Absorption Spectroscopy

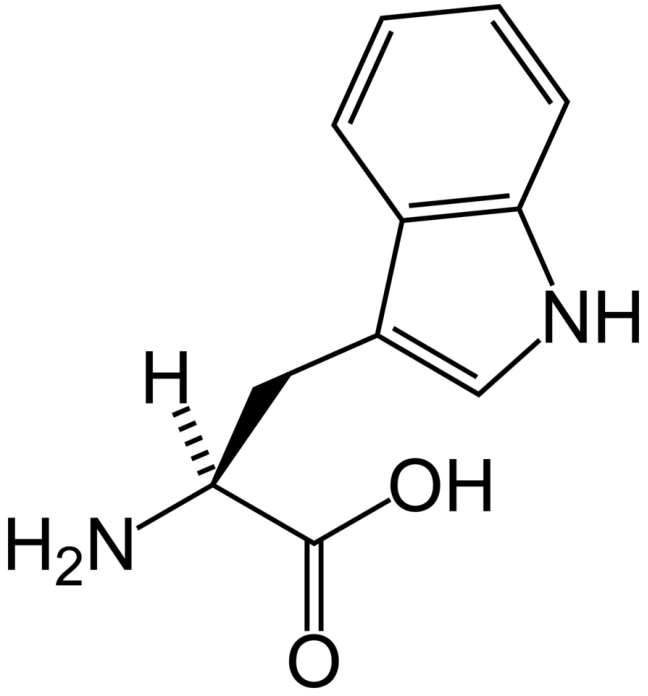
Absorption Spectroscopy of Biopolymers:

For biologists, the absorption properties of nucleic acids and proteins are of most interest. Nucleic acids and proteins have distinct chromophores that give rise to unique absorption curves. Typically, proteins and nucleic acids have chromophores that absorb in the UV (< 300 nm). Some proteins have chromophores in the visible (e.g. GFP, heme proteins, or proteins complexed with transition metals that give rise to unique absorbance spectra). Measurements are typically performed in aqueous solvents, which limits measurements to wavelengths > 170 nm. Buffers, salts etc. need to be chosen with care to avoid background absorbance.

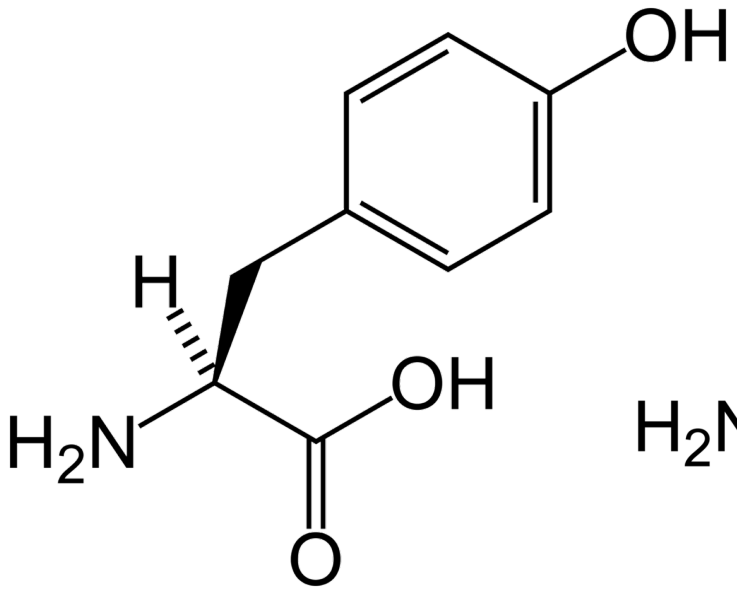
For proteins, the following chromophores are dominant:

- Peptide bond: delocalized π electrons (carbonyl-nitrogen) n to π^* transition (weak intensity in the far-UV at 210-220 nm)
- Peptide bond: delocalized π electrons (carbonyl-nitrogen) π to π^* transition (very strong intensity in the far-UV at ~ 190 nm)
- Amino acids with aromatic side chains that have delocalized π electrons, in order of absorbance strength (λmax weakest to strongest): Phe (258 nm), Tyr (274 nm), Trp (280 nm).
- Disulfide linkages from oxidized sulfur atoms on Cys residues (250-270 nm), as well as His (both are weak).

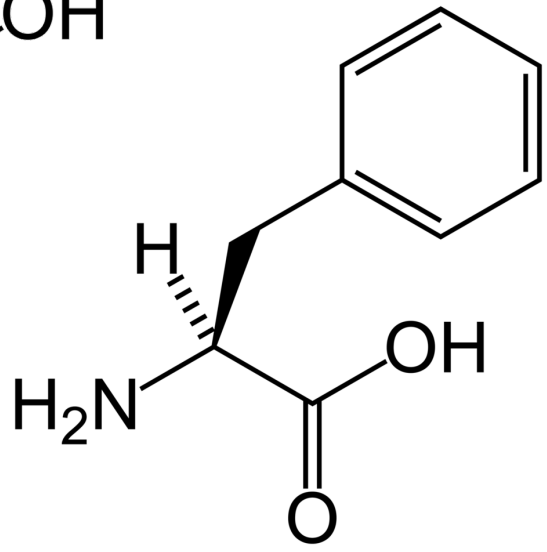
Aromatic Amino Acids



Trp (W)



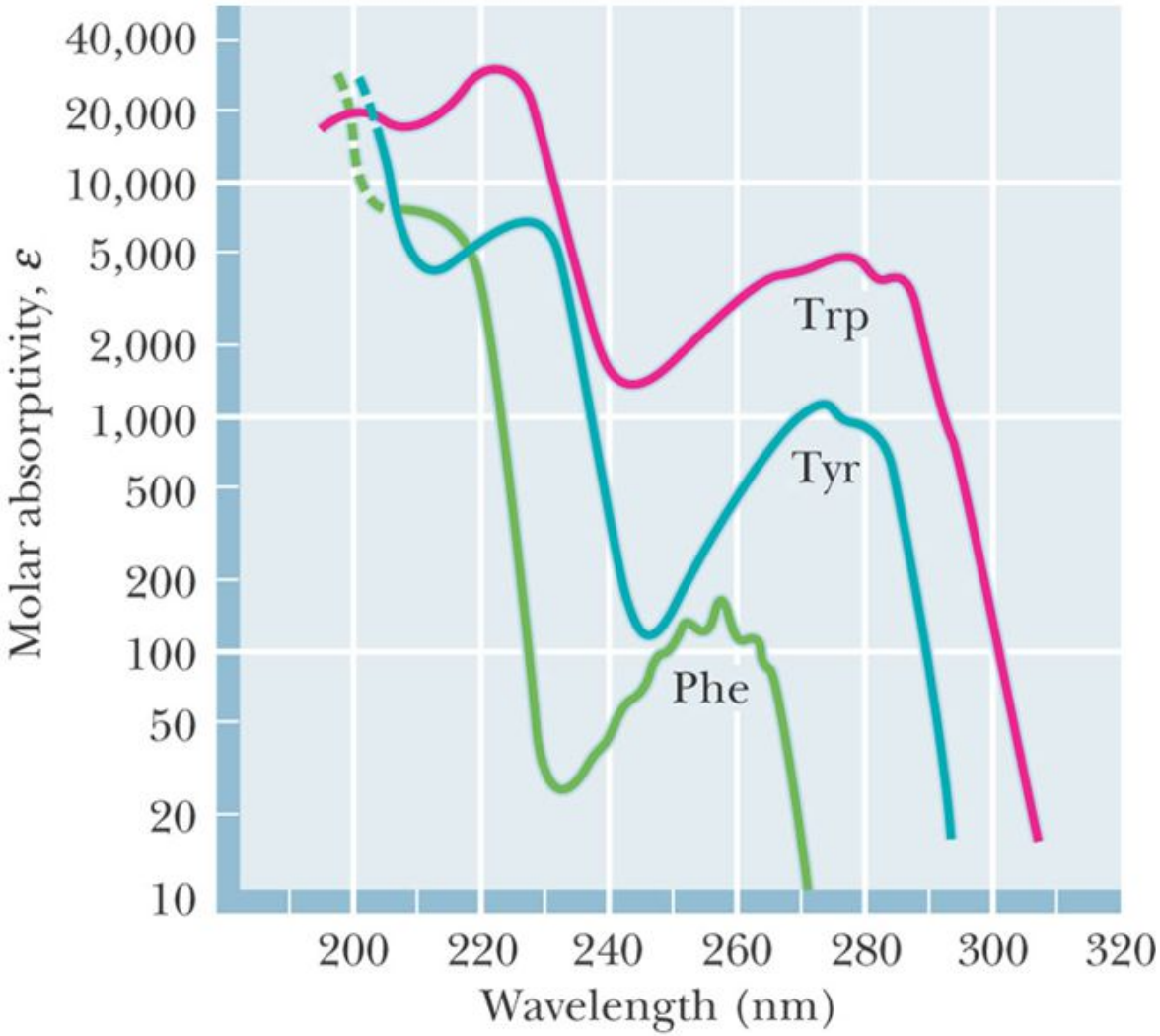
Tyr (Y)



Phe (F)

Spectroscopy

The ultraviolet absorption spectra of the aromatic amino acids at pH 6. (*From Wetlaufer, D.B., 1962. Ultraviolet spectra of proteins and amino acids.* Advances in Protein Chemistry **17**:*303–390.*)



Absorption Spectroscopy

For proteins without prosthetic groups, the number of tryptophan and tyrosine residues can be used to estimate protein concentration by measuring the absorbance of the protein at 280 nm. The molar extinction coefficients of Trp and Tyr are 5,700 and 1,300 OD 280 nm/(mol*cm), so the molar extinction coefficient of the protein can be estimated based on the number of tryptophan and tyrosine residues (*nTrp* and *nTyr*) with this formula:

$$\epsilon_{protein,\,280\,nm} = 5{,}700\,nTrp + 1{,}300\,nTyr$$

To convert into mg/ml, divide by the molar mass, *M*:

$$C_{protein,\,280\,nm} = \frac{5{,}700\,nTrp + 1{,}300\,nTyr}{M}$$

(Note: Phe and oxidized sulfhydryl Cys residues also absorb, but less significantly)

A more sensitive method, due to the typically higher extinction, is to measure the protein concentration by monitoring the absorption at 230 nm. Here the extinction is roughly 300 OD 230/(mol*cm) for each peptide bond.

