

# UV-VIS Spectrophotometry

## A Brief Background to Spectrophotometry

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### Introduction

The spectrophotometer is ubiquitous among modern laboratories. Ultraviolet (UV) and Visible (VIS) spectrophotometry has become the method of choice in most laboratories concerned with the identification and quantification of organic and inorganic compounds across a wide range of products and processes. Applied across research, quality, and manufacturing, with continuing focus on life science and pharmaceutical environments, they are equally as relevant in agriculture, animal husbandry and fishery, geological exploration, food safety, environmental monitoring, and many manufacturing industries to name a few.

Modern spectrophotometers are quick, accurate, and reliable. They require only small demands on the time and skills of the operator. However, the non-specialised end-user who wants to optimise the functions of their instrument, and be able to monitor its performance will benefit from the appreciation of the elementary physical laws governing spectrophotometry, as well as the basic elements of spectrophotometer design. This brief background to spectrophotometry offers an insight to support users of Biochrom's range of spectrophotometers.

### Electromagnetic Spectrum

The electromagnetic spectrum ranges from Gamma radiation, with the smallest wavelength (1 pm), to Low Frequency radiation, with the largest wavelength well beyond conventional radio waves (100 Mm or 100000 km). Human beings can only directly detect a very small portion of this spectrum, with thermal perception of radiant heat being a sensitivity to infrared (IR) radiation, and sight is limited to the VIS spectrum. The spectrum is smoothly continuous and the labelling and assignment of separate ranges are appointed largely as matter of convenience (Figure 1). UV-VIS spectrophotometry concerns the UV range covering of 200-380 nm and the VIS range covering 380-770 nm. Many instruments will offer slightly broader range from 190 nm in the UV region up to 1100 nm in the near infrared (NIR) region.

All electromagnetic radiation travels at the speed of light in a vacuum ( $c$ ), which equals  $3 \times 10^8$  m/s, the distance between two peaks along the line of travel is the wavelength, ( $\lambda$ ), and the number of peaks passing a point per unit time is the frequency ( $\nu$ ). The mathematical relationship between these three quantities is expressed using:

$$c = \lambda\nu$$

Additionally, the laws of quantum mechanics defines the energy of a single particle of light, a photon, as:

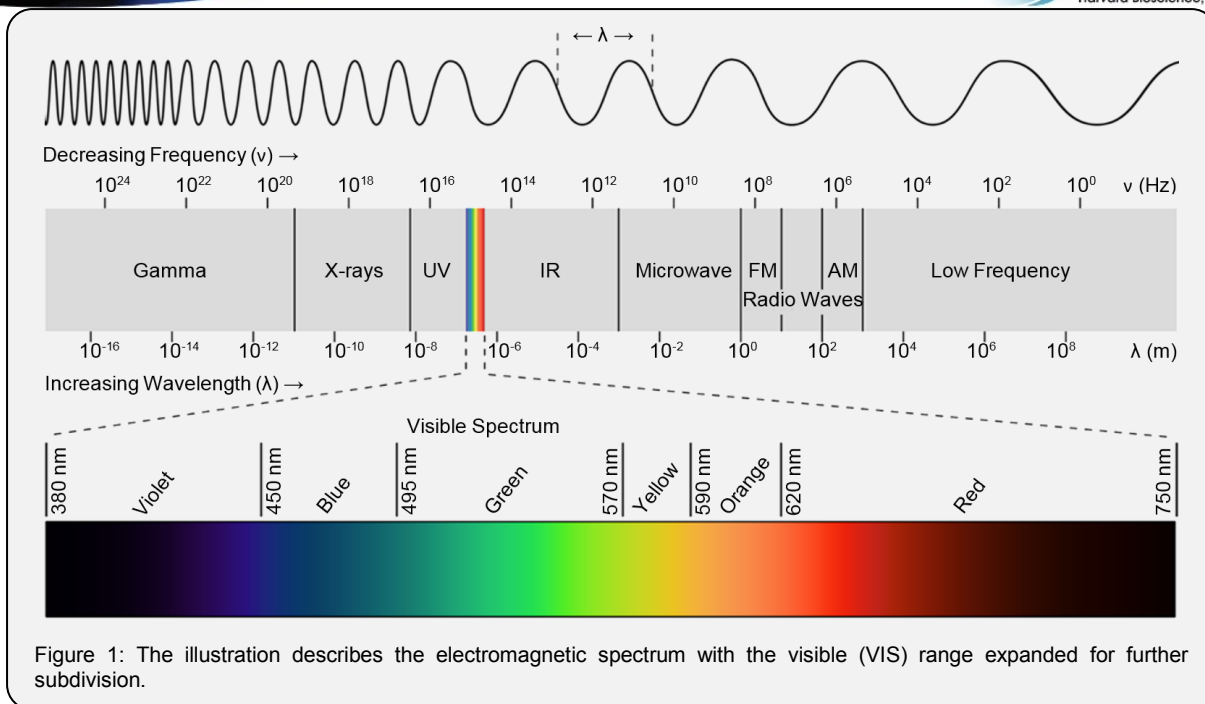
$$E = h\nu$$

Where  $E$  is the energy of the radiation, and  $h$  is Planck's constant. Combining these two equations gives:

$$E = hc/\lambda$$

Which shows that the energy is inversely proportional to wavelength. That is to say that the shorter the wavelength the higher the energy.

In the visible region it is convenient, and the modern convention, to define the wavelength in nm (nanometres), which is  $10^{-9}$  m. However,



historical literature may display alternative units, such as millimicron ( $m\mu$ ) or Angstrom ( $\text{\AA}$ ). These are simply converted using:

$$1 \text{ nm} = 1 \text{ m}\mu = 10 \text{ \AA}$$

## Radiation and the Atom

It is convenient to describe electromagnetic radiation as waves. However to clearly demonstrate the interactions that lead to specific absorption, it is helpful to consider the radiation as discrete packages of energy, or quanta, called photons.

The phenomena of absorbance depends upon the atomic structure, more specifically the atomic orbitals which each of the electrons of those atoms occupy and the associated energy levels of those orbitals. Occupied orbitals are finite and well defined, but an electron can be moved to a more energetic orbital, in a process called electron excitation, providing a quantum of energy equal to the energy difference between the ground and excited state is delivered. Excited states are generally unstable and the electron will rapidly revert to the ground state, in a process termed electron relaxation, losing the acquired energy, described as emission.

Whilst the accepted model of atomic and molecular structure has arisen from the Schrödinger wave mechanical treatment, it is convenient to employ the simpler Rutherford–

Bohr model to explain the electronic phenomena which concerns spectrophotometry.

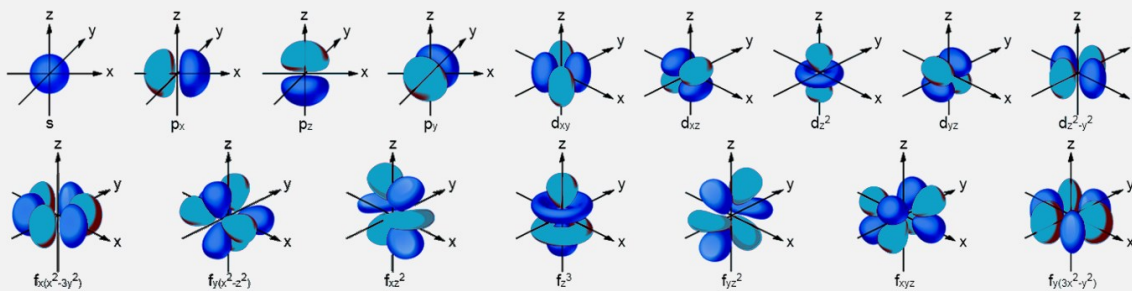
The Rutherford–Bohr model defines an atom as having a number of electron shells,  $n_1$ ,  $n_2$ ,  $n_3$  and so on, in which the increasing values of  $n$  represent higher energy levels and greater distance from the nucleus. Electrons orbit the nucleus in subshells, designated  $s$ ,  $p$ ,  $d$ , and  $f$ , within each shell. Each  $n$ -shell contains a configuration of  $s$ ,  $p$ ,  $d$ , and  $f$  subshells and each subshell can house a maximum of two electrons (Figure 2). No two electrons can have identical energies, but for succinctness they can be grouped related to the  $n$ -shell they occupy,  $1s$ ,  $2s$ ,  $2p$ ,  $3s$ ,  $3p$ ,  $3d$ , and so on.

