



SPECTROPHOTOMETRIC MEASUREMENTS TECHNIQUES FOR FERMENTATION PROCESS

(PART ONE)

BASE THEORY FOR UV-VIS SPECTROPHOTOMETRIC MEASUREMENTS

INTERNAL REPORT

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Abstract

This paper presented some general theoretical aspects regarding spectrophotometric measurements. Spectrophotometry is a method to measure how much a chemical substance absorbs light by measuring the intensity of light as a beam of light passes through sample solution.

The basic principle is that each compound absorbs or transmits light over a certain range of wavelength. This measurement can also be used to measure the amount of a known chemical substance. Spectrophotometry is one of the most useful methods of quantitative analysis in various fields such as chemistry, physics, biochemistry, material and chemical engineering and clinical applications.

1. Introduction

Over a certain range of wavelength, every chemical compound absorbs, transmits, or reflects light (electromagnetic radiation). Spectrophotometry is a measurement of how much a chemical substance absorbs or transmits and a spectrophotometer is an instrument that measures the amount of the intensity of light absorbed after it passes through sample solution. With the spectrophotometer, the concentrations of a substance (the amount of a known chemical substance) can also be determined by measuring the intensity of light detected.

It can be classified into two different types, depending on the range of wavelength of light source:

- *UV-visible spectrophotometer:* uses light over the ultraviolet range (185 - 400 nm) and visible range (400 - 700 nm) of electromagnetic radiation spectrum.
- *IR spectrophotometer:* uses light over the infrared range (700 - 15000 nm) of electromagnetic radiation spectrum.

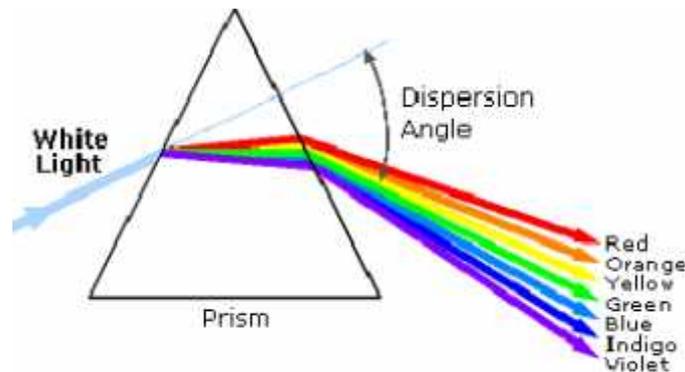
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2. **Visible and Ultraviolet Spectroscopy**



An obvious difference between certain compounds is their color. Although we see sunlight (or white light) as uniform or homogeneous in color, it is actually composed of a broad range of radiation wavelengths in the ultraviolet (UV), visible and infrared (IR) portions of the spectrum. By passing sunlight through a prism, the component colors of the visible portion can be separated, which acts to bend the light in differing degrees according to wavelength [1].

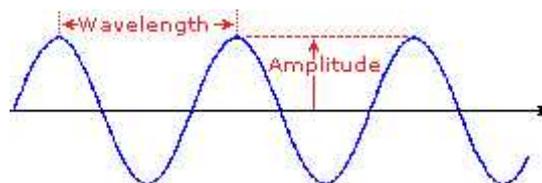
Electromagnetic radiation such as visible light is commonly treated as a wave phenomenon, characterized by a wavelength or frequency.

Wavelength is defined on the left below, as the distance between adjacent peaks (or troughs), and may be designated in meters, centimeters or nanometers (10^{-9} meters).

Frequency is the number of wave cycles that travel past a fixed point per unit of time, and is usually given in cycles per second, or hertz (Hz). Visible wavelengths cover a range from approximately 400 to 800 nm.

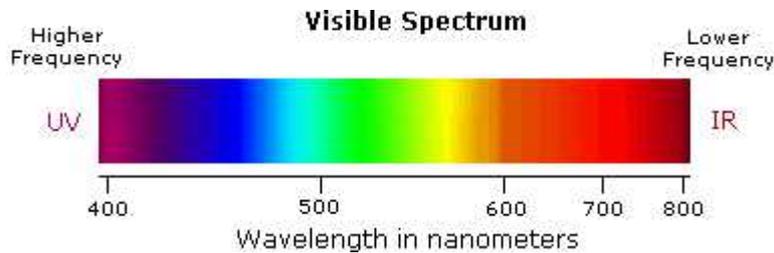
The longest visible wavelength is red and the shortest is violet. Other common colors of the spectrum, in order of decreasing wavelength, may be remembered by the mnemonic: **ROY G BIV**.

