one runs through the sample, the other through a reference made of the substance the sample is prepared in. After passing through a splitter alternating between both beams, they are reflected into the detector.

For **IR spectroscopy** Samples of solids are either prepared in thick suspensions (mulls) such as nujol, and held as layers between NaCl planes or pressed into KBr disks. Non-covalent materials must be used for sample containment and in the optics, as these materials are transparent to infrared. All materials need to be free of water, because of the strong absorption of the O–H vibration.

For **Raman spectroscopy,** aqueous solutions are frequently used, since water possesses a rather featureless weak Raman spectrum. The Raman effect can principally be observed with bright, monochromatic light of any wavelength; however, light between the visible region of the spectrum is normally used due to few unwanted absorption effects. The ideal light source for Raman spectrometers is therefore a laser. Because the Raman effect is observed in light scattered off the sample, typical spectrometers use a 90^{o} configuration

APPLICATIONS

The use of infrared and Raman spectroscopy is mainly in chemical and biochemical research of small compounds such as drugs, metabolic intermediates and substrates. Examples are the identification of synthesised compounds, or identification of sample constituents (e.g. in food)

FLUORIMETRY

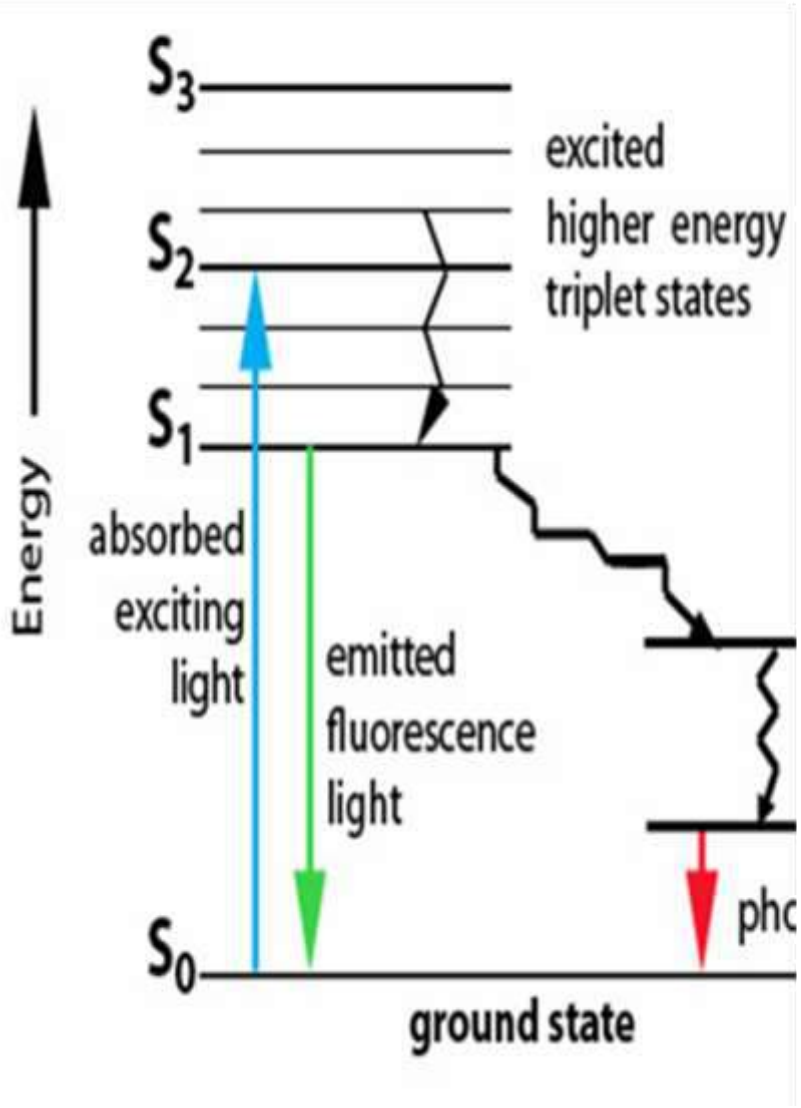
PRINCIPLES

Fluorescenceis an emission phenomenon where an energy transition from a higher to a lower state is accompanied by radiation. Only molecules in their excited forms are able to emit fluorescence; thus, they have to be brought into a state of higher energy prior to the emission phenomenon.

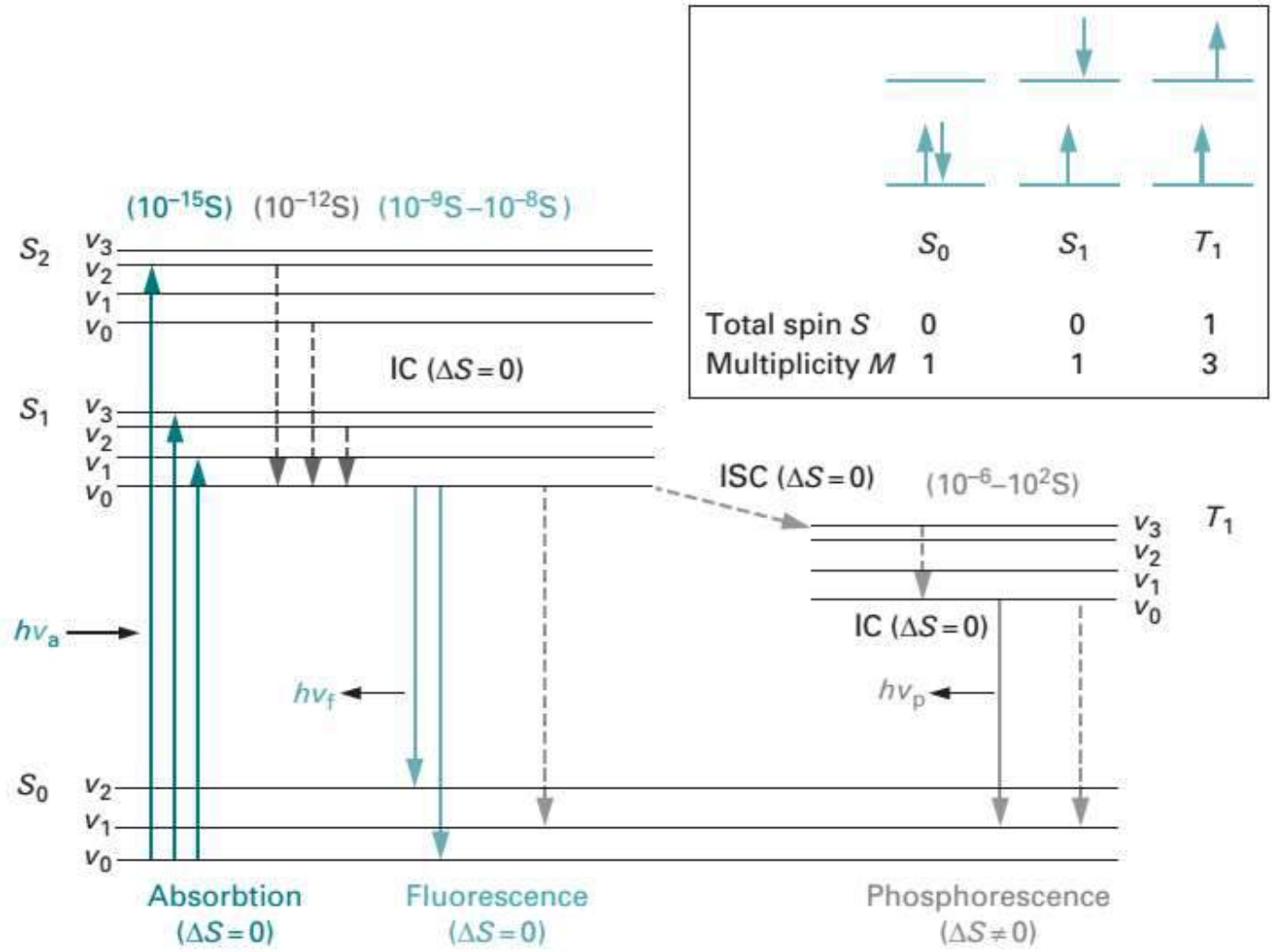
If the vibrational levels of the ground state overlap with those of the electronic excited state, the molecule will not emit fluorescence, but rather revert to the ground state by non-radiative internal conversion. This is the most common way for excitation energy to be dissipated and is why fluorescent molecules are rather rare.

