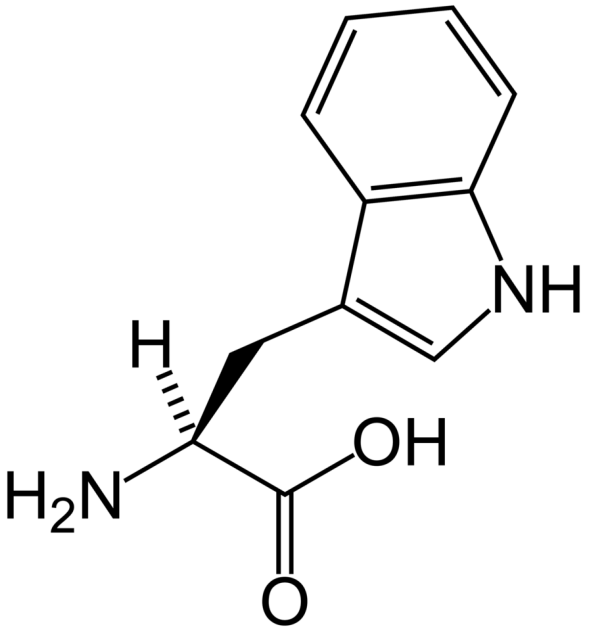
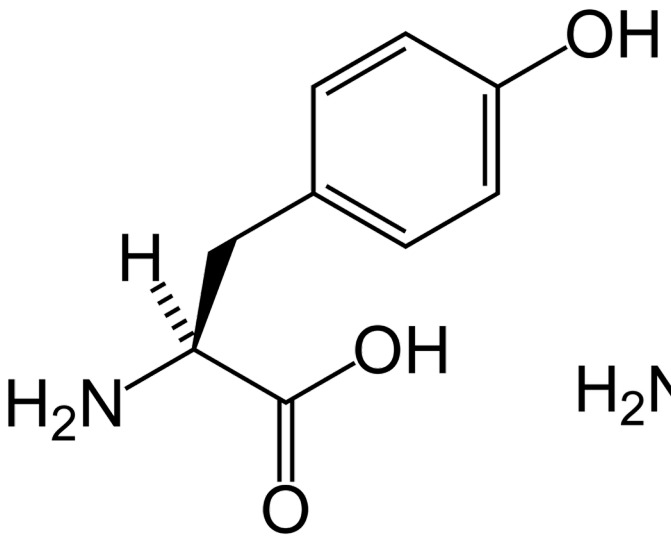
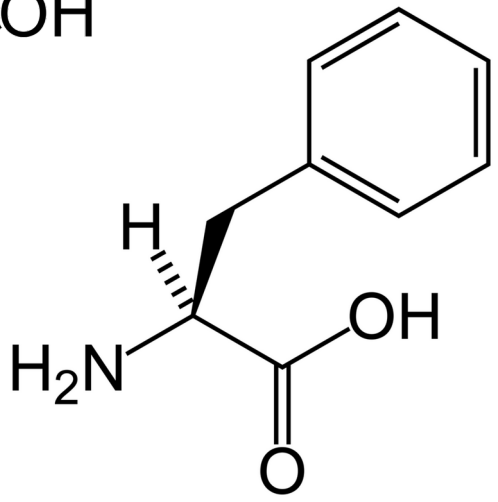
Aromatic Amino Acids



Trp (W)



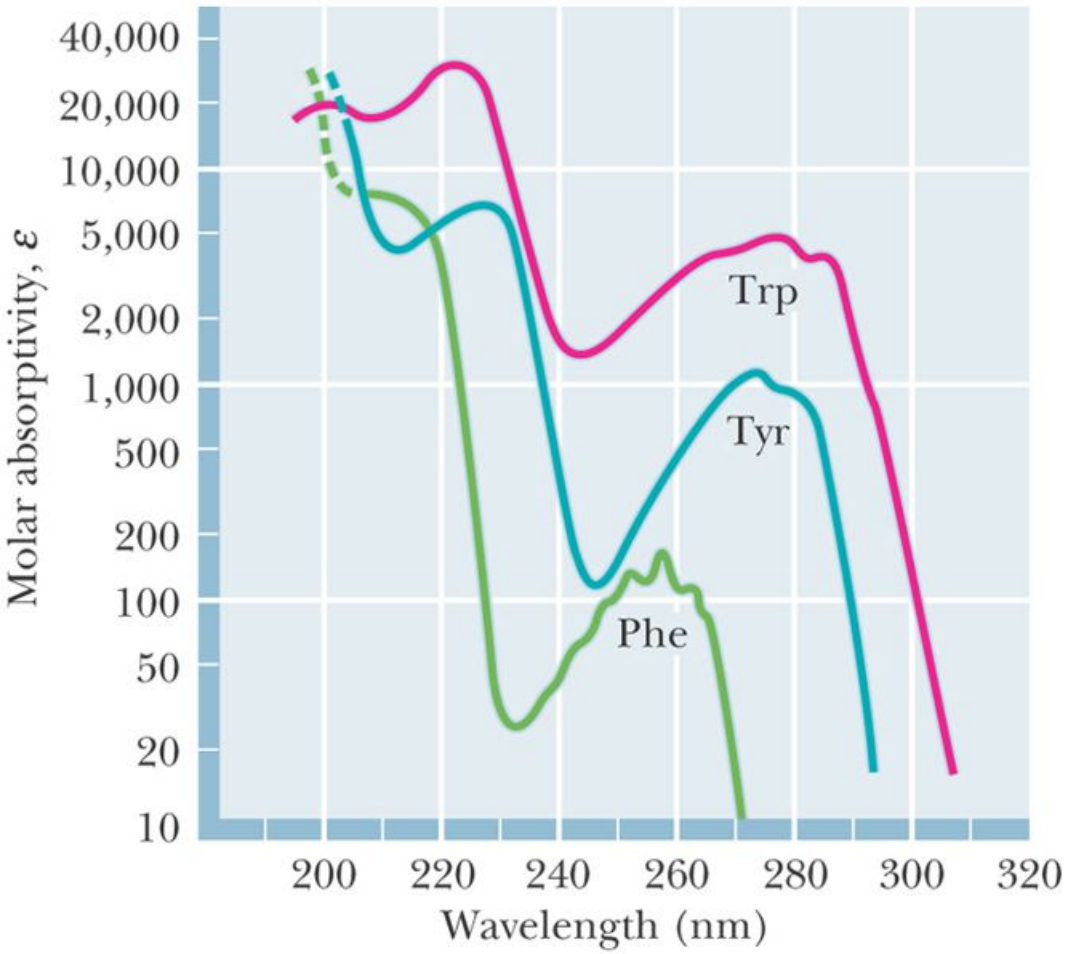
Tyr (Y)



