Laboratory 4
Determination of Protein Concentrations by Spectrophotometry

I. Introduction

All cells contain hundreds of different biomolecules, including proteins, carbohydrates, lipids, and nucleic acids. These terms refer to classes of compounds and there are actually many types of proteins, carbohydrates, etc. The total amounts of these different molecules vary from cell to cell or from tissue to tissue. An initial step that is often done to characterize a particular cell type is to determine the total amounts of the different types of biomolecules per cell. This is usually accomplished by extracting the molecules from a collection or set of cells and then by doing a spectrophotometric assay to measure the total amount of a certain type of molecule quantitatively. This involves the same basic spectrophotometric methods you learned in last week's lab.

At part of this lab, you will:

- make an extract of a plant or animal food source suitable for protein analysis
- construct a standard curve for the quantitative measurement of proteins
- use this curve to calculate the protein concentration of your extract
- determine if the food label on the plant or animal food source is accurate
- learn how to write methods in the style of a scientific journal article

The methods learned during this session will be used several times during this semester. This experiment is adapted from one originally described in Farrell, S. O. and Taylor, L. E. (2005) **Experiments in Biochemistry: a hands on approach**. Brooks/Cole, Florence, KY.

For this experiment, each group will need to bring to the lab a suitable sample for protein analysis. The most convenient samples are animal or plant food products that have significant protein content and are available in liquid or powder form. For example, you can use a liquid protein product such as Ensure or Soy Milk that has relatively low fat content. Alternatively, you can use a powder protein product such as Slimfast Shake Mix or Carnation Instant Breakfast Mix. Be sure that there is nutritional label on the package that indicates the serving size and the number of grams of protein per serving.

II. Pre-Lab Preparation

Read the Introduction, Background Information, and Experimental Procedures before the lab session. Because this lab will utilize a standard curve that was introduced in Laboratory 1, you should read the background information for that lab as well. After preparing for the lab, you should be able to answer the following questions.

A. What is a protein?

B. How do proteins differ from lipids, carbohydrates, or nucleic acids?

C. What is the Beer-Lambert Law?

D. What does the Beer-Lambert Law allow you to do?

E. Under what conditions is the Beer-Lambert Law valid?

F. Under what conditions is the Beer-Lambert Law not valid?

G. What is meant by a standard curve?

H. How is a standard curve constructed?

I. How is a standard curve used to determine the amount of a molecule of interest in an unknown solution?

J. What are the four major methods of determining protein concentrations?

K. What is the basis of the Bradford assay that we are using this week?

L. What wavelength of light is used in the Bradford method to measure the absorbance of solutions containing protein?

III. Background Information

A. Proteins

Proteins are the molecular machines that allow complex cellular processes such as respiration, DNA synthesis, and motility to occur. While all proteins are assembled from the same set of 20 amino acids, it is the length and sequence of an individual protein that determines its structure and activity. Actively-growing cells may contain as many as 2000 different proteins, which vary greatly in their individual concentrations. In most animal and bacterial cells, proteins make up about 50% of the total cellular mass. In most plant cells, proteins comprise a smaller proportion of the cellular mass because of the presence of cellulose and other polysaccharides in the cell wall.

1. Amino Acids

All amino acids are based on a common structure, in which an amino group ($-NH_2$), a carboxylic acid group (-COOH), a hydrogen (-H), and a sidechain or R group are attached to a central carbon atom (Figure 4.1)

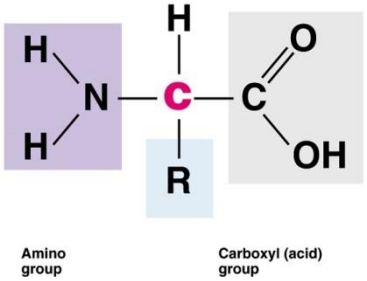


Figure 4.1. General structure of an amino acid. This structure is common to all but one of the α-amino acids. (Proline, a cyclic amino acid, is the exception.) The R group or side chain (blue) attached to the α-carbon (red) is different in each amino acid.

At physiological pHs, the amino group is normally protonated and so appears as $-NH_3^+$; the carboxylic acid group is normally deprotonated and so appears as $-COO^-$.

