Ultra-violet Visible Spectroscopy

by Alain Martelli

I. Theoretical principles

A. Introduction

Many molecules absorb ultraviolet (UV) or visible light. The absorbance of a solution increases as attenuation of the beam increases. Absorbance is directly proportional to the path length, b, and the concentration, c, of the absorbing species, according to the Beer-Lambert Law (see below):

$$A = \varepsilon bc$$

where ε is a constant of proportionality called the *molar absorptivity*. Different molecules absorb radiation of different wavelengths. An absorption spectrum will show a number of absorption bands corresponding to structural groups within the molecule.

1. Electronic transitions

The absorption of UV or visible radiation corresponds to the excitation of outer electrons. There are three types of electronic transitions to be considered:

- Transitions involving π, s and n electrons.
- Transitions involving charge-transfer electrons
- Transitions involving d and f electrons

When an atom or molecule absorbs energy, electrons are promoted from their ground state to an excited state. In a molecule, the atoms can rotate and vibrate with respect to each other. These vibrations and rotations also have discrete energy levels, which can be considered as being packed on top of each electronic level.

Absorption of UV and visible radiation in organic molecules is restricted to certain functional groups (chromophores) that contain valence electrons of low excitation energy. The spectrum of a molecule containing these chromophores is complex, because the superposition of rotational and vibrational transitions with the electronic transitions gives a jumble of overlapping lines that appears as a continuous absorption band.

2. Charge-transfer absorption

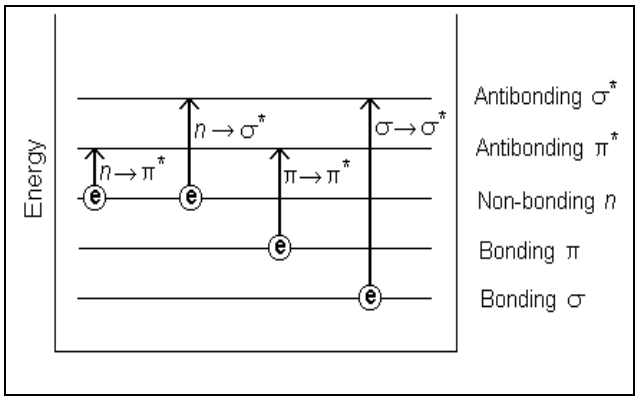
Many inorganic species show charge-transfer absorption; these are called charge-transfer complexes. For a complex to demonstrate charge-transfer behavior, one of its electrons must be able to be donated, and another component must be able to accept the electron. Absorption of radiation then involves the transfer of an electron from the donor to an orbital associated with the acceptor. Molar absorptivities from charge-transfer absorption are large (greater than 10,000 $L.mol^{-1}.cm^{-1}$). Depending on the complex, charge-transfer complexes can absorb almost anywhere in the UV-Vis range.

C. Possible electronic transitions of π, s, and n electrons

<u>$\sigma \rightarrow \sigma^*$ transitions:</u>

An electron in a bonding s orbital can be excited to the corresponding antibonding orbital, though the energy required for this is large. For example, methane (which has

only C-H bonds, and can only undergo s → s* transitions) cannot be seen in typical UV-Vis spectra (200 – 700 nm).



n → s* transitions:

Saturated compounds containing atoms with lone pairs (non-bonding electrons) are capable of n → s* transitions. These transitions usually need less energy than s → s* transitions. They can initiated by light whose wavelength is in the range 150 – 250 nm. The number of organic functional groups with n → s* peaks in the UV region is small.

n → p* and p → p* transitions:

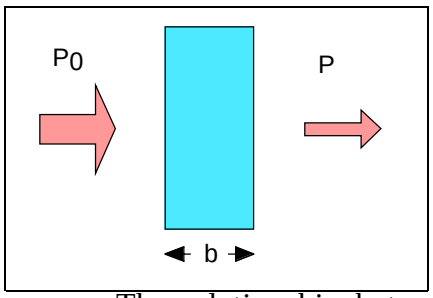
Most absorption spectroscopy of organic compounds is based on transitions of n or p electrons to the p* excited state. This is because the absorption peaks for these transitions fall in an experimentally convenient region of the spectrum (200 – 700 nm). These transitions need an unsaturated group in the molecule to provide the p electrons.

Molar absorptivities from n → p* transitions are relatively low, and range from 10 to 100 $L.mol^{-1}.cm^{-1}$. p → p* transitions normally give molar absorptivities between 1,000 and 10,000 $L.mol^{-1}.cm^{-1}$.

