Spectroscopy techniques

By-

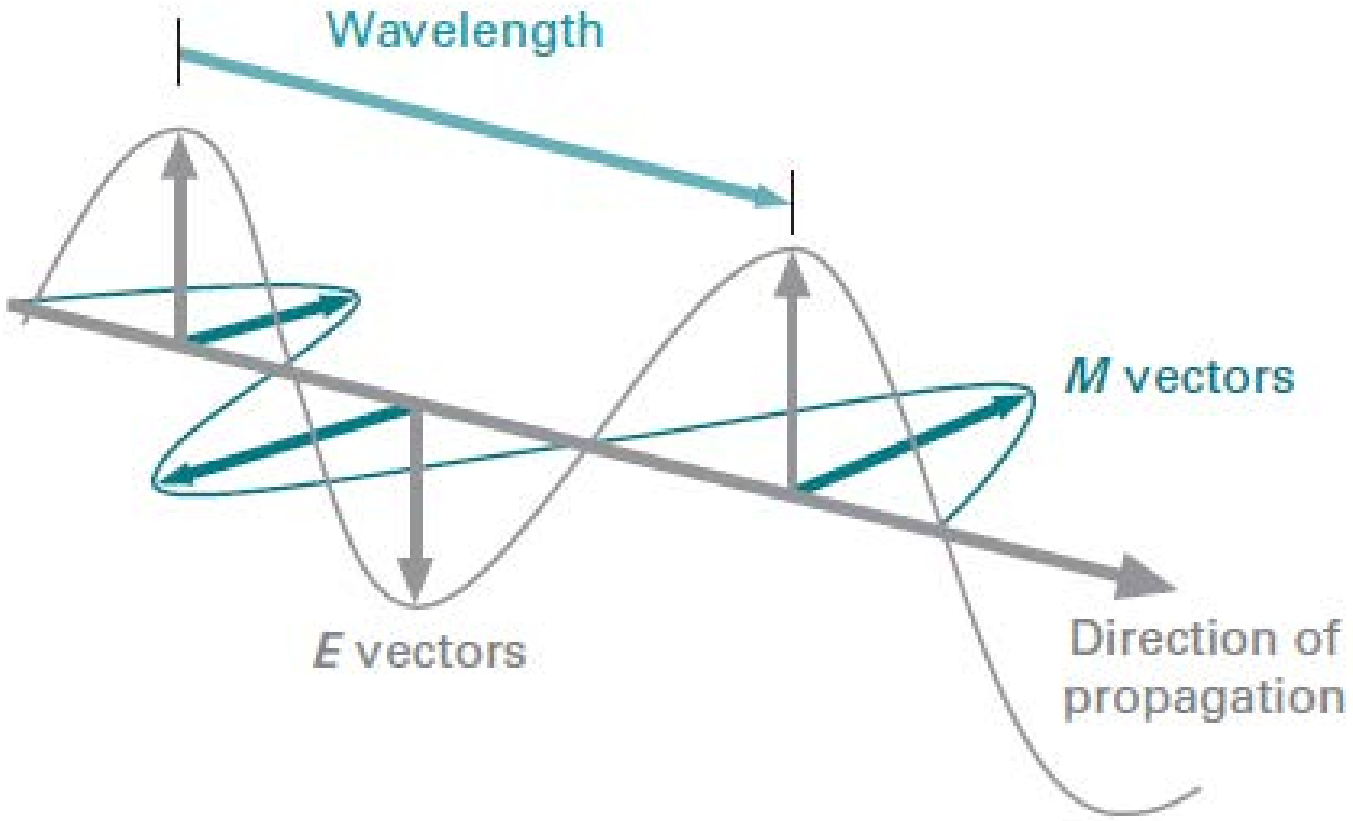
Dr. Ekta Khare

Spectroscopy

- Spectroscopic techniques employ light to interact with matter and thus probe certain features of a sample to learn about its consistency or structure.
- Light is electromagnetic radiation, a phenomenon exhibiting different energies, and dependent on that energy, different molecular features can be probed.