It is the side chain or R group that differentiates one amino acid from another. Figure 4.2 shows the structures of the 20 common amino acids. The amino acids are divided into five groups: 1) those with nonpolar, aliphatic R groups; 2) those with nonpolar, aromatic R groups; 3) those with polar but uncharged R groups; 4) those with basic or positively-charged R groups; and 5) those with acidic or negatively-charged R groups.

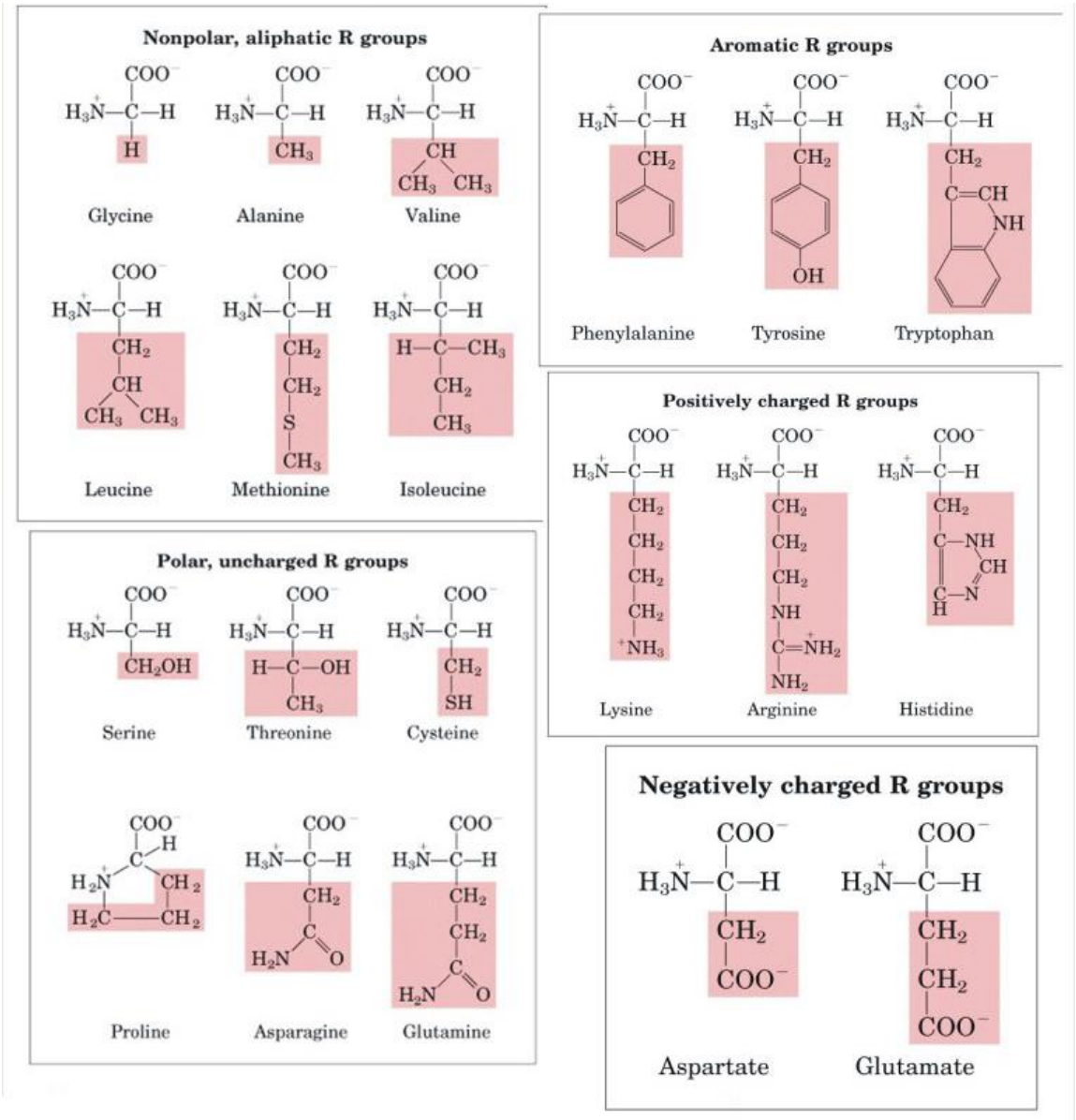


Figure 4.2. The 20 Standard Amino Acids of Proteins

2. Peptides and Proteins

Amino acids are linked together by peptide bonds to form short peptides and longer proteins through a process of condensation or dehydration synthesis (Figure 4.3). A peptide bond is created by removing the components of a molecule of water from the carboxylic acid group of one amino acid and from the amino group of the next amino acid in the chain. Because all amino acids have an amino group and a carboxylic acid group, they can be joined together in any order. The R groups or side chains simply extend out away from the backbone of the chain. The polypeptide chain extends from the first amino acid, which has a free amino group, to the last amino acid, which has a free carboxylic acid group

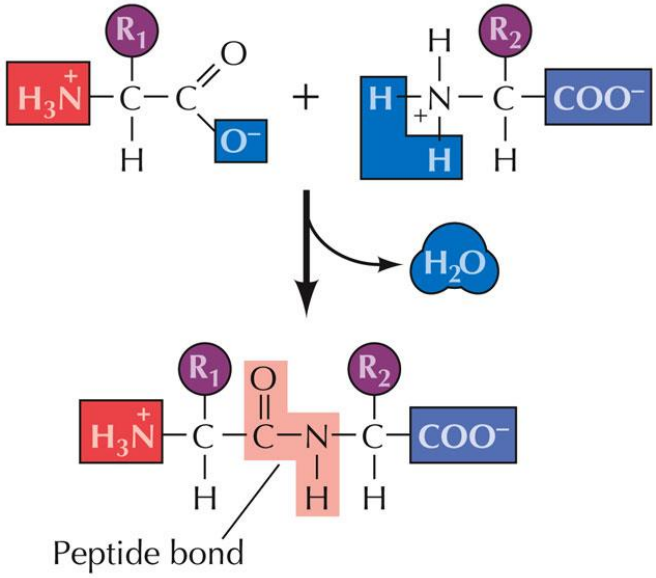


Figure 4.3. Formation and Structure of a Peptide Bond

While a protein can be described in terms of its sequence of amino acids, proteins in a living cell do not exist as simple linear chains. Rather, each chain is folded through weak chemical bonds between the peptide bonds and R groups into a complex three-dimensional conformation.

B. Absorption of Light and the Beer-Lambert Law

