The Basics of Optical Spectroscopy

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1. Introduction & Theory

Spectroscopy is concerned with how light or more generally electromagnetic radiation interacts with matter. In the Pharmaceutical Sciences spectroscopy is used to probe :

1. The identity and molecular structures of drugs, excipients and formulated medicines.
2. The amounts (concentrations) of drugs and excipients.
3. The purity of drugs and excipients.
4. The environment of drugs and excipients.

The results enable a better understanding of how drugs behave chemically, physico-chemically and biologically. Drug formulation studies also benefit from this knowledge. This is necessary for a more complete appreciation of why and how drugs do what they do. The licensing of drugs for administration requires a molecular description of a drug and its properties (activity, stability, metabolism …. etc).

Electromagnetic radiation is considered to be a wave derived from an oscillating electric field. (A varying electric field has an associated varying magnetic field. One is a consequence of the other and they are mutually at 90°. In ordinary spectroscopy, only the electric field is explicitly considered.) A light wave has five important properties :

1. Wavelength (energy)
2. Intensity
3. Speed (refractive index)
4. Polarisation
5. Propagation direction

Can you think of others ?

In practice, all interactions of light waves with matter can be described in terms of these 5 basic properties. However, at the beginning of a course on spectroscopy, the first two properties are the most important.

1.1 Property 1 : Wavelength (Energy)

In the early days of quantum mechanics and the beginnings of modern spectroscopy, a German physicist called Planck explained that the energy associated with electromagnetic radiation is related to its wavelength by the equation which bears his name :

$$E = hc/\lambda \qquad (1)$$

where E = energy, h = Planck's constant, c = velocity of light and λ = wavelength of light. Spectroscopy can be defined as the resonant interaction of electromagnetic radiation (light) with a molecule. As illustrated in Figure 1, energy levels in a molecule (or atom) are discrete (quantised). Provided the wavelength of light matches the energy of the excitation with $E=hc/\lambda$, a resonant interaction can occur. The molecule (or atom) will be excited from the ground state to the excited state and light will be absorbed; a spectroscopic transition will have taken place.

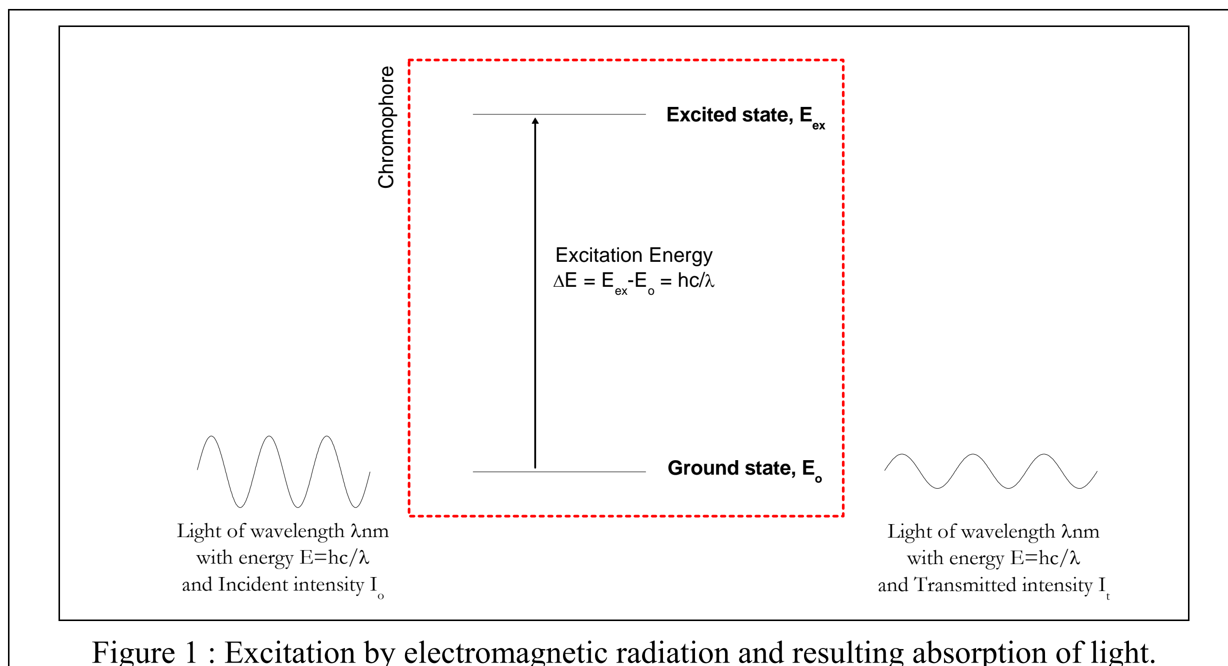

Figure 1 : Excitation by electromagnetic radiation and resulting absorption of light.

According to Planck's equation, electromagnetic radiation of short wavelength carries a lot of energy. Longer wavelengths have lower energy.

i) A light beam having only a single wavelength is called monochromatic, a broad spectrum of wavelengths would be polychromatic.
ii) Radio waves with long wavelengths (metres) have very little energy and can only unalign the spin of an atomic nucleus that has been aligned in a magnetic field.
iii) Microwaves with a little more energy can change the translation/rotation motion of a molecule or the spin orientation of an electron aligned in magnetic field.
iv) Infra-red radiation, with intermediate energy, will cause different chemical bonds to vibrate.
v) UV and Visible light have enough energy to excite electrons. The excitation/absorption process involving electrons is usually localised in a region of the molecule called the chromophore.
vi) X-rays are highly energetic and can ionise molecules making them chemically reactive and carcinogenic.

The precise wavelength where an interaction occurs will depend upon the nature of the interaction. For example, a wavelength λ=270nm is required for the excitation of an electron in benzene; a wavelength of λ=60 microns induces a C=O bond to vibrate and a wavelength λ=3 metres will unaligned a proton nucleus aligned in magnetic field. These different interactions are the basis of sub-dividing electromagnetic radiation into spectral regions.

The wavelength (λ) of light in the UV/Vis region is in nanometres (nm, 10^{-9}m). In the IR, the wavenumber unit is preferred; written as $\overline{v}$ and defined as $\overline{v} = 1/\lambda$ where λ is now in centimetres (cm). Rewriting Planck's equation in terms of wavenumbers gives $E = hc\overline{v}$; wavenumbers are proportional to energy, wavelength is inversely proportional to energy. In the radiofrequency, both wavelengths and frequency are used (eg : λ =3 metres or f = 100MHz from f=c/λ).

