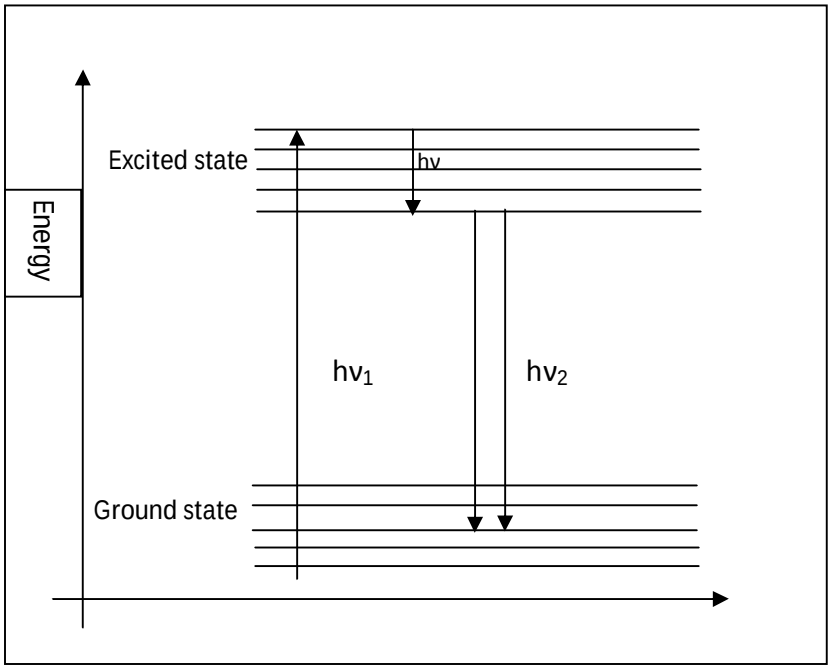
INSTRUMENTAL METHODS OF ANALYSIS (CHM 303)

FLUOROMETRY

Molecular Fluorescence

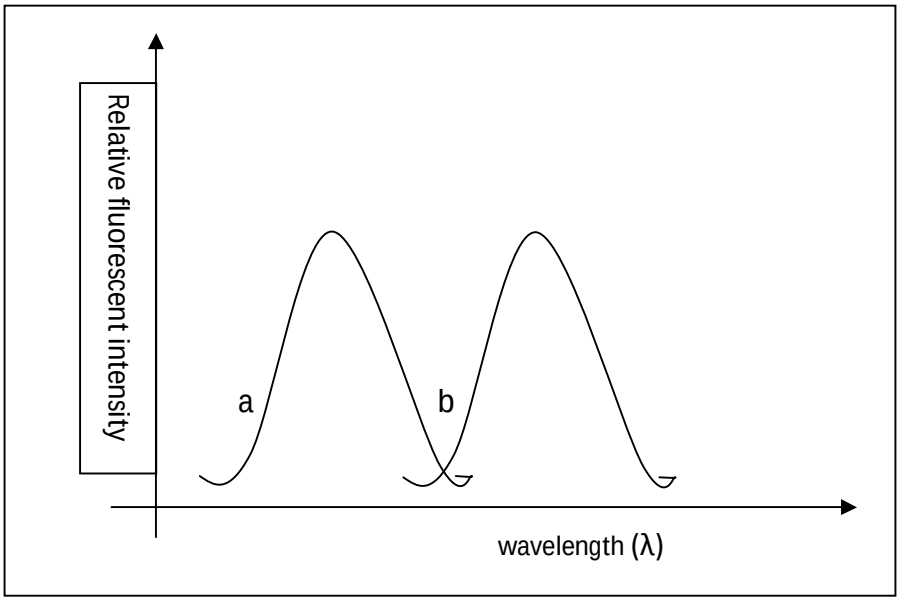
When a molecule absorbs electromagnetic energy, the energy is usually lost as heat by collision process for certain molecules, (5-10%) especially when absorbing high energy radiation like UV, only part of the energy is lost by collision; the electron then drops back to ground state by emitting a photon of lower energy (longer wavelength) than the one absorbed.