As noted in **Laboratory 3 (Spectrophotometric Analysis of Membrane Stability in Beet Root Cells)**, spectrophotometeric assays are based on the direct absorption of light by biomolecules or on the fluorescence of these molecules following exposure to certain wavelengths of light. Absorption or fluorescence can be measured quantitatively in a spectrophotometer or a spectrofluorometer. The Beer-Lambert Law describes the relationship between absorbance, concentration, and the molar extinction coefficient of a particular molecule. Recall that the Beer-Lambert Law indicates that:

$$\mathbf{A} = \log_{10} \frac{\mathbf{I_o}}{\mathbf{I}} = \mathbf{E\,c\,l}$$

where **A** is the absorbance of the solution, $\mathbf{I_o}$ is the intensity of the incident light, and **I** is the intensity of the transmitted light; **E** is the molar extinction coefficient, **c** is the concentration of the absorbing solute, and **l** is the pathlength. If you know the value of E for a particular molecule at a certain wavelength, Beer's law allows you to calculate the concentration of a substance in solution after measuring the absorbance with a spectrophotometer. Before you use a spectrophotometer, it must be properly

calibrated or zeroed. If it is not, the numbers you generate will be meaningless. Beer's law only works if you know that the relationship between absorbance and concentration is linear. This is not always the case. Beer's law also only works if you have a pure substance with a single value of E.

C. Standard Curves

When you do not know the molar extinction coefficient (E) for a particular molecule or are uncertain about the linearity of the relationship between concentration and absorbance, you can still use spectrophotometry to make quantitative measurements if you first construct a standard curve. As noted in **Laboratory 1 (Scientific Calculations),** a standard curve is a graph that shows the relationship between the amount of a particular compound in a solution and the absorbance of that solution. Please review Section III of the Background Information provided for **Laboratory 1: Scientific Calculations and Basic Lab Techniques** for details on making and using a standard curve.