The General Scheme

	Optical Spectroscopy			**Magnetic Resonance Spectroscopy**	
X-ray (10^{-10} metres)	UV - Vis (10^{-7} to 10^{-6}metres) 100nm to 1000nm	Near IR - mid IR - far IR 10^{-6}metres to 10^{-4}metres	Microwave (10^{-4} metres to 1cm)	Microwave (10^{-4} metres to 1cm)	Radiowave (1cm to 10 metres)
X-ray spectroscopy	Electronic Spectroscopy	Vibrational Spectroscopy	Rotational/Translational spectroscopy	Electron spin resonance	Nuclear spin resonance
1. X-ray fluorescence	1. UV/Vis absorption spectroscopy 2. Florescence 3. Optical activity 4. Atomic & ICP spectroscopy 5. Light scattering 6. IR spectroscopy 7. Raman spectroscopy			ESR	NMR
X-ray imaging & crystallography	Optical microscopy Fluorescence imaging IR & Raman imaging				Magnetic resonance imaging (MRI) (*cf* Ultra-sonic scan)

1.2 Property 2 :The Intensity of light

The intensity of electromagnetic radiation is measured by a detector. There are several types of detector largely dependent on the spectral range. In the microwave and radiowave regions special “microphones” are used. In the X-ray, UV, Vis and IR regions detectors are based upon the ejection of electrons from a very reactive Group I metal surface (photomultipliers) or the excitation of an electron into a conductance band (photodiodes). Whatever the detector, the detection of light is generally conceived as a discrete process. The particle model of light is usually invoked and intensity is determined as photons per second. A simple model is to consider light as a “wave packets”. If light is absorbed the situation presented in Figure 2 is considered.

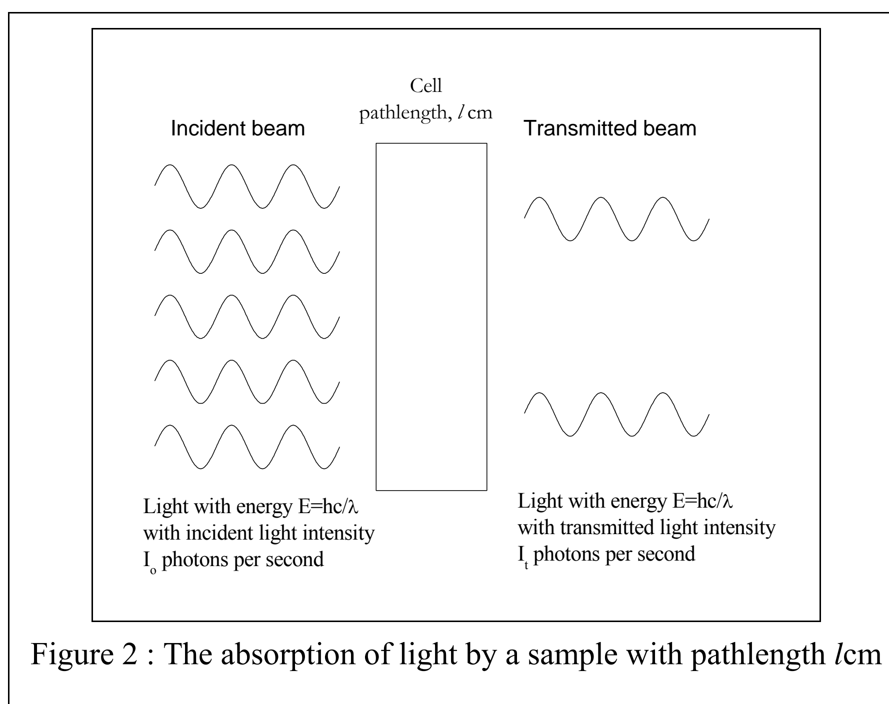

Figure 2 : The absorption of light by a sample with pathlength *l*cm

Laws of Absorption of electromagnetic radiation

Regardless of the spectral region, if absorption takes place, the intensity of light reduces as it passes through a sample. The relationship between the thickness (pathlength) of an absorbing sample and the reduction of light intensity was first quantified independently by Bouger and Lambert and is known as the Lambert, or Bouger-Lambert law. This law says that for a homogeneous absorbing medium, successive equal thicknesses will reduce the intensity by equal fractions, and that the ratio between the intensities of the incident beam, I_o, and the transmitted beam, I_t, is independent of the intensity of the incident beam.
If the incident and transmitted intensities are denoted by I_o, and I_t, then the exponential relation is given by equation (2)

$$I_t = I_o e^{-a.l} \quad \text{or} \quad I_t/I_o = e^{-a.l} \qquad (2)$$

where *l* is the thickness of the sample and a is a sample dependent constant.

This is illustrated in Figure 3 :

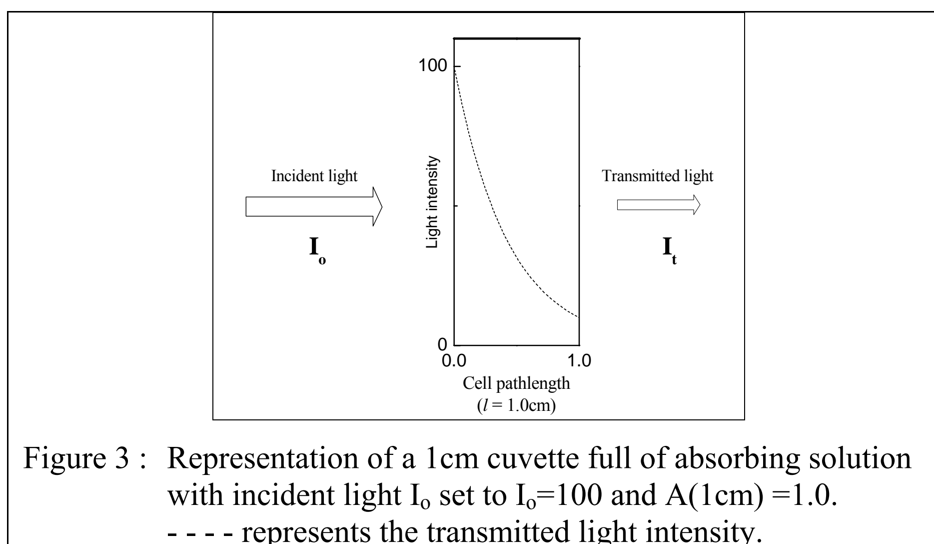

Figure 3 : Representation of a 1cm cuvette full of absorbing solution with incident light I_o set to I_o=100 and A(1cm) =1.0. - - - - represents the transmitted light intensity.

A second, equally important, law was discovered by Beer and relates the ratio of intensities of the incident and transmitted beams to the concentration *c* (or number of absorbing molecules) in a constant pathlength *l*.

$$I_t = I_o e^{-b.c} \quad \text{or} \quad I_t / I_o = e^{-b.c} \qquad (3)$$

where *b* is a constant. Combining these two laws gives :

$$I_t / I_o = e^{-k.c.l} \qquad (4)$$

where k is a constant (composed of a and b) and the expression is known as the Beer-Lambert Law. As it stands in equation (4) the law does not express a direct linear relationship between intensities and concentration or pathlength.

