UV-Visible Spectrophotometry

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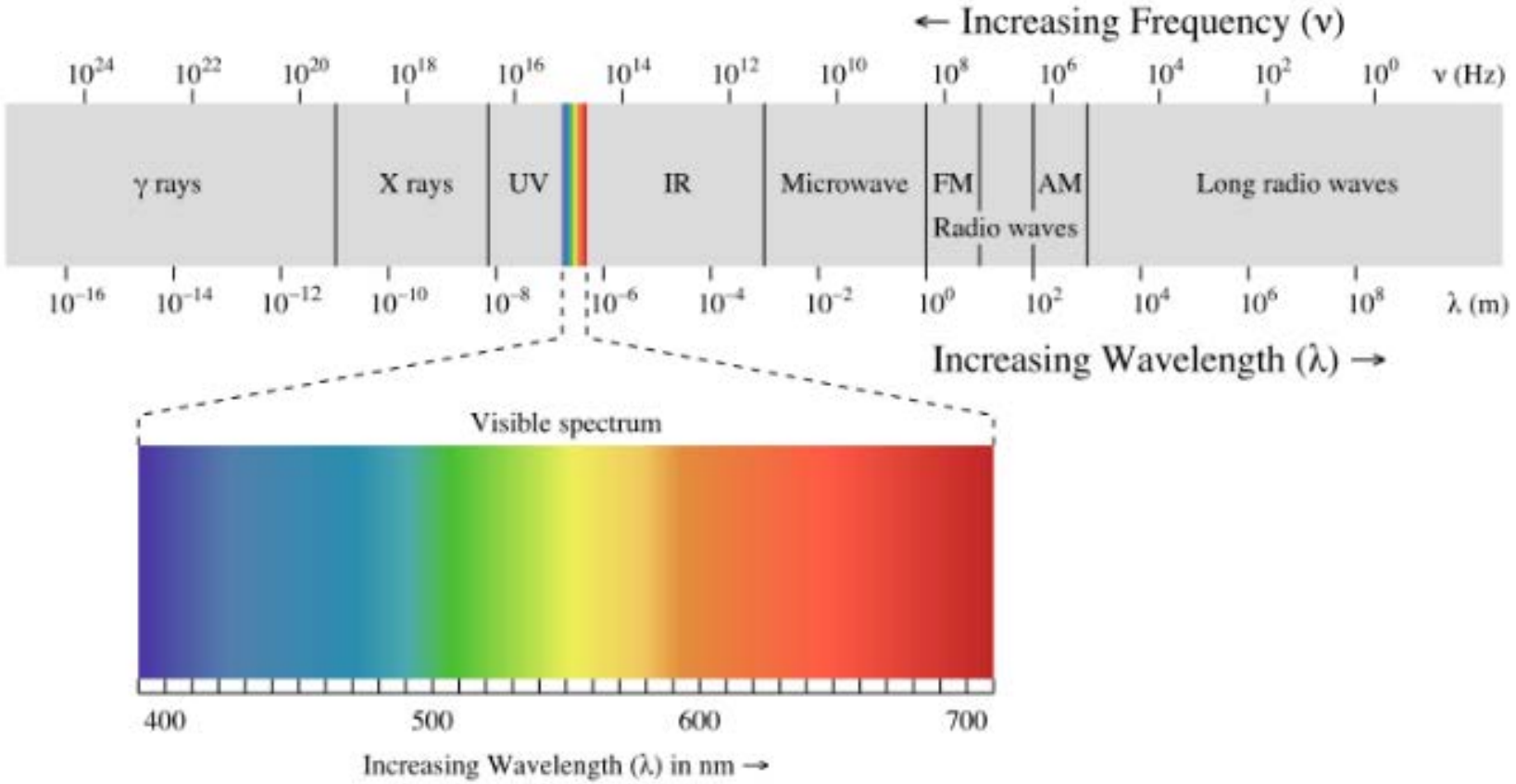
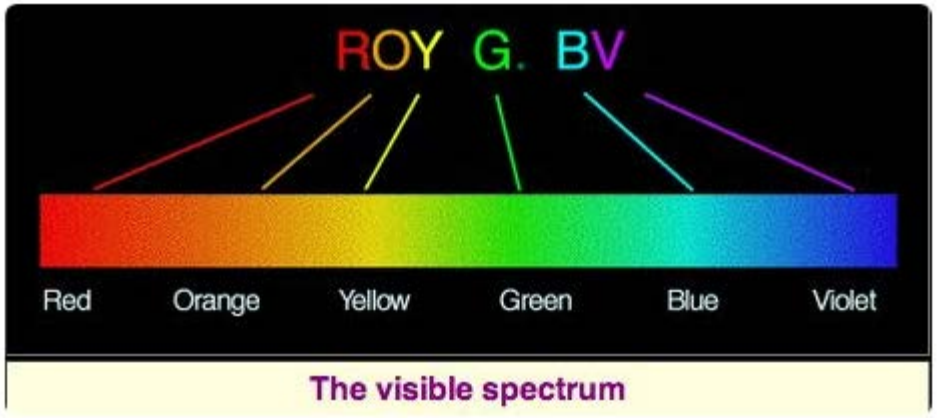
Electromagnetic spectrum

