Introduction

Ultraviolet and visible spectrometers have been in general use for the last 35 years and over this period have become the most important analytical instrument in the modern day laboratory. In many applications other techniques could be employed but none rival UV-Visible spectrometry for its simplicity, versatility, speed, accuracy and cost-effectiveness.

This description outlines the basic principles for those new to UV-Visible spectrometry. It is intended purely as a brief introduction to the technique and it is Thermo Spectronic's policy to continually add to this range of documentation for further details, as they become available.

Definitions and Units

Radiation is a form of energy and we are constantly reminded of its presence via our sense of sight and ability to feel radiant heat. It may be considered in terms of a wave motion where the wavelength, $\lambda$, is the distance between two successive peaks. The frequency, $\nu$, is the number of peaks passing a given point per second. These terms are related so that:

$$c = \nu \lambda$$

where $c$ is the velocity of light in a vacuum.

![Figure 1 The wavelength $\lambda$ of electromagnetic radiation](image)

The full electromagnetic radiation spectrum is continuous and each region merges slowly into the next. For spectroscopy purposes, we choose to characterize light in the ultraviolet and visible regions in terms of wavelength expressed in nanometers. Other units which may be encountered, but whose use is now discouraged, are the Angstrom (Å) and the millimicron (m$\mu$).

$$1\text{nm} = 1\text{m}$\mu$ = 10Å = 10^{-9} \text{meters}$$
Basic UV-Vis Theory, Concepts and Applications

For convenience of reference, definitions of the various spectral regions have been set by the Joint Committee on Nomenclature in Applied Spectroscopy:

<table>
<thead>
<tr>
<th>Region</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Far ultraviolet</td>
<td>10-200</td>
</tr>
<tr>
<td>Near ultraviolet</td>
<td>200-380</td>
</tr>
<tr>
<td>Visible</td>
<td>380-780</td>
</tr>
<tr>
<td>Near infrared</td>
<td>780-3000</td>
</tr>
<tr>
<td>Middle infrared</td>
<td>3000-30,000</td>
</tr>
<tr>
<td>Far infrared</td>
<td>30,000-300,000</td>
</tr>
<tr>
<td>Microwave</td>
<td>300,000-1,000,000,000</td>
</tr>
</tbody>
</table>

The human eye is only sensitive to a tiny proportion of the total electromagnetic spectrum between approximately 380 and 780 nm and within this area we perceive the colors of the rainbow from violet through to red. If the full electromagnetic spectrum shown in Figure 2 was redrawn on a linear scale and the visible region was represented by the length of one centimeter, then the boundary between radio and microwaves would have to be drawn approximately 25 kilometers away!

Figure 2 The electromagnetic spectrum

Radiation Sources

Besides the sun, the most conveniently available source of visible radiation with which we are familiar is the tungsten lamp. If the current in the circuit supplying such a lamp is gradually increased from zero, the lamp filament at first can be felt to be emitting warmth, then glows dull red and the gradually brightens until it is emitting an intense white light and a considerable amount of heat.
Basic UV-Vis Theory, Concepts and Applications

The radiation from normal hot solids is made up of many wavelengths and the energy emitted at any particular wavelength depends largely on the temperature of the solid and is predictable from probability theory. The curves in Figure 3 show the energy distribution for a tungsten filament at three different temperatures. Such radiation is known as 'black body radiation'. Note how the emitted energy increases with temperature and how the wavelength of maximum energy shifts to shorter wavelengths. More recently it has become common practice to use a variant of this - the tungsten-halogen lamp. The quartz envelope transmits radiation well into the UV region. For the UV region itself the most common source is the deuterium lamp and a UV-Visible spectrometer will usually have both lamp types to cover the entire wavelength range.

Quantum Theory

To gain an understanding of the origins of practical absorption spectrometry, a short diversion into quantum theory is necessary. For this purpose, it is best to think of radiation as a stream of particles known as photons instead of the waves considered earlier. Atoms and molecules exist in a number of defined energy states or levels and a change of level requires the absorption or emission of an integral number of a unit of energy called a quantum, or in our context, a photon.

The energy of a photon absorbed or emitted during a transition from one molecular energy level to another is given by the equation

\[ E = h\nu \]

where \( h \) is known as Planck's constant and \( \nu \) is the frequency of the photon. We have already seen that \( c = \nu\lambda \), therefore, \( E = hc/\lambda \).
Thus, the shorter the wavelength, the greater the energy of the photon and vice versa.

A molecule of any substance has an internal energy which can be considered as the sum of the energy of its electrons, the energy of vibration between its constituent atoms and the energy associated with rotation of the molecule.

The electronic energy levels of simple molecules are widely separated and usually only the absorption of a high energy photon, that is one of very short wavelength, can excite a molecule from one level to another.

![Energy levels of a molecule](image)

In complex molecules the energy levels are more closely spaced and photons of near ultraviolet and visible light can effect the transition. These substances, therefore, will absorb light in some areas of the near ultraviolet and visible regions.