Taking logarithms of equation (4) gives:

$$\log_{10} (I_t/I_o) = -2.303.k.c.l \qquad (5)$$

or

$$\log(I_o/I_t) = 2.303.k.c.l \qquad (6)$$

combining the constants gives :

$$A = \log(Io/I_t)= k'.c.l \qquad (7)$$

where k' represents the combined constants (2.303.k). The log ratio, $\log_{10}(I_o/I_t)$, is known as the absorbance, A, and it is this quantity that is directly proportional to both concentration and path length. Thus the Beer-Lambert law was derived saying that absorbance is proportional to concentration (moles/litre) and pathlength. The actual value of k' is very sample and transition (wavelength) dependent. In practice, k' is given the symbol ε and is known as the extinction coefficient. The extinction coefficient, ε, is said to describe the character of a transition. The Beer-Lambert Law is thus written as :

$$A = \varepsilon.c.l \quad (8)$$

Even though there may be an energy gap in the molecule being irradiated with light matching this energy (wavelength given by $E=hc/\lambda$), this does not necessarily mean that light will be absorbed strongly. The extinction coefficient is a measure of the probability of a transition being excited. In electronic spectroscopy (UV/Vis), ε values can be as high as 50000 or as low as 100. In the IR, ε values can be as high as 100 but are more likely to be 10 or less.
The ability for a solution to absorb light is dependent on three factors : concentration, pathlength and extinction coefficient. Relative ease of measurement (instruments, glass cell and optics, water solvent), ε values between 1000 to 50000, coupled with the transparency of solvents permitting long pathlengths of the order of 1cm are the main reasons why UV/Vis spectroscopy is mainly used for determining concentration.

An important restriction to the Beer-Lambert law is that the nature of the absorbing substance must not change as the concentration is varied. If the absorbing substance aggregates or dimerises, as the concentration is increased, then deviations from Beer's law will be observed. This is because the value of ε can be different for different species.

With any new spectrophotometric analysis procedure it is essential to check that the Beer - Lambert law is obeyed for the system or samples being analysed before the assay method is used routinely.

Note :

Consider the definition of absorbance :

$A=\log(I_o/I_t)$

If absorbance, A, is close to zero then I_t is very similar to I_o and the two values are difficult to distinguish. If the absorbance is very high then I_t is very small (close to zero) and difficult to measure. The compromise is to measure A in the range 0.2 to 1.5 where I_o and I_t are both relatively large and yet sufficiently different to distinguish.

Transmittance and Absorbance

Although the Beer-Lambert law shows that **absorbance** is the quantity which is directly proportional to concentration (and pathlength), in the early days of spectroscopy, electronics were not as sophisticated as today and measurements were made in terms of the transmission properties of a sample. IR spectra are still often reported as transmittance spectra despite the ability of the modern FT spectrometer to easily report A values. The transparencies of optical components are also often described in terms of transmittance.

Transmittance (T) is defined simply as the ratio of the transmitted intensity (I_t) and the incident intensity (I_o):

$$T = I_t/I_o \quad (9)$$

The numerical values of transmittance lie between one and zero.

Percentage transmission (%T) is defined as :

$$\%T = 100.(I_t/I_o) \quad (10)$$

Percent transmission (%T) has numerical values between 100 and zero. For qualitative work (especially in the IR region), T and %T measurements have the advantage that weaker features are more readily observed by eye. However, in these computer-based days and using library searching, the Absorbance scale is better. The absorbance scale is a must for quantitative analysis.

Since $A = \log(I_o/I_t)$, scale conversions are simple:

$$A = \log(I_o/I_t) = \log(1/T) = -\log(T) \quad (11)$$

$$A = -\log(\%T/100) = 2 - \log(\%T) \quad (12)$$

In some applications (e. g. stray light corrections) it is necessary to convert from absorbance to transmittance:

$$T = 10^{-A} = -\text{antilog}(A) \quad (13)$$

$$\%T = 100\ (10^{-A}) = 100\ (-\text{antilog } A) \quad (14)$$

2 The Absorption Spectrum

An absorption spectrum is plotted to show how the characteristic absorption of a substance varies with wavelength (wavenumber or frequency). Certain conventions have been adopted for the presentation of absorption spectra. In optical spectroscopy, the abscissa should be always be either the wavelength or wavenumber ($\bar{\nu}$), plotted so that wavelength increases from left to right, and wavenumber decreases from left to right. The ordinate scale may be absorbance (A), log (A) extinction coefficient (ε), log(ε) if the change in ε is large or transmittance (T) / %Transmittance (%T). All these scales are used for particular purposes. Obviously, great care is required when comparing spectra recorded on different scales. Figure 4 illustrates a typical UV/Vis spectrum and shows some of the terms frequently used to describe the features of spectrum.

The wavelengths (λ_{max}) corresponding to the A_{max} values are the wavelengths ($E=hc/\lambda$) required to promote excitations. However, the excitation can be achieved over a range of wavelengths either side of the λ_{max} because the process is not so simple (eg exciting electrons also implies exciting vibrations. Therefore, an absorption feature associated with a particular transition has a width and the term absorption band associated with a particular spectroscopic transition is often used.

