Chapter 1

Introduction to UV-Visible Spectroscopy

1. Introduction to UV-Visible Spectroscopy

1.1 General Principles

All chemicals absorb energy or light from at least one region of the spectrum of electromagnetic radiation. The energy at which absorption occurs depends on the available electronic, vibrational and rotational energy levels of the molecule. When absorption is from the UV-Visible region of the spectrum, transitions occur between electronic energy levels. It is these transitions that form the basis of UV-Visible spectrometry.

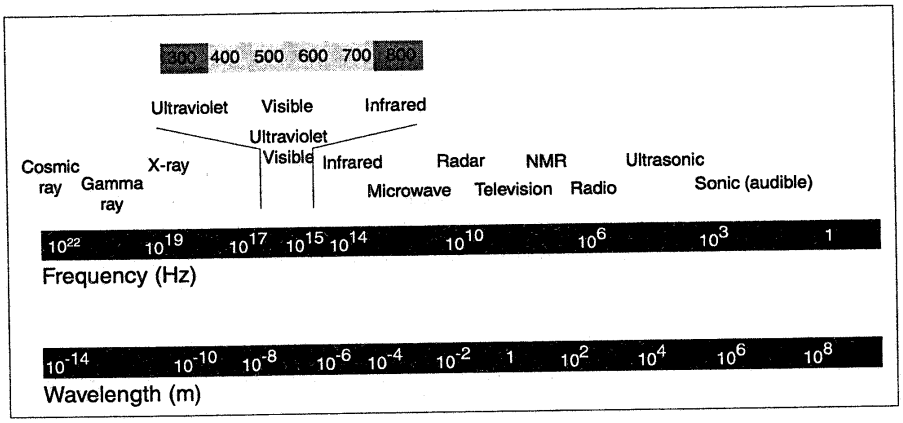


Figure 1-1.: The Electromagnetic Spectrum

1.1.1 Wavelength and Energy

The electromagnetic spectrum can be defined in terms of wavelength or frequency. The wavelength of light is defined as the distance between two crests, or troughs of a wave and is usually expressed by the symbol λ. The unit used in the UV-Visible region of the spectrum is a nanometer (nm). A nanometer is 10^{-9}m. The wavelength of light is related to the energy in terms of the speed of light, c, and Planck's constant, h.

$$E = hc/\lambda$$

This relationship shows that the shorter the wavelength, the greater the energy of light.

1.1.2 Energy Levels of Molecules

Molecules absorb light of particular wavelengths, defined by specific energy levels which are dependant upon their molecular structure. When wavelengths have an energy which is equivalent to the difference between the ground state and excited state energies, part of this wavelength will be absorbed. This selective absorption of light is the basis of UV-Visible spectrometric analysis. A UV-visible spectrum is obtained by measuring the amount of light absorbed as a function of wavelength.

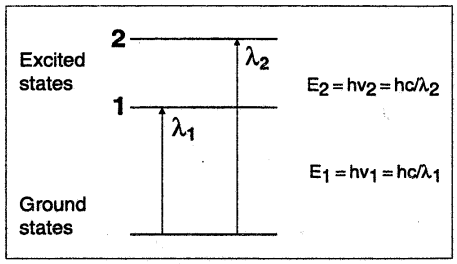


Figure 1-2.: Energy Levels of Molecules

1.1.3 Transmittance and Absorbance

There is no direct method for measuring absorbed light. This information is obtained indirectly by measuring the light which is not absorbed by the sample, i.e., the transmitted light. The Beer-Lambert law relates the absorption intensity to the incident and transmitted intensities as well as the concentration of the absorbing material, such that:

$$Abs = \log 10 \frac{I}{I_0} = \alpha c l$$

where α is the absorption coefficient of the absorbing substance, c is the concentration of the absorbing substance and l is the optical path length of the cell.

The Beer-Lambert law is the basis for all quantitative measurements in UV-Visible spectrometry as it relates absorbance directly to concentration.

