UNIVERSITY OF TERAMO

Spectroscopy

UV-Vis, infrared, fluorescence and flame emission

Analytical Chemistry

BSc in Biotechnology · A.Y. 2026/2027

Prof. Federico Fanti

01

Fundamentals

Electromagnetic radiation and its interaction with matter

Electromagnetic radiation

What it is

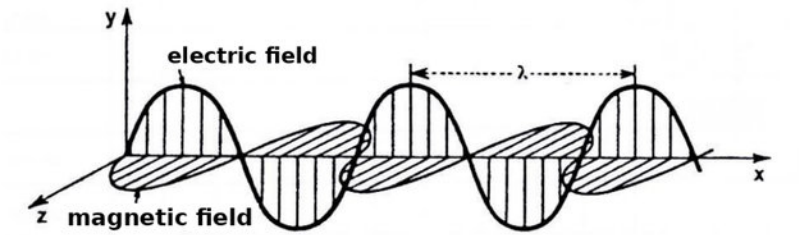
A form of energy that travels even through a vacuum, made of an electric field and a magnetic field oscillating in phase, on planes perpendicular to each other and to the direction of propagation.

Dual nature

It behaves as a wave in diffraction and interference, and as a stream of photons, particles of defined energy, when it is absorbed or emitted by matter.

Spectroscopic methods

They measure the interaction between radiation and the analyte to obtain qualitative and quantitative information.



Electric and magnetic fields oscillating on perpendicular planes.

Wave parameters

$$c = \lambda \nu \quad (\text{in vacuum } c = 3.00 \cdot 10^8 \text{ m s}^{-1})$$

Wavelength λ

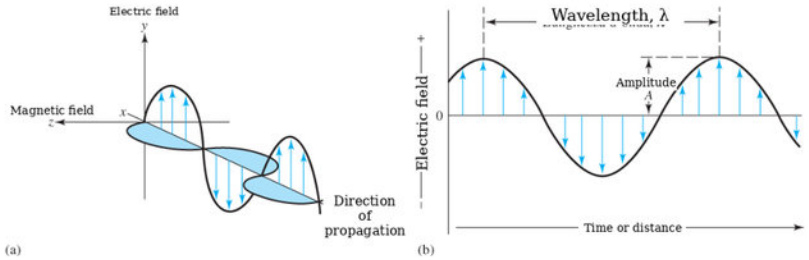
Distance between two successive maxima; measured in nm for UV and visible, in µm for the infrared.

Frequency ν

Number of oscillations per second, in hertz. It depends only on the source and does not change when passing through different media.

Amplitude and power

The amplitude is the maximum value of the electric field; the radiant power P, energy per unit area and time, is proportional to its square.



Amplitude, wavelength and period of an electromagnetic wave.

Photon energy

$$E = h\,\nu = h\,c\,/\,\lambda = h\,c\,\tilde{\nu} \qquad h = 6.63 \cdot 10^{-34}\ \mathrm{J\,s}$$

Energy and frequency

The energy of a photon is directly proportional to frequency and inversely proportional to wavelength: X-rays are far more energetic than microwaves.

Wavenumber $\tilde{\nu}$

The number of waves per centimetre, $\tilde{\nu} = 1/\lambda$ in $\mathrm{cm^{-1}}$. It is proportional to energy and is the unit used in infrared spectroscopy.

An example

A photon at 500 nm carries about $4.0 \cdot 10^{-19}$ J; a mole of photons about 240 kJ, an energy comparable to that of a chemical bond.

The electromagnetic spectrum

A continuum of energies

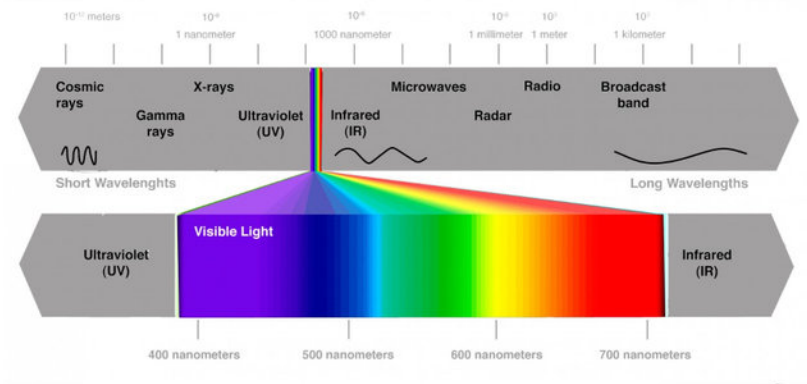
From gamma rays to radio waves the wavelength spans more than 15 orders of magnitude. The part visible to the human eye covers only about 400 to 750 nm.

Regions of analytical interest

UV (190-400 nm) and visible (400-750 nm) for electronic transitions, infrared (2.5-25 µm) for vibrations, radio frequencies for nuclear magnetic resonance.

A rule

Each region of the spectrum probes a different type of transition and therefore a different property of the molecule.



Regions of the electromagnetic spectrum and detail of visible light.

Spectral regions and types of transition

Region	Wavelength	Transition	Technique
γ-rays	< 0.1 nm	Nuclear	Mössbauer
X-rays	0.01-10 nm	Inner-shell electrons	XRF, XRD
Ultraviolet	190-400 nm	Valence and bonding electrons	UV spectrophotometry
Visible	400-750 nm	Valence and bonding electrons	Colorimetry, Vis
Infrared	0.75-1000 µm	Molecular vibrations	NIR, MIR (FTIR)
Microwaves	1 mm-1 m	Molecular rotations, electron spin	ESR
Radio waves	> 1 m	Nuclear spin	NMR

Each spectroscopic technique is defined by the transition it measures.

Energy levels: absorption and emission

Ground state

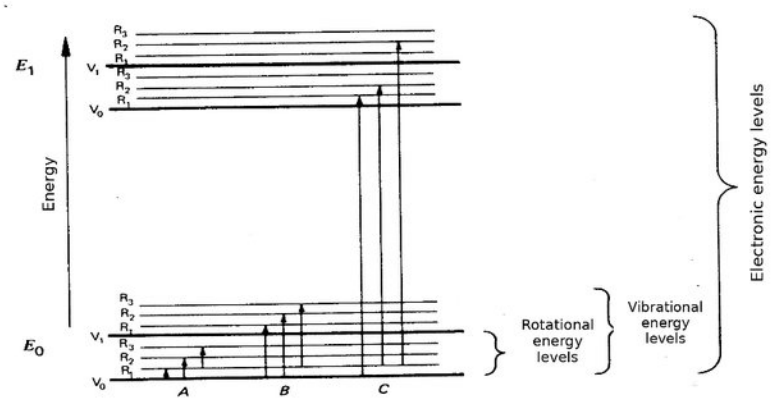
Before stimulation the analyte is mostly in its lowest energy level. The allowed energies are quantised.

Absorption

A photon is absorbed only if its energy matches exactly the difference between two levels: the species moves to the excited state.

Emission

Returning to the ground state, the excited species can release the energy as radiation. Measuring the absorbed or emitted radiation provides information on the analyte.



Electronic, vibrational and rotational levels of a molecule.

Classification of spectroscopic methods

Absorption

The attenuation of a beam passing through the sample is measured. Examples: UV-Vis spectrophotometry, IR spectroscopy, atomic absorption.

Emission

The radiation emitted by thermally or electrically excited species is measured. Examples: flame photometry, ICP-OES.

Photoluminescence

The analyte is excited by absorbing radiation and the radiation re-emitted at longer wavelength is measured. Examples: fluorescence and phosphorescence.

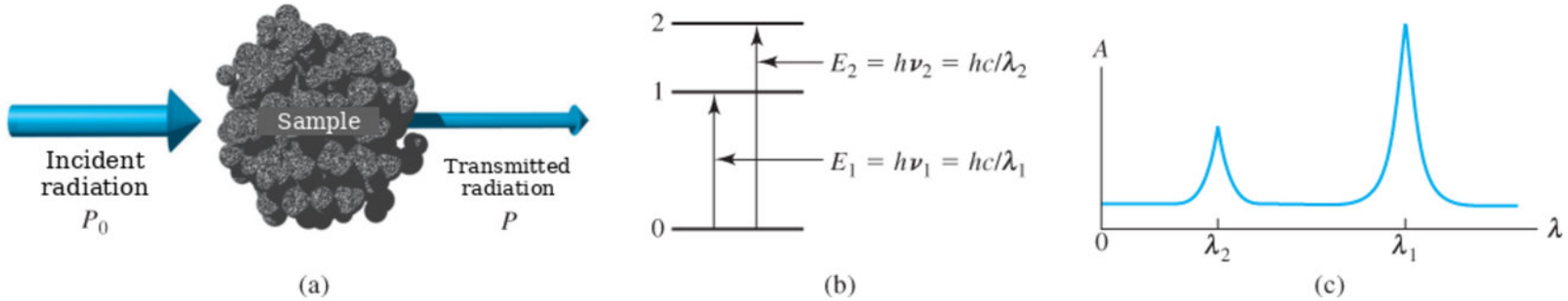
An absorption experiment

The principle

A beam of radiant power P_0 passes through the sample; part of the radiation is absorbed and the transmitted power P is lower than P_0.

The spectrum

Absorption occurs only at energies corresponding to differences between levels of the species: the plot of absorption against λ is the absorption spectrum.



(a) Attenuation of the beam, (b) energy levels involved, (c) resulting absorption spectrum.

02

UV-Vis spectrophotometry

Electronic transitions, the Beer-Lambert law and quantitative analysis

Electronic transitions

In UV-Vis, radiation promotes valence electrons from bonding or non-bonding orbitals to σ* and π* antibonding orbitals.

σ → σ* and n → σ*

They require high energies, below 200 nm: they involve single bonds and saturated heteroatoms and are not useful in routine measurements.

π → π*

Double and triple bonds, aromatic and conjugated systems. Highly probable transitions, with ε from 10^3 to over 10^4 L mol^{-1} cm^{-1}.

n → π*

Non-bonding electrons of O, N, S adjacent to a double bond, as in the carbonyl group. Less probable transitions, with ε typically below 100.