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.*)



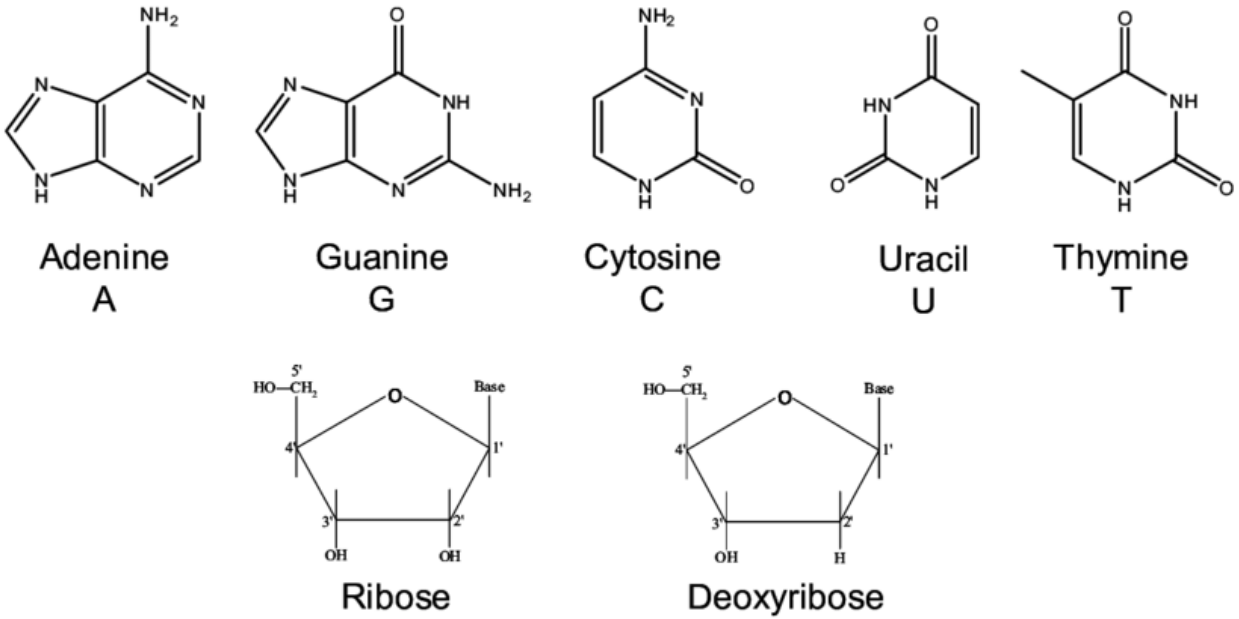
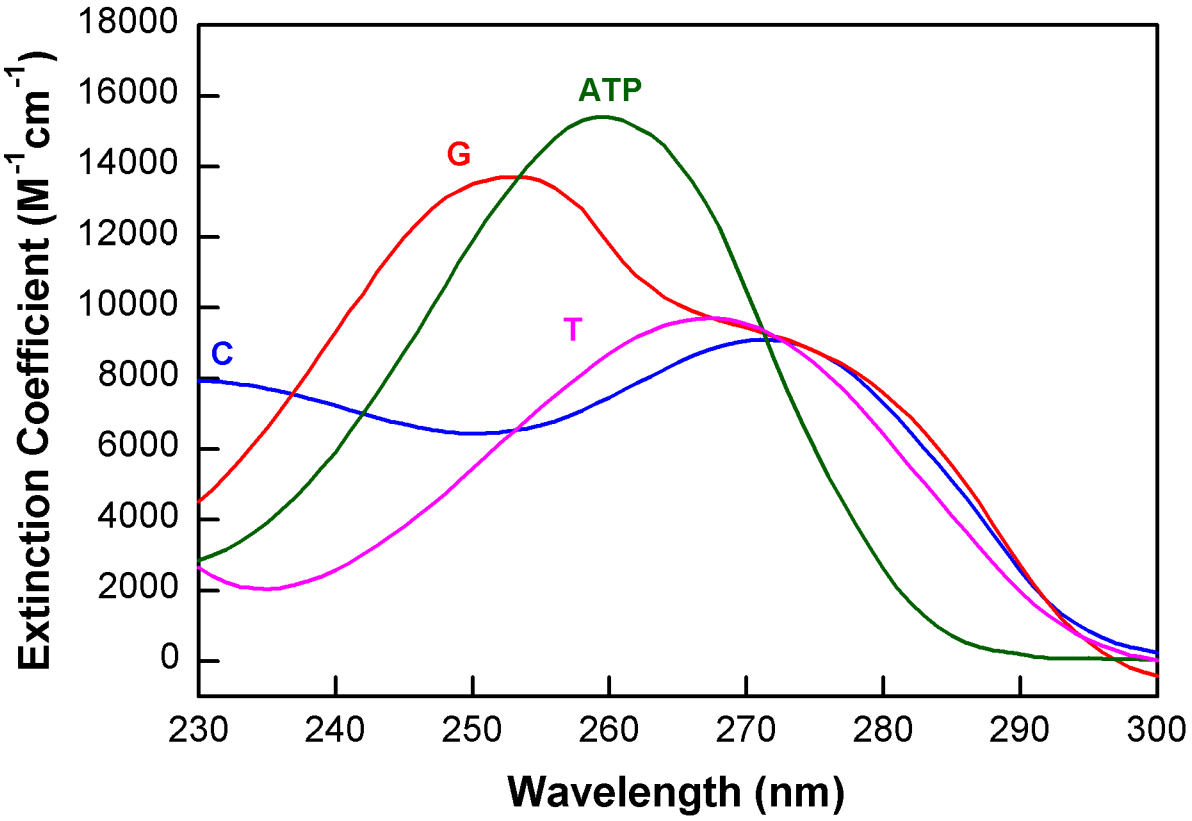


Figure 1.1. Structures of nucleic acid constituents

Absorption Spectra of the Nucleic Acids



Practical use of UV Spectroscopy
Example: Multi-wavelength AUC

Approach:

To exploit the dynamic range of the UV spectrophotometer, measure multiple concentrations of the analyte over a wide wavelength range and globally fit the resulting absorbance traces to an intrinsic absorption spectrum over all wavelengths. Each analyte must be dialyzed into the same buffer, and the spectrophotometer must be blanked with the buffer to get pure analyte spectra.