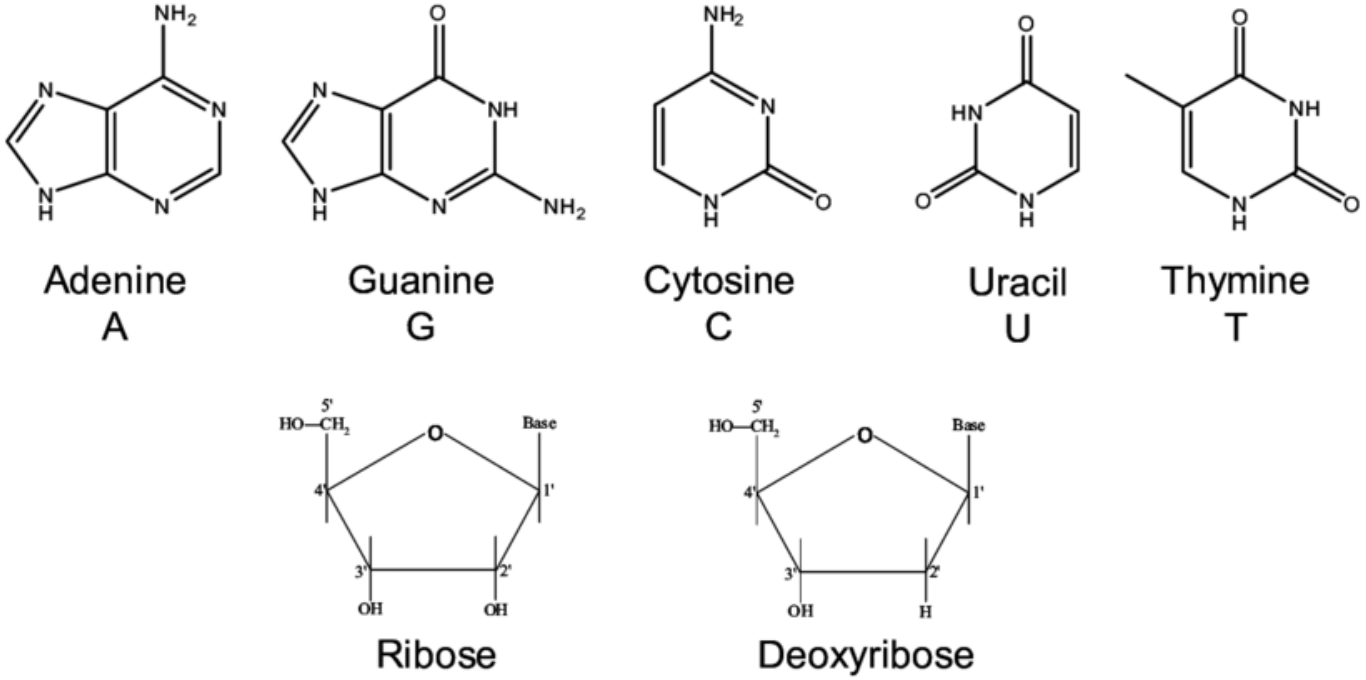
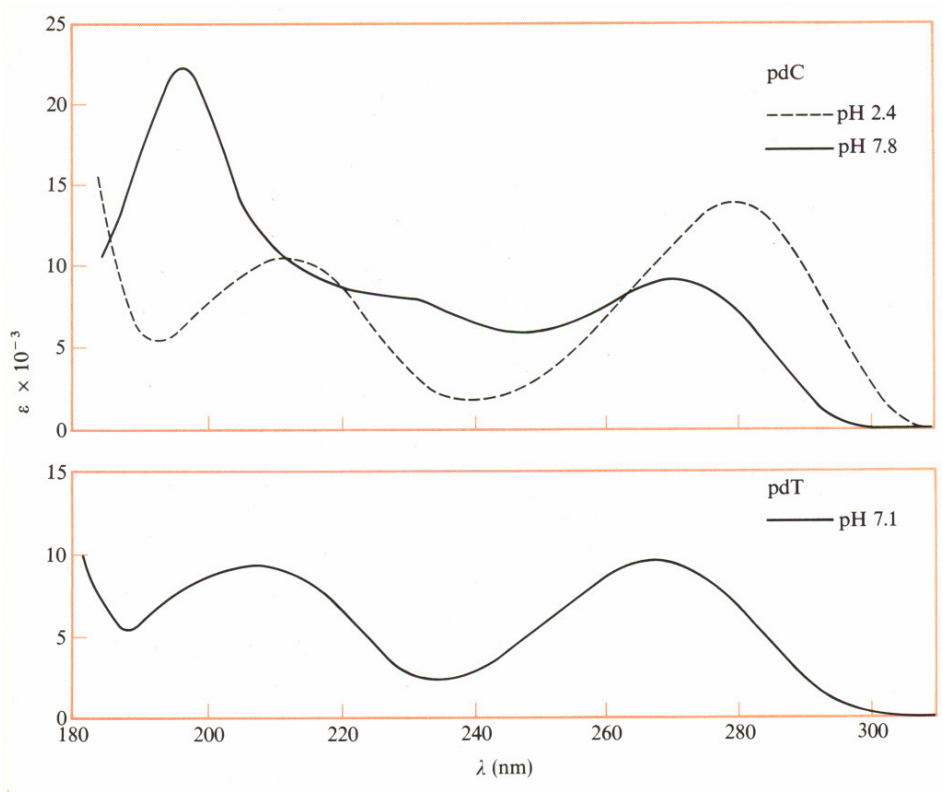
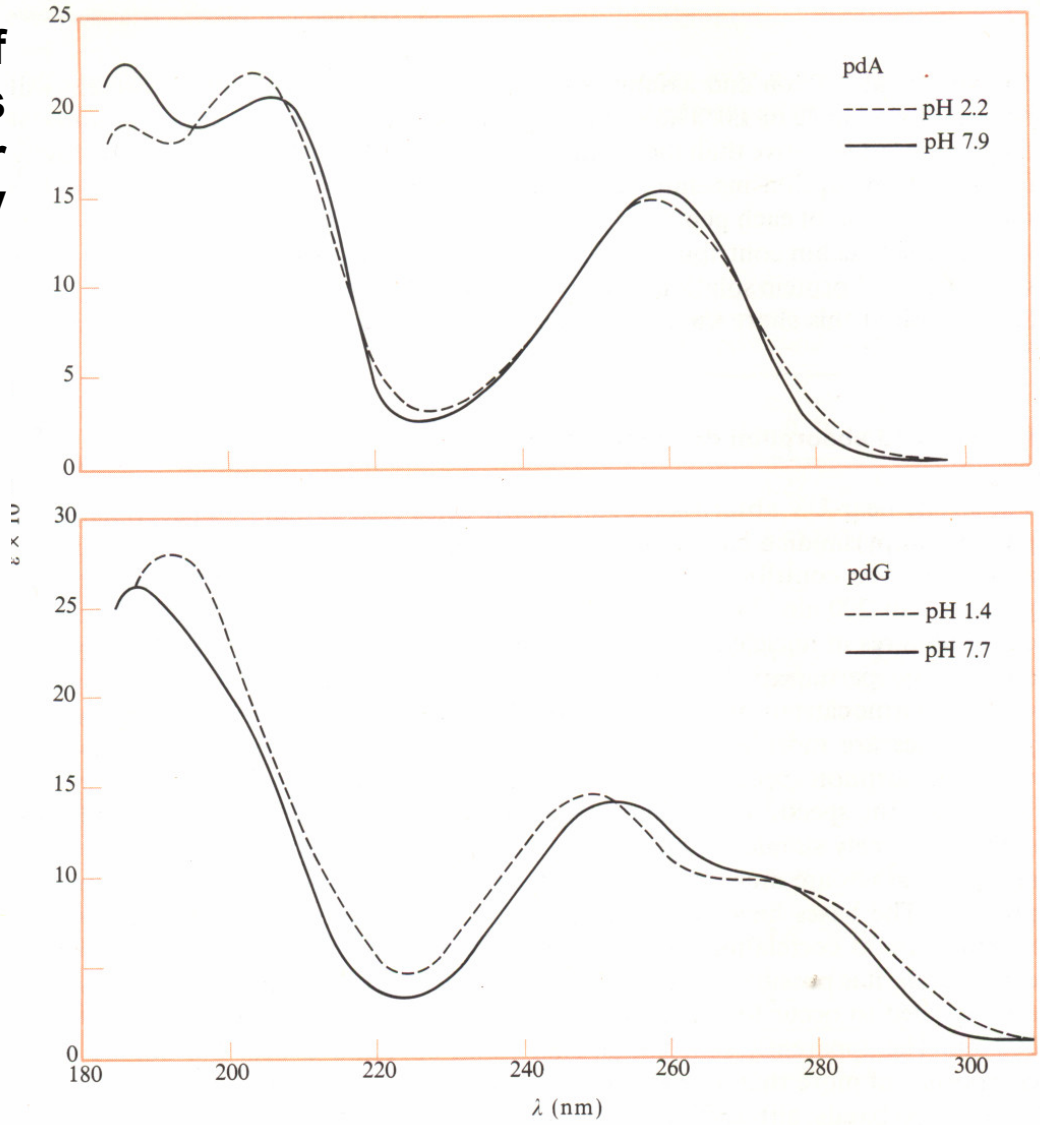


Figure 1.1. Structures of nucleic acid constituents

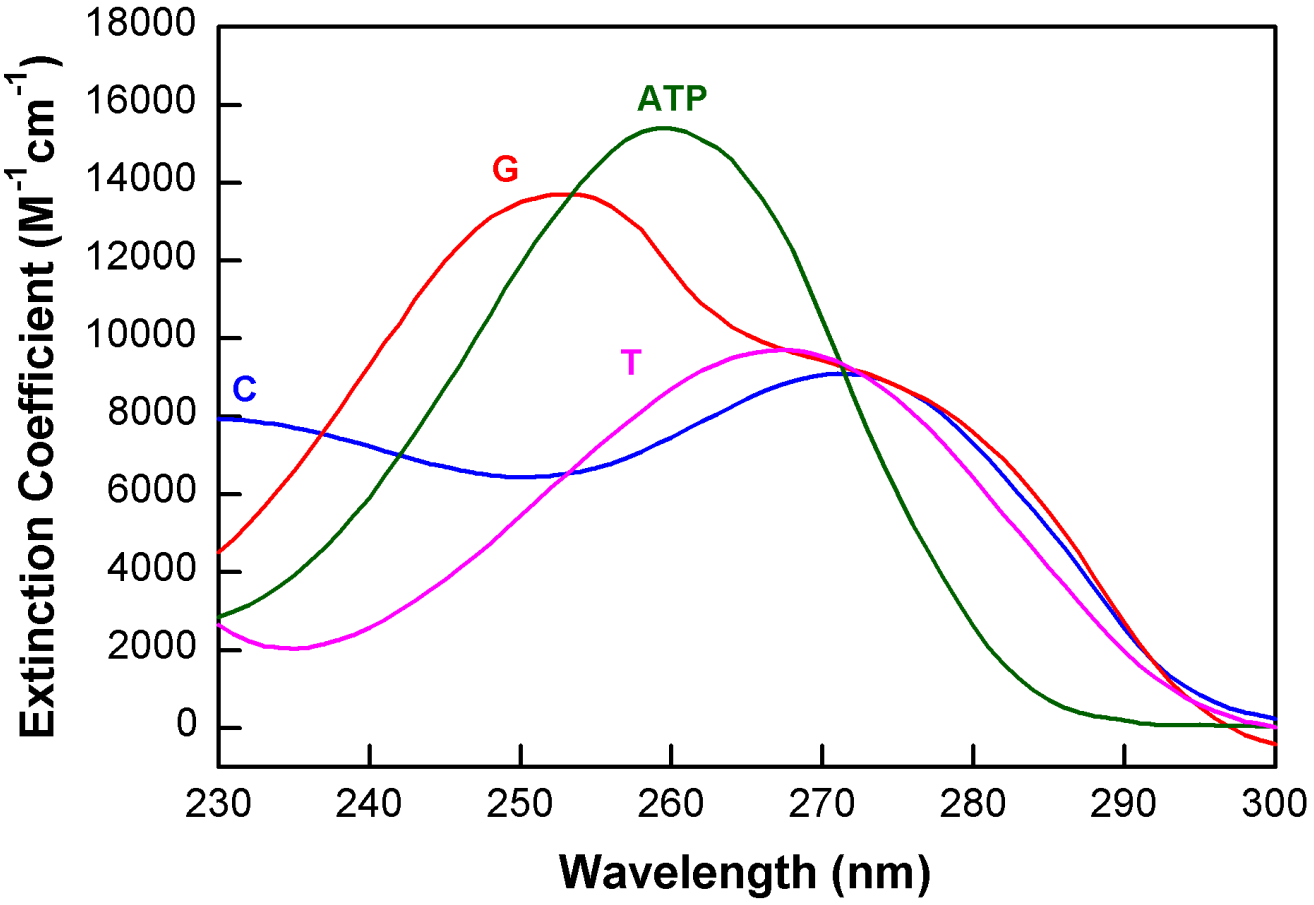
Absorption Spectroscopy

For nucleic acids, the strong extinction of the purine and pyrimidine bases contributes to their absorption in the near UV at around 260 nm, and in the far UV between 190-210 nm.





Absorption Spectra of the Nucleic Acids



Absorption Spectroscopy

Ways in which light interacts with a sample:

- Absorption – light energy is absorbed by the molecule causing electrons to be excited to a higher energy state
- Scattering (elastic) – light bounces off a molecules and can be observed at a different angle
- Optical rotation – the plane of polarized light is changed to a different angle
- What happens to the absorbed energy?
  - Converted to heat (vibration)
  - Fluorescence (light is emitted at a longer wavelength)
  - Raman scattering
  - Inelastic scattering
- Electric and magnetic properties of sample are affected when it interacts with light
- Pulsed light allows us to observe the decay of the excitation

Absorption Spectroscopy

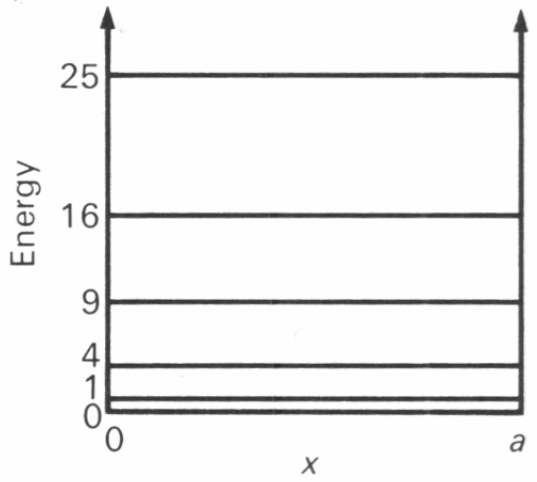


Figure 8.1 The simplest quantum-mechanical system—a particle in a one-dimensional box. The potential energy is zero within the box ($0 \leq x \leq a$) and infinite outside ($x > a$ or $x < 0$). The horizontal lines show the allowed energy levels.

In quantum mechanics the energy of a particle is quantized, and only certain energy levels are allowed. For a particle in a simple one dimensional box with dimensions from *0* to *a*, wave mechanics allows the particle with mass *m* only to have discrete energy levels (*h* is Planck's constant):

$$E_n = \frac{n^2 h^2}{8 m a^2} \qquad n = 1, 2, 3, 4\ldots$$

In molecules, electrons do not reside in one-dimensional boxes, but instead are located in orbitals. Their probabilities are described by wavefunctions.

Absorption Spectroscopy

Useful Tips for Absorbance Spectroscopy

Modern spectrophotometers typically store a reference spectrum in memory and perform the subtraction internally to report true analyte absorbance.

Background absorbance results from cuvette and buffer.

For UV mneasurements, only use quartz cuvettes. Glass and plastic cuvettes absorb in the UV and can only be used in the visible range.

Many buffer components absorb in the UV, in particular beta mercapto-ethanol and di-thiothreotol. TCEP can be used down to 240 nm.

Many buffers absorb to varying degrees below 240 nm, including HEPES, TRIS, acetate, MOPS; sodium and potassium phosphate buffers do not absorb and can be used to quite low wavelengths.

Always consider the total absorbance when subtracting buffer absorbance to make sure you are not measuring outside the dynamic (linear) range of the spectrophotometer.

Absorption Spectroscopy

Like phosphate buffer, the phosphate groups do not contribute to the extinction.

Within experimental error, ATP, ADP and AMP all have the same extinction properties. Absorbance of an individual nucleoside at 260 nm is about 10,000 OD 260 nm/(mol*cm). This strong extinction allows measurement down to concentrations of ~ 3 ug/ml.

