

PharmacoLearn

MPH101T Modern Pharmaceutical Analytical Techniques, M.Pharm Semester 1

Unit 1: UV-Visible Spectroscopy, IR Spectroscopy, Spectrofluorimetry, Flame Emission and Atomic Absorption Spectroscopy

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Modern Pharmaceutical Analytical Techniques, subject code MPH101T, is the common Semester 1 subject of M.Pharm. The same subject is coded MPC101T in Pharmaceutical Chemistry and MPL101T in Pharmacology, and the syllabus is the same for Pharmaceutics, Pharmacology and Pharmaceutical Chemistry students. It carries 4 credits and 60 hours of theory. This subject teaches the advanced instruments used to identify, characterise and quantify drugs and excipients, in bulk drug, in single and combination dosage forms, and in biological samples. A student who completes it should be able to choose the right technique for a given analytical problem, explain how the instrument works, and interpret the spectrum, mass spectrum or chromatogram it produces. The five units move in a logical order: first the spectroscopic methods that work on absorption and emission of radiation, then NMR, then mass spectroscopy, then the separation science of chromatography, and finally electrophoresis, X-ray crystallography, immunological assays and the electroanalytical and thermal methods.

1.1 UV-Visible spectroscopy: introduction and theory

UV-Visible spectroscopy is an absorption spectroscopy. It measures how much ultraviolet radiation (200 to 400 nm) and visible radiation (400 to 800 nm) a sample absorbs at each wavelength. Absorption happens when a photon promotes an electron of the molecule from a lower energy state to a higher energy state. The energy of the photon must exactly match the energy gap between the two states, which is given by $E = h\nu$, where h is Planck constant and ν is the frequency of the radiation.

The electronic transitions involved, in order of decreasing energy, are sigma to sigma star, n to sigma star, pi to pi star and n to pi star. The pi to pi star and n to pi star transitions matter most in drug analysis because most drugs contain double bonds, aromatic rings or lone pair bearing groups such as carbonyls and amines. A group that absorbs UV or visible radiation is called a chromophore, for example C=C, C=O, N=N and the benzene ring. A group that does not absorb itself but shifts or intensifies the absorption of a chromophore is called an auxochrome, for example OH, NH₂ and SH groups attached to the chromophore.

Four terms describe any absorption band:

- Wavelength of maximum absorption (λ_{max}): the wavelength absorbed most strongly, characteristic of the chromophore.
- Molar absorptivity (ϵ): the absorbance of a 1 molar solution in a 1 cm cell at λ_{max} , a measure of how strongly the compound absorbs.
- Bathochromic shift: shift of λ_{max} to longer wavelength (red shift), caused by conjugation or a polar solvent acting on an n to pi star transition in the reverse way described below.
- Hypsochromic shift: shift to shorter wavelength (blue shift). Hyperchromic effect means increased intensity and hypochromic effect means decreased intensity of the band.

1.2 Laws of UV-Visible spectroscopy

Two laws govern quantitative work.

Beer-Lambert Law: Calibration Plot

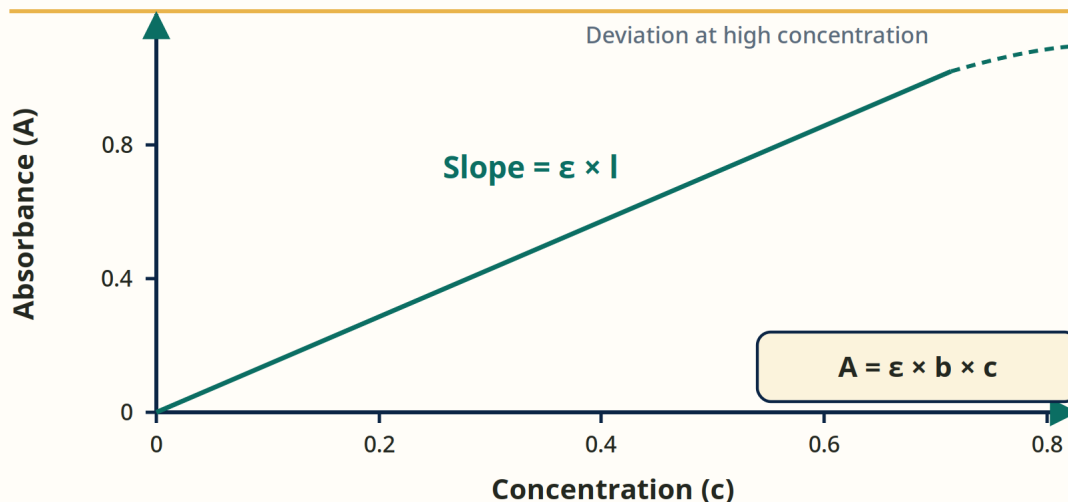


Figure: Beer-Lambert law calibration plot, absorbance is directly proportional to concentration.