- Light is a wave of alternating electric and magnetic fields.
- Like any other wave, light has a few fundamental properties that describe it:
  - One is its *frequency*, measured in *hertz* (Hz), which counts the number of waves that pass by a point in one second.
  - Another closely related property is *wavelength*: the distance from the peak of one wave to the peak of the next.
- 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





Spectrophotometry

- Spectrophotometry is a method to measure how much a chemical substance absorbs light by measuring the intensity of light as a beam of light passes through sample solution.
- 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.

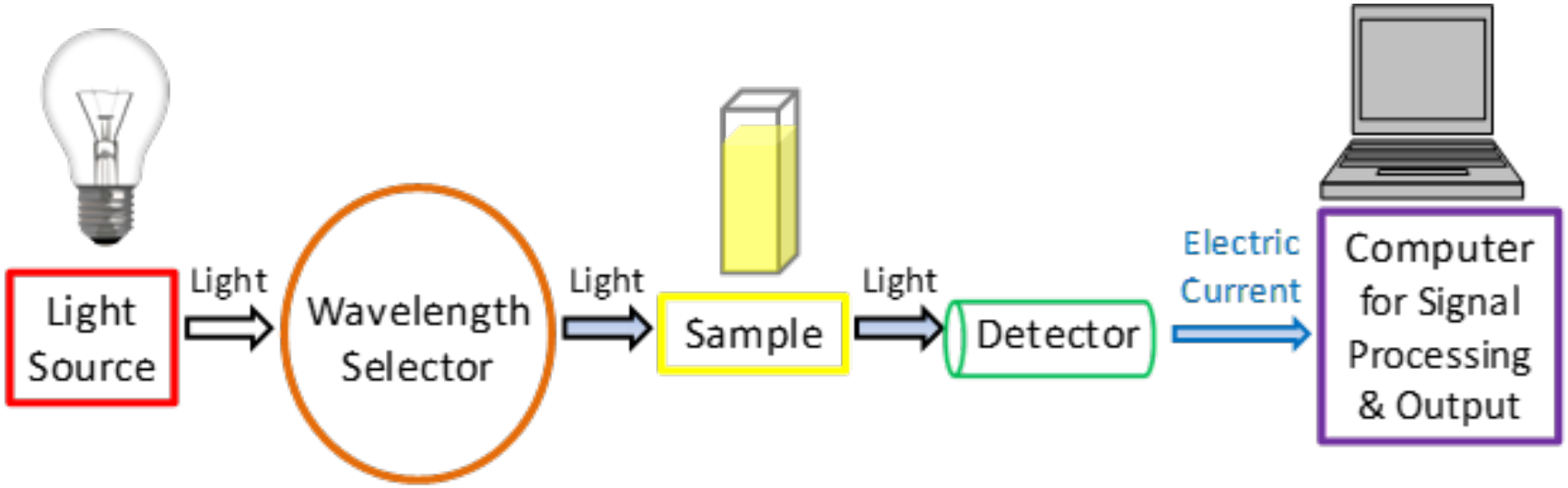
... Spectrophotometry

- 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 spectrophotometry?

- UV-Vis spectrophotometry 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 spectrophotometry to analyze or identify different substances by locating the specific wavelengths corresponding to maximum absorbance.

A simplified schematic of the main components in a UV-Vis spectrophotometer



Light source

- As a light-based technique, a steady source able to emit light across a wide range of wavelengths is essential.
- A single **xenon** lamp is commonly used as a high intensity light source for both UV and visible ranges.
- Xenon lamps are, however, associated with higher costs and are less stable in comparison to tungsten and halogen lamps.
- For instruments employing two lamps, a **tungsten or halogen lamp** is commonly used for visible light, whilst a **deuterium lamp** is the common source of UV light.
- As two different light sources are needed to scan both the UV and visible wavelengths, the light source in the instrument must switch during measurement.
- In practice, this switchover typically occurs during the scan between 300 and 350 nm where the light emission is similar from both light sources and the transition can be made more smoothly.