The vibrational energy states of the various parts of a molecule are much closer together than the electronic energy levels and thus protons of lower energy (longer wavelength) are sufficient to bring about vibrational changes. Light absorption due to only to vibrational changes occurs in the infrared region. The rotational energy states of molecules are so closely spaced that light in the far infrared and microwave regions of the electromagnetic spectrum has enough energy to cause these small changes.
For ultraviolet and visible wavelengths, one should expect from this discussion that the absorption spectrum of a molecule (i.e., a plot of its degree of absorption against the wavelength of the incident radiation) should show a few very sharp lines. Each line should occur at a wavelength where the energy of an incident photon exactly matches the energy required to excite an electronic transition.

In practice it is found that the ultraviolet and visible spectrum of most molecules consists of a few humps rather than sharp lines. These humps show that the molecule is absorbing radiation over a band of wavelengths. One reason for this band, rather than line absorption is that an electronic level transition is usually accompanied by a simultaneous change between the more numerous vibrational levels. Thus, a photon with a little too much or too little energy to be accepted by the molecule for a 'pure' electronic transition can be utilized for a transition between one of the vibrational levels associated with the lower electronic state to one of the vibrational levels of a higher electronic state.

If the difference in electronic energy is 'E' and the difference in vibrational energy is 'e', then photons with energies of E, E+e, E+2e, E-e, E-2e, etc. will be absorbed.

Furthermore, each of the many vibrational levels associated with the electronic states also has a large number of rotational levels associated with it. Thus a transition can consist of a large electronic component, a smaller vibrational element and an even smaller rotational change. The rotational contribution to the transition has the effect of filling in the gaps in the vibrational fine structure.

In addition, when molecules are closely packed together as they normally are in solution, they exert influences on each other which slightly disturb the already numerous, and almost infinite energy levels and blur the sharp spectral lines into bands. These effects can be seen in the spectra of benzene as a vapor and in solution. In the vapor, the transitions between the vibration levels are visible as bands superimposed on the main electronic transition bands.

In solution they merge together and at high temperature or pressure even the electronic bands can blur to produce single wide band such as that enclosed by the dotted line in Figure 6.
General Chemical Origins

When white light falls upon a sample, the light may be totally reflected, in which case the substance appears white or the light may be totally absorbed, in which case the substance will appear black. If, however, only a portion of the light is absorbed and the balance is reflected, the color of the sample is determined by the reflected light. Thus, if violet is absorbed, the sample appears yellow-green and if yellow is absorbed, the sample appears blue. The colors are described as complementary. However, many substances which appear colorless do have absorption spectra. In this instance, the absorption will take place in the infra-red or ultraviolet and not in the visible region. Table 1 illustrates the relationship between light absorption and color.
A close relationship exists between the color of a substance and its electronic structure. A molecule or ion will exhibit absorption in the visible or ultraviolet region when radiation causes an electronic transition within its structure. Thus, the absorption of light by a sample in the ultraviolet or visible region is accompanied by a change in the electronic state of the molecules in the sample. The energy supplied by the light will promote electrons from their ground state orbitals to higher energy, excited state orbitals or antibonding orbitals.

Potentially, three types of ground state orbitals may be involved:

i) $\sigma$ (bonding) molecular as in

```
\[
\begin{array}{c}
\text{C} \\
\text{C}
\end{array}
\]
```

ii) $\pi$ (bonding) molecular orbital as in

```
\[
\begin{array}{c}
\text{C} \\
\text{O} \\
\text{N} \\
\text{C}
\end{array}
\]
```
iii) \( n \) (non-bonding) atomic orbital as in

\[
\begin{align*}
\text{C} & \quad \text{Br} \quad \text{C} & \quad \text{OH} \quad \text{C} & \quad \text{NH}
\end{align*}
\]

In addition, two types of antibonding orbitals may be involved in the transition:

i) \( \sigma^* \) (sigma star) orbital

ii) \( \pi^* \) (pi star) orbital

(There is no such thing as an \( n^* \) antibonding orbital as the \( n \) electrons do not form bonds).

A transition in which a bonding s electron is excited to an antibonding \( \sigma \) orbital is referred to as \( \sigma \) to \( \sigma^* \) transition. In the same way \( \pi \) to \( \pi^* \) represents the transition of one electron of a lone pair (non-bonding electron pair) to an antibonding \( \pi \) orbital. Thus the following electronic transitions can occur by the absorption of ultraviolet and visible light:

\[
\begin{align*}
\sigma & \rightarrow \sigma^*, \\
n & \rightarrow \sigma^*, \\
n & \rightarrow \pi^*, \\
\pi & \rightarrow \pi^*.
\end{align*}
\]

Figure 7 illustrates the general pattern of energy levels and the fact that the transitions are brought about by the absorption of different amounts of energy.

Figure 7 Energy and molecular transitions

Both s to \( \sigma^* \) and \( n \) to \( \sigma^* \) transitions require a great deal of energy and therefore occur in the far ultraviolet region or weakly in the region 180-240nm. Consequently, saturated groups do not exhibit strong absorption in the ordinary ultraviolet region. Transitions of the \( n \) to \( \pi^* \) and \( \pi \) to \( \pi^* \) type occur in molecules with unsaturated centers; they require less energy and occur at longer

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wavelengths than transitions to \( \sigma^* \) antibonding orbitals. Table 2 illustrates the type of transition and the resulting maximum wavelength.