Most molecules are flexible and thus have very high vibrational levels in the ground state. Indeed, most fluorescent molecules possess fairly rigid aromatic rings or ring systems. The fluorescent group in a molecule is called a **fluorophore**

Alexander Jablonski Diagram



- Light from the excitation filter excites the fluorochoromes to a higher energy state
- From the high state it declines slowly releasing energy
- Transition between absorption & emission



Jablonski diagram. Shown are the electronic ground state (S_0), two excited singlet states (S_1, S_2) and a triplet state (T_1). Vibrational levels (v) are only illustrated exemplarily. Solid vertical lines indicate radiative transitions, dotted lines show non-radiative transitions. The inset shows the relationship between electron configurations, total spin number S and multiplicity M.

INSTRUMENTATION

Fluorescence spectroscopy works most accurately at very low concentrations of emitting fluorophores. UV/Vis spectroscopy, in contrast, is least accurate at such low concentrations.

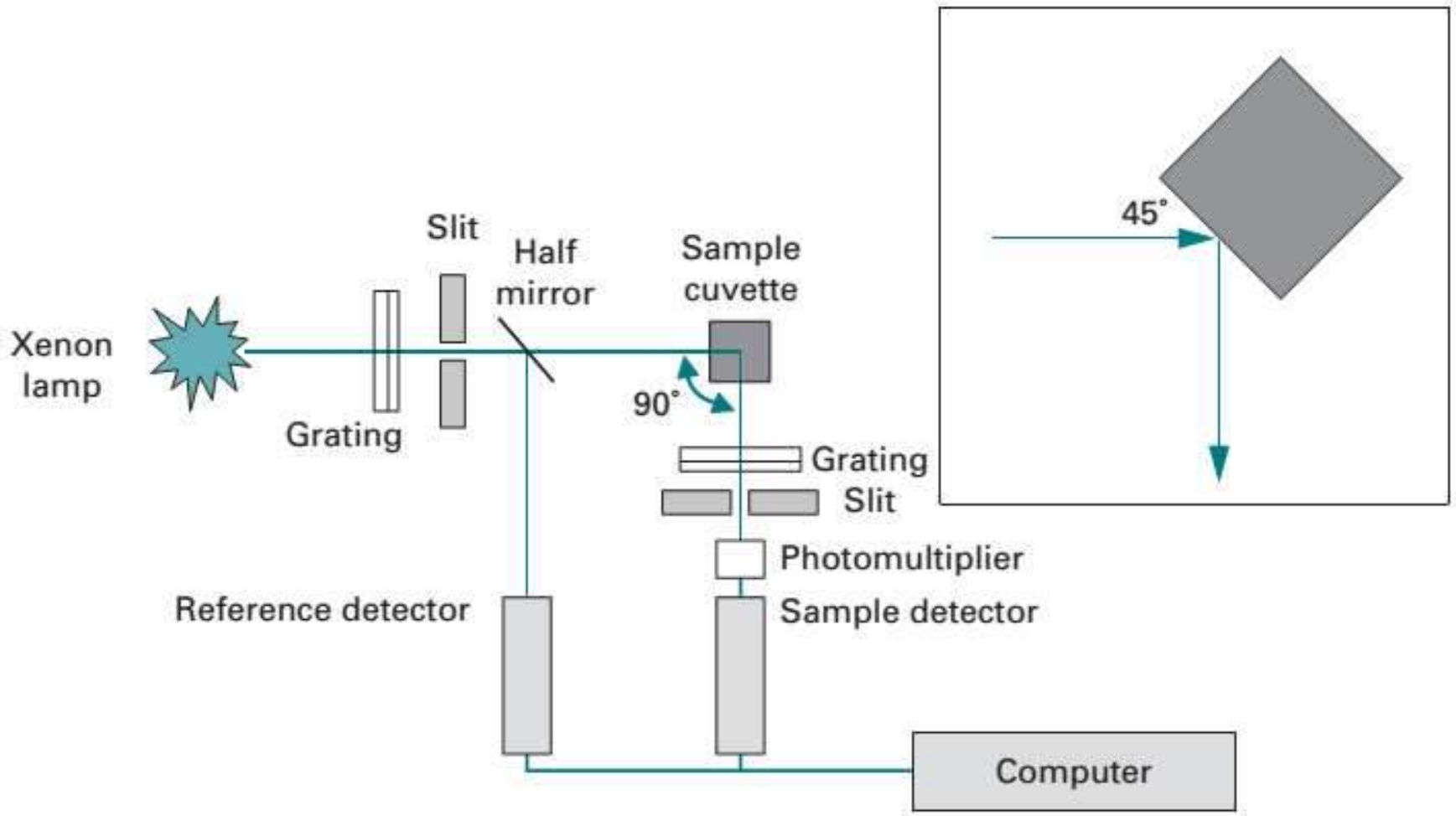
One major factor adding to the high sensitivity of fluorescence applications is the spectral selectivity. Due to the Stokes shift, the wavelength of the emitted light is different from that of the exciting light.