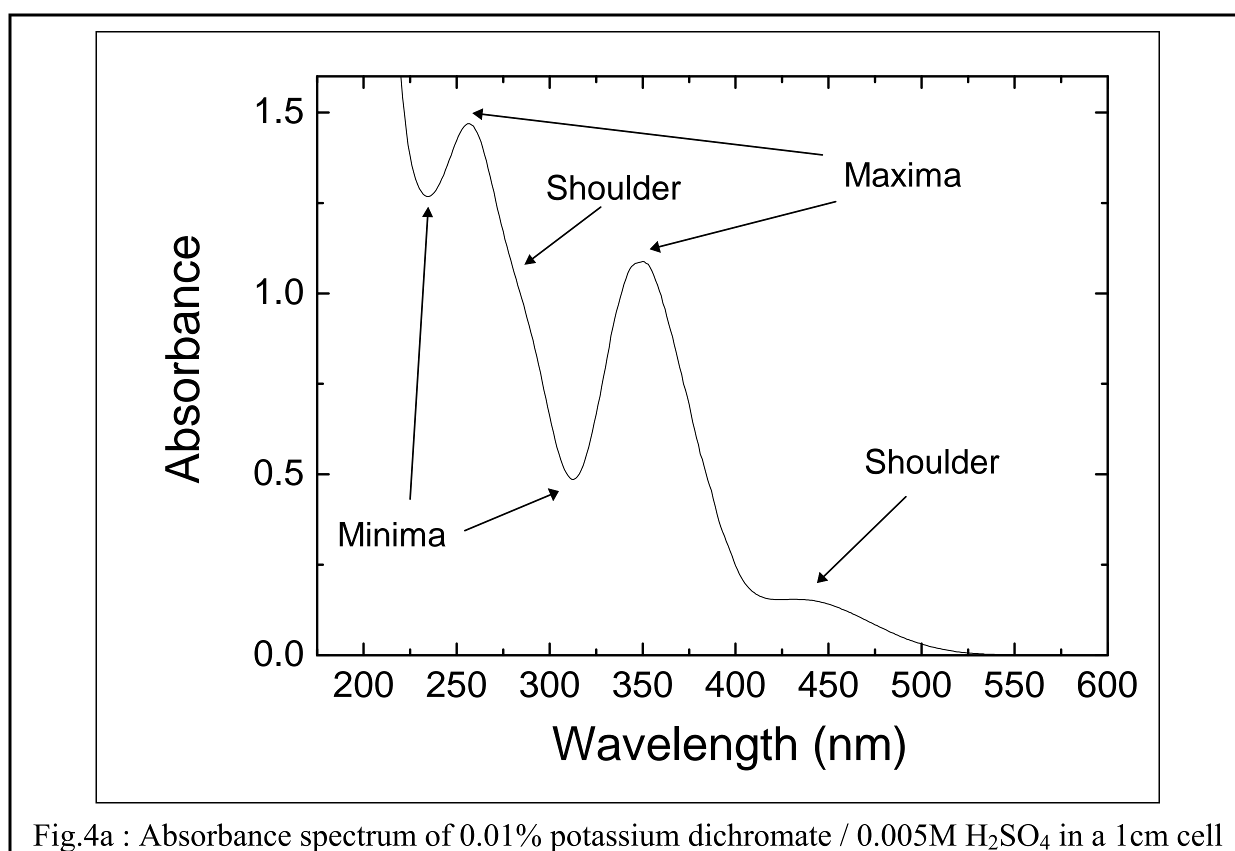

Fig.4a : Absorbance spectrum of 0.01% potassium dichromate / 0.005M H_2SO_4 in a 1cm cell

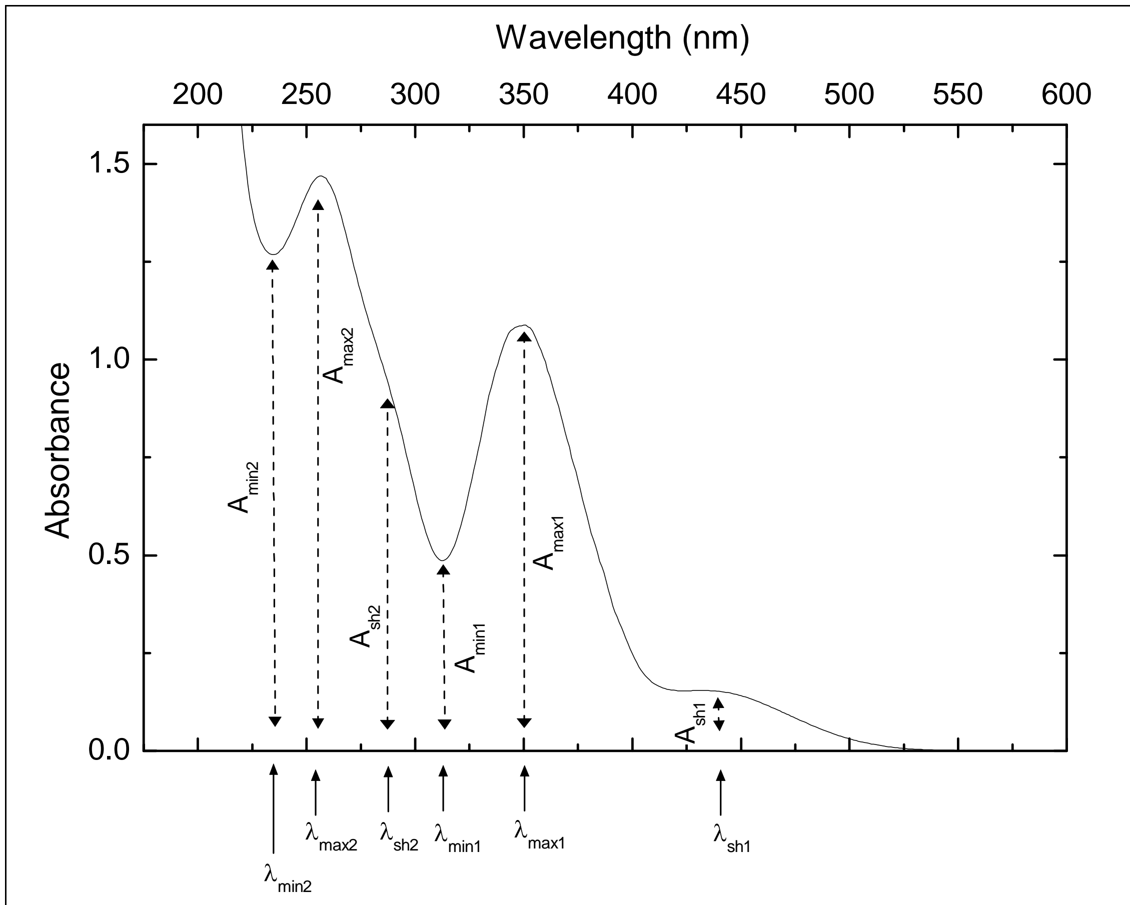

Fig.4b : Absorbance spectrum of 0.01% potassium dichromate / 0.005M H_2SO_4 in a 1cm cell

2.1 The Absorption Coefficients

The three most commonly used absorption coefficients are :

Molar Extinction Coefficient, ε

The Molar Extinction Coefficient, ε, is the most fundamental of all the common absorption coefficients.

$$\varepsilon = A/(c.l) \qquad (15)$$

where A = absorbance, **c = concentration in moles per litre** and *l* = pathlength.
The units of ε are $M^{-1}cm^{-1}$.
The numerical value of ε denotes the (theoretical) absorbance that would be measured for a one molar solution contained in a transparent cuvette of 1 cm path length.
In terms of concentrations quoted as x=mg/ml :

$$\varepsilon = \frac{A \cdot MW}{x(mg/ml) \cdot l(cm)} \qquad (16)$$

where MW = molecular weight.

Specific Absorption Coefficient A[1%, 1cm]

If the molecular weight is not known, as for a new compound or an analyte that is a difficult to fully characterise (natural product or polymer), the Specific Absorption Coefficient is widely used in analytical work. The specific absorbance is simply the numerical value of A that would be obtained for a 1% w/v solution contained in a transparent cuvette of 1 cm pathlength. The concentration units for Specific Absorption Coefficient are **gram per 100 ml or percent w/v**.
The specific absorbance is given the symbol A(1%,1cm) or perhaps better $A_1^1(\lambda nm)$.

Absorptivity α or a

Absorptivity is used mainly in the U.S. literature, the concentration units are **gram per litre**. In many ways this is a more rational coefficient than A(1%,lcm) since it is more simply related to the molar extinction coefficient **ε.** In the UK and the EC the accepted practice is to use (A1%, 1 cm) based upon “gram per 100 ml or percent w/v”.

2.2 The measurement of Absorption coefficients, absorbance and concentration

Nearly all UV/VIS analytical methods depend upon the Beer- Lambert law being obeyed, so that concentrations may be determined simply from the measured absorbance at a specified wavelength by using the equation:

$$c = \frac{A_\lambda}{\varepsilon_\lambda . \ell} \tag{17}$$

where c is molarity

$$x = \frac{A}{A_1^1(\lambda nm) \cdot \ell} \tag{18}$$

where x is the concentration in g/100ml.