By considering atoms of sodium vapour, the effect of subjecting an atom to an appropriate radiation can be demonstrated. Excitation of an electron, in the outermost subshell of a sodium atom, by a photon at 589, 330 or 285 nm will promote its transition to varying excited states; corresponding with the higher energy (shorter wavelength) of the radiation (Figure 3.).

## Radiation and the Molecule

### Electron Transitions

Electrons in the atom can be considered as occupying groups of similar energy levels. The more complicated molecular model shows bonding electrons associated with more than one



		Subshells			
		s	p	d	f
Shell	n1 (2)	1 (2)			
	n2 (8)	1 (2)	3 (6)		
	n3 (18)	1 (2)	3 (6)	5 (10)	
	n4 (32)	1 (2)	3 (6)	5 (10)	7 (14)

Figure 2: The illustrations above show the spatial geometries of atomic subshells. Each electron pair can occupy any space of the same colour, allowing for two pairs in each geometry except for the s subshell. They are shown on xyz axes and the specific nomenclature, based on their orientation about that axis, is shown below each illustration. Within larger atoms subsequent subshells envelope the equivalent subshell of the previous n-shell, but maintain the same geometry. The table left shows the subshell composition of the first four n-shells. The number in each cell defined the number of that given subshell at that n-shell level, and the numbers in brackets define the maximum number of electrons that can be accommodated in each shell and subshell.

nucleus, and are particularly susceptible to subshell transitions.

The electrons concerned, may be present in one of two chemical bond types; sigma ( $\sigma$ ) bonds which result from s-subshell overlap, or the generally weaker pi ( $\pi$ ) bond which results from p-subshell overlap.

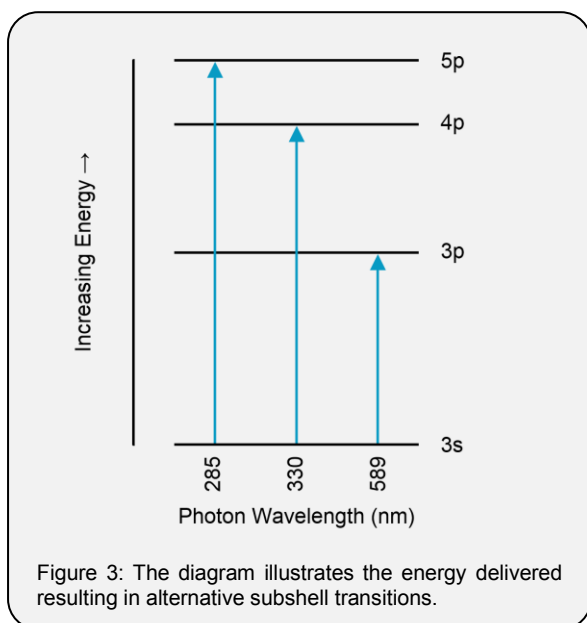


Figure 3: The diagram illustrates the energy delivered resulting in alternative subshell transitions.

Chemical bonds are formed by overlapping atomic shells that result in one of three types of Molecular Orbital (MO); bonding (low energy), antibonding (high energy), or non-bonding. Excitation is most typically associated with transitions induced in electrons involved in bonding MO's, and the atoms involved are usually those containing s and p occupied electrons. Excitation by UV-VIS radiation results in electron

transitions from bonding MO's to their relative antibonding MO's, and from non-bonding MO's to either antibonding MO's (Figure 4).

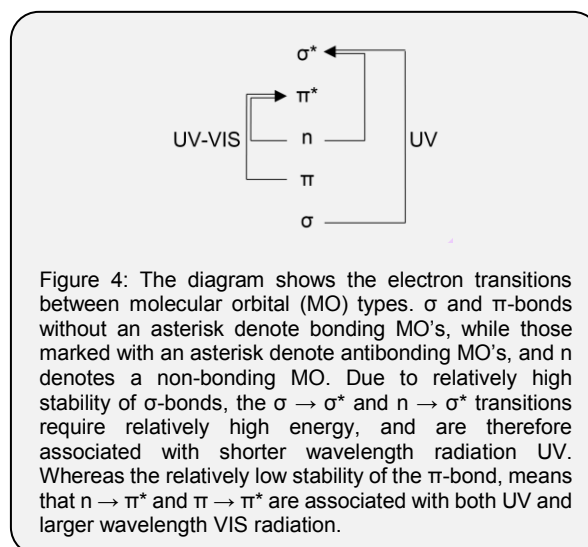


Figure 4: The diagram shows the electron transitions between molecular orbital (MO) types.  $\sigma$  and  $\pi$ -bonds without an asterisk denote bonding MO's, while those marked with an asterisk denote antibonding MO's, and n denotes a non-bonding MO. Due to relatively high stability of  $\sigma$ -bonds, the  $\sigma \rightarrow \sigma^*$  and  $n \rightarrow \sigma^*$  transitions require relatively high energy, and are therefore associated with shorter wavelength radiation UV. Whereas the relatively low stability of the  $\pi$ -bond, means that  $n \rightarrow \pi^*$  and  $\pi \rightarrow \pi^*$  are associated with both UV and larger wavelength VIS radiation.

The presence of a carbon-carbon double bond in the molecule increases the likelihood of  $\pi$ -bonds. Especially if they alternate with single bonds (conjugate double bonds), where one of the bonding MO's is raised in energy and the other lowered relative to the energy of an isolated double bond. The same applies to the antibonding MO's. The effect is greater still if the bond contains a highly electronegative atom, such as nitrogen, which attract electrons more strongly. As a result, the transition probability of molecules with  $\pi$ -bonds is enhanced, the wavelength of maximum excitation moves to a longer, less energetic, wavelength and often the likelihood of transitions to higher excitation states is increased.

The probability that transition will occur is closely related to MO structure. If the MO composition is known, the probability of transition can be calculated with relative certainty and an estimate can be made of the energy required to induce electron transition, indicating an approximate value for the molar absorptivity of the species.

### Vibration and Rotation

The internal molecular structure may respond to radiant energy in addition to electron transitions. In some molecules the bonding electrons also have natural resonant frequencies, giving rise to molecular vibration, while others exhibit a rotation. The differences in energy levels associated with vibration and rotation are much smaller than those involved in electron transitions, therefore excitation resulting from these phenomena will occur at comparatively longer wavelengths; vibrational excitation is typically associated with IR radiation, while rotational excitation are associated with far-IR or even microwave radiation.

Despite vibrational and rotational excitation being primarily associated with spectral regions other than UV-VIS, they do have an effect on electron transitions within this range. The principal effect is of 'broadening', that is the deviation of an observed absorption region from its predicted region.

For most species, especially in solution, excitation does not appear as sharp absorbance points at highly differentiated wavelengths, but rather as bands of absorbance over a range of wavelengths. A principal reason is that absorbance at the electron transition level are frequently accompanied by smaller structures at the vibrational level. In the same way each vibrational structures may have even smaller associated structures at the rotational level, so an absorbance spectrum due to electron transitions may display far more complex structures than expected.

