

The Basics of UV-Vis Spectrophotometry

A primer





Contents

| | | |
|----------|--|-----------|
| 1 | Basic Principles of UV-Vis Measurement | 3 |
| 1.1 | The electromagnetic spectrum | 3 |
| 1.2 | Wavelength and frequency | 3 |
| 1.3 | UV-visible spectra | 3 |
| 1.4 | Transmittance and absorbance | 4 |
| 1.5 | Summary | 4 |
| 2 | How Does a Modern UV-Vis Spectrophotometer Work? | 5 |
| 2.1 | Instrumental design | 7 |
| 3 | Selecting the Optimum Parameters for your UV-Vis Measurements | 13 |
| 3.1 | Optical cell selection | 13 |
| 3.2 | Thermostating your samples | 16 |
| 3.3 | Stirring your sample | 16 |
| 3.4 | Measurements at low temperatures | 16 |
| 3.5 | Solvent transparency | 17 |
| 3.6 | Optimum spectral band width | 18 |
| 3.7 | Stray light | 19 |
| 3.8 | The linear range of a UV-Vis instrument | 19 |
| 3.9 | Other useful information | 20 |
| 3.10 | Wavelength or inverse centimeters | 20 |
| 4 | Overview of Common UV-Vis Applications | 21 |
| 4.1 | Identification—spectra and structure | 21 |
| 4.2 | Confirmation of identity | 22 |
| 4.3 | Quantifying a molecule | 22 |
| 4.4 | Kinetics | 24 |
| 4.5 | Color measurement | 26 |
| 4.6 | Structural changes of compounds | 28 |
| 4.7 | Protein and nucleic acid melting temperature | 28 |
| 4.8 | Multi-component analysis | 30 |
| 4.9 | Software requirements | 32 |
| 5 | Glossary | 34 |

1. Basic Principles of UV-Vis Measurement

1.1 The electromagnetic spectrum

Ultraviolet (UV) and visible radiation are a small part of the electromagnetic spectrum, which includes other forms of radiation such as radio, infrared (IR), cosmic, and X rays.

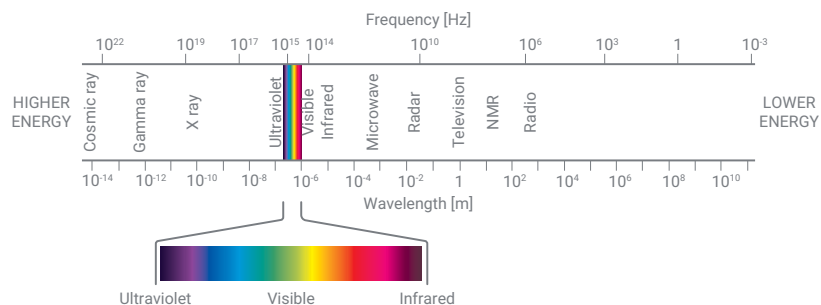


Figure 1. The electromagnetic spectrum, with the visible light section expanded.

The energy associated with electromagnetic radiation is defined as:

$$E = h\nu$$

where E is energy (in joules), h is Planck's constant (6.62×10^{-34} Js), and ν is frequency (in seconds).

Spectroscopy allows the study of how matter interacts with or emits electromagnetic radiation. There are different types of spectroscopy, depending on the wavelength range that is being measured. UV-Vis spectroscopy uses the ultraviolet and visible regions of the electromagnetic spectrum. Infrared spectroscopy uses the lower energy infrared part of the spectrum.

1.2 Wavelength and frequency

Electromagnetic radiation can be considered a combination of alternating electric and magnetic fields that travel through space in a wave motion. Because radiation acts as a wave, it can be classified in terms of either wavelength or frequency, which are related by the following equation:

$$\nu = c/\lambda$$

where ν is frequency (in seconds), c is the speed of light (3×10^8 ms $^{-1}$), and λ is wavelength (in meters).

In UV-Vis spectroscopy, wavelength is usually expressed in nanometers ($1 \text{ nm} = 10^{-9} \text{ m}$). It follows from the equations that radiation with shorter wavelength has higher energy, and, for UV-Vis spectroscopy, the low (short)

wavelength UV light has the highest energy. Sometimes, this energy may be sufficient to cause unwanted photochemical reactions when measuring samples that are photosensitive.

1.3 UV-visible spectra

When radiation interacts with matter, several processes can occur, including reflection, scattering, absorbance, fluorescence/phosphorescence (absorption and re-emission), and photochemical reactions (absorbance and bond breaking). Typically, when measuring samples to determine their UV-visible spectrum, absorbance is measured.

Because light is a form of energy, absorption of light by matter causes the energy content of the molecules (or atoms) in the matter to increase. The total potential energy of a molecule is represented as the sum of its electronic, vibrational, and rotational energies:

$$E_{\text{total}} = E_{\text{electronic}} + E_{\text{vibrational}} + E_{\text{rotational}}$$

The amount of energy a molecule possesses in each form is not a continuum but a series of discrete levels or states. The differences in energy among the different states are in the order:

$$E_{\text{electronic}} > E_{\text{vibrational}} > E_{\text{rotational}}$$

In some molecules and atoms, incident photons of UV and visible light have enough energy to cause transitions between the different electronic energy levels. The wavelength of light absorbed has the energy required to move an electron from a lower energy level to a higher energy level. Figure 2 shows an example of electronic transitions in formaldehyde and the wavelengths of light that cause them.

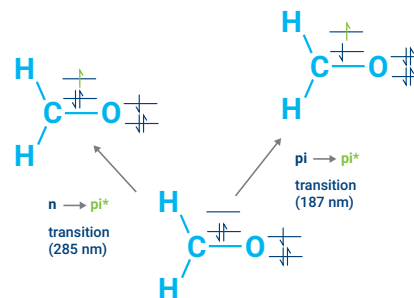


Figure 2. Electronic transitions in formaldehyde. UV light at 187 nm causes excitation of an electron in the C-O bond and light at 285 nm wavelength causes excitation and transfer of an electron from the oxygen atom to the C-O bond.

These transitions result in very narrow absorbance bands at wavelengths highly characteristic of the difference in energy levels of the absorbing species. This is also true for atoms, as depicted in Figure 3.

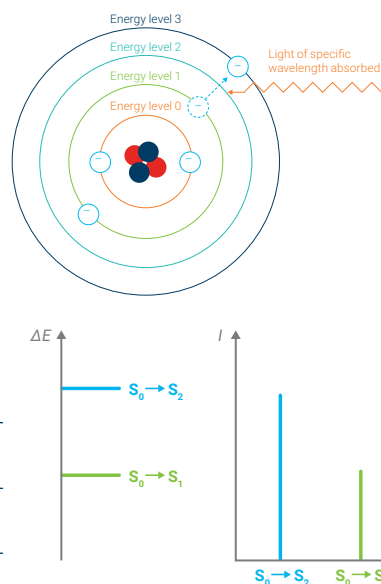


Figure 3. Incident light of a specific wavelength causes excitation of electrons in an atom. The type of atom or ion (i.e. which element it is) and the energy levels the electron is moving between determines the wavelength of the light that is absorbed. Transitions can be between more than one energy level, with more energy i.e. lower wavelengths of light, required to move the electron further from the nucleus.

However, for molecules, vibrational and rotational energy levels are superimposed on the electronic energy levels. Because many transitions with different energies can occur, the bands are broadened (see Figure 4). The broadening is even greater in solutions owing to solvent-solute interactions.

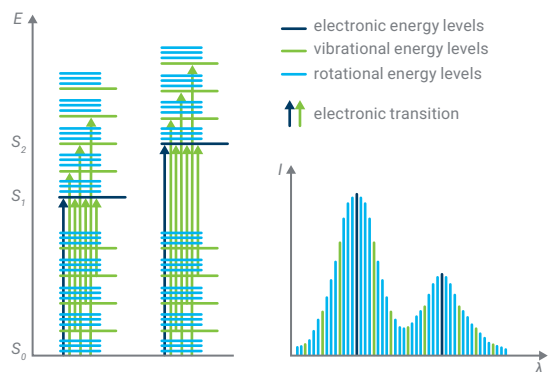


Figure 4. Electronic transitions and UV-visible spectra in molecules (I is intensity and λ is wavelength).

1.4 Transmittance and absorbance

When light passes through or is reflected from a sample, the amount of light absorbed is the difference between the incident radiation (I_0) and the transmitted radiation (I). The amount of light absorbed is expressed as absorbance. Transmittance, or light that passes through a sample, is usually given in terms of a fraction of 1 or as a percentage and is defined as follows:

$$T = I / I_0 \text{ or } \%T = I / I_0 \times 100$$

Absorbance is defined as follows:

$$A = -\log T$$

For most applications, absorbance values are used since the relationship between absorbance and both concentration and path length is normally linear (as per the Beer Lambert law, described in section 1.9).

1.5 Summary

- UV and visible light are part of the electromagnetic spectrum
- In UV-Vis spectroscopy wavelength is expressed in nanometers (nm)
- Light can be reflected, scattered, transmitted or absorbed from matter, and can cause photochemical reactions to occur
- Energy from incident light causes electrons to transition to different energy levels. Electronic transitions also occur between the vibrational and rotational energy levels of molecules
- Absorbance of light is used for most UV-Vis spectroscopy applications. It is defined as $A = -\log T$, where T is transmittance.

2. How Does a Modern UV-Vis Spectrophotometer Work?

Ultraviolet visible (UV-Vis) spectrophotometers use a light source to illuminate a sample with light across the UV to the visible wavelength range (typically 190 to 900 nm). The instruments then measure the light absorbed, transmitted, or reflected by the sample at each wavelength. Some spectrophotometers have an extended wavelength range, into the near-infrared (NIR) (800 to 3200 nm).

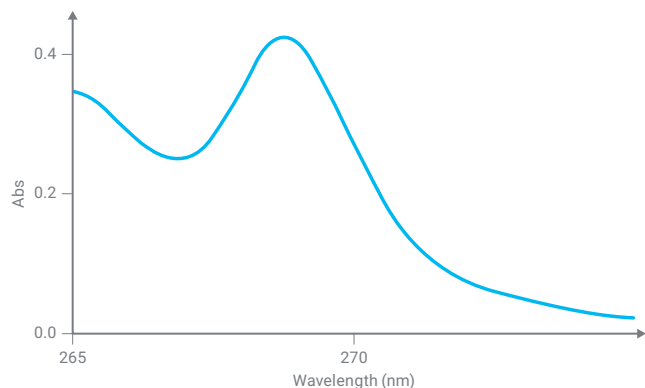


Figure 5. A UV absorbance spectrum, showing an absorbance peak at approximately 269 nm.

From the spectrum obtained, such as the one shown in Figure 5, it is possible to determine the chemical or physical properties of the sample.

In general, it is possible to:

- Identify molecules in a solid or liquid sample
- Determine the concentration of a particular molecule in solution
- Characterize the absorbance or transmittance through a liquid or solid—over a range of wavelengths
- Characterize the reflectance properties of a surface or measuring the color of a material
- Study chemical reactions or biological processes.

Various types of measurements can be performed by combining different accessories and sample holders with the UV-Vis spectrophotometer.

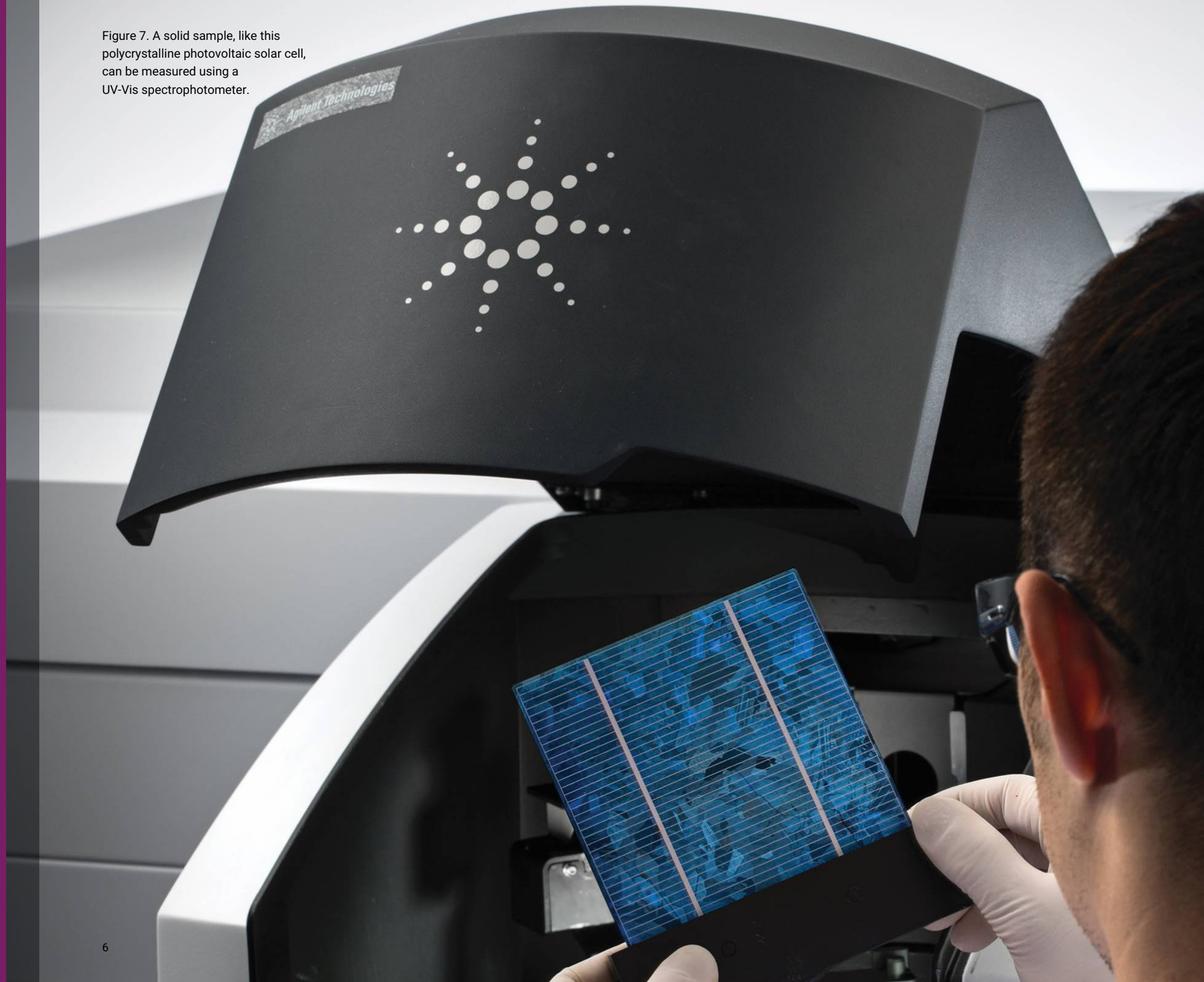
Different accessories exist for different measurement capabilities and sample types, e.g., solids versus liquids, and for different measurement conditions (Figure 6 and 7).