Another feature is that fluorescence is emitted in all directions. By placing the detector perpendicular to the excitation pathway, background of the incident beam is reduced.

The schematics of a typical spectrofluorimeter are two monochromators used as one for tuning the wavelength of the exciting beam and other for analysis of the fluorescence emission.

Due to the emitted light always having a lower energy than the exciting light, the wavelength of the excitation monochromator is set at a lower wavelength than the emission monochromator.

The better fluorescence spectrometers in laboratories have a photon-counting detector yielding very high sensitivity. Temperature control is required for accurate work as the emission intensity of a fluorophore is dependent on the temperature of the solution



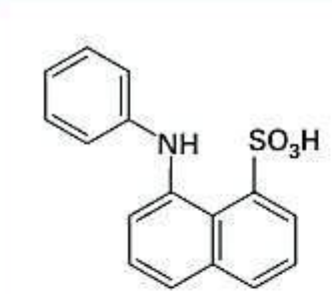
Schematics of a spectrofluorimeter with 'T' geometry (90^{o}). Optical paths are shown as green lines. Inset: Geometry of front-face illumination.

Applications

There are many and highly varied applications for fluorescence despite the fact that relatively few compounds exhibit the phenomenon. The effects of pH, solvent composition and the polarisation of fluorescence may all contribute to structural elucidation. Measurement of fluorescence lifetimes can be used to assess rotation correlation coefficients and thus particle sizes. Non-fluorescent compounds are often labelled with fluorescent probes to enable monitoring of molecular events. This is termed **extrinsic fluorescence** as distinct from intrinsic fluorescence where the native compound exhibits the property. Some fluorescent dyes are sensitive to the presence of metal ions and can thus be used to track changes of these ions in *in vitro* samples, as well as whole cells.

Since fluorescence spectrometers have two monochromators, one for tuning the excitation wavelength and one for analysing the emission wavelength of the fluorophore, one can measure two types of spectra: excitation and emission spectra. For fluorescence **excitation spectrum** measurement, one sets the emission monochromator at a fixed wavelength (λ_{em}) and scans a range of excitation wavelengths which are then recorded as ordinate (x-coordinate) of the excitation spectrum; the fluorescence emission at λ_{em} is plotted as abscissa. Measurement of **emission spectra** is achieved by setting a fixed excitation wavelength (λ_{exc}) and scanning a wavelength range with the emission monochromator. To yield a spectrum, the emission wavelength λ_{em} is recorded as ordinate and the emission intensity at λ_{em} is plotted as abscissa.

Structures of some extrinsic fluorophores



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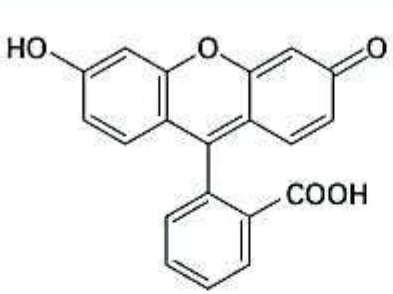
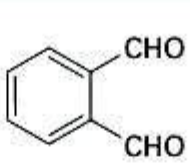
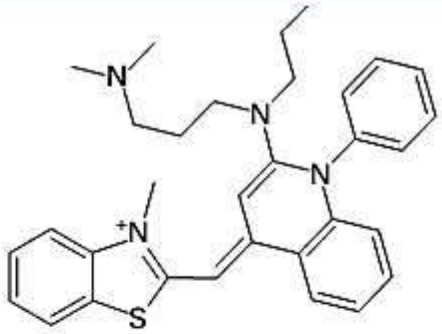


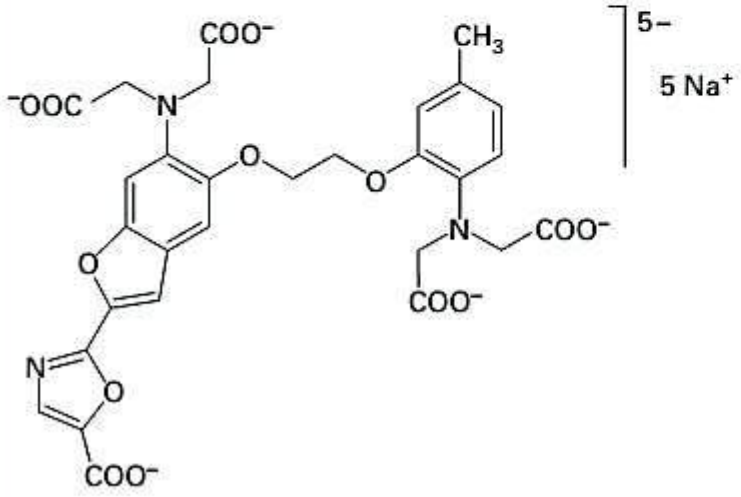
Fluorescein



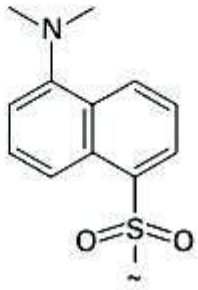
o-Phthalaldehyde



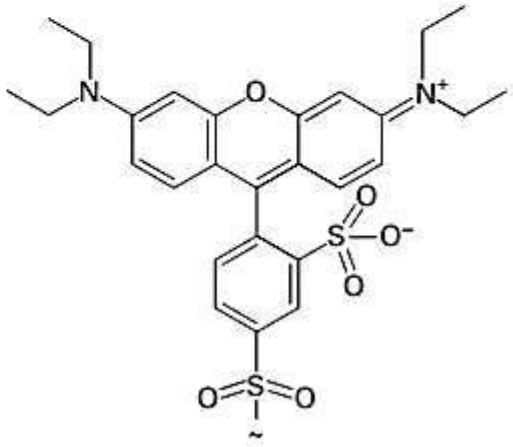
SYBR Green



Fura-2



Dansyl



N-(Lissamine rhodamine B sulfonyl)

QUENCHING

A quencher molecule decreases the quantum yield of a fluorophore by non-radiating processes. The absorption (excitation) process of the fluorophore is not altered by the presence of a quencher. However, the energy of the excited state is transferred onto the quenching molecules.

Quantum yield of a fluorophore is dependent on several internal and external factors. One of the external factors with practical implications is the presence of a quencher. Two kinds of quenching processes can be distinguished:

Dynamic Quenching which occurs by collision between the fluorophore in its excited state and the quencher; and

Static Quenching whereby the quencher forms a complex with the fluorophore. The complex has a different electronic structure compared to the fluorophore alone and returns from the excited state to the ground state by non-radiating processes.