Chromophores and spectral shifts

Chromophores

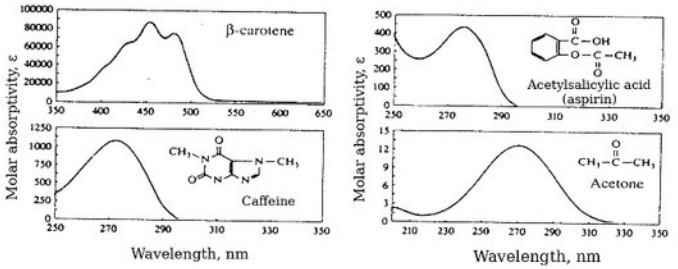
Functional groups responsible for absorption, such as C=C, C=O, N=N and aromatic rings. Conjugation shifts absorption to longer wavelengths and intensifies it.

Position shifts

Bathochromic towards longer λ (red shift), hypsochromic towards shorter λ (blue shift).

Intensity shifts

Hyperchromic if absorption increases, hypochromic if it decreases. Solvent, pH and substituents can produce all these effects.



UV spectra of some typical organic compounds.

Typical chromophores

Chromophore	Example	Solvent	λ_{max} (nm)	ε_{max}
Alkene	$C_6H_{13}CH=CH_2$	n-heptane	177	13,000
Conjugated alkene	$CH_2=CHCH=CH_2$	n-heptane	217	21,000
Alkyne	$C_5H_{11}C\equiv CCH_3$	n-heptane	178 / 196 / 225	10,000 / 2000 / 160
Carbonyl (ketone)	CH_3COCH_3	n-hexane	186 / 280	1000 / 16
Carbonyl (aldehyde)	CH_3CHO	n-hexane	180 / 293	large / 12
Carboxyl	CH_3COOH	ethanol	204	41
Amide	CH_3CONH_2	water	214	60
Azo	$CH_3N=NCH_3$	ethanol	339	5
Nitro	CH_3NO_2	isooctane	280	22
Nitrate	$C_2H_5ONO_2$	dioxane	270	12
Aromatic	Benzene	n-hexane	204 / 256	7900 / 200

Conjugation and colour

Compound	Conjugated double bonds	λ_{max} (nm)	ε_{max} (L mol^{-1} cm^{-1})
Ethylene	1	171	15,000
1,3-Butadiene	2	217	21,000
1,3,5-Hexatriene	3	258	35,000
Lycopene	11	~470	~180,000
β-Carotene	11	~450	~140,000

Each additional conjugated double bond brings HOMO and LUMO closer: the π → π band shifts to longer λ and becomes more intense. Beyond about 8 conjugated double bonds absorption enters the visible and the substance appears coloured.*

Auxochromes and the effect of pH

Auxochromes are groups that do not absorb above 200 nm but, attached to a chromophore, shift its absorption to longer λ and increase its intensity.

Auxochromic groups

–OH, –OR, $–NH_2$, $–NR_2$, –SH, halogens: their non-bonding electron pairs conjugate with the π system of the chromophore.

Phenol and phenolate

Benzene absorbs at 255 nm, phenol at 270 nm. In basic solution phenolate, with an extra electron pair, shifts to 287 nm and absorbs more strongly: bathochromic and hyperchromic effect.

Aniline and anilinium

Aniline absorbs at 280 nm; protonated to anilinium it loses the nitrogen lone pair and returns to about 254 nm, like benzene: hypsochromic and hypochromic effect.

Solvent effects

π → π* transitions

The excited state is more polar than the ground state and is stabilised more by polar solvents: as solvent polarity increases the band shifts a few nanometres to longer λ.

n → π* transitions

In polar protic solvents the non-bonding electrons are stabilised by hydrogen bonds in the ground state: the band shifts to shorter λ. The n → π* band of acetone moves from 279 nm in hexane to 265 nm in water.

Practical consequences

The solvent must always be reported with λ_{max} and ε; spectra can only be compared in the same solvent. The solvent shift also helps to assign the type of transition.

Chromophores of biomolecules

Biomolecule	Chromophore	λ_{max} (nm)	ε (L mol^{-1} cm^{-1})	Analytical use
Proteins	Peptide bond	190-220	~7000 per bond	HPLC detection at 214 nm
Proteins	Tryptophan	280	~5600	Assay at 280 nm
Proteins	Tyrosine	274	~1400	Assay at 280 nm
Nucleic acids	Purine and pyrimidine bases	260	~10^4 per base	Quantitation, purity
Coenzymes	NADH, NADPH	340	6220	Enzyme assays
Flavins	FAD, FMN	450	~11,300	Flavoproteins
Haem proteins	Porphyrin (Soret band)	~415	> 10^5	Haemoglobin, cytochromes
Pigments	Chlorophyll a	430, 662	~10^5	Plant analysis
Pigments	Anthocyanins	~520 (acidic pH)	~$3 \cdot 10^4$	pH-differential method

Absorbed and observed colour

Absorbed range (nm)	Colour absorbed	Colour observed (complementary)
400-435	Violet	Yellow-green
435-480	Blue	Yellow
480-490	Green-blue	Orange
490-500	Blue-green	Red
500-560	Green	Purple
560-580	Yellow-green	Violet
580-595	Yellow	Blue
595-650	Orange	Green-blue
650-750	Red	Blue-green

A solution appears in the colour complementary to the one it absorbs: violet $KMnO_4$ absorbs in the green around 525 nm.

Charge-transfer absorption

The phenomenon

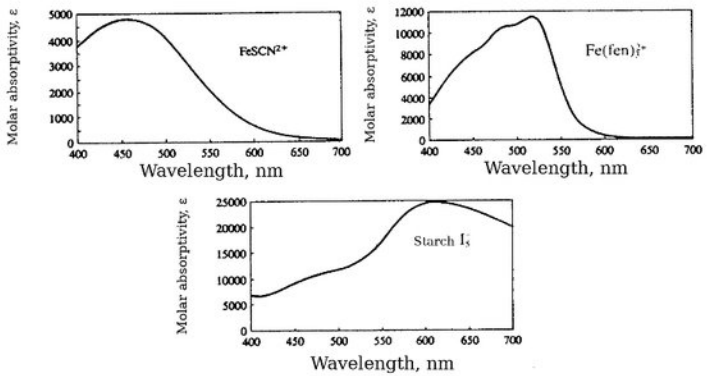
In some complexes an electron moves, on absorbing a photon, from a ligand orbital to a metal orbital or vice versa.

Why it is useful

Charge-transfer bands are very intense, with ε above 10^4 L mol^{-1} cm^{-1}: they give high sensitivity.

Examples

The Fe(II)-o-phenanthroline complex for iron determination and the deep red iron(III) thiocyanate complex.



Spectra of charge-transfer complexes.

Transmittance and absorbance

$$T = P / P_0 \qquad A = \log (P_0 / P) = -\log T$$

Transmittance

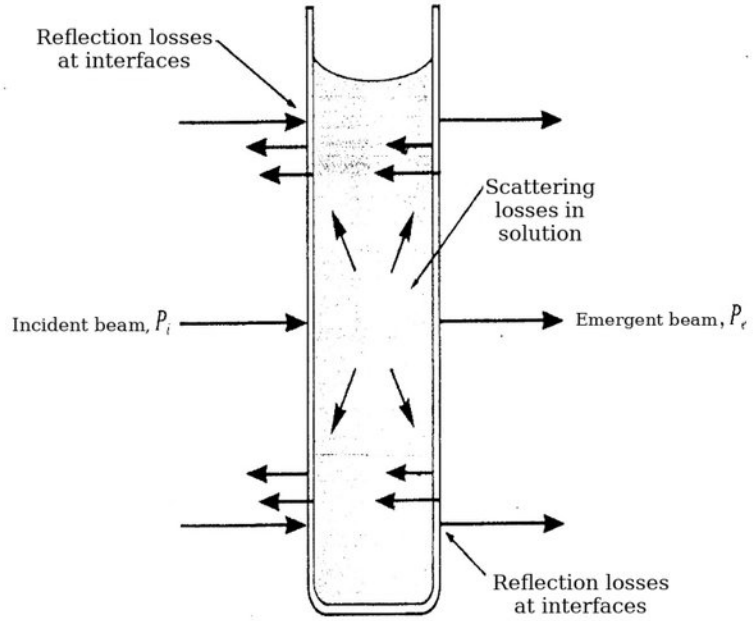
Fraction of the incident radiation that passes through the sample; also expressed as a percentage, %T = 100 · P/P_0.

Absorbance

The logarithm of the ratio P_0/P. It has no unit and, unlike transmittance, is proportional to concentration.

The blank

In practice P_0 is measured with a cuvette containing only the solvent, to compensate for reflection and absorption by cuvette and solvent.



Reflection and scattering losses in a cuvette.

The Beer-Lambert law

$$A = \varepsilon\, b\, c$$

The terms

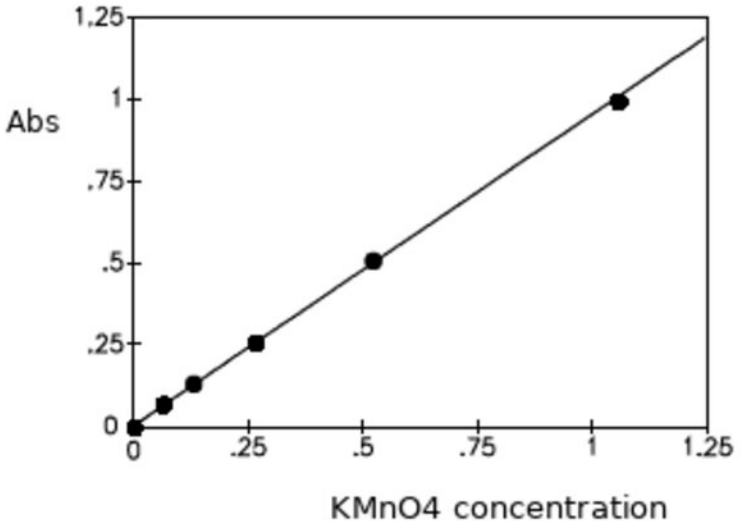
ε is the molar absorptivity in L mol^{-1} cm^{-1}, b the path length in cm, c the concentration in mol/L. With c in g/L the absorptivity a is used.

Linearity

At a fixed wavelength absorbance is proportional to concentration: this is the basis of quantitative analysis with a calibration curve.

Conditions

Monochromatic radiation, dilute solutions (roughly below 0.01 M), absorbing species that do not interact with each other.