Figure 6. A fiber-optic probe accessory can be fitted to a UV-Vis spectrophotometer to measure liquid samples in a range of containers.

UV-Vis spectrophotometry is a versatile technique and has been used for close to a century in a wide range of fields. UV-Vis spectrophotometers are in common use in material testing/research, chemistry/petrochemistry, and biotechnology/pharmaceuticals laboratories.

Figure 7. A solid sample, like this polycrystalline photovoltaic solar cell, can be measured using a UV-Vis spectrophotometer.



2.1 Instrumental design

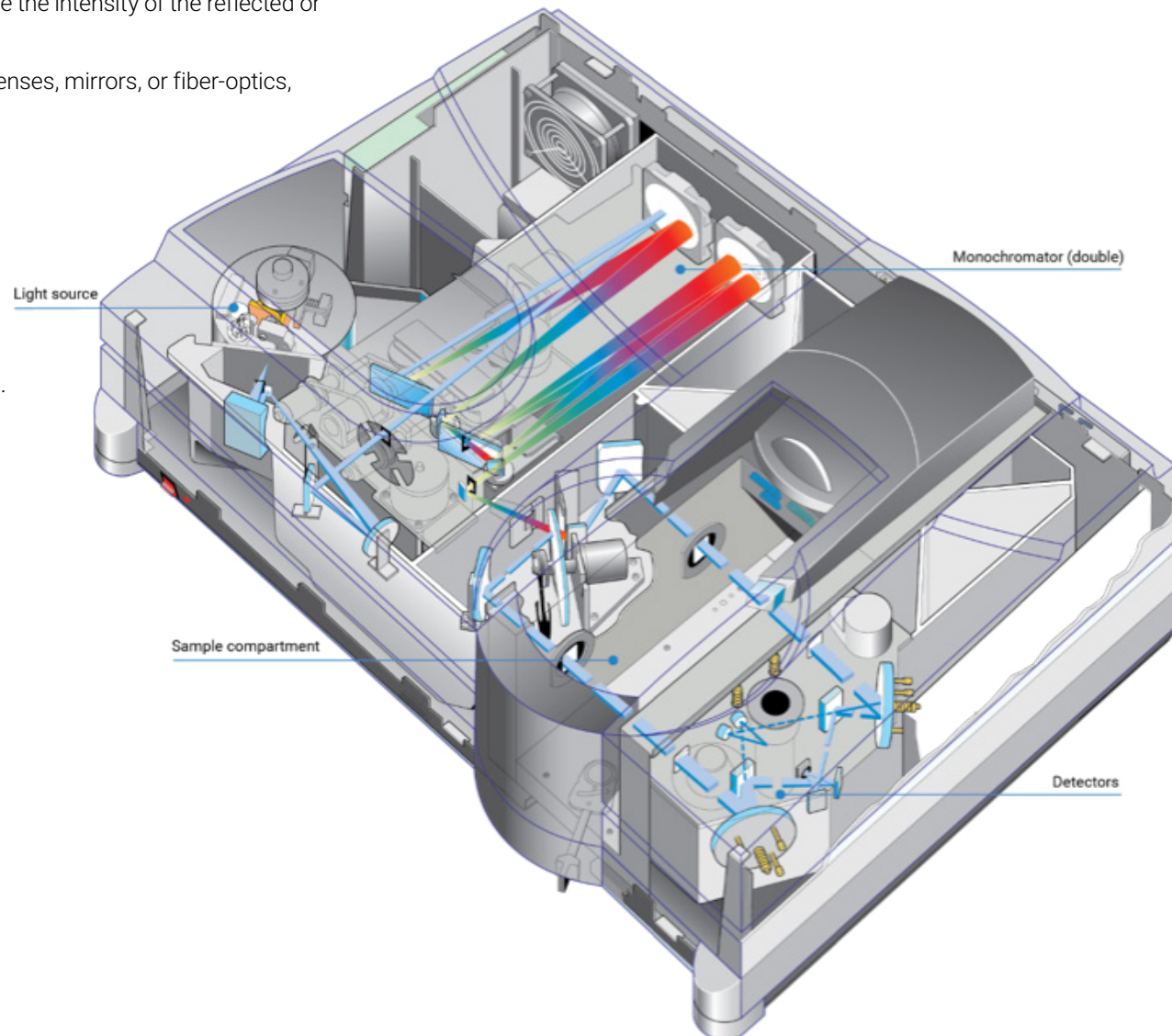
Components

The key components of a spectrophotometer are:

- A light source that generates a broadband of electromagnetic radiation across the UV-visible spectrum
- A dispersion device separates the broadband radiation into wavelengths
- A sample area, where the light passes through or reflects off a sample
- One or more detectors to measure the intensity of the reflected or transmitted radiation

Other optical components, such as lenses, mirrors, or fiber-optics, relay light through the instrument.

Figure 8. Schematic of the internal layout of an Agilent Cary 5000 UV-Vis-NIR spectrophotometer, showing the main components. Note that this is a high-performance instrument. UV-Vis spectrophotometers for routine measurements have a simpler optical design.



Light sources

The ideal light source would yield a constant intensity over all wavelengths with low noise and long-term stability of the output. Unfortunately, such a source does not exist. Two different light sources have historically been used in UV-visible spectrophotometers:

- The deuterium arc lamp was used to provide a good intensity continuum in the UV region and useful intensity in the visible region
- The tungsten-halogen lamp yielded good intensity over the entire visible range and part of the UV spectrum

More recently, a single Xenon flash lamp has been used more widely. The use of a Xenon flash lamp as a single source has significant advantages over the use of the two conventional lamps.

Deuterium (D_2) arc lamp

The deuterium arc lamp uses arc discharge from deuterium gas and yields a good intensity continuum in the UV region and useful intensity in the visible region, 185 to 400 nm (see Figure 9). Although modern deuterium arc lamps have low signal noise, noise from the lamp is often the limiting factor in overall instrument noise performance. Over time, the intensity of light from a deuterium arc lamp decreases steadily. Such a lamp typically has a half-life (the time required for the intensity to fall to half of its initial value) of approximately 1,000 hours. This short half-life means the D_2 lamp needs to be replaced relatively frequently.

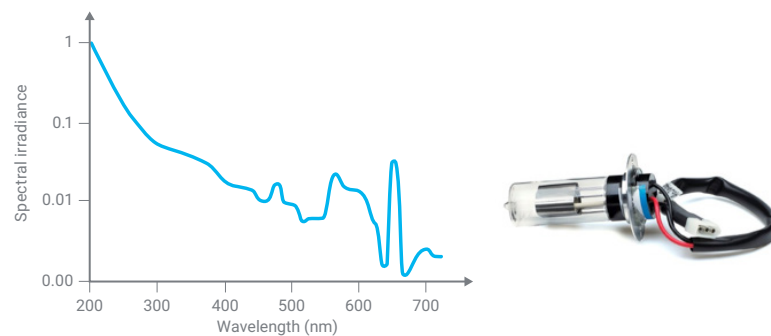


Figure 9. Intensity spectrum of the deuterium arc lamp.

Tungsten-halogen lamp

The tungsten-halogen lamp uses a filament. When a current is passed through the filament, it becomes heated and emits light (see Figure 10). The lamp yields good intensity over part of the UV spectrum and over the entire visible and NIR range (350 nm- 3000 nm). This type of lamp has very low noise and low drift and typically has a functional life of 10,000 h.

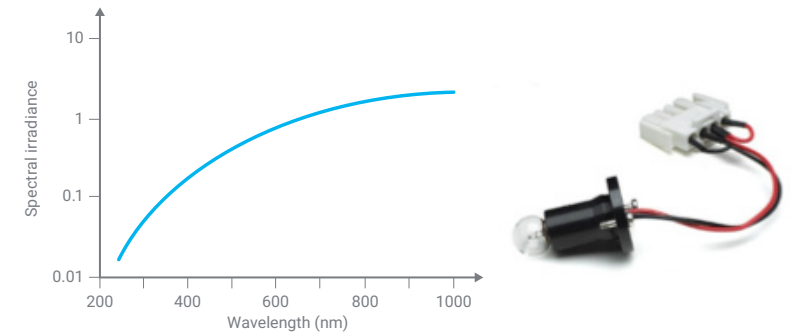


Figure 10. Intensity spectrum of the tungsten-halogen lamp.

In UV-visible spectrophotometers using both a D_2 and a tungsten-halogen lamp, either a source selector is used to switch between the lamps as appropriate, or the light from the two sources is mixed to yield a single broadband source.

Xenon flash lamp

Unlike the D_2 or tungsten-halogen lamps, which provide a constant light source, a Xenon flash lamp emits light for an extremely short time, in flashes. Since it emits only for a short time and only during sample measurement, it has a long life. The sample is only irradiated with light at the time of measurement. This short illumination time makes the Xenon flash lamp suitable for measuring samples that may be sensitive to photobleaching. Photobleaching can be observed on sensitive samples when exposed to a constant long exposure by a continuous light source. The Xenon flash lamp emits high intensity light from 185 – 2500 nm, which means no secondary light source is required (Figure 11). The Xenon flash lamp may be used for many years before requiring replacement, which makes it a popular choice compared to systems using D_2 or tungsten-halogen lamps. An extra benefit is that it does not require warmup time, unlike D_2 or tungsten-halogen lamps.

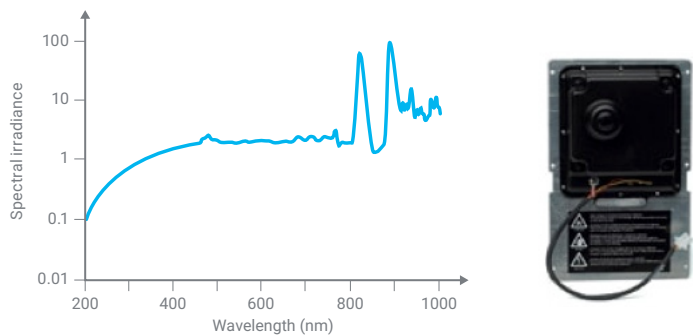


Figure 11. Intensity spectrum of the Xenon lamp.

The monochromator

All the light sources produce a broad-spectrum white light. To narrow the light down to a selected wavelength band, the light is passed through a monochromator. A monochromator consists of:

- An entrance slit,
- A dispersion device, to spread the light into different wavelengths (like a rainbow) and allow the selection of a nominated band of wavelengths, and
- An exit slit where the light of the nominated wavelengths passes through and onto the sample.

An easy way to think about a monochromator is to think of a room, with the sun shining through a window. The sunlight hits a prism that separates the white light into a rainbow. The rainbow falls onto a window on the opposite side of the room. As the prism is turned, light of different colors i.e. different wavelengths, pass out of the room through the window.

Ideally, the output from a monochromator is light of a single wavelength. In practice, however, the output is always a band of wavelengths.

Most spectrophotometers on the market today contain holographic gratings as the dispersion device. These optical components are made from glass, onto which extremely narrow grooves are precisely etched onto the surface. The dimensions of the grooves are of the same order as the wavelength of light to be dispersed. Finally, an aluminum coating is applied to create a reflective surface.

Interference and diffraction of the light falling on the grating is reflected at different angles, depending on the wavelength. Holographic gratings yield a linear angular dispersion with wavelength and are temperature insensitive. However, they reflect light in different orders, which overlap (see Figure 12). As a result, filters must be used to ensure that only the light from the desired reflection order reaches the detector. A concave grating disperses and focuses light simultaneously.

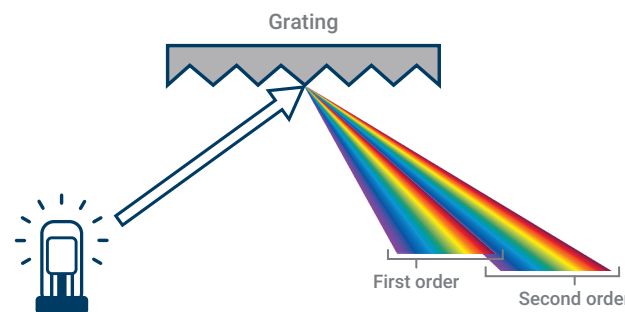


Figure 12. How a holographic grating disperses white light into light of different wavelengths.

Single monochromator spectrophotometers

A single monochromator spectrophotometer is used for general-purpose spectroscopy and can be integrated into a compact optical system.

Figure 13 shows a schematic diagram of a single monochromator optical system. A single monochromator spectrophotometer cannot select the wavelengths of light as narrowly as a double monochromator system, but this ability may not be required for many applications, for example when measuring samples that have broad absorption peaks.

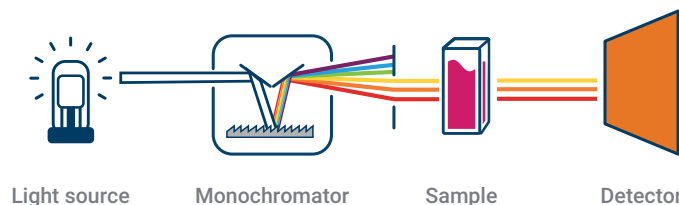


Figure 13. Single monochromator spectrophotometer.



Double monochromator spectrophotometers

A double monochromator is typically found in high-performance instruments. The two monochromators are arranged in series. The source light is split by the first monochromator and then further split by the second. Stray light, light that leaks into the system, is reduced, and the spectral accuracy (the ability to accurately select a particular wavelength) is increased. Figure 14 shows a schematic diagram of the double-monochromator optical system.