Beer law states that when monochromatic radiation passes through a solution of an absorbing substance, the absorbance is directly proportional to the concentration of the absorbing substance. **Lambert law** states that the absorbance is directly proportional to the path length of the cell. Combined, the **Beer-Lambert law** is written as:

$$A = \epsilon \times b \times c$$

where A is absorbance (no units), epsilon is molar absorptivity in $\text{L mol}^{-1} \text{cm}^{-1}$, b is path length in cm and c is concentration in mol/L. Absorbance and transmittance are related by $A = 2 - \log_{10}$ of percentage transmittance, or $A = \log_{10}(I_0/I)$, where I_0 is incident intensity and I is transmitted intensity.

The law holds only under certain conditions, and its deviations are a favourite exam topic:

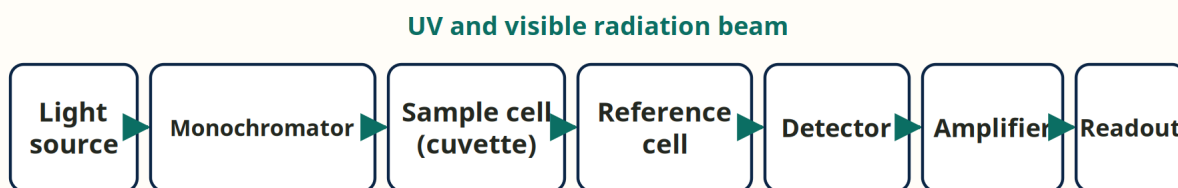
- The radiation must be monochromatic and the solution must be dilute.
- The solute must not associate, dissociate, react with the solvent or change with pH in a way that changes the absorbing species. These are chemical deviations.
- Polychromatic radiation, stray light and mismatched cells cause instrumental deviations, which are worst at high absorbance.
- At high concentration, molecules sit close together, the refractive index changes and apparent absorbance falls below the expected value. This is a real deviation of the law itself.

In practice, absorbance between about 0.2 and 0.8 gives the most accurate readings, and a calibration curve of absorbance against concentration of a standard is used for assays.

1.3 Instrumentation of UV-Visible spectrophotometer

A UV-Visible spectrophotometer has five basic parts. The working flow is:

UV-Visible Spectrophotometer



Double beam design: source drift is corrected by the reference beam

Figure: Parts of a UV-Visible spectrophotometer in the order of the light path.

Radiation source -> Monochromator (wavelength selector) -> Sample cell and Reference cell
-> Detector -> Amplifier and Readout (recorder or display)

- **Radiation sources:** a deuterium lamp (or hydrogen lamp) covers the UV region 200 to 375 nm, and a tungsten filament lamp covers the visible region 375 to 800 nm. A xenon arc lamp covers both regions and is used in some instruments.
- **Monochromator:** it isolates a narrow band of wavelengths. Entry slit, collimating mirror or lens, dispersing element and exit slit are its parts. The dispersing element is either a prism (glass for visible, quartz or fused silica for UV) or, more commonly today, a diffraction grating, which gives better resolution and a linear wavelength scale.
- **Sample holders:** cells or cuvettes of 1 cm path length. Quartz or fused silica cells are essential below 350 nm because glass absorbs UV strongly. Cells must be matched, clean and free of fingerprints on the optical faces.
- **Detectors:** the barrier layer (photovoltaic) cell is simple and needs no external power, the phototube is more sensitive, and the photomultiplier tube (PMT) is the most sensitive and is standard in research grade instruments. Modern instruments use photodiode and photodiode array detectors, which capture the whole spectrum at once.
- **Single beam versus double beam:** in a single beam instrument the blank and the sample are measured one after the other in the same cell position, so the instrument is simple but any drift affects the result. In a double beam instrument the light is split into sample and reference beams measured at the same time, which corrects for source fluctuations and solvent absorption automatically. Most pharmaceutical assays are done on double beam instruments.