Wavelength selection

- In the next step, certain wavelengths of light suited to the sample type and analyte for detection must be selected for sample examination from the broad wavelengths emitted by the light source. Available methods for this include:
- **Monochromators**
  A monochromator separates light into a narrow band of wavelengths.
- It is most often based on diffraction gratings that can be rotated to choose incoming and reflected angles to select the desired wavelength of light.
- **Absorption filters**
  Absorption filters are commonly made of colored glass or plastic designed to absorb particular wavelengths of light.
- **Interference filters**
  Also called dichroic filters, these commonly used filters are made of many layers of dielectric material where interference occurs between the thin layers of materials.

… Wavelength selection

- **Cutoff filters**

  Cutoff filters allow light either below (shortpass) or above (longpass) a certain wavelength to pass through.
- **Bandpass filters**
  Bandpass filters allow a range of wavelengths to pass through that can be implemented by combining shortpass and longpass filters together.
- Monochromators are most commonly used for this process due to their versatility.
- However, filters are often used together with monochromators to narrow the wavelengths of light selected further for more precise measurements and to improve the signal-to-noise ratio.

Sample analysis

- Whichever wavelength selector is used in the spectrophotometer, the light then passes through a sample.
- For all analyses, measuring a reference sample, often referred to as the "blank sample", such as a cuvette filled with a similar solvent used to prepare the sample, is imperative.
- If an aqueous buffered solution containing the sample is used for measurements, then the aqueous buffered solution without the substance of interest is used as the reference.
- When examining bacterial cultures, the sterile culture media would be used as the reference.
- The reference sample signal is then later used automatically by the instrument to help obtain the true absorbance values of the analytes.

Sampling cuvettes

- The majority of plastic cuvettes are inappropriate for UV absorption studies because plastic generally absorbs UV light.
- Glass can act as a filter, often absorbing the majority of UVC (100-280 nm) and UVB (280-315 nm) but allowing some UVA (315-400 nm) to pass through.
- Therefore, quartz sample holders are required for UV examination because quartz is transparent to the majority of UV light.
- Air may also be thought of as a filter because wavelengths of light shorter than about 200 nm are absorbed by molecular oxygen in the air.
- A special and more expensive setup is required for measurements with wavelengths shorter than 200 nm, usually involving an optical system filled with pure argon gas.
- Cuvette-free systems are also available that enable the analysis of very small sample volumes, for example in DNA or RNA analyses.

Detection

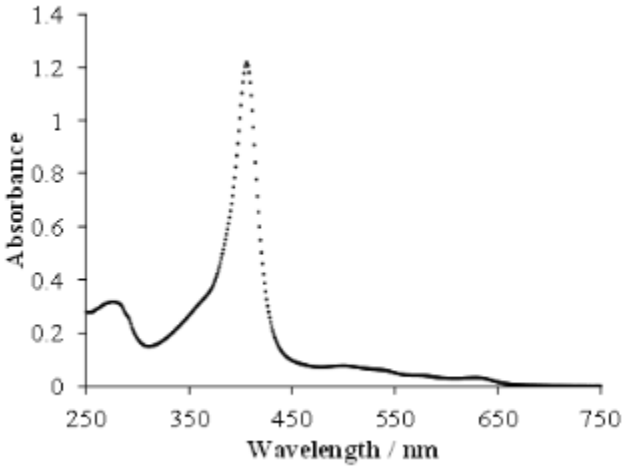
- After the light has passed through the sample, a detector is used to convert the light into a readable electronic signal.
- Generally, detectors are based on photoelectric coatings or semiconductors.
- A **photoelectric coating** ejects negatively charged electrons when exposed to light.
- When electrons are ejected, an electric current proportional to the light intensity is generated.
- A photomultiplier tube (PMT)is one of the more common detectors used in UV-Vis spectroscopy.
- A PMT is based on the photoelectric effect to initially eject electrons upon exposure to light, followed by sequential multiplication of the ejected electrons to generate a larger electric current.
- PMT detectors are especially useful for detecting very low levels of light.

- When **semiconductors** are exposed to light, an electric current proportional to the light intensity can pass through.
- More specifically, photodiodes and charge-coupled devices (CCDs) are two of the most common detectors based on semiconductor technology.

- After the electric current is generated from whichever detector was used, the signal is then recognized and output to a computer or screen.

UV-Vis spectroscopy analysis

- UV-Vis spectroscopy information may be presented as a graph of absorbance, optical density or transmittance as a function of wavelength.
- However, the information is more often presented as a graph of absorbance on the vertical *y* axis and wavelength on the horizontal *x* axis.
- This graph is typically referred to as an absorption spectrum.