### Specific Absorption

Each electron in a molecule has a unique ground state energy, and as the distinct levels it may be promoted to are also unique, there is a finite and predictable set of transitions available to electrons in any given molecule. Each transition, resulting from the absorption of a photon, will have a direct and permanent relationship between the wavelength of the photon and the particular transition that it stimulates, known as specific absorption. A plot of those points along the

wavelength scale, at which a given substance shows absorption 'peaks', or maxima, is called an absorption spectrum (Figure 5).

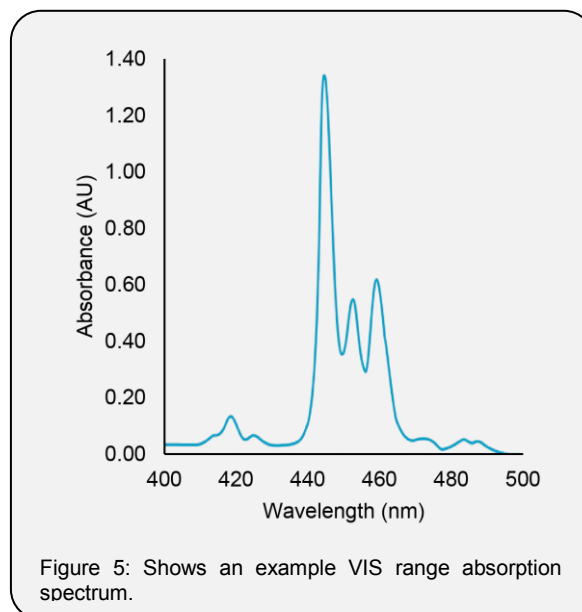


Figure 5: Shows an example VIS range absorption spectrum.

An absorption spectrum of a compound is a useful physical characteristic, for both qualitative (identification) analysis and quantitative (concentration) analysis. In its simplest form, absorption of wavelengths at the red end of the VIS spectrum, and reflection of unabsorbed wavelengths will result in the compound appearing green/blue (Figure 6).

Absorbed Wavelength	Absorbed Colour	Reflected Colour
700 nm	Red	Green
600 nm	Orange-Red	Blue-Green
550 nm	Yellow	Violet
530 nm	Green-Yellow	Red-Violet
500 nm	Green	Red
450 nm	Blue	Orange
400 nm	Violet	Yellow

Figure 6: Shows the colour of absorbed light and its complimentary reflected colour from the VIS spectrum. The given wavelengths are for estimating the spectral region only.

The chemical group that most strongly influences the absorbance of a compound is referred to as the chromophore. As discussed earlier, chromophores which can be excited by UV-VIS radiation involve a multiple bond (such as C=C, C=O or C≡N). They may be conjugated with other groups to form complex chromophores, and increasingly complex chromophores move the associated absorption peak towards longer, less energetic, wavelengths and generally increase the degree of absorbance at the absorption maxima (Figure 7).



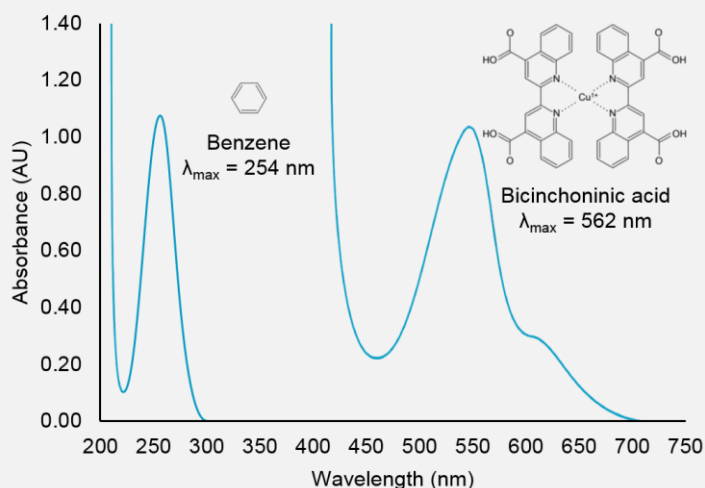


Figure 7: Shows a composite of UV-VIS range absorption spectra based on the comparative complexity of benzene and a bicinchoninic acid (BCA)-copper complex, and the effect it has on their respective absorption maxima ( $\lambda_{\text{max}}$ ).

The correlation between molecule complexity and detectability using UV-VIS spectrophotometry lends itself to the measurement of organic compounds. However, a wide range of inorganic compounds offer themselves to similar methods of analysis. Species with a non-metal atom double bonded to oxygen absorb in the UV region, and there are several inorganic double-bond chromophores that show characteristic absorption peaks. In some instances, measurement of inorganic materials may demand a secondary process, such as complexation with a colour-forming reagent or oxidation. For example, manganese (II) oxide ( $\text{MnO}$ ) oxidised to manganese (VII) oxide ( $\text{Mn}_2\text{O}_7$ ), and measured as the permanganate ion ( $\text{MnO}_4^-$ ).

### Absorption and Concentration

For analytical purposes, two main propositions define the laws of light absorption. The first, Lambert's Law states the proportion of incident light absorbed by a transparent medium is independent of the intensity of the light (Lambert,

1760). Therefore successive layers of equal thickness will transmit an equal proportion of the incident energy. It is defined by the equation:

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

Where  $I$  is the intensity of the transmitted light,  $I_0$  is the intensity of the incident light, and  $T$  is the transmittance. It is typical to express transmittance as a percentage of the incident light, which is defined as follows:

$$\frac{I}{I_0} \times 100 = \%T$$

The second, Beer's Law states the absorption of light is directly proportional to both the concentration of the absorbing medium and the thickness of that medium (Beer, 1852).

A combination of the two laws, the Beer-Lambert Law, defines the relationship between absorbance and transmittance.

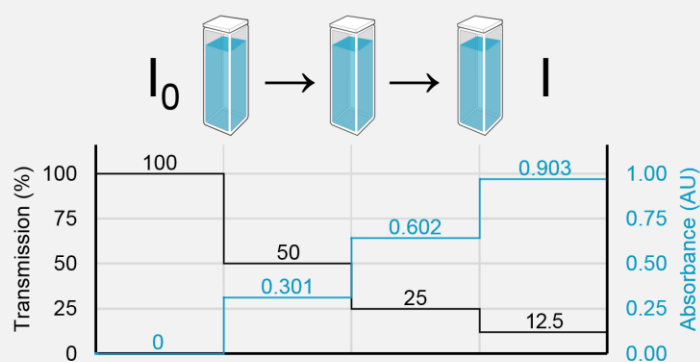
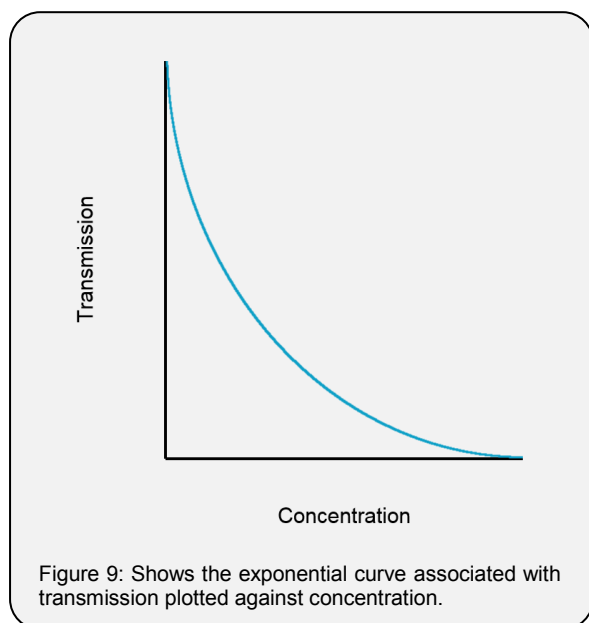


Figure 8: Illustrates the conditions when three samples with identical absorption are introduced into a beam of monochromatic light, each transmitting half of the intensity of the incident (50 %T).

$$A = \log \frac{I}{I_0} = \log \frac{100}{T} = \epsilon cl$$

Where  $A$  is the absorbance, which has no units, although it is often referred to in absorbance units (AU).  $\epsilon$  is the molar attenuation coefficient of the medium ( $M^{-1}cm^{-1}$ ),  $c$  is the molar concentration (M), and  $l$  is the pathlength (cm). It is important to note that the molar attenuation coefficient is a function of wavelength, therefore Beer-Lambert law is only true at a single wavelength, also known as monochromatic light.