The emitted radiation is usually in the visible region and at right angles to the incident radiation; only visible rarely when absorbed radiation is of visible high energy (short wavelength) in U.V region is the emitted radiation in U.V region. Molecules at room temperature are in ground electronic state. It absorbs energy and goes to excited electronic state. The groups of lines represent vibrational state. The entire process takes place in a very short time (≈ 10^{-12} – 10^{-9} s). The absorption requires about 10^{-15} s while the collision takes about 10^{-8} s.



The molecules emit energy hν by collision and drops to the lowest vibrational level of the excited state. The probability of return to ground state with emission of photon is greatest at

this point. The electron thus emits a photon of energy $h\nu_2$ at a longer wavelength which is lower than $h\nu_1$ absorbed. This emitted light is "fluorescence". In some cases, the electron crosses over to a triplet state (i.e. it becomes unpaired) before emitting a photon. This takes a longer time $>10^{-9}$ s. the emitted radiation is then called phosphorescence. Both processes are called luminescence.



a – excitation b – fluorescent

Generally, Beer's law applies to power of radiation transmitted by a substance or solution but modification becomes necessary for fluorescence since radiation is emitted and not transmitted

Beer's law: $\ln T = \ln P/Po = - abc$

$A = \ln 1/T = \ln Po/P = abc.$

Fluorescence

$F = k (Po - P)$ …….. (1)

Where F is the intensity of fluorescent radiation, k is constant (quantum yield), Po is incident radiation and P is transmitted radiation.

I.e. $F \infty (Po - P)$ i.e. radiation is absorbed.

From Beer's law,

$$P = Po\ e^{-abc}$$

Where Po is the power of incident radiation and P is the power of transmitted radiation.

$$Po - P = Po - Po\ e^{-abc} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

$$= Po\ (1 - e^{-abc}) \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

$$F = K\ (Po - P) = KPo\ (1 - e^{-abc}) \ldots\ldots\ldots\ldots\ldots\ldots \quad (4)$$

From (1), if no light is transmitted, i.e. all incident radiation is absorbed,

$$Fo = KPo \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (5)$$

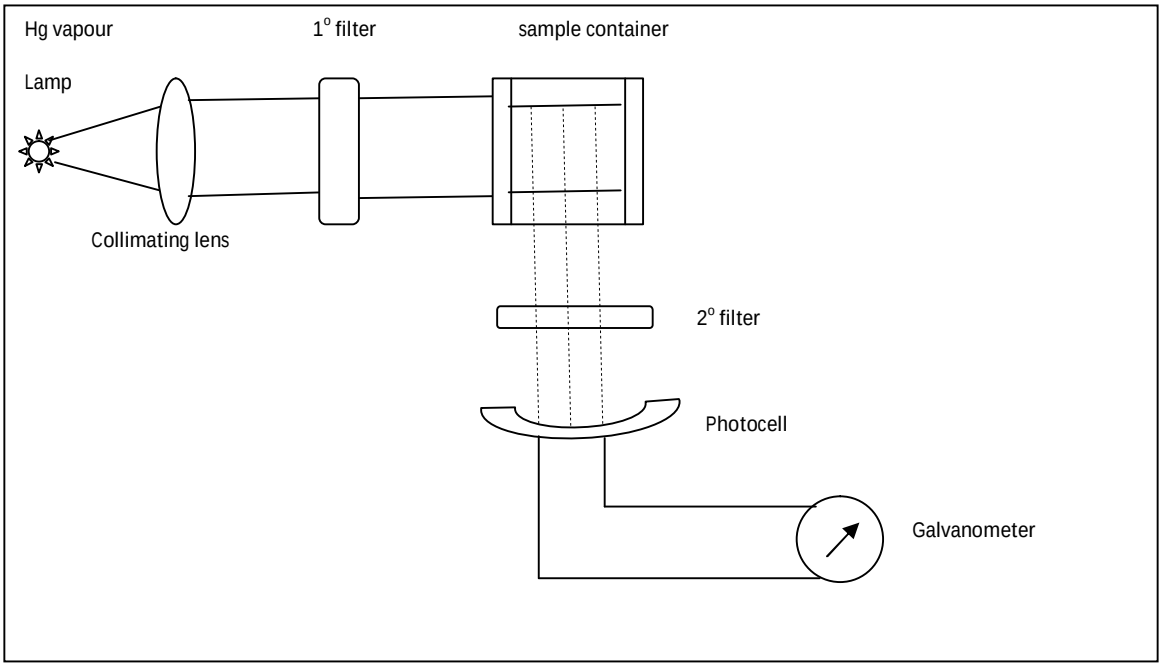
From (4), $F = Fo - Fo\ e^{-abc}$ …… (6)

$$Fo - F/\ Fo = e^{-abc}$$

$$\ln Fo - F/\ Fo = -abc$$

$$\ln Fo/\ Fo - F = abc \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (7)$$

K is the fraction of incident radiation that is absorbed and depends on factors such as the dimensions of the light beam, area of the solution irradiated, transmission band of filter before photocell, spectral response of photocell etc. “a” is constant – absorptivity.