Electromagnetic radiation

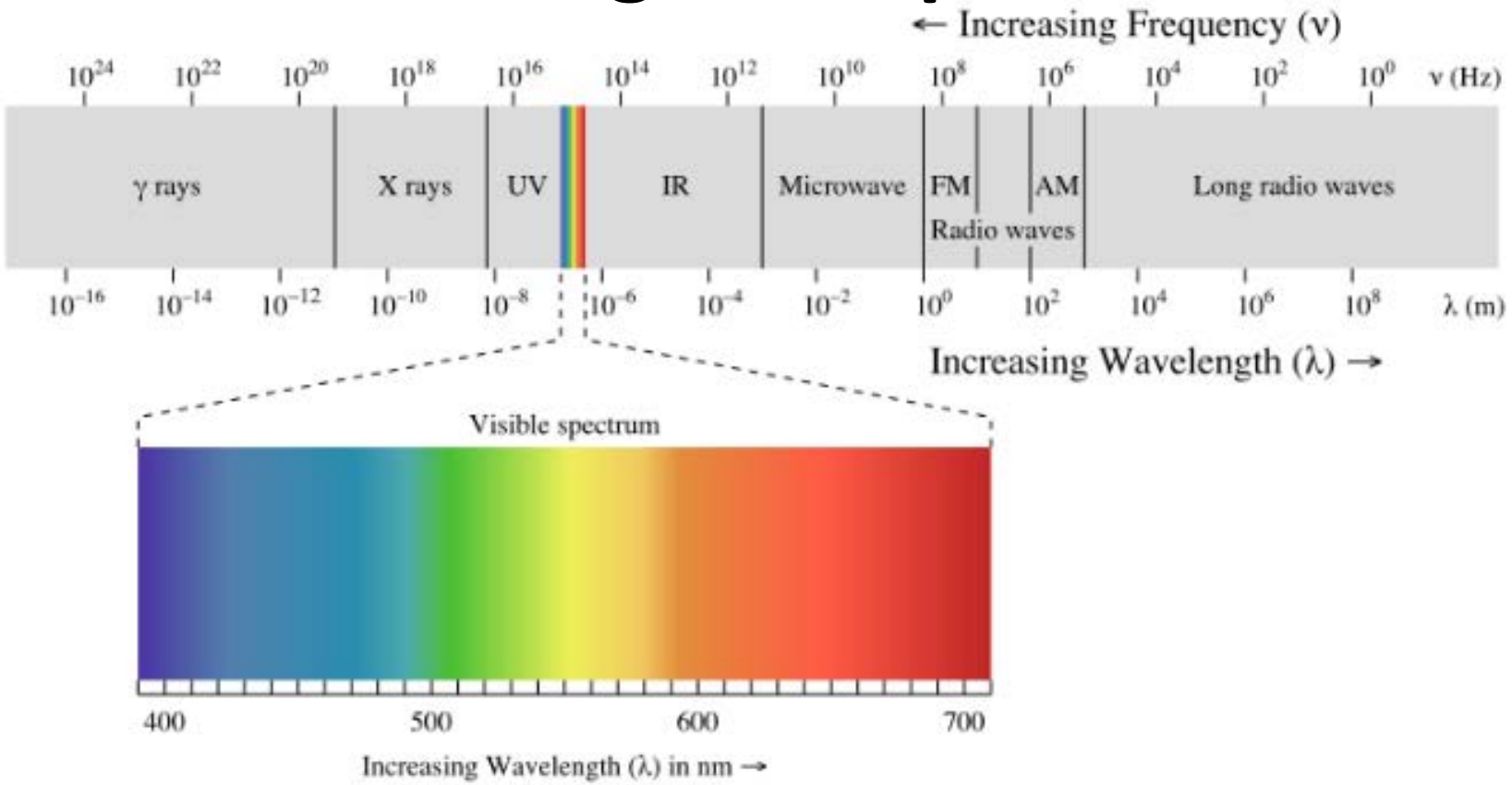
- Electromagnetic radiation is composed of an electric and a perpendicular magnetic vector, each one oscillating in plane at right angles to the direction of propagation.
- The wavelength λ is the spatial distance between two consecutive peaks (one cycle) in the sinusoidal waveform.
- The maximum length of the vector is called the amplitude.
- The frequency ν of the electromagnetic radiation is the number of oscillations made by the wave within the timeframe of 1 s.
- The frequency is related to the wavelength via the speed of light c by $\nu = c\,\lambda^{-1}$.