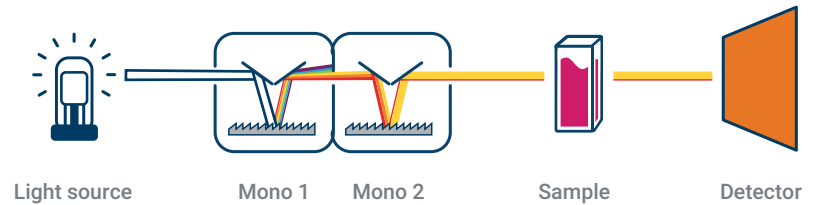


Figure 14. Schematic diagram of a double monochromator spectrophotometer.

Sample compartment

In the sample compartment, the sample is positioned to allow the beam from the monochromator to pass through the sample. For the measurement of absorbance, liquid samples would typically be held in a cuvette with a known, fixed pathlength. A cuvette is a rectangular liquid holder as shown in Figure 15. They are made from glass, quartz, plastic or another material that transmits UV or visible light. Standard cuvettes have a 10 mm pathlength and are made from quartz, to ensure good transmittance of UV wavelengths. Cheaper plastic cuvettes can also be used, but generally do not transmit UV light so are only useful if measurements in the visible wavelength region are required. A multitude of cuvettes for special applications are available – from cuvettes that hold tiny volumes of liquids through to those that have much longer pathlengths, for use with very dilute samples.



Figure 15. Cuvettes for measuring liquid samples. From left to right: A standard 10 mm pathlength, 3 mL cuvette, an ultramicro cell for measuring very low volumes, and a long pathlength cuvette for dilute solutions.

Solid samples can be held in place for simple transmission measurements. They can also be measured at various angles of incidence. For more complex measurements, like diffuse reflectance or transmission, other accessories may be installed into the sample compartment.

Single beam spectrophotometer

The simplest UV-Vis spectrophotometer has a single beam optical system. In a single-beam system, the light from the monochromator passes through the sample and then to the detector. This simple design means less optical components are used, and it makes it possible to reduce the size of the instrument and thus the cost.

However, before a sample can be measured, a baseline or blank sample must be measured. For liquid measurements, the baseline reading is taken to allow for any absorbance of the cuvette and solvent used. With a single beam system, the baseline needs to be measured separately from the sample. The separate readings mean that if there is any variation of light intensity, or system optical performance, between the baseline and sample being read, the measurement may be less accurate. This inaccuracy is a concern for sample measurements that take a long time, or where the blank may vary over time. In practice this means that a baseline/blank measurement should be run frequently and regularly during a measurement session if using a single beam system.

Double beam spectrophotometer

Many UV-Vis systems use a double beam optical system. In the double beam type, the light emitted from the monochromator is split into two beams: a reference beam and a sample beam. The light is usually split with an optical component such as a rotating wheel which has a mirrored segment, or a half-silvered mirror called a beam splitter. Each beam enters the sample chamber through separate optical paths. Since two beams of the same wavelengths are available, the reference/blank and sample can be measured at the same time. This means the sample measurement can be corrected for any instrument fluctuations in real time. This real time correction delivers a highly accurate measurement.

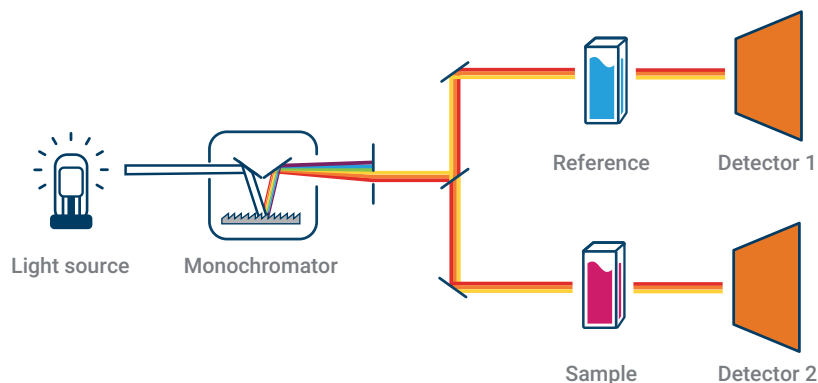


Figure 16. Schematic diagram of double beam optical system, with dual detectors.

Dual beam spectrophotometer

Another, more recent, spectrophotometer design uses a dual-beam optical layout with a sample and reference detector. The reference detector is used to correct lamp brightness fluctuations for each measurement, while the solvent or blank (in the case of a solid sample) is measured in the sample position and then subtracted from the sample spectrum after collection. With improvements in electronics and software, this design keeps the measurement process simple and reduces the chance of user error due to mismatched cuvettes or incorrect sample placement. Dual beam design has the same performance as a routine double beam instrument, while double beam design is now typically reserved for research-grade instruments.

Sample compartment

The sample compartment of a UV-Vis spectrophotometer is typically a black-colored box with a closing lid. The matt black inside the compartment helps to absorb stray light that may enter the compartment.

In the sample compartment, the sample is positioned to allow the beam from the monochromator to pass through the sample. As discussed above, glass, plastic, or quartz cuvettes (Figure 15) are used for liquid samples. Solid samples are held in position by a holder attached to the floor of the sample compartment. The light can also be taken out of the sample compartment using fiber optics. Fiber optics are useful when measuring very large, hot, cold, radioactive, or other dangerous samples. As shown in Figure 6, fiber optics can take the light from the spectrophotometer through a fiber optic probe, to measure solutions outside of the sample compartment. Alternatively, a fiber optic device that allows the measurement of light reflectance, fluorescence or transmission through a solid sample can be used.

The detector

A detector converts the light from the sample into an electrical signal. Like the light source, it should give a linear response over a wide wavelength range, with low noise and high sensitivity. Spectrophotometers normally contain either a photomultiplier tube detector or a photodiode detector. Other specialized detectors are found on high-performance systems to improve wavelength coverage or sensitivity.

Each detector has a different sensitivity and wavelength range. For systems with multiple detectors, the system will switch to the detector corresponding to the required wavelength range for the measurement.

Photomultiplier tube (PMT)

The photomultiplier tube (Figure 17) combines signal conversion with several stages of amplification within the body of the tube. The nature of the cathode material determines spectral sensitivity. A single PMT yields good sensitivity over the entire UV-visible range from 200 to 900 nm. A PMT detector provides high sensitivity at low light levels. For dilute samples, most of the light hitting the sample will pass through to the detector. To accurately detect small differences between blank and sample measurements, the detector must have low signal noise at these high light intensity levels.

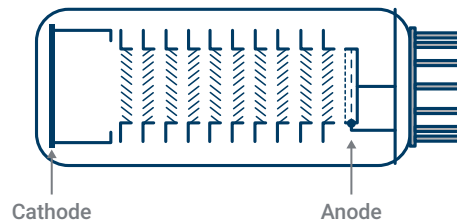


Figure 17. A photomultiplier tube detector.

Silicon diode (Si)

Silicon photodiode detectors (Figure 18) are extensively used as detectors in modern spectrophotometers. Photodiode detectors have a wider dynamic range and are more robust than PMT detectors. In a photodiode, light falling on the semiconductor material allows electrons to flow through it, depleting the charge in a capacitor connected across the material. The amount of charge needed to recharge the capacitor at regular intervals is proportional to the intensity of the light. The limits of detection for silicon-based detectors are approximately 170 to 1100 nm.

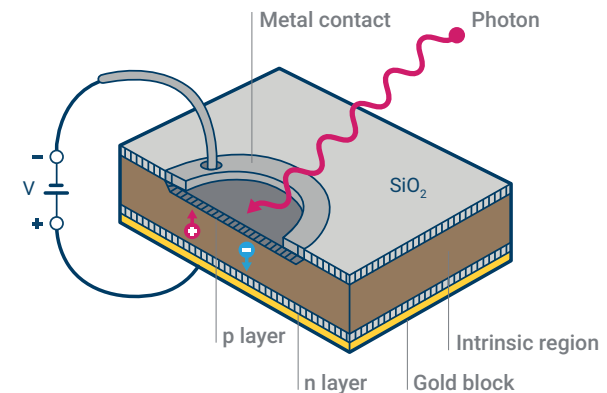


Figure 18. A silicon photodiode detector.

Indium gallium arsenide (InGaAs) photodiode

The InGaAs detector is a specialized detector that provides excellent performance for the visible and the NIR wavelength range. InGaAs detectors are available in narrow band (800 to 1700 nm) and wide band (800 to 2500 nm) options. These detectors are useful for their linear response and sensitivity in the near infrared region.

Lead sulfide (PbS) detector

The most common NIR detector used in spectrophotometers is the PbS detector. This detector is sensitive between 1000 to 3500 nm. In high performance, wide wavelength range spectrophotometers, the PbS detector is often combined with a PMT detector for UV-visible coverage. Where high sensitivity is required at the low NIR frequencies, a PbS detector may be combined with a narrow band InGaAs detector.

3. Selecting the Optimum Parameters for your UV-Vis Measurements

Selecting the most suitable sample holder, solvent and instrument parameters is critical for the success of your measurement.

3.1 Optical cell selection

Liquid samples are usually contained in a cuvette (which is another name for an optical cell, or just 'cell'). Cuvettes come in a variety of designs to suit the application. These include:

- A 'standard' 10 mm pathlength optical cell (refer to Figure 15). Holding around 3.5 mL, the cell has two optical windows, parallel to each other. Typically, the other sides are frosted or grooved to indicate that these sides are to be used for handling the cell. The optical windows should be kept as clean as possible and never touched. Avoid scratching the optical surfaces when not in use. Disposable, limited use, cells are also available. These are manufactured from polystyrene or polymethyl methacrylate (PMMA) and cannot be used at elevated temperatures. Polystyrene does not transmit UV light which allows only measurements between 340 to 800 nm to be performed. PMMA cells can be used down to 300 nm.
- For small volumes, up to around 0.5 mL, a semi-micro cell can be used. These have similar external dimensions to a standard cell but have a narrow channel on the inside to reduce the required sample volume. Ultra-micro cells are also available, holding as little as 0.5 μ L. The black masking either side of the optical window (as shown in Figure 19 and Figure 20) prevents internal reflection within the cuvette. Care needs to be taken to ensure the cells align to the optical height of the beam in the UV-Vis instrument. This is referred to as the z-height. The z-height is a measurement from the base of the cuvette to the centre of the optical path length.

Semi or ultra-micro cells are useful when sample volumes are limited.

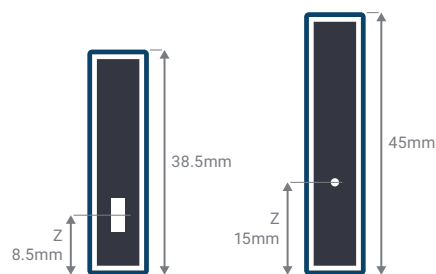


Figure 19. "Masked" cuvettes ensure that the optical beam is passed through the sample. Ensuring the z-height of the cuvette is compatible with your UV-Vis spectrophotometer design is critical.



Figure 20. A 10 mm pathlength, 1.4 mL volume semi-micro cell.

To measure multiple liquid samples a flow-through cell can be used. Flow-through cells are also available in a variety of internal cell volumes and pathlengths. The cells are usually connected to a peristaltic pump and can be connected to an autosampler. The pump pushes the sample through tubing connected to the cell, filling the cell for the measurement. A rinse solution is then pushed through to clean the cell before the next sample is pumped into the cell.



Figure 21. Flow cell, with 4 x 11 mm rectangular apertures, 10 mm pathlength. Shown with connectors and tubing.

Most optical cells are provided with a cap or lid. The cap is designed to both reduce accidental spillage of the sample and evaporation of the sample. The use of a cap is strongly recommended when measuring volatile or dangerous samples.

Cell pathlengths

The pathlength is the distance that the incident light travels through a sample. Cuvettes are available with different pathlengths. The pathlength you should use is dependent upon the absorbance of your sample:

- Concentrated samples with high absorbance (>3 Abs) need a short pathlength cuvette (less than or equal to 5 mm) – or they need to be diluted. Short pathlength cuvettes can also be used to compensate for solvents with high absorbance.
- Dilute samples with low absorbance (<0.2 Abs) need a long pathlength cell (up to 100 mm) which will increase the absorbance reading. This can also help reduce the level of error.

Most UV-Vis spectrophotometer systems are provided with a cuvette holder suitable for a standard cuvette with a 10 mm pathlength. Longer pathlength cuvettes will require an appropriate long pathlength cell holder.

Shorter pathlength cells are available in the same dimensions as the standard 10 mm pathlength cuvette. They will fit into the standard cell holder supplied with the instrument. Cell spacers are also available to securely fit a smaller pathlength cuvette into the standard cuvette holder.

Material

The optical properties of the material used in the cuvette windows needs to be considered. For the widest wavelength range quartz glass is preferred. As quartz glass is more expensive, optical glass or plastic (polystyrene) can be used when measurements below 340 nm (polystyrene) and 350 nm (optical glass) are not required. Polystyrene cells are not suitable for use at elevated temperatures. Care also needs to be taken when using polystyrene cells to ensure that samples/solvents will not damage the cells. Polystyrene cells are often referred to as disposable, or plastic cell. They are easily scratched and are designed for a single use. Table 1 shows the transmission of common materials over the UV to NIR wavelength range.

Table 1. Transparency windows for common cuvette materials.

| Material | Suitable Wavelength (nm) |
|--------------------------|--------------------------|
| Quartz | 170–2700 |
| Infrasil quartz (NIR) | 220–3800 |
| Optical glass | 334–2500 |
| Polystyrene (disposable) | 340–800 |

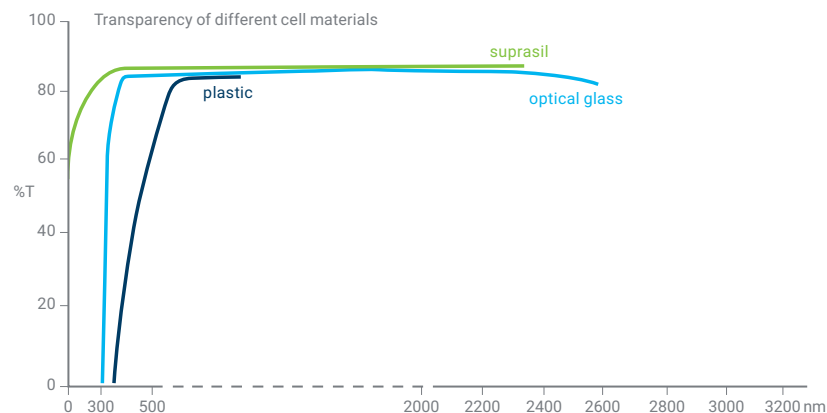


Figure 22. Transparency of common optical cell materials.