This approach implies that

i) An accurate and precise measurement of absorbance is made (spectrometer performance).
ii) A reliable value of the absorption coefficient is available.
iii) The pathlength is accurately known.

Absorbance is defined as $A=\log(I_o/I_t)$. Two measurements are required to determine I_o and I_t. These need to be made separately in a single beam instrument or continuously in a double instrument. The reference beam in a double beam spectrometer is effectively measuring I_o continuously. A single beam instrument (eg IR spectrometer) requires the I_o to be measured separately often at the beginning of a measurement session and must remain stable through the rest of the session – this can be difficult to guarantee.

When measuring the spectrum of a solution it is important to appreciate that the analyte, solvent and cuvette can all contribute to the absorption at a given wavelength :

$$A_{measured} = A_{sample} + A_{solvent} + A_{cuvette}$$

$$A_{sample} = A_{measured} - (A_{solvent} + A_{cuvette}) \tag{19}$$

To determine A_{sample} , the value of ($A_{solvent} + A_{cuvette}$) needs to be determined. In a single beam measurement this achieved with a separate measurement of (cuvette + solvent). In a double beam instrument a matched cuvette with solvent is placed in the reference beam. In the double beam instrument the reduction in light intensity due to the (cuvette + solvent) is automatically compensated for and it can be said that the reference beam is monitoring the effective I_o and the sample beam is monitoring I_t.

The measurement of the reference (baseline) is as important as the measurement of the sample itself.

The composition of the reference solution is very important. It must contain the same ingredients as the sample (e.g. buffers, neutral salts, colour-forming reagents etc….), except

for the component being analysed. Many (otherwise reasonable) spectrophotometric analytical procedures have failed to produce consistent results because insufficient attention has been paid to the reference measurement. Except where otherwise indicated, it is assumed that absorbances for a sample have been measured against the correct reference, and the value of A refers only to the analyte(s) in the sample.

Absorbance Ratios as Tests for Purity/Identity

When an absorption spectrum shows two or more well defined features such as peaks (maxima), minima, or shoulders, it is often possible to use the ratio of absorbances at these points as an aid to identification or as a check on the purity of a sample. Supposing that Figure 2 represents the absorption spectrum of a pure sample of a substance, then several absorbance ratios can be calculated :

$$\left(\frac{A_{\lambda 2}}{A_{\lambda 1}}\right),\ \left(\frac{A_{\lambda 3}}{A_{\lambda 1}}\right),\ \left(\frac{A_{\lambda 4}}{A_{\lambda 1}}\right)\ \ldots\ldots\ldots\ldots\ldots\ \text{etc.} \qquad (20)$$

The peak absorbance $A_{\lambda 1}$ is often known as a 'reference' point. These ratios are absolute properties of the molecule (unlike A alone), because they are <u>independent of concentration</u> and path length.

$$\frac{A_{\lambda 2}}{A_{\lambda 1}} = \frac{\varepsilon_{\lambda 2}\cdot c\cdot \ell}{\varepsilon_{\lambda 1}\cdot c\cdot \ell} = \frac{\varepsilon_{\lambda 2}}{\varepsilon_{\lambda 1}} = \text{constant} \qquad (21)$$

since c and ℓ cancel.

The identity of any sample can then be confirmed by comparing its absorbance ratios with those obtained for the pure material. If the ratio at a given wavelength varies either the sample is impure or is not the same chemical compound as the pure standard.

Absorbance ratios are used particularly for the quality control of nucleic acids (A_{260}/A_{230}) and in diode-array spectroscopy.

Analysis of Mixtures

If two or more absorbing compounds are present in an analytical sample the absorbance of the various species will be additive. At a given wavelength :

$$A_{obs} = A_a + A_b + A_c + \ldots\ldots\ldots\ldots\ldots\ldots\text{etc.} \qquad (22)$$

where A_{obs} is the observed absorbance of the solution mixture; A_a, A_b, $A_{c,}$ refer to the absorbances of the components a, b, c ….etc.
Applying the Beer-Lambert law to a 2-component system gives :

$$A_{obs,\lambda} = \left[\left(\varepsilon_{a,\lambda}\cdot c_a\right) + \left(\varepsilon_{b,\lambda}\cdot c_b\right)\right]\cdot \ell \qquad (23)$$

At a single wavelength, it is not possible to distinguish components a and b.

Distinctions can be made using two or more wavelengths.

$$A_{obs,\lambda 1} = \left[\left(\varepsilon_{a,\lambda 1} \cdot c_a \right) + \left(\varepsilon_{b,\lambda 1} \cdot c_b \right) \right] \cdot \ell$$

$$A_{obs,\lambda 2} = \left[\left(\varepsilon_{a,\lambda 2} \cdot c_a \right) + \left(\varepsilon_{b,\lambda 2} \cdot c_b \right) \right] \cdot \ell \qquad (24)$$

Knowing the pathlength $\boldsymbol{\ell}$, determining $\varepsilon_{a,\lambda 1}$, $\varepsilon_{a,\lambda 2}$ $\varepsilon_{b,\lambda 1}$, $\varepsilon_{b,\lambda 2}$ independently with solutions of the individual components and measuring $A_{obs,\lambda 1}$ and $A_{obs,\lambda 2}$ will allow the solution of the two simultaneous equations to give the concentrations of the two species c_a and c_b.

Choice of Analytical Wavelength

The choice of an appropriate wavelength for the measurement of absorbance is crucial to the success of any spectrophotometric assay and this choice can only be properly made with reference to an absorption spectrum of the sample in question. Two factors influence the choice of the analytical wavelength:-

i) High sensitivity of measured absorbances with respect to concentration,
ii) Minimal interferences from other absorbing species,
iii) Minimal interference to measurements from spectrophotometer characteristics and operation.

The most obvious choice is the wavelength of maximum absorption (e.g. the absorption coefficient has its greatest value, and the Beer's law plot its greatest slope). Consequently, a small change in concentration is more readily detected than at any other wavelength. Also, at this wavelength the absorbance is least sensitive to any mis-setting of the wavelength. This is particularly relevant for measurements at wavelengths on the side of an absorption band. However, λ_{max} may occur at the peak of a very narrow absorption band so that small errors in setting this wavelength may shift the measurement from the peak to the steep sides of the band and so reduce the reproducibility of measurement quite markedly. This kind of behaviour is typical of IR bands which are sharp compared with the bandwidth of the instrument and very precise wavelength setting is required for quantitative accuracy. Ultraviolet spectra rarely show sharp absorption bands and the primary consideration here is to choose a band having a high absorption coefficient.

