SPECTROSCOPY -PRINCIPLES AND APPLICATIONS

UV-VISIBLE SPECTROPHOTOMETER

Introduction to UV spectroscopy

UV spectroscopy is type of absorption spectroscopy in which light of ultra-violet region (200-400 nm.) is absorbed by the molecule. Absorption of the ultra-violet radiations results in the excitation of the electrons from the ground state to higher energy state. The energy of the ultra-violet radiation that are absorbed is equal to the energy difference between the ground state and higher energy states (deltaE = hf).

Generally, the most favoured transition is from the highest occupied molecular orbital (HOMO) to lowest unoccupied molecular orbital (LUMO). For most of the molecules, the lowest energy occupied molecular orbitals are s orbital, which correspond to sigma bonds. The p orbitals are at somewhat higher energy levels, the orbitals (nonbonding orbitals) with unshared paired of electrons lie at higher energy levels. The unoccupied or antibonding orbitals (pie* and sigma*) are the highest energy occupied orbitals.

In all the compounds (other than alkanes), the electrons undergo various transitions. Some of the important transitions with increasing energies are: nonbonding to pie*, nonbonding to sigma*, pie to pie*, sigma to pie* and sigma to sigma*.

Principle of UV spectroscopy

UV spectroscopy obeys the Beer-Lambert law, which states that: when a beam of monochromatic light is passed through a solution of an absorbing substance, the rate of decrease of intensity of radiation with thickness of the absorbing solution is proportional to the incident radiation as well as the concentration of the solution.

The expression of Beer-Lambert law is-
$A = \log (I_0/I) = Ecl$
Where, A = absorbance
I_0 = intensity of light incident upon sample cell
I = intensity of light leaving sample cell
C = molar concentration of solute
L = length of sample cell (cm.)
E = molar absorptivity

From the Beer-Lambert law it is clear that greater the number of molecules capable of absorbing light of a given wavelength, the greater the extent of light absorption. This is the basic principle of UV spectroscopy.

Instrumentation and working of UV spectroscopy

Instrumentation and working of the UV spectrometers can be studied simultaneously. Most of the modern UV spectrometers consist of the following parts-

Light Source- Tungsten filament lamps and Hydrogen-Deuterium lamps are most widely used and suitable light source as they cover the whole UV region. Tungsten filament lamps are rich in red radiations; more specifically they emit the radiations of 375 nm, while the intensity of Hydrogen-Deuterium lamps falls below 375 nm.

Monochromator- Monochromators generally composed of prisms and slits. The most of the spectrophotometers are **double beam spectrophotometers**. The radiation emitted from the primary source is dispersed with the help of rotating prisms. The various wavelengths of the light source which are separated by the prism are then selected by the slits such the rotation of the prism results in a series of continuously increasing wavelength to pass through the slits for recording purpose. The beam selected by the slit is monochromatic and further divided into two beams with the help of another prism.

Sample and reference cells- One of the two divided beams is passed through the sample solution and second beam is passé through the reference solution. Both sample and reference solution are contained in the cells. These cells are made of either silica or quartz. Glass can't be used for the cells as it also absorbs light in the UV region.

Detector- Generally two photocells serve the **purpose of detector in UV spectroscopy**. One of the photocell receives the beam from sample cell and second detector receives the beam from the reference. The intensity of the radiation from the reference cell is stronger than the beam of

sample cell. This results in the generation of pulsating or alternating currents in the photocells.

Amplifier- The alternating current generated in the photocells is transferred to the amplifier. The amplifier is coupled to a small servometer. Generally current generated in the photocells is of very low intensity, the main purpose of amplifier is to amplify the signals many times so we can get clear and recordable signals.

Recording devices- Most of the time amplifier is coupled to a pen recorder which is connected to the computer. Computer stores all the data generated and produces the spectrum of the desired compound.

Concept of Chromophore and Auxochrome in the UV spectroscopy

Chromophore- Chromophore is defined as any isolated covalently bonded group that shows a characteristic absorption in the **ultraviolet or visible region (200-800 nm)**.

Chromophores can be divided into two groups-
a) **Chromophores** which contain p electrons and which undergo pie to pie* transitions. Ethylenes and acetylenes are the example of such chromophores.
b) **Chromophores** which contain both p and nonbonding electrons. They undergo two types of transitions; pie to pie* and nonbonding to pie*. Carbonyl, nitriles, azo compounds, nitro compounds etc. are the example of such chromophores.

c) Auxochromes- An auxochrome can be defined as any group which does not itself act as a chromophore but whose presence brings about a shift of the absorption band towards the longer wavelength of the spectrum. –OH,-OR,-NH_2,-NHR, -SH etc. are the examples of auxochromic groups.

Absorption and intensity shifts in the UV spectroscopy

There are four types of shifts observed in the UV spectroscopy-

a) **Bathochromic effect**- This type of shift is also known as red shift. Bathochromic shift is an effect by virtue of which the absorption maximum is shifted towards the longer wavelength due to the presence of an auxochrome or change in solvents.
The nonbonding to pie* transition of carbonyl compounds observes bathochromic or red shift.

b) **Hypsochromic shift**- This effect is also known as blue shift. Hypsochromic shift is an effect by virtue of which absorption maximum is shifted towards the shorter wavelength. Generally it is caused due to the removal of conjugation or by changing the polarity of the solvents.

c) **Hyperchromic effect**- Hyperchromic shift is an effect by virtue of which absorption maximum increases. The introduction of an auxochrome in the compound generally results in the hyperchromic effect.

d) **Hypochromic effect**- Hyperchromic effect is defined as the effect by virtue of intensity of absorption maximum decreases. Hyperchromic effect occurs due to the distortion of the geometry of the molecule with an introduction of new group.

Applications of UV spectroscopy

1. Detection of functional groups- UV spectroscopy is used to detect the presence or absence of chromophore in the compound. This is technique is not useful for the detection of chromophore in complex compounds. The absence of a band at a particular band can be seen as an evidence for the absence of a particular group. If the spectrum of a compound comes out to be transparent above 200 nm than it confirms the absence of –
a) Conjugation b) A carbonyl group c) Benzene or aromatic compound d) Bromo or iodo atoms.

2. Detection of extent of conjugation- The extent of conjugation in the polyenes can be detected with the help of UV spectroscopy. With the increase in double bonds the absorption shifts towards the longer wavelength. If the double bond is increased by 8 in the polyenes then that polyene appears visible to the human eye as the absorption comes in the visible region.

3. Identification of an unknown compound- An unknown compound can be identified with the help of UV spectroscopy. The spectrum of unknown compound is compared with the spectrum of a reference compound and if both the spectrums coincide then it confirms the identification of the unknown substance.

4. Determination of configurations of geometrical isomers- It is observed that cis-alkenes absorb at different wavelength than the trans-alkenes. The two isomers can be distinguished with each other when one of the isomers has non-coplanar structure due to steric hindrances. The cis-isomer suffers distortion and absorbs at lower wavelength as compared to trans-isomer.