LUMINOMETRY

Emission of electromagnetic radiation from a system can also be achieved by prior excitation in the course of a chemical or enzymatic reaction. Such processes are summarised as **luminescence**.

Bioluminescence describes the same phenomenon, only the reaction leading to a fluorescent product is an enzymatic reaction. The most commonly used enzyme in this context is certainly luciferase.

Luminometry is the technique used to measure luminescence, which is the emission of electromagnetic radiation in the energy range of visible light as a result of a reaction. Chemiluminescence arises from the relaxation of excited electrons transitioning back to the ground state. The prior excitation occurs through a chemical reaction that yields a fluorescent product.

For instance, the reaction of luminol with oxygen produces 3-aminophthalate which possesses a fluorescence spectrum that is then observed as a chemiluminescence.

In other words, the chemiluminescence spectrum is the same as the fluorescence spectrum of the product of the chemical reaction.

ATOMIC SPECTROSCOPY

Atomic spectroscopy is the study of the electromagnetic radiation absorbed and emitted by atoms. Electrons exist in energy levels (i.e. atomic orbitals) within an atom. Electrons may move between orbitals, but in doing so they must absorb or emit energy equal to the energy difference between their atom's specific quantized orbital energy levels.

In atomic spectroscopy, energy absorbed to move an electron to a higher energy level (higher orbital) and/or the energy emitted as the electron moves to a lower energy level is measured. Atomic absorption spectroscopy (AAS) quantifies the absorption of electromagnetic radiation by well-separated neutral atoms, while atomic emission spectroscopy (AES) measures emission of radiation from atoms in excited states.

Because each element has a unique number of electrons, an atom will absorb/release energy in a pattern unique to its elemental identity (e.g. Ca, Na, etc.) and thus will absorb/emit photons in a correspondingly unique pattern.

The type of atoms present in a sample, or the amount of atoms present in a sample can be deduced from measuring these changes in light wavelength and light intensity. In practice, atomic spectroscopy is used primarily to determine mineral elements

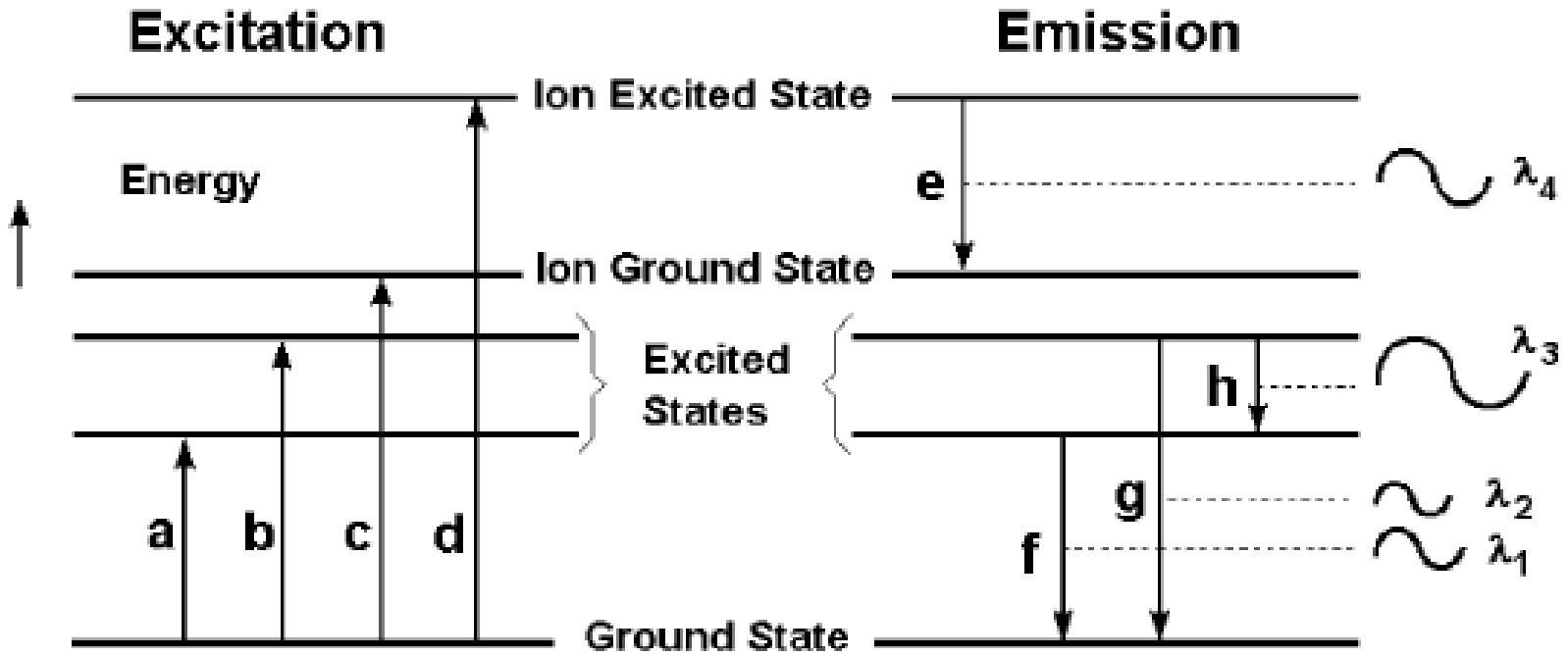


Figure 1-2. Energy level diagram depicting energy transitions where **a** and **b** represent excitation, **c** is ionization, **d** is ionization/excitation, **e** is ion emission, and **f**, **g** and **h** are atom emission.