"b" is the path length (thickness) of cell, "c" is concentration. When abc is small and negligible compared to 1, (≤ 0.01), equation (4) becomes

$$F = 2.303\ KPo\ .\ abc \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (8) \quad \text{(Proved)}$$

$$= K'C \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (9)$$

i.e. $F \propto C$ if $abc \leq 0.01$. K' is constant for a particular substance in a given instrument.

Equation (8) holds only for dilute solutions (when most of the radiation is transmitted, 92%) and breaks down at higher concentration. As usual, there should be no dissociation or association of molecules.

Chemical Structure and Fluorescence

In principle, any molecule that absorbs UV light radiation should fluoresce but not all do. The greater the absorption by a molecule, the greater its fluorescence intensity. Many aromatic and hetero compounds especially if they contain certain substituted groups. Compounds with multiple conjugated double bonds are more favourable to fluoresce. Presence of one or more electron donating group enhances fluorescence e.g. –OH, $-NH_2$, $-OCH_3$ etc. Polycyclic compounds like vitamin K, purines and nucleosides and conjugated polyenes like vitamin A are fluorescent. Groups like $–NO_2$, -COOH, $-CH_2COOH$, -Br, -I, and azo groups tend to inhibit fluorescence. The nature of other substituent may affect the degree of fluorescence. The fluorescence of many molecules is pH dependent as only the ionized or un-ionized form may be fluorescent.

A compound that is not fluorescent may be converted to a fluorescent derivative e.g. non-fluorescent steroids may be converted to fluorescent compounds by dehydrating with conc. H_2SO_4 which convert these cyclic alcohols to phenols. Similarly, dibasic acids e.g. maleic acid can be reacted with β-naphtha in conc. H_2SO_4 to form a fluorescent derivative.

White *et al* developed fluorometric methods for many metals by forming chelates with organic ligands. Antibodies can be made fluorescent by condensing them with fluorescein isocyanate which reacts with free amino groups of the proteins.

Fluorescence Quenching

Some substances quench fluoresce. These substances compete for electronic excitation energy and decrease the quantum yield (i.e. decrease the rate of conversion of absorbed energy to

fluorescent radiation). I^- is a very effective quencher. I^- and Br^- substituent groups decrease quantum yield. These substances may even be determined by measuring the extent of fluorescence quenching. Some molecules do not fluoresce, whose bond dissociation energy is less than that of incident radiation. Instead of getting excited, a bond is broken. Also, coloured species in solution with fluorescing species may interfere by absorbing the fluorescent radiation. This is "inner filter" effect. For example, in Na_2CO_3 solution, $K_2Cr_2O_7$ has absorption peaks at 245 and 348 nm, which overlap with excitation (275 nm) and emission (350 nm) peaks of tryptophan and interferes.

Limitations

This arises because fluorometry is an extremely sensitive technique and can detect at ppb level and the method is in fact limited to trace levels (a few ppm). Problems include instability of dilute solutions due to adsorption onto container surface which leads to significant errors. The problem is negligible in more concentrated solutions. Organic substances at < 1 ppm in organic solvents are adsorbed onto glass surfaces. Addition of small amount of more polar solvent may decrease it.

Quantitative Procedure

A series of standard solutions are prepared with slightly different concentration. The fluorescent intensity (power) is measured and a calibration curve is plotted. The intensity of the sample solution is also measured and concentration is read from calibration curve.

Infrared Spectrophotometry

The infrared region extends from a wavelength of 780nm to 1500nm (1.5μm) for near I.R and 1.5μm to 300μm for the far I.R, but the most useful region is from 2.5μm to 25μm which is most frequently used for analysis.