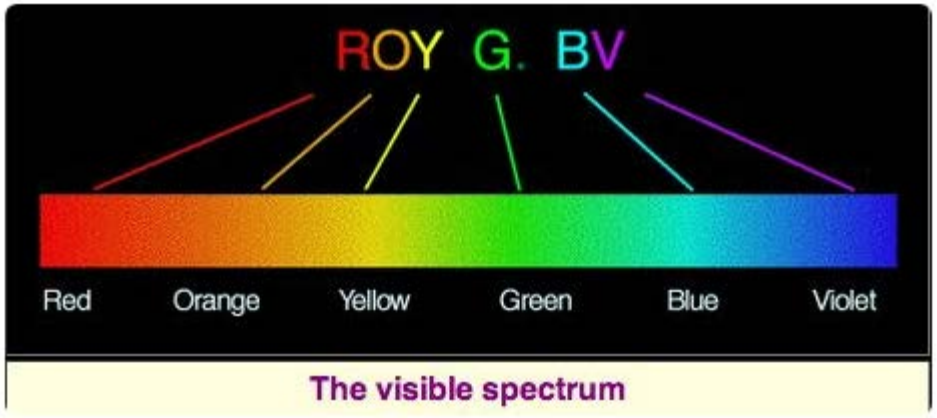

Wavelength
M vectors
E vectors
Direction of propagation

...Electromagnetic spectrum

- The shorter the wavelength, the greater the frequency and the larger the energy.
- The electromagnetic spectrum ranges from gamma (γ) radiation, which has the shortest wavelength, highest frequency, and greatest energy, to radio waves, which has the longest wavelength and lowest frequency and energy.
- Visible light is found in the middle of the EM spectrum, between IR and UV. It has frequencies of about 400 THz (Terahertz) to 800 THz and wavelengths of about 740 nm to 380 nm.
- UV light is in the range of the EM spectrum between visible light and X-rays. It has frequencies of about 8×10^{14} to 3×10^{16} Hz and wavelengths of ~ 380 nm to ~ 10 nm.
- Ultraviolet light (UV) is divided into three regions:
- UV A, wavelength = 400 - 320 nm
- UV B, wavelength = 320 - 280 nm
- UV C, wavelength = < 280 nm

Electromagnetic spectrum





Spectroscopy

- Spectroscopy is a method to measure how much a chemical substance absorbs light by measuring the intensity of light as a beam of light passes through sample solution.
- Light in the near-ultraviolet (UV) and visible (vis) range of the electromagnetic spectrum has an energy of about 150– 400 kJ mol^{-1}.
- A specific amount of energy is needed to promote electrons in a substance to a higher energy state which we can detect as absorption.
- Electrons in different bonding environments in a substance require a different specific amount of energy to promote the electrons to a higher energy state.
- This is why the absorption of light occurs for different wavelengths in different substances.