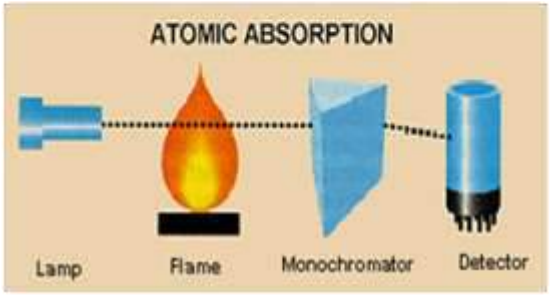
$$E = hc/\lambda$$

E – energy difference between two levels;
h – Plank's constant, 6.626068×10^{-34} m^2kg/s;
c – speed of light, 299 792 458 m/s;
λ – wavelenght, nm

ATOMIC SPECTROSCOPY

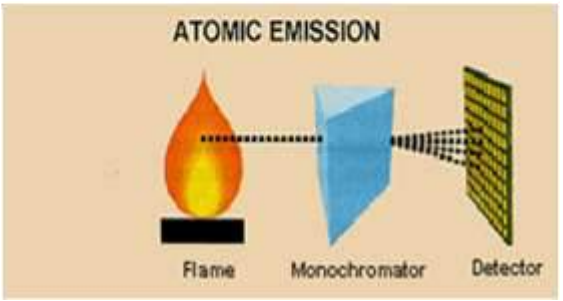
Absorption Spectroscopy:

AAS



Emission Spectroscopy:

FES, ICP-AES(OES)



Atomic spectroscopy is further divided into atomic absorption spectroscopy and atomic emission spectroscopy. In atomic absorption spectroscopy, light of a predetermined wavelength is passed through a collection of atoms. If the wavelength of the source light has energy corresponding to the energy difference between two energy levels in the atoms, a portion of the light will be absorbed. The difference between the intensity of the light emitted from the source (e.g., lamp) and the light collected by the detector yields an absorbance value. This absorbance value can then be used to determine the concentration of a given element (or atoms) within the sample.

The relationship between the concentration of atoms, the distance the light travels through the collection of atoms, and the portion of the light absorbed is given by the Beer–Lambert law. In atomic emission spectroscopy]], the intensity of the emitted light is directly proportional to the concentration of atoms.

ATOMIZATION

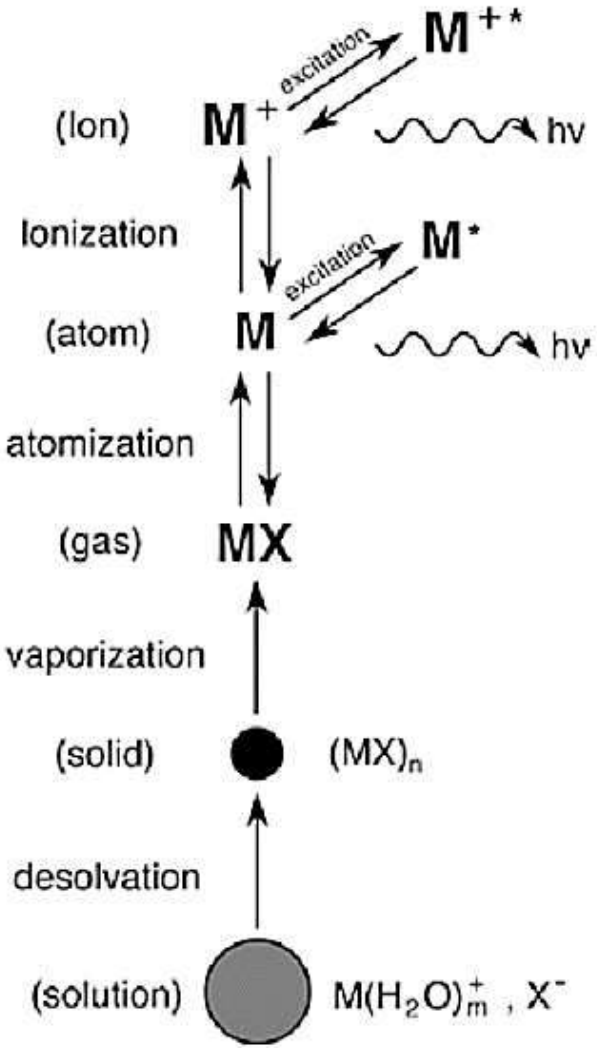
Atomic spectroscopy requires that atoms of the element of interest to be in the atomic state (not combined with other elements in a compound) and to be well separated in space.

Atomization is usually accomplished by exposing a solution containing the analyte (the substance being measured) as a fine mist to high temperatures, typically in a flame or plasma.

The solvent quickly evaporates, leaving solid particles of the analyte that vaporize and decompose to atoms that may absorb radiation (atomic absorption) or become excited and subsequently emit radiation (atomic emission).

Three common methods for atomizing samples, including their atomization temperature ranges are presented in table

Source of energy for atomization	*Approximate atomization temperature range (K)*	*Analytical method*
Flame	2,000–3,400	AAS, AES
Electrothermal	1,500–3,300	AAS (graphite furnace)
Inductively coupled argon plasma	6,000–7,000	ICP-OES, ICP-MS



A schematic representation of the atomization of an element in a flame or plasma. The large circle at the bottom represents a tiny droplet of a solution containing the element (M) as part of a compound

ATOMIC ABSORPTION SPECTROSCOPY

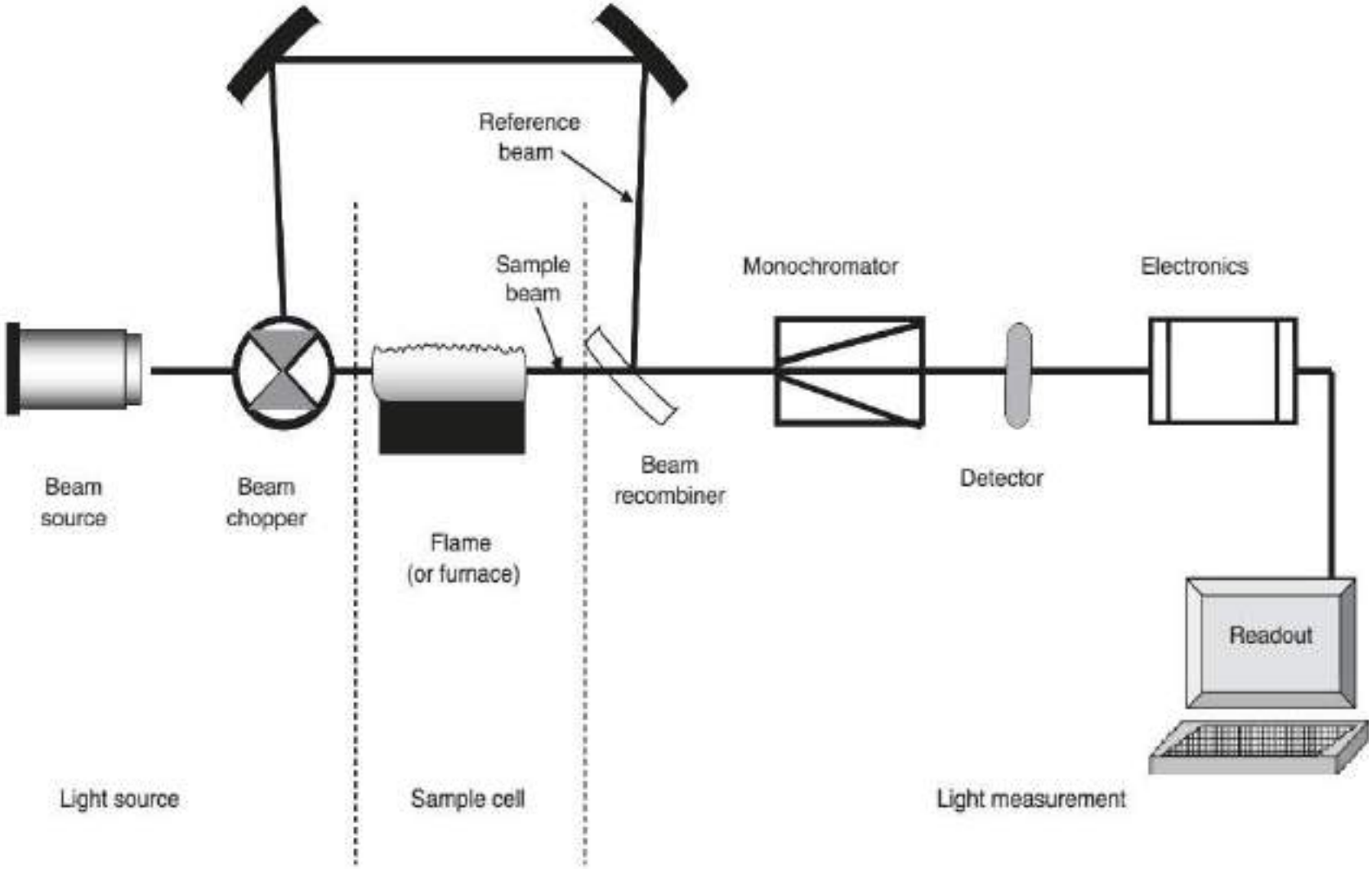
AAS is an analytical method based on the absorption of ultraviolet-visible (UV-Vis) radiation by free atoms in the gaseous state.