Cuvette matching

Some cuvettes are sold as matched pairs and are used for most UV-Vis and UV-Vis-NIR routine analyses. Matched pairs ensure both cuvettes provide a similar absorbance or transmission reading when empty or filled with water. The match code is like a batch number. It reflects the transmission characteristics of the batch (melt) of raw material that the cuvettes are made from.

For a pair of cuvettes to be matched they must be within the acceptable transmission tolerance at a particular wavelength. For example, a pair should transmit within 1.5% of each other at 200 nm. Cuvette manufacturers specify the transmission matching tolerances at measured wavelengths for the materials they supply.

The matching codes are only of real value when comparing new cells. Transmission characteristics change during use because of surface contamination or wear due to cleaning processes or mishandling. Therefore, a brand-new cell will not necessarily match an older used cell of the same match code.

Modern production and fusing techniques have improved flatness, parallelism, and construction tolerances. Together with more attention to quality assurance of raw materials, this has resulted in a virtual elimination of the need for transmission matching in regular standard high grade quartz cells.

Cleanliness

The oils in fingerprints are significant absorbers in the UV region. Fingerprints left on optical surfaces can cause erroneous results. Wipe off all fingerprints and contaminants using a clean soft cloth before using a cell. Ensure that the cloth is free of detergents or lubricants. Lens tissues for glasses or other uses often contain detergents or lubricants which can affect your measurements.

Do not use an ultrasonic cleaner to clean your glass or quartz cuvettes. Each ultrasonic bath generates ultrasonic waves at a different frequency and if your bath operates at the resonant frequency of the cell, the cell will break.

Fluorinated acids such as hydrofluoric acid (HF) in all concentrations should be avoided as they will attack the quartz and glass. Strong basic solutions (pH 9.0 and above) will also degrade the surface of the windows and shorten the life of the cuvette.

Once a blank measurement has been made avoid cleaning the optical windows of your cell. If cleaning is required, take a new blank measurement before continuing your measurements.

Other cuvette handling tips

Some simple steps can be taken to ensure the longevity of cuvettes:

- Consider removing samples from cuvettes immediately after use. This will prevent your sample from drying out and sticking to your cuvette. Take particular care with proteins and strong dyes as they are known to adhere to the inside surfaces of the cuvette. Remove these sample from the cell immediately after testing and rinse thoroughly with your solvent between samples.
- If measuring the same sample over a long period of time, keep the sample capped and at an appropriate temperature to minimize evaporation. Some samples may require continuous stirring.
- Clean all cuvettes thoroughly at the end of each day and either:
 - store them in a suitable container after drying or,
 - store the cuvettes wet in a mildly acidic solution (1% nitric acid or hydrochloric acid), in an acid resistant beaker. Only store one cuvette per beaker to avoid chipping the cuvette. Always rinse with copious amounts of water immediately before use again.



Measuring mobile phone displays

Designers of the screens used on mobile phones, tablets, laptops and televisions strive to make them as thin as possible. Reducing the thickness by even tens of microns can make a significant difference to reducing the overall size of a device. These screens use light emitting diodes (LED) and liquid crystal displays (LCD) to control the colors of the display, with a back light reflector to provide the illumination. Back light reflectors are usually made from a transparent polymer material with a reflector film applied to one surface. They look like a sheet of plastic mirror. Measuring the reflectance and transmission of the back light reflector is a critical part of developing a new design. It is also an important quality control measurement during their manufacture. The reflectance and transmission of a back light reflector is measured with a UV-Vis spectrophotometer. The back light reflector is mounted vertically in the sample compartment and rotated around its central axis, allowing the measurement of its reflectance and transmission at different angles of incidence.

[Here are the details of the measurement](#)

3.2 Thermostatting your samples

Many samples can be measured at room temperature, but there are some circumstances that require samples to be heated or cooled. These include:

- Cooling of volatile samples to reduce evaporation
- Heating of viscous samples to improve sample handling or homogeneity
- Samples that are sensitive to chemical change when heated
- Observing changes in samples as they are heated or cooled.

UV-Vis spectrophotometers can be fitted with accessories to control the temperature of samples. The simplest temperature control systems are suited for fixed temperature measurements. Typically, these systems use a thermostatted water circulator to pass heated water through a manifold holding the sample cuvette. For more precise temperature control a Peltier heater/cooler is embedded into the sample manifold. Peltier devices allow greater temperature control and allow temperature ramping measurements to be undertaken. An air-cooled Peltier system requires less maintenance than a water-cooled Peltier system or a water circulation system. Water circulating systems need periodic maintenance, including checking water hoses for leaks and topping up of the coolant solution. Another advantage to the Peltier system is their quiet operation as no pumping of coolant solution is needed.

When using either temperature control options your system should provide you with temperature monitoring. As a minimum the system should report the temperature of the sample manifold. This is particularly important for an external water heated system. Heat losses from the temperature set on the water bath may occur between the circulator and the sample manifold. For Peltier controlled systems the sample manifold temperature is monitored providing feedback to keep the temperature stable.

When temperature control is critical, taking measurements of the sample directly provides a more accurate reading. Small temperature probes are inserted into the sample, inside the cuvette. The probes are carefully positioned out of the light path. When monitoring the temperature directly in the samples your UV-Vis control software should allow you to record the temperature of each cuvette at each measurement.

3.3 Stirring your sample

Stirring of a thermostatted sample is important and ensures that both solution and temperature homogeneity is always maintained. Stirring is particularly important for viscous samples or to ensure consistently mixed solutions when studying a chemical reaction within the cuvette.

The effectiveness of stirring to achieve thermal (and chemical) homogeneity is strongly dependent upon the sample, solvent, and viscosity of the solution. It is important to note that viscosity changes with temperature and this may influence stirring efficiency, and the measurements, when the temperature is ramped over time.

To stir solutions a magnetic stirring bead or star, is placed at the bottom of the sample cuvette. Specially designed cuvettes with a recessed base are available. This circular recess contains the stirring bead and increases the stirring efficiency.

Care should be taken when developing the analytical method to ensure that the stir speed is suitable for the solutions. If the stirring speed is too slow the sample may not mix properly. If the speed is too high, air bubbles can be trapped in the sample, causing erroneous results.

It is recommended that test experiments are conducted on all samples to find the optimum stir speed for your experiment. When measuring liquid of similar viscosities to water, stir speeds of 800 to 900 rpm generally yield the best temperature uniformity within standard cuvettes. Lower the stirring speed for higher viscosity samples and increase the speed for lower viscosity samples.

3.4 Measurements at low temperatures

When measuring samples at lower than ambient temperature, condensation may form on the outside of cuvettes. This can interfere with the measurement. Condensation can be prevented by purging the sample compartment of the UV-Vis spectrophotometer with a clean dry gas. Some systems have specialized purging ports to allow entry of the gas into the sample compartment without introducing any light. An alternative for measuring cool samples is to use a fiber optic dip probe. A fiber optic coupler is inserted into the sample compartment and directs the light from the system through a fiber optic cable, through a dip probe directly inserted in the sample (as shown in Figure 6). The light is then directed back to the detector through a return fibre optic cable. For high throughput sampling this technique may be preferred when multiple samples are being measured at a fixed temperature.

3.5 Solvent transparency

When measuring liquid samples, or dissolving solid samples for UV analysis, solvent transparency needs to be considered. Solvents are selected based on sample solubility, stability, pH requirements, and the UV-visible cut-off wavelength. For aqueous soluble compounds water is an excellent choice as it allows measurement throughout the UV wavelengths. The use of organic solvents does limit the effective useable UV wavelength range. When selecting a solvent, consider both the solubility of your sample in the solvent as well as the transparency of the solvent in the wavelength range of interest (as shown in Figure 23).

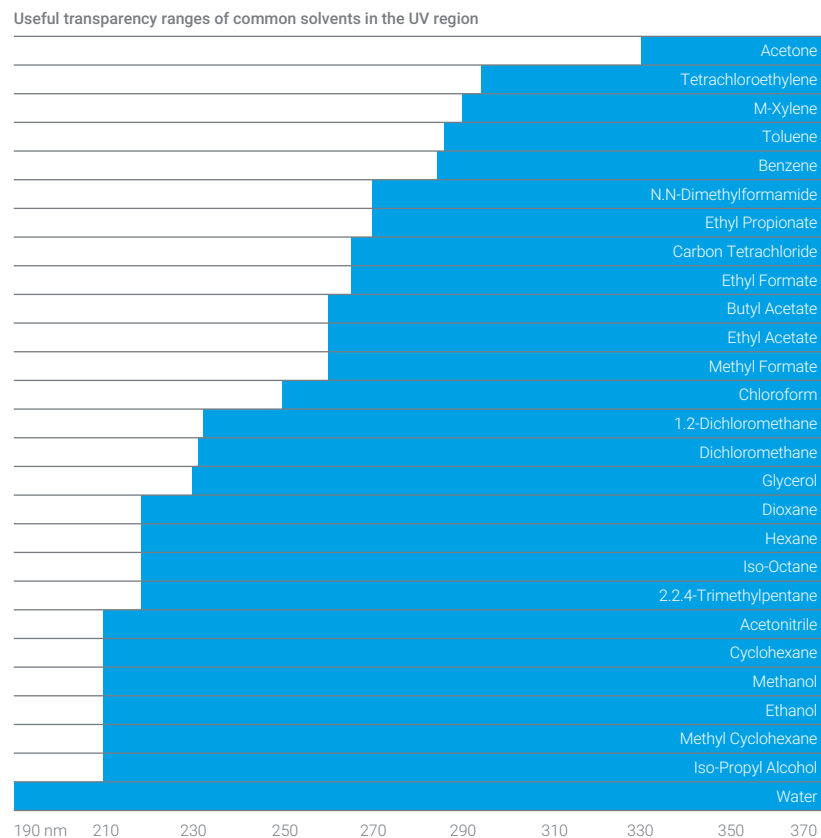


Figure 23. Transparency ranges of common solvents in the UV region. While water is preferred for UV analysis, another solvent may be required if your sample is not soluble in water.



One molecule with a very important role

The protoporphyrin molecule forms the basis of life for many plants and animals. The body stabilizes the molecule by inserting zinc atoms into its ring structure in immature red blood cells (reticulocytes). As the reticulocytes mature, the zinc is replaced by iron. The mature red cells then combine with globin-forming hemoglobin, the oxygen carrying molecule in the blood of many animals and humans.

In plants, magnesium is inserted into the protoporphyrin ring to form chlorophyll, one of the primary compounds needed for photosynthesis.

Protoporphyrin absorbs UV light – no surprise there, given its importance in photosynthesis. The molecule also fluoresces. These characteristics make protoporphyrin ideal for analysis with spectroscopy. In fact, it forms the basis of the primary screening test for childhood lead poisoning (1).

1. *Clinical and Laboratory Standards Institute, Erythrocyte Protoporphyrin Testing; Approved Guideline, Volume 16, No. 8, 1996*

3.6 Optimum spectral band width

When measuring a sample consideration should be given to the measurement resolution required. Most solid or liquid samples analyzed by UV-Vis spectroscopy have naturally broad peaks, in the order of 20 nm or more from side to side. It is good practice to use an instrument with a spectral bandwidth (SBW) setting approximately one tenth of the natural bandwidth of the analyte. The SBW of the instrument is defined as the width of the band of light at one-half the peak maximum (as shown in Figure 24), and sometimes referred to as full width at half maximum (FWHM).

The SBW of the UV-Vis spectrophotometer is related to the physical slit width of the monochromator design.

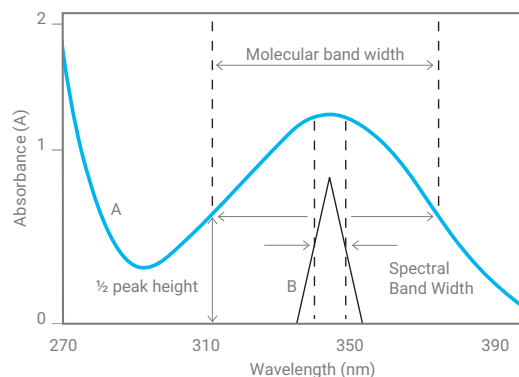


Figure 24. Spectrum A shows a peak maximum close to 345 nm. The spectral bandwidth is shown. The spectral slit width of the UV-Vis spectrophotometer will always be narrower than the required spectral band width.

Depending on the spectrophotometer design, the physical slit can be either a fixed or variable width. For most mid-range UV-Vis spectrophotometers, a fixed spectral bandwidth of 1.5 nm is common and sufficient for resolving the peaks of most liquid and solid samples. Using a larger SBW allows more light through the sample and can give better quality data and less noise, but will not resolve narrow or close together sample peaks. Using a smaller SBW will provide better resolving power but can result in increased data collection times to achieve the same data quality due to less light reaching the sample.

High performance or research grade spectrophotometer systems are more frequently designed to allow the user to select the slit width, and thereby adjust the resolution of the system. This is useful when measuring more challenging samples. The slit width can be maximized to allow greater light throughput in highly absorbent samples where high resolution of the peak is not necessary. Greater light throughput to the detector allows better method repeatability, accuracy, and precision of the results. When high resolution is required the slit width can be reduced (as shown in Figures 25 and 26).

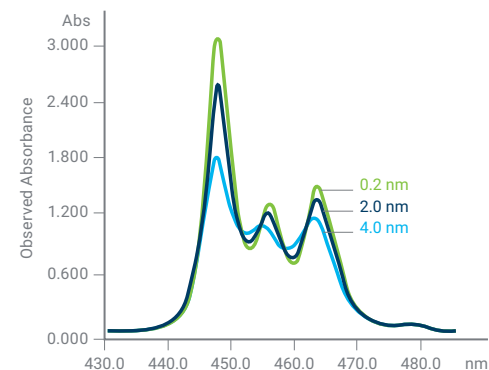


Figure 25. Overlaid scans, each measured using a different instrument slit. As the slit widens the signal-to-noise ratio improves, however resolution decreases.

As a guideline the SBW should be set at one tenth of the molecular band width of the sample (examples in Table 2).