Calibration curve: absorbance as a function of concentration.

Molar absorptivity and sensitivity

A property of the species

The molar absorptivity at the absorption maximum is characteristic of a species in a given solvent and at a given wavelength.

Typical values

For many organic compounds it ranges from below 10 to over 10^4 L mol^{-1} cm^{-1}; some transition-metal complexes reach 10^4-$5 \cdot 10^4$.

Why it matters

High ε means steeper calibration curves and lower detection limits. This is why measurements are made at the maximum, where small changes in λ also have little effect.

Spectra at increasing concentration

Observation

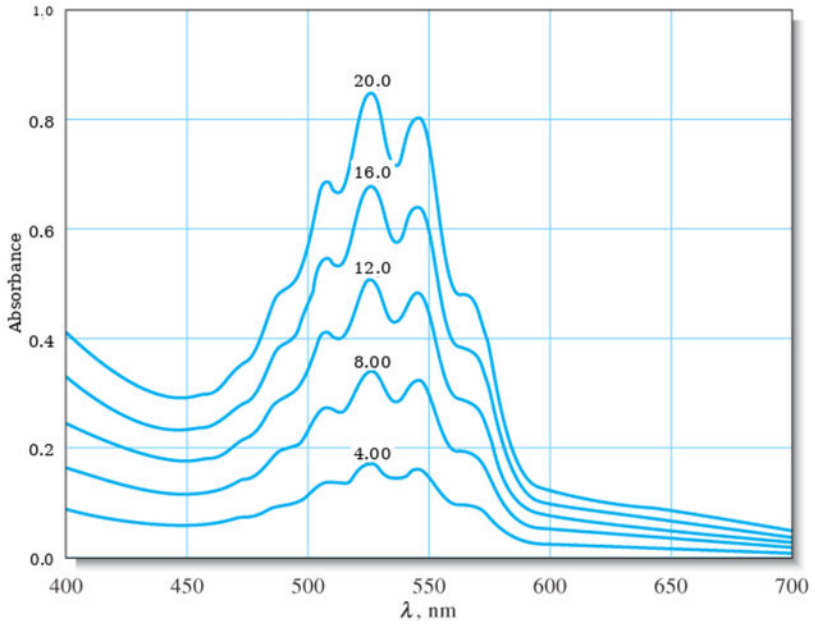
Spectra of the same substance recorded at increasing concentration have the same shape and the same maximum; only the height changes.

Consequence

The position of the maximum identifies the species, the intensity measures its amount. At every wavelength absorbance increases linearly with concentration.

Choice of λ

Measure at the maximum, where sensitivity is highest and the slope of the spectrum is zero.



Absorption spectra at increasing concentration.

Deviations from Beer's law

Real limits

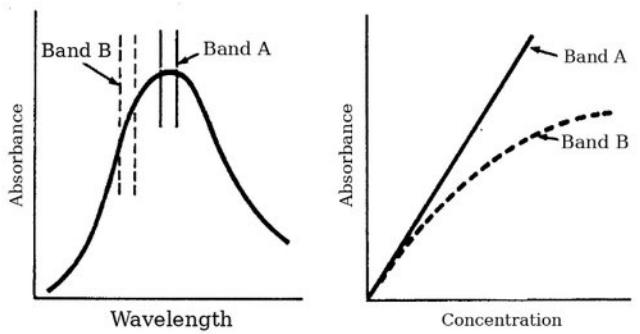
At high concentration molecules interact with each other and the refractive index changes: the calibration curve bends.

Chemical deviations

Association, dissociation or reaction with the solvent produce species with different ε, for example an acid-base indicator in unbuffered solution.

Instrumental deviations

Polychromatic radiation, especially when measuring on the side of a band, and stray light reaching the detector without passing through the sample.



Effect of polychromatic radiation on Beer's law: band A at the maximum, band B on the slope.

Spectrophotometric error

Low absorbance

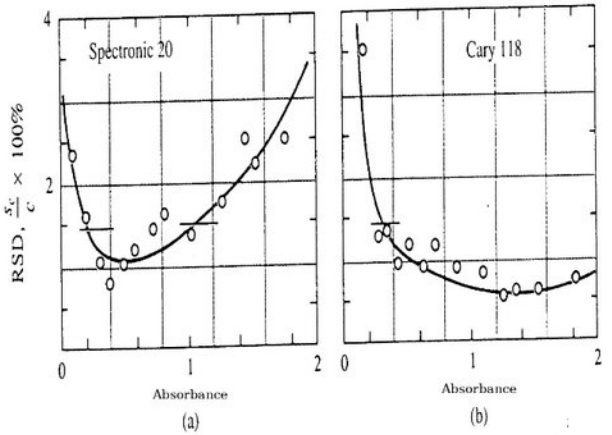
P is almost equal to P_0: the measurement is the small difference between two large quantities and the relative error on concentration is high.

High absorbance

Very little light reaches the detector, the signal is close to the noise and P is measured with poor accuracy.

Optimal range

The relative error is minimal for absorbances between about 0.2 and 0.8. Samples that are too concentrated are diluted, those too dilute are measured with a longer path length.



Relative error on concentration as a function of absorbance for two instruments.

Additivity of absorbances

$$A_\lambda = \varepsilon_{x,\lambda}\, b\, c_x + \varepsilon_{y,\lambda}\, b\, c_y$$

The principle

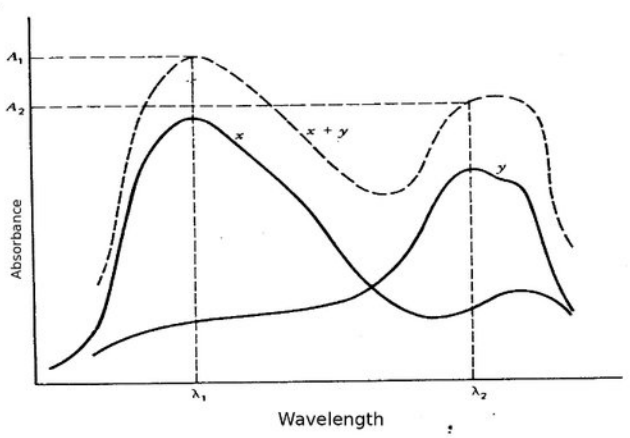
In a mixture of non-interacting species, the total absorbance at each wavelength is the sum of the absorbances of the individual components.

Two components

Measuring at two wavelengths λ_1 and λ_2, with the ε of each species known from pure standards, gives a system of two equations in two unknowns c_x and c_y.

Choice of λ

Choose wavelengths where one component absorbs much more than the other.



Spectra of two pure components and of their mixture.

Worked example: the Beer-Lambert law

Data A NADH solution measured at 340 nm in a 1.00 cm cuvette gives A = 0.415 against the blank. Molar absorptivity of NADH at 340 nm: $\varepsilon = 6220\ \text{L mol}^{-1}\ \text{cm}^{-1}$.

Formula	$c = A / (\varepsilon\, b)$
Calculation	$c = 0.415 / (6220 \times 1.00) = 6.67 \cdot 10^{-5}\ \text{mol/L}$
Units	$c = 66.7\ \mu\text{mol/L}$
Check	$A = 0.415$ falls in the optimal 0.2-0.8 range: the measurement is reliable

Conclusion The same measurement at 340 nm is used in enzyme assays that follow the formation or consumption of NADH over time.

Nucleic acids and proteins

Nucleic acids

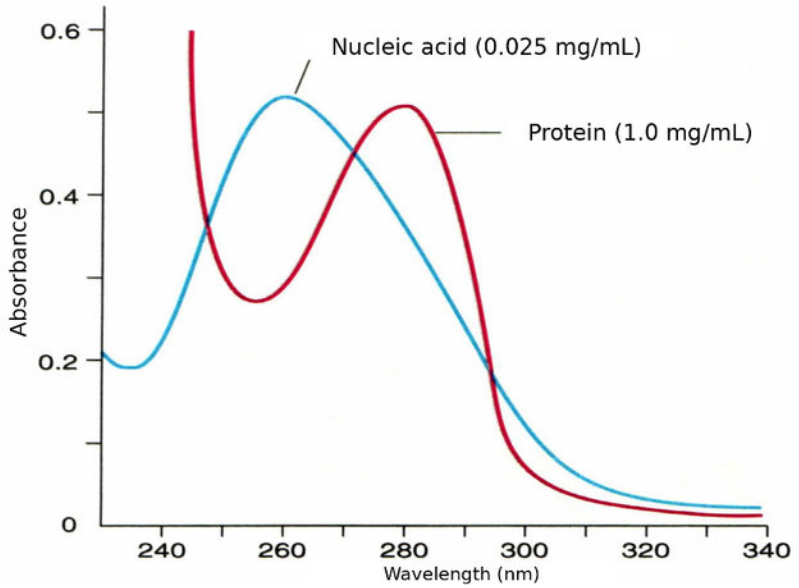
Nitrogenous bases absorb with a maximum at 260 nm. For a 1 cm path, A_{260} = 1 corresponds to about 50 µg/mL of double-stranded DNA and 40 µg/mL of RNA.

Proteins

They absorb at 280 nm thanks to tryptophan and tyrosine; for lower concentrations or mixtures colorimetric assays such as Bradford or BCA are used.

Purity

The A_{260}/A_{280} ratio is about 1.8 for pure DNA and 2.0 for RNA; lower values indicate protein contamination.



Spectra of a nucleic acid (0.025 mg/mL) and a protein (1.0 mg/mL).

Worked example: DNA concentration and purity

Data A DNA extract diluted 1:20 gives, in a 1 cm cuvette, A_{260} = 0.250 and A_{280} = 0.135.

Diluted	$c = 0.250 \times 50\ \mu g/mL = 12.5\ \mu g/mL$
Extract	$c = 12.5 \times 20 = 250\ \mu g/mL$
Purity	$A_{260}/A_{280} = 0.250 / 0.135 = 1.85$

Conclusion A ratio close to 1.8 indicates DNA without significant protein contamination; the extract contains 250 µg/mL of double-stranded DNA.

