

Colorimetric Methods for Determining Protein Concentration.

Goals:

1. Learn how to use colorimetric (Lowry, BCA, and Bradford) methods to determine protein concentration in mg/mL.
2. Use intrinsic biomolecular spectral properties to determine protein concentration:
 - A_{280} method: Trp, Tyr, Cystines.
 - A_{260} absorption by DNA.
 - Visible spectra of heme proteins.

Different Ways to Express Protein Concentration

1. Molarity: mol/L.
2. Amount/vol: mg/mL (mg/dL).
3. % solution: 1% sol'n = 1 g/100 mL.
4. Enzyme activity: $\mu\text{mol Product}$
(min mL E).

Why are these methods important?

- **Determine concentration for a reaction.**
 - **[Protein]** often expressed in mg/mL or molarity.
 - **[Polynucleotide]** (DNA or RNA) often expressed in ug/mL or molarity.
 - **Amount of enzyme** often expressed in units of **Enzyme Activity** = the amount of enzyme needed to convert 1 umol Substrate to Product in 1 min = 1 EU (Enzyme Unit).
- **Many physical methods require knowing precise [protein] before doing experiment.**
 - **SDS-PAGE**
 - **Mass spec.**
 - **Edman's degradation.**
 - **NMR.**
 - **Steady-state kinetics**
 - **Ligand binding studies**

Colorimetric Methods in General:

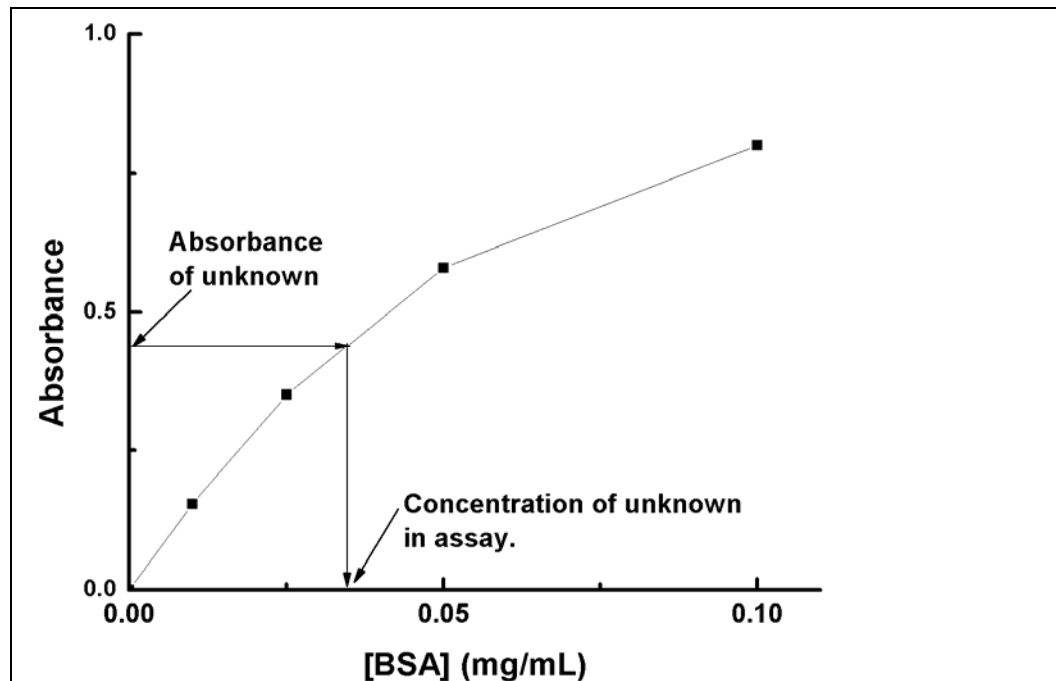
- **Use a colorimetric reagent that changes color upon reaction with a protein.**
- **Two general classes: Cu redox chemistry based or Coomassie blue binding.**
- **Protein denaturation in these methods is absolutely required in order to get maximal color change.**
 - **Denaturation in Biuret, Lowry, and BCA due to high [NaOH].**
 - **Denaturation in Bradford due to high [H₃PO₄].**
- **Therefore all these methods are pretty caustic and suitable precautions should be taken.**

Spectroscopic Methods:

- **Utilize ability of protein (or a prosthetic group) to absorb light in a specific region of UV-visible spectrum.**
- **Proteins are not denatured by these methods.**

Common Procedures for all Colorimetric Methods:

1. Generate range of concentrations for protein standard (usually BSA).
2. Add fixed volume of standard to fixed volume of dye solution.
3. Incubate for specific time and specific temperature.
4. Measure Absorbance at single wavelength specific to method (i.e. specific to dye).
5. Plot Absorbance vs. [BSA standard]_{assay} in mg/mL (= standard curve).
6. Repeat process for the unknown protein.