<u>Contributions to the absorbance spectrum by the environment:</u>

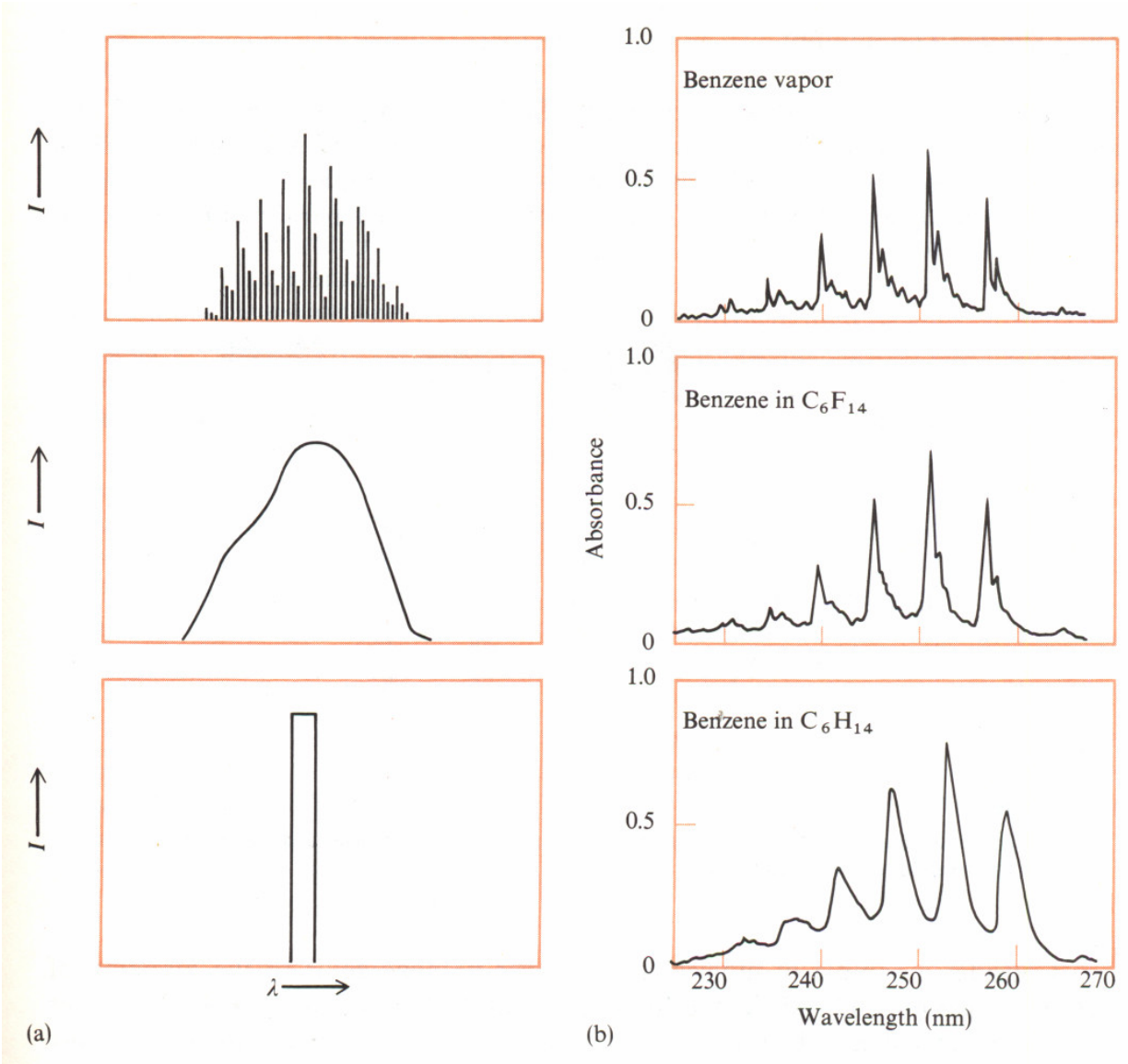
Dipole interactions with the solvent, if the solvent is polar like water, can be significant, and can change the absorption characteristics of chromophores when the solvent is changed to a non-polar solvent. Similarly, if the environment of chromophore changes within the molecule (e.g., a surface solvent-exposed tryptophan becoming buried in a hydrophobic interior of the protein), extinction intensities and absorption bands change. This can be used to monitor changes of a macromolecule, such as in:

- **melting studies**
- **pH changes**
- **oxidation states**
- **Conformational changes such as unfolding**
- **Solvent differences**
- **Complex formation**

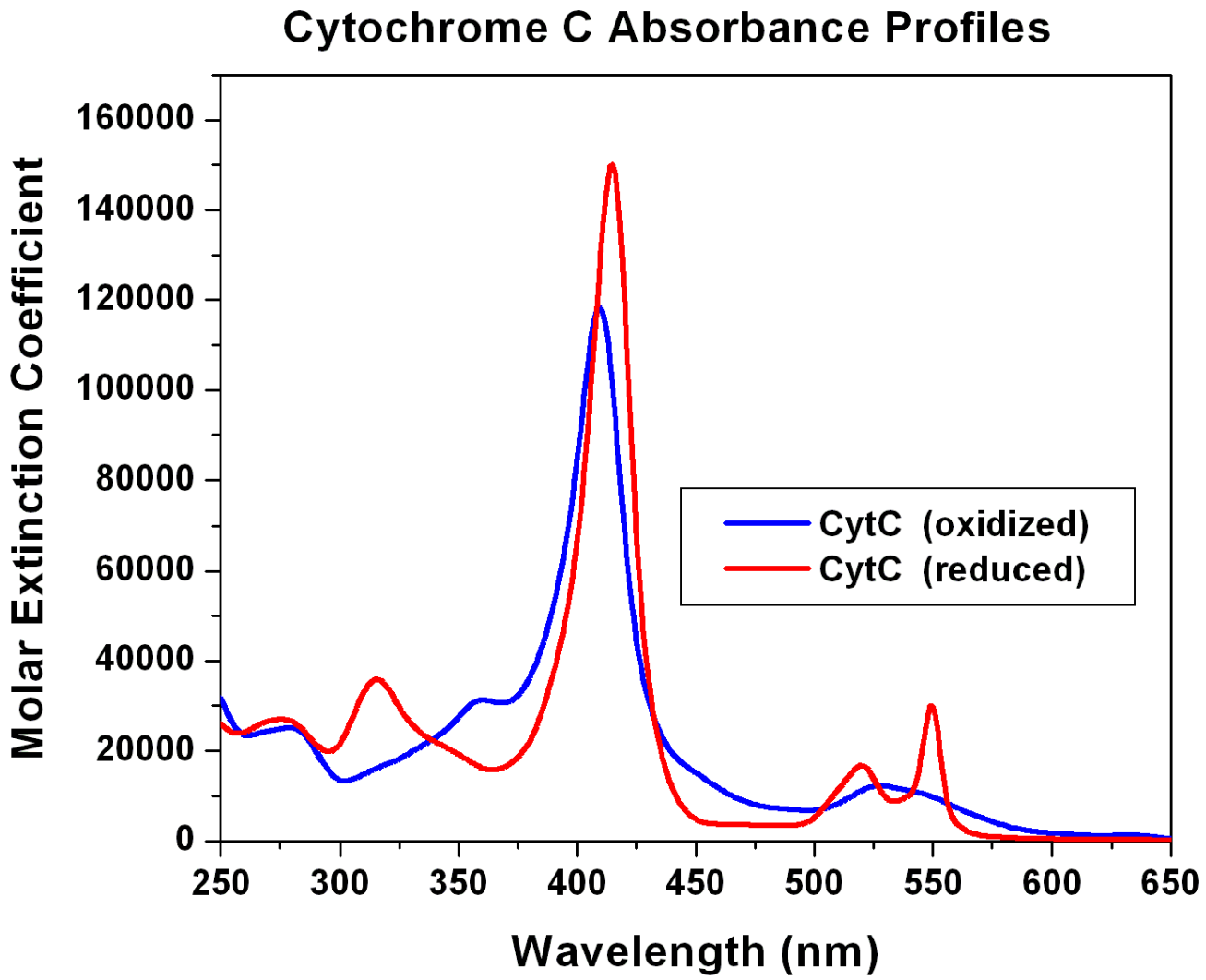
Absorption Spectroscopy

Absorption spectrum of a small molecule (a) and benzene (b) in the gas phase (top), the solution phase (center) and the theoretically calculated spectrum (bottom).

Observed spectra are generally broad envelopes of many closely spaced sharp spectral transitions. Further broadening of the spectrum is caused by solvent properties, Doppler shifts and other effects.



Absorption Spectroscopy



Absorption Spectroscopy

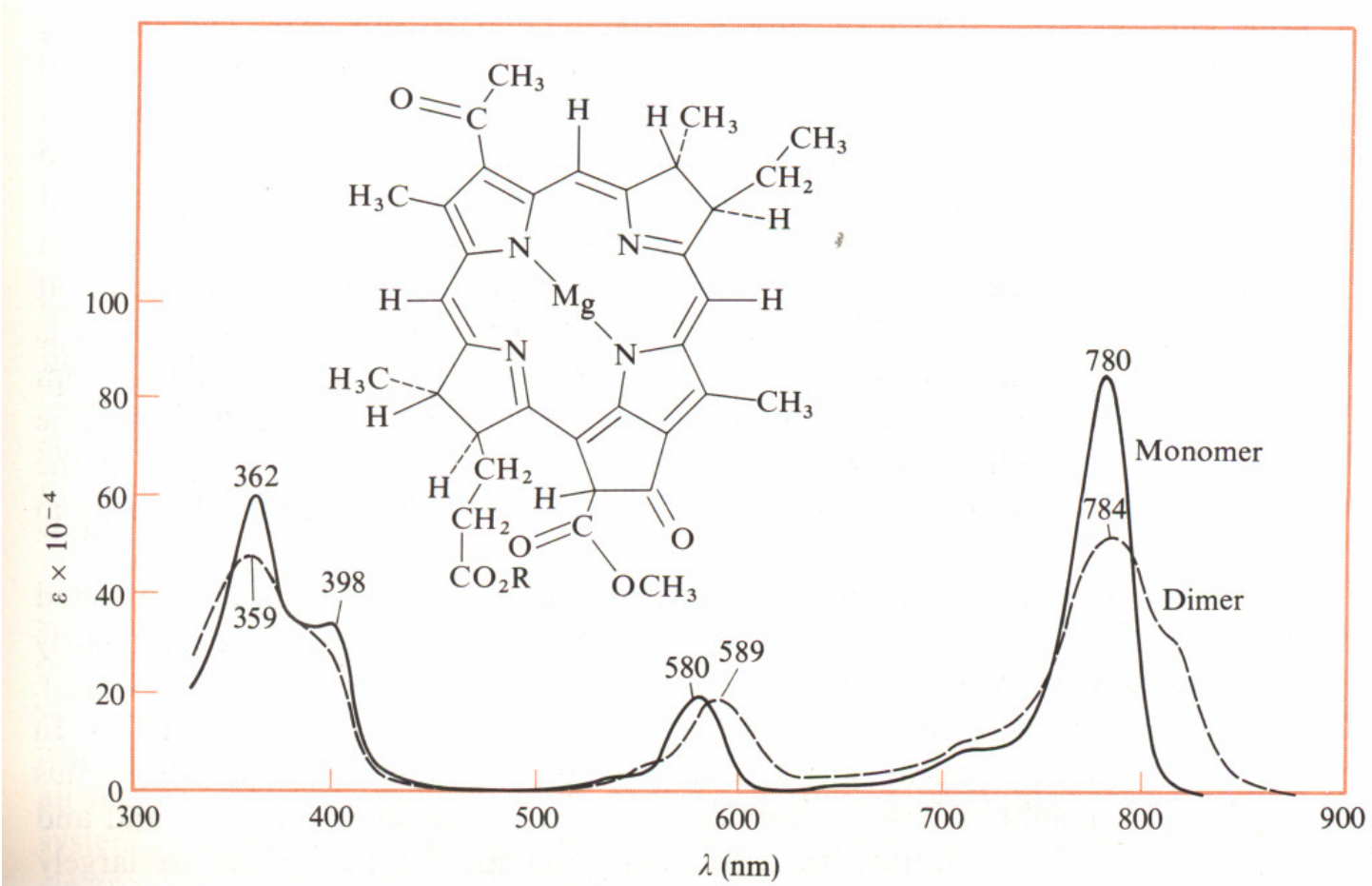


Figure 7-19

Monomer and dimer spectra for solutions of bacteriochlorophyll. A pronounced splitting of the longest-wavelength band in the dimer is visible. [After K. Sauer, J. R. L. Smith, and A. J. Schultz, *J. Am. Chem. Soc.* 88:2681 (1966).]

Absorption Spectroscopy

Hypochromism is the loss of absorption intensity in some regions of the absorbance spectrum.

Hypochromism is caused by the alignment of transition dipoles.

Examples for the effect of hypochromism can be observed most often during binding or aggregation. A very good example is the 30% increased absorbance DNA nucleosides over single stranded to double stranded DNA. The alignment of the bases (stacking) can reduce the absorbance.

Hypochromism depends on $1/r^3$ of the aligned residues giving rise to the dipoles

Absorption Spectroscopy

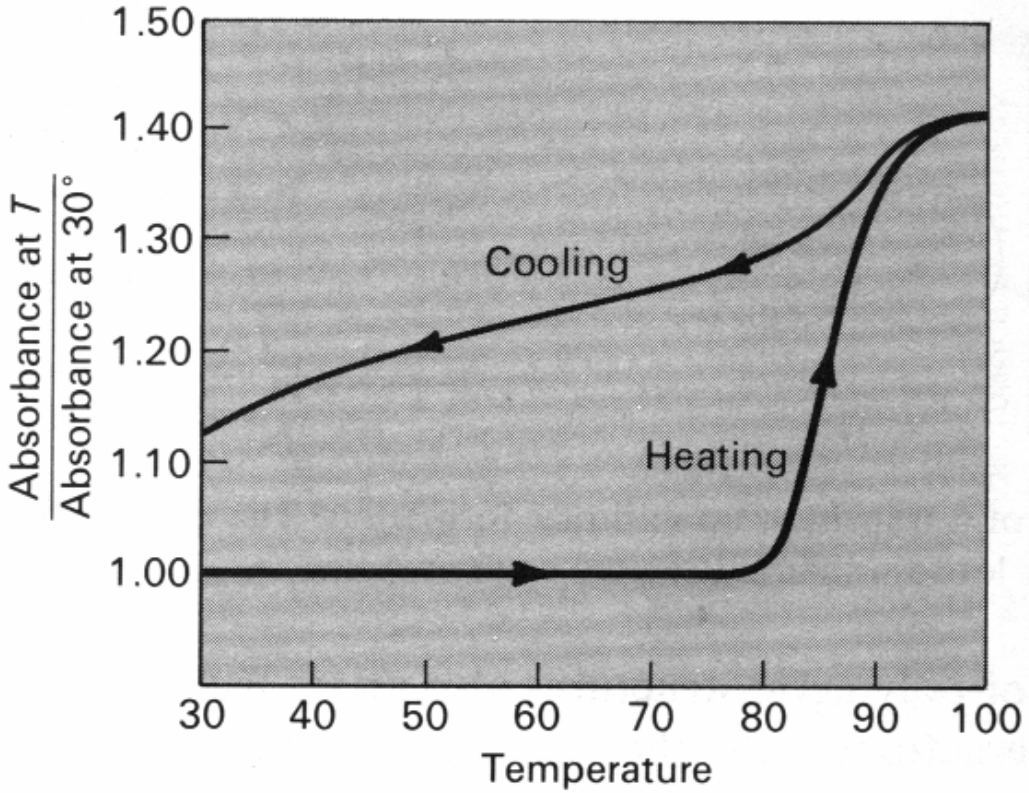


Figure 8.11 The "melting" of DNA as followed by absorption at 260 nm. Note the sharpness of the transition in cycle 1. This is typical of a cooperative process (see Chapter 3). Also note that recovery of the ordered structure is not complete on rapid cooling.