Table 2 Examples of transitions and resulting \( \lambda_{\text{max}} \)

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Transition</th>
<th>( \lambda_{\text{max}} ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethane</td>
<td>( \sigma \rightarrow \sigma^* )</td>
<td>135</td>
</tr>
<tr>
<td>Methanol</td>
<td>( \sigma \rightarrow \sigma^* )</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>( \pi \rightarrow \pi^* )</td>
<td>175</td>
</tr>
<tr>
<td>Benzene</td>
<td>( \pi \rightarrow \pi^* )</td>
<td>254</td>
</tr>
<tr>
<td>Acetone</td>
<td>( \pi \rightarrow \pi^* )</td>
<td>290</td>
</tr>
</tbody>
</table>

It will be seen presently that the wavelength of maximum absorption and the intensity of absorption are determined by molecular structure. Transitions to \( \pi^* \) antibonding orbitals which occur in the ultraviolet region for a particular molecule may well take place in the visible region if the molecular structure is modified. Many inorganic compounds in solution also show absorption in the visible region. These include salts of elements with incomplete inner electron shells (mainly transition metals) whose ions are complexed by hydration e.g. \([\text{Cu(H}_2\text{O)}_4]^{2+}\). Such absorptions arise from a charge transfer process, where electrons are moved from one part of the system to another by the energy provided by the visible light.
Correlation of Molecular Structure and Spectra Conjugation

π to π* transitions, when occurring in isolated groups in a molecule, give rise to absorptions of fairly low intensity. However, conjugation of unsaturated groups in a molecule produces a remarkable effect upon the absorption spectrum. The wavelength of maximum absorption moves to a longer wavelength and the absorption intensity may often increase.

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In general, the greater the length of a conjugated system in a molecule, the nearer the $\lambda_{\text{max}}$ comes to the visible region.

Thus, the characteristic energy of a transition and hence the wavelength of absorption is a property of a group of atoms rather than the electrons themselves. When such absorption occurs, two types of groups can influence the resulting absorption spectrum of the molecule: chromophores and auxochromes.

**Chromophores**

A chromophore (literally color-bearing) group is a functional group, not conjugated with another group, which exhibits a characteristic absorption spectrum in the ultraviolet or visible region. Some of the more important chromophoric groups are:

- Nitro: $\text{NO}_2^-$
- Azoxy: $\text{N} = \text{N}^-$
- Nitroso: $\text{N} = \text{O}$
- Carbonyl: $\text{C} = \text{O}$
- Aza: $\text{N} = \text{N}$
- Thiocarbonyl: $\text{C} = \text{S}$
- Azo amino: $\text{N} = \text{N} = \text{NH}^-$

If any of the simple chromophores is conjugated with another (of the same type or different type) a multiple chromophore is formed having a new absorption band which is more intense and at a longer wavelength that the strong bands of the simple chromophores.

This displacement of an absorption maximum towards a longer wavelength (i.e. from blue to red) is termed a bathochromic shift. The displacement of an absorption maximum from the red to ultraviolet is termed a hypsochromic shift.

**Auxochromes**

The color of a molecule may be intensified by groups called auxochromes which generally do not absorb significantly in the 200-800nm region, but will affect the spectrum of the chromophore to which it is attached. The most important auxochromic groups are $\text{OH}$, $\text{NH}_2$, $\text{CH}_3$ and $\text{NO}_2$ and their properties are acidic (phenolic) or basic.

The actual effect of an auxochrome on a chromophore depends on the polarity of the auxochrome, e.g. groups like $\text{CH}_3^-$, $\text{CH}_2\text{CH}_2^-$ and $\text{Cl}^-$ have very little effect, usually a small red shift of 5-10nm. Other groups such as $\text{-NH}_2$ and $\text{-NO}_2$ are very popular and completely alter the spectra of chromophores such as:
In general it should be possible to predict the effect of non-polar or weakly polar auxochromes, but the effect of strongly polar auxochromes is difficult to predict. In addition, the availability of non-bonding electrons which may enter into transitions also contributes greatly to the effect of an auxochrome.

**Steric Effects**

Steric hindrance will also affect the influence of an auxochrome on a chromophore. Electron systems conjugate best when the molecule is planar in configuration. If the presence of an auxochrome prevents the molecule from being planar then large effects will be noticed in the spectrum; e.g., m- and p-methyl groups in the diphenyls have predictable but slight effects on the spectra compared with that of diphenyl itself. However, methyl groups in the o-position alter the spectrum completely.

Cis and trans isomers of linear polyenes also show differences in their spectra. The all-trans isomer has the longer conjugated system. $\lambda_{\text{max}}$ is at a longer wavelength and $\varepsilon_{\text{max}}$ (molar absorptivity or molar extinction coefficient) is higher than for the all cis or mixed isomer.