Model the UV spectrum with a linear sum of Gaussians:

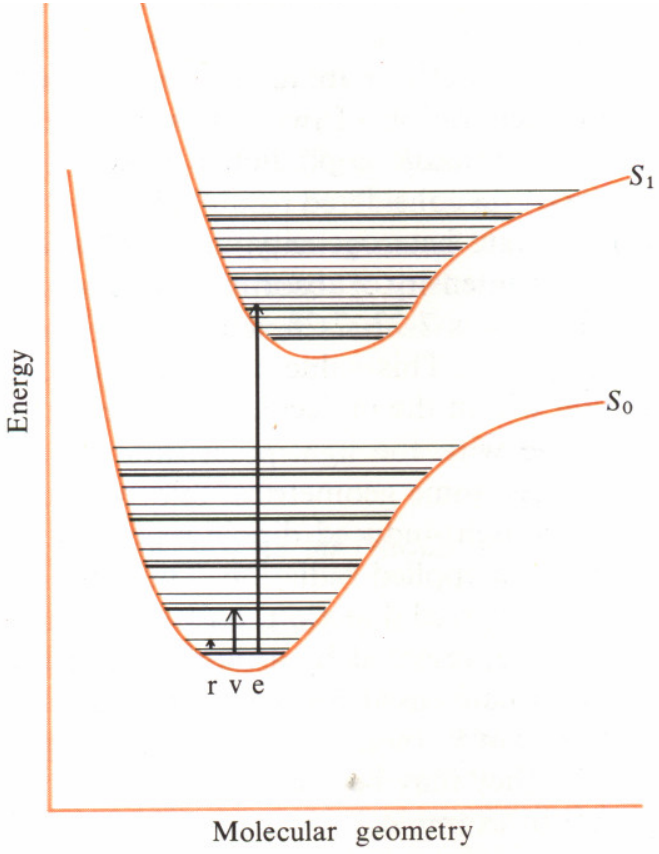
$$\text{Spectrum} = \sum_{i=1}^{n} c_i G_i(\lambda)$$

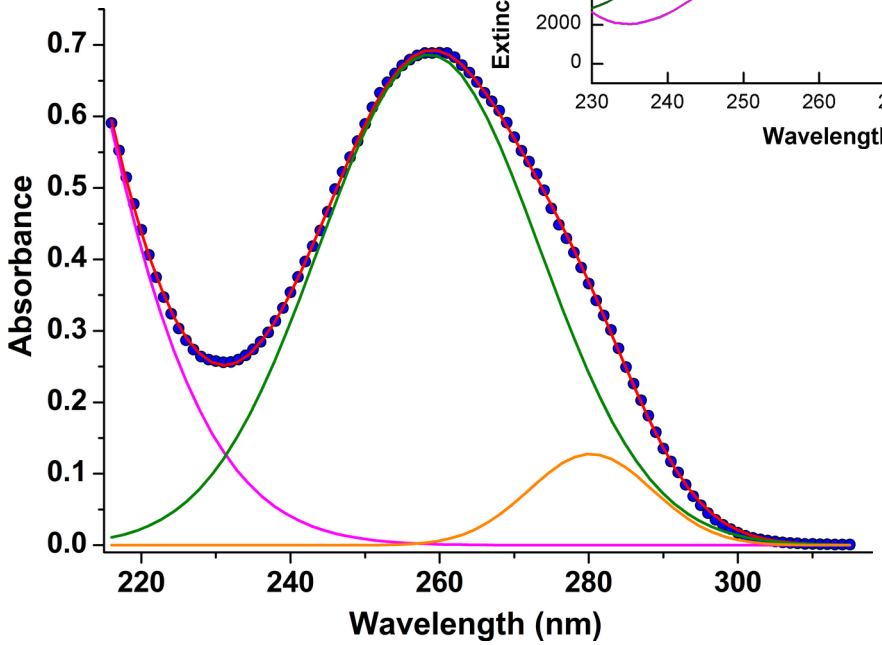
$$\text{where } G(\lambda) = \frac{1}{\sigma\sqrt{2\pi}} \exp\frac{-1}{2}\left(\frac{\lambda-\mu}{\sigma}\right)^2 \text{ and } \lambda = \text{wavelength},$$

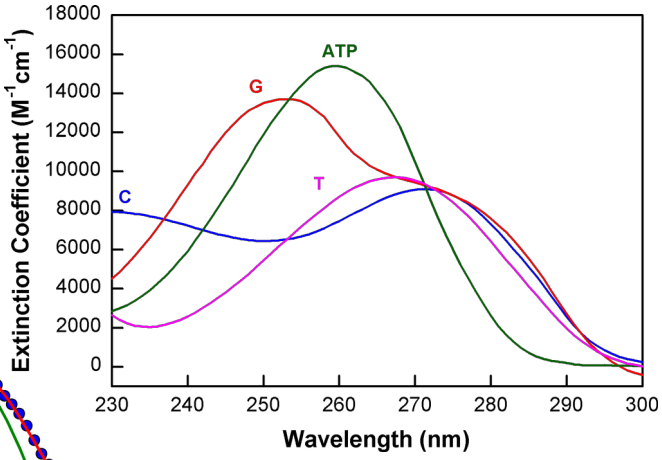
$$\sigma = \text{standard deviation (width), and } \mu \text{ is the mean}$$

Practical use of UV Spectroscopy

Example: DNA fitted by three Gaussian terms:







Practical use of UV Spectroscopy

Example: Multi-wavelength AUC

Decompose an unknown mixture of two or more analytes into a linear combination of intrinsic absorption profiles to determine the amplitude (partial concentration) of each analyte in the mixture:

$$\text{Spectrum (Mixture)} = \sum_{j=1}^{m} a_j \, Spectrum_j$$

Minimize with NNLS (Lawson CL, Hanson RJ. Solving Least Squares Problems. Prentice-Hall, Inc. Englewood Cliffs, New Jersey, 1974).