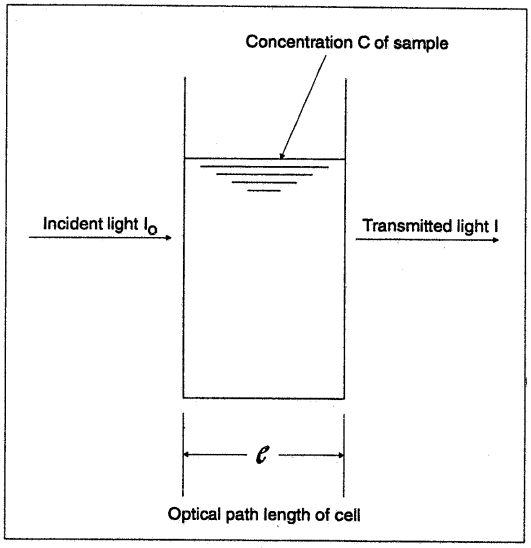


Figure 1-3.: Incident Light/Cell/Transmitted Light

1.2 Basic Construction of a Spectrometer

All UV-Visible spectrometers comprise the following major components:

1. A ***light source*** which provides illumination of the appropriate wavelengths, i.e., 190–1,000 nm
2. A ***monochromator*** which selects the precise wavelength of interest
3. The ***sample compartment*** to house the sample to be studied
4. A ***detector/amplifier*** system, which measures the amount of light transmitted by the sample.

1.2.1 Light Sources

Ideally, light sources should provide uniformly intense, stable radiation over a wide spectral range with comparable output at all wavelengths. They should also be dimensionally stable, of optimum size, durable and inexpensive. While no single source fulfils all of these criteria for the entire wavelength range, there are two commonly available sources which provide the necessary wavelength coverage.

1.2.1.1 Tungsten-Iodine Lamp

The most commonly used source of visible radiation is the tungsten-iodine lamp which can be used for the wavelength range 300–2000 nm. These lamps consist of a compact tungsten filament within a quartz envelope filled with one or more of the halogens. They have the advantage of high energy output, particularly in the 300–400 nm range, due to the superior transmission characteristics of the quartz envelope. Their operating temperature is also a few hundred degrees higher than for conventional bulbs.

1.2.1.2 Deuterium Lamp

Below approximately 300 nm the most satisfactory source of ultraviolet radiation for spectrometric purposes is the deuterium lamp. It provides a stable, continuous source in the UV region between 180–400 nm, due to the excitation of deuterium at low pressure with an electric discharge. The envelope of the lamp is normally constructed of quartz which exhibits excellent ultraviolet transmission.

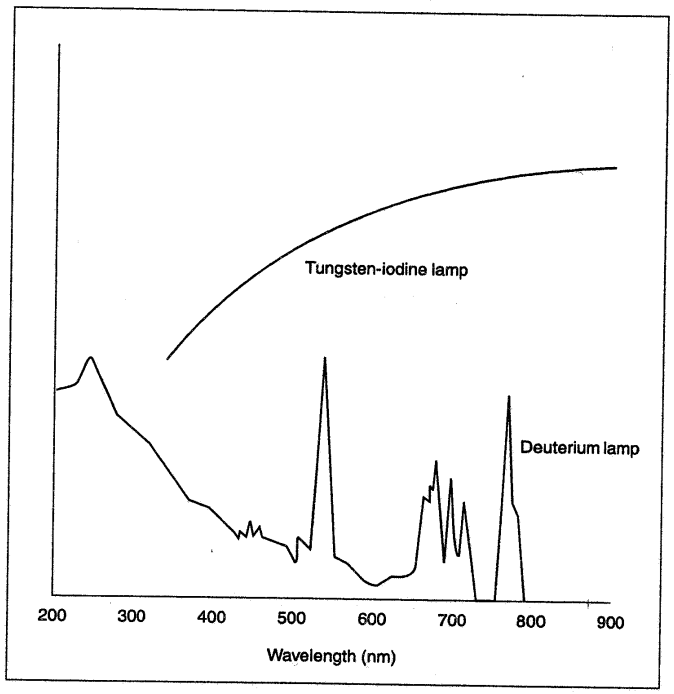


Figure 1-4.: Lamp Profiles

1.2.2 Monochromator

A monochromator is the device that resolves radiation into its component wavelengths and permits the isolation of any desired portion of the spectrum.

Monochromators usually consist of an entrance slit to confine the source radiation to a useable area, mirrors to pass the light through the system, a dispersing element to spread the source radiation into its component wavelengths and an exit slit to select the wavelength with which it is desired to illuminate the sample.

The heart of the monochromator is the dispersing element, i.e. diffraction grating, which separates the radiation according to wavelength. In addition, mirrors are used to direct the radiation from point to point and to provide focusing and collimating capabilities. The slits are the openings through which the radiation enters and leaves the monochromator housing.