In a scenario where three identical samples of 50 %T are placed sequentially in a beam of incident radiation (100 %T), then the intensity after each sample will be halved (Figure 8). The three samples may be considered as known concentrations of an absorbing medium and it is therefore possible to plot transmission against concentration (Figure 9). This graph will follow an exponential curve, and so is of limited value.

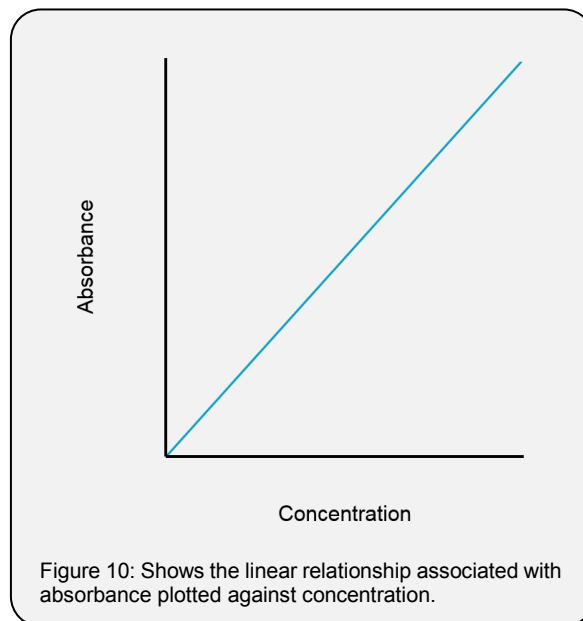


However, providing the light is monochromatic and the Beer-Lambert law is obeyed, it becomes possible to define the process in terms of absorbance units (AU).

For the same example, converting %T to AU then plotting absorbance against concentration shows a linear relationship (Figure 10). Making the results more convenient to be expressed in absorbance, rather than transmission, when measuring samples of unknown concentration, given that linear calibration curves are available.

An alternative use of the linear relationship between absorbance and concentration, is to calculate a factor for a specific molecule of interest

that can then be applied to subsequent sample of unknown concentrations of that same molecule. This avoids the relatively time consuming process of a plotting calibration curve.



To calculate the factor ( $k$ ), the absorbance of a known concentration of the molecule of interest needs to be determined. These two parameters can then be used as detailed in the equation.

$$k = \frac{\text{concentration}}{\text{absorbance}}$$

This factor can then be used in the following equation to calculate the concentration ( $c$ ) from the absorbance ( $A$ ) of a sample of unknown concentration.

$$c = k \times A$$

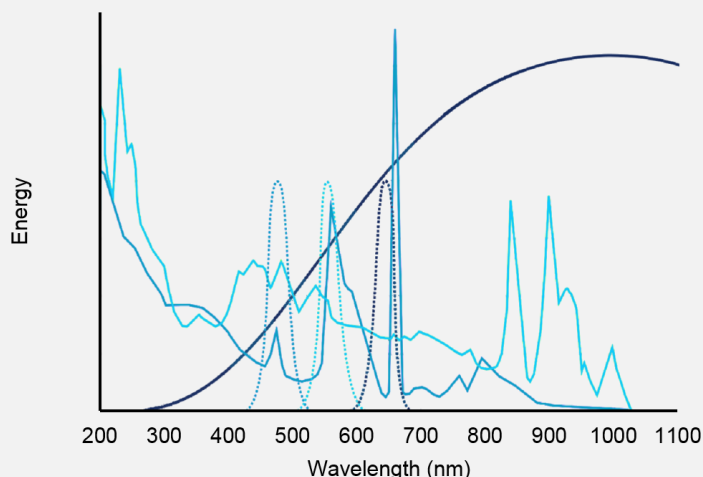
## Instrumentation

In their simplest form, a spectrophotometer has a light source, a monochromator, a sample compartment, and a detector coupled with a measurement system and result readout.

### Light Source

UV light is generally derived from a deuterium arc lamp that provides emission of high intensity and suitable continuity in the 190-380 nm range. A quartz envelope is necessary to transmit the shorter wavelengths of UV radiation.

VIS light is normally supplied by a tungsten based lamp, with tungsten-halogen lamps having



Key	
—	Tungsten-halogen
—	Deuterium
—	Xenon
...	Red LED
...	Blue LED
...	Green LED

Figure 11: Shows approximate emission spectra of tungsten-halogen, deuterium, xenon, and LED light sources as per the key above. In a system that combines deuterium and tungsten lamps, the UV-VIS crossover is usually set to approximately 360 nm.

relatively higher output in the UV-VIS crossover region (Figure 11). The long wavelength limit is usually the cut-off of the glass or quartz envelope of the lamp, but is normally beyond the useful visible limit at 900 nm.

Xenon flash lamp light sources are an alternative to combined deuterium-tungsten systems. Xenon flash lamps cover the UV and VIS range, and have a very long lifetime. However, additional processes to account for higher levels of stray light, as well as less energy in the VIS region, means that the instruments are limited to applications where higher instrument specifications are not required.

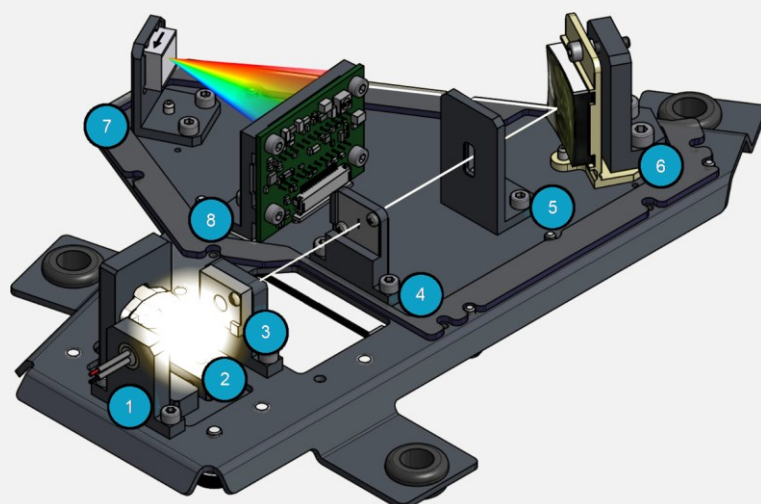
Light Emitting Diodes (LED) have wavelength ranges of approximately 25 nm and follow a Gaussian distribution. As a result they are unfit for general applications, but can be used for specialised applications or as reference sources to calibrate against broader wavelength range light sources.

### Monochromator

The monochromator is responsible for producing a selectable beam of monochromatic (single wavelength) radiation from the wide range of wavelengths provided by the light source. They comprise of any number or combination of Lenses, filters, gratings, mirrors, and slits (Figure 12).

Two basic methods of wavelength selection exist; filters, and dispersing elements such as diffraction gratings.