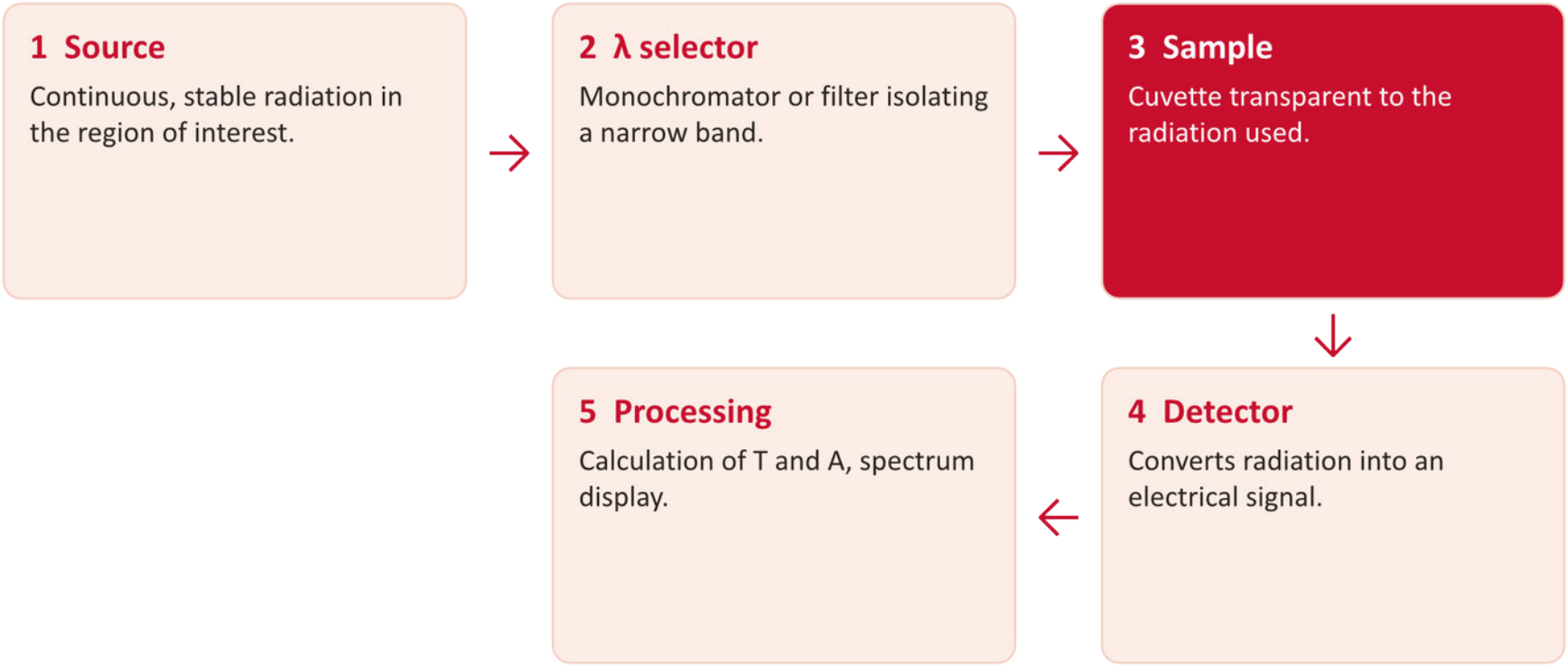
03

Instrumentation

Sources, wavelength selectors, cuvettes and detectors

Components of a spectrophotometer

Every absorption spectrophotometer combines the same functional blocks; the materials change with the spectral region.



Sources

UV

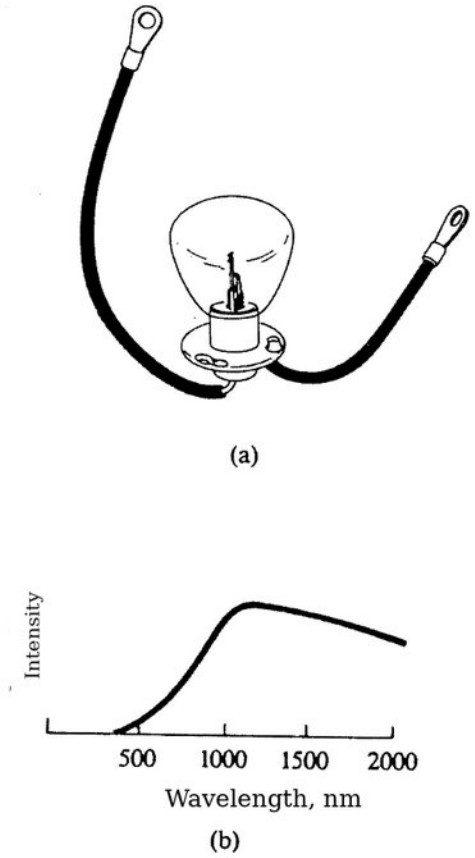
Deuterium lamp, with continuous emission between about 160 and 380 nm.

Visible and near IR

Tungsten or tungsten-halogen filament lamp, from about 320 to 2500 nm. UV-Vis spectrophotometers fit both lamps.

Other sources

Xenon lamp, intense across the UV-Vis and used in fluorescence; in the IR heated solids such as the Globar (silicon carbide) and the Nernst glower; lasers and LEDs for specific applications.



(a) Tungsten filament lamp; (b) its emission spectrum.

Prism monochromator

Function

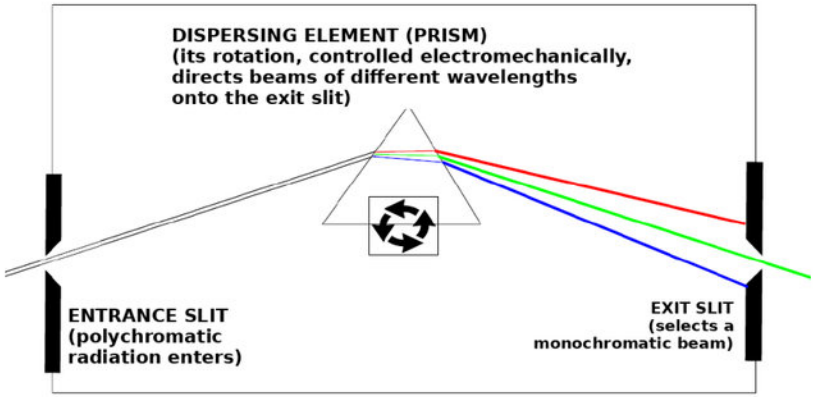
Separates polychromatic radiation into its components and allows a narrow band of wavelengths to be selected.

Principle

The refractive index of the material varies with wavelength: each λ is deviated at a different angle.

Limits

Dispersion is not linear and is smaller at longer wavelengths. The material must be transparent: quartz for the UV, glass for the visible.



Entrance slit, dispersing element and exit slit.

The reflection grating

$$n\,\lambda = d\,(\sin i + \sin r)$$

Structure

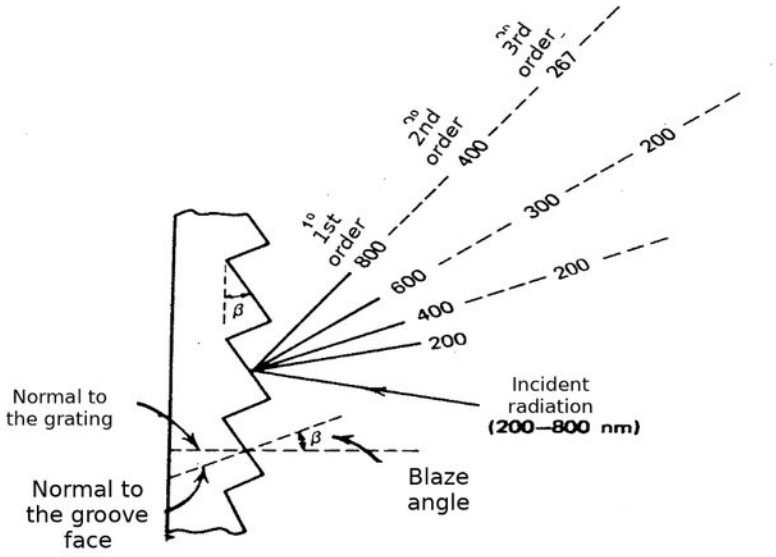
A reflecting surface, usually aluminium, ruled with a large number of parallel, equally spaced grooves, from hundreds to thousands per millimetre.

Principle

Radiation reflected by the different grooves interferes constructively only at certain angles. n is the diffraction order, d the groove spacing, i and r the angles of incidence and reflection.

Advantages

Practically linear dispersion. Higher orders are removed with filters; it is the λ selector of modern instruments.



Diffraction of radiation by a reflection grating.

The grating monochromator

Components

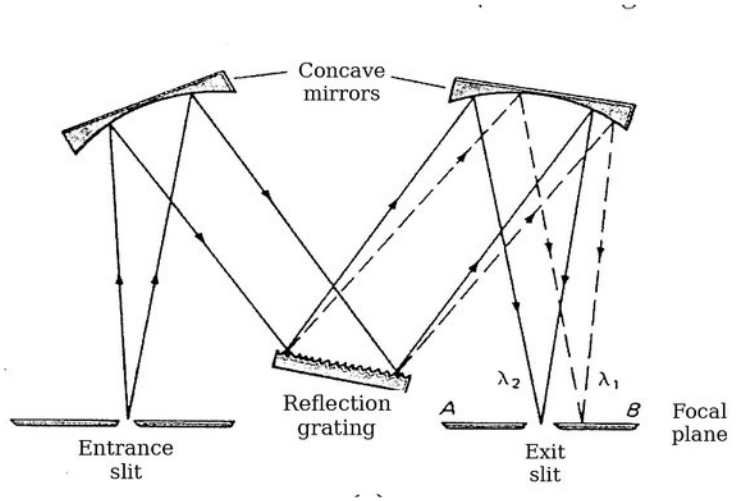
Entrance slit, concave mirror that collimates the beam, grating, second mirror that focuses the dispersed radiation onto the exit slit.

Selecting λ

Rotating the grating brings the desired wavelength onto the exit slit.

Czerny-Turner design

The most common configuration: the two concave mirrors correct aberrations and keep the instrument compact.



Grating monochromator with concave mirrors.