5. Determination of the purity of a substance- Purity of a substance can also be determined with the help of UV spectroscopy. The absorption of the sample solution is compared with the absorption of the reference solution. The intensity of the absorption can be used for the relative calculation of the purity of the sample substance.

INFRA RED SPECTROSCOPY

Theoretical Principles

Introduction

The term "infra red" covers the range of the electromagnetic spectrum between 0.78 and 1000 mm. In the context of infra red spectroscopy, wavelength is measured in "wavenumbers", which have the units cm^{-1}.

wavenumber = 1 / wavelength in centimeters

It is useful to divide the infra red region into three sections; *near*, *mid* and *far* infra red;

Region	**Wavelength range** (mm)	**Wavenumber range** (cm^{-1})
Near	0.78 - 2.5	12800 - 4000
Middle	2.5 - 50	4000 - 200
Far	50 -1000	200 - 10

The most useful I.R. region lies between 4000 - 670cm^{-1}.

Theory of infra red absorption

IR radiation does not have enough energy to induce electronic transitions as seen with UV. Absorption of IR is restricted to compounds with small energy differences in the possible vibrational and rotational states.

For a molecule to absorb IR, the vibrations or rotations within a molecule must cause a net change in the dipole moment of the molecule. The alternating electrical field of the radiation (remember that electromagnetic radation consists of an oscillating electrical field and an oscillating magnetic field, perpendicular to each other) interacts with fluctuations in the dipole moment of the molecule. If the frequency of the radiation matches the vibrational frequency of the molecule then radiation will be absorbed, causing a change in the amplitude of molecular vibration.

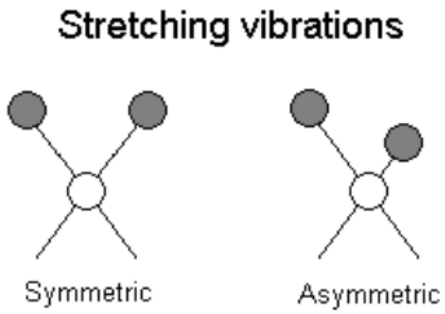
Molecular rotations

Rotational transitions are of little use to the spectroscopist. Rotational levels are quantized, and absorption of IR by gases yields line spectra. However, in liquids or solids, these lines broaden into a continuum due to molecular collisions and other interactions.

Molecular vibrations

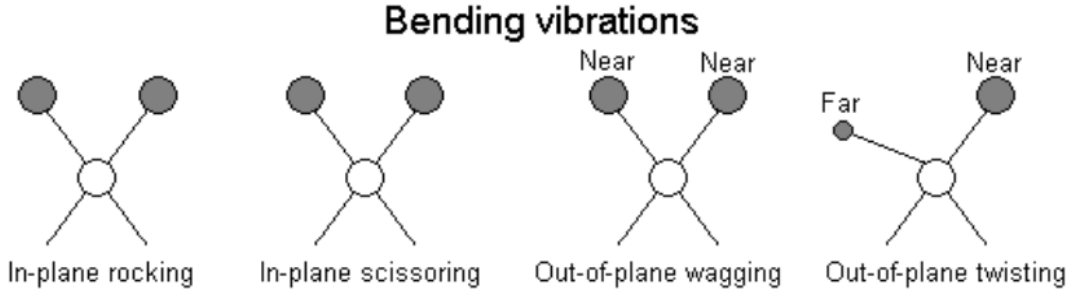
The positions of atoms in a molecules are not fixed; they are subject to a number of different vibrations. Vibrations fall into the two main catagories of *stretching* and *bending*.

Stretching: Change in inter-atomic distance along bond axis



Bending: Change in angle between two bonds. There are four types of bend:

- Rocking
- Scissoring
- Wagging
- Twisting



Vibrational coupling

In addition to the vibrations mentioned above, interaction between vibrations can occur (*coupling*) if the vibrating bonds are joined to a single, central atom. Vibrational coupling is influenced by a number of factors;

- Strong coupling of stretching vibrations occurs when there is a common atom between the two vibrating bonds
- Coupling of bending vibrations occurs when there is a common bond between vibrating groups
- Coupling between a stretching vibration and a bending vibration occurs if the stretching bond is one side of an angle varied by bending vibration
- Coupling is greatest when the coupled groups have approximately equal energies
- No coupling is seen between groups separated by two or more bonds

INSTRUMENTAL COMPONENTS

Sources

An inert solid is electrically heated to a temperature in the range 1500-2000 K. The heated material will then emit infra red radiation.

The Nernst glower is a cylinder (1-2 mm diameter, approximately 20 mm long) of rare earth oxides. Platinum wires are sealed to the ends, and a current passed through the cylinder. The Nernst glower can reach temperatures of 2200 K.

The Globar source is a silicon carbide rod (5mm diameter, 50mm long) which is electrically heated to about 1500 K. Water cooling of the electrical contacts is needed to prevent arcing. The spectral output is comparable with the Nernst glower, execept at short wavelengths (less than 5 mm) where it's output becomes larger.

The incandescent wire source is a tightly wound coil of nichrome wire, electrically heated to 1100 K. It produces a lower intensity of radiation than the Nernst or Globar sources, but has a longer working life.

Detectors

There are three catagories of detector;

- Thermal
- Pyroelectric
- Photoconducting

Thermocouples consist of a pair of junctions of different metals; for example, two pieces of bismuth fused to either end of a piece of antimony. The potential difference (voltage) between the junctions changes according to the difference in temperature between the junctions

Pyroelectric detectors are made from a single crystalline wafer of a pyroelectric material, such as triglycerine sulphate. The properties of a pyroelectric material are such that when an electric field is applied across it, electric polarisation occurs (this happens in any dielectric material). In a pyroelectric material, when the field is removed, the polarisation persists. The degree of polarisation is temperature dependant. So, by sandwiching the pyroelectric material between two electrodes, a temperature dependant capacitor is made. The heating effect of incident IR radiation causes a change in the capacitance of the material. Pyroelectric detectors have a fast response time. They are used in most Fourier transform IR instruments.

Photoelectric detectors such as the mercury cadmium telluride detector comprise a film of semiconducting material deposited on a glass surface, sealed in an evacuated envelope. Absorption of IR promotes nonconducting valence electrons to a higher, conducting, state. The electrical resistance of the semiconductor decreases. These detectors have better response characteristics than pyroelectric detectors and are used in FT-IR instruments - particularly in GC - FT-IR.