Once a standard curve has been created, it can be used to determine the amount of the compound of interest in an unknown solution. The absorbance of this unknown solution is first measured using the same instrument at the same wavelength as the standards. You can use the equation for the linear region of the standard curve to create a conversion factor for relationship between absorbance and amount of unknown in a solution.

Within the linear region of a standard curve, the straight line has the formula:

$$\mathbf{y = mx + b}$$

where **m** is the slope of the line and **b** is the Y intercept. If **b** = 0, that is, the line goes through the origin at 0,0, you can use the slope of the line (**m**) as the conversion factor since it directly gives the relationship between **x** and **y**. You can then divide the absorbance of the unknown sample by the conversion factor to determine the corresponding amount.

For example, suppose you construct a standard curve for compound Z by setting up a series of tubes containing varying amounts of Z in µg and by measuring the absorbance of each tube. Suppose you then determine the slope of the line and get the equation y = 0.062 x. This means that y (the absorbance) increases by 0.062 for increment in x (the number of µg). In other words, there is 0.062 A/µg. If you then measure the absorbance of an unknown solution containing Z and find that the absorbance is 0.198, you can calculate that the number of µg of Z in this tube is:

$$\mathbf{0.198 \times \frac{1\ \mu g}{0.062} = 3.19\ \mu g}$$

D. Spectrophotometric Assays for Proteins

There are four commonly-used spectrophotometric assays for proteins. The first involves measurement of the absorbance of the extract at 280 nm. This absorbance value reflects the total amount of the aromatic amino acids phenylalanine, tryptophan, and tyrosine. The second involves measurement of the absorbance of the solution at 750 nm after reaction of the proteins with the Lowry or Folin-Ciocalteau reagent. This reaction involves the binding of Cu^{2+} ions to peptide bonds, the oxidation of certain amino acids such as cysteine and tyrosine and the simultaneous reduction of the copper to Cu^{+1}, and reaction of

the Cu^{+1} with phosphomolybdate. The third involves measurement of the absorbance of the solution at 562 nm after reaction of the proteins with the bicinchoninic acid (BCA) reagent. This reaction is similar to the Lowry procedure but uses a different reagent to visualize the resulting Cu^{+1}. The fourth involves measurement of absorbance at 595 nm after reaction of the proteins with the Bradford or Coomassie Blue reagent. This reaction involves binding of a dye to the protein chains. There are advantages and disadvantages to each of these methods, and one method is often selected to meet a specific experimental need.

In this experiment, **we will use the Bradford Method** because it is particularly easy to do. This method was first described by Bradford in 1976. It is based on the binding of the dye Coomassie Blue G-250 in a phosphoric acid solution to proteins (Figure 4.5).

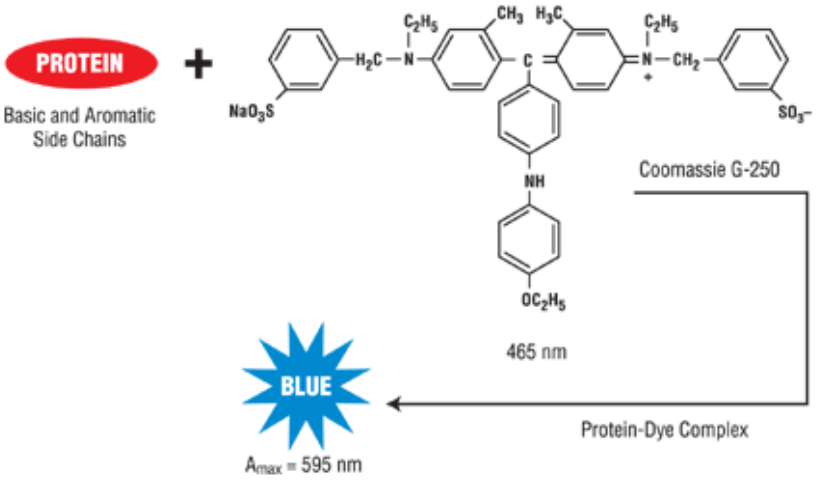


Figure 4.5. Structure of Coomassie Dye. Coomassie G-250 binds to basic and aromatic side chains to form a blue protein-dye complex.