Filters of coloured glass or gelatine are the simplest form of selection, but they are limited in usefulness due to cost-effective filters being restricted to the VIS region, as well as having poor wavelength resolution: Typically they cannot isolate wavelength ranges smaller than 30-40 nm. More sophisticated interference filters can achieve wavelength resolutions of 10 nm or less,



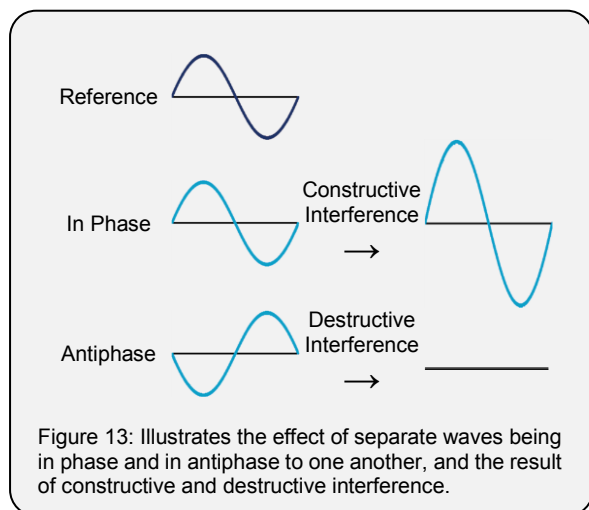
Key	
1	LED
2	Tungsten lamp
3	Lens and filter
4	Entry slit
5	Field stop slit
6	Mirror
7	Grating
8	Detector

Figure 12: Shows an example optical bench with the positions of the various elements highlighted as per the key above.

but are still inappropriate for most routine laboratory applications, in spite of low cost and technical simplicity. More often they are used in combination with, and upstream, of more sophisticated dispersing elements to remove relatively large regions of unwanted radiation.

Traditional transmissive diffraction gratings, a plate with multiple parallel slits, have been replaced by reflective diffraction gratings, mirrors with multiple parallel grooves on their surface, but the term diffraction grating without distinction is commonplace. The series of parallel grooves, referred to as lines, on a reflective diffraction grating can be considered separate mirrors, and the diffracted light from neighbouring lines overlap with one another resulting in interference.

If diffracted waves are in phase, they undergo constructive interference resulting in amplification. Increasing the intensity of the diffracted light. If the diffracted waves are in antiphase, then they undergo destructive interference cancelling each other out, decreasing the intensity of detracted light (Figure 13). As different wavelengths from the same light source would be in phase and in antiphase at different angles to the incident beam, their amplified and cancelled out projected light occur at different position on a projected surface, separating the wavelengths and creating a spectrum.



The angle where maxima occur can be found in the following equation:

$$\frac{d \sin \theta_m}{\lambda} = m$$

Where  $\theta_m$  is the angle between the diffracted light and the diffraction grating's normal vector, the line perpendicular the plane of the diffraction grating,  $d$  is the distance between the centres of adjacent lines, and  $m$  is an integer representing the

propagation-mode of interest, described as the order.

When light is normally incident on the grating, the reflected light will have maxima at angles  $\theta_m$ :

$$d \sin \theta_m = m\lambda$$

To incorporate the angle of the incident radiation ( $\theta_i$ ), relative to the grating's normal vector, the grating equation becomes:

$$d(\sin \theta_i + \sin \theta_m) = m\lambda$$

When rearranged for to make the angle maxima the subject, the equation is:

$$\theta_m = \arcsin\left(\frac{m\lambda}{d} - \sin\theta_i\right)$$

Rotating the grating in the incident beam changes its relative angle, changing the output wavelength at a given point ( $\lambda$ ), thus provides wavelength selectability of light.

The light that corresponds to reflection is called the zero order. Diffraction maxima that occur at angles other than the zero order are represented by non-zero integers, 1, 2, 3, and so on, referred to as first, second, third, etcetera order. These orders can be both positive and negative, resulting in diffracted orders on both sides of the zero order beam (Figure 14).

### Optical Geometry

As measurements are ratio dependent ( $I/I_0$ ), a reference measurement, creating a baseline imparted by the reference solution, is taken before the sample measurement is recorded. The reference intensity ( $I_0$ ) varies with wavelength, due to many factors such as source energy, monochromator transmission, slit width, and detector response, so it is necessary to create a new reference baseline for each separate wavelength at which a sample measurement is to be made. Microprocessors in modern instruments have enabled the facility to store multiple baselines, set at each wavelength, allowing options to overcome this requirement. As a result, single beam instruments do not have this traditional disadvantage compared to more expensive double beam instruments (Figure 15).

The incorporation of xenon flash lamps in an instrument requires a split beam configuration. This is due to the high intensity flashes not being of equal magnitude. Approximately 70 % of the energy from the monochromator is passed



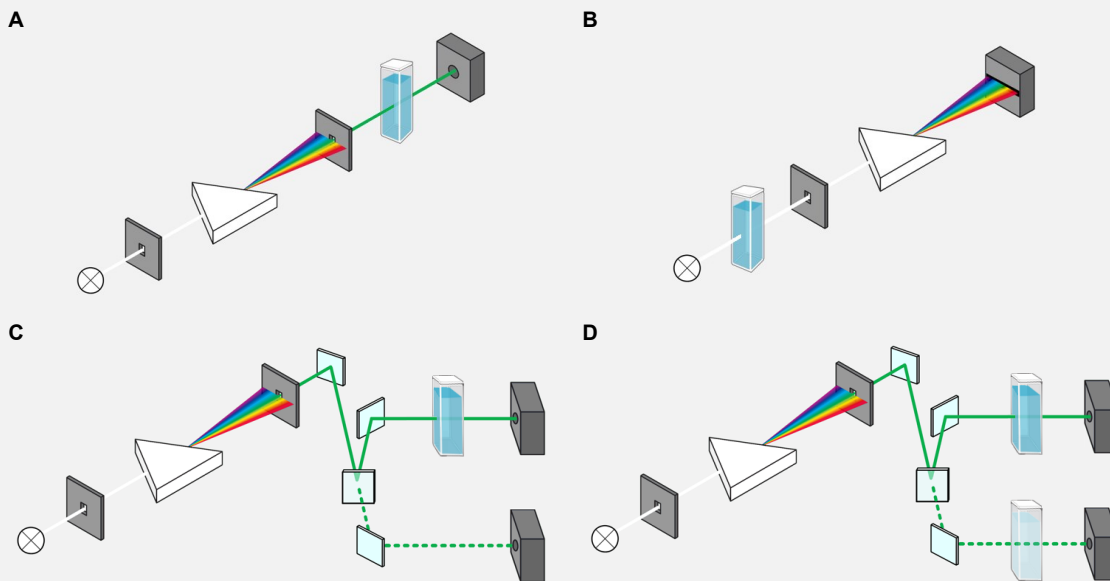


Figure 15: Shows representations of example optical geometries, with an example of both standard (A) and reverse (B) single beam configurations. As well as a split beam (C) configuration with a test cell position, versus a double beam (D) configuration with a test cell position and a reference cell position.

through the sample, with the rest going to a separate reference detector, enabling a means of taking into account fluctuations in energy. This stabilises the system without additional expensive elements being required.

For increased accuracy, the preferred optical configuration is a double beam system. Double beam operation is achieved by dividing the beam, using a partial mirror or chopper, into two separate light paths which are directed through the sample and reference positions. The wavelength dependent functions of the instrument are significantly reduced to give improved performance, as the system is capable of compensating for light source and detector variations, as well as time dependant baseline changes to the test and reference samples.

An additional aspect to an optical geometry is standard or reverse optical configuration. In a standard optical configuration, the sample is downstream of the monochromator and so received monochromatic light. In a reverse optical configuration, the sample is upstream of the monochromator and the transmitted light is then passed through the monochromator. A visual clue to which configuration an instrument employs is whether the sample compartment has a lid or not, as the light that travels through a sample holder of a reverse optical system is yet to be monochromated, therefore ambient light will not greatly affect the measurement. Another point to note is that reverse optical systems tend to be used with photodiode array (PDA) and charge

coupled device (CCD) detectors. So the dispersive element is in a fixed position.