Table 2. Recommended spectral band width settings for common UV-Vis measurement types.

| Representative Compound | Peak nm | Band Width nm | Optimum (SBW) nm |
|-----------------------------------|----------|---------------|------------------|
| Amino Acids | | | |
| tryptophan | 279 | 45 | 4.5 |
| tyrosine | 275, 195 | 40, 10 | 4.0, 1.0 |
| phenylalanine | 258 | 2.2 | 0.2 |
| Nucleotides | | | |
| adenosine | 260 | 28 | 2.8 |
| thymine | 265 | 30 | 3 |
| Proteins | | | |
| cytochrome c, oxidized | 410 | 25 | 2.5 |
| rhodopsin | 500, 278 | ~90, 25 | 9, 2.5 |
| ribonuclease | 278 | 20 | 2 |
| Pigments and Dyes | | | |
| β -carotene | 480 | 35 | 3.5 |
| chlorophyll a | 660 | 20 | 2 |
| Coenzymes | | | |
| Nicotinamide adenine dinucleotide | 260 | 35 | 3.5 |
| NADH | 340, 260 | 50, 25 | 5, 2.5 |
| Simple Organics | | | |
| benzene, vapor | 253 | <<0.1 | <<0.01 |
| benzene, solution | 253 | 2 | 0.2 |
| anthracene | 375 | 3 | 0.3 |

When optimizing spectral resolution, the data interval of the collection also needs to be considered. A minimum of three data points across the peak should be collected. While a smaller data interval can provide better resolution there will be a trade-off between how long the data takes to collect and the data interval.

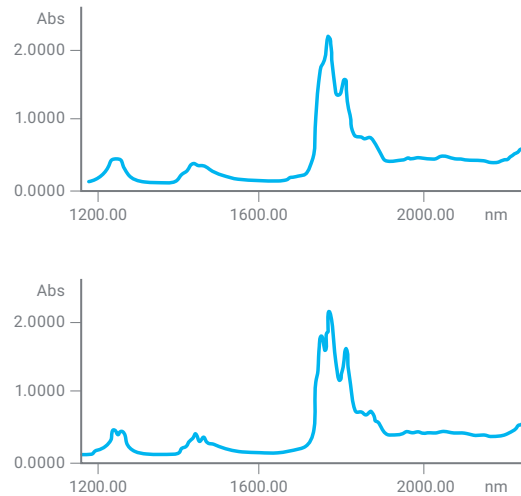


Figure 26. These two scans demonstrate the effect on resolution of varying the SBW. At a SBW of 15 nm (upper graph), little structural detail is observed in the spectrum. At a SBW of 3 nm (lower graph), the peaks are much more defined.

3.7 Stray light

Stray light or stray radiant energy (SRE) is defined as the percentage of radiation reaching the detector whose wavelengths are outside the selected spectral band. It is caused by poor instrument design (light leaking into the instrument from the laboratory lights or daylight through windows, or the light not being well separated by the monochromator) or from damage to the instrument. Most systems are provided with instrument performance checks that identify stray light issues. This is done using a test solution. The solutions used to test stray light levels are non-transmitting at the indicated wavelengths (they do transmit at other wavelengths), so the observed transmittance is due only to stray light.

Stray light causes decreased absorbance readings and changes the observed peak shape (as shown in Figure 27). As a result, stray light causes deviation from the Beer-Lambert law (as shown in Figure 28), making concentration measurements unreliable. The stray light performance of a UV-Vis instrument also determines the maximum absorbance the instrument can measure.

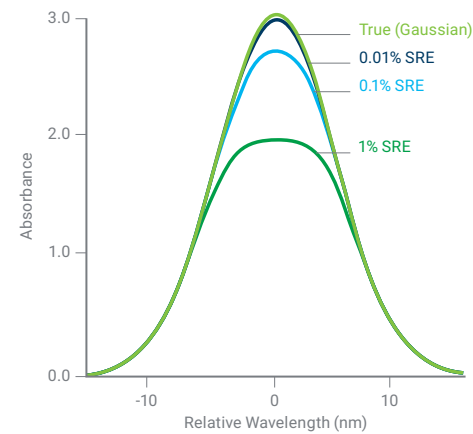


Figure 27. The maximum absorbance of peaks will be lower and the curve will flatten as stray light in the instrument increases.

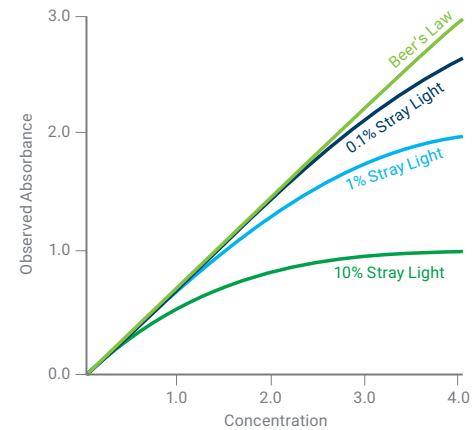


Figure 28. A UV-Vis system with poor stray light performance will show deviations from Beer's Law. This makes concentration calculations unreliable.

3.8 The linear range of a UV-Vis instrument

Both instrument design and the measurement parameters used will determine the maximum absorbance an instrument will be able to measure at a specific wavelength. At high absorbance very little light is reaching the detector which decreases the signal to noise ratio (refer to the characteristic 'fringe' on the spectrum in Figure 29). Understanding the limits of your system allows you to avoid measuring samples or performing calibrations which are outside the capabilities of your instrument. For liquid samples, diluting the sample is a way to get the measurement into the linear range of the instrument. Alternatively, you can use a short pathlength cuvette.

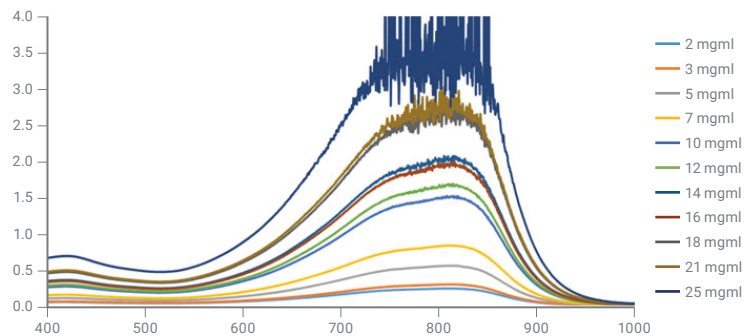


Figure 29. As sample absorbance increases progressively less light reaches the detector. This increases noise in the results and an obvious spiky signal will be observed in scanning mode.

3.9 Other useful information

Absorbance (A or Abs) is frequently measured in UV-Vis spectroscopy due to the linear relationship between concentration and absorbance as described by the Beer-Lambert law. For other applications, the percentage of light transmitted or absorbed may be more meaningful. When comparing the optical properties of a material for example it may be more useful to compare the percent transmission or absorbance difference.

Most UV-Vis spectrophotometer systems will enable you to convert your collected data between the commonly used parameters. The relationship between these parameters is shown in Table 3.

Table 3. The relationship between percent transmission and absorbance can be hard to visualize. The table shows that a sample measuring 7 Abs transmits just 0.00001% of the light through the samples.

| %T | T | Abs | %A | LogA |
|---------|-----------|-----|----------|-------|
| 100 | 10 | 0 | 0 | - |
| 50 | 0.5 | 0.3 | 50 | -0.52 |
| 10 | 0.1 | 1 | 90 | 0 |
| 1 | 0.01 | 2 | 99 | 0.3 |
| 0.1 | 0.001 | 3 | 99.9 | 0.48 |
| 0.01 | 0.0001 | 4 | 99.99 | 0.60 |
| 0.001 | 0.00001 | 5 | 99.999 | 0.70 |
| 0.0001 | 0.000001 | 6 | 99.9999 | 0.78 |
| 0.00001 | 0.0000001 | 7 | 99.99999 | 0.85 |

3.10 Wavelength or inverse centimeters

Most UV-Vis measurements are reported against wavelengths measured in nanometres (1×10^{-9} m). In some older literature the reciprocal length or wavenumber (cm^{-1}) is used. Wavenumber is often used in infrared (IR) spectroscopy measurements. Using a wavenumber scale is useful as it conveys the change in energy levels of the incident radiation. A lower wavelength gives a larger wavenumber and a higher energy (as shown in Table 4).

The use of wavenumber for infrared spectroscopy also allows for easier visualization of spectral differences as the wavelength gets progressively shorter.

For UV-Vis spectroscopy, wavelength is generally preferred as a convenient way to visualize the displayed spectrum over a spectral range.

Most UV-Vis spectrophotometer systems will enable you to collect a spectrum in either wavelength or wavenumber.

Table 4. Conversion between wavelength (nm) and wavenumber (cm^{-1}).

| λ nm | cm^{-1} |
|--------------|------------------|
| 3300 | 3030 |
| 3000 | 3300 |
| 2500 | 4000 |
| 2000 | 5000 |
| 1500 | 6666 |
| 1000 | 10000 |
| 800 | 12500 |
| 600 | 16667 |
| 400 | 25000 |
| 200 | 50000 |
| 175 | 57143 |

4. Overview of Common UV-Vis Applications

4.1 Identification—spectra and structure

UV-visible spectra generally show only a few broad absorbance peaks. Compared with techniques such as infrared spectroscopy, which produces many narrow peaks, UV-visible spectroscopy provides a limited amount of qualitative information. With only a few broad peaks, it's difficult to identify a compound based on a characteristic spectrum.

Most absorption by organic compounds results from the presence of π (that is, unsaturated) bonds. A chromophore is a molecular group usually containing a π bond. When inserted into a saturated hydrocarbon (which exhibits no UV-visible absorbance spectrum), it produces a compound with absorption between 185 and 1000 nm. Table 5 lists some chromophores and the wavelengths of their absorbance maxima.

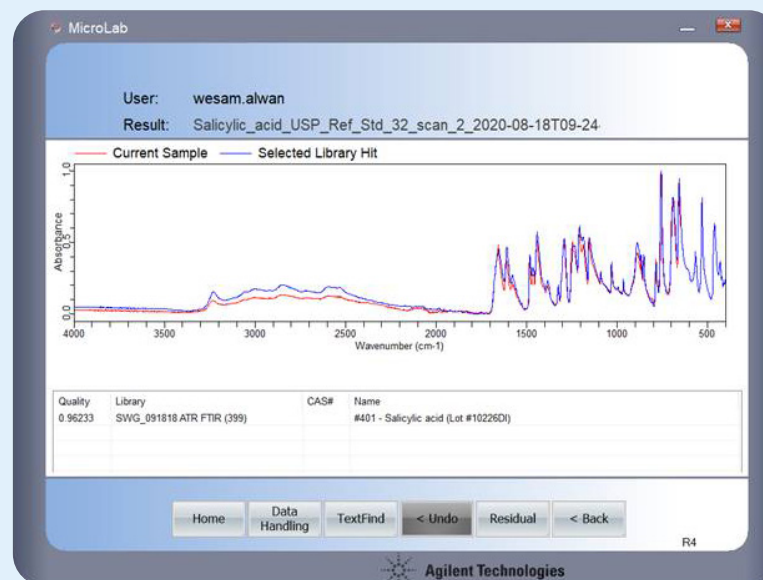
Table 5. Selected chromophores and the wavelength of their absorbance maxima.

| Chromophore | Formula | Example | λ_{max} (nm) |
|---------------------|-------------------------------|--------------|-----------------------------|
| Carbonyl (ketone) | $\text{RR}'\text{C}=\text{O}$ | Acetone | 271 |
| Carbonyl (aldehyde) | $\text{RHC}=\text{O}$ | Acetaldehyde | 293 |
| Carboxyl | RCOOH | Acetic acid | 204 |
| Amide | RCONH_2 | Acetamide | 208 |
| Ethylene | $\text{RCH}=\text{CHR}$ | Ethylene | 193 |
| Acetylene | $\text{RC}=\text{CR}$ | Acetylene | 173 |
| Nitrile | $\text{RC}=\text{N}$ | Acetonitrile | <160 |
| Nitro | RNO_2 | Nitromethane | 271 |

The presence of an absorbance band at a particular wavelength often is a good indicator of the presence of a chromophore. However, the wavelength position of the absorbance maximum is not fixed but depends partially on the molecular environment of the chromophore and on the solvent in which the sample is dissolved. Other parameters, such as pH and temperature, also may cause changes in both the intensity and the wavelength of the absorbance maxima.

FTIR spectra can be used to identify compounds

Fourier Transform Infrared (FTIR) spectra contain a lot more detail than UV-Vis spectra. A spectrum like the one shown here (red) can be matched against a library of FTIR spectra to identify the compound. In this case, a pharmaceutical—salicylic acid (the library spectrum of which is shown in blue).



Conjugating the double bond with additional double bonds increases both the intensity and the wavelength of the absorption band. For some molecular systems, such as conjugated hydrocarbons or carotenoids, the relationship between intensity and wavelength has been systematically investigated. Transition metal ions also have electronic energy levels that cause absorption of 400–700 nm in the visible region.

4.2 Confirmation of identity

Although UV-visible spectra do not enable absolute identification of an unknown, they are used to confirm the identity of a substance through comparison of the measured spectrum with a reference spectrum. Where spectra are highly similar, derivative spectra may be used. As shown in Figure 30, the number of bands increases with higher orders of derivatives. These complex derivative spectra can be useful in qualitative analysis, either for characterizing materials or for identification purposes. For example, the absorbance spectrum of the steroid testosterone shows a single, broad, featureless band centered at around 330 nm, whereas the second derivative shows six distinct peaks. The resolution enhancement effect may be of use as well in identifying an unknown. Figure 30 shows a computer simulation. When two Gaussian bands with a 40 nm natural spectral bandwidth (NBW), separated by 30 nm, are added in absorbance mode, a single band with a maximum midway between the two component bands results. The two components are not resolved. Using a fourth derivative of the spectrum, these two bands are clearly visible, with maxima centered close to the λ_{\max} of the component bands.