The most common scanning monochromator is the Czerny-Turner monochromator. The Czerny-Turner mounting consists of an entrance slit, collimating mirror, a plane grating, a focusing mirror and an exit slit. Wavelength scanning is achieved by rotation of the grating.

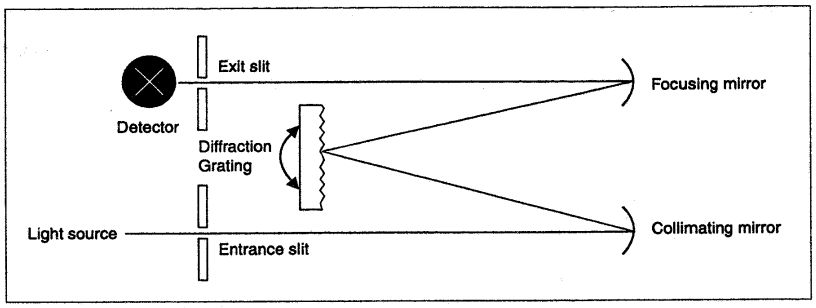


Figure 1-5.: Czerny-Turner Monochromator

The relationship between the wavelength and the angle at which it is reflected is most conveniently expressed in terms of d, the distance separating the grooves.

$$n\lambda = 2d \sin \theta,$$

where θ is the angle at which the radiation hits the grating.

A diffraction grating produces a whole series of overlapping spectra at angles determined by the value of n. Where n is 1 the spectrum is known as first order, where n is 2, as second order etc. Filters are used to remove the unwanted higher spectral orders.

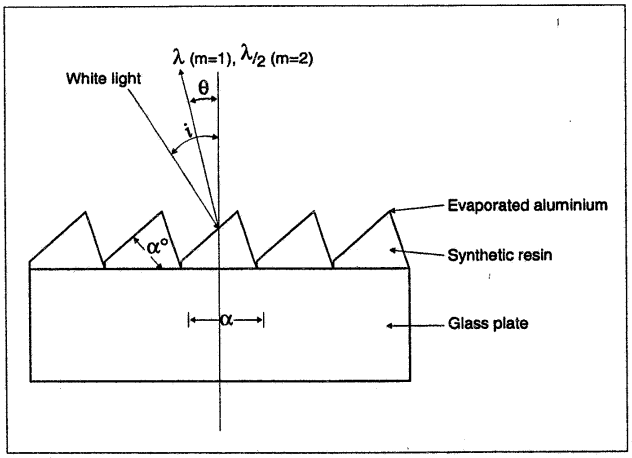


Figure 1-6.: Grating and Angle Information

High performance instruments contain two dispersing elements; i.e., two diffraction gratings. This arrangement markedly reduces the amount of stray light and provides greater dispersion and spectral resolution. Furthermore, higher-order wavelengths are removed by the second dispersive element.

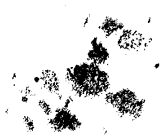
1.2.3 Sample Compartment

The sample compartment has a lid to protect the detector from strong external light. It accommodates sample cells and is sealed to protect optical components.

Sample cells, or cuvettes, are available in a wide variety of shapes and sizes to suit a particular spectrometric measurement. A number of different materials are used in the manufacture of cuvettes, normally quartz, optical glass or moulded plastic. The choice of material will depend upon the wavelength range for the analysis and the nature of the sample.

Quartz cells are required for work in the ultraviolet region below 350 nm. These cuvettes can be employed across the whole UV-Visible wavelength range, 190–1,000 nm. Optical glass can only be employed in the region above 300 nm, whereas moulded plastic cells can only be used in the visible region, >350 nm. The advantage of this type of cell is their low cost.

The most common size of cell is the 10 mm rectangular cell, which has an optical path length of 10 mm and path width of 10 mm. These cells typically hold 2–3 mL of sample. When sample volume is an important issue, micro cells are available, providing a smaller working volume. Generally, micro cells reduce the volume by narrowing the path width of the cell.



1.2.4 Detectors

The purpose of the detector is to convert the radiant energy falling upon them into an electrical signal which can then be processed. Two types of detectors are commonly used in UV-Visible spectrometers—the silicon photo diode and the photo multiplier tube.

1.2.4.1 Silicon Diode

A silicon diode utilizes the photo voltaic effect to generate an electric voltage when radiation strikes its semi-conductive surface. Spectral sensitivity is low in the ultraviolet region, but it is higher and more stable in the visible and near-infrared regions.