1.4 Choice of solvents and solvent effects

The solvent must dissolve the sample, must not absorb in the region being measured, and must not react with the solute. Every solvent has a UV cut off wavelength below which it cannot be used; for example water and ethanol are transparent down to about 210 nm, while chloroform absorbs below about 245 nm and acetone below about 330 nm, so acetone is a poor solvent for UV work.

Solvent polarity shifts the bands in a predictable way:

- For π to π^* transitions, a polar solvent stabilises the more polar excited state more than the ground state, the energy gap falls, and the band shows a bathochromic (red) shift.

- For $n \rightarrow \pi^*$ transitions, a polar solvent, especially a protic solvent like ethanol or water, hydrogen bonds with the lone pair and stabilises the ground state, the energy gap rises, and the band shows a hypsochromic (blue) shift.

pH is a special solvent effect of great practical use. Phenols in alkaline medium form the phenoxide ion, which absorbs at longer wavelength with higher intensity, while aniline in acid medium forms the anilinium ion, which loses the auxochrome effect and absorbs like benzene. This pH dependent shift is used in difference spectroscopy and in identifying ionisable drugs.

1.5 Applications of UV-Visible spectroscopy

- Quantitative assay of single drugs and of multi component formulations, using simultaneous equations, absorbance ratio method, dual wavelength method and derivative methods when spectra overlap.
- Determination of dissociation constant (pK_a) of ionisable drugs from absorbance at different pH values.
- Determination of stoichiometry and stability constant of metal complexes by Job method of continuous variation and mole ratio method.
- Dissolution testing of tablets and capsules, where UV is the standard detector.
- Detection of impurities in a pharmacopoeial limit test sense, reaction monitoring, and as the detector of choice in HPLC (covered in Unit 4).
- Qualitative identification by λ_{max} and by comparison with a reference spectrum.

1.6 Difference and derivative spectroscopy

Difference spectroscopy records the difference in absorbance between two forms of the same sample, usually the sample at two different pH values or the sample against a reference containing the interfering excipients. Interferences that are identical in both cells cancel out, so the analyte can be measured even in a coloured or turbid formulation. It is used for assays in presence of degradation products and for pK_a determination.

Derivative spectroscopy converts the normal (zero order) spectrum into its first, second or higher derivative with respect to wavelength. Overlapping bands that look like one broad hump separate into sharp features, and a shoulder becomes a clear peak. In practice it is used to resolve binary mixtures without a prior separation, to remove turbidity and background interference because a constant background disappears in the first derivative, and for fingerprint comparison. The amplitude of a derivative peak is proportional to concentration, so quantification is done by measuring the derivative amplitude at a wavelength where the other component crosses zero.

1.7 IR spectroscopy: theory and modes of molecular vibration

Infrared spectroscopy works on a different principle: molecules absorb IR radiation (conventionally 4000 to 400 cm^{-1} in the mid IR region) when the radiation frequency matches a vibrational frequency of a bond, and only if that vibration produces a change in the dipole moment of the molecule. Homonuclear molecules such as O_2 and N_2 , whose vibration causes no dipole change, are IR inactive.

A molecule must first be understood as balls (atoms) joined by springs (bonds). The approximate vibrational frequency is given by Hooke law: stronger bonds and lighter atoms vibrate at higher frequency (higher wavenumber), which is why C-H stretching appears near 3000 cm^{-1} while C-Cl stretching appears near 700 cm^{-1} .

The modes of molecular vibration are:

- **Stretching vibrations**, in which the bond length changes: symmetric stretching (both atoms move in the same sense) and asymmetric stretching (one bond shortens while the other lengthens).
- **Bending vibrations**, in which the bond angle changes: in plane bending, scissoring and rocking, and out of plane bending, wagging and twisting.

Not every vibration gives a separate peak. Some are degenerate (same energy, one band), some are too weak, some fall outside the instrument range, and some produce no dipole change. This is why the number of observed bands is usually less than the theoretical number of vibrational modes, which for a non linear molecule is $3N - 6$ and for a linear molecule is $3N - 5$, where N is the number of atoms.

Two regions of the IR spectrum should be read differently. The **functional group region** (4000 to 1500 cm^{-1}) shows the characteristic stretching bands of groups such as OH, NH, C=O and C=C and is used to identify which functional groups are present. The **fingerprint region** (1500 to 400 cm^{-1}) is crowded and unique for each compound, and identity is confirmed by matching the whole fingerprint of the sample with that of a reference standard, exactly as pharmacopoeias prescribe.