**Visible Spectra**

In general a compound will absorb in the visible region if it contains at least five conjugated chromophoric and auxochromic groups; e.g.,

The ability to complex many metals, particularly the transition elements, with complex organic and inorganic molecules which absorb in the visible region provides the basis for their quantitative spectrometric analysis. The absorptions are due to movement of electrons between energy levels of the organo-metal complex. These complexing systems are termed spectrometric reagents. The most common are dithizone, azo reagents (PAN, thoron, zincon), dithiocarbamate, 8-hydroxyquinoline, formaldoxime and thiocyanate. In addition, many inorganic ions in solution also absorb in the visible region e.g. salts of Ni, Co, Cu, V etc. and particularly elements with incomplete inner electron shells whose ions are complexed by hydration e.g. $(\text{Cu(H}_2\text{O)}_4)^{2+}$. Such absorptions arise from a charge transfer process where electrons are moved from one part of the system to another due to the energy provided by the visible light.
The effect on the absorption spectrum of a compound when diluted in a solvent will vary depending on the chemical structures involved. Generally speaking, non-polar solvents and non-polar molecules show least effect. However, polar molecules exhibit quite dramatic differences when interacted with a polar solvent. Interaction between solute and solvent leads to absorption band broadening and a consequent reduction in structural resolution and $\varepsilon_{\text{max}}$. Ionic forms may also be created in acidic or basic conditions. Thus care must be taken to avoid an interaction between the solute and the solvent.

Figure 9 illustrates the effect of iso-octane and ethanol on the spectrum of phenol, a change from hydrocarbon to hydroxylic solvent. The loss of fine structure in the latter is due to broad band h-bonded solvent-solute complexes replacing the fine structure present in the iso-octane. The fine structure in the latter solvent illustrates the principle that non-solvating or non-chelating solvents produce a spectrum much closer to that obtained in the gaseous state.

[Figure 9 Spectra of Phenol in Iso-octane and in Ethanol]

Commercially available solvents of 'spectroscopic purity' are listed in Table 3 accompanied by their cut-off wavelengths, based on a 10mm pathlength. Water and 0.1N solutions of hydrochloric acid and sodium hydroxide are commonly used solvents for absorption spectrometry. Again care has to be taken to avoid interaction. Where methodology requires buffering, solutions have to be non-absorbing and generally both the composition and pH will be specified. However, if this information is not available lists can be found in the literature. For reactions in the 4.2 to 8.8 pH region, mixtures of 0.1N dihydrogen sodium phosphate and 0.1N hydrogen disodium phosphate are generally used.
### Table 3 Commonly used solvents and their 'cut-off' wavelengths

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Cut-off (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso-octane</td>
<td>202</td>
</tr>
<tr>
<td>Ethyl alcohol</td>
<td>205</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>200</td>
</tr>
<tr>
<td>Acetone</td>
<td>325</td>
</tr>
<tr>
<td>Tetrachloroethylene</td>
<td>290</td>
</tr>
<tr>
<td>Benzene</td>
<td>280</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>265</td>
</tr>
<tr>
<td>Chloroform</td>
<td>245</td>
</tr>
<tr>
<td>Ethyl ether</td>
<td>220</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>210</td>
</tr>
<tr>
<td>Methyl alcohol</td>
<td>210</td>
</tr>
</tbody>
</table>

### General Interactions of Light and Matter

When a beam of radiation strikes any object it can be absorbed, transmitted, scattered, reflected or it can excite fluorescence. These processes are illustrated in Figure 10. With scattering it can be considered that the radiation is first absorbed then almost instantaneously completely re-emitted uniformly in all directions, but otherwise unchanged. With fluorescence a photon is first absorbed and excites the molecule to a higher energy state, but the molecule then drops back to an intermediate energy level by re-emitting a photon. Since some of the energy of the incident photon is retained in the molecule (or is lost by a non-radiative process such as collision with another molecule) the emitted photon has less energy and hence a longer wavelength than the absorbed photon. Like scatter, fluorescent radiation is also emitted uniformly in all directions.
The processes concerned in absorption spectrometry are absorption and transmission. Usually the conditions under which the sample is examined are chosen to keep reflection, scatter and fluorescence to a minimum. In the ultraviolet and visible regions of the electromagnetic spectrum, the bands observed are usually not specific enough to allow a positive identification of an unknown sample, although this data may be used to confirm its nature deduced from its infrared spectrum or by other techniques. Ultraviolet and visible spectrometry is almost entirely used for quantitative analysis; that is, the estimation of the amount of a compound known to be present in the sample. The sample is usually examined in solution.

### Lambert's (or Bouguer's) Law

Lambert's Law states that each layer of equal thickness of an absorbing medium absorbs an equal fraction of the radiant energy that traverses it.

The fraction of radiant energy transmitted by a given thickness of the absorbing medium is independent of the intensity of the incident radiation, provided that the radiation does not alter the physical or chemical state of the medium.

If the intensity of the incident radiation is $I_0$ and that of the transmitted light is $I$, then the fraction transmitted is:

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

The percentage transmission is

$$\%T = \frac{I}{I_0} \times 100$$
If a series of colored glass plates of equal thickness are placed in parallel, each sheet of which absorbs one quarter of the light incident upon it, then the amount of the original radiation passed by the first sheet is:

\[(1 - \frac{1}{4})/1 \times 100 = 75\%\]

and by the second sheet is 56.25\%, i.e. 75\% of 75\%, and by the third sheet is 42.19\%, i.e. 75\% of 56.25\%, and by the nth sheet is \((0.75)^n \times 100\%\).