1.2.4.2 Photo Multiplier Tube

A photo multiplier tube is a combination of a photo tube and a high gain amplifier. The advantage of a photo multiplier tube detector is that its sensitivity can be varied by adjusting the applied voltage. It ensures the highest spectral sensitivity in the wavelength range from 200–600 nm, although it provides limited practical sensitivity in the wavelength range beyond 900 nm.

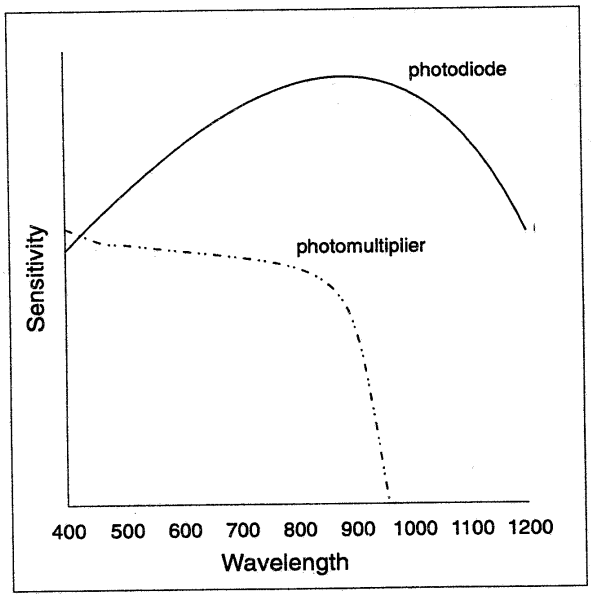


Figure 1-7.: Detector Sensitivity

1.3 Applications of UV-Visible Spectrometry

The technique of UV-Visible spectrometry is the most common in analytical science and is used in a wide variety of applications, including clinical, environmental, pharmaceutical, education and research. Since spectrometry carries the ability to select a measuring wavelength, it is widely used when both selectivity of measurement and sensitivity are required.

The applications of UV-Visible Spectrometry are numerous. A few of the more common types of analysis are discussed. This is not intended as an exhaustive review, but rather as a guide to the types of analysis that can be performed with a UV-Visible spectrometer.

1.3.1 Qualitative Analysis

The object of qualitative analysis for the compound under investigation can involve the following:

- the identification of a pure compound,
- the determination of the presence or absence of a particular species in a mixture, or
- the identification of certain functional groups (such as carbonyl, aromatic, nitro, or conjugated diene).

Identification of a pure compound involves an empirical comparison of the details of the spectrum (maxima, minima and inflection points) of the unknown with those of the pure compound. A close match is considered good evidence of chemical identity, particularly if the spectrum contains a number of sharp and well-defined peaks. However, the absorption bands of UV-Visible spectra tend to be broad (typically 20–100 nm) and hence lacking in detail.

A further qualitative application involves the detection of highly absorbing impurities in non-absorbing media. If an absorption peak for the contaminant has sufficiently high absorptivity, the presence of trace amounts can be readily established.

Factors which affect qualitative determinations include choice of solvent and slit width.

In choosing a solvent, consideration must be given to both its transparency and its possible effects upon the absorbing system. The nature of the solvent may affect the spectral detail or the absorption maxima. Clearly it is important to employ identical solvents when comparing absorption spectra for identification purposes.

The following table presents some common solvents and the approximate wavelengths below which they cannot be used due to absorption effects.

Solvent	*Cutoff Wavelength (nm)*	*Solvent*	*Approximate transparency minimum (nm)*
Water	180	Carbon tetrachloride	260
Ethanol	220	Diethly ether	210
Hexane	200	Acetone	330
Cyclohexane	200	Dioxane	320
Benzene	280	Cellosolve	320

In order to obtain an absorption spectrum useful for qualitative comparison, absorbance data should be collected with the narrowest possible band width. Otherwise, significant details of the spectrum may be lost. This effect is demonstrated below.