Types of instrument

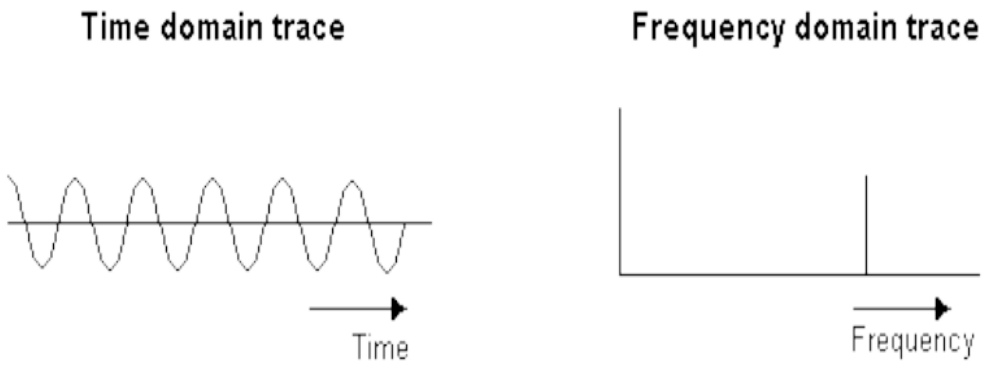
Dispersive infra red spectophotometers

These are often double-beam recording instruments, employing diffraction gratings for dispersion of radiation.

Radiation from the source is flicked between the reference and sample paths. Often, an *optical null* system is used. This is when the detector only responds if the intensity of the two beams is unequal. If the intensities are unequal, a light attenuator restores equality by moving in or out of the reference beam. The recording pen is attached to this attenuator.

Fourier-transform spectrometers

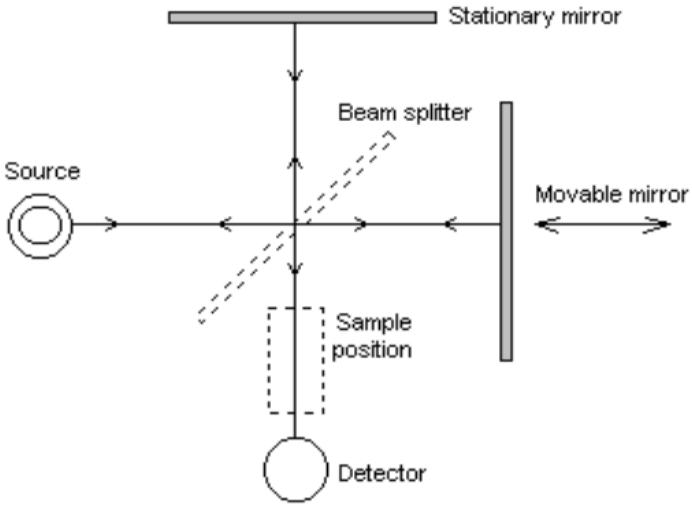
Any waveform can be shown in one of two ways; either in *frequency domain* or *time domain*.



Dispersive IR instruments operate in the frequency domain. There are, however, advantages to be gained from measurement in the time domain followed by computer transformation into the frequency domain.

If we wished to record a trace in the time domain, it could be possible to do so by allowing radiation to fall on a detector and recording its response over time. In practice, no detector can respond quickly enough (the radiation has a frequency greater than 10^{14} Hz). This problem can be solved by using interference to modulate the i.r. signal at a detectable frequency. The *Michelson interferometer* is used to produce a new signal of a much lower frequency which contains the same information as the original IR signal. The output from the interferometer is an *interferogram*.

The Michelson interferometer



Radiation leaves the source and is split. Half is reflected to a stationary mirror and then back to the splitter. This radiation has travelled a fixed distance. The other half of the radiation from the source passes through the splitter and is reflected back by a **movable** mirror. Therefore, the path length of this beam is variable. The two reflected beams recombine at the splitter, and they interfere (e.g. for any one wavelength, interference will be constructive if the difference in path lengths is an exact multiple of the wavelength. If the difference in path lengths is half the wavelength then destructive interference will result). If the movable mirror moves away from the beam splitter at a constant speed, radiation reaching the detector goes through a steady sequence of maxima and minima as the interference alternates between constructive and destructive phases.

If monochromatic IR radiation of frequency, $f\ (\ ir\ )$ enters the interferometer, then the output frequency, f_m can be found by;

$$f_m = \frac{v}{1.5 \times 10^{11}} \times f(ir)$$

where v is the speed of mirror travel in mm/s

Because **all** wavelengths emitted by the source are present, the interferogram is extremely complicated.

The moving mirror must travel smoothly; a frictionless bearing is used with electromagnetic drive. The position of the mirror is measured by a laser shining on a corner of the mirror. A simple sine wave interference paatern is produced. Each peak indicates mirror travel of one half the wavelength of the laser. The accuracy of this measurement system means that the IR frequency scale is accurate and precise.

In the FT-IR instrument, the sample is placed between the output of the interferometer and the detector. The sample absorbs radiation of particular wavelengths. Therefore, **the interferogram contains the spectrum of the source minus the spectrum of the sample**. An interferogram of a reference (sample cell and solvent) is needed to obtain the spectrum of the sample.

After an interferogram has been collected, a computer performs a *Fast Fourier Transform*, which results in a frequency domain trace (i.e intensity vs. wavenumber) that we all know and love.

The detector used in an FT-IR instrument must respond quickly because intensity changes are rapid (the moving mirror moves quickly). Pyroelectric detectors or liquid nitrogen cooled photon detectors must be used. Thermal detectors are too slow.

To acheive a good signal to noise ratio, many interferograms are obtained and then averaged. This can be done in less time than it would take a dipersive instrument to record one scan.

Advantages of Fourier transform IR over dispersive IR;

- Improved frequency resolution
- Improved frequency reproducibility (older dispersive instruments must be recalibrated for each session of use)
- Higher energy throughput
- Faster operation
- Computer based (allowing storage of spectra and facilities for processing spectra)
- Easily adapted for remote use (such as diverting the beam to pass through an external cell and detector, as in GC - FT-IR)

Uses and applications

Infrared spectroscopy is a simple and reliable technique widely used in both organic and inorganic chemistry, in research and industry. It is used in quality control, dynamic measurement, and monitoring applications such as the long-term unattended measurement of CO_2 concentrations in greenhouses and growth chambers by infrared gas analyzers.

It is also used in forensic analysis in both criminal and civil cases, for example in identifying polymer degradation. It can be used in determining the blood alcohol content of a suspected drunk driver.

IR-spectroscopy has been successfully used in analysis and identification of pigments in paintings[5] and other art objects[6] such as illuminated manuscripts.

A useful way of analysing solid samples without the need for cutting samples uses ATR or attenuated total reflectance spectroscopy. Using this approach, samples are pressed against the face of a single crystal. The infrared radiation passes through the crystal and only interacts with the sample at the interface between the two materials.

With increasing technology in computer filtering and manipulation of the results, samples in solution can now be measured accurately (water produces a broad absorbance across the range of

interest, and thus renders the spectra unreadable without this computer treatment).Some instruments will also automatically tell you what substance is being measured from a store of thousands of reference spectra held in storage.