DEMO

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 measurements, 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

Keep in mind the logarithmic nature of absorbance:

1.0 OD means that only 10% of the light is seen by the detector, 90% is absorbed. At 2.0 OD only 1% of the light reaches the detector, 99% is absorbed.

Some problems:
What is the absorbance when 50% of the light is absorbed by the sample?

$$A = -\log_{10}\left(\frac{I}{I_0}\right) = -\log_{10}(0.5) = 0.301\,OD$$

What is the molar concentration of protein that absorbs 0.75 OD at a wavelength where its extinction coefficient is 24,300 OD/(mol*cm)? Assume a 1 cm pathlength cell.

$$A = C\epsilon l \qquad C = \frac{A}{\epsilon l} = \frac{0.75}{24{,}300} = 30.86\,\mu M$$

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 mn. Buffers, salts etc. need to be chosen with care to avoid background absorbance.

Absorption Spectroscopy

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).**

Absorption Spectroscopy

Table 7-2

Spectroscopic properties of proteins containing prosthetic groups

Protein	Prosthetic group	Longest-wavelength absorption band		Second-longest absorption band	
		λ_{max} (nm)	ε_{max} ($\times 10^{-4}$)	λ_{max} (nm)	ε_{max} ($\times 10^{-4}$)
Amino acid oxidase, rat kidney	FMN	455	1.27	358	1.07
Azurin, *P. fluorescens*	Cu^{II}	781	0.32	625	0.35
Ceruloplasmin, human	8 Coppers (3 distinct classes)	794	2.2	610	1.13
Cytochrome *c*, reduced, human	Fe^{II}-heme	550	2.77	—	——
Ferredoxin, *Scenedesmus*	(2 Fe^{III}, 2 sulfide) cluster	421	0.98	330	1.33
Flavodoxin, *C. pasteurianium*	FMN	443	0.91	372	0.79
Monoamine oxidase, bovine kidney	Flavins plus Cu	455	4.7	—	——
Pyruvic dehydrogenase, *E. coli*	FAD	460	1.27	438	1.46
Rhodopsin, bovine	Retinal-Lys	498	4.2	350	1.1
Reubredoxin, *M. aerogenes*	(Fe^{III}, 4 Cys) tetrahedron	570	0.35	490	0.76
Threonine deaminase, *E. coli*	4 Pyridoxal phosphates	415	2.6	—	——
Xanthine oxidase	Fe, Mo	550	2.2	—	——

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:

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

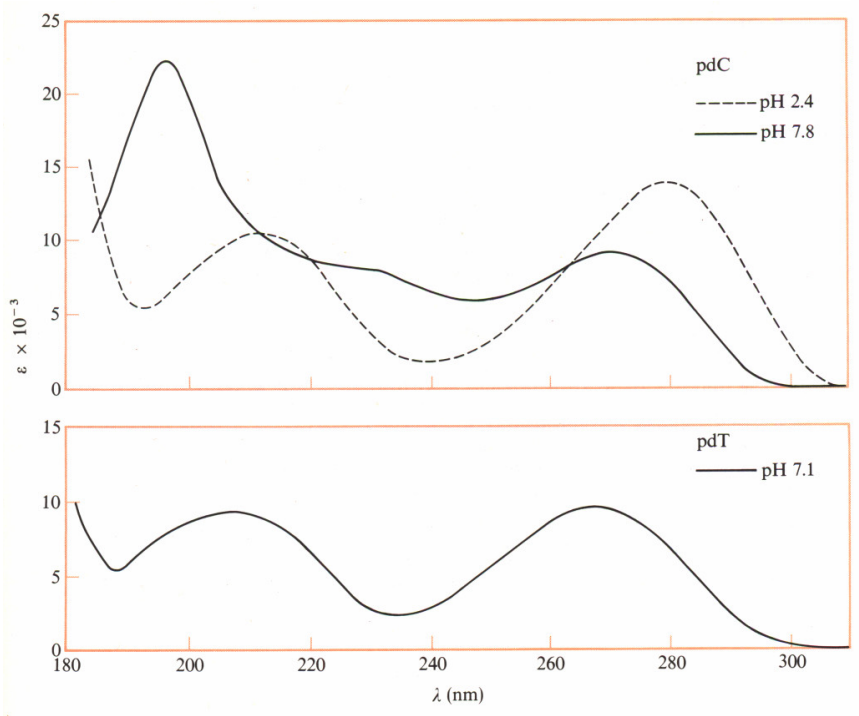
To convert into mg/ml, divide by the molar mass, *M*:

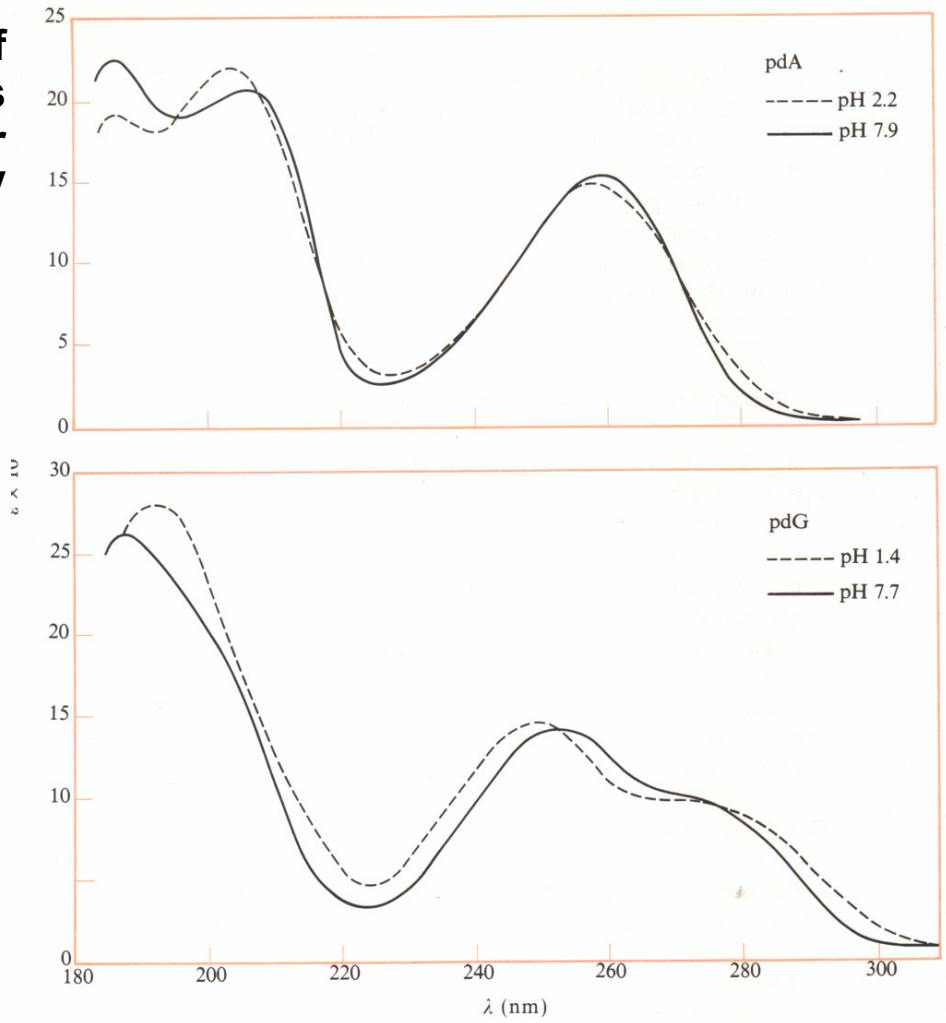
$$C_{protein,\,280\,nm} = \frac{5{,}700\,nTrp + 1{,}300\,nTyr}{M}$$

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.

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 Spectroscopy

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

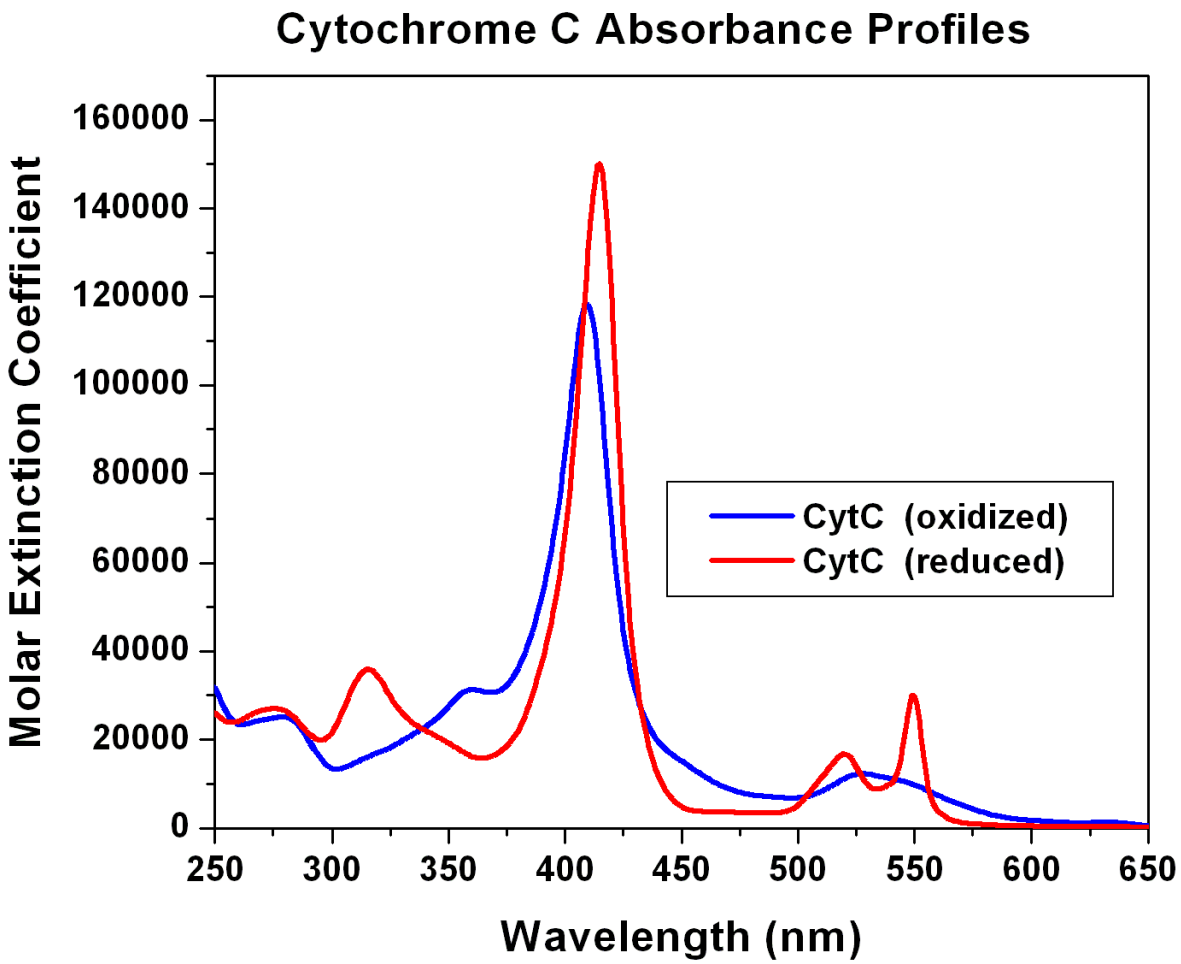
Within experimental error, ATP, ADP and AMP all have the same extinction properties. Absorbance of an individual nucleoside at 260 nm is about 10,000 OD 260 nm/(mol*cm). This strong extinction allows measurement down to concentrations of ~ 3 ug/ml.