The wavelengths of what we perceive as particular colors in the visible portion of the spectrum are displayed and listed below.

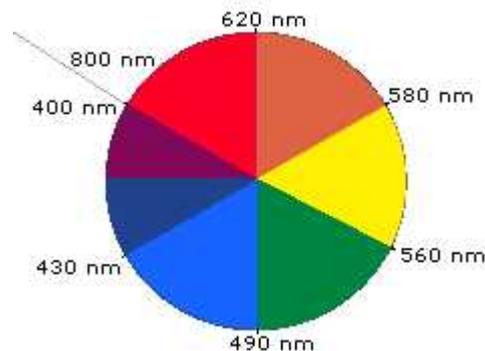




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- **Violet:** 400 - 420 nm
- **Indigo:** 420 - 440 nm
- **Blue:** 440 - 490 nm
- **Green:** 490 - 570 nm
- **Yellow:** 570 - 585 nm
- **Orange:** 585 - 620 nm
- **Red:** 620 - 780 nm



When white light passes through or is reflected by a colored substance, a characteristic portion of the mixed wavelengths is absorbed. The remaining light will then assume the complementary color to the wavelength(s) absorbed. This relationship is demonstrated by the color wheel. Here, complementary colors are diametrically opposite each other. Thus, absorption of 420-430 nm light renders a substance yellow, and absorption of 500-520 nm light makes it red. Green is unique in that it can be created by absorption close to 400 nm as well as absorption near 800 nm

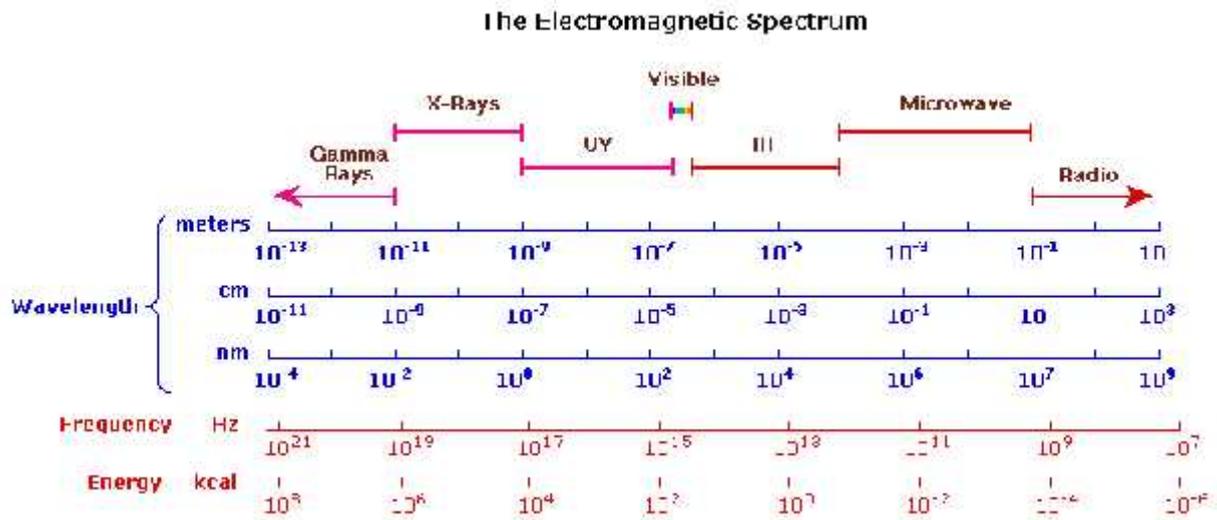
2.1. *The Electromagnetic Spectrum*

The visible spectrum constitutes but a small part of the total radiation spectrum. Most of the radiation that surrounds us cannot be seen, but can be detected by dedicated sensing instruments [2,3,4]. This *electromagnetic spectrum* ranges from very short wavelengths



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(including gamma and x-rays) to very long wavelengths (including microwaves and broadcast radio waves).