...UV-Visible Spectroscopy

- Molecules with electrons in delocalized aromatic systems often absorb light in the near-UV (150–400 nm) or the visible (400–800 nm) region.
- Absorption spectrophotometry is usually performed with molecules dissolved in a transparent solvent, such as in aqueous buffers.
- The absorbance of a solute depends linearly on its concentration and therefore absorption spectrophotometry is ideally suited for quantitative measurements.
- The wavelength of absorption and the strength of absorbance of a molecule depend not only on the chemical nature but also on the molecular environment of its chromophores.
- Spectroscopic measurements are very sensitive and nondestructive, and require only small amounts of material for analysis.

What is UV-Vis spectroscopy?

- UV-Vis spectroscopy is an analytical technique that measures the amount of discrete wavelengths of UV or visible light that are absorbed by or transmitted through a sample in comparison to a reference or blank sample.
- This property is influenced by the sample composition, potentially providing information on what is in the sample and at what concentration.
- Light can be described by its wavelength, which can be useful in UV-Vis spectroscopy to analyze or identify different substances by locating the specific wavelengths corresponding to maximum absorbance.

Principles

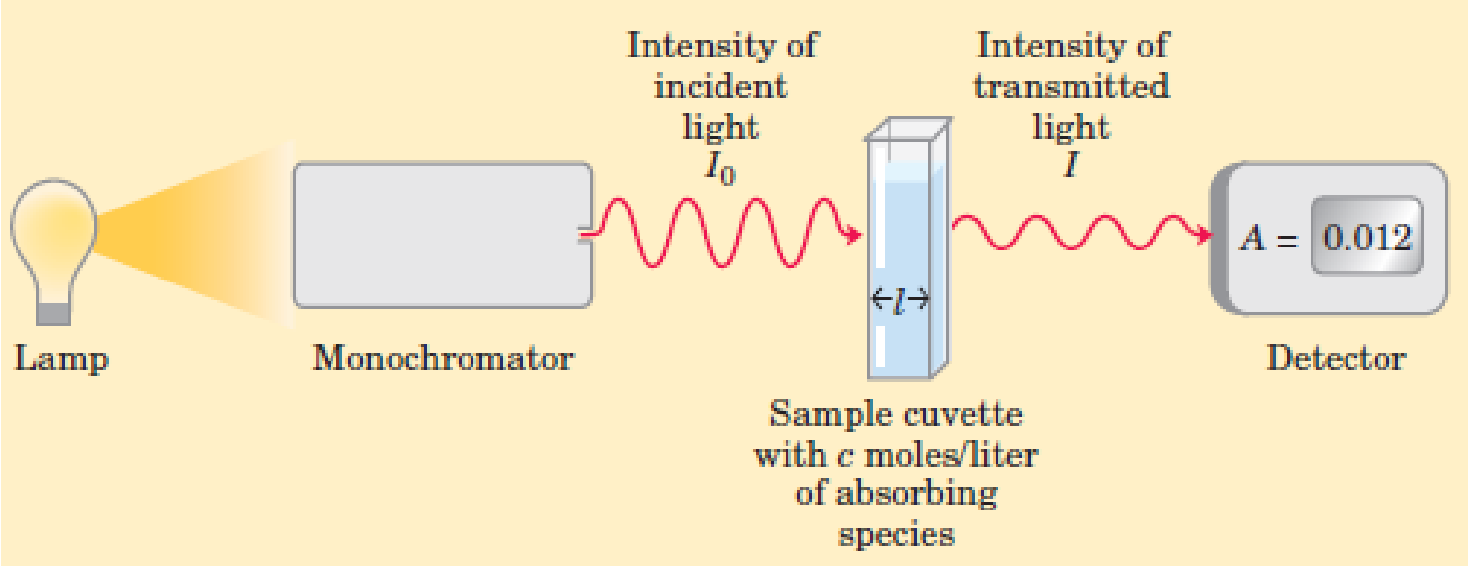
- The chance for a photon to be absorbed by matter is given by an extinction coefficient which itself is dependent on the wavelength λ of the photon.
- If light with the intensity I_0 passes through a sample with appropriate transparency and the path length (thickness), the intensity I drops along the pathway in an exponential manner.
- The ratio $T= I/I_0$ is called transmission.
- Biochemical samples usually comprise aqueous solutions, where the substance of interest is present at a molar concentration c.
- The fraction of the incident light absorbed by a solution at a given wavelength is related to the thickness of the absorbing layer (path length) and the concentration of the absorbing species (Fig. 1).
- These two relationships are combined into the Lambert-Beer law,