The instrumental characteristics must also be considered. For example, the strongest band in a spectrum may occur at 200 nm, but at this short wavelength the photometric accuracy of many instruments (and UV detectors in HPLC) is poor so that reproducibility is low. The best option is to choose another band at longer wavelength for the measurements. In HPLC, λ=254nm is a popular choice of analytical wavelength. This is not because of the sample character but because traditionally Mercury (Hg) lamps give a very intense light output at this wavelength which can be easily selected with a filter.

3. **The UV/Vis Absorption Spectrometer**

The actual design of a spectrometer depends upon the spectral range being studied and is very manufacturer dependent. This section describes a typical UV/Vis absorption spectrometer with the layout presented in Figure 5.

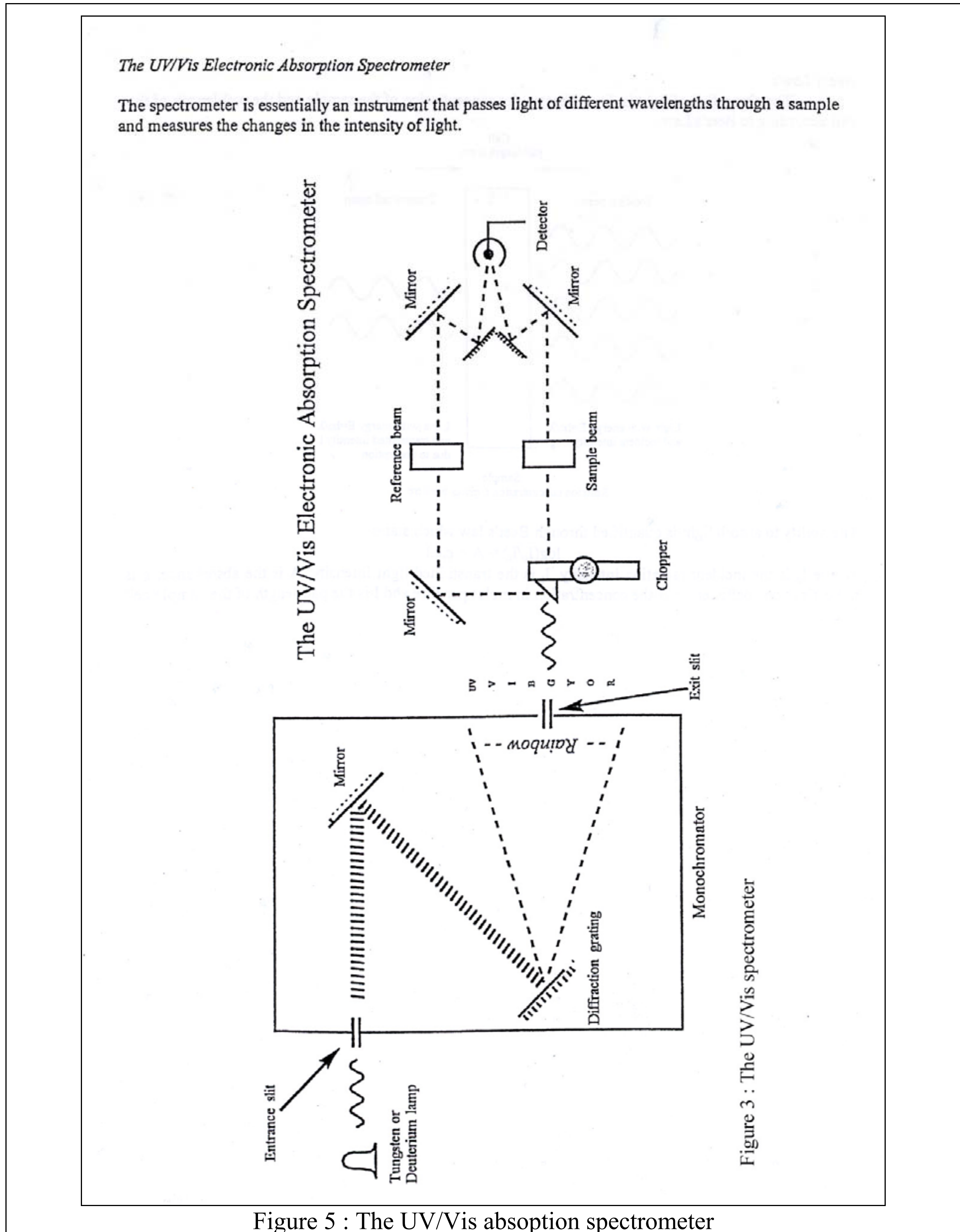

Figure 5 : The UV/Vis absoption spectrometer

Light from a Deuterium or a tungsten lamp is focussed onto the entrance slit of a monochromator. The UV discharge lamp (deuterium lamp), providing UV light (360-190nm), operates on the same principle as the neon strip light (but in miniature). The tungsten lamp, providing visible and near IR light is a miniature version of a domestic filament lamp of the type used in projector. The polychromatic light coming from the entrance slit is reflected by a mirror onto a diffraction grating that splits the light into its wavelength components. A "rainbow" is cast on the exit slit that only allows through light of a single wavelength (monochromatic light) (see spectral bandwidth). Remember the colours of the rainbow (ROYGBIV followed by UV wavelengths). The UV runs from 190 to 360nm; the visible runs from 360 to 750nm; the wavelength range 750 to 2500nm is known as the near IR. The mid-IR runs from 2500nm ($4000cm^{-1}$) to 20000nm ($500cm^{-1}$). A controlled rotation of the diffraction grating by the wavelength drive motor moves the "rainbow" across the exit slit. Accordingly, the wavelength of light coming from the exit slit of a monochromator can be controlled.

The intensity I_t needs to measured with respect to I_o. In principle they could be measured separately in two different measurements (single beam spectrometer). However, I_o could fluctuate during the measurement and I_t and hence A will vary accordingly. Hence it is best to effectively measure I_o and I_t simultaneously. This is most conveniently achieved by sending the light beam along two channels, a reference beam to continuously measure I_o and a sample beam to continuously measure I_t.(double beam spectrometer) An optical beam splitter or mechanical chopper is required after the monochromator exit slit. A rotating mechanical chopper sends the monochromatic light from the monochromator exit slit alternately along two paths known as the sample and reference beams. The "chopper" is a rotating disc carrying a hole and a mirror that allows the light to pass through to the sample chamber or reflects the light towards the reference chamber.