Two types of atomization are commonly used in AAS: flame atomization and electrothermal (graphite furnace) atomization.

Once the sample is atomized in the flame, the quantity of the analyte element is measured by determining the attenuation (decrease in intensity) of a beam of radiation passing through the flame, due to atomic absorption of incident radiation by the analyte element.

For the measurement to be specific for the analyte element, the radiation source ideally should emit radiation of the exact discreet wavelengths that only the analyte element is capable of absorbing.

The amount of radiation absorbed by the analyte element is principled by Beer's law



Schematic representation of a double-beam atomic absorption spectrophotometer

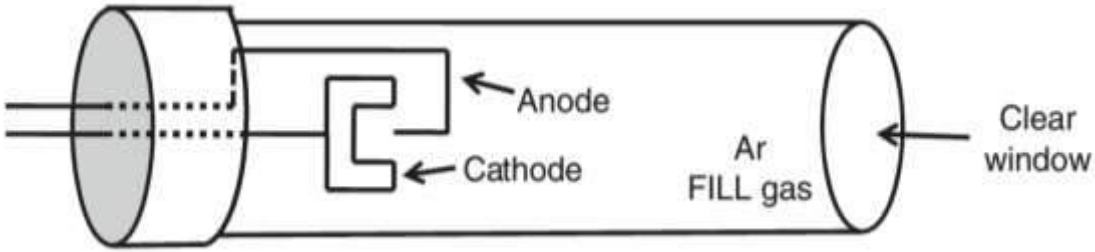
Instrumentation for Atomic Absorption Spectroscopy

Atomic absorption spectrometers, typically with a double- beam design, consist of the following components:

1. **Radiation source:** a hollow cathode lamp (HCL) or an electrode-less discharge lamp (EDL)
2. **Atomizer:** usually a nebulizer-burner system or a graphite furnace
3. **Monochromator:** usually an UV-Vis grating monochromator
4. **Detector:** a photomultiplier tube (PMT) or a solid- state detector (SSD)
5. **Readout device:** an analog or a digital readout

Radiation Source

A hollow cathode lamp(HCL) consists of a hollow tube filled with argon or neon gas, an anode made of tungsten, and a cathode made of the metallic form of the element being measured. When voltage is applied across the electrodes, the lamp emits radiation characteristic of the metal in the cathode.



Schematic representation of a hollow cathode lamp

For example, if the cathode is made of iron, an iron spectrum will be emitted. As the radiation passes through the flame containing the atomized sample, only iron atoms (not atoms of other elements) will absorb this radiation because the emitted wavelengths from the HCL are specific for iron atoms. Of course, this means that it is necessary to use a different lamp for each element analyzed.

Atomizers

Flame and graphite furnace atomizers are the two common types of atomizers used in AAS.

Flame Atomizer :

- The flame atomizer consists of a nebulizer and a burner. The nebulizer is designed to convert the sample solution into a fine mist or aerosol. This is accomplished by aspirating the sample through a capillary into a chamber through which oxidant and fuel are flowing.
- The chamber contains baffles that remove larger droplets, leaving a very fine mist. Only about 1 % of the total sample is carried into the flame by the oxidant-fuel mixture. The larger droplets fall to the bottom of the mixing chamber and are collected as waste.
- The burner head contains a long, narrow slot that produces a flame that may be 5–10 cm in length. This gives a long path length that increases the sensitivity of the measurement. Flame characteristics may be manipulated by adjusting oxidant/fuel ratios and by choice of oxidant and fuel. Air-acetylene and nitrous oxide-acetylene are the most commonly used oxidant-fuel mixtures although other oxidants and fuels may be used for some elements.

Graphite Furnace:

- The graphite furnaceis typically a cylindrical graphite tube connected to an electrical power supply. The sample is injected into the tube through an inlet using a microliter syringe with sample volumes ranging from 0.5 to 100 µL.
- During operation, the system is flushed with an inert gas to prevent the tube from burning and to exclude air from the sample compartment.
- The tube is heated electrically in stages: first the sample solvent is evaporated, then the sample is ashed, and finally the temperature is rapidly increased to ~2000–3000 K to quickly vaporize and atomize the sample.

Interferences in Atomic Absorption Spectroscopy

Two types of interferences are encountered in AAS: spectral and non-spectral.