Criteria for choosing a specific assay:

- Highly reproducible.
- Simple and quick to perform.
- Inexpensive.
- Does not use a lot of protein – related to sensitivity.
- Is not sensitive to reagents (i.e. contaminants) that might be present in solution.

REMEMBER: colorimetric assays measure TOTAL PROTEIN in mg/ml, so they are useful for heterogenous solutions = Samples do not need to be pure. The A_{280} method (Thursday) requires pure, homogeneous protein preparations.

Complications due to Real Life Contaminants (Reagents):

- Reducing agents: **BME** and DTT.
- Detergents: **SDS**, LDAO, octyl-glucoside, lauryl maltoside.
- Amines and NH_4^+ : Tris and **AmSO_4** (= $(\text{NH}_4^+)_2\text{SO}_4$).
- Others: **EDTA**, **glycerol**, steroids, DNA...

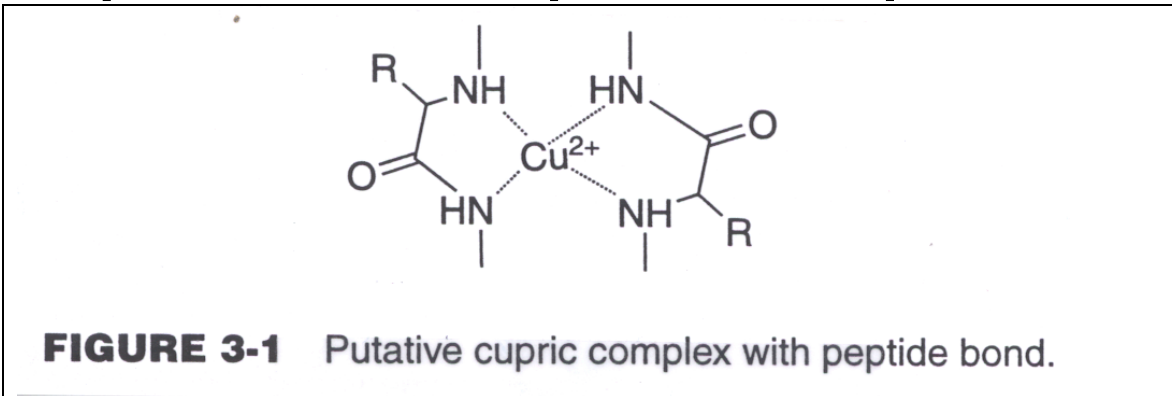
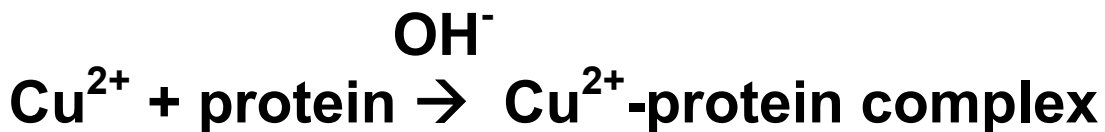
Specific Protein Assays:

Biuret Method (550 nm):

- The “Grandfather” of them all.
- Least sensitive (100 – 1000 fold less than Lowry, BCA, or Bradford).
- Is the basis for the Lowry and BCA methods.

Two reactions are involved:

(1) Chelation reaction:



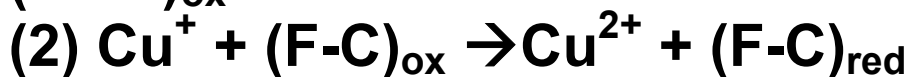
(2) Redox reaction:



The production of Cu^+ in this oxidation reaction is responsible for the increased sensitivity of the Lowry and BCA methods.

Lowry Method: (750 nm):

- Much more sensitive than Biuret assay.
- Couples redox reaction of Biuret (i.e. generation of Cu^+) with a second redox reaction responsible for enhanced color change:



F-C = Folin's Reagent (phospho-Mo-Tungstate acid).