While free Coomassie Blue G-250 has an absorption maximum at 465 nm and solutions of it are brown in color, the dye-protein complex has an absorption maximum at 595 nm and solutions containing these complexes are blue in color (Figure 4.5). The Bradford method is particularly easy to use and only takes about five minutes. Unlike the A_{280}, Lowry, and BCA methods, this assay is less sensitive to differences in the amino acid composition of proteins and to interfering substances. However, the response is linear over only a very limited range of protein concentrations and therefore it is always necessary to make a standard curve. In addition, the Coomassie Blue dye tends to stick tightly to glassware, and so the tubes or cuvettes used in this assay must usually be cleaned with ethanol and washed before they can be reused.

Any protein can be used to construct a standard curve for the Bradford assay, but the most commonly-used protein is bovine serum albumin (BSA), which is found in serum from cows. BSA is normally used to transport fatty acids through the blood and is commercially available at relatively low cost. It should be noted that the use of the standard curve based on a particular protein is only a matter of convenience. **In an extract of plant seeds such as pinto beans or in an extract of chicken breast muscle, there is no BSA!** The statement that a particular solution has a protein concentration of 2.58 mg/ml only indicates that the solution contains as much "apparent protein" as a 2.58 mg/ml solution of BSA. The solution is a mixture of many different proteins in varying amounts, which only show as much reaction as the corresponding amount of the standard protein.

E. Basic Procedure for the Bradford Assay

To do the Bradford protein assay, you will add a series of solutions to a brand new 13 x 100 mm test tube. New tubes are used to avoid any contamination introduced into the tubes from previous experiments. The following steps should be done in the order given:

1. add water to the tube with a micropipetter (0 to 100 µl)
2. add the BSA standard or another protein solution to the tube with a micropipetter (5-100 µl)
3. mix the complete sample (100 µl) by inversion
4. add 3.0 ml Bradford reagent and mix by inversion
5. incubate at room temperature for 10 minutes
6. read absorbance at 595 nm

F. Example of a Protein Assay

To illustrate how a standard curve is made and used, consider the following example. As part of an enzyme purification procedure, it was necessary to determine the protein concentrations of a series of fractions or samples containing the enzyme of interest. The BCA reagent was used in this case. A protein standard curve was first created by varying volumes of a bovine serum albumin solution to a series of tubes containing a total volume of 2.0 ml. Table 4.1 shows the absorbance values at 562 nm for different amounts of BSA.

Table 4.1. Absorbance at 562 for different amounts of BSA

µg of protein	A(562)
0	0.000
2	0.022
5	0.065
10	0.106
20	0.178
30	0.299
40	0.380
50	0.472

To make a standard curve, the absorbance of each solution was plotted as a function of the amount of protein (Figure 4.6)

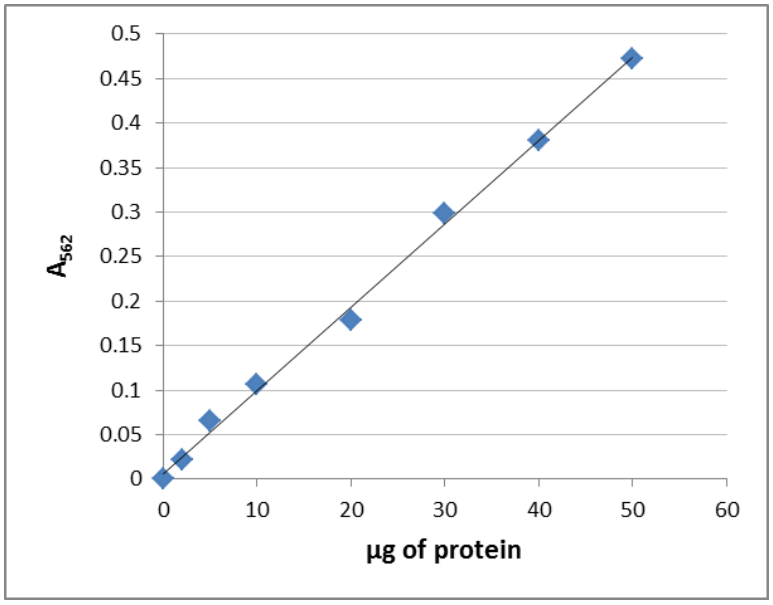


Figure 4.6. Standard curve of absorbance versus the micrograms of Bovine Serum Albumin used in a sample Bradford Assay.