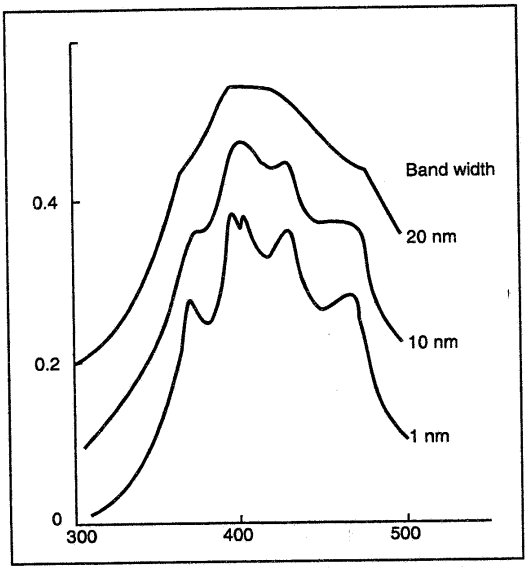


Figure 1-8.: Effect of the band width on spectra of identical solutions

1.3.2 Quantitative Analysis

Quantitative analysis is undertaken to determine the quantity (concentration) of one or more species in a mixture. A large range of quantitative methods can be employed for the determination of organic and inorganic substances. These methods are all based on the assumptions of the Beer-Lambert law.

Quantitative analysis can be further categorized as either a single component or multi component analysis.

1.3.2.1 Single Component Analysis

Generally, a calibration curve should be obtained by measuring the absorbance of standard samples of known concentrations. The absorbance value of the unknown sample is measured and its concentration can then be determined from the calibration curve.

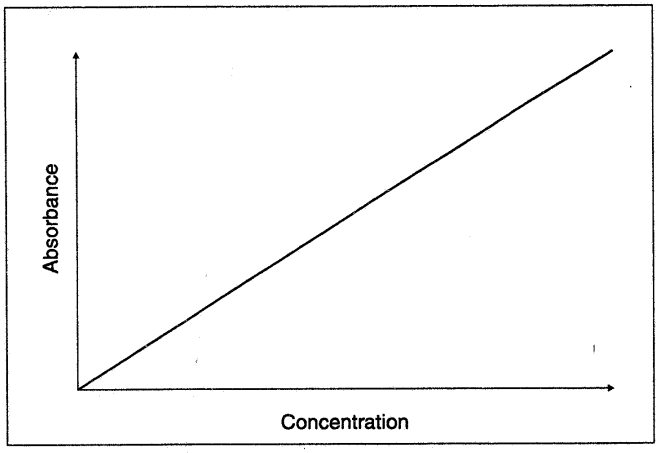


Figure 1-9.: Calibration Curve

Quantitative analysis can be applied to absorbance at single or multiple wavelengths, or using the peak height or area of a wavelength scan.

Common variables that influence the absorption spectrum of a substance include the nature of the solvent, the pH of the solution, the temperature, high electrolyte concentrations and the presence of interfering substances. The effects of these variables must be known and a set of analytical conditions must be chosen to ensure that the absorbance will not be influenced by small, uncontrolled variations in their magnitudes.

1.3.2.2 Multicomponent Analysis

The analysis of a number of components in a sample or mixture is of importance in a number of industries such as pharmaceutical, food, dye and paint manufacturing. As these industries are subject to strict regulations regarding product safety and quality, reliable and accurate methods which will reduce testing time and costs are required.

A UV-Visible multi-component analysis mathematically separates each component of the sample. This requires the measurement of spectra for known standards of all the components which are present at significant levels in the sample.

Accurate multi-component determination by UV-Visible spectrometry requires that the following conditions be satisfied:

- All components of the mixture can be identified and absorbed within the wavelength range of the instrument.
- The absorbances of the components in the mixture follows the Beer-Lambert law.
- There is some degree of spectral difference between the components. The greater the similarity between the spectra of the individual components, the more difficult the analysis.
- The spectrum of the mixture is the sum of the spectra of the components, i.e., the components must not interact to cause photometric or wavelength shifts.
- There should be no interaction between components and the solvent.
- Very large or small absorbances should be avoided.
- There should be no absorbances in the analytical wavelength region due to impurities.

If any of these assumptions do not hold, then the multi-component assumption is invalid.

1.3.3 Kinetics

Rates of chemical reactions determine how quickly a reaction mixture will reach its equilibrium state. The rate can depend upon a number of controllable factors such as temperature, pH, pressure and the presence of a catalyst.

As the basic data of chemical kinetics is the concentrations of the reactants and products as a function of time, UV-Visible spectrometry is the perfect method for monitoring the rate of change when the products or reactants absorb UV or visible light.

1.3.3.1 Enzyme Kinetics

Many rate equations are encountered which are more complex than the reaction orders mentioned above. Such rate equations are illustrated by reactions that occur in biological systems, or are affected by enzymes that occur in such systems.