The energy associated with a given segment of the spectrum is proportional to its frequency. The bottom equation describes this relationship, which provides the energy carried by a photon of a given wavelength of radiation.

$$v = c/\lambda \quad v = \text{frequency, } \lambda = \text{wavelength, } c = \text{velocity of light } (c = 3 \cdot 10^{10} \text{ cm/sec})$$

$$\Delta E = h\nu \quad E = \text{energy, } \nu = \text{frequency, } h = \text{Planck's constant } (h = 6.6 \cdot 10^{-27} \text{ erg sec})$$

In *visible spectrophotometry*, the absorption or the transmission of a certain substance can be determined by the observed color

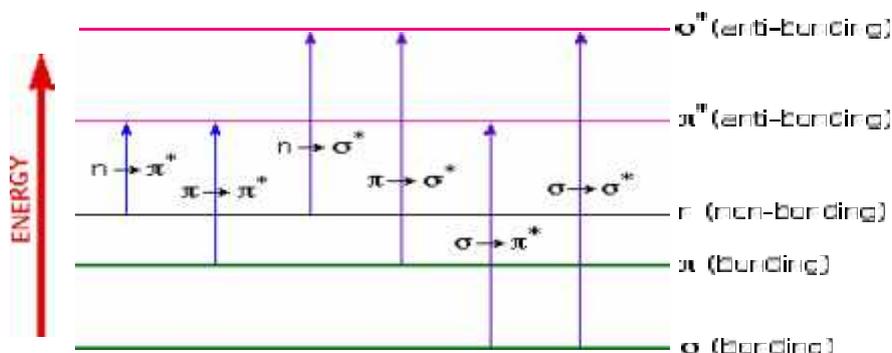
On the other hand, if all visible wavelengths are transmitted (i.e., absorbs nothing), the solution sample appears white. If a solution sample absorbs red light (~700nm), it appears green because green is the complementary color of red. Visible spectrophotometers, in practice, use a prism to narrow down a certain range of wavelength (to filter out other wavelengths) so that the particular beam of light is passed through a solution sample.

- **UV-Visible Absorption Spectra**

To understand why some compounds are colored and others are not, and to determine the relationship of conjugation to color, we must make accurate measurements of light absorption at different wavelengths in and near the visible part of the spectrum [4,5].



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The visible region of the spectrum comprises photon energies of 36 to 72 kcal/mole, and the near ultraviolet region, out to 200 nm, extends this energy range to 143 kcal/mole. Ultraviolet radiation having wavelengths less than 200 nm is difficult to handle, and is seldom used as a routine tool for structural analysis.

The energies noted above are sufficient to promote or excite a molecular electron to a higher energy orbital. Consequently, absorption spectroscopy carried out in this region is sometimes called "electronic spectroscopy".

As a rule, energetically favored electron promotion will be from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO), and the resulting species is called an excited state.

When sample molecules are exposed to light having an energy that matches a possible electronic transition within the molecule, some of the light energy will be absorbed as the electron is promoted to a higher energy orbital. An optical spectrometer records the wavelengths at which absorption occurs, together with the degree of absorption at each wavelength. The resulting spectrum is presented as a graph of absorbance (A) versus wavelength.

Absorbance usually ranges from 0 (no absorption) to 2 (99% absorption), and is precisely defined in context with spectrometer operation.

Because the absorbance of a sample will be proportional to the number of absorbing molecules in the spectrometer light beam (e.g. their molar concentration in the sample tube), it is necessary to correct the absorbance value for this and other operational factors if the spectra of different compounds are to be compared in a meaningful way. The corrected absorption value is called "molar absorptivity", and is particularly useful when comparing the spectra of different compounds and determining the relative strength of light absorbing functions (chromophores).

The presence of chromophores in a molecule is best documented by UV-Visible spectroscopy, but the failure of most instruments to provide absorption data for wavelengths below 200 nm makes the detection of isolated chromophores problematic.[4,5] Fortunately, conjugation generally moves the absorption maxima to longer wavelengths, as in the case of isoprene, so conjugation becomes the major structural feature identified by this technique.



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Once it is known the intensity of light after it passes through the cuvette, can be related it to transmittance (T). Transmittance is the fraction of light that passes through the sample. This can be calculated using the equation:

$$\text{Transmittance}(T) = \frac{I_t}{I_o}$$

Where I_t is the light intensity after the beam of light passes through the cuvette and I_o is the light intensity before the beam of light passes through the cuvette. Transmittance is related to absorbance by the expression:

$$\text{Absorbance}(A) = -\log(T) = -\log\left(\frac{I_t}{I_o}\right)$$

Where absorbance stands for the amount of photons that is absorbed. With the amount of absorbance known from the above equation, you can determine the unknown concentration of the sample by using Beer-Lambert Law. Figure 1 illustrates transmittance of light through a sample.