Bandwidth and slits

Effective bandwidth

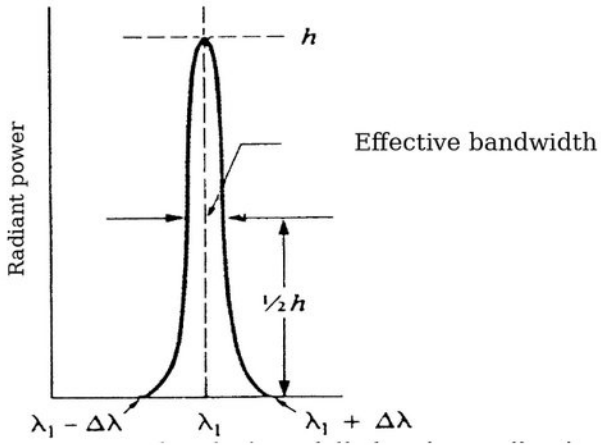
The monochromator does not isolate a single λ but a band, whose width at half height depends on the dispersion of the grating and on the opening of the exit slit.

The trade-off

Narrow slits improve resolution and the linearity of Beer's law, but reduce the power reaching the detector and increase noise.

Typical values

In benchtop spectrophotometers the bandwidth is 1-2 nm, more than sufficient for the broad bands of molecules in solution.



Distribution of wavelengths leaving the monochromator.

Absorption and interference filters

Absorption filters

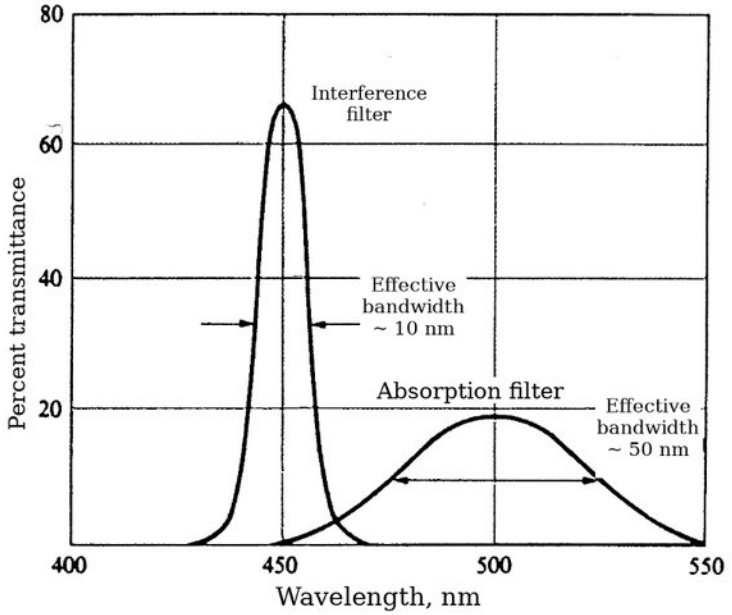
Coloured glasses transmitting a broad band, tens of nanometres wide: cheap and robust.

Interference filters

Thin dielectric layers exploiting constructive interference: narrower bands, around 10 nm, and higher transmission, but higher cost.

Where they are used

In instruments working at fixed wavelengths, such as photometers and microplate readers for immunoenzymatic assays.



Transmission curves of an interference filter and an absorption filter.

Cuvettes

Material

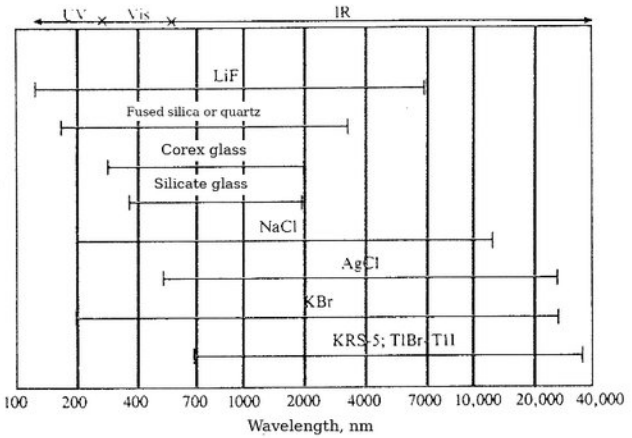
Must be transparent in the measurement region: quartz or fused silica in the UV, glass and plastic only in the visible, NaCl or KBr windows in the infrared.

Path length

The standard is 1 cm. Longer cuvettes increase sensitivity; micro-cuvettes and microvolume instruments reduce the amount of sample needed.

Good practice

Clean walls without fingerprints, matched cuvettes for blank and sample, constant orientation in the holder.



Transmission range of materials used for cuvettes and windows.

Detectors: phototubes and photodiodes

Photon detectors

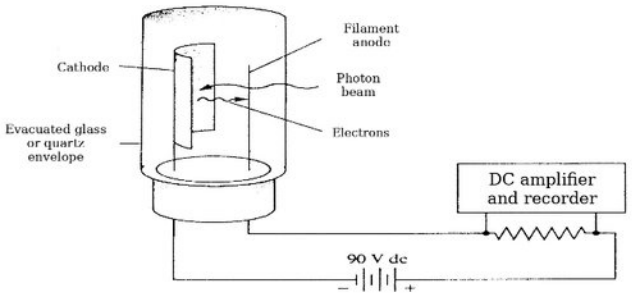
They contain a surface that, when struck by photons, emits electrons (photoemission) or becomes conductive (photoconduction). They work up to about 2 µm.

Phototube

A photoemissive cathode and an anode in an evacuated bulb: the current produced is proportional to the radiant power.

Silicon photodiodes

Compact, inexpensive and robust semiconductor detectors; arranged in an array they form diode-array detectors. In the IR, thermal detectors or specific photoconductors are used.



Phototube with amplification circuit.

The photomultiplier

Structure

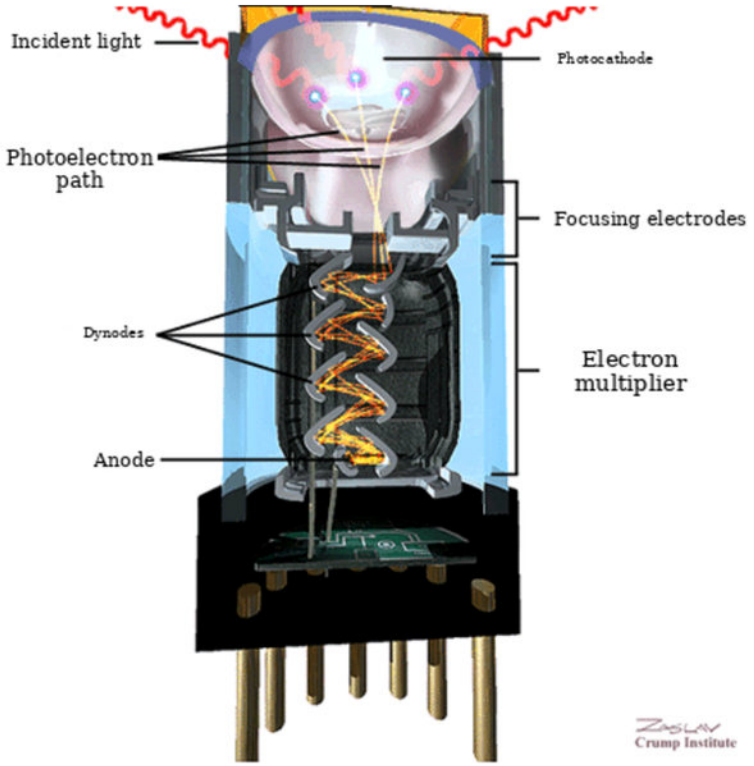
A photocathode, a series of dynodes at increasing potential and an anode, in an evacuated tube.

Amplification

Each electron striking a dynode releases several more: after 9-12 dynodes the signal is amplified 10^6-10^7 times.

Use

The most sensitive detector for UV-Vis: essential in fluorescence and for very weak signals. It must be protected from intense light, which damages it.



Photomultiplier: photocathode, dynodes and anode.

Single-beam spectrophotometer

Layout

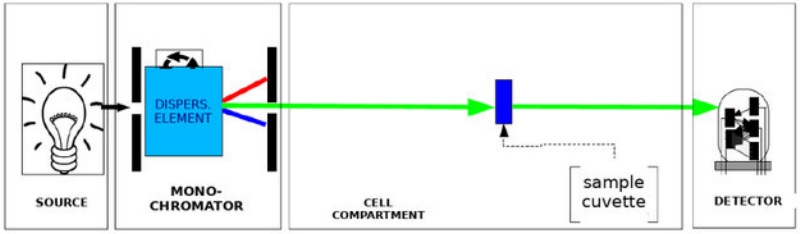
Source, monochromator, cell compartment and detector are aligned along a single optical path.

Procedure

The blank is measured first to set P_0 (A = 0), then the sample in the same position.

Pros and cons

Simple and inexpensive, but any drift of source or detector between the two measurements introduces an error: it requires stable instruments and frequent blanks.



Layout of a single-beam spectrophotometer.

Double-beam spectrophotometer

Layout

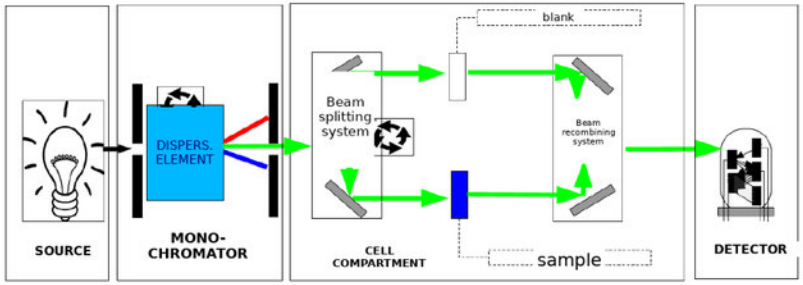
After the monochromator a mirror system, often a rotating chopper, splits the beam: one beam passes through the blank, the other through the sample.

Advantage

Blank and sample are measured almost simultaneously: fluctuations of source and detector cancel out.

Use

The preferred configuration for recording full spectra and for precise measurements over long periods.



Layout of a double-beam spectrophotometer.

Diode-array detector (DAD)

Reversed optics

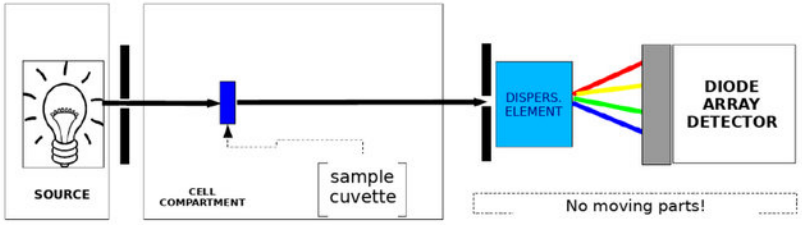
Polychromatic radiation passes through the sample and only then is dispersed by the grating onto an array of hundreds of photodiodes.