Notice that the amount of protein in µg is plotted on the X axis and the absorbance at 562 nm is plotted on the Y axis. The data points fall on a straight line from 0 to 50 µg of protein. You can use the line to create a conversion factor relating absorbance to amount. Since 10 µg of protein meets the line at an absorbance value of 0.1, the conversion factor is:

$$\frac{0.1\ A(562)}{10\ \mu g} = \frac{0.01\ A(562)}{1\ \mu g}$$

Suppose now that 10 µl of one of the fractions gives an absorbance of 0.251 under the same conditions. **What is the protein concentration in mg/ml?**

You can answer this question either by reading the numbers off of the graph or by using the conversion factor.

From the graph, 0.251 corresponds to about 26 µg.

$$\frac{26\ \mu g}{10\ \mu l} = \frac{2.6\ \mu g}{1\ \mu l} \times \frac{1000\ \mu l}{1\ ml} \times \frac{1\ mg}{1000\ \mu g} = \frac{2.6\ mg}{ml}$$

From the conversion factor,

$$0.251\ A(562) \times \frac{1\ \mu g}{0.01\ A(562)} = 25.1\ \mu g$$

$$\frac{25.1\ \mu g}{10\ \mu l} = \frac{2.51\ \mu g}{1\ \mu l} \times \frac{1000\ \mu l}{1\ ml} \times \frac{1\ g}{1000\ \mu g} = \frac{2.51\ mg}{ml}$$

Remember that 1 µg/µl is the same as 1 mg/ml, so you don't really need to multiply through by all of the metric conversion factors!

Suppose also that 5 µl of another fraction gave an absorbance of 0.097, 10 µl of this fraction gave an absorbance of 0.178, 20 µl of this fraction gave an absorbance of 0.317, and 50 µl of this fraction gave an absorbance of 0.653. **What is the average value of the protein concentration for this fraction?** Again, you can either read the protein amounts off of the graph or use the conversion factor. The example below uses the conversion factor.

Analysis of the 5 µl fraction

$$0.097\ A(562) \times \frac{1\ \mu g}{0.01 A(562)} = 9.7\ \mu g$$

$$\frac{9.7\ \mu g}{5\ \mu l} = \frac{1.94\ \mu g}{1\ \mu l} = \frac{1.94\ mg}{ml}$$

Analysis of the 10 µl fraction

$$\mathbf{0.178\ A(562) \times \frac{1\ \mu g}{0.01A(562)} = 17.8\ \mu g}$$

$$\mathbf{\frac{17.8\ \mu g}{10\ \mu l} = \frac{1.78\ \mu g}{1\ \mu l} = \frac{1.78\ mg}{ml}}$$

Analysis of the 20 µl fraction

$$\mathbf{0.317\ A(562) \times \frac{1\ \mu g}{0.01A(562)} = 31.7\ \mu g}$$

$$\mathbf{\frac{31.78\ \mu g}{20\ \mu l} = \frac{1.58\ \mu g}{1\ \mu l} = \frac{1.58\ mg}{ml}}$$

The average of these three values is:

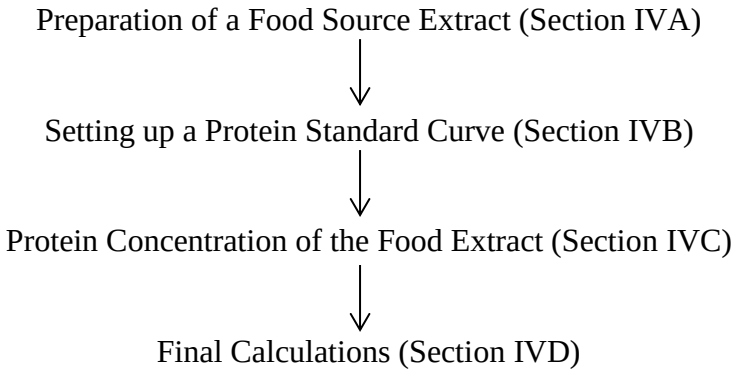
$$\mathbf{\frac{1.94 + 1.78 + 1.58}{3} = 1.77\ mg/ml}$$

In doing this calculation, you **cannot** use the absorbance of 0.653 for the 50 µl sample because it is beyond the range of the standard curve. Even though the line appears to be linear, you have no way of knowing that it continues indefinitely. **In fact, most standard curves will deviate from linearity at high absorbance values or high amounts.**

IV. Experimental Procedures

This experiment has several parts but they must be done sequentially. Again, for this experiment, each group will need to bring to the lab a suitable sample for protein analysis. The most convenient samples are liquid or powder animal or plant food products that have significant protein content. Be sure that there is nutritional label on the side of the can that indicates the serving size and the number of grams of protein per serving.