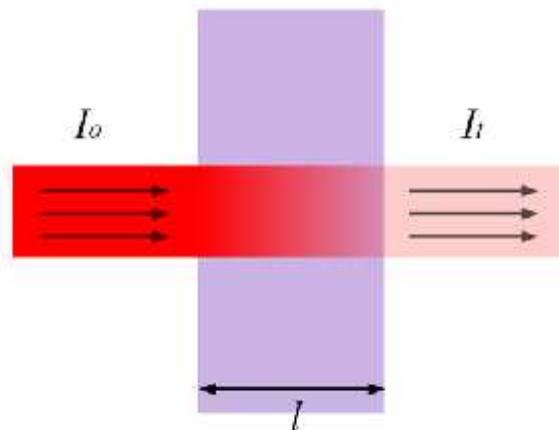


Figure 1. Transmittance (illustrated by Heesung Shim)[4]

If the sample compound does not absorb light of a given wavelength, $I = I_o$. However, if the sample compound absorbs light then I is less than I_o , and this difference may be plotted on a graph versus wavelength, as shown on the right.

If no absorption has occurred, $T = 1.0$ and $A = 0$. Most spectrometers display absorbance on the vertical axis, and the commonly observed range is from 0 (100% transmittance) to 2 (1% transmittance). The wavelength of maximum absorbance is a characteristic value, designated as λ_{max} .



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Different compounds may have very different absorption maxima and absorbances. Intensely absorbing compounds must be examined in dilute solution, so that significant light energy is received by the detector, and this requires the use of completely transparent (non-absorbing) solvents. The most commonly used solvents are water, ethanol, hexane and cyclohexane. Solvents having double or triple bonds, or heavy atoms (e.g. S, Br & I) are generally avoided. Because the absorbance of a sample will be proportional to its molar concentration in the sample cuvette, a corrected absorption value known as the *molar absorptivity*.

2.2. Beer-Lambert Law

Beer-Lambert Law (also known as Beer's Law) states that there is a linear relationship between the absorbance and the concentration of a sample [9]. For this reason, Beer's Law can only be applied when there is a linear relationship.

The method is most often used in a quantitative way to determine concentrations of an absorbing species in solution, using the Beer-Lambert law:

$$A = \log_{10} \frac{I_0}{I} = \epsilon \cdot c \cdot L$$

where:

A is the measured absorbance, (no units),

I_0 is the intensity of the incident light at a given wavelength,

I is the transmitted intensity,

L the pathlength through the sample,

c the concentration of the absorbing species, and

For each species and wavelength ϵ is a constant known as the *molar absorptivity* or extinction coefficient; this constant is a fundamental molecular property in a given solvent, at a particular temperature and pressure, and has units of $\frac{1}{M \cdot cm}$ or often $\frac{AU}{M \cdot cm}$.

The absorbance and extinction are sometimes defined in terms of the natural logarithm instead of the base-10 logarithm.

Because the absorbance of a sample will be proportional to its molar concentration in the sample cuvette, a corrected absorption value known as the molar absorptivity is used when comparing the spectra of different compounds.

So, the molar absorptivity is defined as:

$$\epsilon = \frac{A}{c \cdot l}$$

where:

ϵ is the molar absorptivity

A is the absorbance

C is the sample concentration in moles/liters

l is the length of the path through the cuvette in cm



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Molar absorptivities may be very large for strongly absorbing compounds ($\epsilon > 10,000$) and very small if absorption is weak ($\epsilon = 10$ to 100).

The Beer-Lambert Law is useful for characterizing many compounds but does not hold as a universal relationship for the concentration and absorption of all substances. A 2nd order polynomial relationship between absorption and concentration is sometimes encountered for very large, complex molecules such as organic dyes.

Beer's Law can be written as well as:

$$A = \epsilon lc$$

where

A is the measure of absorbance (no units),

ϵ is the molar extinction coefficient or molar absorptivity (or absorption coefficient),

l is the path length, and

c is the concentration.

The molar extinction coefficient is given as a constant and varies for each molecule. Since absorbance does not carry any units, the units for ϵ must cancel out the units of length and concentration. As a result, ϵ has the units: $L \cdot mol^{-1} \cdot cm^{-1}$.

The path length is measured in centimeters. Because a standard spectrometer uses a cuvette that is 1 cm in width, l is always assumed to equal 1 cm. Since absorption, ϵ , and path length are known, we can calculate the concentration c of the sample.

3. UV-VIS spectrophotometer

3.1. Devices and mechanism

The basic structure of spectrophotometers is represented in figure 2. It consists of a light source, a collimator, a monochromator, a wavelength selector, a cuvette for sample solution, a photoelectric detector, and a digital display or a meter.