1.8 Sample handling in IR spectroscopy

Sample preparation depends on the state of the sample, and a wrong choice gives a useless spectrum:

- **Solids**: the KBr pellet method (about 1 mg sample ground with 100 mg dry KBr and pressed into a transparent disc) is the most common; the Nujol mull method (sample ground in liquid paraffin and placed between salt plates) is used when KBr may react or absorb moisture, noting that Nujol itself shows C-H bands; films cast from solution are used for polymers.
- **Liquids**: a thin film between two sodium chloride or potassium bromide plates, or a liquid cell of fixed path length. Water cannot be used as a solvent because it absorbs IR strongly and dissolves the salt plates.
- **Gases**: long path length gas cells (about 10 cm) with IR transparent windows.
- **FT-IR sampling accessories**: attenuated total reflectance (ATR) allows direct measurement of solids, pastes and liquids with almost no preparation and is now the routine method in quality control laboratories.

1.9 Instrumentation: dispersive IR and FT-IR

A **dispersive IR spectrophotometer** resembles a UV instrument: an IR source (Nernst glower, Globar or incandescent wire), a monochromator with gratings or prisms of salt optics, sample and reference compartments, and a detector such as a thermocouple, bolometer, Golay cell or pyroelectric detector. The spectrum is scanned wavelength by wavelength, so it is slow.

FT-IR: Michelson Interferometer

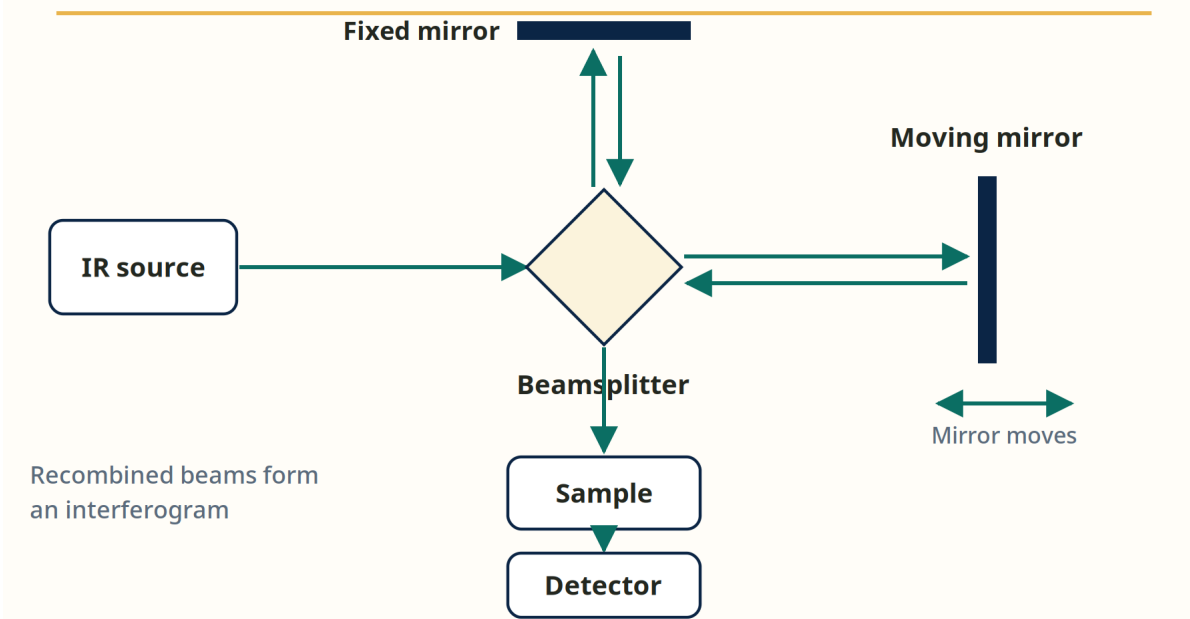


Figure: Michelson interferometer of an FT-IR spectrometer.