Not all molecules can absorb in I.R region but only those with a change in dipole moment (polarity) of the molecule. A diatomic molecule must have a permanent dipole although bigger molecules do not. For example, NΞ N, H-H, O=O, without dipoles cannot absorb in I.R region. C Ξ O has dipole moment and will absorb CO_2 is symmetrical and has no net dipole and not expected to absorb in I.R but by vibration, it develops dipole and absorbs. In the vibration mode (a), there exist symmetry and no absorbance while in mode (b), there exist no symmetry and it absorbs.

O <= C => O O <=C <= O

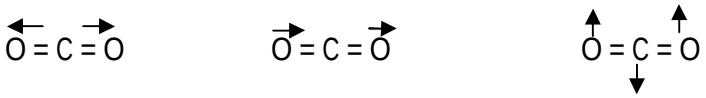
(a) (b)

I.R. Spectra and Molecular Structure

Vibrating groups absorbing in the I.R. region do so within certain wavelength region but the exact wavelength depends on neighbouring groups. The peaks are sharper than in UV or visible regions and easier to identify. Each molecule has a complete absorption spectrum unique to it which is equivalent to a fingerprint of the molecule. Hence, the molecule can be identified.

Vibrational Transitions

Two types of molecular vibrations occur; stretching and bending, e.g. CO_2.



I **II** **III**

I and II are stretching while III is bending. I will not lead to IR absorption while II and III will. Bending may involve movement of a group of atoms within a molecule relative to the rest of the molecule. Different types of bending occur: twisting, rocking, wagging, scissoring e.t.c.

IR absorption due to II and III of CO_2 occur at "fundamental frequencies". These are frequencies at which intense absorption bands occur for complex molecules (ν_1, ν_2 etc). Less intense bands called "overtones" may occur at multiples of the fundamental frequencies, e.g. ($2\nu_1$, $2\nu_2$ etc). There could also be combination tones at frequencies corresponding to sums of fundamental frequencies e.g. ($\nu_1 + \nu_2$) etc.

Structure of Frequency

Vibrational frequency of the bond between given atoms or groups of atoms is characteristic and not affected by molecular environment. It is therefore possible to obtain structural or functional group information from IR spectra e.g. carbonyl group =C=O in alkanals and alkanones absorbs at wave number 1700 cm^{-1} or wavelength 5.9μm. Structural changes can cause minor shifts and changes in the absorption bands, which can provide additional information. Correlation is possible between absorption frequency and types of bonds or chemical groups.

There are four general regions in the IR spectrum;

1. Hydrogen stretching region (2.7 – 4.0 μm = λ). This includes stretching bands of O-H, N-H, C-H and S-H bands.
2. Triple bond stretching region (λ = 4.5 – 5.0 μm). This includes stretching bands of C Ξ C and C ΞN. Cumulated double bonds (C = C = C) also absorb in this region.

3. Double bond stretching region (λ = 5.4 – 6.4 μm). This include C = C, C = O, C = N with C = O at 5.9 μm.

Acids (-C=O-OH) and esters (-C=O-OR) absorb at lower wavelength while amides (-C=O-NH_2) absorb at longer wavelength with two peaks, hence discrimination is possible. These three regions are the "functional group" region. The fourth region is the "fingerprint" region. This is the single bond stretching and bending region. This includes not only C-H and N-H stretching and bending but also vibrations of single bonds that connect groups such as methyl (-CH_3), (-CH_2) and amine (-NH_2) groups. Absorption in this region is very much dependent on molecular environment and is unique for each molecule and leads to identification of the molecule.

IR is mainly used for qualitative analysis i.e. for identification and structural analysis. It can also be used for quantitative analysis of complex mixtures of similar compounds because some absorption peaks for each compound occur at definite and selective wavelength and have intensities directly proportional to the concentration of absorbing species.

Cells

Most common cells are cells of NaCl windows. The solvent used must not attack the windows of the cell. NaCl cells must be protected from moisture and are stored in desiccators. They require periodic polishing to remove "fogging" due to moisture condensation.

AgCl windows are used for wet samples or aqueous solutions. These are soft and must be protected from light as they slowly darken in visible light.

When samples therefore exist as pure liquid, they are run without dilution. This allows for identification or confirmation of an unknown or new compound. The cell length must be short (0.01 – 0.05 mm) to keep the absorbance within the optimum region. If solution of sample is to be prepared, a fairly high concentration is required because no solvent is completely transparent in the IR region and this keeps solvent absorbance at a minimum. So, short path length ≤ 0.1mm.