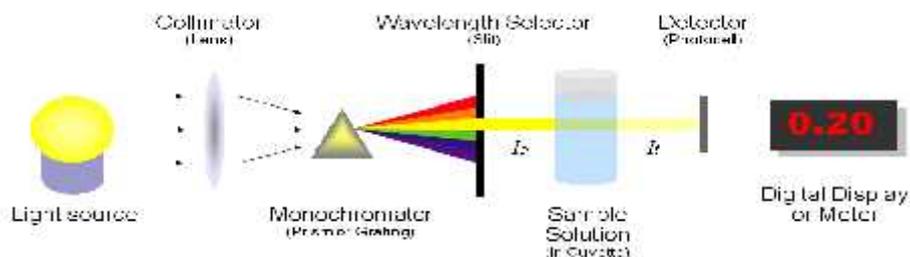


Figure 2: Basic structure of spectrophotometers (illustrated by Heesung Shim)

A spectrophotometer, in general, consists of two devices; a spectrometer and a photometer. A spectrometer is a device that produces, typically disperses and measures light. A photometer indicates the photoelectric detector that measures the intensity of light.

Spectrometer: It produces a desired range of wavelength of light. First a collimator (lens) transmits a straight beam of light (photons) that passes through a monochromator



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(prism) to split it into several component wavelengths (spectrum). Then a wavelength selector (slit) transmits only the desired wavelengths.

Photometer: After the desired range of wavelength of light passes through the solution of a sample in cuvette, the photometer detects the amount of photons that is absorbed and then sends a signal to a galvanometer or a digital display, as illustrated in Figure 1.

An UV/Vis **spectrophotometer** measures the intensity of light passing through a sample (I), and compares it to the intensity of light before it passes through the sample (I_0) expressed in absorbance (A) or transmittance (T).

The ratio $\frac{I_0}{I}$ is called the reflectance, and is usually expressed as a percentage (%R).

A beam of light from a visible and/or UV light source (colored red) is separated into its component wavelengths by a prism or diffraction grating (figure 3). Each monochromatic (single wavelength) beam in turn is split into two equal intensity beams by a half-mirrored device.

One beam, the sample beam (colored magenta), passes through a small transparent container (cuvette) containing a solution of the compound being studied in a transparent solvent. The other beam, the reference (colored blue), passes through an identical cuvette containing only the solvent. The intensities of these light beams are then measured by electronic detectors and compared.

The intensity of the reference beam, which should have suffered little or no light absorption, is defined as I_0 . The intensity of the sample beam is defined as I . Over a short period of time, the spectrometer automatically scans all the component wavelengths in the manner described. The ultraviolet (UV) region scanned is normally from 200 to 400 nm, and the visible portion is from 400 to 800 nm.

The basic parts of a spectrophotometer are a light source, a holder for the sample, a diffraction grating in a monochromator or a prism to separate the different wavelengths of light, and a detector. The radiation source is often a Tungsten filament (300-2500 nm), a deuterium arc lamp, which is continuous over the ultraviolet region (190-400 nm), Xenon arc lamp, which is continuous from 160-2,000 nm; or more recently, light emitting diodes (LED), [7], for the visible wavelengths.

The detector is typically a photomultiplier tube, a photodiode, a photodiode array or a charge-coupled device (CCD). Single photodiode detectors and photomultiplier tubes are used with scanning monochromators, which filter the light so that only light of a single wavelength reaches the detector at one time.

The scanning monochromator moves the diffraction grating to "step-through" each wavelength so that its intensity may be measured as a function of wavelength. Fixed monochromators are used with CCDs and photodiode arrays. As both of these devices consist of many detectors grouped into one or two dimensional arrays, they are able to collect light of different wavelengths on different pixels or groups of pixels simultaneously.

A spectrophotometer can be either *single beam* or *double beam*.

In a single beam instrument, all of the light passes through the sample cell. I_0 must be measured by removing the sample. This was the earliest design and is still in common use in both teaching and industrial labs.