$$\log \frac{I_0}{I} = \varepsilon c l$$

- Where, I_0 is the intensity of the incident light, I is the intensity of the transmitted light, ε is the molar extinction coefficient (in units of liters per mole-centimeter), c is the concentration of the absorbing species (in moles per liter), and l is the path length of the light absorbing sample (in centimeters).
- The expression log (I_0 /I) is called the **absorbance,** designated A.
- It is important to note that each successive millimeter of path length of absorbing solution in a 1.0 cm cell absorbs not a constant amount but a constant fraction of the light that is incident upon it.
- However, with an absorbing layer of fixed path length, the absorbance, A, is directly proportional to the concentration of the absorbing solute.
- The molar extinction coefficient varies with the nature of the absorbing compound, the solvent, and the wavelength, and also with pH if the light-absorbing species is in equilibrium with an ionization state that has different absorbance properties.
- The Beer–Lambert law is valid for low concentrations only.
- Higher concentrations might lead to association of molecules and therefore cause deviations from the ideal behaviour.

FIGURE 1 The principal components of a spectrophotometer.

- A light source emits light along a broad spectrum, then the monochromator selects and transmits light of a particular wavelength.
- The monochromatic light passes through the sample in a cuvette of path length *l and is absorbed by* the sample in proportion to the concentration of the absorbing species.
- The transmitted light is measured by a detector.



Absorption or light scattering – optical density

- In some applications, for example measurement of turbidity of cell cultures (determination of biomass concentration), it is not the absorption but the scattering of light that is actually measured with a spectrophotometer.
- Extremely turbid samples like bacterial cultures do not absorb the incoming light.
- Instead, the light is scattered and thus, the spectrometer will record an apparent absorbance (sometimes also called attenuance).
- In this case, the observed parameter is called optical density (OD).
- Instruments specifically designed to measure turbid samples are nephelometers or Klett meters.
- However, most biochemical laboratories use the general UV/Vis spectrometer for determination of optical densities of cell cultures.

Instrumentation

- UV/Vis spectrophotometers are usually dual-beam spectrometers where the first channel contains the sample and the second channel holds the control (buffer) for correction.
- Alternatively, one can record the control spectrum first and use this as internal reference for the sample spectrum.
- The latter approach has become very popular as many spectrometers in the laboratories are computer-controlled, and baseline correction can be carried out using the software by simply subtracting the control from the sample spectrum.
- **The light source** is a tungsten filament bulb for the visible part of the spectrum, and a deuterium bulb for the UV region.
- Since the emitted light consists of many different wavelengths, a **monochromator**, is placed between the light source and the sample.

...Instrumentation

- Wavelength selection can also be achieved by using coloured filters as monochromators that absorb all but a certain limited range of wavelengths.
- This limited range is called the bandwidth of the filter.
- Filter-based wavelength selection is used in colorimetry, a method with moderate accuracy, but best suited for specific colorimetric assays where only certain wavelengths are of interest.
- If wavelengths are selected by prisms or gratings, the technique is called spectrophotometry (Fig. 12.6).