Solids may be run as suspension or thick slurry in a viscous liquid having approximately same refractive index to reduce light scattering. The sample is ground in the liquid, usually Nujol (a mineral oil). If Nujol masks any C-H band, then chlorofluorocarbon greases are used. This method is only for qualitative work since the slurry is difficult to reproduce for quantitative work. Alternatively, the sample may be ground with KBr (transparent in IR region) and pressed into a pellet for mounting.

For gases, a long path length (≈10cm) is required.

ATOMIC SPECTROMETRY

Emission

The principle depends on measurement of emitted radiant energy from sample solution aspirated into the flame.

The instrument consists of the nebulizer, the burner, the optical system, detector, amplifier, scale or recorder.

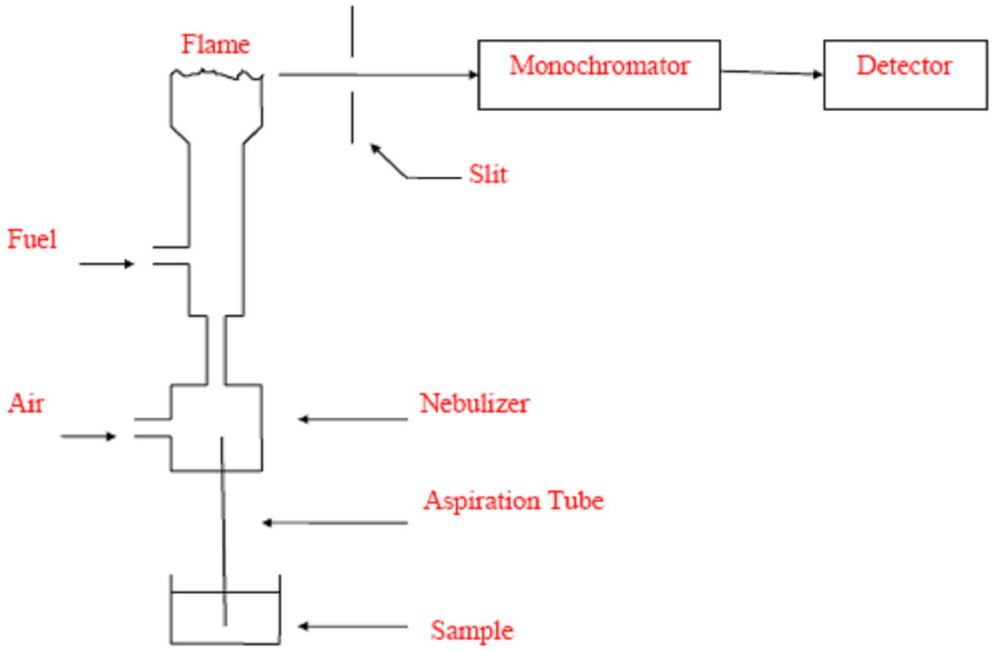
Nebulizer: This breaks the solution into a fine spray and introduces the spray into the flame at a stable and reproducible rate. It must not be attacked by corrosive solutions and must be easily cleaned. It is usually made of plastic.

Burner: It must provide a steady flame.

Optical System: This collects light from the steadiest part of the flame, renders it monochromatic and focuses it onto the surface of a photosensitive detector. It consists of a concave mirror placed behind the flame with its centre of curvature in the flame thereby almost doubling the intensity of emitted radiation. For simple routine use, absorption filters used for elements with simple spectrum and in absence of background emission from flame transmits wide band as it cannot absorb radiation close to analytical line. Interference filters are better but monochromators containing quartz prism or diffraction grating and narrow slit-width, with very sensitive detecting circuits and amplifiers are best.

Flame Photometer

A simple flame photometer consists of a nebulizer, flame, lens, a screen with a slit, filter, detector and galvanometer.



Air is drawn in at a given pressure and passed into a nebulizer where it creates a partial vacuum resulting in suction which sucks in the sample solution as a fine spray into a small mixing of the chamber at the burner. Here it mixes with the fuel gas at a specific pressure into the flame. Emitted radiation from the flame passes through a lens which renders it parallel and through a slit to produce a narrow beam. It then passes through a filter which allows only the line of the test element to pass through to the detector (photocell) and a galvanometer which gives the reading. The flame is surrounded by a chimney to protect it from drought. It is primarily used for analysis of sodium, potassium, calcium and lithium.