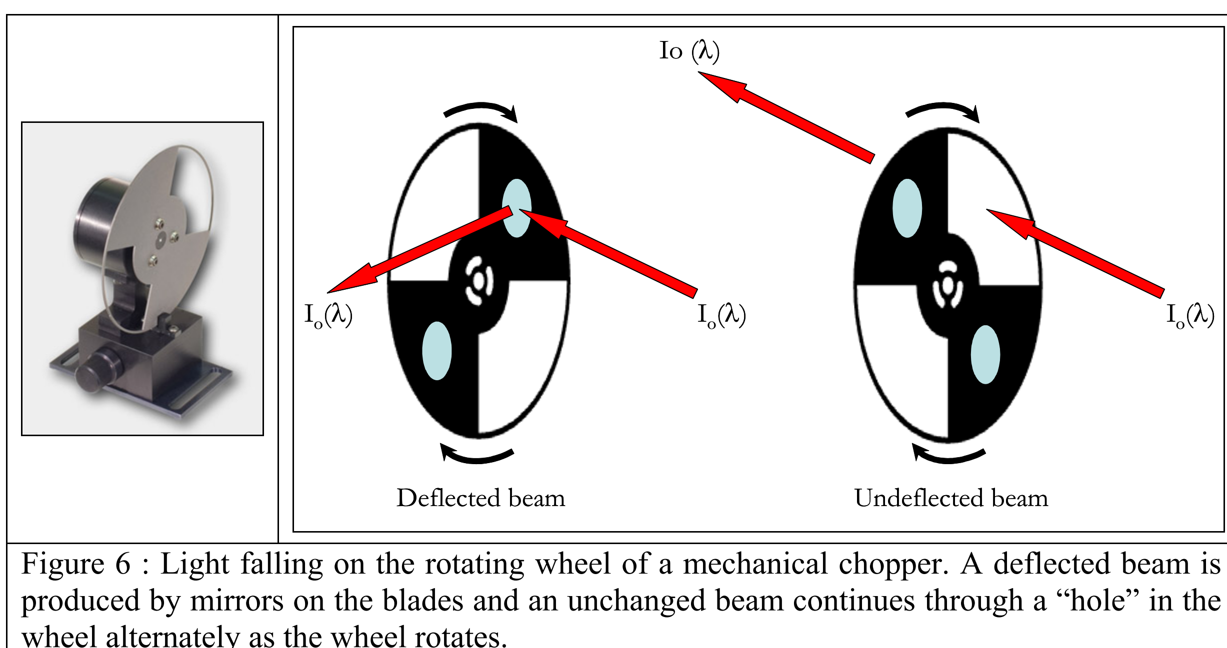

Figure 6 : Light falling on the rotating wheel of a mechanical chopper. A deflected beam is produced by mirrors on the blades and an unchanged beam continues through a "hole" in the wheel alternately as the wheel rotates.

After passing though the sample or reference cuvette the light is focussed onto a single light detector. During the period that the detector registers the reference beam, I_o is effectively measured. I_t is effectively detected during the period the light passes through the sample (see Figure 7).

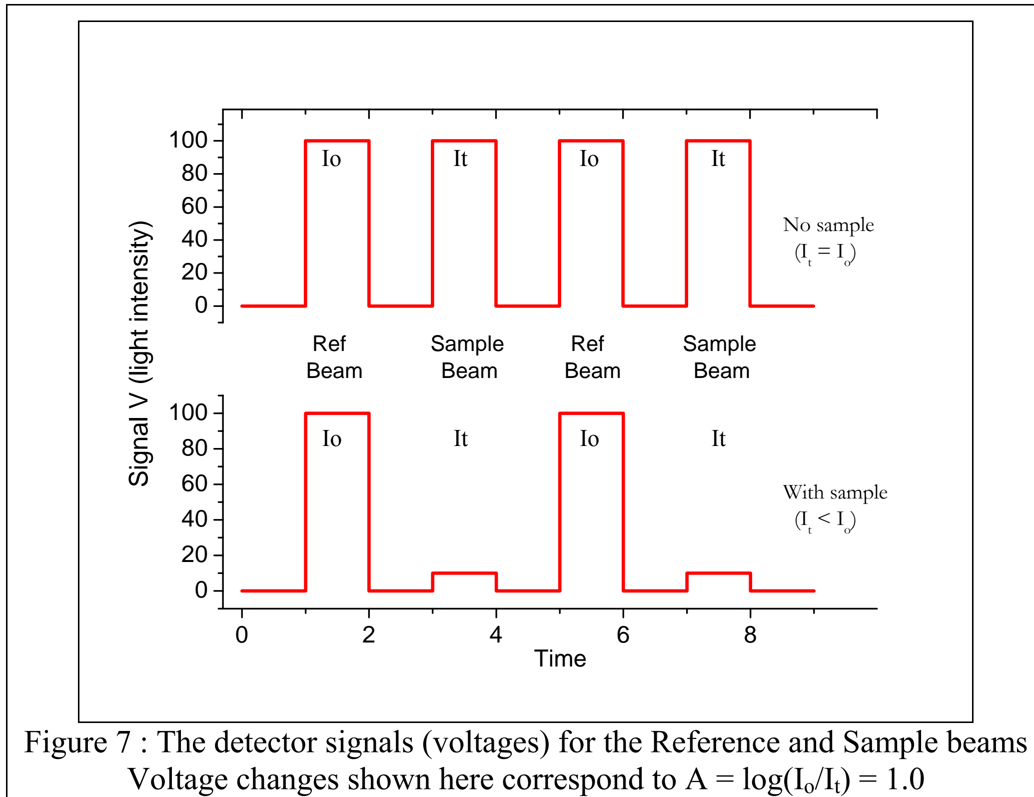

Figure 7 : The detector signals (voltages) for the Reference and Sample beams
Voltage changes shown here correspond to $A = \log(I_o/I_t) = 1.0$

The detector electronics now allow the determination of the absorbance at a particular wavelength as :

$$A = \log\left(\frac{I_o}{I_t}\right) = \log\left(\frac{V_{reference}}{V_{sample}}\right) = \log\left(V_{reference}\right) - \log\left(V_{sample}\right) \qquad (25)$$

where $V_{reference}$ and V_{sample} are the voltages produced by the detector during the reference and sample time periods respectively.

3.1 Spectrophotometric Calibrations

To ensure that measurements with a spectrometer are reliable, the spectrometer needs to be calibrated. Typically a spectrometer is tested to ensure that its performance complies with the requirements of an official accepted document. In Pharmaceutical Science, normally this implies ensuring that the performance of a spectrometer is Pharmacopeia Compliant.

Read the BP & EP sections on this topic to see what a Pharmacopeia requires.

There are 6 major features that need to be addressed when ensuring the quality of a spectrum:

i) Wavelength calibration
ii) Absorbance calibration
iii) Spectral bandwidth calibration
iv) Stray light determination
v) Time scale of measurement
vi) Data resolution

i) Wavelength calibration

Suitable wavelength standards for the ultraviolet region are holmium and didymium glasses. These rare-earth elements have a number of sharp bands distribute throughout the ultraviolet and visible regions so that calibration in different parts of the wavelength scale is simple.
The sharp lines in the absorption spectra of gases (benzene, NH_3) are often used. For very high precision work, the emission lines from Hg discharge lamps are used.
Polystyrene film is used for wavelength calibration in the IR region, but only certain sharp bands are used. More precise standards, such as indene (liquid film), and the vibrational-rotational spectra of certain gases (NH_3 , CO, H_2O), may be used in fundamental studies on molecular structure.