The solvent in which the absorbing species is dissolved also has an effect on the spectrum. Peaks resulting from n → p* transitions are shifted to shorter wavelengths (blue shift) with increasing solvent polarity. This arises from increased solvation of the lone pair, which lowers the energy of the n orbital. Often (but not always), the reverse (red shift) is seen for p → p* transitions. This is caused by attractive dipole forces between the solvent and the absorber, which lower the energy levels of both the excited and unexcited states. This effect is greater for the excited state, and so the energy difference between the excited and unexcited states is slightly reduced. This results in a small red shift. This effect also influences n → p* transitions, but is overshadowed by the blue shift resulting from solvation of lone pairs.

D. The Beer-Lambert Law

The diagram below shows a beam of monochromatic radiation of radiant power P_0 directed at a sample solution. Absorption takes place and the beam of radiation leaving the sample has a radiant power P. The amount of radiation absorbed may be measured in a number of ways:



Transmittance, $T = P/P_0$

%Transmittance, $\%T = 100 \times T$

Absorbance:

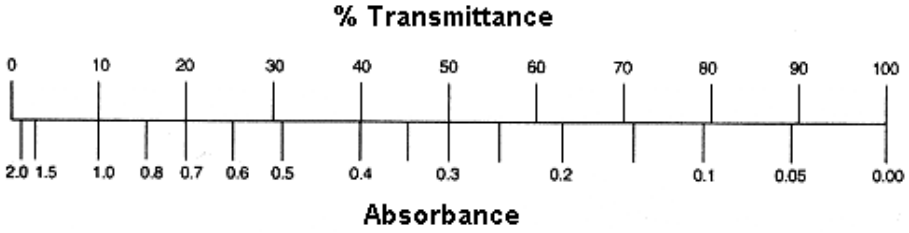
$A = \log_{10} (P_0 / P)$

$= \log_{10} (1 / T)$

$= \log_{10} (100 / \%T)$

$= 2 - \log_{10} \%T$

The relationship between absorbance and transmittance is illustrated in the following diagram:



So if all incoming radiation is absorbed, then percent of transmittance is zero and absorption is infinite.

The Beer-Lambert Law is:

$$A = \varepsilon bc$$

Where **A** is absorbance (unitless), ε is the molar absorptivity with units of L. $mol^{-1}.cm^{-1}$, **b** is the path length (in cm) of the sample (that is, the length of the cuvette), and **c** is the concentration of the compound in solution, expressed in mol. L^{-1}.

The reason why we prefer to express the law with this equation is so that absorbance is directly proportional to the other parameters. This way, as long as the law is obeyed, we can easily determine the concentration or the molar absorptivity of a substance by measuring its absorbance at a particular wavelength. Note that at high concentration the Beer-Lambert Law is not obeyed. It can be considered true only for absorbances between 0 and 2.0, though if you want to be really precise, quantify based on absorbances between 0.1 and 1.6.

The molar absorptivity is a constant for a particular substance at a particular wavelength. So, if the concentration of the solution is halved, so is the absorbance.

II. Ultraviolet-visible spectroscopy in molecular biology

A. UV for quantification of nucleic acid concentration

The concentration of DNA, RNA, oligonucleotides, or even mononucleotides can be measured directly in aqueous solutions. Aqueous buffers with low ion concentrations (e.g. TE buffer) are ideal. The concentration is determined by measuring absorbance at 260 nm (subtracting the "blank" absorbance) and then simply calculating concentration via a standard factor as per the Beer-Lambert law.

An absorption of 1.0 is equivalent to approximately:

50 µg/mL double-stranded DNA (dsDNA)
33 µg/mL single-stranded DNA (ssDNA)
40 µg/mL single-stranded RNA
30 µg/mL for ssDNA oligonucleotides.

For more precise calculation methods, see the separate section on DNA technical information or use the biopolymer calculator on our website.

The purity of a nucleic acid sample can be assessed by calculating the ratio between absorbances at 260 nm and 280 nm. This ratio (A_{260}/A_{280}) is used to estimate purity because proteins absorb more strongly at 280 nm. Pure DNA should have a ratio of approximately 1.8, whereas pure RNA should give a value of approximately 2.0. Absorption at 230 nm reflects contamination of the sample by substances such as carbohydrates, peptides, phenols or aromatic compounds. The ratio A_{260}/A_{230} should be approximately 2.2 for pure nucleic acid samples.