<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 Spectroscopy

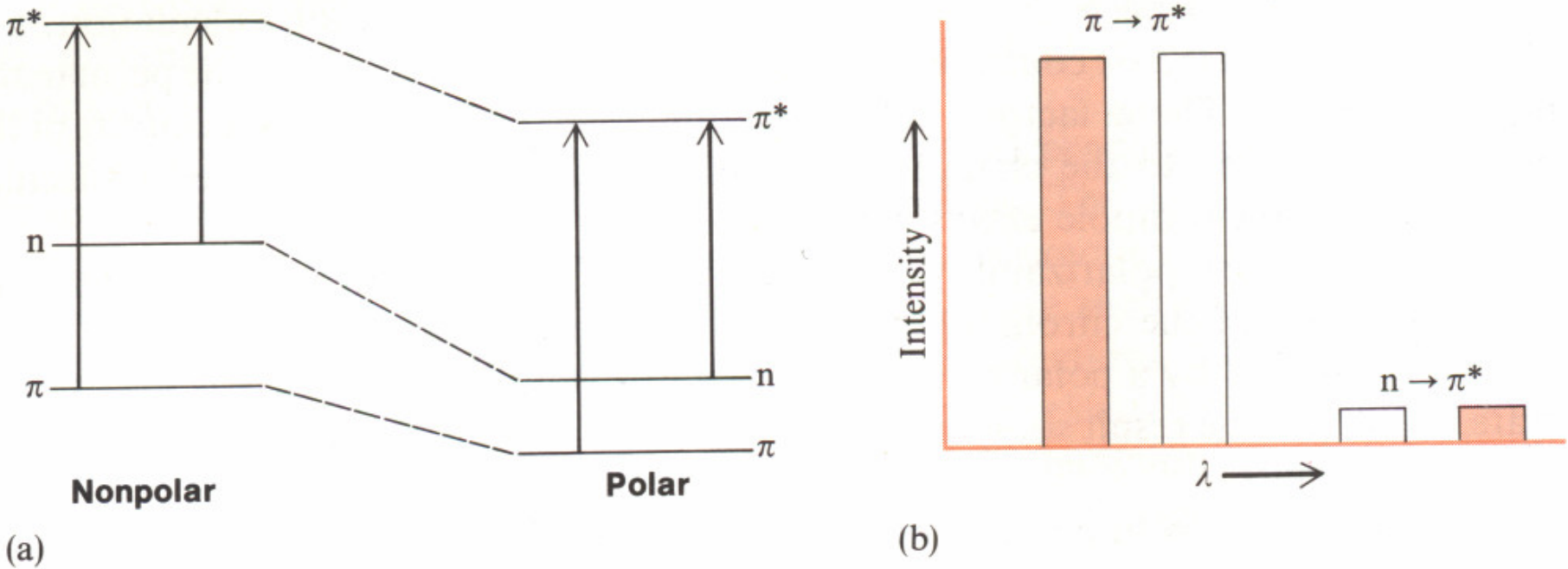


Figure 7-15

Typical effects of solvent polarity on transition energies for $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions. **(a)** Energy levels. **(b)** Corresponding schematic spectra for polar (*unshaded bars*) and nonpolar (*shaded bars*) solvents.

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

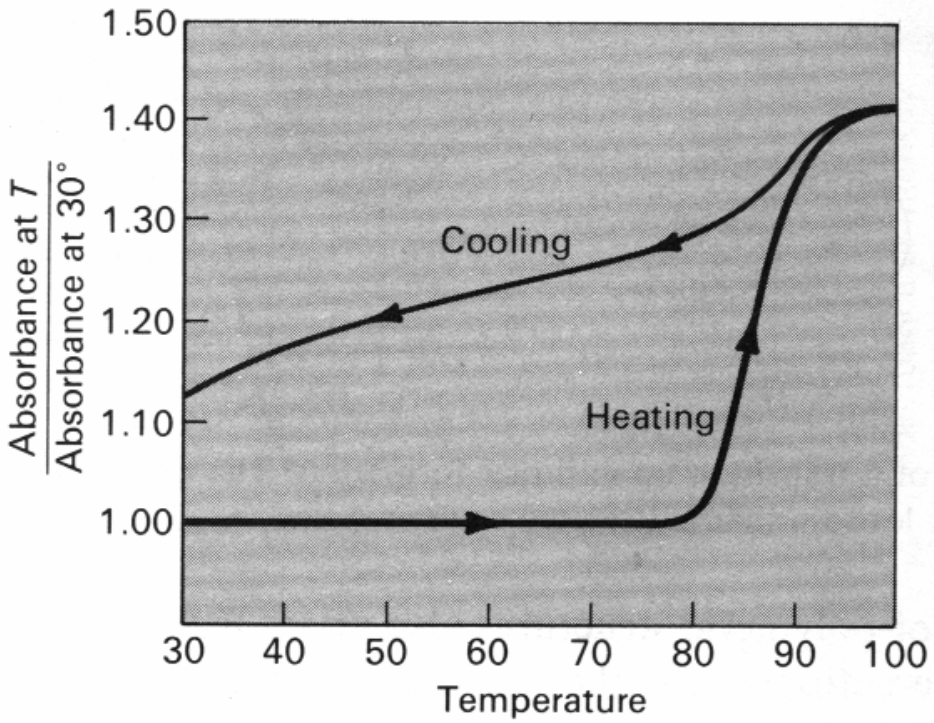
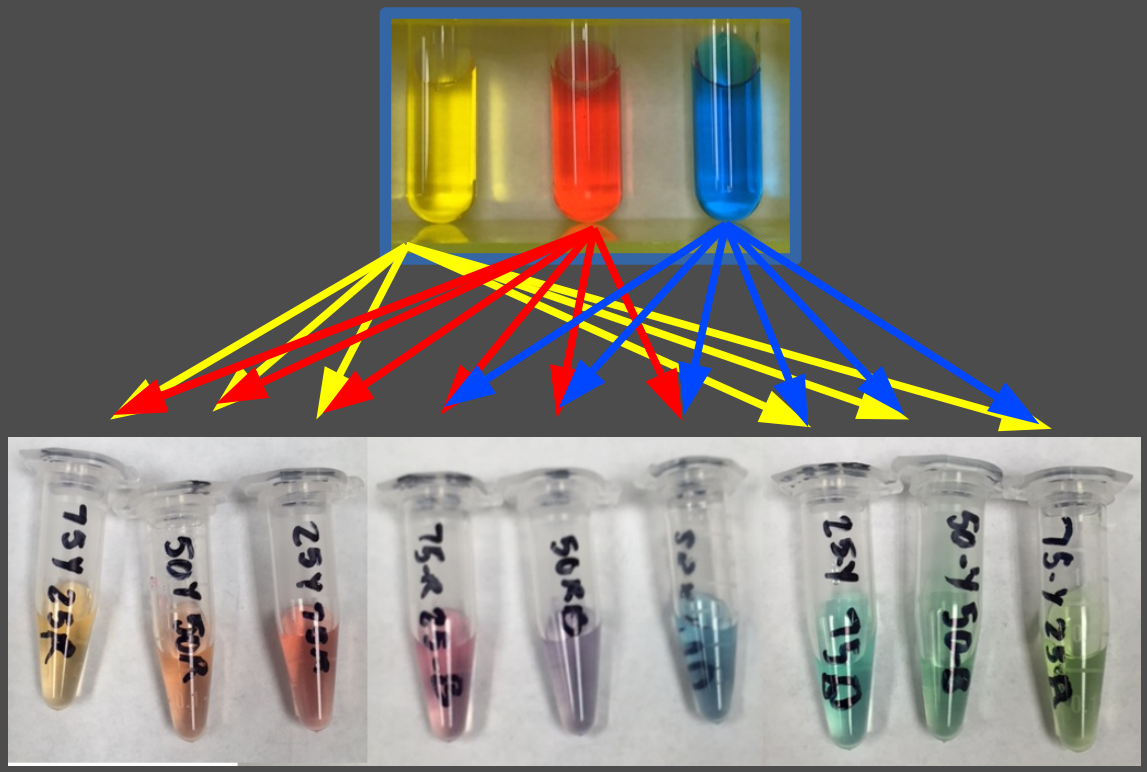