Flame and Flame Temperature:

- It transforms sample from liquid or solid state to gaseous state.
- It decomposes molecular compounds into simpler molecules or atoms.
- It excites atoms to emit radiation.

Gases in flame are CO, H_2, CO_2 and H_2O (and N_2, if air is one of the gases), with smaller quantities of H, O and OH

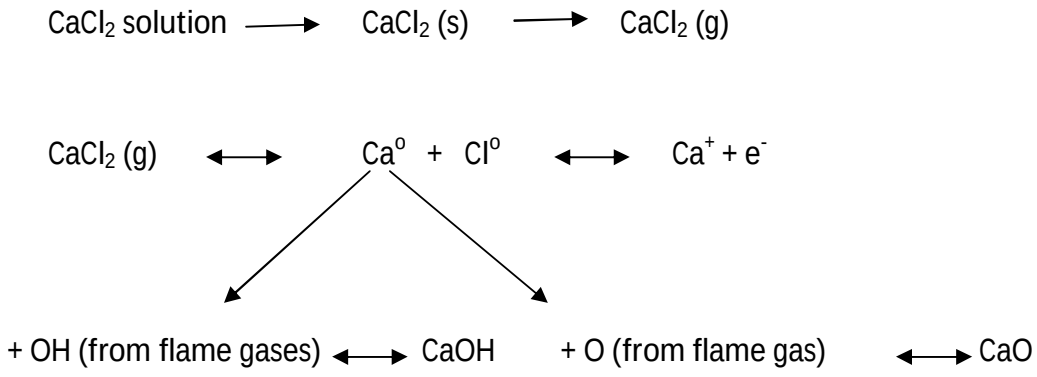
Temperature of flame depends on fuel gas and supporter.

Fuel gas	Air	Oxygen
Illuminating gas	1700	2700
Propane	1925	2800
Butane	1900	2900
Hydrogen	2100	2780
Acetylene	2200	3050
Cyanogens	2330	4550

Emission Spectra

On aspiration into flame following in rapid succession;

- Water or solvent vaporized, leaving minute particles of dry salt.
- Salt vaporized; part or all gaseous molecules dissociated into ground state atoms.
- Some atoms combine with radicals or atoms in flame gases.
- Vapours of ground state metal atoms or molecules containing the atoms absorb energy from flame – excited. Some ionization may occur.
- Species return to ground state – emit excess energy. E1 – E2 = hv. Return may be in one step or in several steps. Most prominent line is equivalent to the lowest excited level and ground state.



Applications

Simple flame photometers using butane air flame with element filters are used to routinely determine easily excitable elements, K, Na, and Ca – elements with low ionization energies.

However, with hotter flames like oxyacetylene flame and use of spectrophotometers and very narrow slit widths, more elements (up to 70) can be determined.

Spectral Interference

In relatively cool flames, refractory molecular oxides and hydroxides form. Molecules have energies and energy levels of rotational, vibrational and electronic excitation. Each electronic transition is accompanied by a whole lot of vibrational and rotational ones - a broad emission spectrum rather than a narrow band. The bands interfere with and make measurement of analytical line difficult and inaccurate if adjacent to or overlapping analytical line. Examples of such molecules are CaOH, SrOH, BaOH, MnOH, CaO etc. Also, background emission from flame due to –OH, CO, O_2, CH, C_2, and H_2O.

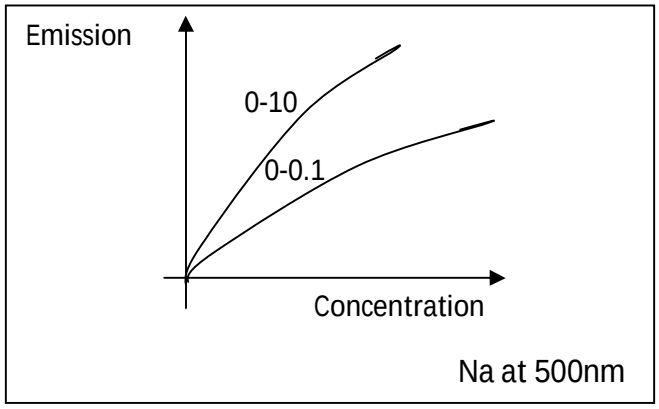
Usually prevalent with filters and reduced by monochromators. If improved, resolution cannot correct it, another line must be found.