A **Fourier transform IR (FT-IR) spectrometer** replaces the monochromator with a Michelson interferometer. All frequencies reach the detector together as an interferogram, and a mathematical Fourier transform converts it into the spectrum. Its advantages are the classic exam answer:

- **Fellgett advantage (multiplex):** all wavelengths are measured simultaneously, so the spectrum is obtained in seconds with a better signal to noise ratio.
- **Jacquinot advantage (throughput):** no narrow slits, so more radiation reaches the detector and sensitivity is higher.
- **Connes advantage:** a helium neon laser references the mirror position, giving very accurate and reproducible wavenumber scale.

Because of these, FT-IR has replaced dispersive instruments almost completely.

1.10 Factors affecting vibrational frequencies

The observed position of an IR band shifts from its expected value because of:

- **Coupled vibrations:** two bonds sharing an atom interact, for example the two C=O stretches of an anhydride give two bands instead of one.
- **Hydrogen bonding:** intermolecular hydrogen bonding lowers the stretching frequency and broadens the band (the broad OH band of alcohols and carboxylic acids), while intramolecular hydrogen bonding sharpens it and shifts it in a concentration independent way.
- **Electronic effects:** electron withdrawing groups increase the bond force constant and raise the frequency (inductive effect), while conjugation lowers the C=O frequency because the double bond gets partial single bond character (mesomeric effect). Example: a saturated ketone absorbs near 1715 cm⁻¹, an alpha beta unsaturated ketone near 1685 cm⁻¹, an ester near 1735 cm⁻¹ and an amide near 1650 cm⁻¹.
- **Bond angle and ring strain:** in small rings the carbonyl frequency rises, for example cyclobutanone absorbs at higher wavenumber than cyclohexanone.

- **Field effect:** through space interaction of close substituents, seen in some ortho substituted compounds, and Fermi resonance, in which a fundamental band interacts with an overtone of another vibration and splits, as in the aldehyde C-H doublet near 2820 and 2720 cm⁻¹.

1.11 Applications and data interpretation of IR spectroscopy

IR is mainly a qualitative and structural tool. Its uses are identification of drugs by fingerprint matching against pharmacopoeial reference spectra, detection of functional groups during structure elucidation (read together with NMR and mass spectra in Units 2 and 3), distinction of polymorphs because different crystal forms of the same drug give different IR spectra, study of drug excipient compatibility, and rapid raw material identification in quality control using ATR. A typical interpretation sequence is: check the carbonyl region first (1820 to 1630 cm⁻¹, the strongest and most informative band), then OH and NH stretching (3600 to 3200 cm⁻¹), C-H stretching (3300 to 2850 cm⁻¹, position tells sp, sp² or sp³ carbon), C=C and C=N (1680 to 1450 cm⁻¹), and finally confirm with the fingerprint region.

1.12 Spectrofluorimetry: theory of fluorescence

Fluorescence is a type of photoluminescence. A molecule absorbs UV or visible radiation, an electron is promoted to an excited singlet state, it first loses some energy by vibration and collision (internal conversion) to reach the lowest vibrational level of the first excited singlet state, and then returns to the ground state by emitting a photon. Because some energy is lost before emission, the emitted light is always of longer wavelength (lower energy) than the absorbed light. This gap is called the Stokes shift, and it is what makes fluorescence measurement so sensitive: the signal is read against a dark background at a different wavelength, at 90 degrees to the excitation beam.

Related terms: **phosphorescence** is emission from a triplet state after intersystem crossing, it is slower and longer lived; **quantum yield** is the ratio of photons emitted to photons absorbed; **fluorescence lifetime** is the average time the molecule spends in the excited state, typically nanoseconds. A rigid, planar, highly conjugated molecule with electron donating groups usually fluoresces strongly, while heavy atoms, nitro groups and flexible structures reduce it.

1.13 Factors affecting fluorescence, and quenchers

- **Structure:** rigidity and planarity increase fluorescence; free rotation wastes energy as heat.
- **Substituents:** electron donating groups (OH, NH₂, OCH₃) increase fluorescence, electron withdrawing groups (NO₂, COOH, halogens, especially iodine and bromine through the heavy atom effect) decrease it.
- **pH:** many fluorophores fluoresce in only one ionisation state; quinine fluoresces strongly in acid, while fluorescein fluoresces in alkaline medium. Assays must buffer the pH strictly.
- **Solvent and temperature:** increasing temperature increases collisions and decreases fluorescence, so temperature control matters. Dissolved oxygen is a strong quencher.
- **Concentration:** at high concentration the emitted light is reabsorbed by the sample itself (inner filter effect) and the calibration curve bends; fluorescence is linear only in dilute solution.