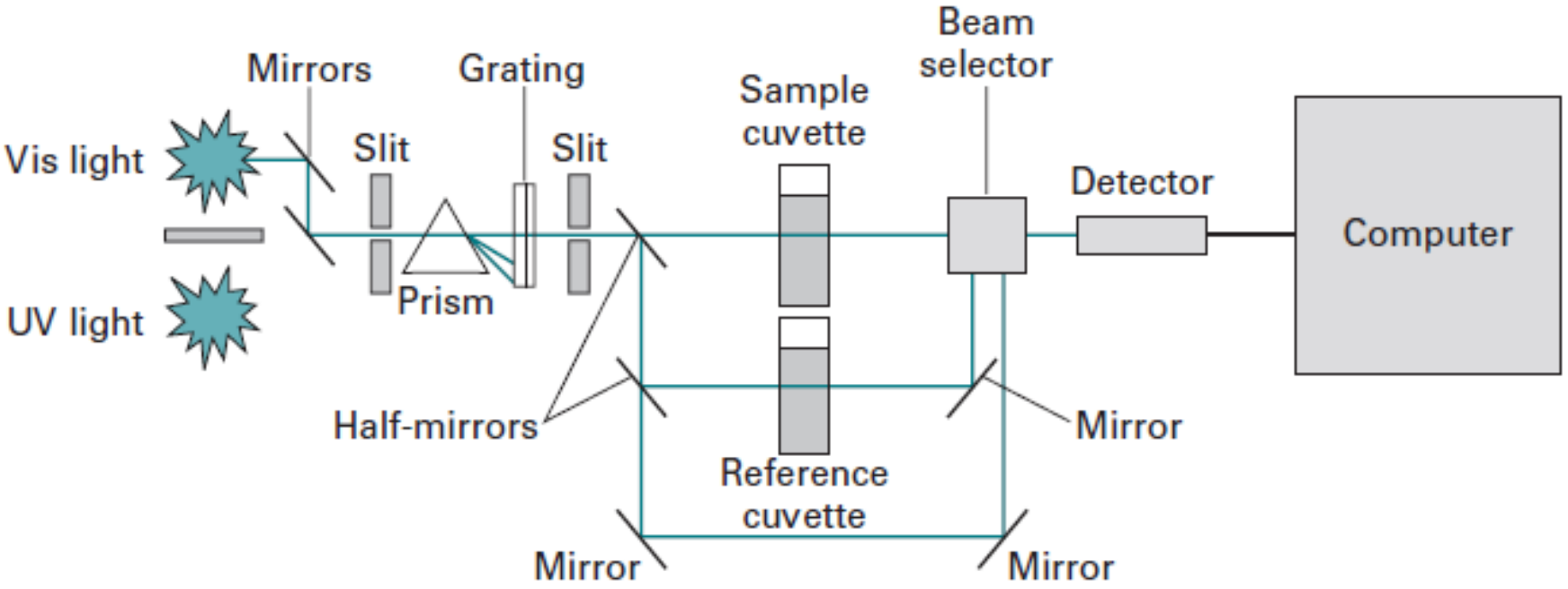


Fig. 12.6 Optical arrangements in a dual-beam spectrophotometer. Either a prism or a grating constitutes the monochromator of the instrument. Optical paths are shown as green lines.

... Instrumentation

- In a dual-beam instrument, the incoming light beam is split into two parts by a half mirror.
- One beam passes through the sample, the other through a control (blank, reference).
- This approach obviates any problems of variation in light intensity, as both reference and sample would be affected equally.
- The measured absorbance is the difference between the two transmitted beams of light recorded.
- Depending on the instrument, a second detector measures the intensity of the incoming beam, although some instruments use an arrangement where one detector measures the incoming and the transmitted intensity alternately.
- The latter design is better from an analytical point of view as it eliminates potential variations between the two detectors.
- At about 350nm most instruments require a change of the light source from visible to UV light.
- This is achieved by mechanically moving mirrors that direct the appropriate beam along the optical axis and divert the other.

- Since borosilicate glass and normal plastics absorb UV light, such cuvettes can only be used for applications in the visible range of the spectrum (up to 350 nm).
- For UV measurements, quartz cuvettes need to be used.
- However, disposable plastic cuvettes have been developed that allow for measurements over the entire range of the UV/Vis spectrum.