Background Emission

This is emission both from flame and sample matrix. This must be corrected to avoid serious errors. If only from flame gases, aspirate pure solvent to zero. If monochromator, used, background measured in presence of test element and subtract background signal.

Self – Absorption

Excited atoms release excess energy in discrete amounts. The radiant energy travels some distance in flame before getting out. Collides with other ground state atoms and get absorbed which leads to decrease in signal. Self – absorption increases as the number of ground state atoms in flame increases. Work at low concentration.



Ionization

Some atoms ionized rather than being excited if flame is hot enough. Increase in the number of atoms and decrease in signal.

$$M \longleftrightarrow Mn^{+} + ne$$

Add a second easily ionizable element e.g. Na or K. Excess electrons drive equilibrium to left hand side. More metal atoms, higher signal. This is more easily observed in acetylene-air or oxy-acetylene flames.

Acetylene air

Effect of Na on K emission

	+20 ppm Na	+100 ppm Na	+1000 ppm Na	+2000 ppm Na	+5000 ppm Na
K 5 ppm	+17	+56	+92	+96	+97

Add large amount of easily ionizable element to standardize the sample.

Effect of Anions (Refractory Compound Formation)

Some anions from acids or salts depress signals of metallic emission. Significant above 0.1M H_2SO_4, HNO_3 and in particular H_3PO_4 are very prominent. For example, Ca and other alkaline earth metals depressed by $PO4^{3-}$ and Al_2O4. The compound formed is refractory and does not volatilize or decompose.

Use releasing agent e.g. La^{3+} or protective chelation – polyhydroxy alcohols (glycerol) or EDTA.

Procedure for Analysis

Calibration curve and standard addition.

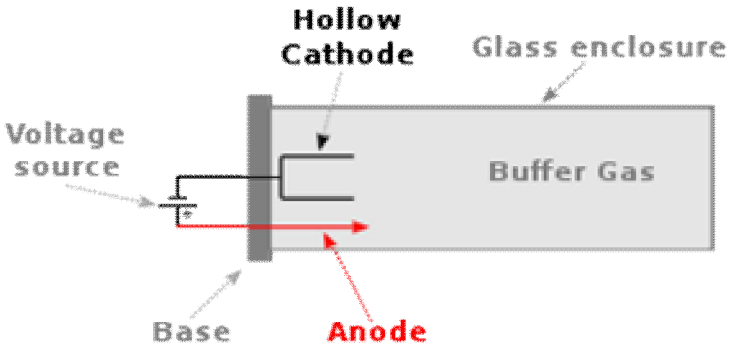
ATOMIC ABSORPTION SPECTROPHOTOMETRY

Principle

Measurement of light absorbed at wavelength of a resonance line by unexcited atoms of element. It is useful for elements which cannot be excited by flame. It can also be used for

some excitable ones since about 99% of all atoms remain unexcited in normal air – acetylene flame. The flame is like a trough or sample cell of the absorbing gas and the absorption follows Beer's law i.e. proportional to path length of flame and concentration of atomic vapour in flame.

Instrumentation



Source

This provides the resonance line of element. It is usually a hollow cathode lamp which emits the specific monochromatic wavelength.

Cylindrical hollow cathode made of the element to be measured or an alloy of it. Anode is tungsten. It is enclosed in glass tube with quartz window (most wavelengths in UV region) reduced pressure and filled with inert gas, Ar or Ne. high voltage across tube, electrons released by anode, ionize gas , positive gas ions accelerated to cathode. They bombard the cathode, cause metal to sputter and vapourise. Metal atoms excited by collision with more ions. They return to ground state and emit characteristic wavelength. Filler gas also emits line but not close enough to interfere. It is passed through flame and get absorbed. Most absorbed is usually but not always the resonance line. This is equivalent to transition from ground state to lowest excited state.

Multi element Lamp

These are alloys of several elements. They emit lines of all the elements. They are used for two or three elements but have shorter life span. Radiation lines from hollow cathode lamp narrower width than absorption line of atom in flame.