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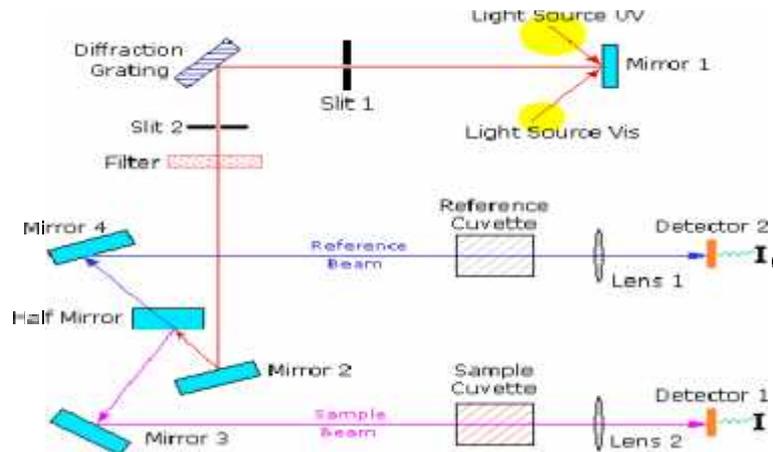


Figure 3. Diagram of a single-beam UV/VIS spectrophotometer

In a double-beam instrument, the light is split into two beams before it reaches the sample. One beam is used as the reference; the other beam passes through the sample. The reference beam intensity is taken as 100% Transmission (or 0 Absorbance), and the measurement displayed is the ratio of the two beam intensities.

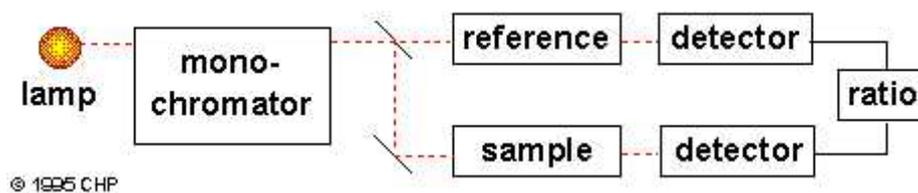


Figure 4. Schematic of a dual-beam UV-VIS spectrophotometer [11]

The dual-beam design greatly simplifies this process by simultaneously measuring P and P_0 of the sample and reference cells, respectively. Most spectrometers use a mirrored rotating chopper wheel to alternately direct the light beam through the sample and reference cells [11].

The detection electronics or software program can then manipulate the P and P_0 values as the wavelength scans to produce the spectrum of absorbance or transmittance as a function of wavelength. Some double-beam instruments have two detectors (photodiodes), and the sample and reference beam are measured at the same time. In other instruments, the two beams pass through a beam chopper, which blocks one beam at a time. The detector alternates between measuring the sample beam and the reference beam in synchronism with the chopper.



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There may also be one or more dark intervals in the chopper cycle. In this case, the measured beam intensities may be corrected by subtracting the intensity measured in the dark interval before the ratio is taken.

Array-detector spectrophotometers allow rapid recording of absorption spectra. Dispersing the source light after it passes through a sample allows the use of an array detector to simultaneously record the transmitted light power at multiple wavelengths. There are a large number of applications where absorbance spectra must be recorded very quickly. Some examples include HPLC detection, process monitoring, and measurement of reaction kinetics.

These spectrometers use photodiode arrays (PDAs) or charge-coupled devices (CCDs) as the detector. The spectral range of these array detectors is typically 200 to 1000 nm. The light source is a continuum source such as a tungsten lamp. All wavelengths pass through the sample. The light is dispersed by a diffraction grating after the sample and the separated wavelengths fall on different pixels of the array detector. The resolution depends on the grating, spectrometer design, and pixel size, and is usually fixed for a given instrument. Besides allowing rapid spectral recording, these instruments are relatively small and robust. Portable spectrometers have been developed that use optical fibers to deliver light to and from a sample.

These instruments use only a single light beam, so a reference spectrum is recorded and stored in memory to produce transmittance or absorbance spectra after recording the sample spectrum.

Samples for UV/Vis spectrophotometry are most often liquids, although the absorbance of gases and even of solids can also be measured. Samples are typically placed in a transparent cell, known as a cuvette. Cuvettes are typically rectangular in shape, commonly with an internal width of 1cm. (This width becomes the path length, L , in the Beer-Lambert law.)

Test tubes can also be used as cuvettes in some instruments. The type of sample container used must allow radiation to pass over the spectral region of interest.

The most widely applicable cuvettes are made of high quality fused silica or quartz glass because these are transparent throughout the UV, visible and near infrared regions. Glass and plastic cuvettes are also common, although glass and most plastics absorb in the UV, which limits their usefulness to visible wavelengths, [9,10,11,12].

Specialized instruments have also been made. These include attaching spectrophotometers to telescopes to measure the spectra of astronomical features. UV-visible microspectrophotometers consist of a UV-visible microscope integrated with a UV-visible spectrophotometer.