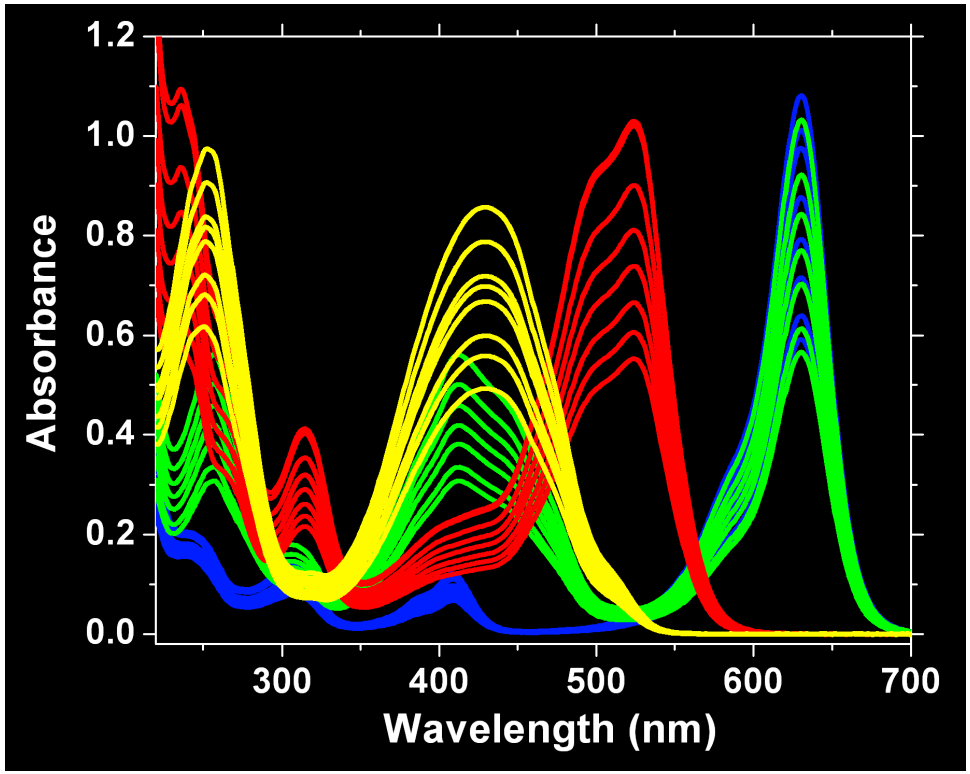
Absorption Spectroscopy

Deconvolution of multiple basis spectra to quantify individual components:

When known absorbance spectra are sufficiently different and hypochromism is negligible, the individual components can be mathematically separated to determine relative amounts of each constituent.

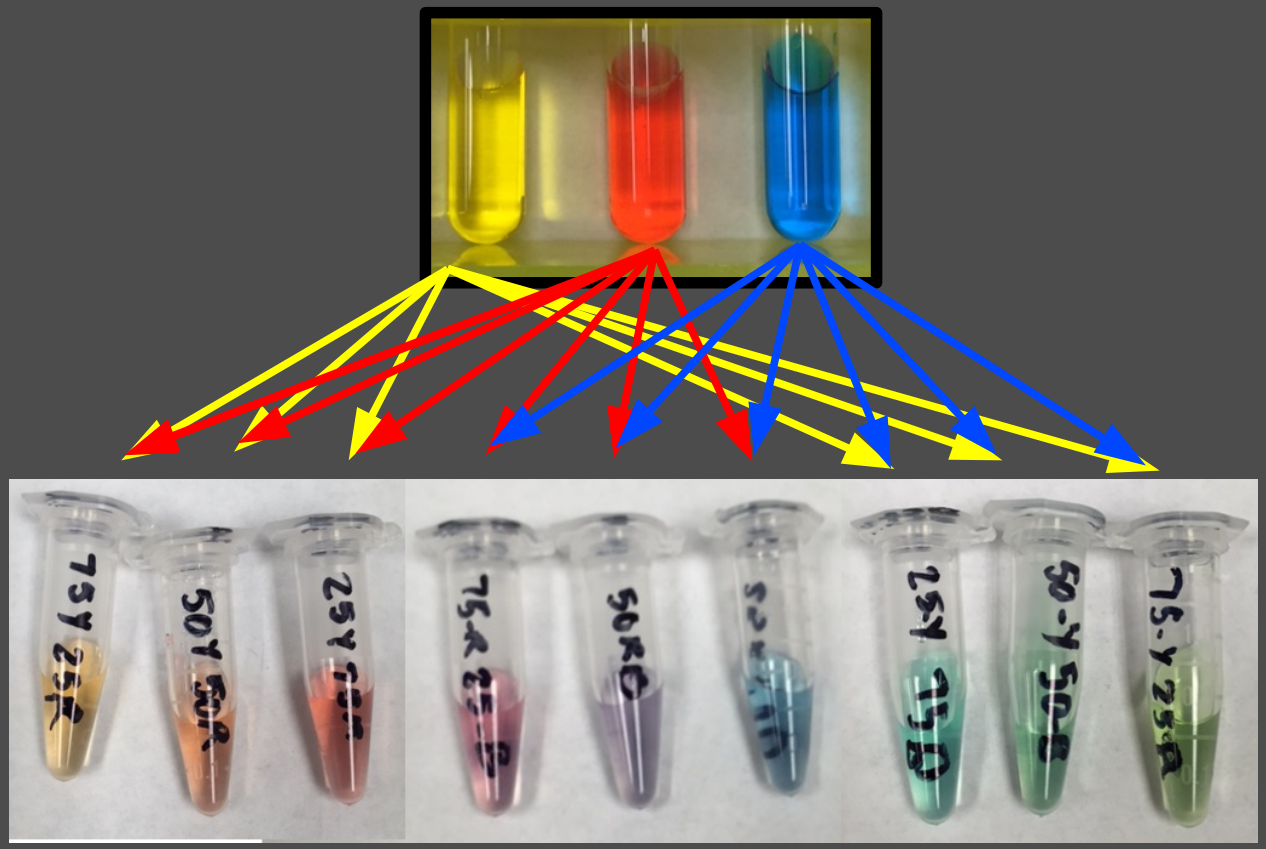
$$F_{base} = \sum G_i(\sigma_i, \lambda_i, c_i) = \sum c_i e^{\frac{-(x-\lambda_i)^2}{2\sigma_i^2}}$$

$$F_{mix} = \sum_i a_i F_{base,i}$$

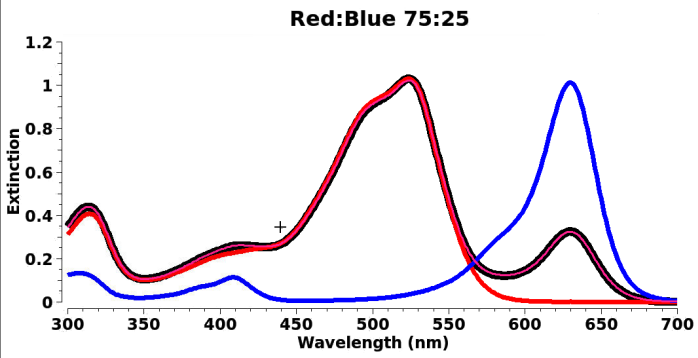


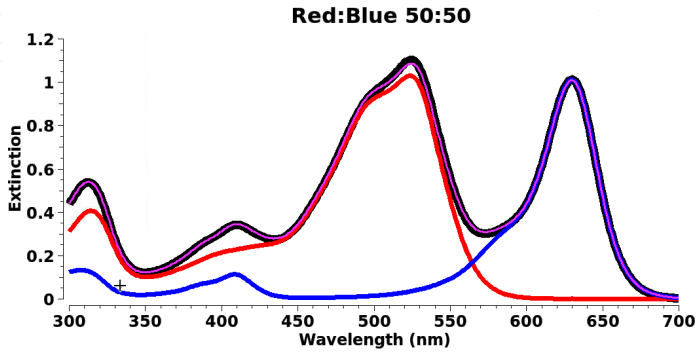
Absorption Spectroscopy

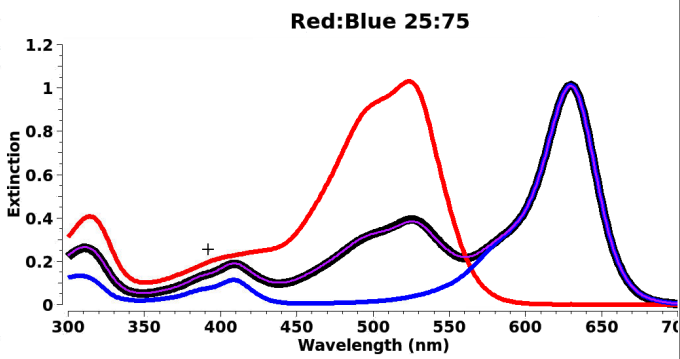
Experiment: Prepare food color solutions from primary colors, and measure their pure absorbance spectra. Take multiple concentrations and globally fit each color to a sum of Gaussian terms. Then mix the colors with known ratios and try to determine the composition by performing a linear decomposition of the summation spectra into their basis spectra.

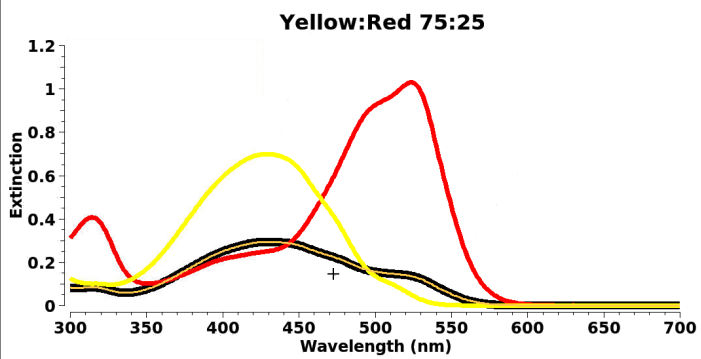


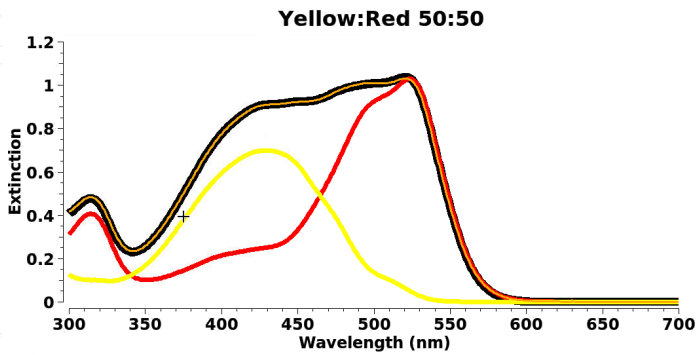
Absorption Spectroscopy

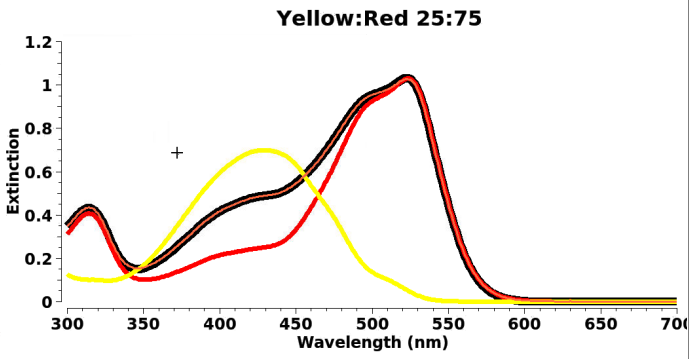


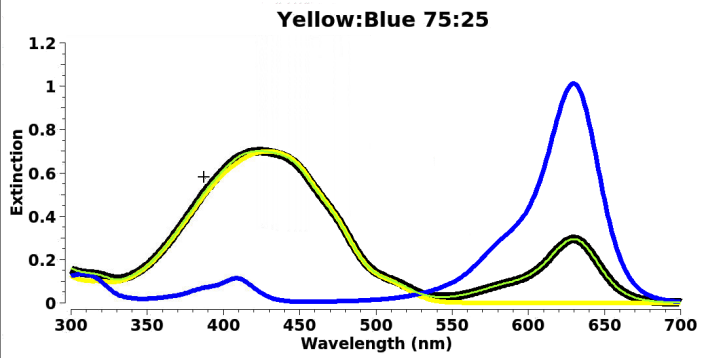


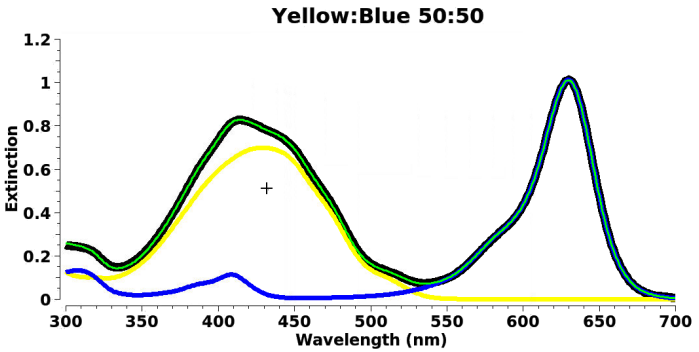


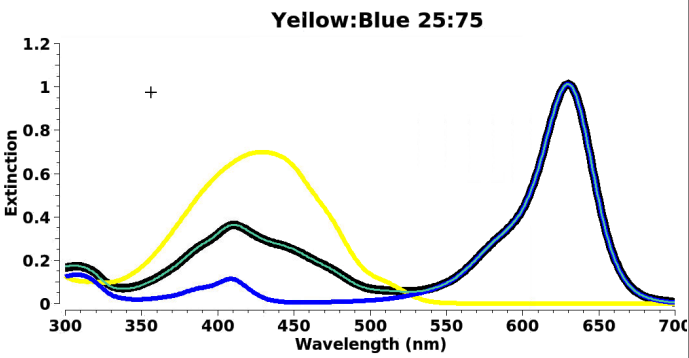












Group	Color	Expected	Calculated	Control	% Error	RMSD (%)
25Y75R	Red	75	73.82	0.00	1.6	0.24
	Yellow	25	26.18		4.7	
50Y50R	Red	50	49.69	0.00	0.6	0.32
	Yellow	50	50.31		0.6	
75Y25R	Red	25	22.99	0.00	8	0.21
	Yellow	75	77.01		2.7	
25R75B	Red	25	25.28	0.00	1.1	0.35
	Blue	75	74.72		0.4	
50R50B	Red	50	50.6	0.00	1.2	0.50
	Blue	50	49.4		1.2	
75R25B	Red	75	75.44	0.00	0.6	0.23
	Blue	25	24.56		1.8	
25Y75B	Yellow	25	27.8	0.0016	11.2	0.17
	Blue	75	72.2		3.7	
50Y50B	Yellow	50	52.12	0.0011	4.2	0.22
	Blue	50	47.88		4.2	
75Y25B	Yellow	75	77.21	0.0018	2.9	0.21
	Blue	25	22.79		8.8	
33R33B33Y	Red	33	32.72	____	0.8	0.62
	Yellow	33	35.32		7	
	Blue	33	32.72		0.8	
Green	Yellow	?	39.42	0.00		1.4
	Blue	?	60.58			