Now imagine a container with parallel glass walls 10mm apart filled with an absorbing solution. If monochromatic light is incident on one face and 75\% of the light is transmitted, Lambert's Law states that if a similar cell is put next to the first the light transmitted will be reduced to 56.25\%. If the contents of the two containers are evaporated to half their volume, thereby doubling their concentration, and then measured in a single container, it will be found that the transmission will again be reduced to 56.25\%.

It can be immediately seen that to determine the concentration of an unknown sample the percentage transmittance of a series of solutions of known concentration or 'standards' can be plotted and the concentration or the unknown read from the graph. It will be found that the graph is an exponential function which is obviously inconvenient for easy interpolation.

**The Beer-Lambert Law**

The Beer-Lambert Law states that the concentration of a substance in solution is directly proportional to the 'absorbance', \(A\), of the solution.

Absorbance \(A = \text{constant} \times \text{concentration} \times \text{cell length}\)

The law is only true for monochromatic light, that is light of a single wavelength or narrow band of wavelengths, and provided that the physical or chemical state of the substance does not change with concentration.

When monochromatic radiation passes through a homogeneous solution in a cell, the intensity of the emitted radiation depends upon the thickness \((l)\) and the concentration \((C)\) of the solution.

\[I_0 \text{ is the intensity of the incident radiation and } I \text{ is the intensity of the transmitted radiation. The ratio } I/I_0 \text{ is called transmittance. This is sometimes expressed as a percentage and referred to as } \%\text{transmittance.}\]
Mathematically, absorbance is related to percentage transmittance $T$ by the expression:

$$A = \log_{10}(\frac{I_o}{I}) = \log_{10}(100/T) = kcL$$

where $L$ is the length of the radiation path through the sample, $c$ is the concentration of absorbing molecules in that path, and $k$ is the extinction coefficient - a constant dependent only on the nature of the molecule and the wavelength of the radiation.

Now, in the example above, the transmittance of our sample fell from 75 to 56.25% when the concentration doubled. What happens to the absorbance in the same circumstance?

$$A = \log_{10}(\frac{100}{T}) = \log_{10}(100) - \log_{10} (T) = 2 - \log_{10} (T)$$

When $T = 75\%$, $A = 2 - 1.875 = 0.125$

When $T = 56.25\%$ $A = 2 - 1.750 = 0.250$

Quite clearly as the absorbance doubles for twice the concentration, it is far more convenient to work in absorbance than transmittance for the purposes of quantitative analysis.

It is useful to remember that

- $0\%T = \infty A$
- $0.1\% = 3.0A$
- $1.0\%T = 2.0A$
- $10\%T = 1.0A$
- $100\% = 0A$

Absorbance in older literature is sometimes referred to as ‘extinction’ or ‘optical density’ (OD).

Figure 11 (a) %T vs concentration (b) Absorbance vs concentration
If, in the expression $A = kcl$, $c$ is expressed in molar$^{-1}$ and $l$ in m, then $k$ is replaced by the symbol $\tau$ and is called the molar absorption coefficient. The units of $\tau$ are mol$^{-1}$m$^2$. $\tau$ was formerly called the molar extinction coefficient and concentrations were often expressed as mol l$^{-1}$, mol dm$^{-3}$ or M and the cell length in cm to give units mol$^{-1}$cm$^{-1}$, mol$^{-1}$dm$^3$cm$^{-1}$ and M$^{-1}$cm$^{-1}$ respectively.

Alternatively, if the relative molecular mass (molecular weight) of the substance is unknown, then a 1% w/v solution is prepared and the absorbance is measured in a 1 cm cell. In this case, $k$ is replaced by $E^{1\%}$. Sometimes the wavelength is included: $E^{1\%}(325\text{ nm})$.

$C$ Sometimes is expressed in g dm$^{-3}$ (g l$^{-1}$) and $l$ in cm. In this case, $k$ is replaced by $A$ (sometimes $E$). $A$ is known as the specific absorption coefficient.

**General Applications**

Having looked, albeit very briefly, at the theory and origins of UV-Visible spectra, let us now investigate how we can apply the technique to chemical analysis starting with consideration of sample state.

With solid sample it is usually found that the material is in a condition unsuitable for direct spectrometry. The refractive index of the material is high and a large proportion of the radiation may be lost by random reflection or refraction at the surface or in the mass. Unless the sample can be easily made as an homogenous polished block or film, it is usual to eliminate these interfaces by dissolving it in a transparent solvent.