Applications of UV-Vis spectroscopy

- **DNA and RNA analysis**
- When preparing DNA or RNA samples, for example for downstream applications such as sequencing, it is often important to verify that there is no contamination of one with the other, or with protein or chemicals carried over from the isolation process.
- The 260 nm/280 nm absorbance (260/280) ratio is useful for revealing possible contamination in nucleic acid samples.
- Pure DNA typically has a 260/280 ratio of 1.8, while the ratio for pure RNA is usually 2.0.
- Pure DNA has a lower 260/280 ratio than RNA because thymine, which is replaced by uracil in RNA, has a lower 260/280 ratio than uracil.
- Samples contaminated with proteins will lower the 260/280 ratio due to higher absorbance at 280 nm.

Wavelength used in absorbance analysis in nanometers	What does UV absorbance at this wavelength indicate the presence of?	What causes UV absorbance at this wavelength?
230	Protein	Protein shape[10]
260	DNA and RNA	Adenine, guanine, cytosine, thymine, uracil
280	Protein	Mostly tryptophan and tyrosine

DNA and RNA analysis

- The 260 nm/230 nm absorbance (260/230) ratio is also useful for checking the purity of DNA and RNA samples and may reveal protein or chemical contamination.
- Proteins can absorb light at 230 nm, thus lowering the 260/230 ratio and indicating protein contamination in DNA and RNA samples.
- Guanidinium thiocyanate and guanidinium isothiocyanate, two of the common compounds used in purifying nucleic acids, strongly absorb at 230 nm which will lower the 260/230 absorbance ratio too.

Absorbance ratio	Typical values
260/280	1.8 absorbance ratio typical for pure DNA 2.0 absorbance ratio typical for pure RNA
260/230	Absorbance ratio varies; 2.15 to 2.50 typical for RNA and DNA[11]

Pharmaceutical analysis

- In particular, processing UV-Vis spectra using mathematical derivatives allows overlapping absorbance peaks in the original spectra to be resolved to identify individual pharmaceutical compounds.
- For example, benzocaine, a local anesthetic, and chlortetracycline, an antibiotic, can be identified simultaneously in commercial veterinary powder formulations by applying the first mathematical derivative to the absorbance spectra.
- Simultaneous quantification of both substances was possible on a microgram per milliliter concentration range by building a calibration function for each compound.

Bacterial culture

- UV-Vis spectroscopy is often used in bacterial culturing.
- OD measurements are routinely and quickly taken using a wavelength of 600 nm to estimate the cell concentration and to track growth.
- 600 nm is commonly used and preferred due to the optical properties of bacterial culture media in which they are grown and to avoid damaging the cells in cases where they are required for continued experimentation.

Beverage analysis

- The identification of particular compounds in drinks is another common application of UV Vis spectroscopy.
- Caffeine content must be within certain legal limits, for which UV light can facilitate quantification.
- Certain classes of colored substances, such as anthocyanin found in blueberries, raspberries, blackberries, and cherries, are easily identified by matching their known peak absorbance wavelengths in wine for quality control using UV Vis absorbance.

Other applications

- An enzyme catalyses the conversion of one or several substrates to one or several products. The rate of the catalysed reaction or the activity of the enzyme can be determined by measuring either the decrease in substrate concentration or the increase in product concentration as a function of the reaction time.
- In wastewater treatments, UV-Vis spectroscopy can be used in kinetic and monitoring studies to ensure certain dyes or dye by-products have been removed properly by comparing their spectra over time.
- Tracking changes in the wavelength corresponding to the peak absorbance is useful in examining specific structural protein changes and in determining battery composition.
- Shifts in peak absorbance wavelengths can also be useful in more modern applications such as characterization of very small nanoparticles.
- The applications of this technique are varied and seemingly endless.