Infrared spectroscopy is also useful in measuring the degree of polymerization in polymer manufacture. Changes in the character or quantity of a particular bond are assessed by measuring at a specific frequency over time. Modern research instruments can take infrared measurements across the range of interest as frequently as 32 times a second. This can be done whilst simultaneous measurements are made using other techniques. This makes the observations of chemical reactions and processes quicker and more accurate.

Infrared spectroscopy has also been successfully utilized in the field of semiconductor microelectronics:[8] for example, infrared spectroscopy can be applied to semiconductors like silicon, gallium arsenide, gallium nitride, zinc selenide, amorphous silicon, silicon nitride, etc.

The instruments are now small, and can be transported, even for use in field trials.In February 2014, NASA announced a greatly upgraded database, based on IR spectroscopy, for tracking polycyclic aromatic hydrocarbons (PAHs) in theuniverse. According to scientists, more than 20% of the carbon in the universe may be associated with PAHs, possible starting materials for the formation of life. PAHs seem to have been formed shortly after the Big Bang, are widespread throughout the universe, and are associated with new stars and exoplanets.

FLUOROSCENCE SPECTROSCOPY

Fluorescence spectroscopy (also known as fluorometry or spectrofluorometry) is a type of electromagnetic spectroscopy which analyzes fluorescence from a sample. It involves using a beam of light, usually ultraviolet light, that excites the electrons in molecules of certain compounds and causes them to emit light; typically, but not necessarily, visible light. A complementary technique is absorption spectroscopy. In the special case of single molecule fluorescence spectroscopy, intensity fluctuations from the emitted light are measured from either single fluorophores, or pairs of fluorophores.

Devices that measure fluorescence are called fluorometers

Theory

Molecules have various states referred to as energy levels. Fluorescence spectroscopy is primarily concerned with electronic and vibrational states. Generally, the species being examined has a ground electronic state (a low energy state) of interest, and an excited electronic state of higher energy. Within each of these electronic states are various vibrational states.[1]

In fluorescence , the species is first excited, by absorbing a photon, from its ground electronic state to one of the various vibrational states in the excited electronic state. Collisions with other molecules cause the excited molecule to lose vibrational energy until it reaches the lowest vibrational state of the excited electronic state. This process is often visualized with a Jablonski diagram.

The molecule then drops down to one of the various vibrational levels of the ground electronic state again, emitting a photon in the process.[1] As molecules may drop down into any of several vibrational levels in the ground state, the emitted photons will have different energies, and thus frequencies. Therefore, by analysing the different frequencies of light emitted in fluorescent spectroscopy, along with their relative intensities, the structure of the different vibrational levels can be determined.

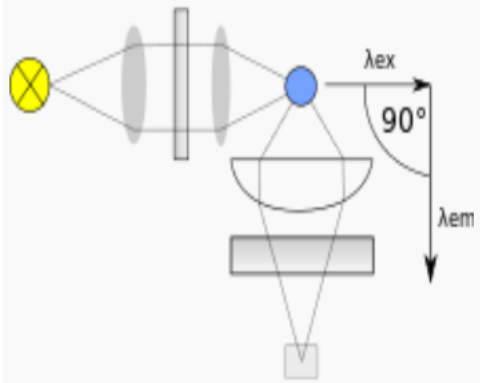
For atomic species, the process is similar; however, since atomic species do not have vibrational energy levels, the emitted photons are often at the same wavelength as the incident radiation. This process of re-emitting the absorbed photon is "resonance fluorescence" and while it is characteristic of atomic fluorescence, is seen in molecular fluorescence as well.[2]

In a typical fluorescence (emission) measurement, the excitation wavelength is fixed and the detection wavelength varies, while in a fluorescence excitation measurement the detection wavelength is fixed and the excitation wavelength is varied across a region of interest. An **emission map** is measured by recording the emission spectra resulting from a range of excitation wavelengths and combining them all together. This is a three dimensional surface data set: emission intensity as a function of excitation and emission wavelengths, and is typically depicted as a contour map.

Instrumentation

Two general types of instruments exist: filter fluorometers that use filters to isolate the incident light and fluorescent light and spectrofluorometers that use adiffraction grating monochromators to isolate the incident light and fluorescent light.

Both types use the following scheme: the light from an excitation source passes through a filter or monochromator, and strikes the sample. A proportion of the incident light is absorbed by the sample, and some of the molecules in the sample fluoresce. The fluorescent light is emitted in all directions. Some of this fluorescent light passes through a second filter or monochromator and reaches a detector, which is usually placed at 90° to the incident light beam to minimize the risk of transmitted or reflected incident light reaching the detector.



A simplistic design of the components of a fluorimeter

Various light sources may be used as excitation sources, including lasers, LED, and lamps; xenon arcs and mercury-vapor lamps in particular. A laser only emits light of high irradiance at a very narrow wavelength interval, typically under 0.01 nm, which makes an excitation monochromator or filter unnecessary. The disadvantage of this method is that the wavelength of a laser cannot be changed by much. A mercury vapor lamp is a line lamp, meaning it emits light near peak wavelengths. By contrast, a xenon arc has a continuous emission spectrum with nearly constant intensity in the range from 300-800 nm and a sufficient irradiance for measurements down to just above 200 nm.

Filters and/or monochromators may be used in fluorimeters. A monochromator transmits light of an adjustable wavelength with an adjustable tolerance. The most common type of monochromator utilizes a diffraction grating, that is, collimated light illuminates a grating and exits with a different angle depending on the wavelength. The monochromator can then be adjusted to select which wavelengths to transmit. For allowing anisotropy measurements the addition of two polarization filters are necessary: One after the excitation monochromator or filter, and one before the emission monochromator or filter.

As mentioned before, the fluorescence is most often measured at a 90° angle relative to the excitation light. This geometry is used instead of placing the sensor at the line of the excitation light at a 180° angle in order to avoid interference of the transmitted excitation light. No monochromator is perfect and it will transmit somestray light, that is, light with other wavelengths than the targeted. An ideal monochromator would only transmit light in the specified range and have a high wavelength-independent transmission. When measuring at a 90° angle, only the light scattered by the sample causes stray light. This results in a better signal-to-noise ratio, and lowers the detection limit by approximately a factor 10000,[3] when compared to the 180° geometry. Furthermore, the fluorescence can also be measured from the front, which is often done for turbid or opaque samples .[4]

The detector can either be single-channeled or multichanneled. The single-channeled detector can only detect the intensity of one wavelength at a time, while the multichanneled detects the intensity of all wavelengths simultaneously, making the emission monochromator or filter unnecessary. The different types of detectors have both advantages and disadvantages.

The most versatile fluorimeters with dual monochromators and a continuous excitation light source can record both an excitation spectrum and a fluorescence spectrum. When measuring fluorescence spectra, the wavelength of the excitation light is kept constant, preferably at a wavelength of high absorption, and the emission monochromator scans the spectrum. For measuring excitation spectra, the wavelength passing though the emission filter or monochromator is kept constant and the excitation monochromator is scanning. The excitation spectrum generally is identical to the absorption spectrum as the fluorescence intensity is proportional to the absorption.[5]

Analysis of data

At low concentrations the fluorescence intensity will generally be proportional to the concentration of the fluorophore.