B. Simple UV for quantification of protein concentration

Measuring absorbance at 280 nm (A_{280}) can be used to measure protein concentrations up to approximately 4 mg/mL (A_{280} = 3.0). The easiest way to get the factor used to calculate concentration from absorbance is to use the biopolymer calculator on our website. For larger proteins, there is likely an equation somewhere with which to estimate concentration from A_{280}.

While the A_{280} method is simple and rapid, A_{280} can be influenced by the parallel absorption of non-proteins (e.g. DNA). This method is thus less sensitive and requires higher protein concentrations than other methods, such as colorimetric dye tests detailed below. Thus A_{280} can be used confidently only with demonstrably pure protein solutions.

C. Colorimetric determination of protein concentration (dye tests)

Quantitative measurement of the protein concentration can be reliably achieved on the basis of reactions between protein functional groups and various dye-forming reagents. After reaction, the intensity of the dye correlates directly to the concentration of the reacting groups. Dye intensity can be measured exactly at the appropriate wavelength.

1. Bradford protein assay

Materials:

- Lyophilized bovine plasma gamma globulin or bovine serum albumin (BSA)
- Coomasie Brilliant Blue 1
- 0.15 M NaCl
- Spectrophotometer and tubes
- Micropipettes

Procedure, Standard Assay (20-150 µg protein; 200-1500 µg/mL):

- Prepare a series of protein standards using BSA diluted with 0.15 M NaCl to final concentrations of 0 (blank, NaCl only), 250, 500, 750 and 1500 µg BSA/mL. Also prepare serial dilutions of the unknown sample to me measured.
- Add 100 µL of each of the above to a separate test tube
- Add 5.0 mL of Coomasie Blue to each tube and mix by vortex or inversion.
- Adjust the spectrophotometer to a wavelength of 595 nm, and record the blank using the tube from step 3 which contains no BSA.
- Wait 5 minutes and read each of the standards and each of the samples at **595 nm** wavelength.
- Plot the absorbance of the standards versus their concentration. Compute the extinction coefficient and calculate the concentrations of the unknown samples.

Procedure, Micro Assay (1-10 µg protein):

- Prepare standard concentrations of BSA of 1, 5, 7.5 and 10 µg/mL. Prepare a blank of NaCl only, as above. Prepare a series of sample dilutions.

- Add 100 µL of each of the above to separate tubes (use microcentrifuge tubes) and add 1.0 mL of Coomasie Blue to each tube.
- Turn on and adjust the spectrophotometer to a wavelength of **595 nm**, and blank the spectrophotometer using the NaCl solution in the 1.5 mL cuvette.
- Wait 2 minutes and read the absorbance of each standard and sample at **595 nm**.
- Plot the absorbance of the standards versus their concentration. Compute the extinction coefficient and calculate the concentrations of the unknown samples.

2. Lowry protein assay

Materials:

- 0.15% (w/v) sodium deoxycholate
- 72% (w/v) trichloroacetic acid (TCA)
- Copper tartrate/carbonate (CTC)
- 20% (v/v) Folin-Ciocalteu reagent
- Bovine Serum Albumin (BSA)
- Spectrophotometer and tubes
- Micropipettes

Procedure:

- Prepare standard dilutions of BSA of 25, 50, 75 and 100 µg/mL. Prepare appropriate serial dilutions of the sample to be measured.
- Place 1.0 mL of each of the above into separate tubes. Add 100 µL of sodium deoxycholate to each tube.
- Wait 10 minutes and add 100 µL of TCA to each tube.
- Centrifuge each tube for 15 minutes at 3,000 G and discard the supernatant.
- Add 1.0 mL of water to each tube to dissolve the pellet. Add 1.0 mL of water to a new tube to be used as a blank.
- Add 1.0 mL of CTC to each tube (including the blank), vortex and allow to set for 10 minutes.
- Add 500 µL Folin-Ciocalteu to each tube (including the blank), vortex and allow to set for 30 minutes.
- Turn on and zero the spectrophotometer to a wavelength of **750 nm**. Use the blank from Step 7 to adjust for 100% T.
- Read each of the standards and samples at 750 nm.
- Plot the absorbance of the standards versus their concentration. Compute the extinction coefficient and calculate the concentrations of the unknown samples.

Notes:

The Lowry method depends on the presence of tyrosine within the protein to be measured. The standard protein must contain approximately the same number of tyrosine residues as the sample, or the procedure will be inaccurate.

If there are no tyrosine residues in the sample to be measured, the Lowry method of protein determination is useless and you should try the Bradford assay instead. In general, the Bradford assay is the method of choice for protein determinations.