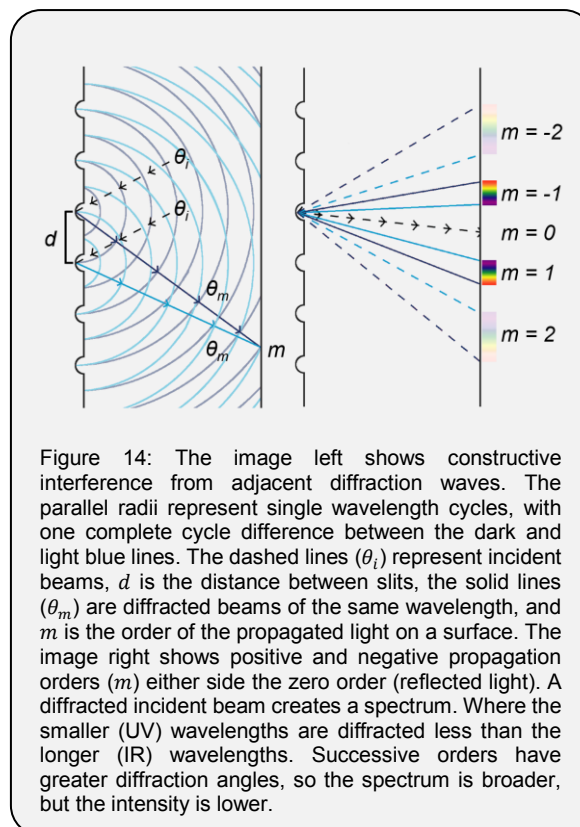


Figure 14: The image left shows constructive interference from adjacent diffraction waves. The parallel radii represent single wavelength cycles, with one complete cycle difference between the dark and light blue lines. The dashed lines ( $\theta_i$ ) represent incident beams,  $d$  is the distance between slits, the solid lines ( $\theta_m$ ) are diffracted beams of the same wavelength, and  $m$  is the order of the propagated light on a surface. The image right shows positive and negative propagation orders ( $m$ ) either side the zero order (reflected light). A diffracted incident beam creates a spectrum. Where the smaller (UV) wavelengths are diffracted less than the longer (IR) wavelengths. Successive orders have greater diffraction angles, so the spectrum is broader, but the intensity is lower.

It is worth noting that instrument optical configurations, defined as double and split beam, can differ between manufacturers. Some describe double beam as having a single detector while a split beam has two, one for each beam, others

describe a double beam as having two cell holder positions, one for each beam, while a split beam has a single cell holder position.

### Sample Handling

The vast majority of measurements are made on samples in solution. However, gases and solids can also be measured, but most instruments are designed to accommodate standard cuvettes (also known as cells) in the sample compartment. Important considerations are the design, construction and material of the cuvette being used for obtaining accurate and reliable measurements, as are operator practice and sample preparation.

Cuvettes are typically made of glass or quartz (fused silica) for VIS or UV-VIS wavelengths of interest respectively. Their joints are fused rather than cemented, to resist the action of solvents, and are finished to ensure that the optical windows (the sides through which the beam passes) are highly polished, parallel to one another and flat, and that the pathlength (distance between inner surfaces of windows) is has minimal deviation.

The holder that locates the cuvette in the beam must ensure precise and reproducible positioning with respect to the beam path through the cuvette.

The most commonly used cuvette has a pathlength of 10 mm, but longer or shorter pathlengths are useful if lower or higher concentrations, respectively, which fall outside the instrument range at a 10 mm pathlength are being measured. Micro cuvettes; for small sample volumes, gas cuvettes, and flow cuvettes, as well as masked and unmasked versions, are all available to extend the usefulness of an instrument. Disposable Polystyrene, used in VIS applications, and Poly-methyl methacrylate (PMMA), used in UV-VIS applications, cuvettes are also available. Preferred to avoid cross contamination, or in high throughput applications to remove arduous washing procedures (Figure 16).

### Detectors

There are two types of detectors found in Biochrom spectrophotometers. They are avalanche photodiodes and arrays detectors. The material used to make a detector defines its properties. Silicon based detectors are most common in UV-VIS spectrophotometry they are sensitive to 190-1100 nm.

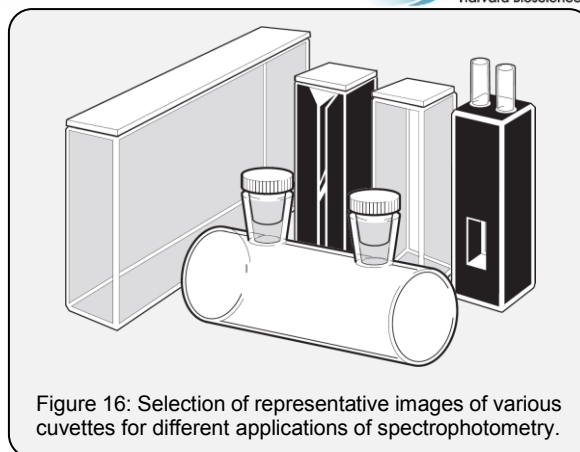


Figure 16: Selection of representative images of various cuvettes for different applications of spectrophotometry.

Avalanche photodiode consist of narrow strips of doped silicon in reverse bias. P-type (positive) doped semiconductor material connected to a negative terminal of an electrical source, and n-type (negative) doped semiconductor material connected to a positive terminal of an electrical source, receive radiation through them. This causes impact ionisation, where electrons move from the n-type anode to the vacant electron holes in the p-type cathode, producing a photocurrent which can be detected and measured. They have a wider wavelength range but are less sensitive. Being solid state devices they are mechanically robust, and benefit from reduced power supply and control circuit demand (Figure 17).

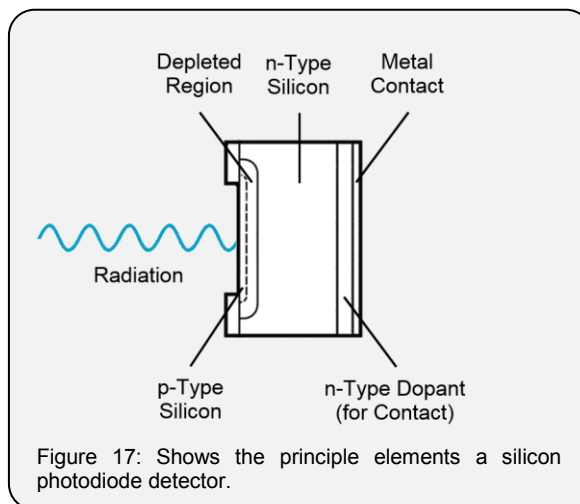


Figure 17: Shows the principle elements a silicon photodiode detector.

Array detectors include photo diode array (PDA) and Charge-coupled device (CCD) array. They are an assembly of individual photodiode elements in linear or matrix form and can be mounted so that the complete spectrum is focused on to an array of appropriate size. No wavelength change mechanism is required making taking and displaying measurements very quick. The resolution is limited by the physical size of individual detector elements. Furthermore, additional optical geometry considerations are

required to remove the effects of stray light from the second order spectra.

### Measuring Systems

The ultimate function of a spectrophotometer is to provide the end-user a value that is proportional to the absorption by a sample at a given wavelength. Many instrument manufacturers offer two variants of the same instrument. One with digital displays (LED or LCD) and the other without a display, which are designed to be controlled and capture the data through additional PC software.

Most instruments allow end-users to define specific parameters such as wavelength, output units, and any relevant factors (dilution, molar and mass coefficients, molecular weight). The instrument will apply the parameters removing the requirement for manual calculation. Furthermore, from this information provided by the end-user, the instrument will automatically select the light source and any filters required to meet the requirements of the measurement. Additional components can often be introduced, automatic cell changers for example, allow for multiple samples to be loaded at once, and with the parameters provided by the end-user, will be automatically positioned into the beam path, and present the appropriate output against the relevant sample.