Liquids may be contained in a vessel made of transparent material such as silica, glass or plastic, known as a cell or cuvette. The faces of these cells through which the radiation passes are highly polished to keep reflection and scatter losses to a minimum. Gases may be contained in similar cells which are sealed or stoppered to make them gas tight. With the sample now ready for measurement, the $I_0$ (incident intensity) can be set by moving the sample out of the beam and letting the light fall directly on the detector. On today’s modern instrumentation, $I_0$ setting is generally accomplished by an ‘autozero’ command. In practice, such a method does not account for the proportion of radiation which is reflected or scattered at the cell faces. It also does not account for the radiation which is absorbed by any solvent and thus does not effectively pass through the sample. Therefore it is usual to employ a reference or blank cell, identical to that containing the sample, but filled only with solvent and to measure the light transmitted by this reference as a true or practical $I_0$.

Having established the $I_0$ or reference position, the procedure adopted for the analysis will depend on the analytical information required. In general terms there are two major measurement techniques; how much analyte is in the sample (quantitative analysis) and which analyte is in the sample (qualitative analysis).

**Quantitative Analysis**

For quantitative analysis, it is normally chosen to use radiation of a wavelength at which $k$, the extinction coefficient, is a maximum, i.e. at the peak of the absorption band, for the following reasons:

- The change in absorbance for a given concentration change is greater, leading to greater sensitivity and accuracy in measurement.
The relative effect of other substances or impurities is smaller.

The rate of change of absorbance with wavelength is smaller, and the measurement is not so severely effected by small errors in the wavelength setting.

The extinction coefficient must have units relating to the units used for concentration and pathlength of the sample.

It is usually written as:

<table>
<thead>
<tr>
<th>$E_{1% 1\text{ cm}}$</th>
<th>the value of $k$ for a 1% sample concentration of 1 cm thickness.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\varepsilon$</td>
<td>the molar absorptivity previously described as the molar extinction coefficient; the value of $k$ for a sample concentration of 1 gram molecule per liter and a 1 cm thickness.</td>
</tr>
</tbody>
</table>

To carry out an analysis of a known material it should be possible in theory to measure the absorption of a sample at its absorption maximum, measure the thickness, obtain values of $\varepsilon$ from tables and calculate the concentration. In practice, values of $E$ depend on instrumental characteristics, so published values from tables are not accurate enough, and a reliable analyst will usually plot the absorbance values of a series of standards of known concentration; the concentrations of actual samples can then be read directly from the calibration graph. Using today's modern software driven instruments, the analyst will usually only require one 'top' standard which can be used to calibrate the instrument so that it displays the concentration of subsequent samples directly in the required units. If the calibration curve is non-linear due to either instrumental or chemistry considerations, then some instruments will automatically construct a 'best fit' calibration when presented with a number of standards.

Rate Measurements

Rate studies involve the measurement of the change in concentration of a participant in the reaction as a function of time. Whereas we have previously considered the measurement of a sample whose absorption characteristics are constant, spectrometric rate measurement involves the measurement of a fall or rise in absorption at a fixed wavelength. This measurement provides information on the change in concentration of either the reactants or products. The most widely encountered application of spectrometric rate measurements is for study of enzymes (proteins that are present in all living tissue). Enzymes cannot be measured directly but their catalytic properties allow their estimation from the speed of the reactions which they catalyse. Enzymes have many uses as reagents or as labels that can be attached to other molecules to permit their indirect detection and measurement. However, their widest use is in the field of clinical diagnostics as an indicator of tissue damage. When cells are damaged by disease, enzymes leak into the bloodstream and the amount present indicates the severity of the tissue damage. The relative proportions of different enzymes can be used to diagnose disease, say of the liver, pancreas or other organs which otherwise exhibit similar symptoms. The reaction most used in these clinical assays is the reduction of nicotinamide adenine dinucleotide (NAD) to NADH$_2$. The spectra of NAD and NADH$_2$ are shown in Figure 12 and it can be seen that if the absorbance is monitored at the NADH$_2$ peak at 340nm the reading will increase as the reaction progresses towards NADH$_2$. 


Other reactions can be monitored by coupling them together so that the product of one reaction feeds the next which can then be optically monitored.

The general equation for an enzyme reaction is:

\[
A \stackrel{\text{enzyme X}}{\longrightarrow} B
\]

In this reaction enzyme X catalyses the conversion of compound A to B. A coupled reaction utilises NAD in a secondary reaction, the completion of which depends on the concentration of a product of the primary reaction. e.g.

\[
A \rightarrow B \quad \text{B} + \text{NADH}_2 \xrightarrow{\text{indicator enzyme}} \quad C + \text{NAD}
\]

(Primary reaction) \quad (Indicator or secondary reaction)

This equation, however, will only hold true when the reaction between B and NADH\(_2\) is virtually instantaneous. Consider the following coupled reaction:

\[
\text{ALT} \quad \text{L-alanine} + \alpha\text{-ketoglutarate} \rightarrow \text{pyruvate}
\]

\[
\text{LDH} \quad \text{pyruvate} + \text{NADH}_2 \rightarrow \text{lactate} + \text{NAD}
\]

Reaction 1

The initial reaction is the conversion of L-alanine and \(\alpha\)-ketoglutarate to pyruvate in the presence of alanine transaminase. The indicator reaction is the conversion of pyruvate (and NADH\(_2\)) to lactate which is catalysed by the enzyme lactate dehydrogenase.
The effect of this reaction on absorbance is illustrated in Figure 13. The actual decrease in absorbance caused by the oxidation of NADH₂ can be seen in Figure 12. The rate of the reaction is determined by the average drop in absorbance over a short period of time following the addition of α-ketoglutarate. Other reactions involve the reduction of NAD to NADH₂ with a resultant increase in absorbance.