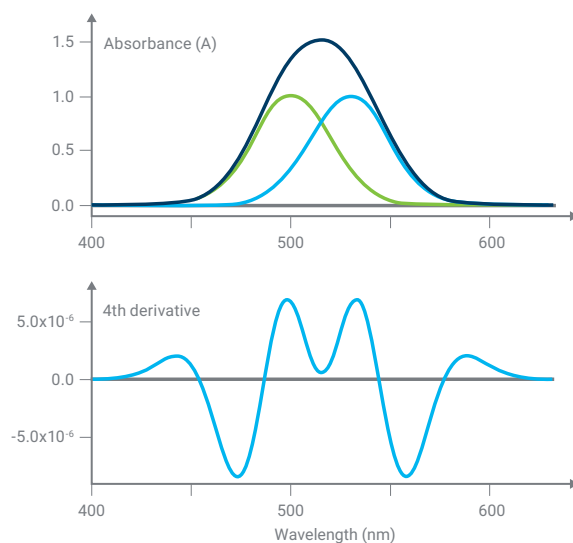


Figure 30. Resolution enhancement using derivative analysis. The original, overlapping peaks resulted in a single, broad peak. By taking the 4th derivative of the spectrum, a spectrum with much higher resolution of the peaks results.

4.3 Quantifying a molecule

Beer's law

If 100 photons of light enter a cuvette and only 50 emerge from the other side, the transmittance is 0.5, or 50%. If these 50 photons then pass through an identical cuvette, only 25 will emerge, and so forth. Figure 31 shows the plot of transmittance against path length of the cuvette.

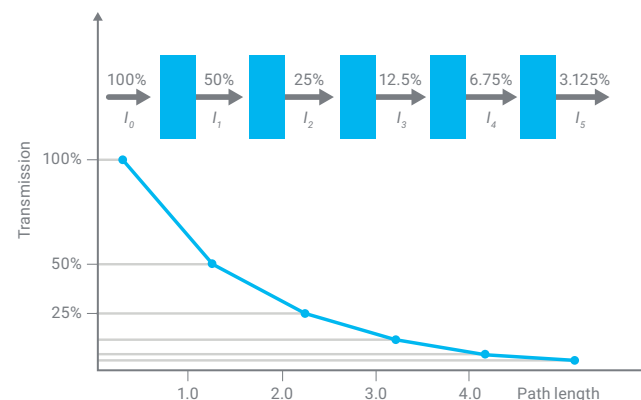


Figure 31. Transmittance and path length—the Bouguer-Lambert law.

Lambert (1760) generally is credited with the first mathematical expression of this effect, although it now appears that Bouguer first stated it in 1729.

The equation is:

$$T = I/I_0 = e^{-kb}$$

Where:

- I_0 is the incident intensity
- I is the transmitted intensity
- e is the base of natural logarithms
- k is a constant
- b is the path length (usually in centimeters).

Beer's law is identical to Bouguer's law, except that it is stated in terms of concentration. The amount of light absorbed is proportional to the number of absorbing molecules through which the light passes. Figure 32 shows a plot of transmittance against concentration.

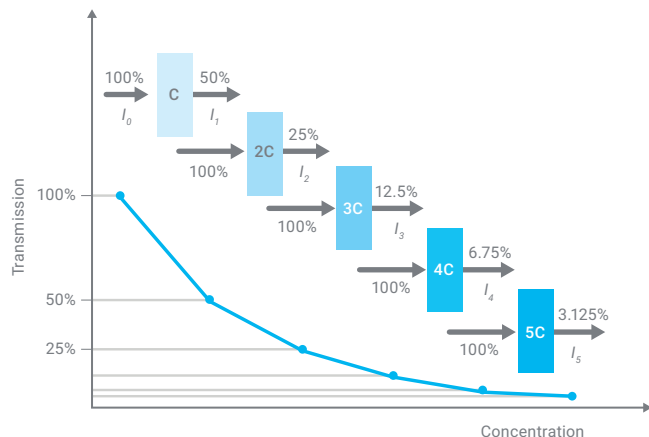


Figure 32. Transmittance and concentration—Beer's law

Combining the two laws gives the Beer-Bouguer-Lambert law:

$$T = I/I_0 = e^{-kbc}$$

Where c is the concentration of the absorbing species (usually expressed in grams per liter or milligrams per liter). This equation can be transformed into a linear expression by taking the logarithm and is usually expressed in the decadic form:

$$A = -\log T = -\log(I/I_0) = \log(I_0/I) = \Sigma bc$$

Where A is the absorbance and Σ is the molar absorption or extinction coefficient. This expression is commonly known as Beer's law. Figure 33 shows a plot of absorbance against concentration.

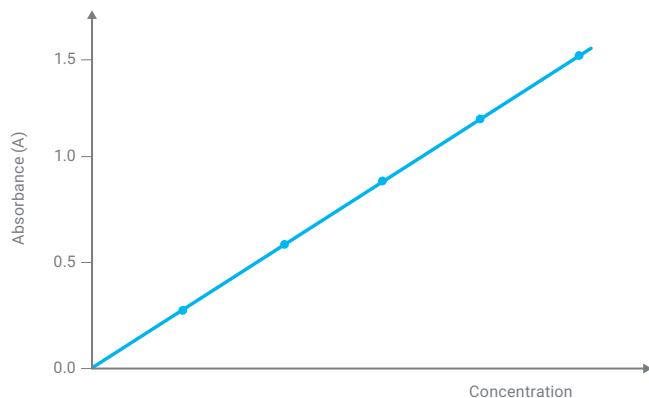


Figure 33. The Beer-Bouguer-Lambert law describes a linear relationship between absorbance of incident light and the concentration of the molecule.

The extinction coefficient (ϵ) is characteristic of a given substance under a precisely defined set of conditions, such as wavelength, solvent, and temperature. In practice, the measured extinction coefficient also depends partially on the characteristics of the instrument used. For these reasons, predetermined values for the extinction coefficient usually are not used for quantitative analysis. Instead, a calibration or working curve for the substance to be analyzed is constructed using one or more standard solutions with known concentrations of the analyte.

For electronic transitions, the difference in energy between ground and excited states is relatively large. Therefore, at room temperature, it is highly likely that all molecules are in the electronic ground state. Absorption and return to ground state are fast processes, and equilibrium is reached very quickly. Thus, absorption of UV-visible light is quantitatively highly accurate. The simple linear relationship between absorbance and concentration and the relative ease of measurement of UV-visible light have made UV-visible spectroscopy the basis for thousands of quantitative analytical methods. Assuming Beer's law is obeyed for the zero-order spectrum, a similar linear relationship exists between concentration and amplitude for all orders of derivative spectra:

Zero order: $A = \epsilon bc$

First derivative: $dA/d\lambda = (d\epsilon/d\lambda)bc$

nth derivative: $d^n A/d\lambda^n = (d^n \epsilon/d\lambda^n)bc$

at λ , where A is absorbance, ϵ is the extinction coefficient, b is the sample path length, and c is the sample concentration.

For single-component quantification, the selection of wavelengths is more difficult with derivative spectra than with absorbance spectra since both positive and negative peaks are present. The even-order derivatives have a peak maximum or minimum at the same λ_{max} as the absorbance spectrum, but for the odd-order derivatives, this wavelength is a zero-crossing point. Taking the difference between the highest maximum and the lowest minimum gives the best signal to noise (S/N) but may result in increased sensitivity to interference from other components.

For accurate results, the sample to be analyzed must contain only the absorbing component for which the calibration has been performed.

If the sample is a solution, a pure sample of the solvent should be used as a blank. It may be possible to correct for an interfering component with a second wavelength.

Concentration measurements

One of the most common applications for the UV-Vis spectrophotometer is for simple quantification of concentration. Coupled with a fiber optic dip probe accessory, a UV-vis spectrophotometer can be used to take measurements directly in the sample container, without the need to decant to a cuvette.

[Find out more.](#)



4.4 Kinetics

Analysis of reaction kinetics is fundamental for understanding how reactions occur in chemistry and biochemistry. UV-Vis spectrophotometry is an ideal technique for this application as the sample is not destroyed. It can be used when the change in reactant or products produces a change in absorbance at a specific wavelength over time. With a fast scanning UV-Vis system, multiple scans can be taken during the reaction to allow the reaction to be visualised and aid in the selection of wavelengths selected for the rate calculation.

Single point kinetics

Single point kinetics analysis is the simplest method of determining a reaction rate. A single wavelength is selected, usually the maximum absorbance of the analyte of interest, and, after initiating the reaction, the absorbance is continuously monitored at that wavelength. This results in a plot of absorbance versus time, as shown for four samples in Figure 34. Monitoring the change in absorbance over time allows you to study a reaction – when the absorbance stops changing, this is usually an indication that the reaction is complete. Figure 34 shows four different samples over 30 minutes.

The advantage of a single point measurement is that for fast reactions near continuous data can be collected as the system does not need to move to another wavelength during measurement. The data collection speed of the instrument and the signal averaging time is the limiting factor in the amount of data that can be collected.

Multiple wavelengths can also be monitored for kinetics measurements. This may provide you with an insight into the creation of a reaction product at one wavelength and depletion at another.

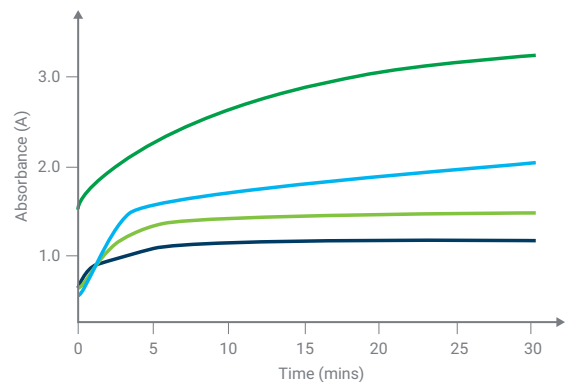


Figure 34. Monitoring the change in absorbance over time allows you to study a reaction – when the absorbance stops changing, this is usually an indication that the reaction is complete. This graph shows four different samples over 30 minutes.

Scanning kinetics

Scanning a wavelength range over time can provide additional information when performing kinetics measurements. Apart from providing a visual impression of the reaction as it occurs (as shown in Figure 34), this measurement allows the flexibility to select any number of wavelengths over the scan range to perform a reaction rate calculation. This could include analysing the consumption of reactants or the production of reaction products as the reaction progresses. When performing scanning kinetics it is important to ensure that the UV-Vis system can rapidly scan the wavelength range selected. Slower scanning will limit the amount of data collected at any wavelength during the reaction.

Rapid-mix kinetics

To monitor the reaction rate of two rapidly reacting solutions may require the use of a specialised rapid-mix or stopped flow accessory. When fitted to a UV-Vis spectrophotometer, these accessories provide accurate delivery of two or more solutions to a flow cell inside the UV-Vis, where mixing occurs. The stopped flow accessory triggers the UV-Vis system to start the analysis as soon as the solutions are mixed in the flow cell. Options include the ability to change the mixing ratio of the solutions and the ability to thermostat both the flow cell and reactants. As the collection speed is critical it is important to select a UV-Vis system with a data collection rate that is fast enough to collect a series of data points within the reaction time frame. The example shown in Figure 36 shows a reaction monitored over three seconds.

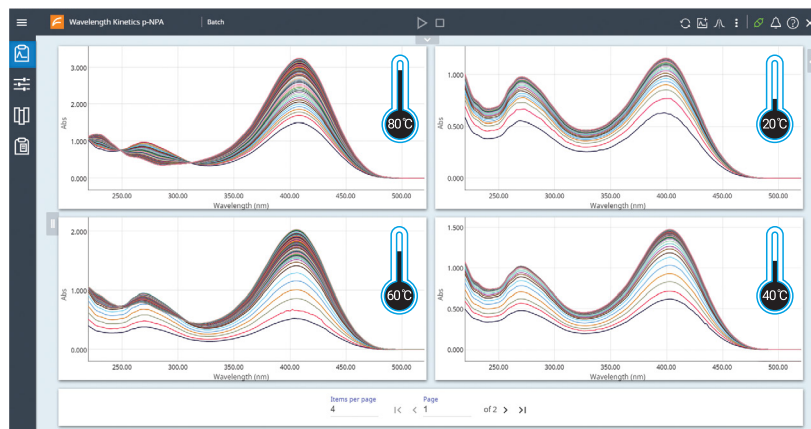


Figure 35. Scanning kinetics: UV-vis spectrophotometers provide powerful insight into reaction chemistry. The impact of temperature on chemical reactions can be observed quickly and easily with rapid scanning at multiple temperatures. [Here's a scanning kinetics experiment.](#)

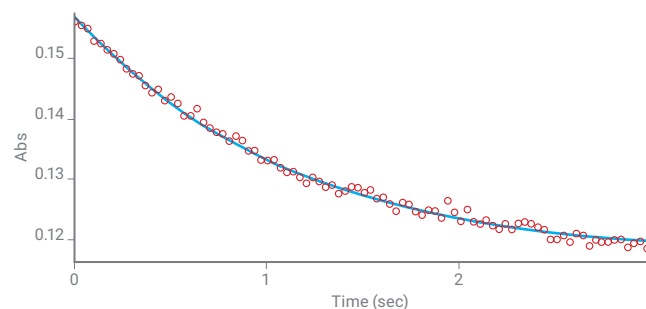


Figure 36. Using a modern UV-Vis spectrometer and a rapid mix accessory allows measurement of reactions over the time span of mere seconds. [Here's an example experiment.](#)

Kinetics measurement considerations

The rate of a reaction can be influenced by temperature. For this reason, it can be important to maintain a sample at a constant temperature, for example, body temperature (37 degrees) is usually selected for biological reactions. Peltier cooled/heated cuvettes or water thermostatted cuvette holders are commonly used with a UV-Vis system for this purpose. These accessories can either keep a sample at a specific temperature or change (ramp) the temperature over time.

The temperature accuracy and reproducibility need to be considered carefully, as slight variations in temperature or the temperature change rate can have a significant impact on results. Some systems provide the ability to measure the temperature of the samples from directly within the cuvette (rather than just measuring the temperature of the cuvette holder). When combined with feedback to the temperature control system, this typically provides better temperature control of the sample. Temperature probes in the sample can also be used to record the sample temperature along with absorbance data.

Consistent and reproducible stirring of samples is also important. When measuring reactions at room temperature, stirring ensures the reactants are mixed consistently during the reaction. For thermostatted samples, stirring ensures there is no temperature gradient across the sample.

When measuring complex systems, the use of a fiber optic probe may be preferred. A fiber optic cable allows the light from the spectrophotometer to be directed to a sample outside the UV-Vis spectrophotometer. This can be useful for measurements of a flowing manufacturing process, where the sample is at extremes of temperature or pressure, or when the sample is physically unable to fit inside the spectrophotometer sample compartment.

A [demonstration of kinetics experiments](#) can be viewed on the agilent.com site.