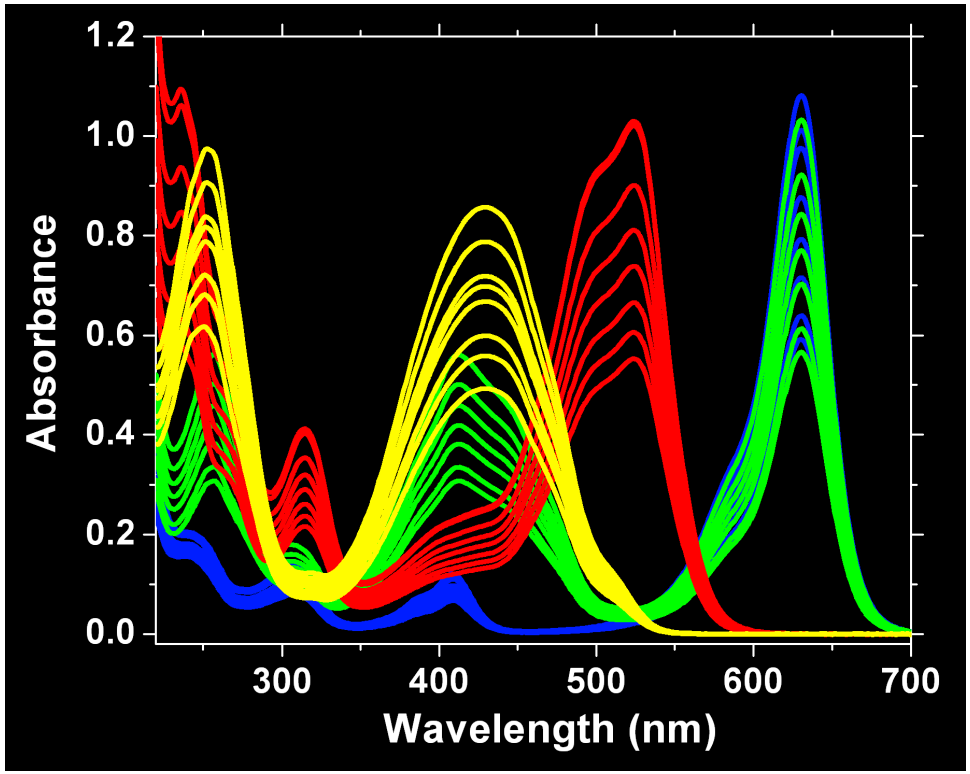
Absorption Spectroscopy

In order to be able to deconvolute the signal from the mixture into individual basis spectra, the basis spectra should be *orthogonal*, or *linearly independent*. A metric for orthogonality can be obtained if one considers the absorbance spectra to be vectors, and determins the angle between the respective vectors. This can be done with the following formula:

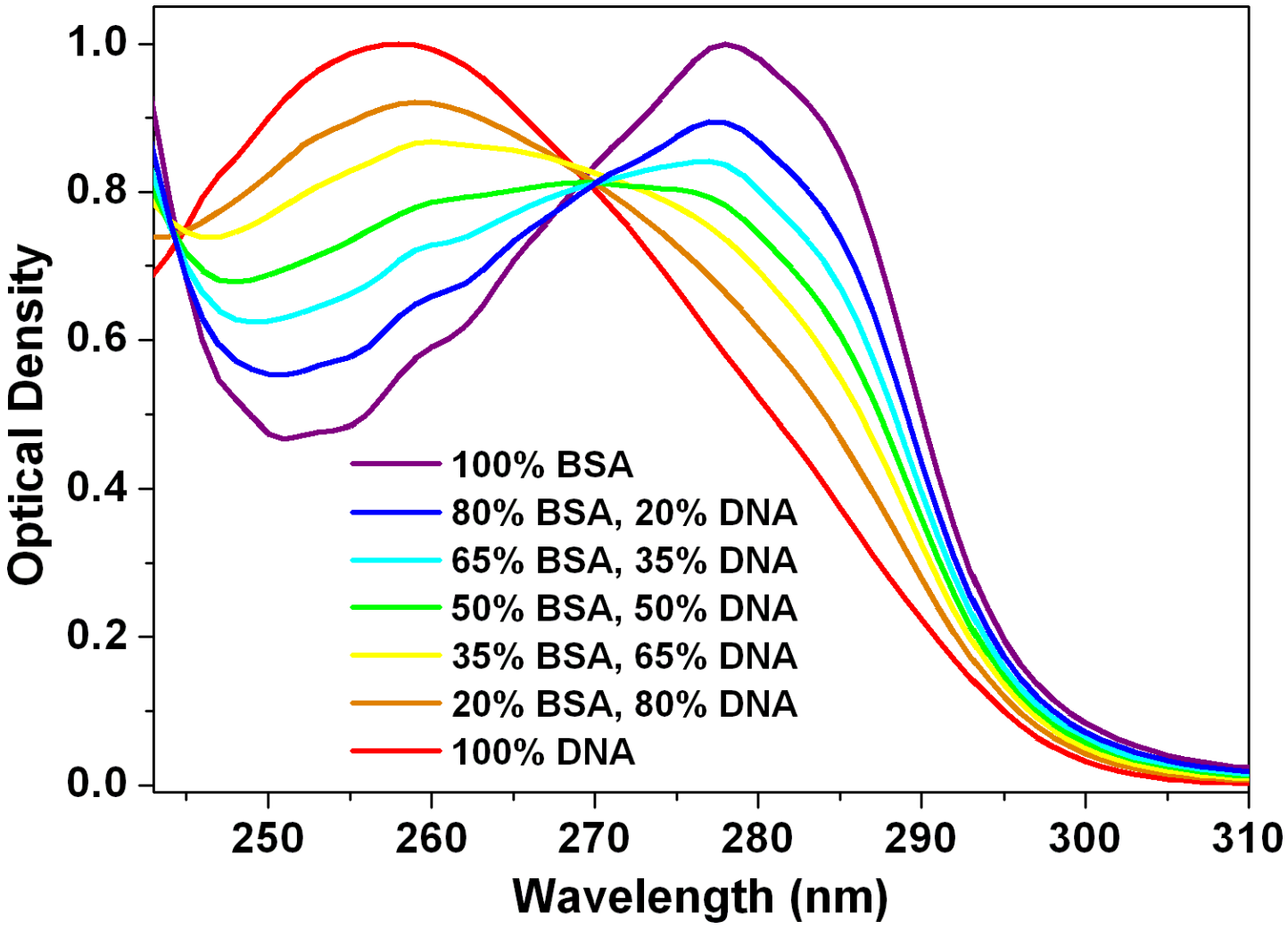
$$\theta = \cos^{-1}\left[\frac{u \cdot v}{\|u\| \cdot \|v\|}\right]$$

Colors	Angles (in degrees)
Red-blue	84.1
Red-green	71.5
Red-yellow	69.8
Blue-yellow	84.7
Blue-green	32.5
Yellow-Green	52.4
50B50Y-Green	13.4

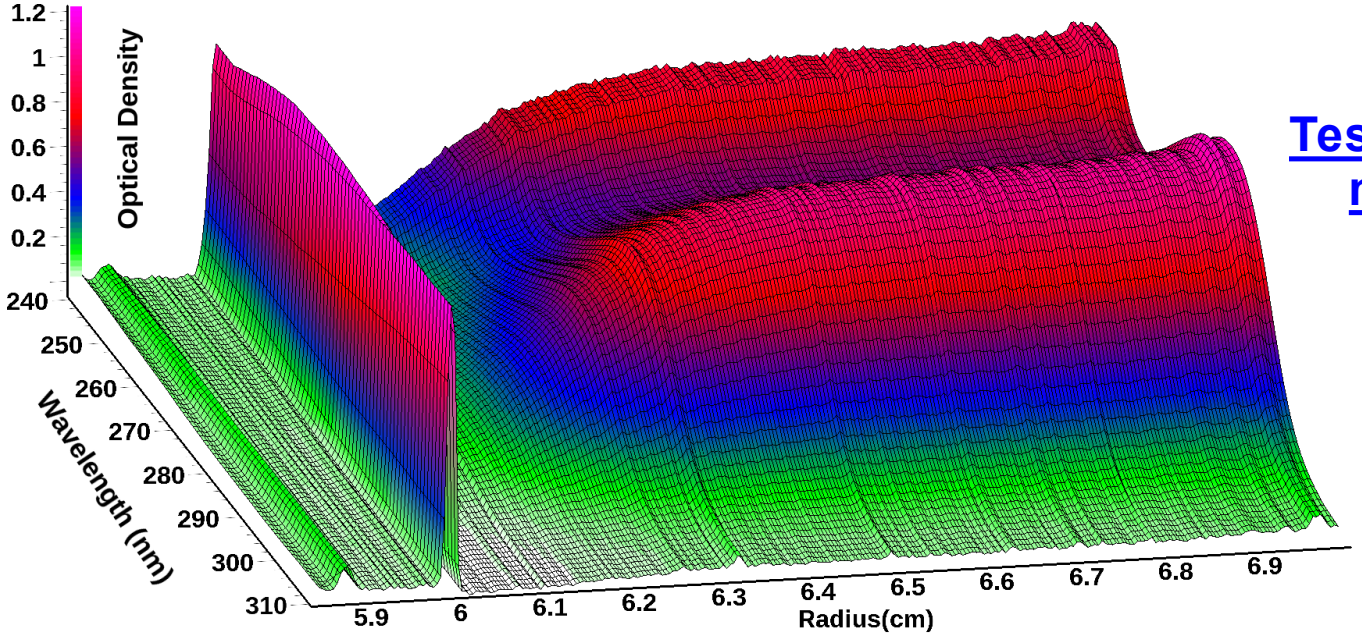
Measured angles between color vectors



Absorption Spectroscopy



UltraScan Analysis of MWL Protein/Nucleic Acid Mixtures



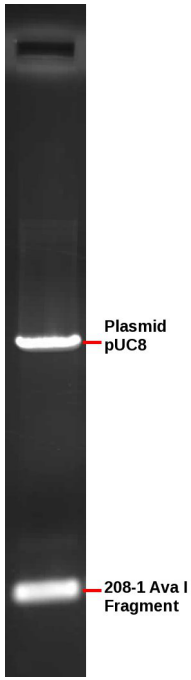
Example 1:

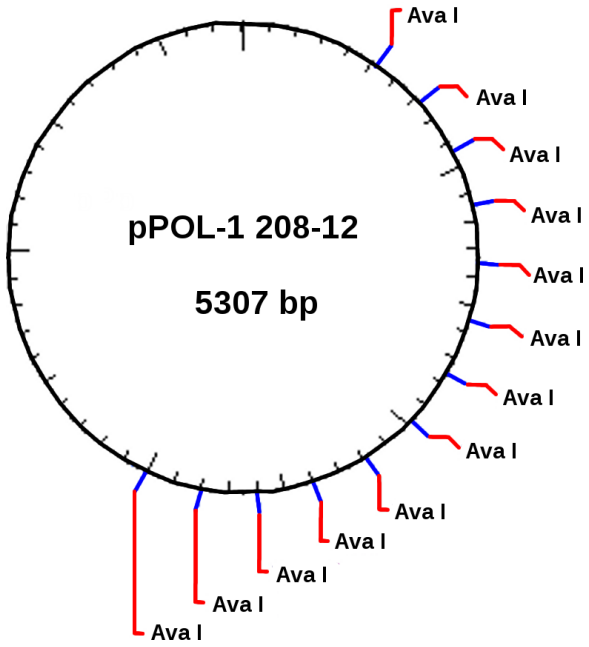
Testing reliability of the method with mixtures of BSA and a DNA plasmid digest:

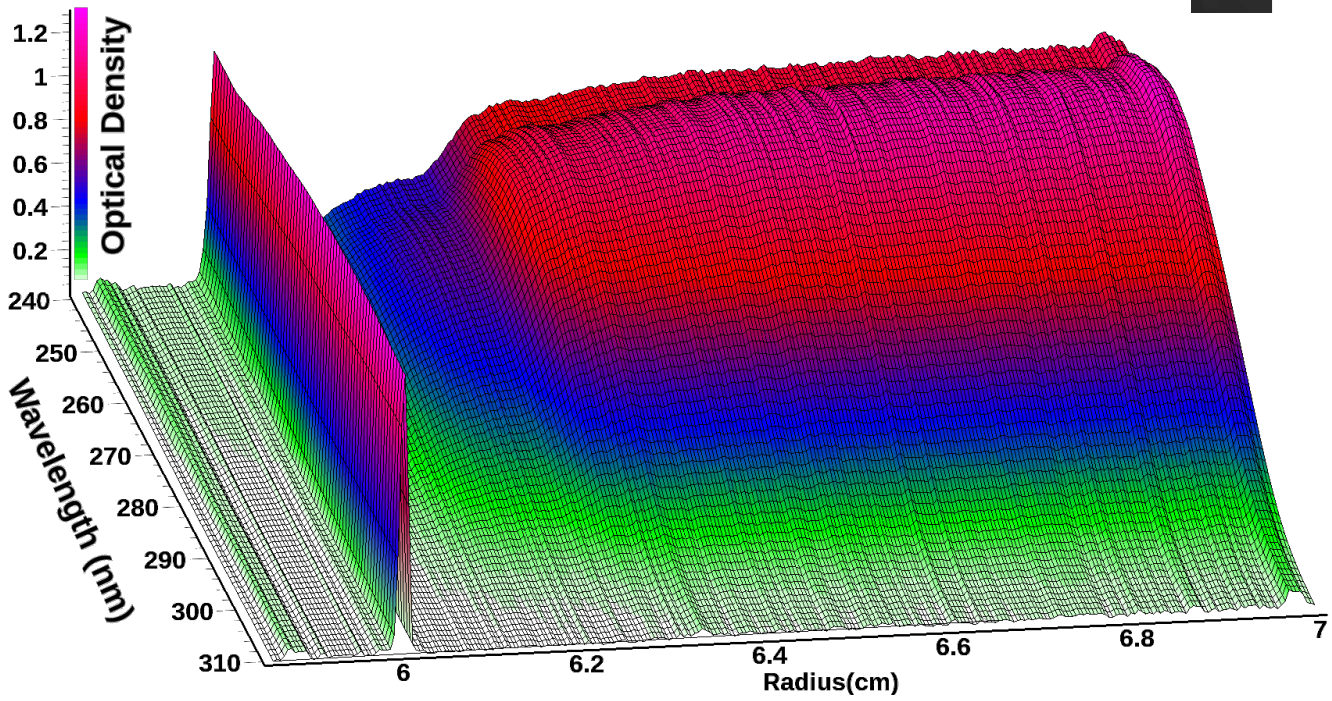
1 x 2811 bp

12 x 208 bp

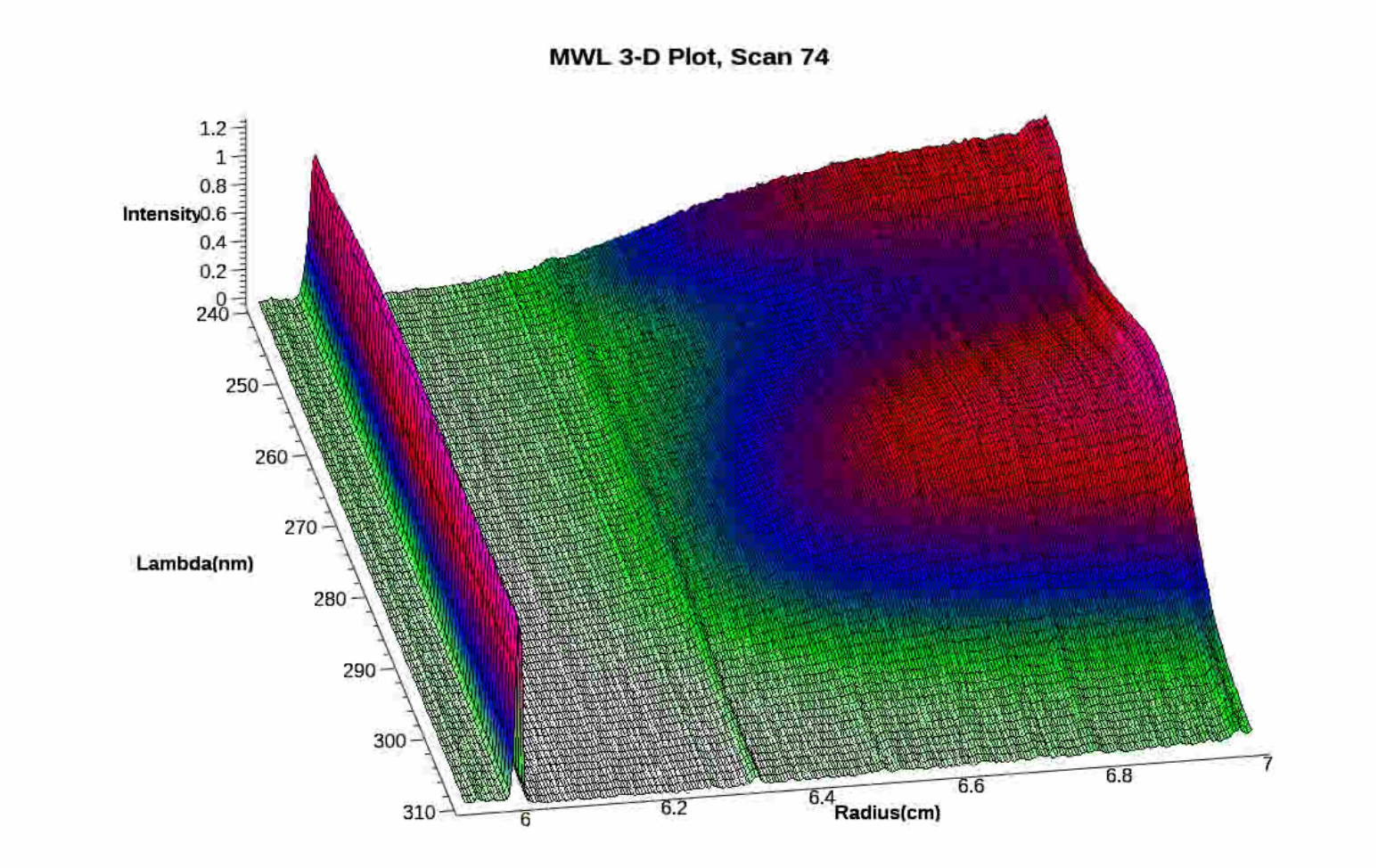
12 x 12 bp



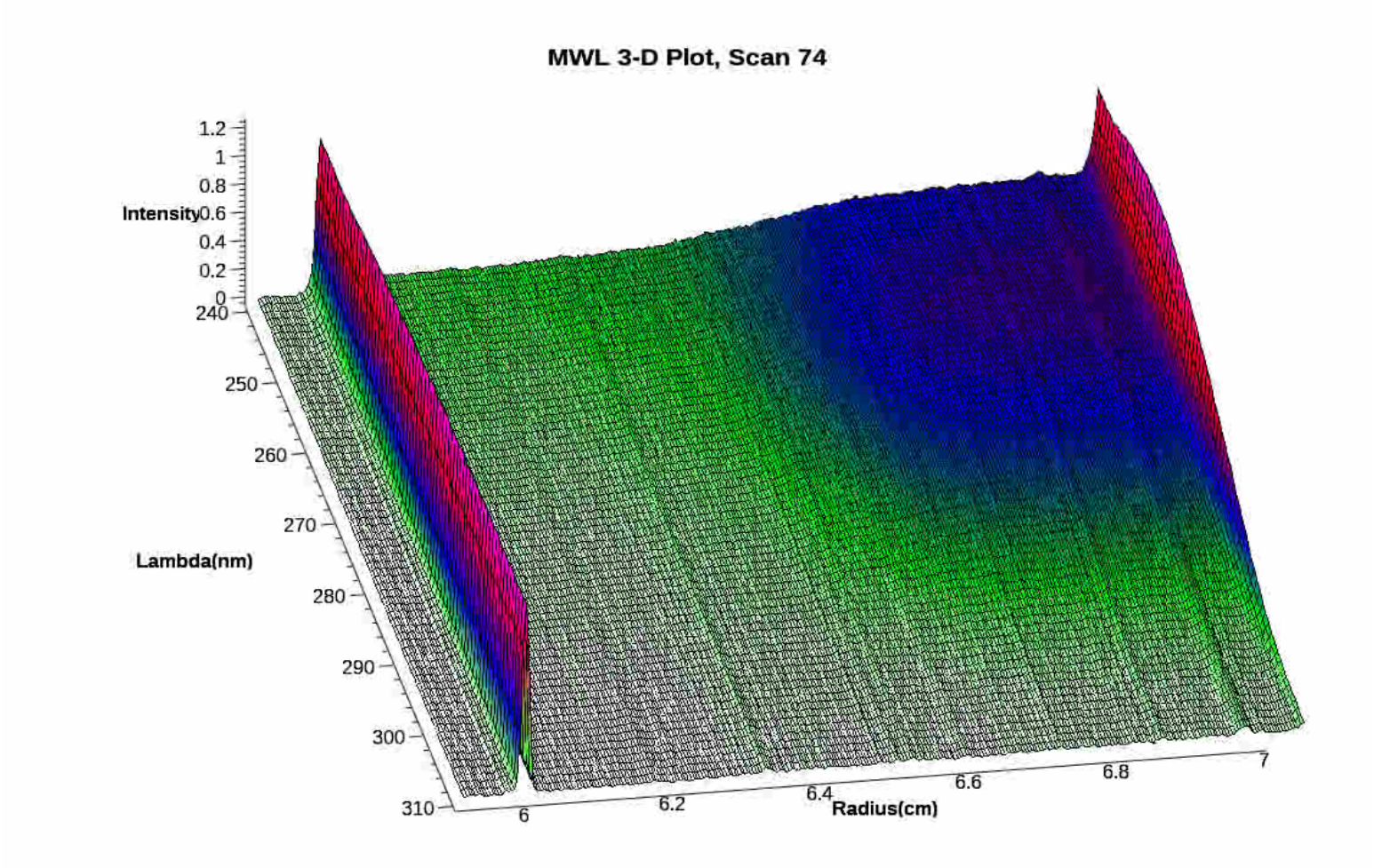




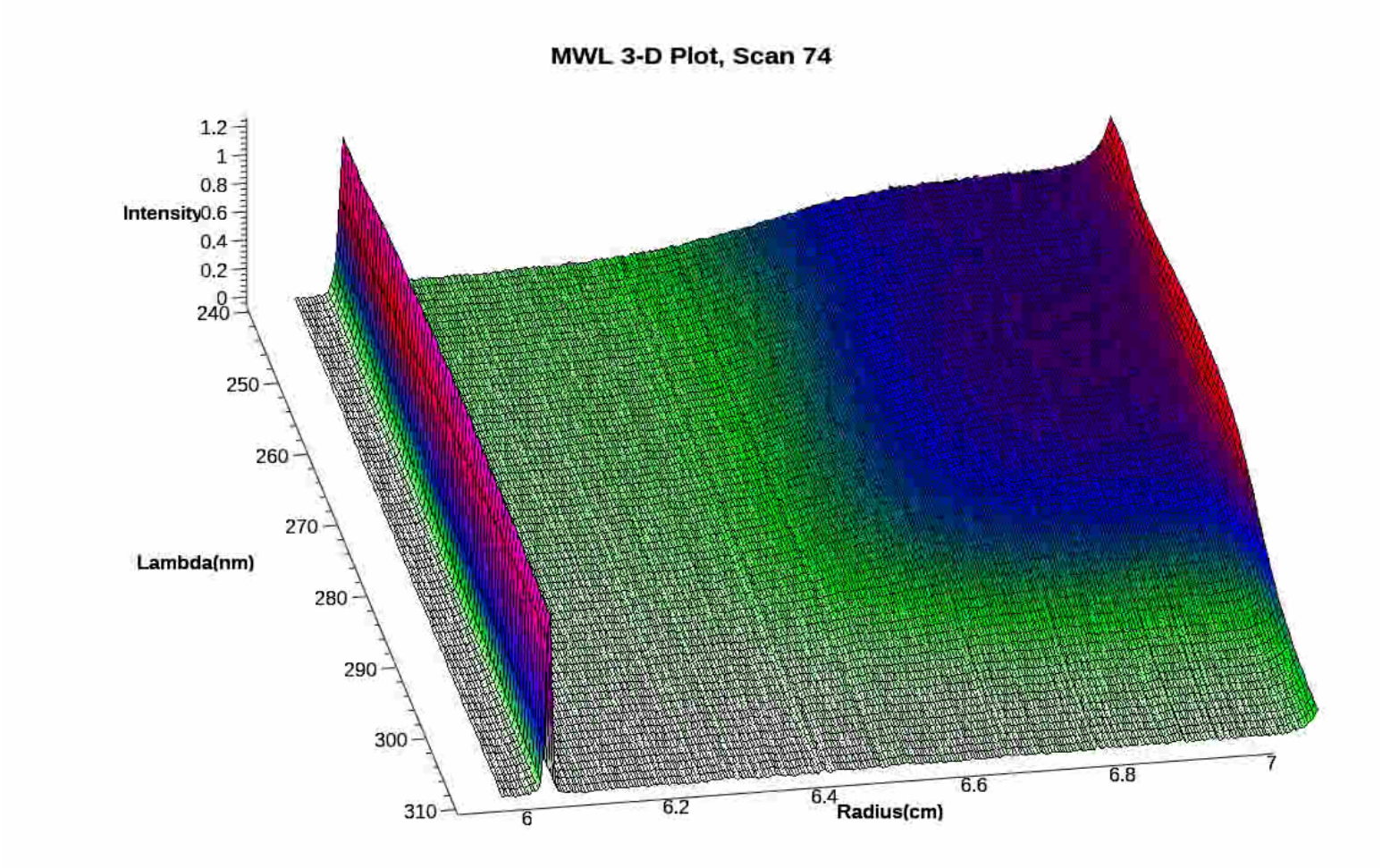
Multi-wavelength Sedimentation Velocity: BSA



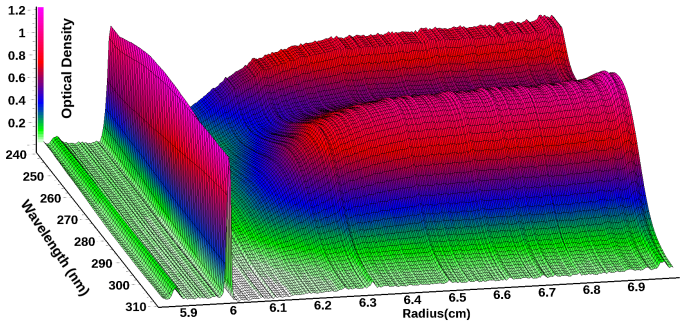
Multi-wavelength Sedimentation Velocity: DNA

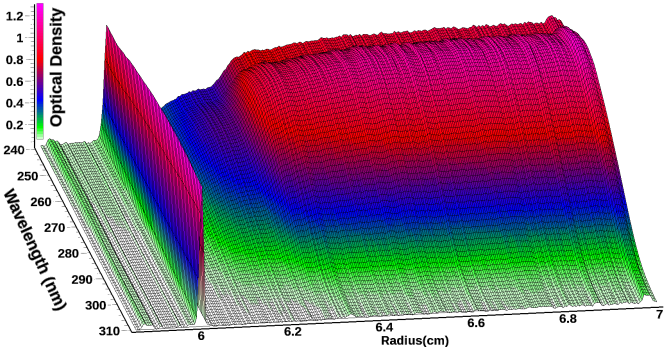


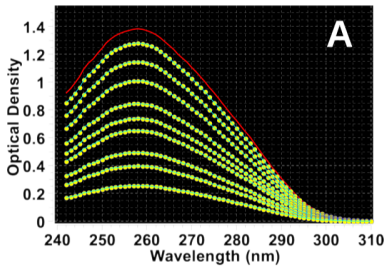
Multi-wavelength Sedimentation Velocity: DNA-BSA Mixture