![Graph showing enzyme reaction based on oxidation of NADH to NAD](image)

**Figure 13 Enzyme reaction based on oxidation of NADH to NAD**

The conversion of glucose-6-phosphate to 6-phosphogluconalactone (a reaction catalysed by glucose-6-phosphate dehydrogenase in the presence of NADH₂) is an example of this type of reaction.

The equation for this reaction would be as follows:

\[
\text{G-6-phosphate dehydrogenase} \\
\text{G-6-phosphate + NAD} \leftrightarrow \text{G-6-phosphogluconalactone + NADH₂}
\]

**Reaction 2**

![Graph showing enzyme reaction based on reduction of NAD to NADH](image)

**Figure 14 Enzyme reaction based on reduction of NAD to NAD**
The change on absorbance is shown as in Figure 14. In case of reaction 1, i.e. alanine transaminase, the rate of reaction is calculated by measuring the average decrease in absorbance over a period of time and expressing it as an average drop in absorbance per minute. With reaction 2, the increase in absorbance is measured.

A variety of parameters will influence the rate of reaction. The majority of these i.e. substrate concentration, enzyme concentration and pH have to be accounted for in the chemistry. However, one of the most important is temperature and it is important to precisely control the temperature of the sample and reagents immediately prior to and during measurement. For this requirement, a whole range of thermostatted cell holders and temperature control systems is available.

Analysis of Mixtures

It is relatively rare to find a practical problem in which one has a mixture to be analysed with only one component which absorbs radiation. When there are several such components which absorb at the same wavelength their absorbances add together, and it is no longer true that the absorbance of the sample is proportional to the concentration of one component (see Figure 15).

In these cases, several approaches can be adopted with the most important being chemical reaction and multi-wavelength measurements.

![Figure 15 Error caused by superimposed absorption in a mixture](image)

Chemical Reaction

A common method of analysis is to change the required component by adding a chemical reagent which reacts with it specifically to form a highly absorbing compound. An example of this is shown in Figure 16. A quantity of the reagent added to the mixture reacts only with one component and both increases its absorption and changes the wavelength of the absorption maximum so that there is no longer interference between the components. The analysis is then reduced to a simple case and its
sensitivity is improved. Many hundreds of such specific reagents are now available for all sorts of analyses and sample matrices and are thoroughly detailed in the literature.

![Figure 16 Improving sensitivity by adding a chemical reagent](image)

**Multiwavelength Measurements**

In a mixture of components, the observed absorption at any wavelength is the sum of the individual absorption spectra of the components thus:

Measure absorbance at wavelength 1

\[ A_1 = E_{AaL} + E_{BbL} + E_{CcL} \ldots \]

Similarly at a wavelength 2

\[ A' = E'_{AaL} + E'_{BbL} + E'_{CcL} \ldots \]

where \( E_A \) and \( E'_A \) are the absorptivity of components A at wavelength 1 and wavelength 2 and A is its concentration, etc.

The cell pathlength \( L \) is generally constant and therefore cancels. If we then take measurements at a number of different wavelengths equal to the number of components, and if the value of the absorptivities are known by measurements of the pure components at each of the wavelengths concerned, we can solve the simultaneous equations to find the required concentrations.

The multi-wavelength or multicomponents analysis technique has seen a resurgence of interest over the last few years. This has been due to data processing techniques. A variety of algorithms is available and the analyst is generally required to input the number of components, measurement wavelengths and concentration values of the standards. Having measured the
standards, samples can be processed and results presented appropriately. Programs of this type, including measurement parameters, can then be stored on disk and recalled and run with a minimum of operator intervention, providing results on complex mixtures containing up to ten components.

**Instrumentation**

In earlier sections we have seen that the purpose of a spectrophotometer is to provide a beam of monochromatic radiation to illuminate a sample and so measure the ratio \( I / I_0 \). Any spectrophotometer will consist of the component parts illustrated in Figure 17.

![Schematic diagram of a spectrophotometer](image)

**Figure 17 Schematic diagram of a spectrophotometer**

There are many combinations of sources, monochromators, measuring systems etc. which can be assembled to form integrated spectrophotometers with varying degrees of accuracy and suitability for particular applications. The various optical components which comprise a spectrometer will not be discussed here.
Definitions

Absorbance Accuracy
Absorbance accuracy is the agreement between the absorbance displayed on the spectrometer and the true absorbance value as given by a standard solution or calibration gauze.

Absorbance Reproducibility
This is the agreement (concordance) of a series of absorbance values obtained when the same solution is scanned, i.e. it is how closely the absorbance values agree.

Accuracy
The accuracy of a determination may be defined as the agreement (concordance) between it and the true or most probable value.