4.5 Color measurement

Color is an important property of a material. The color of matter is related to its absorptivity or reflectivity of specific wavelengths of light. The human eye sees the complementary color to that which is absorbed, as shown in Figure 37 and Figure 38.

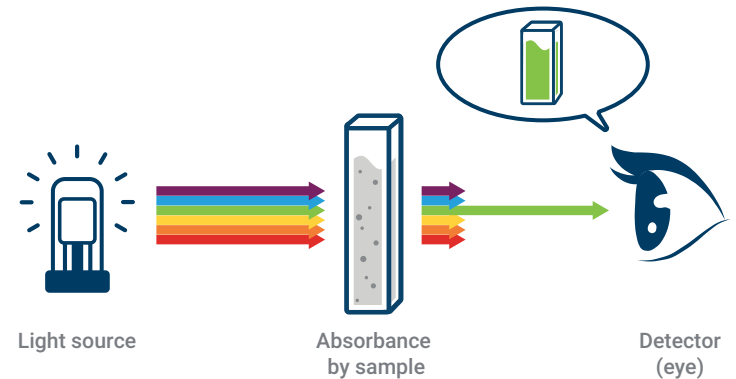


Figure 37. Transmission and color. Much like the spectrophotometer detector, our eye sees the light transmitted through or bounced off a surface. We perceive this as the color of the object.

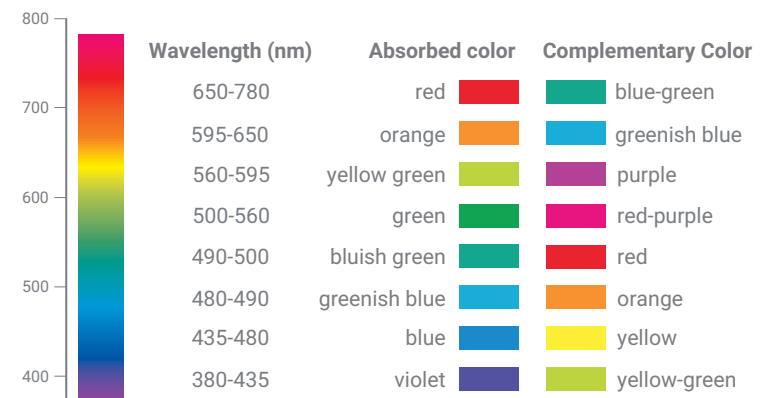


Figure 38. The wavelengths of light associated with different colors (left) and the color of absorbed light and the associated complementary color the human eye sees (right).

In practice, both the generation and sensation of color are highly complex and depend on many factors, including:

- The spectrum of the light falling on the object (consider the difference in colors seen at sunset versus the middle of the day)
- The surface structure of a solid material (the scales of a fish or the feathers of a bird are two examples where the physical structure of the surface changes the color seen)
- The viewing angle (some surfaces, such as pearlescent paints, change color as the angle you are viewing the surface at changes)

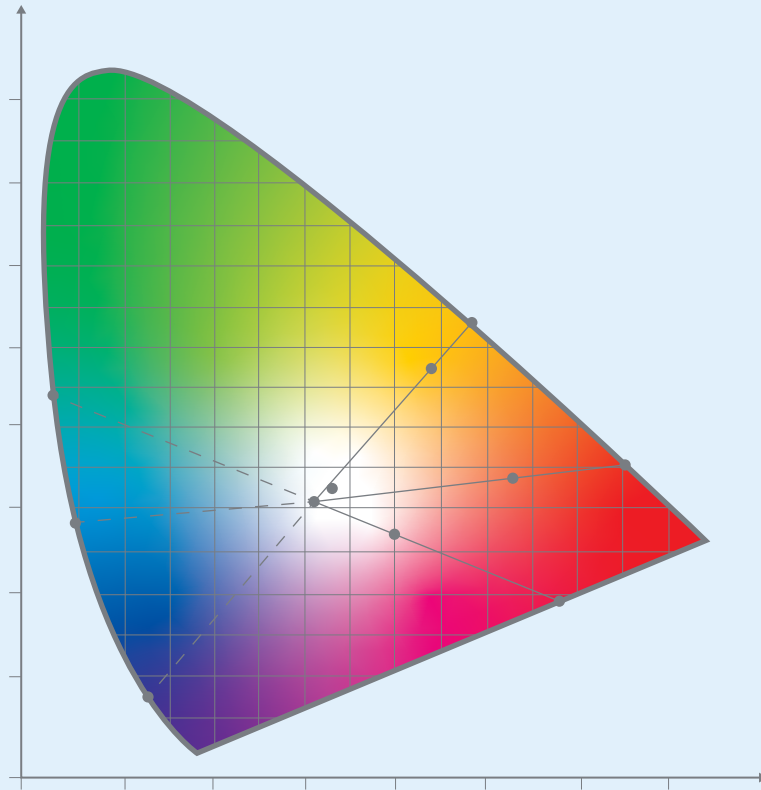
Specialized color measurement systems, such as the CIE L*a*b, and instrumentation to measure color have been developed. When equipped with the appropriate software, most spectrophotometers can be used to measure color. Color perception is also influenced by the surface and its ability to produce specular (mirror like) reflectance or diffuse (scattering) reflectance. Because of these factors, color measurement may require special accessories which allow specular and diffuse reflectance to be collected and observed at different viewing angles.

A color measuring instrument will take the UV-Vis spectrum of a sample and convert it into three color coordinates that locate the color in three-dimensional color space (refer to the image in the side panel). The three coordinates define the sample's lightness, chroma and hue. Lightness is a measure of how light or dark a color is. Chroma is a measure of 'color purity', and hue is the dominant spectral color -similar to the colors seen in a rainbow.

As well as being used for color matching measurements e.g. measuring paint colors on a manufactured item, a UV-Vis spectrophotometer can also be used to measure a change of color in a solution. UV-Vis measurements are often used for this purpose to assess whether a reaction has taken place or is proceeding, without visual inspection. Color based assays are one of the widest used applications for UV-Vis spectrophotometry.

Measuring color

UV-Vis spectrophotometers are used to measure the color of liquids and solids. By measuring the visible light that is transmitted through a solution or material, or the light that is reflected from a surface, the color of the liquid or material can be calculated. A series of three numbers is used to indicate the coordinates of the measured color in a 'color space'. [Find out more.](#)





How black is black? Ask a deep-sea fish

Researchers have found many deep-sea fishes with ultra-black skin – that which reflects less than 0.5% of incident light. The fish use their ultra-black skin to remain undetected while they hunt for prey. In the deep, dark sea creatures often use the glow of bioluminescence to help them see their prey or their attackers. Ultra-black skin absorbs the light of bioluminescence, allowing the fish to remain out of sight.

Researchers from the Smithsonian National Museum of Natural History and Duke University measured the reflection of the fish skin, which absorbs 99.5% of light. This compares to the superb bird of paradise (99.95%) and the blackest material ever made - Vantablack, which absorbs 99.96%.

The mechanism used by the fish to absorb light could have applications in solar panels, telescopes, camera and camouflage systems.

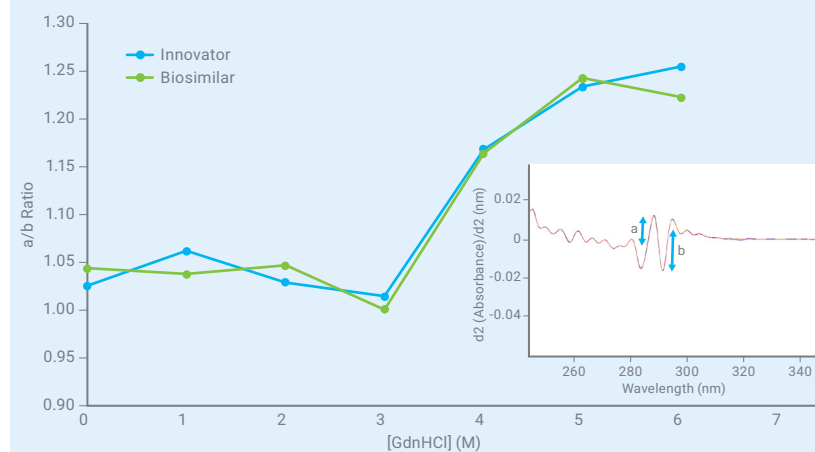
[Read the research results](#)

4.6 Structural changes of compounds

UV-visible spectroscopy can be used to determine many physicochemical characteristics of compounds. These measurements can identify a compound or determine specific properties.

Conformational studies

UV-Vis spectroscopy can provide insight into protein structure. UV-Vis spectrophotometry is also non-destructive, so precious samples will not be sacrificed. This makes UV-Vis an ideal for use prior to analysis by techniques such as LC or mass spectroscopy. This is demonstrated in the comparison of an innovator and biosimilar monoclonal antibody pair. [Find out more.](#)



4.7 Protein and nucleic acid melting temperature

UV-Vis spectroscopy is commonly used in the life sciences for analysis of biomolecules such as proteins and nucleic acids. The absorbance spectra of proteins is due to the absorbance of the aromatic amino acids tryptophan, tyrosine, and phenylalanine. Multicomponent analysis can be used to determine how many of each aromatic amino acid are present in an intact protein.

A protein at room temperature has a specific tertiary structure or conformation that in turn creates a specific electronic environment for the aromatic amino acids. Another application of UV-Vis spectroscopy exposes proteins to heat or chemical denaturants. This will, at a certain temperature or concentration, cause the protein to unfold or melt and lose its structure.

In this process, the electronic environment of the aromatic amino acids changes, which in turn results in spectral changes or shifts.

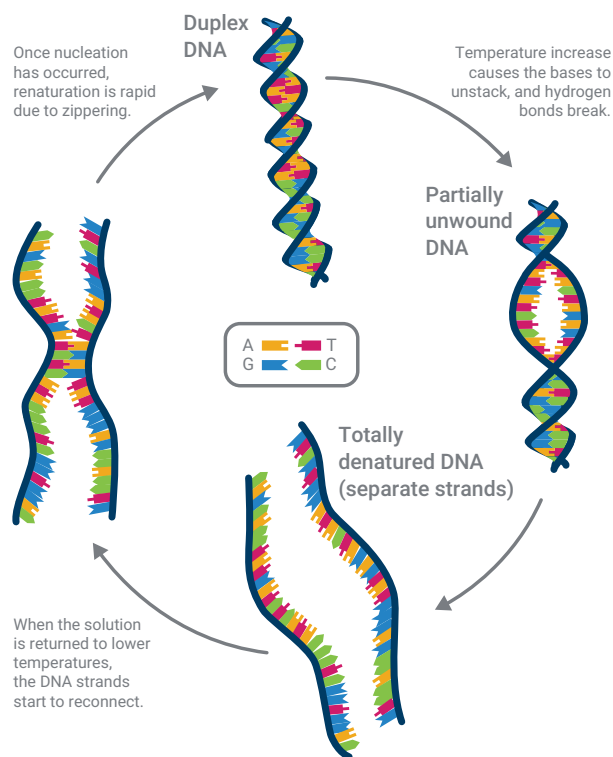


Figure 39. The strands of the DNA double helix unwind as the DNA is heated. This increases the absorbance of UV light at 260 nm. As the DNA is cooled, the strands rejoin.

Deoxyribonucleic acid (DNA) in its native state comprises two strands of deoxyribose molecules helically wound around the same axis. The strands are linked by hydrogen bonds between the purine and pyrimidine bases—adenine is joined to thymine (A-T), and guanine to cytosine (G-C). These bases are primarily responsible for the UV absorbance of DNA and the other types of nucleic acids, with a peak maximum at 260 nm. As in any multicomponent system, the observed absorption of any nucleic acid molecule should equal the sum of the individual absorbances:

$$A_{DNA} = A_{adenine} + A_{guanine} + A_{cytosine} + A_{thymine}$$

However, the observed absorbance is always significantly less than expected because the hydrogen bonding between the bases changes their electronic environment. When a molecule is heated, the hydrogen bonds break, the double helix unwinds, and the absorbance increases so that it approaches that expected from the sum of all bases (refer to Figure 39). This denaturation process is known as melting or thermal melt. In a thermal melt experiment, the temperature of a double stranded nucleic acid solution is increased in a stepwise fashion, and the absorbance at 260 nm at each temperature is measured and plotted as a melting curve (as shown in Figure 40). The midpoint of the temperature range over which the melting occurs is the T_m value. The T_m value of a particular nucleic acid sample depends primarily on the percentage of G-C pairs in the sample, each of which contains three hydrogen bonds (in contrast, each A-T pair contains two hydrogen bonds). The higher the percentage of G-C pairs in the sample, the higher the observed T_m .

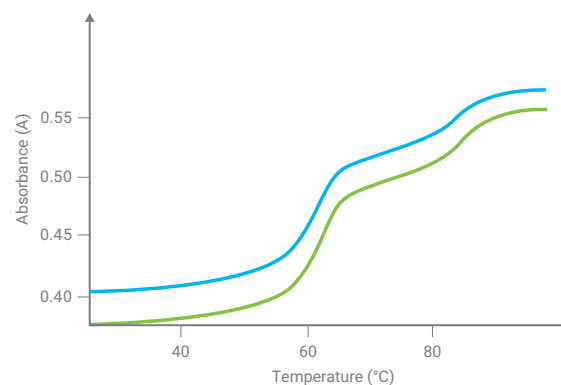


Figure 40. Measuring the absorbance at 260 nm, whilst increasing the temperature results in this characteristic graph of a DNA 'melt'. The change in absorbance indicates the multiple transitions as the DNA helix unwinds.

To perform protein and DNA melt analyses the UV-Vis spectrophotometer must have a means to change the sample temperature accurately and reproducibly. Recent advances in spectrophotometric instrumentation offer significant reductions in elapsed times for thermal melt measurements, as well as higher temperature accuracy than previously possible. UV-Vis thermal melt analysis systems are available with integrated in-cuvette temperature probes, which can be used to accurately control the temperature of the solutions during the experiment. In-cuvette stirring is also provided to ensure samples are heated homogeneously. When there are large numbers of samples for analysis a multicell holder is built into or

provided with the instrument. Most Peltier driven temperature accessories require a circulating water bath to cool the Peltier elements. Again, recent innovations have enabled the use of air-cooled peltiers in the cell block. The required temperature range is typically between 0 and 110 °C and along with the inbuilt temperature monitoring and stirring provide the ability to ramp temperatures of the sample during analysis as rapidly as possible.