Burner and Nebulizer

Nebulizer breaks the solution into a fine spray and introduces the spray into the flame at a steady and reproducible rate.

There are two types of nebulizer:

1. The one with total internal combustion or direct aspiration; aspirates all the solution into the flame.
2. The one with premix chamber in which the solution goes through a chamber where the large drops are removed and only the fine droplets mix with the flame gases and go into the flame.

The advantages and the disadvantages are as follows.

Total Internal Combustion

Disadvantages

a) It has a shorter path length.
b) Large droplets are not completely vapourized; leaves solid particles in light path which scatter light which is recorded as absorption (error).
c) Nebulization efficiency is greatly affected by viscosity of sample.

Advantages

a) Absorption is proportional to gas flow than in pre-mix.
b) Viscous liquid and high solids can be aspirated.

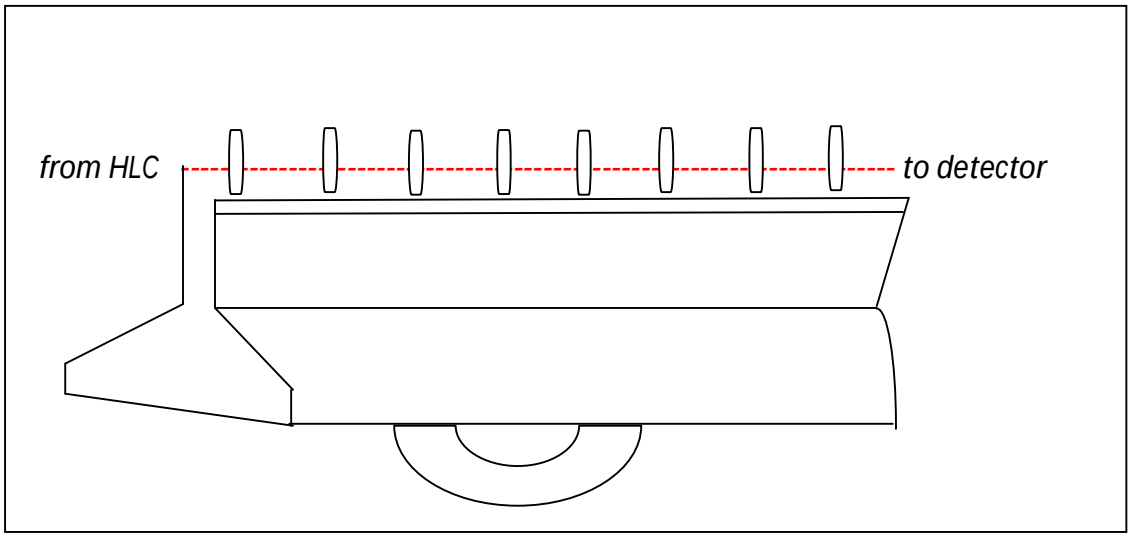
Pre-mix

Advantages

a) Fine droplets which are easily vapourized.
b) Nebulization efficiency is greater.
c) Path length is longer.
d) Combustion is very quiet while total internal combustion is noisy.

Therefore, pre-mix is better.

Burner head, mostly longer than optical path length.



The flame is like a cell of ground state atoms which absorb resonance line from the hollow cathode lamp while the rest of the signal goes on to the detector which measures the absorbance.

Flames are the same as emission.

Oxy – acetylene 3060^{o}C

Nitrous oxide-acetylene 2955^{o}C. They are the highest used.

Interferences

1. **Spectral Interference**
   Similar to emission. Refractory molecular band emission with d.c. source but is eliminated with a.c. If molecule absorbs source radiation, positive interference in AAS is minimized by using line source.
   Light scatter by solid particles result to positive interference especially less than 300 nm with high salt solution. Measure absorbance at a line close to line of element to get background absorption – subtract, since interference over broad area.

2. **Ionization Interference**
   Similar to emission.

3. **Refractory Compound Formation**
   Similar to emission.
   Compounds formed with flame gases e.g. MO, MOH etc. use hotter flames to decompose e.g. nitrous oxide-acetylene or air-acetylene.

Application

Get solution of sample. If interferences absent, chemical form does not matter. Hence, it is used in biological samples blood, urine, csf etc. Aspirate directly or after suitable dilution into flame to prevent clogging of burner.