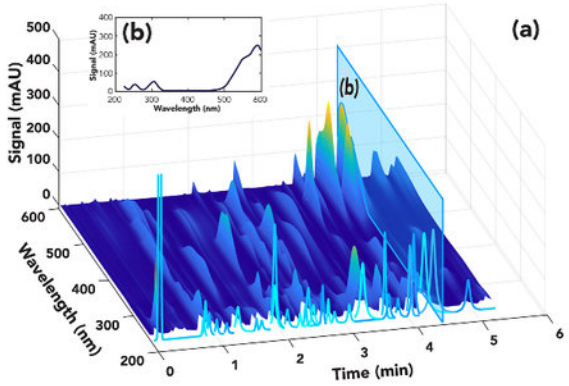
Advantages

It records the whole spectrum in a fraction of a second, with no moving parts, and can monitor several wavelengths simultaneously.

In HPLC

It provides chromatograms at any λ and the spectrum of each peak, useful for identifying compounds and checking peak purity.





Layout of a diode-array detector; three-dimensional absorbance-time-wavelength chromatogram.

Quantitative analysis: the calibration curve

Qualitative and quantitative

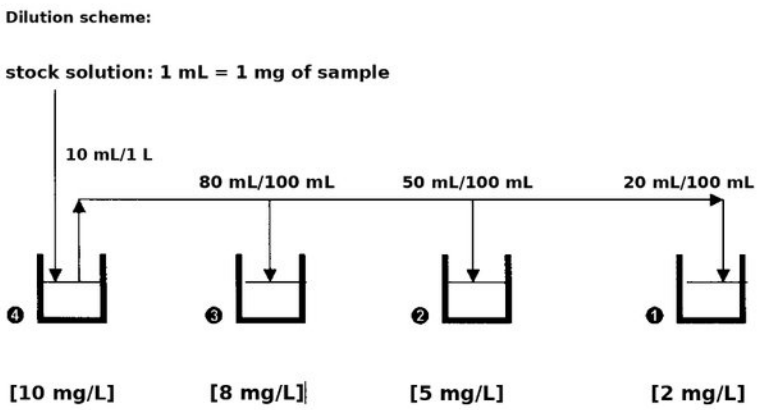
The wavelength of the absorbed radiation is characteristic of the substance; the intensity of absorption depends on its amount.

Preparing standards

From a stock solution of known concentration, standards of increasing concentration covering the range expected in samples are prepared by dilution.

Using the line

The standards are measured, the A versus c curve is built and the concentration of samples is obtained by interpolation.



Preparation of calibration standards from a stock solution.

UV-Vis as an HPLC detector

Flow cell

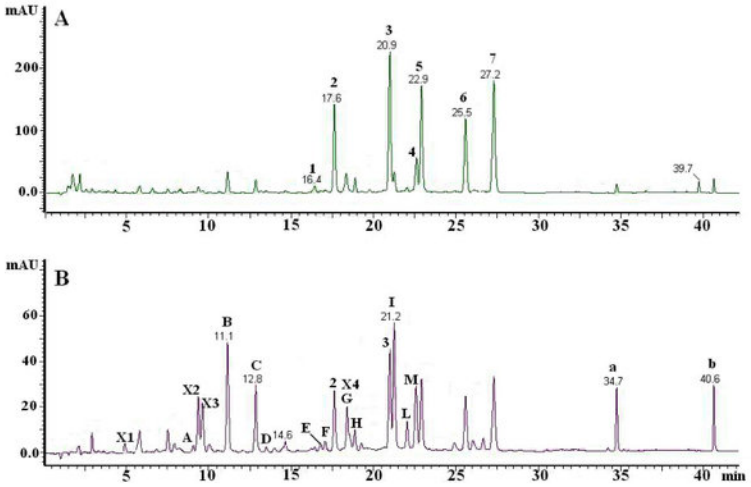
The eluate passes through a cell of a few microlitres; the small volume avoids broadening the peaks separated by the column.

Choice of λ

Use the analyte maximum, taking into account the UV cut-off of mobile-phase solvents: acetonitrile is transparent down to about 190 nm, methanol to about 205 nm.

Selectivity

Changing wavelength enhances or suppresses some peaks: the two chromatograms of the same sample look different.



The same sample recorded at two different wavelengths.

04

Infrared spectroscopy

Molecular vibrations, IR spectra and FTIR

Regions of the infrared

Near IR (NIR)

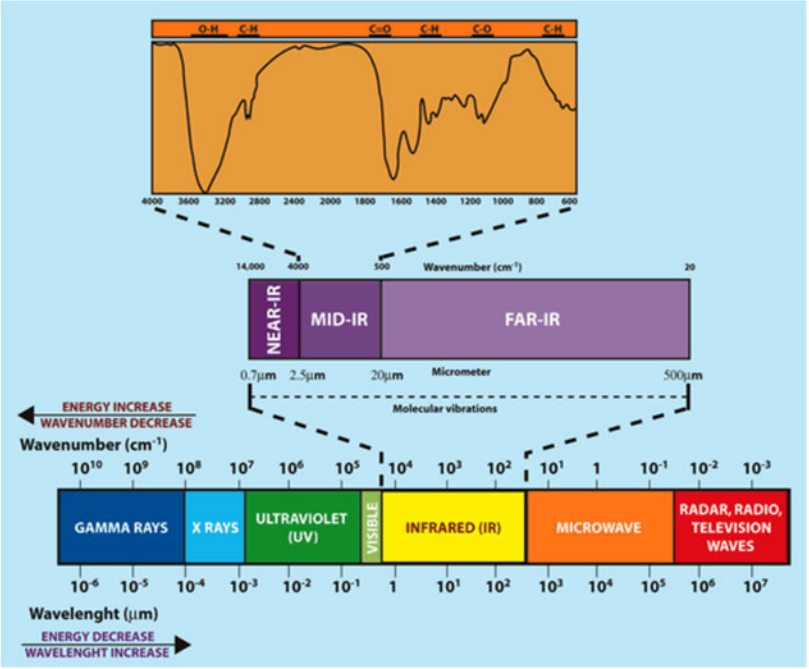
About 13,000-4000 cm^{-1}: overtones and combination bands, weak but useful for fast quantitative analysis of foods and raw materials.

Mid IR (MIR)

4000-400 cm^{-1}: fundamental vibrations of bonds. The region used for structural identification.

Far IR (FIR)

400-10 cm^{-1}: vibrations of heavy atoms and crystal lattices, of more specialist interest.



Position of the infrared in the electromagnetic spectrum.

Vibrational modes

Vibrational spectroscopy

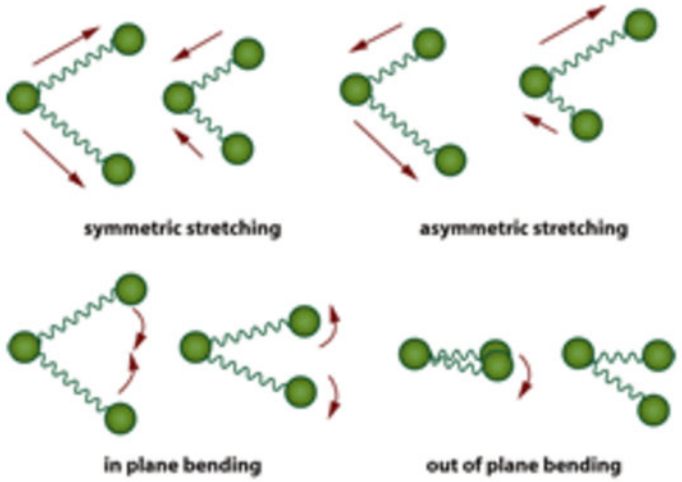
IR radiation is not energetic enough for electronic transitions, but it excites bond vibrations.

Stretching

Periodic change in bond length along its axis; it can be symmetric or asymmetric.

Bending

Change in bond angle: in plane (scissoring and rocking) or out of plane (wagging and twisting). It requires less energy than stretching.



Stretching and bending modes of a CH_2 group.

The harmonic oscillator

$$\tilde{\nu} = (1 / 2\pi c)\sqrt{(k / \mu)} \qquad \mu = m_1 m_2 / (m_1 + m_2)$$

The model

Two bonded atoms behave like two masses joined by a spring. The vibrational frequency depends on the force constant k of the bond and on the reduced mass μ.

Stronger bonds

k increases with bond order: C≡C absorbs at about 2200 cm^{-1}, C=C at 1650, C–C below 1200.

Lighter atoms

A small reduced mass raises the frequency: bonds to hydrogen (O–H, N–H, C–H) absorb above 2800 cm^{-1}; C–D absorbs at lower frequency than C–H.

IR activity: the dipole moment

The rule

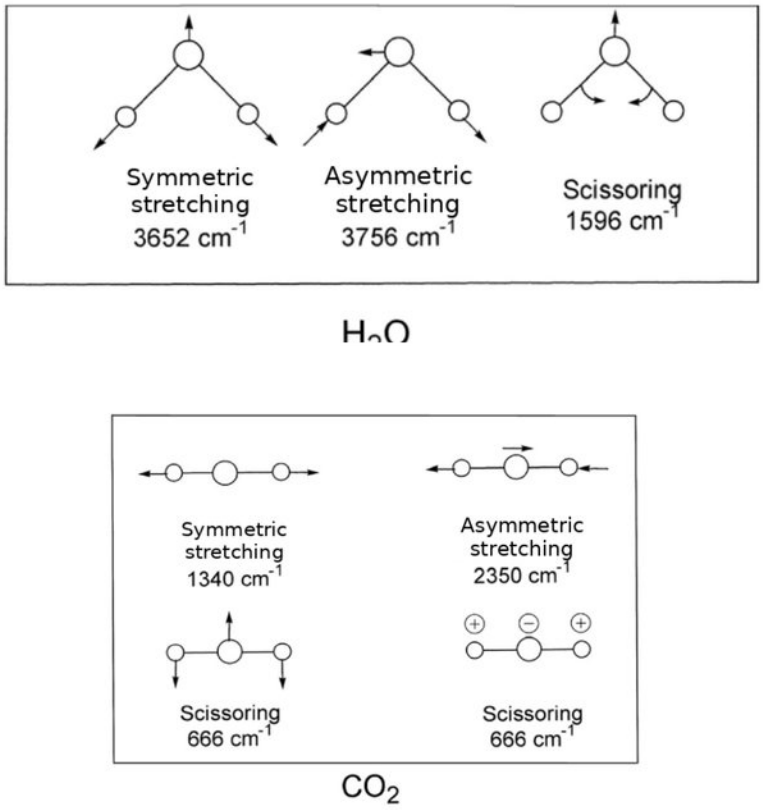
A vibration absorbs in the IR only if it changes the dipole moment of the molecule: the vibrating molecule generates an oscillating electric field that exchanges energy with the radiation.