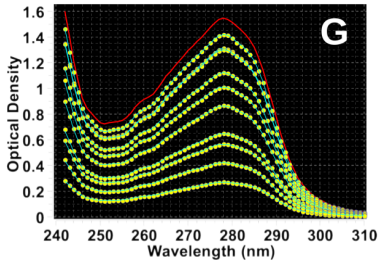


Use L_j Spectra for Spectral Decomposition



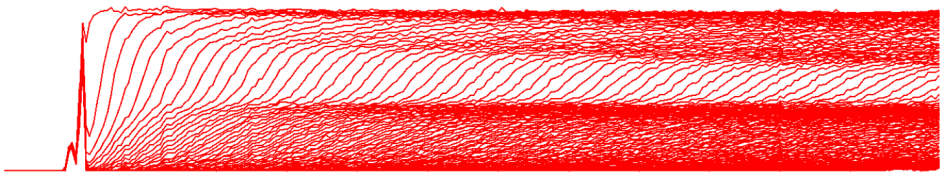


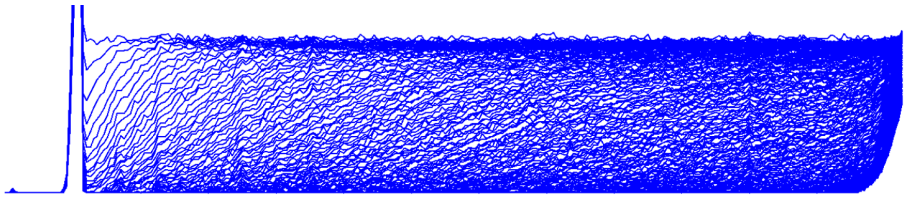




$$C_{MWL} = x_a \begin{bmatrix} \epsilon_{a1} \\ \epsilon_{a2} \\ \cdots \\ \epsilon_{ai} \end{bmatrix}_{r,t} + x_b \begin{bmatrix} \epsilon_{b1} \\ \epsilon_{b2} \\ \cdots \\ \epsilon_{bi} \end{bmatrix}_{r,t}$$

L_{DNA} L_{BSA}

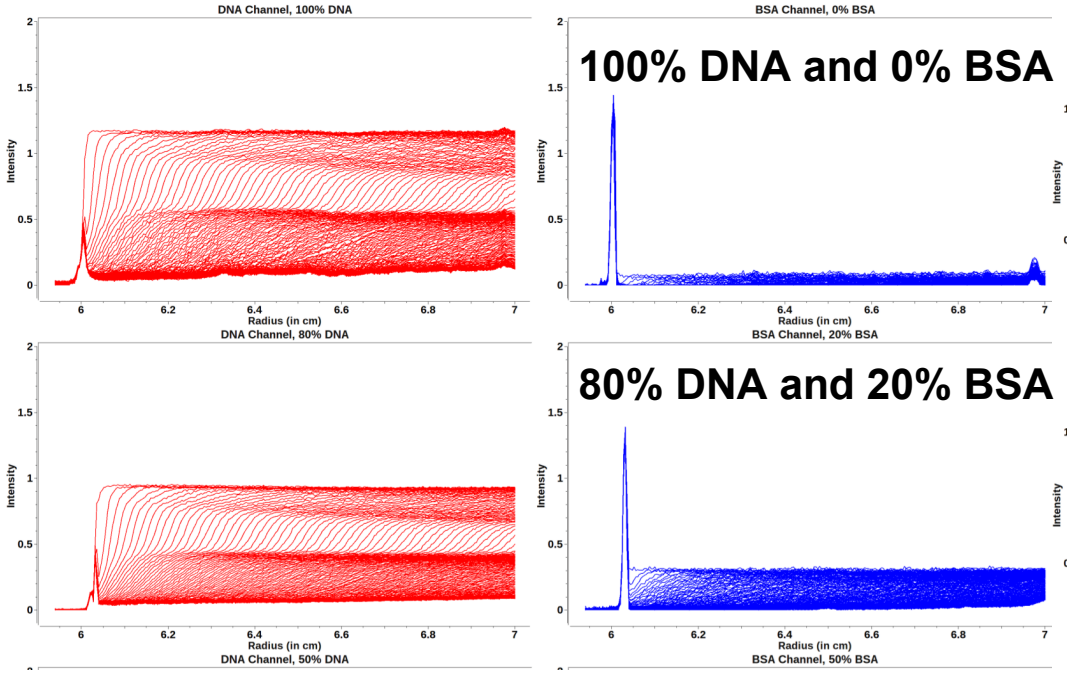


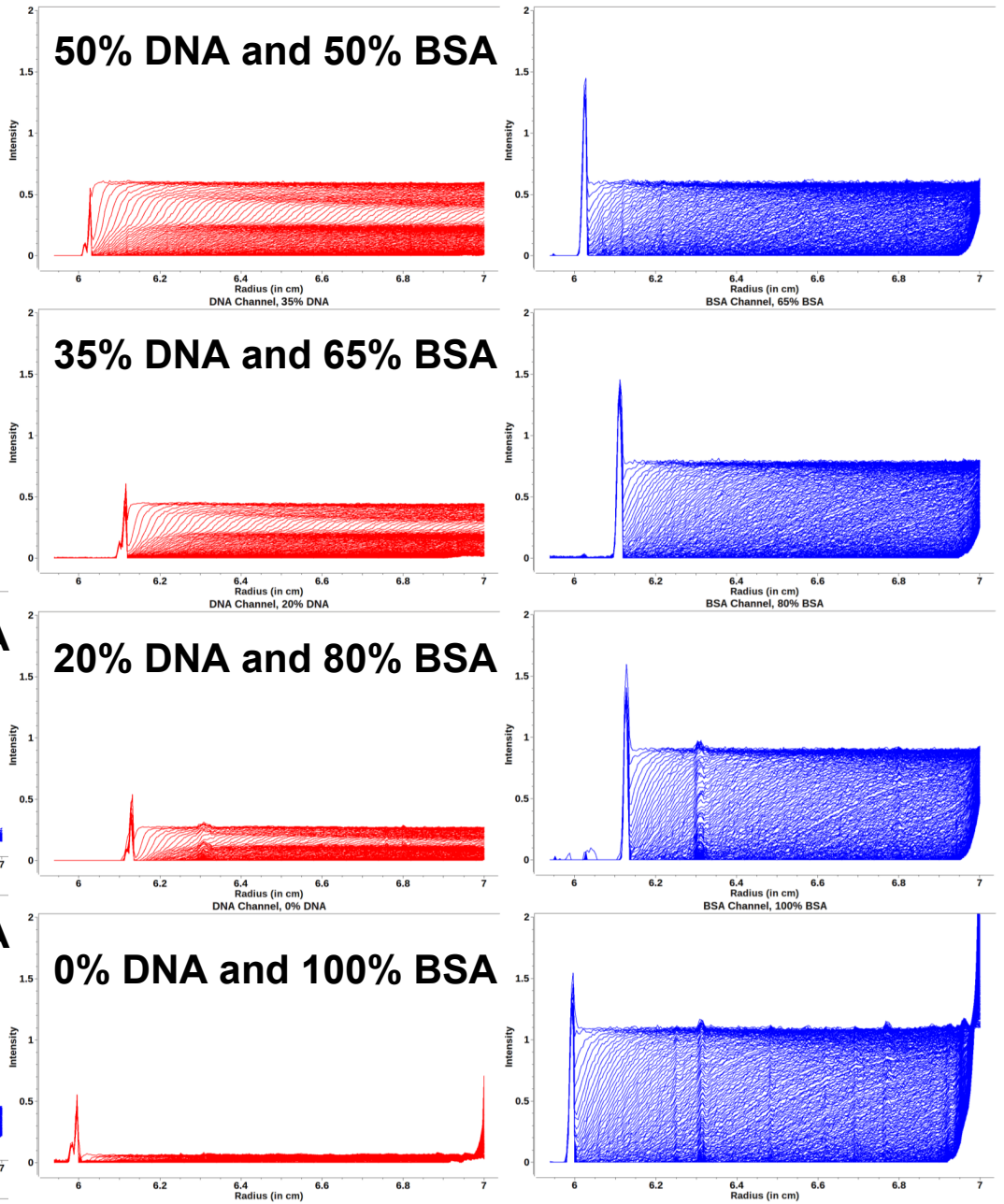


Multi-Wavelength Decomposition of a range of DNA/BSA mixtures using pure 100% DNA and 100% BSA spectra:

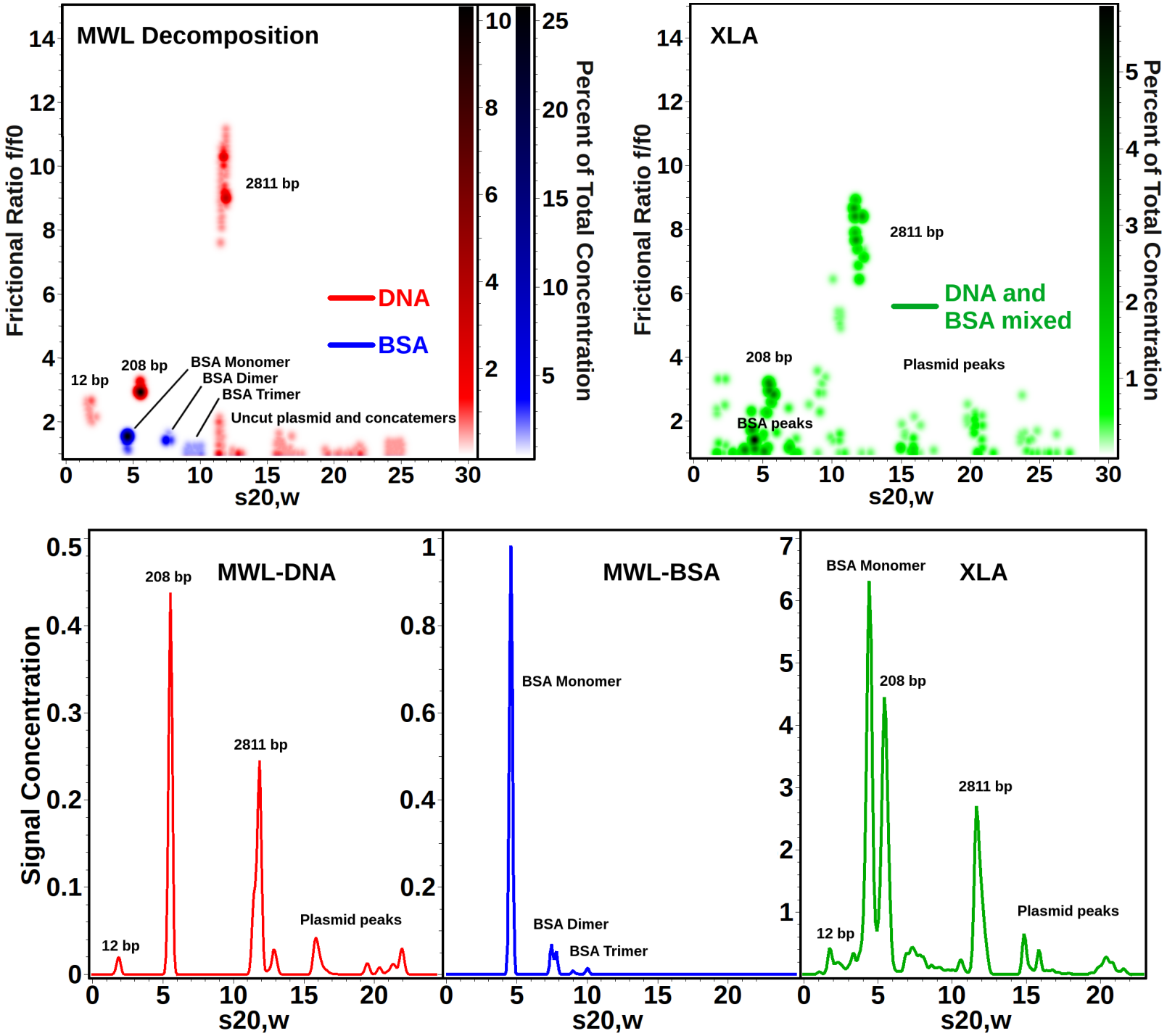
$$C_{MWL} = x_a \begin{bmatrix} \epsilon_{a1} \\ \epsilon_{a2} \\ \dots \\ \epsilon_{ai} \end{bmatrix}_{r,t} + x_b \begin{bmatrix} \epsilon_{b1} \\ \epsilon_{b2} \\ \dots \\ \epsilon_{bi} \end{bmatrix}_{r,t}$$

L_{DNA} L_{BSA}





Global Genetic Algorithm Analysis



Circular Dichroism

Circular Dichroism is a spectroscopic technique that uses circularly polarized light to discriminate between asymmetric (optically active) molecules.

Any light wave has an electrical and a magnetic vector, which are perpendicular to each other, and perpendicular to the direction of propagation (x axis in the image below):

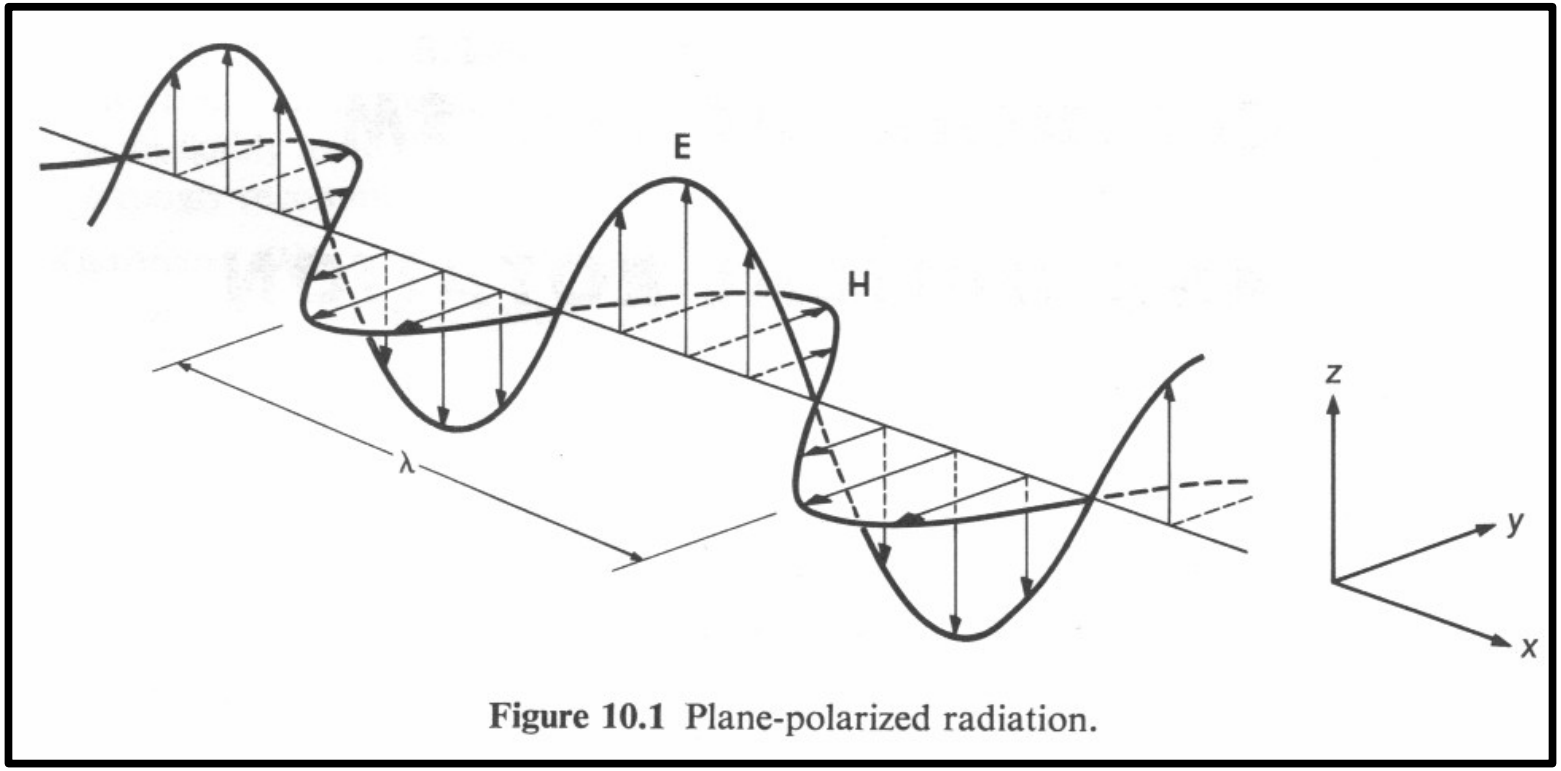


Figure 10.1 Plane-polarized radiation.