Figure 8.11 The "melting" of DNA as followed by absorption at 260 nm. Note the sharpness of the transition in cycle 1. This is typical of a cooperative process (see Chapter 3). Also note that recovery of the ordered structure is not complete on rapid cooling.

Absorption Spectroscopy

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.

Minji Kim



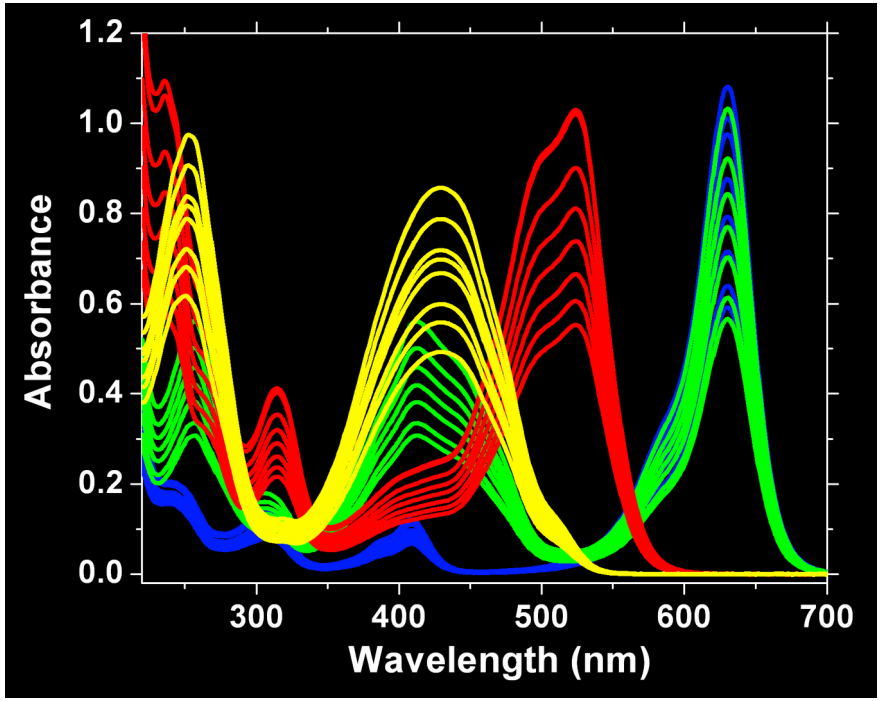
Absorption Spectroscopy

Deconvolution of multiple basis spectra to quantify individual components:

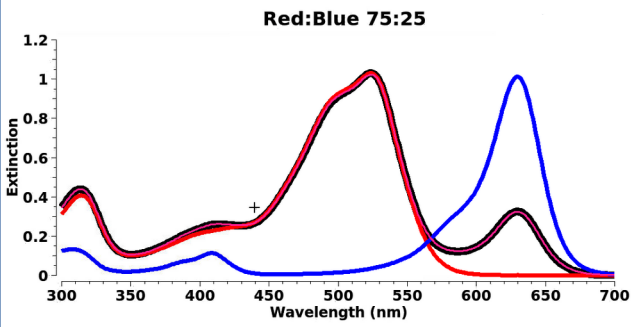
When known absorbance spectra are sufficiently different and hypochromism is negligible, the individual components can be mathematically separated to determine relative amounts of each

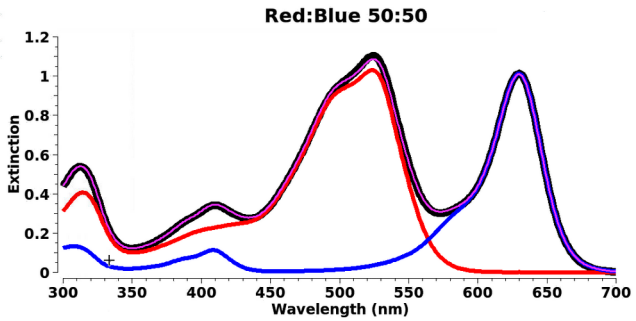
$$F_{base} = \sum G_i(\sigma_i, \lambda_i, c_i) = \sum c_i e^{\frac{-(x-\lambda_i)^2}{2\sigma_i^2}}$$