H_2O

All three modes are IR active: symmetric stretching (3652 cm^{-1}), asymmetric stretching (3756 cm^{-1}) and scissoring (1596 cm^{-1}).

CO_2

Symmetric stretching (1340 cm^{-1}) does not change the dipole moment and is IR inactive; asymmetric stretching (2350 cm^{-1}) and bending (666 cm^{-1}) are active.



Normal vibrational modes of H_2O and CO_2.

Factors affecting the frequency

Hydrogen bonding

It weakens the X–H bond and shifts stretching to lower frequency, broadening the band considerably: free O–H is sharp near 3600 cm^{-1}, the associated O–H of carboxylic acids is very broad between 3300 and 2500.

Inductive and resonance effects

Electron-withdrawing groups attached to a carbonyl raise its frequency (acyl chlorides ~1800 cm^{-1}); electron-donating groups by resonance lower it (amides ~1650 cm^{-1}).

Conjugation

It delocalises the π electrons and reduces double-bond character: a conjugated carbonyl absorbs about 20-40 cm^{-1} lower (1715 → 1685 cm^{-1}).

Regions of the IR spectrum

4000-2500 cm^{-1}

Stretching of bonds to hydrogen: O–H, N–H, C–H.

2500-2000 cm^{-1}

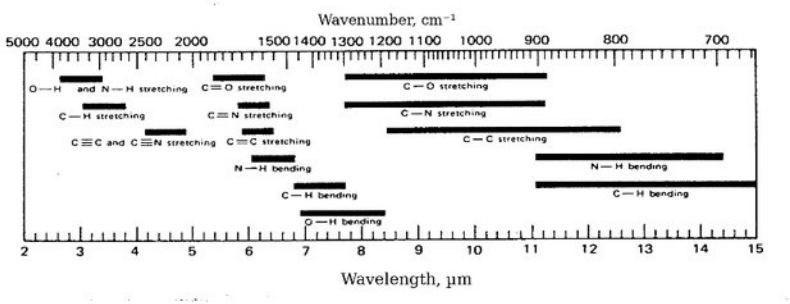
Stretching of triple bonds and cumulenes: C≡C, C≡N, O=C=O.

2000-1500 cm^{-1}

Stretching of double bonds: C=O around 1700, C=C and C=N, aromatic rings.

1500-400 cm^{-1}

Fingerprint region: single-bond stretching and bending, characteristic of each molecule.



Correlation chart of the main IR absorption bands.

Reading an IR spectrum

The plot

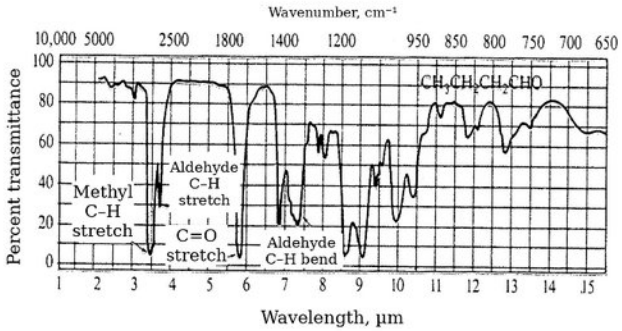
Transmittance (or absorbance) against wavenumber, by convention decreasing from left to right. Absorption bands appear as downward peaks.

Position and intensity

Position depends on the force constant of the bond; intensity on the change in dipole moment. Bands are described as strong, medium or weak and broad or sharp.

Sample phase

Gases show complex vibration-rotation bands; in liquids and solids collisions prevent free rotation and bands are simpler.



IR spectrum of n-butanal (butyraldehyde) with the main bands.

The dispersive IR spectrophotometer

Layout

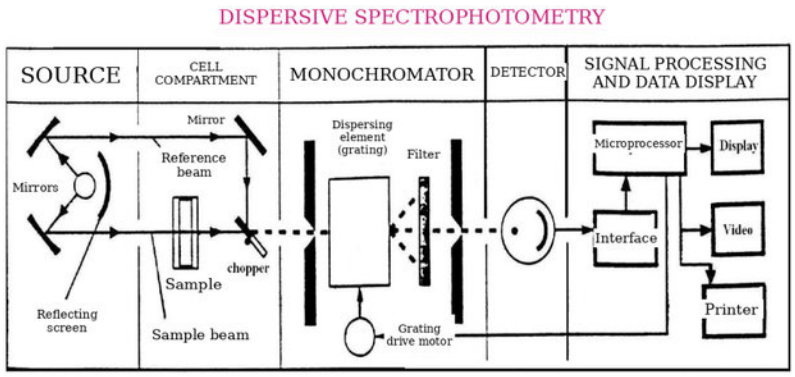
Source (Globar or Nernst glower), double-beam system with chopper, grating monochromator and thermal detector.

Special features

Mirror optics and alkali-halide windows, because glass and quartz absorb in the IR. The sample is placed before the monochromator.

Today

Dispersive instruments have been almost entirely replaced by FTIR spectrometers, which are faster, more sensitive and more accurate.



Block diagram of a dispersive IR spectrophotometer.

FTIR: the Fourier-transform spectrometer

The interferometer

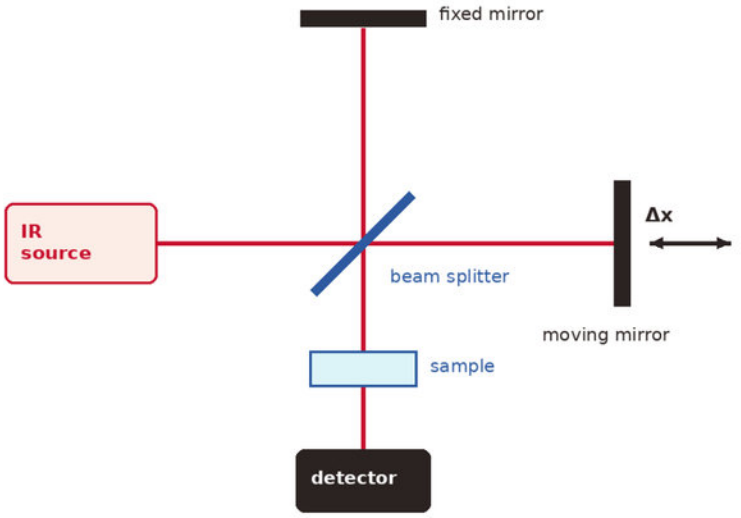
A beam splitter sends the beam to a fixed mirror and a moving mirror; the two recombined beams interfere differently depending on the mirror position.

Interferogram and spectrum

The detector records all frequencies together as a function of mirror position; the Fourier transform converts this interferogram into the spectrum.

Advantages

Multiplex (all frequencies in every scan), higher power at the detector because there are no slits, laser-calibrated wavenumber. A spectrum is acquired in a few seconds.



Michelson interferometer: the signal recorded as a function of Δx is the interferogram.

Michelson interferometer, the heart of an FTIR spectrometer.

Sample preparation

KBr pellets

The solid is ground with dry KBr and pressed into a transparent disc. It requires skill and is sensitive to moisture, which adds O–H bands.

Films and cells

Liquids are analysed as a film between two NaCl or KBr windows; solutions in cells with solvents that absorb little in the region of interest.

ATR (attenuated total reflectance)

The sample is pressed onto a diamond or ZnSe crystal and the radiation penetrates only a few micrometres: solids, liquids and pastes are analysed without preparation. It is now the most used technique.

Applications and limits

Identification

The spectrum in the fingerprint region is practically unique to each molecule: comparison with databases identifies pure compounds and verifies raw materials.

Quantitative analysis

Beer's law also applies in the IR. NIR and MIR, combined with chemometrics, are used to determine moisture, protein, fat and sugars in foods in seconds.

Limits

Complex mixtures give overlapping spectra; water absorbs strongly and interferes with aqueous samples; sensitivity is lower than UV-Vis and fluorescence.

05

Emission spectroscopy

Fluorescence, phosphorescence and flame emission

EMISSION

Luminescence

Luminescence is the emission of radiation by an excited species. Its type depends on the source of the excitation energy.

Photoluminescence

Excitation occurs by absorption of UV-Vis radiation. It includes fluorescence and phosphorescence.

Chemiluminescence

The energy comes from a chemical reaction, as in the oxidation of luminol; when the reaction is enzymatic it is called bioluminescence, as with luciferase.

Other forms

Electroluminescence, radioluminescence, thermoluminescence. Fluorescent molecules are usually aromatic or heterocyclic and are called fluorophores.

The Jablonski diagram

Absorption and relaxation

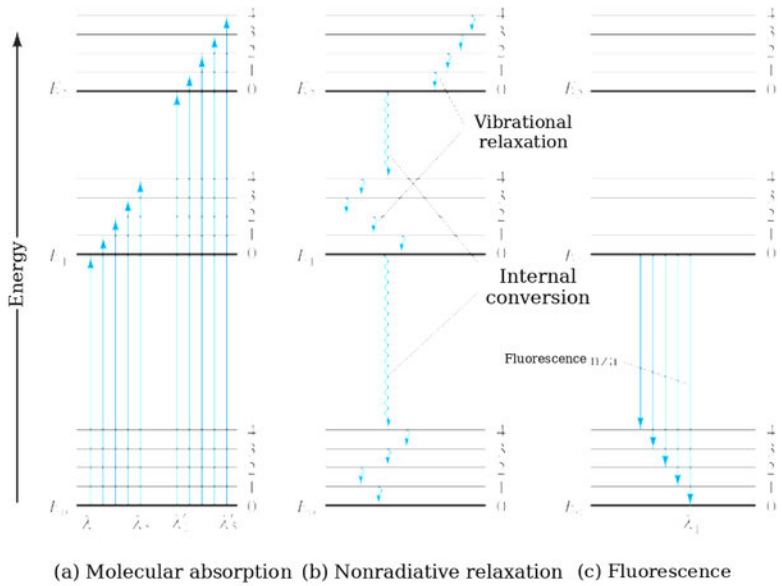
After absorption the molecule reaches an excited vibrational level and quickly loses energy by vibrational relaxation and internal conversion, down to the lowest level of S_1.

