

APPENDIX III. Spectroscopy

1. Absorption Spectroscopy

If white light is passed through a colored solution, certain wavelengths of light are selectively absorbed, and the resulting color observed is due to the wavelengths of the transmitted light. UV/Vis absorption spectroscopy is useful if the sample absorbs light in the 200-350 nm range (ultraviolet, UV) or in the 350-750 nm range (visible). The figure below (Fig. 1) shows the absorption spectrum of riboflavin. Riboflavin appears yellow to the eye. Maximal absorption (also known as lambda max) of light occurs at several wavelengths in the ultraviolet region (below about 370 nm), and at 450 nm in the visible region. Since the absorption in the visible region is at the blue end, the transmitted light is that of the other end of the spectrum (yellow).

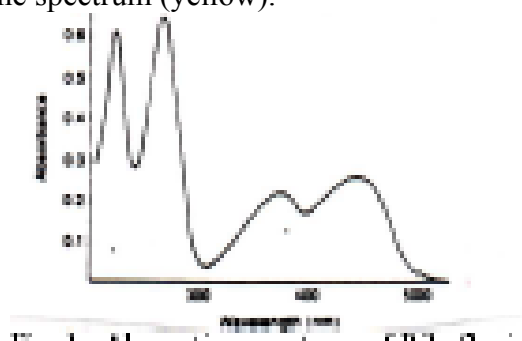


Fig. 1. Absorption spectrum of Riboflavin

The quantitative measurement of the amount of light absorbed is based on two laws. Lambert's law states that the proportion of light absorbed is independent of its intensity, and each successive portion of the medium absorbs an equal fraction of the light passing through it. This is expressed mathematically as:

$$\log (I/I_0)=Kl$$

where I_0 and I are the intensities of the incident and transmitted light, respectively, K is a constant, and l is the path length in cm. $\log (I/I_0)$ is the absorbance (A). This law is always true. The second law applied here, Beer's law, has some exceptions, usually because of a change in the state of the solute upon absorbing light. This could involve changes in ionization or aggregation. Beer's law relates concentration to the factor K . $K=\epsilon c$, where ϵ is the extinction coefficient of the absorbing solute (units are reciprocal concentration times reciprocal length) and c is its concentration. Combining these two laws gives the Beer's-Lambert law:

$$A=\epsilon cl$$

Absorption is dimensionless. If c has units of M , ϵ has units of $M^{-1} \text{ cm}^{-1}$; if c has units of mg/ml , ϵ has units of $\text{ml mg}^{-1} \text{ cm}^{-1}$. The extinction coefficient reflects the relative

tendency of a solute to absorb light of a certain frequency, and is specific only for a specific solute, solvent, and wavelength of light.

UV/Vis absorption spectroscopy can be used to quantitatively measure enzyme activity if a substrate or product of the enzyme-catalyzed reaction absorbs light. This type of assay follows the activity of the enzyme indirectly by monitoring the change in substrate or product directly. A good example of this is the redox coenzyme NAD⁺/NADH, which changes its absorption properties upon oxidation and reduction (Fig. 2). The different absorption of light by NAD⁺ and NADH can be utilized to follow the activity of an enzyme involved in their interconversion.

An analysis of the spectra of both (Fig. 2) suggests that their absorptions differ most at ~340 nm wavelength light. This wavelength therefore provides the largest “window” to study their interconversion. By following the change in absorption at 340 nm over time as a function of enzyme activity, one can extract quantitative rate data using the Beer’s-Lambert law and the extinction coefficient for NADH.

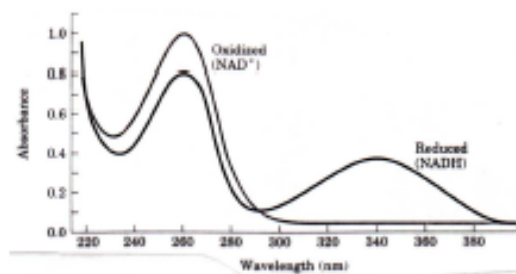


Fig. 2. Absorption spectra of NAD⁺ and NADH

2. Cary 50-UV/Vis spectrometer Standard Operating Procedure

General

- Plug in , turn on and log into PC/spectrometer system
- Access Cary WinUV software

Fixed wavelength measurement

- Select "Simple reads" icon

Under "Set up" select "Read at wavelength" - set desired wavelength
Close window

Important note: there are five different styles of cuvette in use in the current biochemistry laboratory. Quartz cuvettes are to be used for readings in the UV range (200-350 nm).

There are both 1 mL and 3 mL sized cuvettes. Disposable (plastic) cuvettes are to be used for readings in the visible range (350-750). There are both 1 mL and 3 mL sized plastic cuvettes.

- Place cuvette containing blank solution into sample holder #1 insuring a 1 cm path length.
- Click "zero" button to blank instrument
- Set blank solution aside.
- If your measurements require a disposable cuvette simply insert the cuvette containing the sample and press "Read".
- If your measurements require a quartz cuvette pipette your sample into the same cuvette used for the blank.
- Measure sample by clicking "Read" button.
- Throw away disposable cuvettes or carefully clean quartz cuvettes using deionized water. Store properly.

The simplest way to handle data is to copy and paste into Excel. Once in Excel, select column A and under the "Data" tab select the "text to columns" command. Choosing "Fixed width" in the Wizard works well. Follow directions on the screen.

- Save all data to the "Student Shared" folder on the server.

Wavelength Scan

- Click "Scan" icon
- Under "set up" - type in the desired start and stop wavelengths
choose "fast" under scan controls
click "baseline" tab - choose baseline correction
click "auto store" tab - select storage off
click OK
- Insert blank sample and click "zero"
- Click "baseline" - follow screen instructions
- Remove blank and load sample into the same cuvette (quartz) or insert disposable cuvette containing sample.

- Click "start" follow screen instructions
- Save data to server as a *.CVS file (these open in Excel)

Helpful hint. You can record the exact absorbance at a particular wavelength while in scan mode by doing the following:

- Under the "view" pulldown menu select "report"
- Under the "commands" pulldown menu select "rapid result"
- Type in the desired wavelegth, press "result"
- Absorbance reading appears below in the report window