Unlike in UV/visible spectroscopy, 'standard', device independent spectra are not easily attained. Several factors influence and distort the spectra, and corrections are necessary to attain 'true', i.e. machine-independent, spectra. The different types of distortions will here be classified as being either instrument- or sample-related. Firstly, the distortion arising from the instrument is discussed. As a start, the light source intensity and wavelength characteristics varies over time during each experiment and between each experiment. Furthermore, no lamp has a constant intensity at all wavelengths. To correct this, a beam splitter can be applied after the excitation monochromator or filter to direct a portion of the light to a reference detector.

Additionally, the transmission efficiency of monochromators and filters must be taken into account. These may also change over time. The transmission efficiency of the monochromator also varies depending on wavelength. This is the reason that an optional reference detector should be placed after the excitation monochromator or filter. The percentage of the fluorescence picked up by the detector is also dependent upon the system. Furthermore, the detector quantum efficiency, that is, the percentage of photons detected, varies between different detectors, with wavelength and with time, as the detector inevitably deteriorates.

Two other topics that must be considered include the optics used to direct the radiation and the means of holding or containing the sample material (called a cuvette or cell). For most UV, visible, and NIR measurements the use of precision quartz cuvettes is necessary. In both cases, it is important to select materials that have relatively little absorption in the wavelength range of interest. Quartz is ideal because it transmits from 200 nm-2500 nm; higher grade quartz can even transmit up to 3500 nm, whereas the absorption properties of other materials can mask the fluorescence from the sample.

Correction of all these instrumental factors for getting a 'standard' spectrum is a tedious process, which is only applied in practice when it is strictly necessary. This is the case when measuring the quantum yield or when finding the wavelength with the highest emission intensity for instance.

As mentioned earlier, distortions arise from the sample as well. Therefore some aspects of the sample must be taken into account too. Firstly, photodecomposition may decrease the intensity of fluorescence over time. Scattering of light must also be taken into account. The most significant types of scattering in this context are Rayleigh and Raman scattering. Light scattered by Rayleigh scattering has the same wavelength as the incident light, whereas in Raman scattering the scattered light changes wavelength usually to longer wavelengths. Raman scattering is the result of a virtual electronic state induced by the excitation light. From this virtual state, the molecules may relax back to a vibrational level other than the vibrational ground state.[6] In fluorescence spectra, it is always seen at a constant wavenumber difference relative to the excitation wavenumber e.g. the peak appears at a wavenumber 3600 cm^{-1} lower than the excitation light in water.

Other aspects to consider are the inner filter effects. These include reabsorption. Reabsorption happens because another molecule or part of a macromolecule absorbs at the wavelengths at which the fluorophore emits radiation. If this is the case, some or all of the photons emitted by the fluorophore may be absorbed again. Another inner filter effect occurs because of high concentrations of absorbing molecules, including the fluorophore. The result is that the intensity

of the excitation light is not constant throughout the solution. Resultingly, only a small percentage of the excitation light reaches the fluorophores that are visible for the detection system. The inner filter effects change the spectrum and intensity of the emitted light and they must therefore be considered when analysing the emission spectrum of fluorescent light.[5][7]

Tryptophan fluorescence

The fluorescence of a folded protein is a mixture of the fluorescence from individual aromatic residues. Most of the intrinsic fluorescence emissions of a folded protein are due to excitation of tryptophan residues, with some emissions due to tyrosine and phenylalanine; but disulfide bonds also have appreciable absorption in this wavelength range. Typically, tryptophan has a wavelength of maximum absorption of 280 nm and an emission peak that is solvatochromic, ranging from ca. 300 to 350 nm depending in the polarity of the local environment [8] Hence, protein fluorescence may be used as a diagnostic of the conformational state of a protein.[9]Furthermore, tryptophan fluorescence is strongly influenced by the proximity of other residues (*i.e.*, nearby *protonated* groups such as Asp or Glu can causequenching of Trp fluorescence). Also, energy transfer between tryptophan and the other fluorescent amino acids is possible, which would affect the analysis, especially in cases where the Förster acidic approach is taken. In addition, tryptophan is a relatively rare amino acid; many proteins contain only one or a few tryptophan residues. Therefore, tryptophan fluorescence can be a very sensitive measurement of the conformational state of individual tryptophan residues. The advantage compared to extrinsic probes is that the protein itself is not changed. The use of intrinsic fluorescence for the study of protein conformation is in practice limited to cases with few (or perhaps only one) tryptophan residues, since each experiences a different local environment, which gives rise to different emission spectra.

Tryptophan is an important intrinsic fluorescent probe (amino acid), which can be used to estimate the nature of microenvironment of the tryptophan. When performing experiments with denaturants, surfactants or other amphiphilic molecules, the microenvironment of the tryptophan might change. For example, if a protein containing a single tryptophan in its 'hydrophobic' core is denatured with increasing temperature, a red-shifted emission spectrum will appear. This is due to the exposure of the tryptophan to an aqueous environment as opposed to a hydrophobic protein interior. In contrast, the addition of a surfactant to a protein which contains a tryptophan which is exposed to the aqueous solvent will cause a blue-shifted emission spectrum if the tryptophan is embedded in the surfactant vesicle ormicelle.[10] Proteins that lack tryptophan may be coupled to a fluorophore.

With fluorescence excitation at 295 nm, the tryptophan emission spectrum is dominant over the weaker tyrosine and phenylalanine fluorescence.

Applications

Fluorescence spectroscopy is used in, among others, biochemical, medical, and chemical research fields for analyzing organic compounds. There has also been a report of its use in differentiating malignant skin tumors from benign.

Atomic Fluorescence Spectroscopy (AFS) techniques are useful in other kinds of analysis/measurement of a compound present in air or water, or other media, such as CVAFS which is used for heavy metals detection, such as mercury.

Fluorescence can also be used to redirect photons, see fluorescent solar collector.

Additionally, Fluorescence spectroscopy can be adapted to the microscopic level using microfluorimetry In analytical chemistry, fluorescence detectors are used with HPLC.

ATOMIC ABSORPTION SPECTROPHOTOMETRY

Atomic absorption spectroscopy (AAS) is a spectroanalytical procedure for the quantitative determination of chemical elements using the absorption of optical radiation (light) by free atoms in the gaseous state.

In analytical chemistry the technique is used for determining the concentration of a particular element (the analyte) in a sample to be analyzed. AAS can be used to determine over 70 different elements in solution or directly in solid samples used in pharmacology, biophysics and toxicology research.

Atomic absorption spectroscopy was first used as an analytical technique, and the underlying principles were established in the second half of the 19th century by Robert Wilhelm Bunsen and Gustav Robert Kirchhoff, both professors at the University of Heidelberg, Germany.