Secondary routines such as instrument calibration and validation are often available on demand, either through the instruments on-board display or through interfacing with an external PC.

### Good Operating Practice

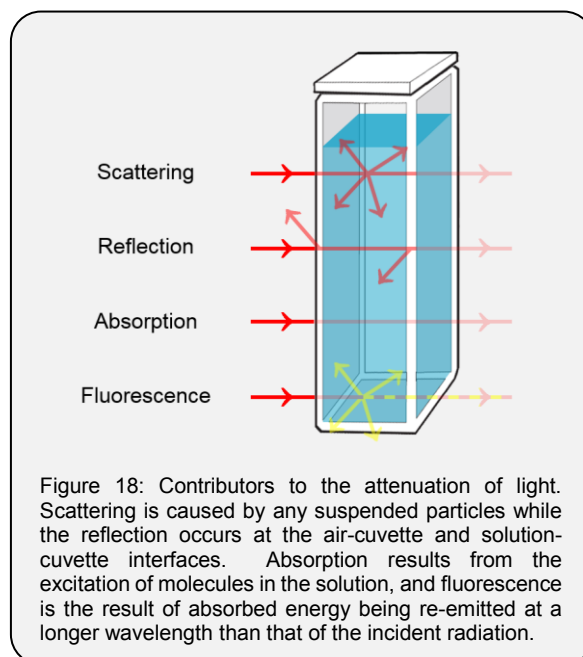
Good laboratory practice should always be applied in the preparation of samples for spectrophotometric assays. The cleanliness of all materials and equipment, especially the sample cuvettes, is imperative, as are weighing and volumetric accuracy.

Cuvettes used for reference and sample measurements should be optically matched where possible. Most cuvette manufacturers can supply cuvettes with optical characteristics matched over a defined wavelength range. Where this is not feasible, a plot of absorbance against concentration should be made, the curve will have an intercept ( $k$ ) which can be applied as a correction as defined below.

$$A = \epsilon lc + k$$

Successive sample measurements can be made using the same cuvette, but it should be effectively cleaned, including sample solvent washes and drying.

Equally important is that the orientation of the cuvette in the beam path is carefully controlled for continuity of the cuvette surfaces and pathlength that influences the measurement. Several processes can contribute to the total attenuation of light as it passes through a cuvette and sample, including reflection, scattering, absorbance, and fluorescence (Figure 18).



In practice, the effects of reflection are restricted to less than significant levels by the considerations described previously, they are the use of quality sample cuvettes, matched where possible, and by careful sample handling. Fluorescence effects can be reduced by chemical inhibition, or by appropriate cut-off filters.

Temperature variations can be a source of error also, therefore new reference measurements should be taken to update the baseline and counteract any potential shift over significant periods of time.

An effort to maintain the solvent pH between measurements should be made. As an example, a change in pH from acidic to basic results in a structural change of phenol red from its yellow form to its red form. The related redistribution of electrons in the molecular orbits means the absorption spectrum of the sample changes with the change in pH (Figure 19).



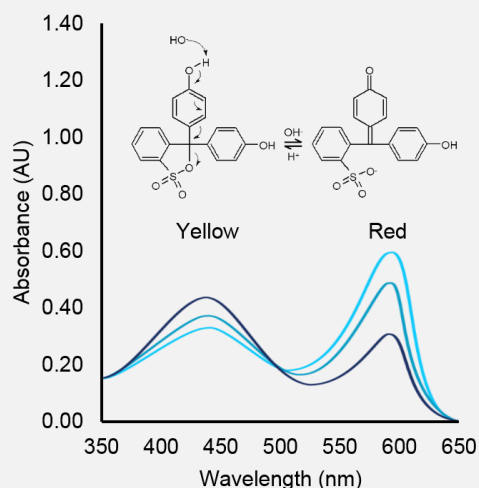


Figure 19: Shows the yellow acidic ( $\lambda_{\text{max}} = 443 \text{ nm}$ ) and red basic ( $\lambda_{\text{max}} = 592 \text{ nm}$ ) forms of phenol red at different pH.

A final point to note is that buffer components can impart their own absorbance. Detergents such as IGEAL, Triton x-100, and NP-9 exhibit absorbance in the UV region. As a result alternative detergents such as Brij-35, CHAPS, or Tween 20 are recommended. There are also reduced (hydrogenated) Triton x-100 commercial alternatives that report lower UV absorbance. Where their use cannot be avoided, concentrations should be kept at a minimum so that the absorbance imparted by the analyte is the largest component of the total absorption.

#### Limitations of Beer-Lambert Law

The linear relationship between absorbance and concentration is demonstrated by Beer-Lambert law. However, under certain circumstances this relationship breaks down. These deviations can be grouped into two categories. Deviations due to the limitations of the law itself, and deviations due to specific characteristics of the sample which is being analysed.

Beer-Lambert law is capable of describing absorption behaviour of solutions of relatively low solutes concentration (<10 mM). Deviations from the law itself occur when the sample concentration is higher, the analyte may behave differently due to interactions with the solvent and other solute molecules, potentially forming hydrogen bond interactions. Solute molecules forced into close proximity as a result of high concentration can influence the charge distribution of neighbouring molecules, potentially result in a shift in the wavelength absorption maxima.

The size of a molecule also affects the degree deviation. Larger molecules, with more potential electron transitions available, will exhibit higher absorbance and therefore show deviations even at very low concentrations. For example methylene blue absorptivity at 436 nm fails to observe Beer-Lambert law even at concentrations as low as 10 $\mu\text{M}$ .

High concentrations can also alter a samples refractive index ( $\eta$ ), which could affect the absorbance value obtained. Corrections to the equation defining Beer-Lambert law have been applied as detailed in the equation.

$$A = \epsilon c l (\eta^2 + 2)^2$$

In practice, corrections should not be required below concentrations of 10 mM.

To conclude, end-user action can be taken to minimise the effects of sample born deviations. The linear relationship between absorbance and concentration as defined by Beer-Lambert law holds true between 0.1 and 1.5 AU. At very low absorbance, measurements can be noisy, and at higher absorbance,  $\geq 2.0$  AU, significant deviations from linearity can occur, resulting in considerable under-estimation of the concentration. Therefore, to minimise the deviations based on the limitations of Beer-Lambert law, it is recommend to keep data in the range of 0.1 to 1.0 AU. If an adjustment is required for the measurement to be within a preferred absorbance range, it is better to change the sample pathlength rather than the concentration of the sample itself wherever possible.

### Sources of Error

For confidence in the measurements obtained from an instrument, it is important to be aware of the sources of error associated with that instrument and with the sample itself.

#### Instrument Sources of Error

Spectral bandwidth is the resolution of a spectrophotometer, described as the ability of an instrument to separate light into distinct, finite wavelength regions and distinguish them from one another. Defined as the wavelength interval in which nominal incident radiation at half its maximum value, or full width at half maximum (FWHM) (Figure 20). It is usually limited by the spectral purity and intensity of the monochromatic light and the detector's sensitivity at that wavelength.



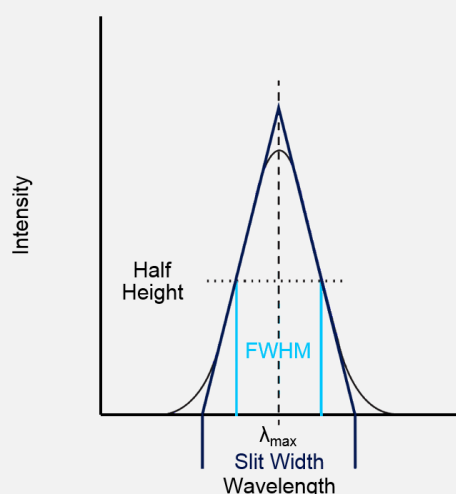


Figure 20: Intensity distribution of wavelengths emerging from the monochromator. The natural bandwidth (FWHM) is shown in light blue and the spectral bandwidth (slit width) is shown in dark blue.