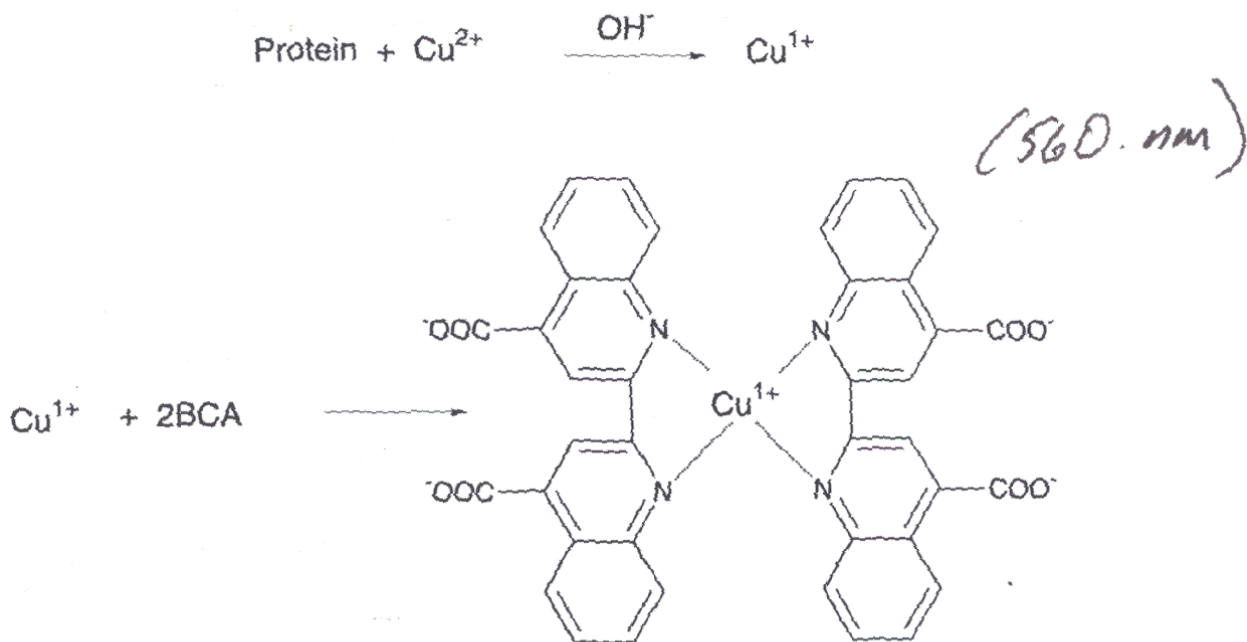
- F-C_{ox} : little absorbance at 750.
- F-C_{red} : polymerizes to dark blue hetero-poly Mo complex.
- λ_{max} for $(\text{F-C})_{\text{red}} = 750 \text{ nm}$.
- Lowry method is VERY good for proteins containing chromophores such as hemes and flavins.

Disadvantages:

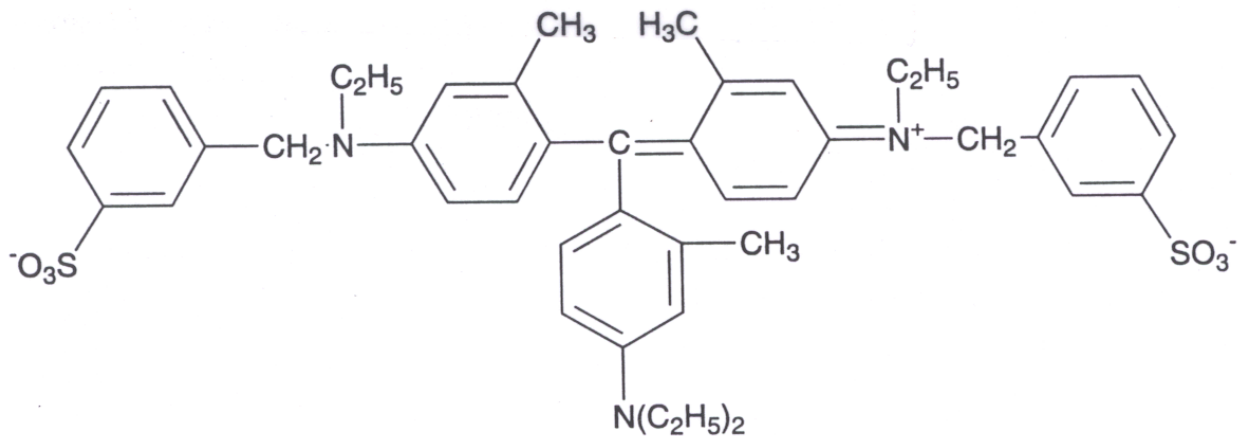
- Sensitive to variety of contaminants.
- Non-linear concentration dependence.
- Correct mixing (there are two reagents) and timing are critical.
- Must generate std. curve each time.