Enzymes are proteins which catalyze biological reactions, and enzyme kinetics is the study of the rates and mechanisms of these reactions. The compound on which an enzyme acts is called its substrate.

The general principles of chemical reaction kinetics apply to enzyme-catalyzed reactions, but they also show a distinctive feature not usually observed in non-enzymatic reactions. This feature is known as *saturation with the substrate*.

At a low substrate concentration, the initial reaction rate is approximately proportional to the substrate concentration. As a result, the reaction is first order with respect to the substrate. However, as the substrate concentration increases, the reaction ceases to be first order until the reaction rate is independent of substrate concentration and becomes a constant. At this point the enzyme is said to be saturated with the substrate.

In enzyme kinetics, the rate equation for a one substrate enzyme-catalyzed reaction is called the Michaelis-Menten equation:

$$V_0 = V_{max}[S] / K_M + [S]$$

Where V_0 = initial reaction rate

$[S]$ = substrate concentration

V_{max} = maximum reaction rate

K_M = Michaelis-Menten Constant, which is the substrate concentration when the initial reaction rate is equal to 1/2 V_{max}

The Michaelis-Menten equation can be algebraically transformed into various forms to enable the calculation of the K_m and V_{max}. One of the more common transformations is the Lineweaver-Burk equation:

$$\frac{1}{V_o} = \frac{K_{max}}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}$$

Figure 1-10 shows the graphical representation of the Lineweaver-Burk equation.

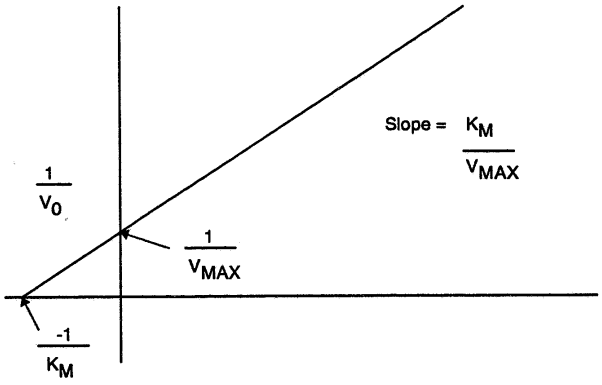


Figure 1-10.: Lineweaver-Burk plot

1.3.4 Color Analysis

Color plays an important role in our lives, giving us information of whether plants are healthy, and influencing our choice of food, clothes and furnishings. As describing colors precisely has often proved difficult, this has led to the development of standard systems to accurately describe color.

The basis of all color co-ordinate systems is the CIE system, developed in 1931. This system mathematically defines a three co-ordinate system, known as tristimulus X,Y,Z values. These values relate to the function of an illuminant (source of light), a specified observer and the geometry of illumination.

The tristimulus values are determined as follows from the reflectance or transmittance of the sample over the wavelength range 380–780 nm:

$$X = k \sum_{380}^{780} R(\lambda)S(\lambda)\bar{x}(\lambda)$$

$$Y = k \sum_{380}^{780} R(\lambda)S(\lambda)\bar{y}(\lambda)$$

$$Z = k \sum_{380}^{780} R(\lambda)S(\lambda)\bar{z}(\lambda)$$

where $S(\lambda)$ is a tabulated value of the standard observer, $R(\lambda)$ is the spectral reflectance of the sample, $\bar{x}(\lambda)$, $\bar{y}(\lambda)$ and $\bar{z}(\lambda)$ are the color matching functions for the standard observer and k is a normalization constant.

A number of alternate scales have been developed that are based on the CIE system. However, the most common used scale is the 1976 CIE L*a*b* scale. When color is represented in this system, L* represents the lightness (or value) whilst a* and b* indicate red-green and yellow-blue respectively. Hue and chroma can also be expressed in terms of a* and b*.