The modern form of AAS was largely developed during the 1950s by a team of Australian chemists. They were led by Sir Alan Walsh at the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Division of Chemical Physics, inMelbourne, Australia.

Atomic absorption spectrometry has many uses in different areas of chemistry such as:

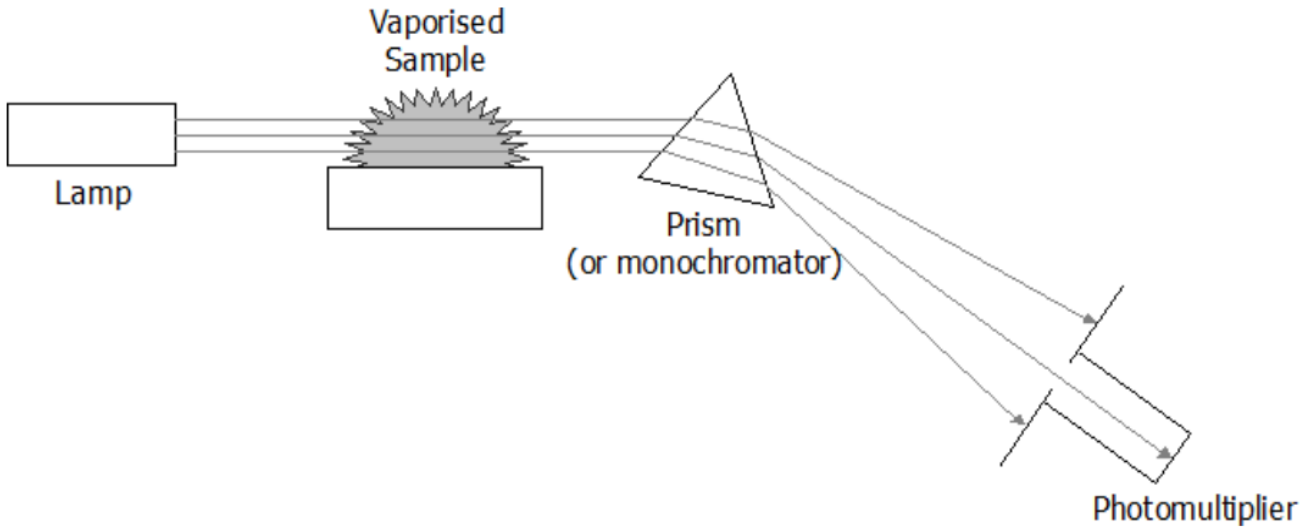
- Clinical analysis: Analyzing metals in biological fluids and tissues such as whole blood, plasma, urine, saliva, brain tissue, liver, muscle tissue, semen
- Pharmaceuticals: In some pharmaceutical manufacturing processes, minute quantities of a catalyst that remain in the final drug product
- Water analysis: Analyzing water for its metal content.

Principles

The technique makes use of absorption spectrometry to assess the concentration of an analyte in a sample. It requires standards with known analyte content to establish the relation between the measured absorbance and the analyte concentration and relies therefore on the Beer-Lambert Law.

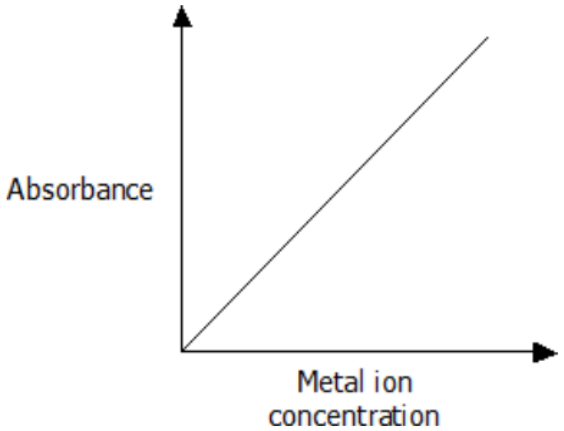
In short, the electrons of the atoms in the atomizer can be promoted to higher orbitals (excited state) for a short period of time (nanoseconds) by absorbing a defined quantity of energy (radiation of a given wavelength). This amount of energy, i.e., wavelength, is specific to a particular electron transition in a particular element. In general, each wavelength corresponds to only one element, and the width of an absorption line is only of the order of a few picometers (pm), which gives the technique its elemental selectivity. The radiation flux without a sample and with a sample in the atomizer is measured using a detector, and the ratio between the two values (the absorbance) is converted to analyte concentration or mass using the Beer-Lambert Law.

- **Atomic absorption spectroscopy (AAS)**: A technique used to identify the presence and concentration of substances by analysing the spectrum produced when a substance is vaporised and absorbs certain frequencies of light.
- AAS is used particularly for detecting the concentrations of metal ions in solutions.
- AAS is performed using an atomic absorption spectrometer.



Schematic diagram of an atomic absorption spectrometer

- To determine the concentration of a certain metal ion in a sample, the following steps occur within an atomic absorption spectrometer:
  - A hollow cathode lamp, with the cathode made of the metal to be tested for, emits light of a certain frequency.
  - The light produced by the lamp is passed through the sample to be tested vaporised in a flame.
  - The degree of light absorption is proportional to the concentration of the metal in the sample.
  - The intensity of the light that passes through the flame is measured by a photomultiplier tube.
  - By comparing the intensity with that produced from a control sample containing none of the metal ions being tested for, the degree of absorption, or absorbance, can be determined.
  - The absorbance is then compared to that of a series of diluted standard solutions in order to determine the concentration.
  - This involves the use of a calibration graph.

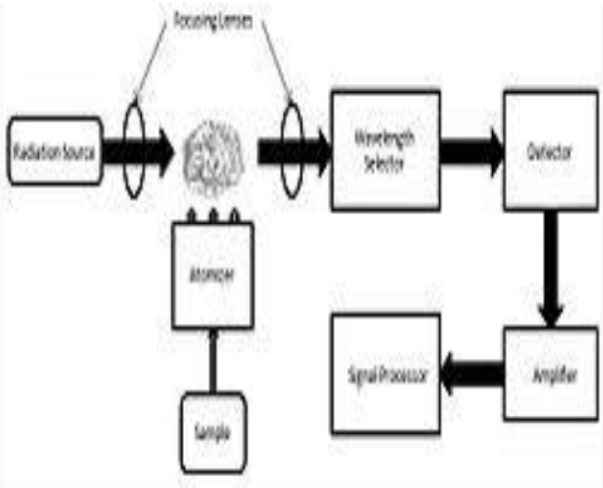


General layout of a calibration graph

- The standard solutions should produce a straight-line graph.
- The absorbance recorded for the sample being tested can be matched with a concentration using the graph.