Fluorescence

Emission from S_1 to S_0, between states of the same spin; it occurs in 10^{-9}-10^{-7} s.

Phosphorescence

After intersystem crossing to the triplet state T_1, emission to S_0 is spin-forbidden and therefore slow, from milliseconds to seconds.



(a) Absorption, (b) nonradiative relaxation, (c) fluorescence.

Quantum yield and structure

$$\Phi = \text{molecules that emit / molecules excited}$$

Competing processes

Fluorescence competes with nonradiative relaxation. Many molecules that could fluoresce do not, because nonradiative deactivation is faster than emission.

Values

Highly efficient fluorophores such as fluorescein have Φ close to 1 under suitable conditions; non-fluorescent species have Φ practically zero.

Structural rigidity

Rigid, planar molecules fluoresce more: rigidity reduces the motions that dissipate energy as heat. Complexation with a metal can also make a ligand fluorescent.

Excitation and emission spectra

Stokes shift

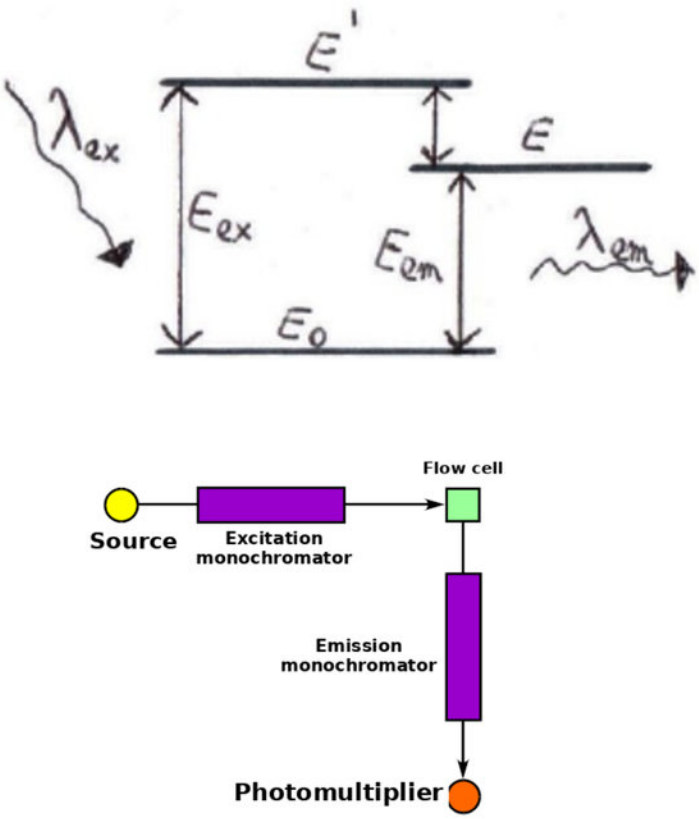
The emitted radiation has lower energy, and therefore longer wavelength, than the absorbed radiation, because some energy is lost by vibrational relaxation.

Two spectra

The excitation spectrum is recorded by varying λ_{ex} at fixed emission and resembles the absorption spectrum; the emission spectrum is recorded by varying λ_{em} at fixed excitation.

Selectivity

Choosing both λ_{ex} and λ_{em} gives greater selectivity than absorption: few compounds share both.



Origin of the Stokes shift and layout of a fluorescence detector.

Fluorescence intensity and concentration

$$F = K'(P_0 - P) \approx 2.303\, K' \varepsilon b c P_0 = k c$$

Linearity

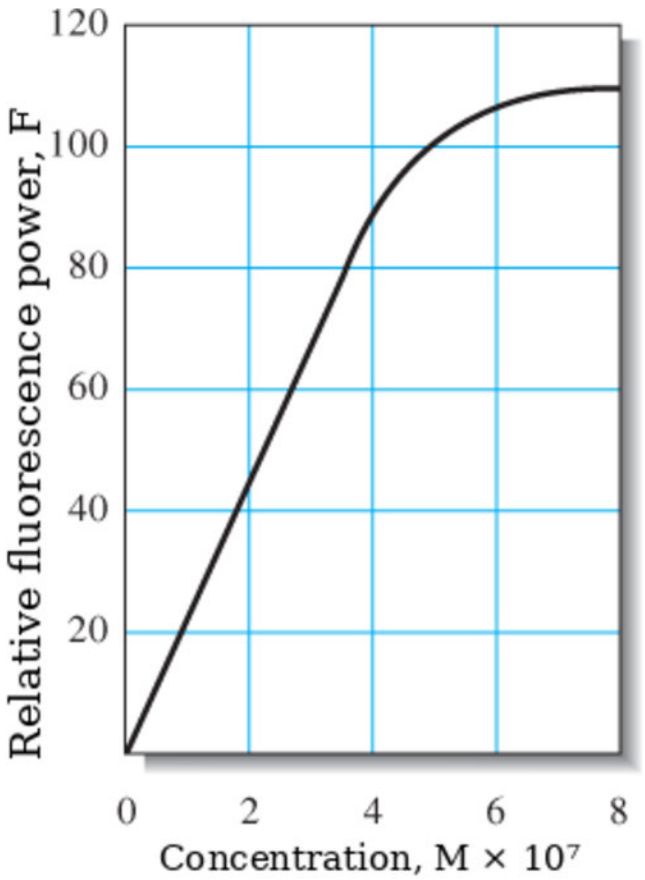
Fluorescence power is proportional to the absorbed radiation. At low absorbance, below about 0.05, F is proportional to concentration and to source power.

Inner-filter effect

At high concentration the excitation beam is absorbed before reaching the centre of the cell (primary filter) and emitted light is reabsorbed (secondary filter): the curve bends and then falls.

Quenching

Oxygen, halide ions and other species deactivate the excited state and reduce intensity.



Fluorescence intensity as a function of concentration.

Fluorometers and spectrofluorometers

90° geometry

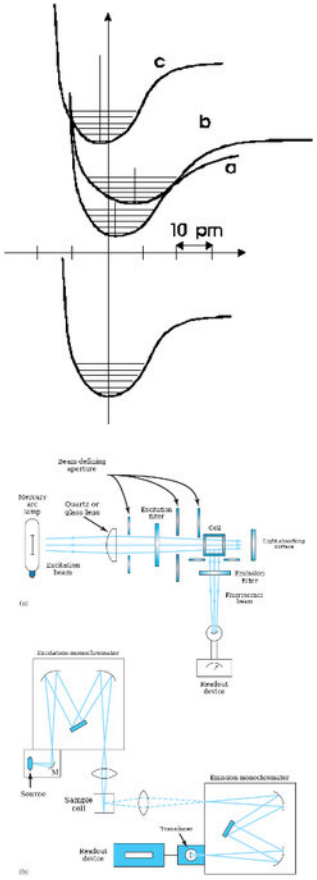
Fluorescence is emitted in all directions: the detector is placed at 90° to the excitation beam so that it does not see the source.

Fluorometer

Uses filters to select excitation and emission: simple, inexpensive and suited to routine quantitative analysis.

Spectrofluorometer

Has two grating monochromators and records excitation and emission spectra. Xenon source, photomultiplier detector.



Potential-energy curves of the ground and excited states; filter fluorometer and two-monochromator spectrofluorometer.

Fluorescence in biotechnology

Analysis

Amino acids, proteins, coenzymes, vitamins, nucleic acids, porphyrins and many metabolites are determined by fluorescence, directly or after derivatisation; the fluorescence detector is widely used in HPLC and electrophoresis.

Ion probes

Indicators such as Fura-2 change spectrum when they bind Ca^{2+}: they make it possible to follow free calcium concentration over time in individual neurons.

Molecular biology

Fluorescent proteins such as GFP, real-time quantitative PCR, flow cytometry and fluorescence microscopy rest on the same principles.

Flame emission spectroscopy

$$I_E = K \cdot c$$

Principle

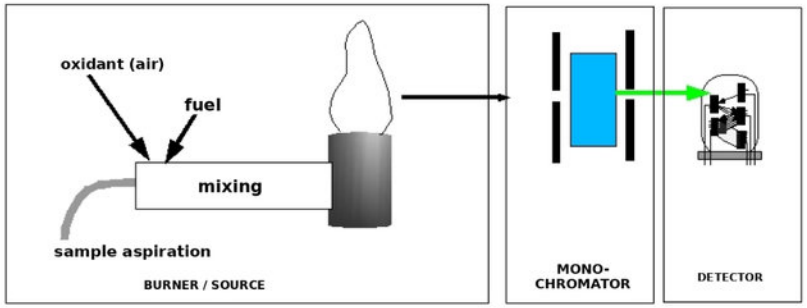
The solution is nebulised into a flame; thermally excited atoms emit at characteristic wavelengths, as narrow lines.

Applications

Quantitative determination of alkali and alkaline-earth metals (Li, Na, K, Ca), easily excited at the temperature of an air-propane or air-acetylene flame (about 2000-2500 K).

Quantification

Emission intensity is proportional to concentration within the working range: a calibration curve is built with standards.



Flame photometer: nebuliser and burner, monochromator, detector.

SUMMARY

The techniques compared

	UV-Vis	IR	Fluorescence	Flame emission
Transition	Electronic	Vibrational	Electronic (emission from S_1)	Atomic electronic
Main use	Quantitative	Identification	Trace quantitation	Alkali metals
Sensitivity	Medium	Low	Very high	High for Na, K
Selectivity	Low-medium	Very high	High	High (lines)
Biotech example	DNA at 260 nm	Raw-material checks	Probes and qPCR	Na and K in fluids

The choice of technique depends on the information required: identity, amount, concentration level and nature of the matrix.