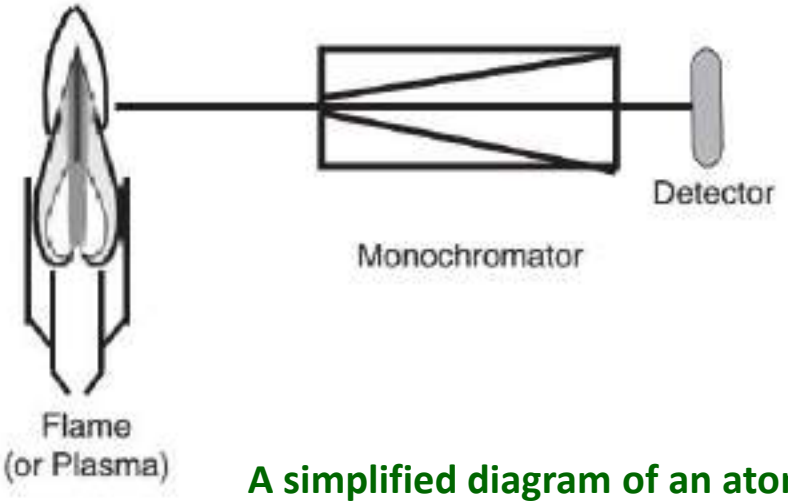
Spectral interferences are caused by the absorption of radiation by other elemental or molecular species at wavelengths that overlap with the spectral regions of the analyte present in the sample.

Non-spectral interferencesare caused by sample matrices and conditions that affect the atomization efficiency and/or the ionization of neutral atoms in the atomizer.

ATOMIC EMISSION SPECTROSCOPY (AES)

In contrast to AAS, the source of the measured radiation in AES is the excited atoms in the sample, not radiation from a source transmitted through a sample.

Sufficient energy is first applied to the sample to excite atoms to higher-energy levels; emissions of wavelengths characteristic of individual elements are then measured when electrons from excited atoms move back to the ground state or a lower-energy state.



A simplified diagram of an atomic emission spectrometer

Energy for excitation may be produced by heat (usually from a flame), light(from a laser), electricity (arcs or sparks), or radio waves(ICP).

The two most common forms of AES used in food analysis are **flame emission spectroscopy** and inductively coupled **plasma-optical emission spectroscopy(ICP-OES**).

Note: AES is also commonly called optical emission spectroscopy (OES), when combined with ICP.

Principles of Flame Emission Spectroscopy

- Flame emission spectrometers employ a nebulizer burner system to atomize and excite the atoms of the elements being measured.
- The flame with the excited atoms serves as the radiation source, so an external source (the HCL with the beam chopper) is not required. Otherwise instrumentation for flame emission spectroscopy is essentially identical to that for AAS.
- Many modern atomic absorption spectrometers can also be operated as flame emission spectrometers
- Flame photometer sare economical emission spectrometers equipped with interference filters and are specifically designed for the analysis of the alkali and alkaline earth metals in biological samples.
- Low flame temperatures are used so that only easily excited elements such as sodium, potassium, and calcium produce emissions.

Principles of Inductively Coupled Plasma- Optical Emission Spectroscopy

ICP-OES differs from flame emission spectroscopy in that it uses an argon plasma as the excitation source. A plasma is defined as a gaseous mixture containing significant concentrations of cations and electrons.

Temperatures in argon plasmas could be as high as 10,000 K, with analyte excitation temperature typically ranging from 6,000 to 7,000 K.

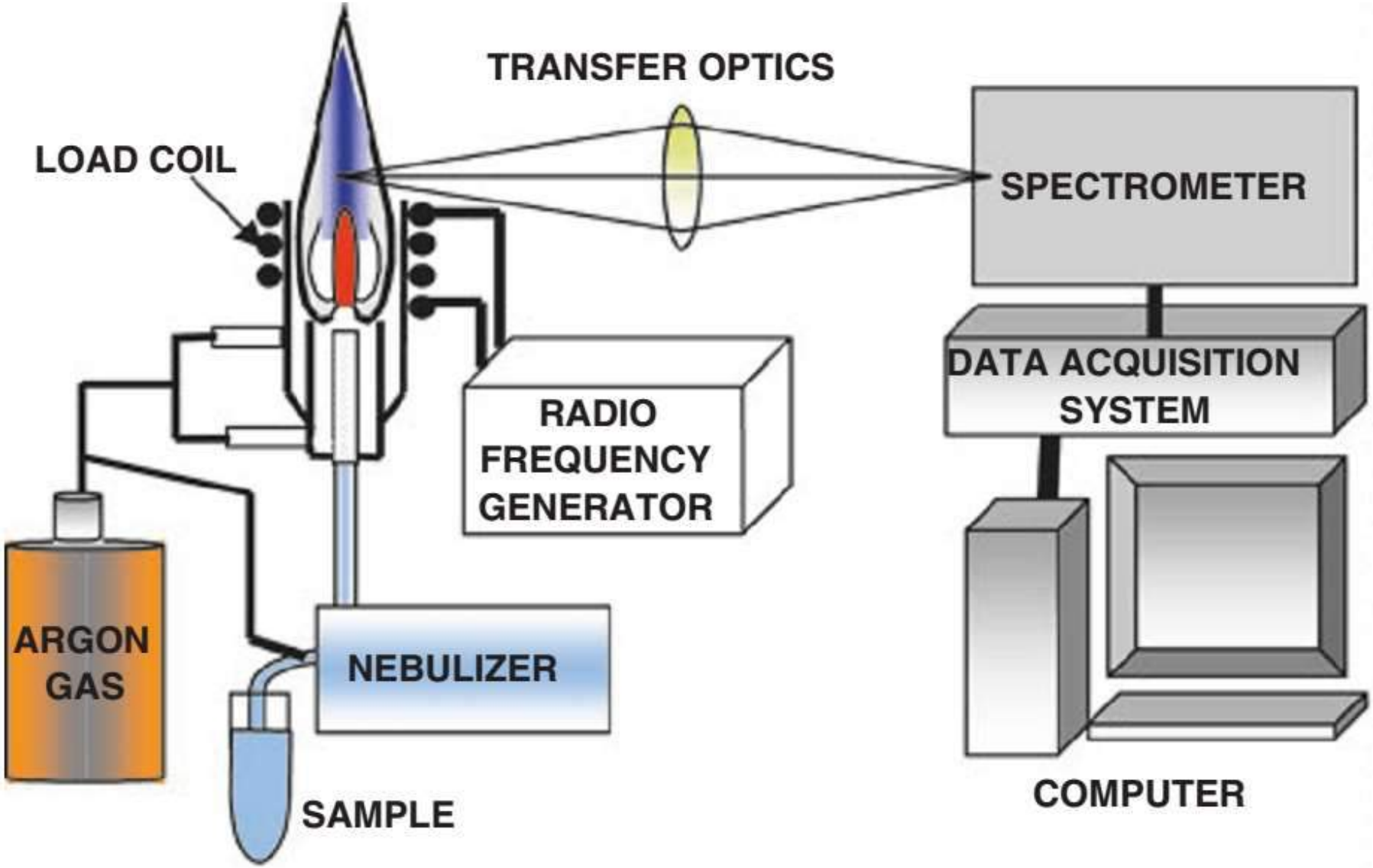
The extremely high temperatures and the inert atmosphere of argon plasmas are ideal for the atomization, ionization, and excitation of the analyte atoms in the sample.