BCA Method (560 nm):

- Same redox rxn of Biuret generates Cu^+ .
- Cu^+ forms coordination complex with BCA reagent (BCA = BiCinchoninic Acid or 4,4'-dicarboxy-2,2'-biquinoline).

**FIGURE 3-2** BCA reaction.

- Assay is very sensitive.
- Detergents do not interfere.
- Interfering agents: reducing agents, EDTA, carbohydrates, catecholamines, oxidizable AA's (Trp, Tyr, Cys: interfere with oxid'n of protein), lipids.

Bradford Method (595 nm):**Coomassie Blue + protein → blue complex****FIGURE 3-3** Coomassie® Brilliant Blue G-250.

Bradford method is perhaps the most commonly used protein colorimetric assay.

Advantages:

- **Simple one-step.**
- **5 minute incubation time at room temp.**
- **Sensitive and accurate.**
- **Is not AA dependent (as are Lowry and BCA).**

Disadvantages:

- **Non-linear concentration dependence.**
- **Stains glassware and glass cuvettes (use disposable plastics).**
- **Detergents can interfere.**

Remember: All colorimetric methods are absolutely dependent upon protein denaturation!

Why do you think this is necessary?

Overall Experimental Procedure:

Prepare BSA stds at different concentrations using 1.5 mg/mL BSA stock.

Prepare a blank for assay.

- **Blank = H₂O (or buffer) + reagent.**
- **Treat EXACTLY as protein assay (Time and Temp).**

On Cary 50: Use SIMPLE READS.

- **Set λ to appropriate value.**
- **Zero (= “blank”) using Blank Solution. This takes into account absorbance of reagent and cuvette.**
- **Read Absorbance for standards, then for unknown (undiluted and diluted).**
- **Contaminant effects (β ME, AmSO₄, EDTA, glycerol, and SDS) on assay: Thoroughly rinse cuvette between readings!!!!**

The A_{280} Method and the Effect of DNA.

For proteins that DO NOT contain an absorbing prosthetic group (heme, flavin, etc.) the A_{280} method provides a quantitative method to determine [protein] using the absorption properties of specific amino acids.

Advantages:

- Does not destroy sample (as do colorimetric assays).
- Simple and relatively fast.

Disadvantages:

- Sample must be pure in order to quantitate for a specific protein.
- Method requires accurate amino acid composition (how many) not sequence.
- Method depends upon solvent (H_2O vs. GdnHCl).
- Requires more protein than colorimetric (but can be recovered).
- Common UV absorbing contaminants can interfere with method:
 - DNA ($\lambda_{max} = 260$ nm). (cf. DNA section below).
 - Flavins and NAD(H).

The A_{280} method is based, primarily, upon the UV absorbing properties of aromatic amino acids.

- Many textbooks state that UV absorption is due to: Trp, Tyr, and Phe.
- The same texts will then show the following figure without actually examining the data:

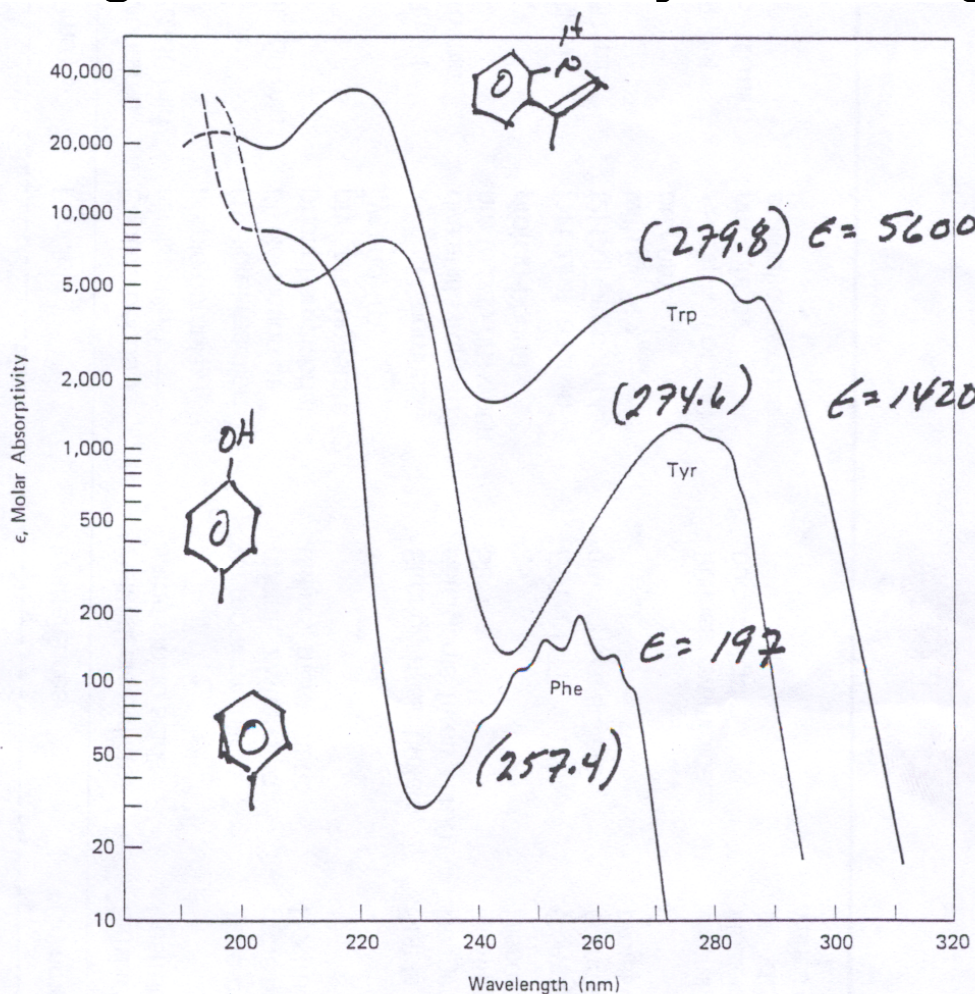


Figure 1-3

Absorption spectra of the aromatic amino acids at pH 6. Incorporation of these amino acids into peptides has little direct effect on the absorbance of their side chains. (From D. B. Wetlaufer, *Adv. Prot. Chem.* 17:303-390, 1962; © Academic Press.)

The most thorough and widely accepted treatment of use of the A_{280} methods and its use as a diagnostic tool: Pace et. al. (Protein Science (1995) 4: 2411-2423).

Purpose: To enable researchers to predict the Molar extinction coefficient for a protein, in H_2O and GdnHCl, based on the number of and absorption properties of Trp, Tyr, and Cystine (RSSR) residues.

AA	ϵ_{280} ($M^{-1} cm^{-1}$)	%Total ϵ_{280}
Trp	5500	77.3
Tyr	1490	21
Cystine	125	1.8

Overall:

$$\epsilon_{280} (M^{-1} cm^{-1}) = (\#Trp)(5500) + (\#Tyr)(1490) + (\#Cystine)(125)$$

Note: method requires knowledge of ACCURATE amino acid composition (ExPASy is a good source).

Second question addressed in Pace et. al.: What is the effect of solvent on absorption properties of Trp and Tyr?

Why important?

AA	% Buried	%Exposed
Trp	87	13
Tyr	76	24
Cystine	92	8

Statistically, significant populations of the side chains are exposed to both hydrophobic non-polar) and aqueous environments. The ability to develop a generalized equation is only valid if the ϵ_{280} does not vary significantly with solvent.

Discussion of Figure 1 to be done in class.

Table 5: Observed and Predicted Absorption Coefficients.

Table 5 presents both Molar (ϵ , $M^{-1}cm^{-1}$) and 1% coefficients ($A(280,1\%)$).

ϵ ($M^{-1}cm^{-1}$): the absorbance a 1 M solution would have.

$A(280,1\%)$: the absorbance that a 1% solution would have.

- useful for comparing results from Colorimetric assays (mg/mL).

1% solution = 1 g/100 mL = 10 g/L = 10 mg/mL.