Nucleic acid thermal melt analysis

Sample temperature control is a key requirement of the UV-Vis thermal melt system. Rapid and reproducible heating can be assisted by the use of an in-cuvette temperature probe.

[Find out more.](#)



4.8 Multi-component analysis

Multicomponent analyses using UV-visible spectra have been performed for almost as long as single-component analyses. However, because the techniques used in multicomponent analysis often gave incorrect results they were not widely applied. A well-designed modern UV-Vis spectrophotometer yields more precise data, and modern curve-fitting techniques give more accurate results and—perhaps more importantly—indicate when results are incorrect.

Principle of additivity

According to Beer's law, absorbance is proportional to the number of molecules that absorb radiation at the specified wavelength. This principle is true if more than one absorbing species is present. All multicomponent quantitative methods are based on the principle that the absorbance at any wavelength of a mixture is equal to the sum of the absorbance of each component in the mixture at that wavelength.

The simple approach to multicomponent analysis is based on measurements at a number of wavelengths—equal to the number of components in the mixture. The wavelengths chosen are usually those of the absorbance maximum of each component. For calibration,

the absorbance of standards of known concentrations of pure components are measured to determine the extinction coefficient for each component at each wavelength selected. The absorbance of the mixture at each wavelength is the sum of the absorbance of each component at that wavelength, which in turn depends on the extinction coefficient and the concentration of each component. Thus for two components x and y, the equations are:

$$A'_{(x+y)} = A'_x + A'_y = e'_x bc_x + e'_y bc_y$$

and

$$A''_{(x+y)} = A''_x + A''_y = e''_x bc_x + e''_y bc_y$$

Where:

- A' is absorbance at wavelength λ'
- A'' is absorbance at wavelength λ''
- e' is molar absorptivity at wavelength λ'
- e'' is molar absorptivity at wavelength λ''
- c is concentration
- b is path length.

These equations are easily solved to determine the concentration of each component. If measurements were always perfect, accurate results could be obtained even for complex mixtures of components with very similar spectra. In practice, however, measurement errors always occur. Such errors can significantly affect the accuracy of results when there is major spectral overlap. Figure 41 shows a simulated two-component mixture with no overlap of the spectra at the absorbance maxima.

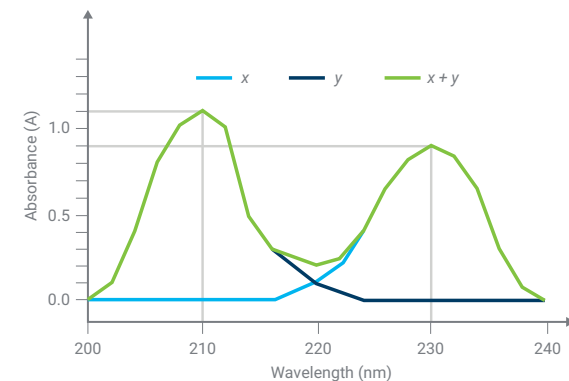


Figure 41. A two-component mixture with little spectral overlap.

In contrast, Figure 42 shows a simulated two-component mixture with significant overlap of the spectra at the absorbance maxima.

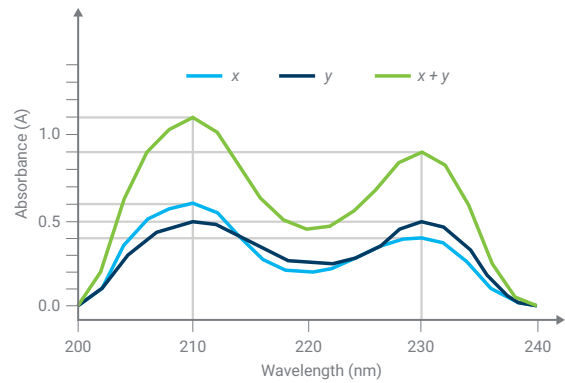


Figure 42. A two-component mixture with significant spectral overlap.

| With little spectral overlap | With substantial spectral overlap |
|------------------------------|-----------------------------------|
| $A'(x+y) = 1.1 + 0.0 = 1.1$ | $A'(x+y) = 0.6 + 0.5 = 1.1$ |
| $A''(x+y) = 0.0 + 0.9 = 0.9$ | $A''(x+y) = 0.4 + 0.5 = 0.9$ |

For a mixture of x and y where $c_x = c_y = 1$, the measured absorbances should be:

If a 10% error occurs in the measurement of $A'_{(x+y)}$ and $A''_{(x+y)}$, that is, $A'_{(x+y)} = 0.99$ (-10%) and $A''_{(x+y)} = 0.99$ (+10%), the quantitative calculation yields the results shown in Table 6:

Table 6. Comparison of multicomponent analysis results for examples with little and substantial spectral overlap.

| Component | Nominal concentration | Calculated concentration | Little spectral overlap | | Substantial spectral overlap | |
|-----------|-----------------------|--------------------------|-------------------------|--------------------------|------------------------------|---------|
| | | | % error | Calculated concentration | % error | % error |
| x | 1 | 0.9 | -10% | 0.0 | -100% | |
| y | 1 | 1.1 | +10% | 1.98 | +98% | |

Least squares method

The effect of random noise can be reduced by using additional spectral information, that is, a series of data points can be used for quantification instead of only two. In this so-called overdetermined system, a least squares fit of the standard spectra to the spectrum of the measured sample yields quantitative results (1,2). Figure 43 depicts a spectrum for the two-component mixture shown in Figure 42 with a 10% random error at each measurement point.

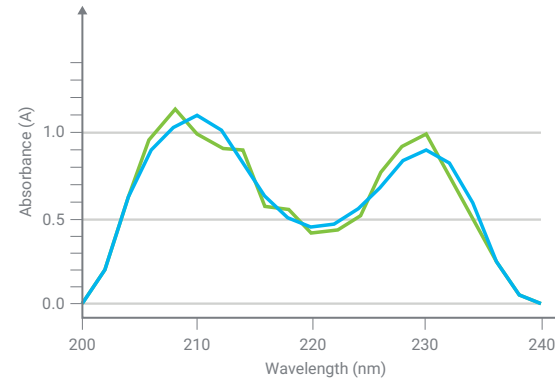


Figure 43. Mixture spectrum with 10% random error at each wavelength.

With 21 data points (2 nm intervals over 200 to 240 nm), the quantitative results from the least squares method have an error of < 1% compared with an error of approximately 100% from the usual measurements at two wavelengths, as shown in Table 7.

Table 7. Comparison of multicomponent analysis results from simple simultaneous equations and least squares methods.

| Component | Nominal concentration | Calculated concentration | Using 210 and 230 nm only | | Using 200–240 nm | |
|-----------|-----------------------|--------------------------|---------------------------|--------------------------|------------------|---------|
| | | | % error | Calculated concentration | % error | % error |
| x | 1 | 0.0 | -100% | 1.003 | +0.3% | |
| y | 1 | 1.98 | +98% | 0.995 | -0.5% | |

This method enables the analysis of more complex mixtures and of simple mixtures of components with similar spectra. The residual from the least squares calculation is a good indicator of how well the standard spectra fit the sample spectra and is therefore a good indicator of the probable accuracy of the results.

An example of multicomponent analysis is the quantification of five hemoglobins in blood with minimum sample preparation (3). Figure 44 shows the absorption spectra of hemoglobin derivatives. This analysis was previously performed using various analytical techniques, including spectroscopy and titrations.

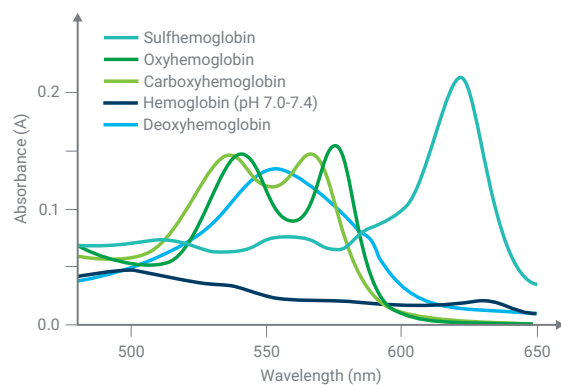


Figure 44. Absorption spectra of hemoglobin derivatives.

Other methods

Other statistical approaches to multicomponent analysis include the partial least squares (PLS), principle component regression (PCR), and multiple least squares (MLS) methods. In theory, these methods offer some advantages over those described above, however the calibration process can be much more complex.

Sample requirements

The simple simultaneous equations and least squares methods yield accurate results only if calibration is performed using pure standards or mixtures of standards for each component in the sample that contributes to the UV-visible spectrum. The unknown sample must not have any additional absorbing capacity.

Instrumental requirements

Single-component quantification is normally performed by measuring with the same instrument a standard or series of standards followed by an unknown. This calibration process should eliminate instrumental bias, making absolute wavelength accuracy and absolute photometric accuracy relatively unimportant. On the other hand, photometric reproducibility is essential for precise results. If measurements are performed only at the absorbance maximum, wavelength reproducibility is also of little importance because the rate of change of absorbance with wavelength is low. However, if a wavelength on the side of the band is used, wavelength reproducibility becomes particularly important. Finally, the instrumental linear range is critical, as the calibration process relies on a linear relationship. Accurate multicomponent analyses require excellent signal to noise performance, especially if the simple simultaneous equations method is used. In the least squares method, data from the sides of absorbance bands is incorporated into the calculation, making excellent wavelength reproducibility essential as well. Moreover, because more data is required, fast scanning is necessary for productivity.

4.9 Software requirements

Specialized multicomponent software is available to help with the creation of data models to analyse collected data. These software packages can either be incorporated into the instrument software control and reporting software or as stand-alone packages. Most UV-Vis systems available can export data in a standard format that can be imported into a multicomponent software package for processing.

References:

1. Kisner, H.; Brown, W.; Kavarnos, G. "Multiple analytical frequencies and standards for the least-squares analysis of serum lipids. **Anal. Chem.** **1983**, *55*, 1703.
2. Maris M.; Brown, C.; Lavery, D. Nonlinear multicomponent analysis by infrared spectrophotometry. **Anal. Chem.** **1983**, *55*, 1694.
3. Zwart, A.; van Kampen, E.; Zijlstra, W. Multicomponent analysis of hemoglobin derivatives with a reversed-optics instrument. **Clin. Chem.**, **1984**, *30*, 373.



5. Glossary

absorbance: 1. characteristic of a substance to absorb light 2. Unit for light absorbance, represented as A or Abs.

arc (lamp): Creates light by an electric or voltaic arc through an inert gas.

baseline: This is a measurement collected under the same parameters as the sample measurement, but without the sample in place. A blank is usually used for a baseline measurement as this allows the contributions of the instrument, the solvent, the cuvette etc to be subtracted from the final sample measurement.

blank: The solvent or substrate of the sample, without the absorbing species. When measuring liquid samples, this will be the solvent (often water) in a cuvette. The absorbance of the blank can then be subtracted from that of the sample to determine the absorbance due purely to the sample.

carotenoids: Chromophore linked to photosynthesis in some plants, algae and some bacteria.

chromophore: Part of a molecule which absorbs light

CRM: abbreviation. Certified reference material. In reference to standards supplied that have been certified to a primary standard for comparison.

cuvette: Commonly referred to as a cell, the cuvette is the container that holds liquid samples. Cuvette are available in different volumes and pathlengths. The cuvette material determines their optical transparency.

dispersion: In optical design refers to the ability of the optical device to split light into its constituent wavelengths. E.g. white light on a prism creates a rainbow effect through dispersion.

fluorescence: A form of luminescence and characteristic of some molecules to absorb light at a frequency and emit short lived light of another wavelength.

GMP: abbreviation. Good manufacturing practice. Commonly used in pharmaceutical and other regulated manufacturing industries.

holographic (optics): Holographic optics are created by etching an interference pattern on an optic surface. Holographic optics can be used in place of lenses, mirrors, and other optical devices. Their design makes them easy to accurately replicates and small and light.

noise: In spectrophotometer terms, noise refers to the background electrical signal contributed by the instrument itself. If this is too great, it can overshadow the measurement signal, making it hard to differentiate between the two signals. An easy way to think about this is when you are looking at the stars from a city location, compared to a remote location. The background light ('noise') contributed by the city lights makes it hard to see the stars. In a remote location, there is little background light, so the light from the stars can be easily seen.

peltier: A Peltier is a heating/cooling device operated by a thermoelectric coupling. The device transfers heat from one side of the device to the other. It can provide accurate temperature control of a sample.

pharmacopeia: Regulatory document listing pharmaceutical details and required or recommended testing procedures for the pharmaceutical industry.

phosphorescence: A form of luminescence related to fluorescence. It is characteristic of some molecules to absorb light at a frequency and emit a delayed, light of another wavelength.

photochemical (reactions): Chemical reaction caused by the absorption of light.

photosensitivity: Sensitivity of a substance to react when exposed to light.

QA/QC: abbreviation. Quality control or quality assurance

quantitative (measurement): Measurement providing measurement defining or describing the same. E.g. identify a molecule in solution.

quantitative (measurement): Measurement resulting in a numerical value. e.g. concentration.

rare earth oxides: Holmium, didymium and samarium oxides are referenced by standards organizations and pharmacopoeia to be used for wavelength validity measurements.

reflection: Describes the path of light a sample which is deflected at the angle of incidence.

scattering: Effect of light bouncing from a surface at random angles.

SOP: abbreviation. Standard operating procedure. Document written to ensure measurement can be formed safely and repeatably.

spectrum: plural, spectra. Range of wavelengths. The electromagnetic spectrum. Also refers to a usually graphical output of wavelength vs intensity (or absorbance, as measured by a spectrophotometer).

SST: abbreviation. System suitability tests. Test to determine the system is fit for purpose.

transmittance: Percentage of incident light that is transmitted through a sample.

zero: This is equivalent to the 'Tare' function on a set of scales – it sets the instrument reading to 0 Abs.

Learn more:

www.agilent.com/chem/

Buy online:

www.agilent.com/chem/store

Get answers to your technical questions and
access resources in the Agilent Community:

community.agilent.com

U.S. and Canada

1-800-227-9770

agilent_inquiries@agilent.com

Europe

info_agilent@agilent.com

Asia Pacific

inquiry_lsca@agilent.com

DE.8623263889

This information is subject to change without notice.

© Agilent Technologies, Inc. 2020
Published in the USA, October 1, 2020
5980-1397EN