Beer–Lambert's law

- The **absorbance** (*A*) is equal to the logarithm of a fraction involving the intensity of light before passing through the sample (I_o) divided by the intensity of light after passing through the sample (*I*).
- The fraction *I* divided by I_o is also called transmittance (*T*), which expresses how much light has passed through a sample.
- However, Beer–Lambert's law is often applied to obtain the concentration of the sample (*c*) after measuring the absorbance (*A*) when the molar absorptivity (ε) and the path length (*L*) are known.
- Typically, ε is expressed with units of L mol^{-1} cm^{-1}, *L* has units of cm, and *c* is expressed with units of mol L^{-1}. As a consequence, *A* has no units.

...Beer–Lambert's law

- Beer–Lambert's law is especially useful for obtaining the concentration of a substance if a linear relationship exists using a measured set of standard solutions containing the same substance.
- Equation 1 shows the mathematical relationships between absorbance, Beer–Lambert's law, the light intensities measured in the instrument, and transmittance.

$$A = \varepsilon\, L\, c = \log_{10}\left(\frac{I_0}{I}\right) = \log_{10}\left(\frac{1}{T}\right) = -\log_{10}(T)$$

Equation 1: A set of equations showing the relationships between absorbance *A*, Beer–Lambert's law, the light intensities measured in the instrument, and transmittance.

... UV-Vis spectrophotometry analysis

- Cuvettes designed for a 1 cm path length are standard and are most common.
- Sometimes, very little sample is available for examination and shorter path lengths as small as 1 mm are necessary.
- Where quantitation is required, absorbance values should be kept below 1, within the dynamic range of the instrument.
- This is because an absorbance of 1 implies that the sample absorbed 90% of the incoming light, or equivalently stated as 10% of the incoming light was transmitted through the sample.
- With such little light reaching the detector, some UV-Vis spectrophotometers are not sensitive enough to quantify small amounts of light reliably.
- Two simple possible solutions to this problem are to either dilute the sample or decrease the path length.
- Recording a baseline spectrum using a “blank” reference solution is essential.
- If the instrument was absolutely perfect in every way, the baseline would have zero absorbance for every wavelength examined.
- In a real situation, however, the baseline spectrum will usually have some very small positive and negative absorbance values.
- For best practice, these small absorbance values are often automatically subtracted from the sample absorbance values for each wavelength of light by the software to obtain the true absorbance values.

… UV-Vis spectrophotometry analysis

- In UV-Vis spectrophotometry, the wavelength corresponding to the maximum absorbance of the target substance is chosen for analysis. This choice ensures maximum sensitivity because the largest response is obtained for a certain analyte concentration.
- For reliability and best practice, UV-Vis spectrophotometry experiments and readings should be repeated.
- When repeating the examination of a sample, in general, a minimum of three replicate trials is common, but many more replicates are required in certain fields of work.
- A calculated quantity, such as the concentration of an unknown sample, is usually reported as an average with a standard deviation.
- A low deviation or variation indicates a higher level of precision and reliability.

Strengths of UV-Vis spectroscopy

- No single technique is perfect and UV-Vis spectroscopy is no exception.
- The technique does, however, have a few main strengths listed below that make it popular:

  - The technique is **non-destructive**, allowing the sample to be reused or proceed to further processing or analyses.

  - Measurements can be made **quickly**, allowing easy integration into experimental protocols.

  - Instruments are **easy to use**, requiring little user training prior to use.

  - Data analysis generally requires **minimal processing**, again meaning little user training is required.

  - The instrument is generally **inexpensive** to acquire and operate, making it accessible for many laboratories.

Limitations of UV-Vis spectroscopy

- **Stray light**
  In a real instrument, wavelength selectors are not perfect and a small amount of light from a wide wavelength range may still be transmitted from the light source, possibly causing serious measurement errors.
- Stray light may also come from the environment or a loosely fitted compartment in the instrument.
- **Light scattering**
  Light scattering is often caused by suspended solids in liquid samples, which may cause serious measurement errors.
- The presence of bubbles in the cuvette or sample will scatter light, resulting in irreproducible results.

… Limitations of UV-Vis spectroscopy

- **Interference from multiple absorbing species**
  A sample may, for example, have multiple types of the green pigment chlorophyll.
- The different chlorophylls will have overlapping spectra when examined together in the same sample.
- For a proper quantitative analysis, each chemical species should be separated from the sample and examined individually.
- **Geometrical considerations**
  Misaligned positioning of any one of the instrument's components, especially the cuvette holding the sample, may yield irreproducible and inaccurate results.
- Therefore, it is important that every component in the instrument is aligned in the same orientation and is placed in the same position for every measurement.
- Some basic user training is therefore generally recommended to avoid misuse.

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.