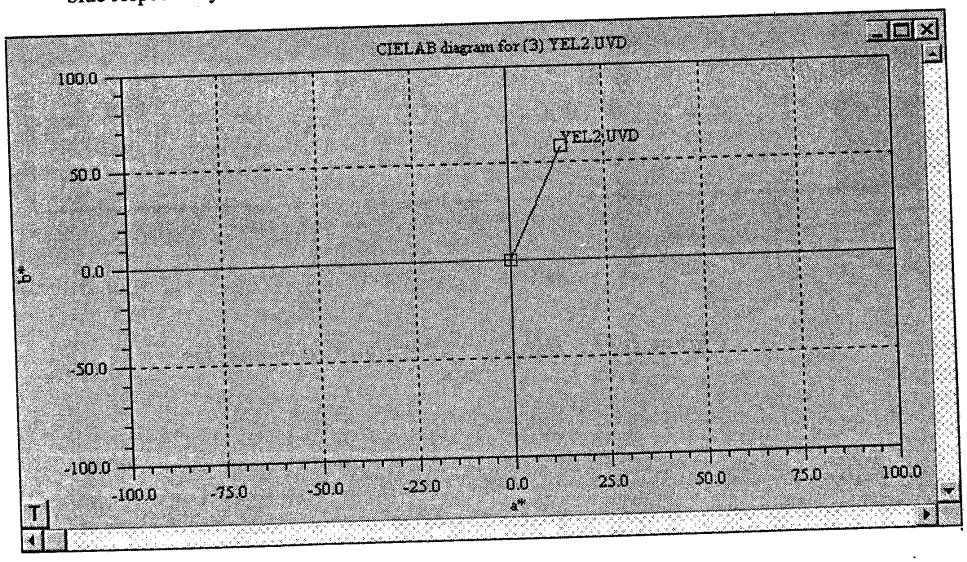


*Figure 1-11.: CIE L*a*b* diagram*

1.3.5 DNA Melt Analysis

When solutions of DNA are exposed to extremes of pH or heat or to solutes such as urea or amides, the double helical structure of DNA undergoes a transition into a randomly single-stranded form known as denatured DNA. During denaturation the interactions between successive base pairs are interrupted.

When DNA denatures, significant changes occur in a number of its physical properties, such as an increase in buoyant density, decrease in viscosity and an increase in the UV absorption at 260 nm. This last effect is known as the hyperchromic effect and provides a convenient method for monitoring the denaturation of DNA by UV-Visible spectrophotometry.

The most common method of studying denaturation of DNA involves increasing the temperature of a DNA solution so that the DNA gradually denatures by strand separation. A UV-Visible Spectrometer with a temperature control accessory is ideal for such studies as the temperature can be readily controlled and monitored and the denaturation can be monitored by observing absorption changes at 260 nm.

Characterisation of thermal denaturations is based on the determination of T_m. The T_m is defined as the mid-point of the thermal transition curve. The Tm value of DNA is dependent upon the proportion of G-C base pairs, which have three hydrogen bonds. The higher the content of G-C pairs, the more stable the structure and the higher the T_m value. The G-C content in DNA can be determined from T_m for DNA using the relationship:

$$GC\% = 2.44 (Tm - 69.3)$$

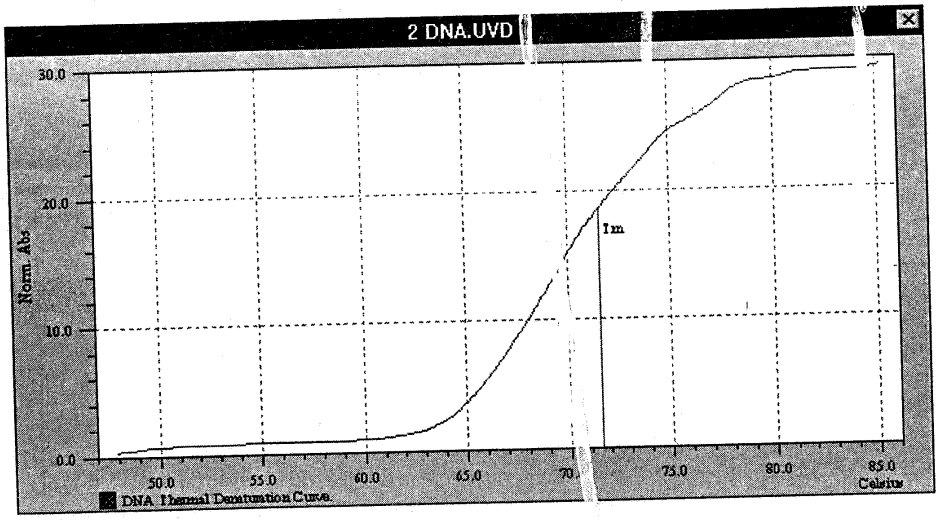


Figure 1-12.: DNA Denaturation Curve and Tm