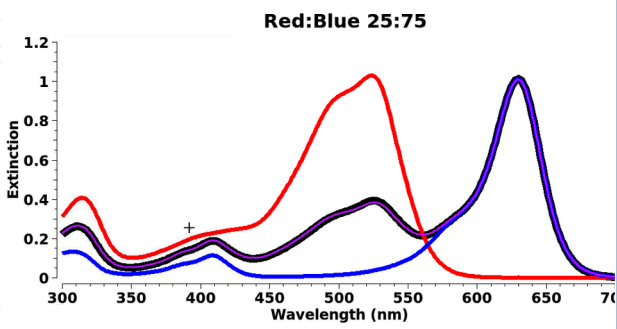
$$F_{mix} = \sum_i a_i F_{base,i}$$

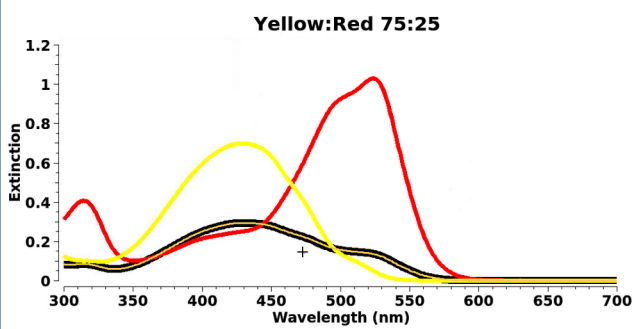


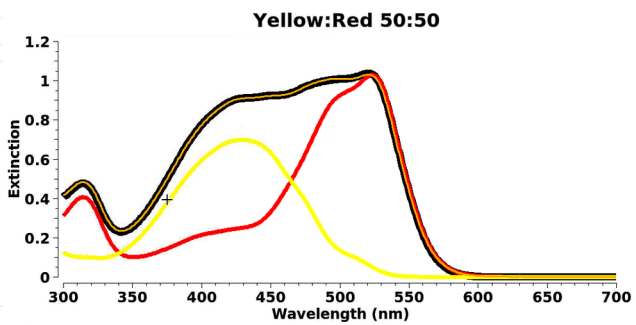
Absorption Spectroscopy

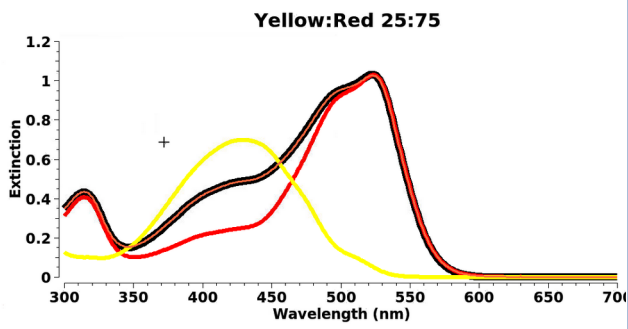


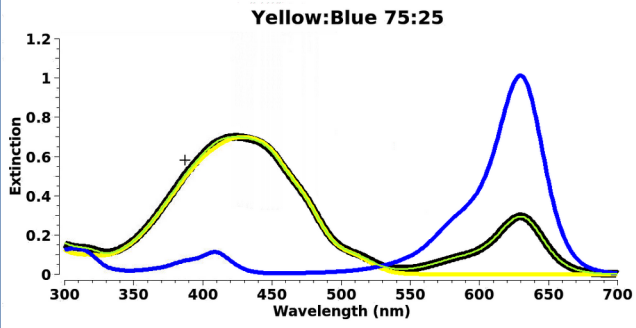


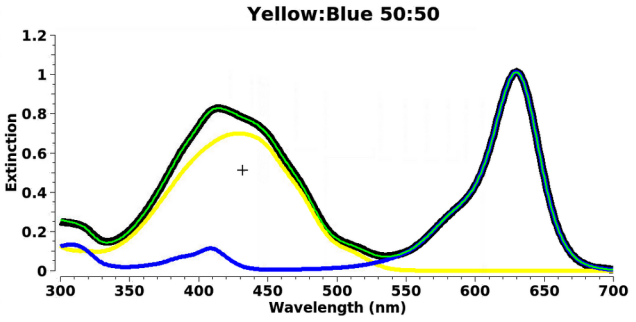


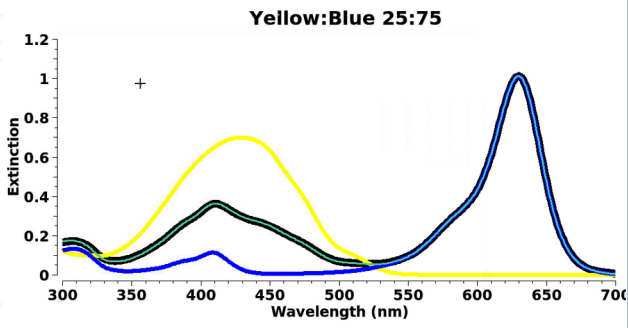












Group	Color	Expected	Calculated	Control	% Error	RMSD (%)
25Y75R	Red	75	73.82	0.00	1.6	0.24
	Yellow	25	26.18		4.7	
50Y50R	Red	50	49.69	0.00	0.6	0.32
	Yellow	50	50.31		0.6	
75Y25R	Red	25	22.99	0.00	8	0.21
	Yellow	75	77.01		2.7	
25R75B	Red	25	25.28	0.00	1.1	0.35
	Blue	75	74.72		0.4	
50R50B	Red	50	50.6	0.00	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			

Homework Assignment

Install UltraScan on your personal computer:

Step 1: Download the software from:
https://www.ultrascan3.aucsolutions.com/download.php

Step 2: Register the software

Step 3: Download the CSV files and load them into the fitter

Step 4: Prepare an intrinsic spectral profile for protein and DNA from a global fit of the available dilutions, normalized to and OD of 1 at 280 nm and 260 nm, respectively

Step 5: fit the mixtures and report the fractions of protein and DNA