Quenching is any process that decreases fluorescence intensity. **Quenchers** include oxygen, halide ions, heavy metal ions, nitro compounds and the analyte itself at high concentration (self quenching). Dynamic (collisional) quenching happens during the excited state, static quenching happens when the quencher forms a non fluorescent ground state complex with the fluorophore.

1.14 Instrumentation and applications of spectrofluorimetry

The fluorimeter flow is:

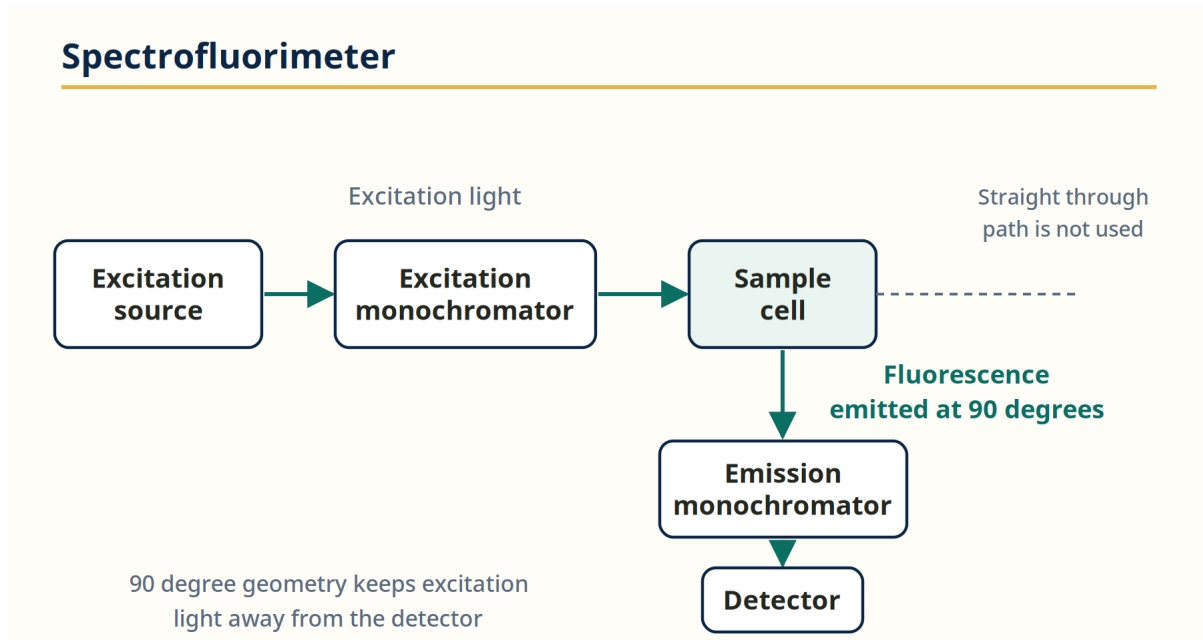


Figure: Spectrofluorimeter, emission is measured at 90 degrees to the excitation beam.

Excitation source (xenon arc or mercury lamp) -> Excitation monochromator
-> Sample cell -> (at 90 degrees) Emission monochromator -> Photomultiplier detector -> Readout

The 90 degree arrangement prevents excitation light from reaching the detector. A spectrofluorophometer has two monochromators, for selecting excitation and emission wavelengths separately, and quartz cells with all four faces polished. Applications: assay of low dose and inherently fluorescent drugs (quinine, riboflavin, tetracyclines after complexation), derivatisation based assays for non fluorescent drugs, analysis in biological fluids at nanogram levels where UV lacks sensitivity, stability studies, and as a highly sensitive and selective detector in HPLC. The main limitation is that only a minority of drugs fluoresce naturally, so many assays need a fluorescent tagging step.

1.15 Flame emission spectroscopy and atomic absorption spectroscopy

Both methods work on free atoms, not molecules, so the sample must first be converted into an atomic vapour, a process called atomisation, usually in a flame.