A complete spectrum of the absorption at all wavelengths of interest can often be produced directly by a more sophisticated spectrophotometer. In simpler instruments the absorption is determined one wavelength at a time and then compiled into a spectrum by the operator [13,14].

Practical considerations

The Beer-Lambert law has implicit assumptions that must be met experimentally for it to apply otherwise there is a possibility of deviations from the law to be observed, [5, 6,16]. For instance, the chemical makeup and physical environment of the sample can alter its



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extinction coefficient. The chemical and physical conditions of a test sample therefore must match reference measurements for conclusions to be valid.

Spectral bandwidth

A given spectrometer has a spectral bandwidth that characterizes how monochromatic the light is. It is important to have monochromatic source of radiation for analysis of the sample, [16,18].

If this bandwidth is comparable to the width of the absorption features, then the measured extinction coefficient will be altered. In most reference measurements, the instrument bandwidth is kept below the width of the spectral lines. When a new material is being measured, it may be necessary to test and verify if the bandwidth is sufficiently narrow. Reducing the spectral bandwidth will reduce the energy passed to the detector and will, therefore, require a longer measurement time to achieve the same signal to noise ratio.

Wavelength error

In liquids, the extinction coefficient usually changes slowly with wavelength. A peak of the absorbance curve (a wavelength where the absorbance reaches a maximum) is where the rate of change in absorbance with wavelength is smallest, [15,18]. Measurements are usually made at a peak to minimize errors produced by errors in wavelength in the instrument, that is errors due to having a different extinction coefficient than assumed.

Stray light

Another important factor is the *purity* of the light used. The most important factor affecting this is the stray light level of the monochromator, [15,18].

The detector used is broadband, it responds to all the light that reaches it. If a significant amount of the light passed through the sample contains wavelengths that have much lower extinction coefficients than the nominal one, the instrument will report an incorrectly low absorbance. Any instrument will reach a point where an increase in sample concentration will not result in an increase in the reported absorbance, because the detector is simply responding to the stray light. In practice the concentration of the sample or the optical path length must be adjusted to place the unknown absorbance within a range that is valid for the instrument. Sometimes an empirical calibration function is developed, using known concentrations of the sample, to allow measurements into the region where the instrument is becoming non-linear.

As a rough guide, an instrument with a single monochromator would typically have a stray light level corresponding to about 3 AU, which would make measurements above about 2 AU problematic. A more complex instrument with a double monochromator would have a stray light level corresponding to about 6 AU, which would therefore allow measuring a much wider absorbance range.

Absorption flattening

At sufficiently high concentrations, the absorption bands will saturate and show absorption flattening [15,18]. The absorption peak appears to flatten because close to 100% of the light is already being absorbed. The concentration at which this occurs depends on the particular compound being measured. One test that can be used to test for this effect is to vary the path length of the measurement. In the Beer-Lambert law, varying concentration and path length has an equivalent effect—diluting a solution by a factor of 10 has the same effect as



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shortening the path length by a factor of 10. If cells of different path lengths are available, testing if this relationship holds true is one way to judge if absorption flattening is occurring.

Solutions that are not homogeneous can show deviations from the Beer-Lambert law because of the phenomenon of absorption flattening. This can happen, for instance, where the absorbing substance is located within suspended particles. The deviations will be most noticeable under conditions of low concentration and high absorbance. The last reference describes a way to correct for this deviation.

Measurement uncertainty sources

The above factor contribute to the measurement uncertainty of the results obtained with UV/Vis spectrophotometry. If UV/Vis spectrophotometry is used in quantitative chemical analysis then the results are additionally affected by uncertainty sources arising from the nature of the compounds and/or solutions that are measured. These include spectral interferences caused by absorption band overlap, fading of the color of the absorbing species (caused by decomposition or reaction) and possible composition mismatch between the sample and the calibration solution, [8,15].

3.2. Applications

Spectrophotometry is widely used for quantitative analysis in various areas (e.g., chemistry, physics, biology, biochemistry, material and chemical engineering, clinical applications, industrial applications, etc). Any application that deals with chemical substances or materials can use this technique [15,16,17,18]. In biochemistry, for example, it is used to determine enzyme-catalyzed reactions. In clinical applications, it is used to examine blood or tissues for clinical diagnosis [20,21,22]. There are also several variations of the spectrophotometry such as atomic absorption spectrophotometry and atomic emission spectrophotometry.

In chemistry, spectrophotometry is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength. It is more specific than the general term electromagnetic spectroscopy in that spectrophotometry deals with visible light, near-ultraviolet, and near-infrared, but does not cover time-resolved spectroscopic techniques.