Black body Radiation
A black body is a hypothetical body or surface which will absorb all radiation incident upon it. Conversely, this black body radiates electromagnetic energy proportional to the fourth power of its absolute temperature. The hotter the body the shorter the wavelengths of its radiation; thus the colour of a glowing body depends on its temperature.

Bond
A bond is an interaction between two or more atoms or groups of atoms which holds the atoms together. This interaction results from the sharing of electrons in (normally) incomplete shells of adjacent atoms.

The predicted paths (orbitals) of the bonding electrons about the nucleus give each atom a particular shape which affects the way it bonds with the adjacent atoms. These different bond orientations (orbitals) are termed:

\( \sigma \) (sigma)
\( \sigma^* \) (sigma star)
\( \pi \) (pi)
\( \pi^* \) (pi star)
\( n \)

A molecule can have a combination of different types of bond orientations.

Bonding occurs where there is a single electron in an orbital intended for two electrons. Antibonding occurs where both electrons are present in an orbital and this inhibits bonding.

Chelate
A substance which incorporates molecules or ions bonded to a metal ion.
Complex
A type of compound in which molecules or ions form bonds with a metal atom or ion.

Conjugation
Conjugated compounds are compounds with alternating double and single bonds.

Enzyme
An enzyme is a protein which catalyses a specific biochemical reaction. It mediates the conversion of one or more substances (the substrate(s)) to another by combining with the substrate(s) to form an intermediate complex.

Extinction Coefficient
The expression of the absorbptivity of a compound of a standard concentration measured in a 10 mm pathlength cuvette. It can be expressed as either:

\[
\varepsilon_{1\%}\text{cm}^{-1}
\]

or This is the Absorbance of a 1% w/v solution of a compound measured in 10 mm pathlength. It is related to molar absorbptivity by:

\[
\varepsilon_{1\%}\text{cm}^{-1} = 100 \times \varepsilon / M
\]

Or

\[
\varepsilon
\]

the molar absorbptivity coefficient. This is the Absorbance of a molar solution in a 10 mm pathlength cuvette. See Molar absorbptivity.

Hydration
To cause to take up or combine with water or the elements of water.

Ion
An atom or group of atoms which carries an electrical charge as a result of having gained or lost an electron.

Isomer
Any one of the possible compounds arising from the different ways of grouping the atoms in a given molecular formula. Compounds can have the same formula but different spatial arrangements of atoms.
Cis isomer

![Cis isomer diagram]

Groups attached on the same side of a bond; e.g.,

Trans isomer

![Trans isomer diagram]

Groups attached on opposite sides; e.g.,

Molar Absorptivity (e)

The absorbance at a specified wavelength of a solution of a compound of unit molar concentration measured in a 10 mm pathlength.

It has dimensions of $\text{M}^{-1}\text{cm}^{-1}$.

Monochromator

This is a device which isolates a very narrow band of wavelengths of the light coming from a source.

Noise

Noise is the general term used to describe the irregular or random trace obtained when the signal from a spectrophotometer is recorded.

Orbital

An orbital is a region round a nucleus where there is a high probability of finding an electron.

Polar

This is a condition where a molecule has no net electrical charge and yet it exhibits polarity - it is more negative at one end than the other. This comes about because some elements bind their outer electrons more tightly than others: with a large atom, the inner shells of electrons shield the core charge to some extent and the outer electrons tend to be further away from the core with a large atom than with a small one.

Precision

Precision may be defined as the agreement (concordance) of a series of measurements of the same quantity; i.e., precision expresses the reproducibility or repeatability of a measurement.
Probability Theory

It is not possible to specify the location and trajectory of an electron in the region of an atom since any attempt to observe the electron will change its location or movement. It is, however, possible to state a probability that the electron will be in a particular region. This probability theory allows the orbitals of the various electrons in a shell to be specified.

Reactant

A compound taking part in a chemical reaction.

Reagent

A solvent or solution which reacts with another. The term is usually applied to common laboratory chemicals used for experiment and analysis, e.g. sodium hydroxide and hydrochloric acid.

Refractive Index

A measure of the amount by which a substance refracts a wave. It is equal to the ratio of the speed of transmission of the wave in a reference medium or vacuum to its velocity in the substance.

Rotational Energy States

Just as a fixed quantum of energy is required to change the energy level of an electron, so also is a fixed quantum of energy required to change the rotation of a molecule. The allowed energy quanta for a given molecule depend on bond lengths and angles within the molecule.

Steric

This relates to the effect which the shape of a molecule has on its reactions. A particular example occurs in molecules containing large groups which hinder the approach of a reactant (steric hindrance).

Stray Light

This is the measurable radiation, at the detector, of any wavelength outside the narrow band expected to be transmitted by the monochromator.

Vibrational Energy States

Just as a fixed quantum of energy is required to change the energy level of an electron, so also is a fixed quantum of energy required to change the vibration of an atom in a molecule. The allowed energy quanta for a given molecule depend on bond forces and atomic masses within the molecule.

Wavelength Accuracy

This is the agreement between the value displayed on the spectrophotometer and the true value.

Wavelength Reproducibility

This is the agreement (concordance) of a series of wavelength values obtained when a spectrum is repeatedly scanned.