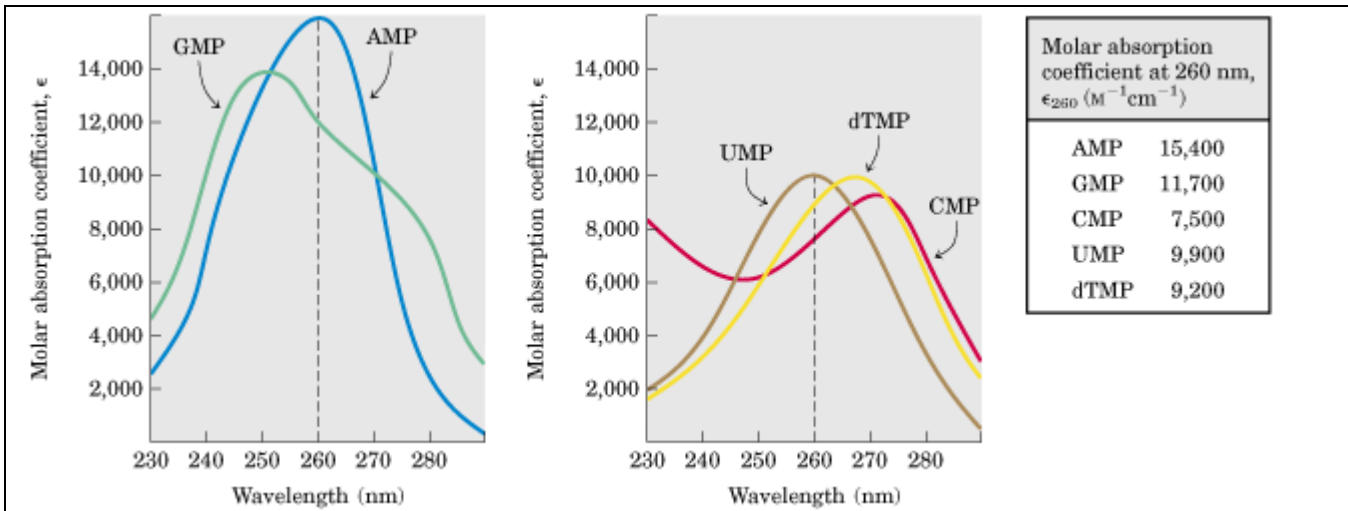
- to compare with colorimetric data, necessary to have an $A(280,0.1\%)$.

For BSA, $A(280,1\%) = 6.6$ so $A(280,0.1\%) = 0.66$.

- units of $A(280,0.1\%)$: $(mL/mg)(cm^{-1})$.
- $A(280,0.1\%)$: the absorbance a 0.1% OR 1 mg/mL protein solution would have.

• Effect of DNA on the A₂₈₀ Method.

Nucleic acid bases strongly absorb UV light at 260 nm.



From Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual:

For double stranded DNA:

50 ug/mL ds DNA gives OD₂₆₀ = 1.0

Comparing ds DNA to BSA (protein):

A(280,0.1%) = 0.66

So what [BSA] will give an OD₂₈₀ = 1?

OD₂₈₀ = 1 = (0.66 mL/mg)(x mg/mL)

So x = 1.5 mg/mL

To compare: $\frac{1.5 \text{ mg/mL BSA}}{0.05 \text{ mg/mL ds DNA}} = 30.3$

On a per wt. Basis, DNA absorbs ~ 30.3 times more strongly than a typical protein.

In purifying proteins from bacteria (either native or recombinant), DNA is a MAJOR contaminant. If attempting to determine the amount of protein from A_{280} of cell lysates, the A_{260} due to DNA bases will sometimes “overwhelm” the protein absorbance.

Plotting the data from Table A8-5 should enable you answer the question:

Is it easier to detect a little DNA in a protein solution or a little protein in a solution containing DNA?

A semiquantitative way to compensate for DNA contamination is Groves equation:

$$[\text{protein}] \text{ (mg/mL)} = 1.55A_{280} - 0.76A_{260}.$$

Non-Protein Chromophores

There are many proteins that contain visible light absorbing chromophores: hemes, flavins, FeS clusters, Cu^{2+} .

The spectral properties of these chromophores (relatively high extinction coefficients) make them ideal for:

- Protein concentration determination.
- Determining chemical state of the chromophore (redox, ligand binding, etc.)
- Conformational state changes upon complex formation with other proteins.
- Following purification of the protein.

Often the purity of these proteins can be determined from:

$$A_{\text{chromophore}}/A_{280} = \text{Purity Index} = R_{\text{factor}}$$

As the protein is purified, A_{280} decreases, so if $A_{\text{chromophore}}$ is constant, PI increases to some maximal value, diagnostic for that protein.