Questions :

i) What is a lanthanide or rare-earth element ?
ii) What is responsible for the Holmium spectrum in the visible region ?

ii) Photometric (absorbance) calibration

Photometric (absorbance) calibration standards are more difficult to select. In the UV region standard solutions of pure chemicals such as potassium dichromate, potassium nitrate and potassium chromate are probably the most widely used and the most useful. The most important properties required of an absorbance standard are that it should :

i) be reproducibly pure, weighed out and dissolved in a given volume accurately in pure solvent and measured using a cell with known pathlength,
ii) obey Beer's law,
iii) show fairly broad, absorption peaks to minimise absorbance errors due to poor wavelength setting.

Photometric standards for the IR region are very difficult to specify: probably the most effective and reproducible standards are wire grids, or rotating sectors of known transmittance. Calibrated smoked glass filters or wire grids known as neutral density filters are also often used to calibrate absorbance in the UV/Vis.

Questions :
What is the relationship between the visible absorption spectrum and colour ?

iii) Spectral band width

The wavelength passing through the sample (and the reference) is controlled by the exit slit of the monochromator. This is essentially a mechanical selection of a range of wavelengths from the “rainbow”. Accordingly, a monochromator cannot produce pure monochromatic light with a single wavelength. There is a spread about a central wavelength. This is the spectral bandwidth. A wavelength spread of ±0.5nm is called a spectral bandwidth of 1nm (SBW=1nm). For example, a wavelength of 250nm with a wavelength spread from 249.5nm to 250.5nm represents a SBW=1nm.
The spectral bandwidth affects the observed resolution of a spectrum. Larger slits (large SBW) can lead to the broadening (or loss) of fine features with reduction of detail and a reduction in the observed A_{max}. Fortunately, the UV/Vis absorption bands of the vast majority drugs are broad and SBW=1.0nm is sufficient. The European Pharmacopoeia currently claims that SBW=1.7nm is good enough for the pharmaceutical sciences.
Narrower slits are preferred for the sharp spectral features used to calibrate wavelength.
The issue of spectral bandwidth is particularly important in the IR where all spectral features are relatively sharp. In the IR, the wavenumber unit is used and spectral bandwidths of the order of 1.0, 2.0, 4.0 and 8.0cm^{-1} need to be considered.

Opening the slits means that the light intensity available for detection is greater (entrance slit = exit slit; double SBW means 4 time more light). The instrument is now more sensitive but its performance is compromised. In lower cost spectrometers and in chromatography sensitivity is the main issue. SBW≥4nm is now employed. This is not good enough for GLP and other QC spectroscopic determinations of concentration.

iv) Stray Light

Stray light refers to light passing through the sample that is not the wavelength specified by the monochromator. It is light of the wrong wavelength that should not be there (nothing is perfect) and is usually light that is not absorbed.
Referring to Figure 8, consider an incident light beam with intensity I_o=100 which is 99% pure (I_o(true)=99) with 1% stray light.

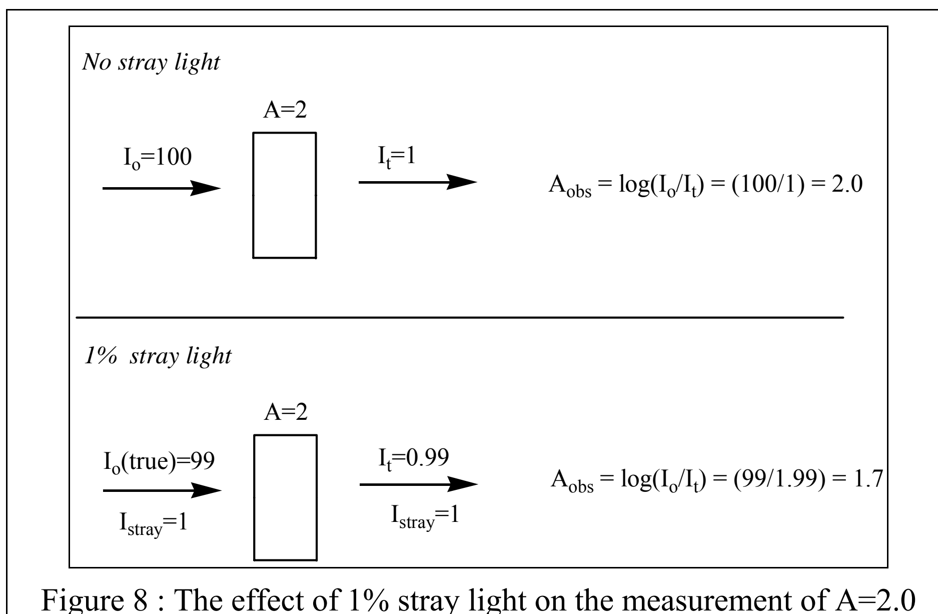

Figure 8 : The effect of 1% stray light on the measurement of A=2.0

Stray light :

a) is particularly import for the measurement of high absorbance values either from the analyte itself or the matrix (eg. solvent, body fluid). A good spectrometer has a stray light figure of <0.1%. The light must be >99.9% “pure”. This means perfect optics which may involve extra light filters or two monochromators in series (double monochromator) and therefore greater cost.
b) is particularly relevant at the extreme wavelengths of a spectrometer where lamps and/or detectors begin to fail particularly towards 200nm in the UV.
c) is one of the most common causes of non-linearity and non-reproducibility in quantitative spectrophotometry and is undoubtedly the most difficult source of error to measure and control.
d) has many origins, such as holes in the monochromator casing, anomalous reflections and scattering from cheaper or dust-covered mirrors and diffraction gratings.

v) Time sale of a measurement

How long does it take to scan a spectrum ? How fast can the wavelength be changed ?
Is one hour needed or can the spectrum be run in 1 second. In chromatography it is necessary to run spectra whilst the sample is the flow cell (<1 second).

These questions are very much related to the instrument response time. The answer is to run the spectrum fast enough for convenience but slow enough to ensure that there are no spectral distortions. In the UV/Vis a scan speed of ~60nm per minute is normal. In the IR, the spectrum scan time is set by the oscillation of the mirror of the interferometer and the question concerns how many spectra need to be scanned, accumulated and averaged to give a good result. In the IR normally a single scan is enough with a total processing time of about 1 minute.

vi) Data resolution

In the computer world, spectral data is stored as digital files of two sets of numbers : absorbances (or intensities) and wavelengths. These two sets of numbers are recalled from the data storage and plotted as “y” against “x” to produce the spectrum “graph”. A Microsoft Excel spreadsheet would be a convenient storage venue. The question now raised is how many data points need to be stored in order to redraw the spectrum. To reproduce a single spectral feature at least 20 x/y data pairs are required. In practice, a UV/Vis spectrum can be well described recording a data point every 0.2 or 0.5nm. Fewer data points can produced distorted spectrum with a saw-tooth appearance.
For a spectrum from 300 to 200nm this implies about 500 to 200 points. In the IR data resolution is typically 1cm^{-1} which for a spectrum from 4000 to 500cm^{-1} implies the storage of 3500 data points. Spectral measurements like Mass spectrometry or NMR can produce very large data files for the faithful storage and retrieval of spectra.