The spectral bandwidth at the detector is dictated by the exit slit of the monochromator, and influences both photometric and wavelength accuracy. Generally narrower slit widths will reduce error, on the condition that the overall energy level remains adequate and electronic noise levels are not significant. Most instruments using diffraction gratings take advantage of the linear dispersion and have fixed slit widths. Higher end instruments may offer variable bandwidths, but each is defined by its own physical slit, with a fixed size which are swapped in place of each other. This gives the user a means of trading energy for spectral resolution (Figure 21).

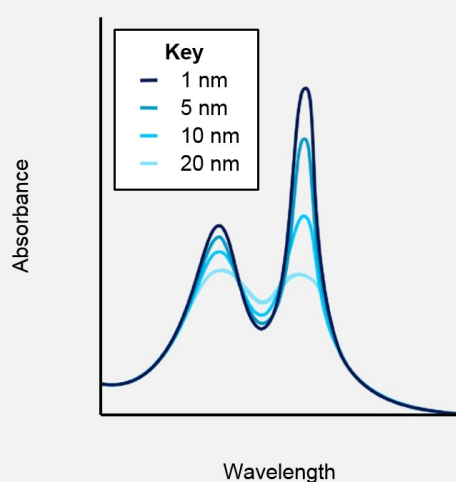


Figure 21: The effect of spectral bandwidth on measured absorbance peaks.

The natural bandwidth is the FWHM of an analyte's absorbance peak. The ratio between the spectral and natural bandwidth is a determining factor in an absorption measurement. As this ratio increases, the deviation of observed absorbance from true absorbance will be greater. The natural bandwidth of most commonly encountered compounds in UV-VIS spectrophotometry, particularly large biological molecules, are within the range of 5 to 50 nm so lack fine spectral detail. Generally, a ratio between spectral and natural bandwidth of 0.1 or less will generate absorbance measurements at 99.5 % accuracy or better. For ratios above 0.1, the accuracy deteriorates. Thus an instrument with a fixed bandwidth of between 3 and 5 nm is ideal for biological molecule measurement. A narrower bandwidth is required for measurements involving rare earth and transition metal complexes, and conjugated organic species, where critical fine detail may be present.

Instrument error is usually stray light. However, quality instruments will measure absorbance across their photometric range with a high degree of accuracy. Although Beer-Lambert law is strictly followed when a monochromatic incident beam is applied to a sample, in practice a single wavelength is not always emitted from the monochromator. Instead, a small range of continuous wavelengths are emitted. For a molecule that has molar attenuation coefficients  $\epsilon'$  and  $\epsilon''$  at wavelengths  $\lambda'$  and  $\lambda''$  within the emitted range, if  $\epsilon'$  and  $\epsilon''$  are equal the absorbance will follow the linearity of Beer-Lambert law. However if  $\epsilon'$  and  $\epsilon''$  are not equal the absorbance will deviate from the linearity, and furthermore the greater the difference between  $\epsilon'$  and  $\epsilon''$  the greater this deviation will be.

It is important to measure a sample at the molecule of interest's absorbance maxima. As absorbance follows a Gaussian distribution, and the greater the deviation from the absorbance maxima's corresponding wavelength the larger the range of discrepancy in the absorbance value obtained (Figure 22). Therefore, to avoid erroneous measurements caused by wavelength inaccuracies, it is advisable to measure at the absorbance maxima where the rate of change is minimal.

Stray light is perhaps the most influential cause of error. It is defined as radiation from the instrument that is outside the nominal wavelength selected. The wavelength range of the stray light is usually very different from the wavelength band selected. It is known that the incident beam exiting from the monochromator is contaminated with minute quantities of scattered or stray radiation, usually

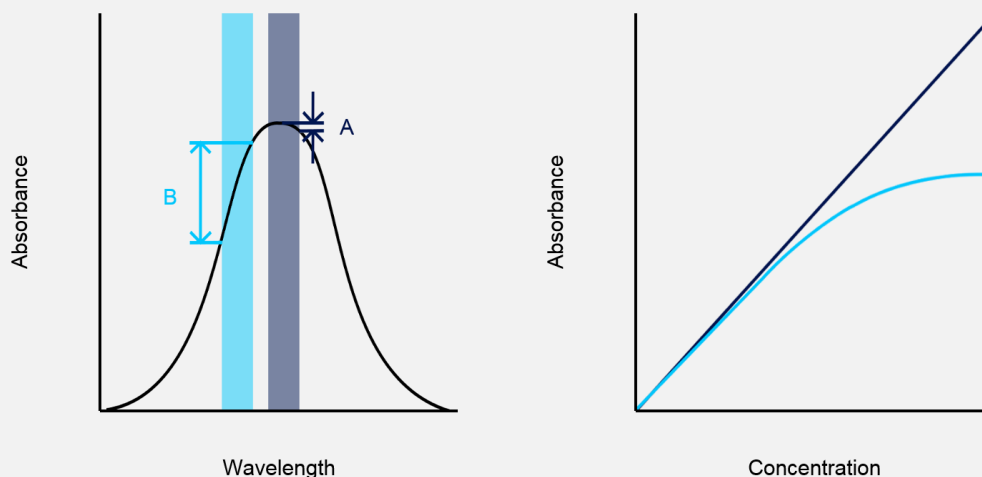


Figure 22: The left-most image shows the low level of variability across the natural bandwidth at the absorbance maxima (A) compared the high level of variability when offset from the absorbance maxima (B). The near-left image shows how any discrepancy increases with the sample concentration.

due to reflection, refraction, and diffraction from the optical surfaces, exacerbated by contaminating particles and aberrations. Its effect is such, that as the absorbance of the incident light increases with analyte concentration, the transmitted incident light decreases while any stray light remains the same. Therefore, any light detected is an increasing proportion of stray light. Which eventually masks further increase of analyte concentration, and deviation from the linearity of Beer-Lambert law is observed (Figure 23).

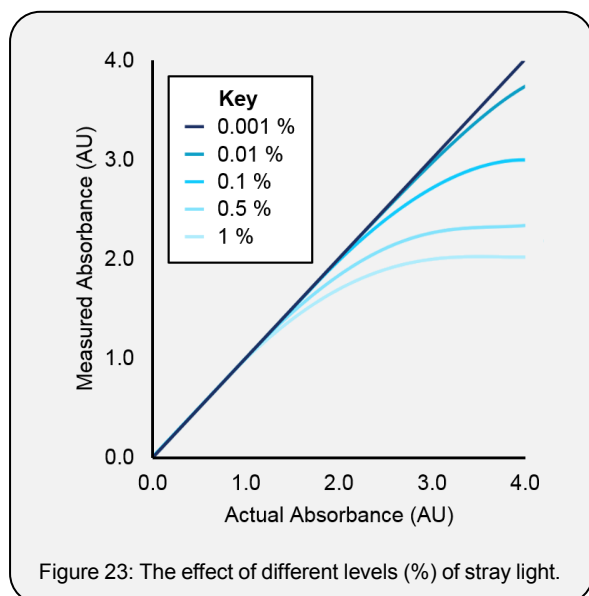


Figure 23: The effect of different levels (%) of stray light.

Instrument error affecting the absorbance accuracy is predominately from the inherent noise in the detector. In practice, the error is negligible except where the incident beam energy is low,

furthermore, it can be minimised by component choice.

#### Non-instrument Sources of Error

Non-instrumental errors most commonly derive from the nature of the solution to be examined. The samples physical characteristics, temperature and concentration, need to be taken into consideration, as well as the potential for additional light absorbing compounds at the wavelength of interest. Absorbance under these conditions is additive, and accurate measurements of the analyte may no longer be possible.

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