A spectrophotometer is commonly used for the measurement of transmittance or reflectance of solutions, transparent or opaque solids, such as polished glass, or gases. However they can also be designed to measure the diffusivity on any of the listed light ranges that usually cover around 200nm – 2500 nm using different controls and calibrations. Within these ranges of light, calibrations are needed on the machine using standards that vary in type depending on the wavelength of the photometric determination.

The use of spectrophotometers spans various scientific fields, such as physics, materials science, chemistry, biochemistry, and molecular biology. They are widely used in many industries including semiconductors, laser and optical manufacturing, printing and forensic examination, and as well in laboratories for the study of chemical substances [10, 11, 12, 13, 20, 21]. Ultimately, a spectrophotometer is able to determine, depending on the control or calibration, what substances are present in a target and exactly how much through calculations of observed wavelengths.

There are two major classes of devices: single beam and double beam. A double beam spectrophotometer compares the light intensity between two light paths, one path containing a reference sample and the other the test sample.



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A single beam spectrophotometer measures the relative light intensity of the beam before and after a test sample is inserted. Although comparison measurements from double beam instruments are easier and more stable, single beam instruments can have a larger dynamic range and are optically simpler and more compact. Additionally, some specialized instruments, such as spectrophotometer built onto microscopes or telescopes, are single beam instruments due to practicality [9, 20, 21].

Historically, spectrophotometers use a monochromator containing a diffraction grating to produce the analytical spectrum. The grating can either be movable or fixed. If a single detector, such as a photomultiplier tube or photodiode is used, the grating can be scanned stepwise so that the detector can measure the light intensity at each wavelength (which will correspond to each "step"). Arrays of detectors, such as charge coupled devices (CCD) or photodiode arrays (PDA) can also be used. In such systems, the grating is fixed and the intensity of each wavelength of light is measured by a different detector in the array.

Additionally, most modern mid-infrared spectrophotometers use a Fourier transform technique to acquire the spectral information. The technique is called Fourier transform infrared spectroscopy.

When making transmission measurements, the spectrophotometer quantitatively compares the fraction of light that passes through a reference solution and a test solution. For reflectance measurements, the spectrophotometer quantitatively compares the fraction of light that reflects from the reference and test samples. Light from the source lamp is passed through a monochromator, which diffracts the light into a "rainbow" of wavelengths and outputs narrow bandwidths of this diffracted spectrum. Discrete frequencies are transmitted through the test sample. Then the photon flux density (watts per metre squared usually) of the transmitted or reflected light is measured with a photodiode, charge coupled device or other light sensor.

The transmittance or reflectance value for each wavelength of the test sample is then compared with the transmission (or reflectance) values from the reference sample.

In short, the sequence of events in a modern spectrophotometer is as follows:

1. The light source is imaged upon the sample
2. A fraction of the light is transmitted or reflected from the sample
3. The light from the sample is imaged upon the entrance slit of the monochromator
4. The monochromator separates the wavelengths of light and focuses each of them onto the photodetector sequentially.

Many older spectrophotometers must be calibrated by a procedure known as "zeroing." The absorbancy of a reference substance is set as a baseline value, so the absorbancies of all other substances are recorded relative to the initial "zeroed" substance.

The most common spectrophotometers are used in the UV and visible regions of the spectrum, and some of these instruments also operate into the near-infrared region as well.

Visible region 400–700 nm spectrophotometry is used extensively in colorimetry science.

Traditional visible region spectrophotometers cannot detect if a colorant or the base material has fluorescence. Where a colorant contains fluorescence, a bi-spectral fluorescent spectrophotometer is used. There are two major setups for visual spectrum spectrophotometers, d/8 (spherical) and 0/45.



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The names are due to the geometry of the light source, observer and interior of the measurement chamber. Scientists use this instrument to measure the amount of compounds in a sample. If the compound is more concentrated more light will be absorbed by the sample; within small ranges, the Beer-Lambert law holds and the absorbance between samples vary with concentration linearly.

Conclusions

UV-VIS Spectrophotometry is widely used for quantitative analysis in various areas (e.g., chemistry, physics, biology, biochemistry, material and chemical engineering, clinical applications, industrial applications, etc). Any application that deals with chemical substances or materials can use this technique. In biochemistry, for example, it is used to determine enzyme-catalyzed reactions. The use of spectrophotometers spans various scientific fields, such as physics, materials science, chemistry, biochemistry, and molecular biology. They are widely used in many industries including biotechnology.

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