- **Trace element**: Also known as a micronutrient, an element required in minute amounts for normal growth of organisms.
- Trace elements work in organisms by helping enzymes to function.
- The concentration of trace elements in animals and plants is normally in the range of 1 to 100 parts per million.
- Before the development of AAS in the 1950s, commonly used analytical methods were not sufficiently sensitive to detect the low concentrations of these elements, and their presence went unnoticed.
- When scientist began to use AAS on organisms and soils, the existence of these trace elements were first recognised.
- AAS has also been used to help demonstrate both the necessity and function of these elements.
- Thus, AAS has had a great impact on scientific understanding of the effects of trace elements.
- In the case of the ill health of an organism, AAS can be used to detect whether required trace elements are present in sufficient quantities in the organism and its environment.
- If a trace element deficiency is observed, then it can be rectified by providing the organism with that particular nutrient.
- This is especially useful in the field of agriculture, where specific practical applications of AAS have included:
  - The discovery of a cobalt deficiency in seemingly good pastureland in coastal southwestern Australia where animal health could not be maintained.
  - The discovery of a molybdenum deficiency in the soils of arid parts of Victoria where legume crops could not be supported.

Instrumentation



Atomic absorption spectrometer block diagram

In order to analyze a sample for its atomic constituents, it has to be atomized. The atomizers most commonly used nowadays are flames and electrothermal (graphite tube) atomizers. The atoms should then be irradiated by optical radiation, and the radiation source could be an element-specific line radiation source or a continuum radiation source. The radiation then passes through amonochromator in order to separate the element-specific radiation from any other radiation emitted by the radiation source, which is finally measured by a detector.

Atomizers

The atomizers most commonly used nowadays are (spectroscopic) flames and electrothermal (graphite tube) atomizers. Other atomizers, such as glow-discharge atomization, hydride atomization, or cold-vapor atomization might be used for special purposes.

Flame atomizers

The oldest and most commonly used atomizers in AAS are flames, principally the air-acetylene flame with a temperature of about 2300 °C and the nitrous oxide system (N_2O)-acetylene flame with a temperature of about 2700 °C. The latter flame, in addition, offers a more reducing environment, being ideally suited for analytes with high affinity to oxygen.

A laboratory flame photometer that uses a propane operated flame atomizer

Liquid or dissolved samples are typically used with flame atomizers. The sample solution is aspirated by a pneumatic analytical nebulizer, transformed into an aerosol, which is introduced into a spray chamber, where it is mixed with the flame gases and conditioned in a way that only the finest aerosol droplets (< 10 µm) enter the flame. This conditioning process is responsible that only about 5% of the aspirated sample solution reaches the flame, but it also guarantees a relatively high freedom from interference.

On top of the spray chamber is a burner head that produces a flame that is laterally long (usually 5–10 cm) and only a few mm deep. The radiation beam passes through this flame at its longest axis, and the flame gas flow-rates may be adjusted to produce the highest concentration of free atoms. The burner height may also be adjusted, so that the radiation beam passes through the zone of highest atom cloud density in the flame, resulting in the highest sensitivity.

The processes in a flame include the following stages:

- **Desolvation** (drying) – the solvent is evaporated and the dry sample nano-particles remain;

- **Vaporization** (transfer to the gaseous phase) – the solid particles are converted into gaseous molecules;
- **Atomization** – the molecules are dissociated into free atoms;
- **Ionization** – depending on the ionization potential of the analyte atoms and the energy available in a particular flame, atoms might be in part converted to gaseous ions.

Each of these stages includes the risk of interference in case the degree of phase transfer is different for the analyte in the calibration standard and in the sample. Ionization is generally undesirable, as it reduces the number of atoms that are available for measurement, i.e., the sensitivity.

In flame AAS a steady-state signal is generated during the time period when the sample is aspirated. This technique is typically used for determinations in the mg L^{-1} range, and may be extended down to a few µg L^{-1} for some elements.

Electrothermal atomizers

Elements in Graphite-Furnace-AAS - Overview

Element	Prefered Tube Type	Modifier	Pyrolysis Temperature	Pyrolysis T. w/o modifier	Atomisation Temperature	Remarks
Ag	platform	Pd/Mg(NO$_3$)$_2$	800	250	1700	Absorption effects at various materials possible, complexing reagents might be used
Al	platform	Mg(NO$_3$)$_2$	1200	1000	2400	danger of contamination via flask materials or lab environment
As	platform	Pd/Mg(NO$_3$)$_2$	1100	300	2100	
Au	platform	Pd/Mg(NO$_3$)$_2$	800	250	1700	Absorption effects at various materials possible, complexing reagents might be used
B	wall	Ca(NO$_3$)$_2$	1200	1000	2500	
Ba	wall	Ca(NO$_3$)$_2$	1200	1000	2500	
Be	platform	Mg(NO$_3$)$_2$	1200	1000	2300	
Bi	platform	Pd/Mg(NO$_3$)$_2$	1100	400	1900	
Ca	wall		1100	1000	2500	High risk of contamination
Cd	platform	NH4H2PO$_4$	700	250	1400	
		Pd/Mg(NO3)2	800	250	1700	Supports evaporation of chlorides
Co	platform	Mg(NO$_3$)$_2$	1200	1000	2200	
Cr	platform	Mg(NO$_3$)$_2$	1400	1000	2300	
Cu	platform	Pd/Mg(NO$_3$)$_2$	1200	900	2100	
Fe	platform	Mg(NO$_3$)$_2$	1200	900	2100	
K	platform		900		1500	High risk of contamination
Mn	platform	Pd/Mg(NO$_3$)$_2$	1200	800	2000	
Mo	wall		1200	1000	2450	
Na	platform			850	1500	High risk of contamination
Ni	platform	Mg(NO$_3$)$_2$	1200	1000	2300	
Pb	platform	NH4H2PO4	800	300	1700	
		Pd/Mg(NO3)2	900	300	1900	Supports evaporation of chlorides
Sb	platform	Pd/Mg(NO$_3$)$_2$	1200	300	2100	
Se	platform	Pd/Mg(NO$_3$)$_2$	1200	300	2100	
Si	platform	Pd/Mg(NO$_3$)$_2$	1200	1000	2400	
Sn	platform	Pd/Mg(NO$_3$)$_2$	1200	700	2300	
Te	platform	Pd/Mg(NO$_3$)$_2$	1000	300	2000	
Ti	wall		1300	1200	2500	
Tl	platform	Pd/Mg(NO$_3$)$_2$	700	300	1700	evaporates in chloric matrices at 200°C
V	wall		1300	1200	2500	

high volatility
low volatility
med. volatility

GFAA method development

Graphite tube

Electrothermal AAS (ET AAS) using graphite tube atomizers was pioneered by Boris V. L'vov at the Saint Petersburg Polytechnical Institute, Russia,[4] since the late 1950s, and investigated in parallel by Hans Massmann at the Institute of Spectrochemistry and Applied Spectroscopy (ISAS) in Dortmund, Germany.[5]

Although a wide variety of graphite tube designs have been used over the years, the dimensions nowadays are typically 20–25 mm in length and 5–6 mm inner diameter. With this technique liquid/dissolved, solid and gaseous samples may be analyzed directly. A measured volume (typically 10–50 µL) or a weighed mass (typically around 1 mg) of a solid sample are introduced into the graphite tube and subject to a temperature program. This typically consists of stages, such as:

- Drying – the solvent is evaporated
- Pyrolysis – the majority of the matrix constituents are removed
- Atomization – the analyte element is released to the gaseous phase
- Cleaning – eventual residues in the graphite tube are removed at high temperature.