Atomic Absorption Spectrometer (AAS)

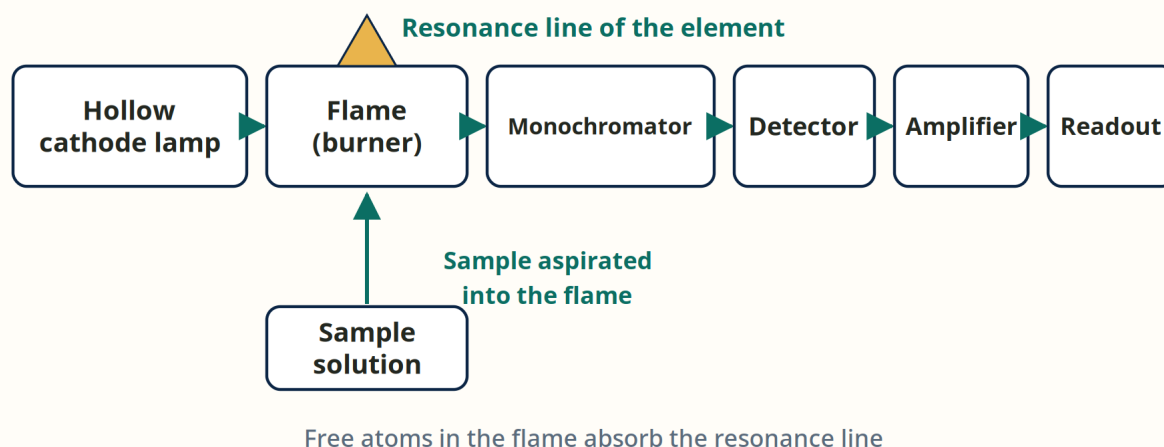


Figure: Atomic absorption spectrometer, the sample solution is aspirated into the flame.

Flame emission spectroscopy (flame photometry): the sample solution is aspirated into a flame, the solvent evaporates, the salt vaporises and dissociates into free atoms, and thermal energy of the flame excites some atoms. As they return to the ground state they emit light at characteristic wavelengths, whose intensity is proportional to concentration. It is the classic method for sodium, potassium, lithium and calcium. Instrumentation: sample aspiration and nebuliser, burner with fuel and oxidant gases (air propane for a cooler flame, air acetylene for hotter work), filter or monochromator to isolate the emission line, photocell detector and readout.

Atomic absorption spectroscopy (AAS): here the free atoms in the flame or in a graphite furnace absorb radiation from a lamp that emits the exact resonance line of the element being measured. The lamp is a **hollow cathode lamp** made of the same element, which is why AAS is highly specific but essentially one element at a time. The absorbance follows the Beer-Lambert type relationship with the number of ground state atoms, which is far larger and more stable than the number of excited atoms, so AAS is more sensitive and less temperature dependent than flame emission. In electrothermal (graphite furnace) AAS the sample is heated electrically in steps (drying, ashing, atomisation), giving detection at parts per billion level from microlitre samples. A background correction system, commonly deuterium lamp or Zeeman correction, corrects for non specific absorption by smoke and salts.

Interferences in flame methods are classified as: spectral interference (overlap of lines or bands), ionisation interference (easily ionised elements lose atoms as ions, suppressed by adding an excess of another easily ionised element such as potassium or caesium), chemical interference (formation of refractory compounds, for example calcium phosphate, removed by adding a releasing agent such as lanthanum or strontium, or by a protective chelating agent such as EDTA), and physical interference (viscosity and surface tension changing the aspiration rate, corrected by matrix matching of standards). Applications of AAS: assay of metallic impurities and catalysts residues, determination of elements such as zinc, iron, copper, lead and cadmium at trace level, and analysis of metals in biological samples. Unlike UV and IR, these atomic methods tell nothing about molecular structure, only which element and how much of it is present.

1.16 Unit 1 comparison tables

Feature	UV-Visible	IR	Fluorescence
Radiation range	200 to 800 nm	4000 to 400 cm ⁻¹	Excitation in UV or visible, emission at longer wavelength
Molecular event	Electronic transition	Bond vibration	Emission after electronic excitation
Main output	Lambda max and absorbance	Wavenumber of bands	Emission intensity at an emission wavelength
Best use	Quantitative assay	Identification, functional groups, fingerprint	Very sensitive assay of fluorescent drugs
Typical sensitivity	Microgram per mL	Milligram amounts	Nanogram per mL

Feature	Flame emission	Atomic absorption
Atoms measured	Excited atoms emitting light	Ground state atoms absorbing light
Light source	The flame itself	Hollow cathode lamp of the same element
Temperature dependence	High, flame must be stable	Low, more robust
Common use	Sodium, potassium, lithium, calcium	Trace metals and metallic impurities