Circular Dichroism

Circularly polarized light makes v rotations per second. The wavelength is the distance required to make one full rotation, with $c = \lambda v$. Circularly polarized light can be either left- or right-handed. By convention, light is said to be right polarized when the electric vector, when seen towards the source, is rotating in a clockwise direction.

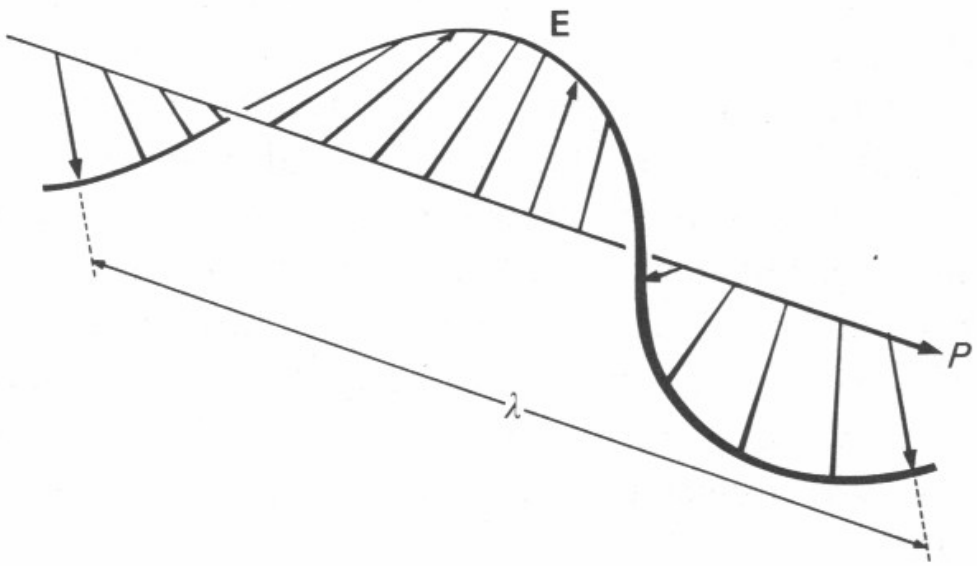


Figure 10.2 Circularly polarized radiation. Only the electric field is shown. By the convention used, this is right circular polarization, since an observer at point *P* would see the field rotating in a clockwise direction as the waves passed.

Circular Dichroism

Asymmetric molecules tend to absorb left- and right-handed circularly polarized light with different amplitudes. *Circular Dichroism* (CD) is defined to be the difference between the left- and right-handed extinction coefficients for the absorption of circularly polarized light:

$$\Delta\epsilon = \epsilon_L - \epsilon_R$$

Unlike regular absorption bands, CD bands can be negative or positive.

CD absorption is also about a 1000 fold weaker, requiring much more sensitive instrumentation.

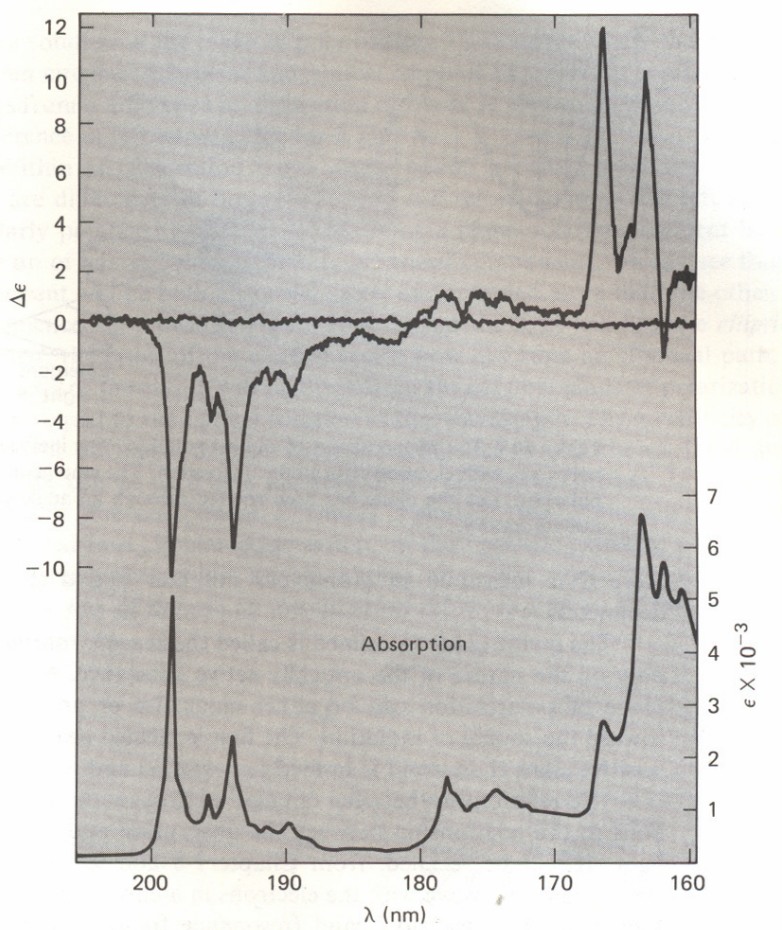


Figure 10.6 Absorption spectrum and CD spectrum of (+)-3-methylcyclopentane. The figure is included to emphasize three points: (1) CD bands can be of either positive or negative sign; (2) $\Delta\epsilon$ is several orders of magnitude smaller than ϵ; and (3) some bands which exhibit intense absorption are weak in CD, and vice versa; compare the band at about 178 nm to that at about 194 nm. After W. C. Johnson, *Rev. Sci. Instrum.*, **42**, 1283–1286 (1971). Reprinted by permission of the author and the American Institute of Physics.

Circular Dichroism

The alpha-carbon in the protein backbone is an asymmetric atom (except in glycine), which makes it optically active, and the backbone dominates the CD spectrum of proteins.

The CD spectrum is most interesting in the region between 160-220 nm, though typically the spectrum is more easily interpreted in the range above 180 nm.

For proteins, the CD spectrum of different secondary structures (alpha-helix, beta-sheet, random coil) are very different, as shown for poly-L-lysine in the figure on the right.

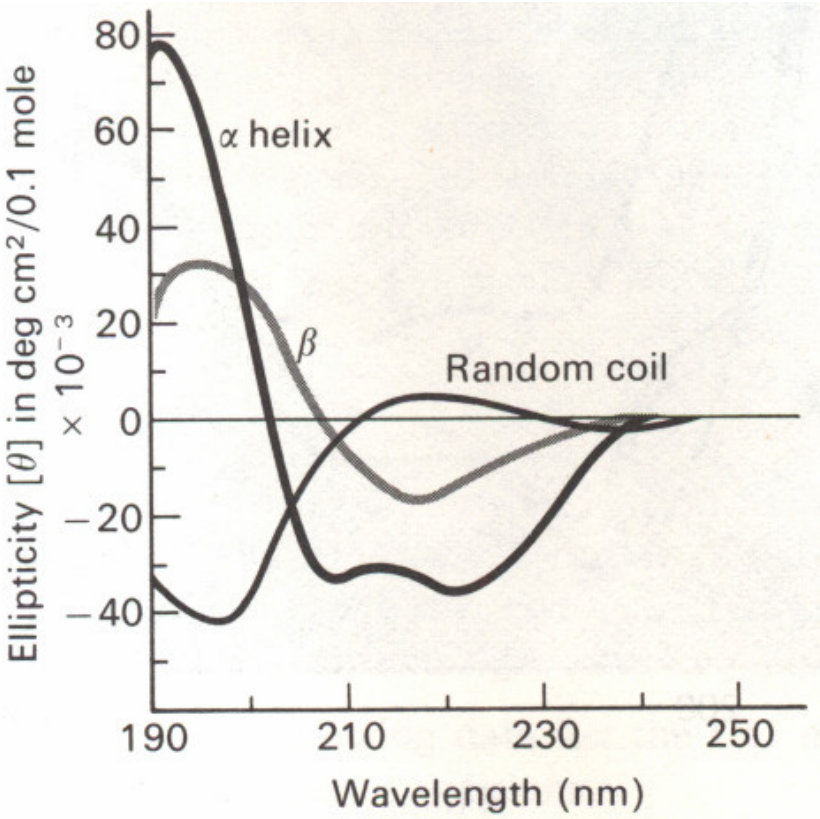


Figure 10.16 Circular dichroism of a polypeptide (poly-L-lysine) in various conformations. After N. Greenfield and G. D. Fasman, *Biochemistry*, **8**, 4108 (1969). Copyright © 1969 by the American Chemical Society.

Circular Dichroism

Using data from X-ray diffraction data, basis spectra have been deduced that can be used in linear decompositions to quantify the degree of random coil, alpha helix and beta sheet in any given protein, making CD a powerful technique to predict secondary structure.

The Figure on the right shows how successively adding basis spectra improved the decomposition fit for the CD spectrum of the protein papain.

However, decomposition techniques have their limitations due to significant differences in the CD spectra of "model" compounds for each conformation. With X-ray crystallography more and more accessible, higher resolution can be obtained with crystallography now.

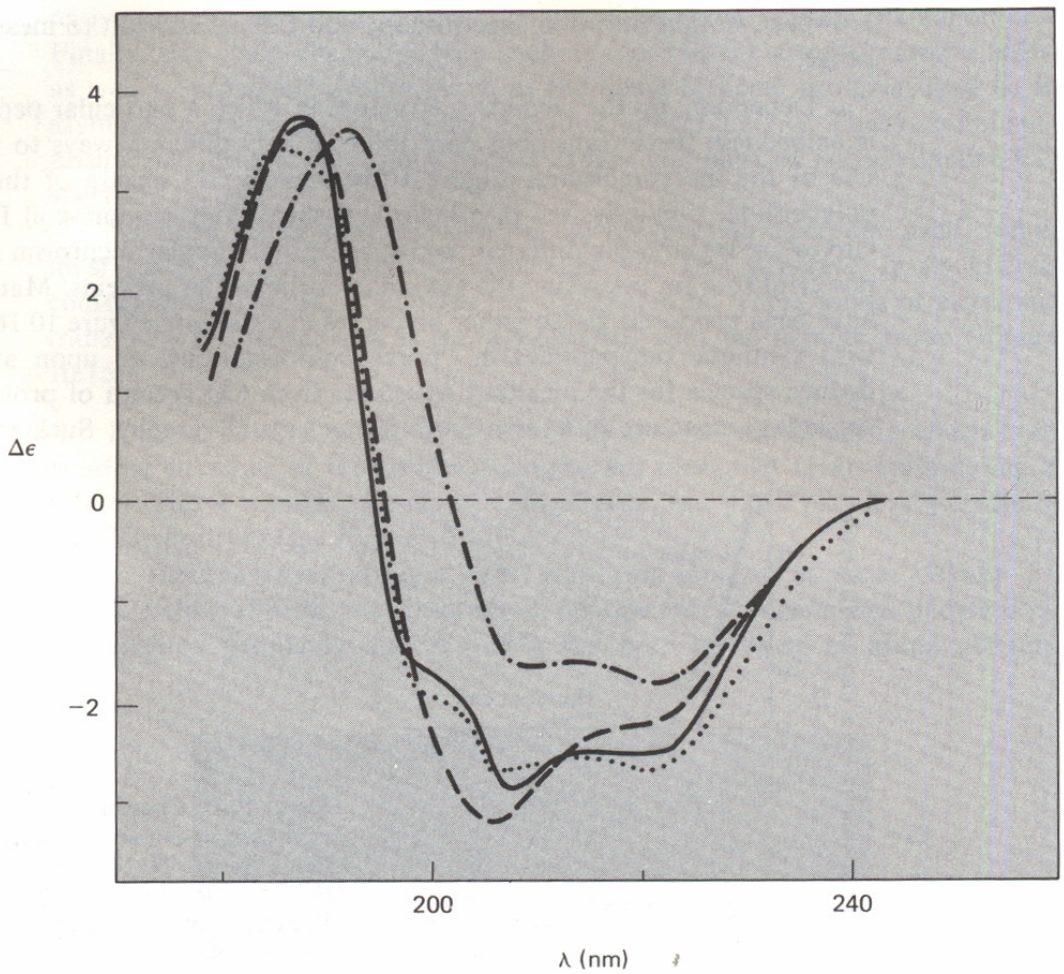


Figure 10.17 Fitting of the CD spectrum of the protein papain (—) using one (–·–·–), three (– – –), or five (·····) of the "basis" spectra of Hennessy and Johnson. Adapted from J. P. Hennessy and W. C. Johnson, *Biochemistry*, **20**, 1085–1094 (1981). Reprinted by permission of the authors and the American Chemical Society.