The graphite tubes are heated via their ohmic resistance using a low-voltage high-current power supply; the temperature in the individual stages can be controlled very closely, and temperature ramps between the individual stages facilitate separation of sample components. Tubes may be heated transversely or longitudinally, where the former ones have the advantage of a more homogeneous temperature distribution over their length. The so-called stabilized temperature platform furnace (STPF) concept, proposed by Walter Slavin, based on research of Boris L'vov, makes ET AAS essentially free from interference.[*citation needed*] The major components of this concept are:

- Atomization of the sample from a graphite platform inserted into the graphite tube (L'vov platform) instead of from the tube wall in order to delay atomization until the gas phase in the atomizer has reached a stable temperature;
- Use of a chemical modifier in order to stabilize the analyte to a pyrolysis temperature that is sufficient to remove the majority of the matrix components;
- Integration of the absorbance over the time of the transient absorption signal instead of using peak height absorbance for quantification.

In ET AAS a transient signal is generated, the area of which is directly proportional to the mass of analyte (not its concentration) introduced into the graphite tube. This technique has the advantage that any kind of sample, solid, liquid or gaseous, can be analyzed directly. Its sensitivity is 2–3 orders of magnitude higher than that of flame AAS, so that determinations in the low μg L^{-1} range (for a typical sample volume of 20 μL) and ng g^{-1} range (for a typical sample mass of 1 mg) can be carried out. It shows a very high degree of freedom from interferences, so that ET AAS might be considered the most robust technique available nowadays for the determination of trace elements in complex matrices.[*citation needed*]

Specialized atomization techniques

While flame and electrothermal vaporizers are the most common atomization techniques, several other atomization methods are utilized for specialized use.

Glow-discharge atomization

A glow-discharge device(GD) serves as a versatile source, as it can simultaneously introduce and atomize the sample. Theglow discharge occurs in a low-pressure argon gas atmosphere between 1 and 10 torr. In this atmosphere lies a pair of electrodes applying a DC voltage of 250 to 1000 V to break down the argon gas into positively charged ions and electrons. These ions, under the influence of the electric field, are accelerated into the cathode surface containing the sample, bombarding the sample and causing neutral sample atom ejection through the process known as sputtering. The atomic vapor produced by this discharge is composed of ions, ground state atoms, and fraction of excited atoms. When the excited atoms relax back into their ground state, a low-intensity glow is emitted, giving the technique its name.

The requirement for samples of glow discharge atomizers is that they are electrical conductors. Consequently, atomizers are most commonly used in the analysis of metals and other conducting samples. However, with proper modifications, it can be utilized to analyze liquid samples as well as nonconducting materials by mixing them with a conductor (e.g. graphite).

Hydride atomization

Hydride generation techniques are specialized in solutions of specific elements. The technique provides a means of introducing samples containing arsenic, antimony, tin, selenium, bismuth, and lead into an atomizer in the gas phase. With these elements, hydride atomization enhances detection limits by a factor of 10 to 100 compared to alternative methods. Hydride generation occurs by adding an acidified aqueous solution of the sample to a 1% aqueous solution of sodium borohydride, all of which is contained in a glass vessel. The volatile hydride generated by the reaction that occurs is swept into the atomization chamber by an inert gas, where it undergoes decomposition. This process forms an atomized form of the analyte, which can then be measured by absorption or emission spectrometry.

Cold-vapor atomization

The cold-vapor technique an atomization method limited to only the determination of mercury, due to it being the only metallic element to have a large enough vapor pressure at ambient temperature. Because of this, it has an important use in determining organic mercury compounds in samples and their distribution in the environment. The method initiates by converting mercury into Hg^{2+} by oxidation from nitric and sulfuric acids, followed by a reduction of Hg^{2+} with tin(II)

chloride. The mercury, is then swept into a long-pass absorption tube by bubbling a stream of inert gas through the reaction mixture. The concentration is determined by measuring the absorbance of this gas at 253.7 nm. Detection limits for this technique are in the parts-per-billion range making it an excellent mercury detection atomization method.

Radiation sources

We have to distinguish between line source AAS (LS AAS) and continuum source AAS (CS AAS). In classical LS AAS, as it has been proposed by Alan Walsh, the high spectral resolution required for AAS measurements is provided by the radiation source itself that emits the spectrum of the analyte in the form of lines that are narrower than the absorption lines. Continuum sources, such as deuterium lamps, are only used for background correction purposes. The advantage of this technique is that only a medium-resolution monochromator is necessary for measuring AAS; however, it has the disadvantage that usually a separate lamp is required for each element that has to be determined. In CS AAS, in contrast, a single lamp, emitting a continuum spectrum over the entire spectral range of interest is used for all elements. Obviously, a high-resolution monochromator is required for this technique, as will be discussed later.

Hollow cathode lamp (HCL)

Hollow cathode lamps

Hollow cathode lamps (HCL) are the most common radiation source in LS AAS. Inside the sealed lamp, filled with argon or neon gas at low pressure, is a cylindrical metal cathode containing the element of interest and an anode. A high voltage is applied across the anode and cathode, resulting in an ionization of the fill gas. The gas ions are accelerated towards the cathode and, upon impact on the cathode, sputter cathode material that is excited in the glow discharge to emit the radiation of the sputtered material, i.e., the element of interest. Most lamps will handle a handful of elements, i.e. 5-8. A typical machine will have two lamps, one will take care of five elements and the other will handle four elements for a total of nine elements analyzed.

Electrodeless discharge lamps

Electrodeless discharge lamps (EDL) contain a small quantity of the analyte as a metal or a salt in a quartz bulb together with an inert gas, typically argon, at low pressure. The bulb is inserted into a coil that is generating an electromagnetic radio frequency field, resulting in a low-pressure inductively coupled discharge in the lamp. The emission from an EDL is higher than that from an HCL, and the line width is generally narrower, but EDLs need a separate power supply and might need a longer time to stabilize.

Deuterium lamps

Deuterium HCL or even hydrogen HCL and deuterium discharge lamps are used in LS AAS for background correction purposes. The radiation intensity emitted by these lamps decreases significantly with increasing wavelength, so that they can be only used in the wavelength range between 190 and about 320 nm.

Xenon lamp as a continuous radiation source

Continuum sources

When a continuum radiation source is used for AAS, it is necessary to use a high-resolution monochromator, as will be discussed later. In addition, it is necessary that the lamp emits radiation of intensity at least an order of magnitude above that of a typical HCL over the entire wavelength range from 190 nm to 900 nm. A special high-pressure xenon short arc lamp, operating in a hot-spot mode has been developed to fulfill these requirements.