The nearly complete atomization of the sample minimizes chemical interferences. The relatively uniform temperatures in plasm as (compared to nonuniform temperatures in flames) and the relatively long residence time give good linear responses over a wide concentration range (up to 6 orders of magnitude)

Instrumentation for Inductively Coupled Plasma-Optical Emission Spectroscopy

Inductively coupled plasma-optical emission spectrometers typically consist of the following components:

1. Argon plasma torch
2. Monochromator, polychromator, or echelle optical system
3. Detector(s), a single or multiple PMT(s) or solid- state array detector(s)
4. Computer for data collection and treatment

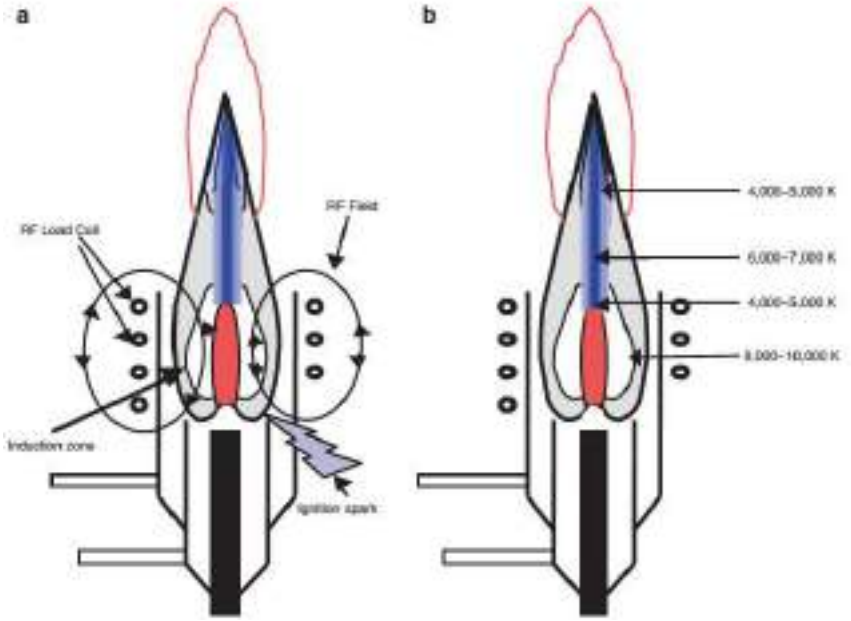


Major components and typical layout of ICP-OES instrument

ARGON PLASMA TORCH

The plasma torch consists of three concentric quartz tubes centered in a copper coil, called the load coil. During operation of the torch, a stream of argon gas flows through the outer tube, and radio frequency(RF) power is applied to the load coil, creating a magnetic field oscillating at the frequency of the RF generator (usually 27 MHz or 40 MHz).

The plasma is started by ionizing argon atoms with an electric spark to form argon ions and electrons. The oscillating magnetic field couples with the argon ions and electrons, forcing them to flow in an annular (ring- shaped) path.



The ICP plasma: (a) the process by which the plasma is formed and sustained and (b) the temperature distribution of the plasma

Heating does not involve burning fuel to directly heat and atomize the sample, as is the case with flame AAS (argon is a noble gas and will not combust). Rather, heating is accomplished by transferring RF energy to free electrons and argon ions in a manner similar to the transfer of microwave energy to water in a microwave oven.

These high-energy electrons in turn collide with argon atoms, generating even more electrons and argon ions and causing a rapid increase in temperature to approximately 10,000 K. The process continues until about 1 % of the argon atoms are ionized. At this point the plasma is very stable and self-sustaining for as long as the RF field is applied at constant power.

The transfer of energy to a system through the use of electromotive forces generated by magnetic fields is known as inductive coupling, hence the name ICP

APPLICATIONS OF ATOMIC ABSORPTION AND EMISSION SPECTROSCOPY

1. Atomic absorption and emission spectroscopy are widely used for the quantitative measurement of mineral elements in foods. In principle, any food may be analyzed with any of the atomic spectroscopy methods. In most cases, it is necessary to ash the food to destroy organic matter and to dissolve the ash in a suitable solvent (usually water or dilute acid) prior to analysis.

2. Some liquid products may be analyzed without ashing, provided appropriate precautions are taken to avoid interferences. For example, vegetable oils may be analyzed by dissolving the oil in an organic solvent such as acetone or ethanol and aspirating the solution directly into a flame atomic absorption spectrometer.

3. Milk samples may be treated with trichloroacetic acid to precipitate the protein; the resulting supernatant is analyzed directly. A disadvantage of this approach is that the sample is diluted in the process and the analyte can become entrapped or complexed to the precipitated proteins. This may be a problem when analytes are present in low concentrations. An alternative approach is to use a graphite furnace for atomization. For example, an aliquot of an oil may be introduced directly into a graphite furnace for atomization.

4. The choice of method will depend on several factors, including instrument availability, cost, precision/sensitivity, and operator skill.

Advantages and disadvantages of AAS, ICP-OES and ICP-MS

	Flame AAS	*Graphite furnace AAS*	*ICP-OES*	*ICP-MS*
Detection limit[a]	Good detection limits with many elements at the part per billion (ppb) level	Better than flame AAS and better than ICP-OES for some elements	Better than flame AAS	Overall best detection limits compared to other techniques
Elemental analytical capability	Single	Single	Multiple	Multiple
Approximate analytical working range	3 orders of magnitude	2 orders of magnitude	6 orders of magnitude (could be higher with dual-view models)	9 orders of magnitude
Cost	Low	Low to medium	Medium	High
Use of explosive fuel gas	Yes (Flame AAS instruments should not be unattended while in operation.)	No	No	No
User-friendliness	Some skills required but relatively easy to use	Some skills required	Easy to use once the computer interface is set up and operation is automated	Method development requires more expertise compared to other techniques
Ideal application	Analyzing a limited number of elements in a given sample	Analyzing a limited number of elements, and requiring better detection limits than Flame AAS	Multiple elements in a large number of samples	Multiple elements at ultra-trace concentrations in a large number of samples
Isotopic analysis	N/A	N/A	N/A	Isotopic analysis possible because isotopes of the same element have different mass-to-charge ratios

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