3. Biuret protein assay

Materials:

- Biuret Reagent
- Bovine serum albumin (BSA)
- Spectrophotometer and tubes

Procedure:

- Prepare standard dilutions of BSA containing 1, 2.5, 5.0, 7.5 and 10 mg/mL. Prepare serial dilutions of the unknown samples.
- Add 1.0 mL of each of the standards, each sample, and 1.0 mL of distilled water to separate tubes. Add 4.0 mL of Biuret reagent to each tube. Mix by vortexing.
- Incubate all of the tubes at 37 ºC for 20 minutes.
- Turn on and adjust a spectrophotometer to read at a wavelength of **540 nm**.
- Cool the tubes from Step 3, blank the spectrophotometer and read all of the standards and samples at **540 nm**.
- Plot the absorbance of the standards versus their concentration. Compute the extinction coefficient and calculate the concentrations of the unknown samples.

Notes:

The Biuret reaction was one of the first for the determination of protein concentration. It remains as a rapid determination, but is not very accurate. It is useful during protein separation procedures since there are fewer salt interference reactions than with the Bradford or Lowry techniques. The color formed is stable for only 1 or 2 hours and consequently all spectrophotometer readings must be made as soon as possible after the incubation step.

D. Measuring bacterial cell density by optical density

The density of bacterial suspensions may be measured spectrophotometrically at 595 or 600 nm without the addition of dyes. This applies very handily to the preparation of competent cells, which must be in a specific phase of growth, or for inducing protein expression in a bacterial culture.

III. Use of the Spectrophotometer

There are two UV-Vis spectrophotometers available for use, a departmental one in the instrument center and one housed in the Schepartz lab. Your TA will supervise your operation of the instrument until you are comfortable with the technique. Using a UV-Vis is quite simple, but there are a few important things to keep in mind to protect the machine and ensure high quality data.

There are designated cuvettes for use by Chemical Biology Laboratory students. For UV absorbance, use the quartz cuvette (volume = 100 mL). Be extremely careful because they are fragile and expensive! Wash them with distilled water and then ethanol, and use Kimwipes if you need to wipe them. If they are very dirty, they may need a bath in concentrated acid (hydrochloric acid or nitric acid); consult your TA for assistance. You should always clean a cuvette before and after each use. For bacterial cell densities (OD_{600}), you can use disposable cuvettes (volume = 3 mL).

For the practical use of the Schepartz B640 spectrophotometer, you can refer to the useful QUICK REFERENCE provided in the operating instruction manual (first page in the manual). You will find all the information you need to run the different options (Rediread, Rediscan, Fixed wavelength, Wavelength scan, Time drive, Graphic manipulations).

DO NOT FORGET TO SWITCH OFF THE LAMP WHEN YOU ARE DONE!!!

Technical information about the B640 spectrophotometer

Visible lamp from Beckman: *tungsten-halogen lamp* - part# 945672 (42$)
UV lamp from Beckman: *deuterium lamp* - part# 514366 (700$)

Cuvette materials used for UV-visible spectroscopy

Optical material	Transmittance range (nm)	Refractive index at 600 nm	Relative rupture strength (sapphire = 100)
Methacrylate	250 - 1100		
UV-grade fused silica	200 - 2500	1.4580	10.9
Synthetic fused silica	230 - 2500	1.4580	10.9
Crystalline quartz (Si_2O_2)	240 - 2500	1.5437	2.3
Quartz, extremely low OH	190 - 2500	1.5437	2.3
Flint glass (SF 10)	380 - 2350	1.7268	3.8
Flint glass (SF 8)	355 - 2350	1.6878	3.8
BK 7 glass	315 - 2350	1.5165	3.7
Optical crown glass	320 - 2300	1.5226	3.7
Borosilicate crown glass	360 - 2350	1.4736	3.7
Pyrex	360 - 2350	1.4736	3.8
Tempax	360 - 2350	1.4736	3.8
Sapphire (Al_2O_3)	150 - 5000	1.7677	100.0
Sodium chloride	250 nm - 16 mm	1.5400	0.5
Suprasil 300	190 - 3600	1.54	3.8
Diamond	220 - 4000	2.40	83.7
Spectrosil	170 - 2700	1.54	3.8
infrasil	220 - 3800	1.54	3.8

Solvent UV cutoffs

Solvent	UV cutoff (nm)
Acetonitrile	190
Water	190
Cyclohexane	195
Isooctane	195
n-hexane	201

Ethanol (95%)	205
Methanol	205
Trimethyl phosphate	210
Acetone	220
Chloroform	240
Xylene	280