The following is a flow chart for this experiment.



A. Preparation of a Food Source Extract

The purpose of this part of the experiment is to prepare an extract of your food source in a simple buffer solution so that its protein content can be determined.

1. Look closely at the nutritional label on the side of the package of food you brought to the lab. Note the serving size and the number of grams of protein per serving.

2. Open the package and measure out one serving size. Depending on the product, it might have a certain weight in grams or ounces (remember 1.0 g = 0.0353 ounces) or a certain volume in liters or cups (one 8 ounce cup = 250 ml). Balances and measuring materials will be available for you to use. If it looks like there is a very large amount of material, use only one-fourth or one-eighth of a serving size. Consider that the total volume of liquid you will add is 100 mL

3. Transfer the material to a clean beaker.

4. Add 100 mL of 0.1 M potassium phosphate buffer, pH 7.0 to the food material.

5. Stir the material for two minutes or until no clumps remain if using a protein powder.

6. Decant the suspension into a graduated cylinder and measure the volume in milliliters.

7. Then transfer the suspension to a clean flask and save it for the protein assay in Section C. **This is your protein extract.**

Record the following information on your food source:

1. What was the food source that you used?

   _____

2. What was the designated serving size? What part of a serving did you use?

   _____ _____

3. What was the labeled protein content in grams/serving?

   _____

4. How many ml of extract did you obtain after homogenizing one or part of one serving? If you used less that an entire serving note that also.

   _____

B. Setting up a Protein Standard Curve

The purpose of this part of the experiment is to prepare a protein standard curve, using bovine serum albumin (BSA) as the protein standard and the Bradford Reagent. You will set up a series of tubes with varying amounts of BSA and a constant amount of Bradford reagent. By plotting the number of micrograms (μg) of BSA on the X-axis and the corrected absorbance on the Y-axis, you will be able to generate a standard curve that can then be used to determine the protein concentrations of your unknown solution.

1. At the beginning of the lab, turn on the Genesys 20 spectrophotometer and allow it to warm up for 15 minutes. Set the wavelength to 595 nm.

2. You will be provided with a 1.0 mg/ml stock solution of bovine serum albumin (BSA). **Remember that 1.0 mg/ml is the same concentration as 1.0 μg/μl.**

3. Set up 17 13 x 100 mm glass tubes as shown in the following table. Notice that you will be testing volumes of BSA from 5 to 60 µl in duplicate. Notice also that water will be added to the BSA to give a total sample volume of 100 µl. 3.0 ml of the Bradford reagent then will be added to each tube. **(THE ORDER IN WHICH YOU ADD THESE IS IMPORTANT!!)**

Tube	Water (µl)	1.0 mg/ml BSA (µl)	Bradford Reagent (ml)
1	100	0	3.0
2	95	5	3.0
3	95	5	3.0
4	90	10	3.0
5	90	10	3.0
6	85	15	3.0
7	85	15	3.0
8	80	20	3.0
9	80	20	3.0
10	70	30	3.0
11	70	30	3.0
12	60	40	3.0
13	60	40	3.0
14	50	50	3.0
15	50	50	3.0
16	40	60	3.0
17	40	60	3.0

4. Using micropipetters, add the water to the tubes **first**. Then add the BSA solution. It will help the accuracy if you **use a new tip for each sample and wipe off the outside of the tip quickly with a Kim-Wipe. Also, after you add the BSA, then using the same tip, draw the liquids up and down several times (going only to the first stop on your pipette) this will help to rinse the inside of the tip and to mix the water and BSA together.**

5. When all of the samples have been prepared, add 3.0 ml of Bradford Reagent to each tube using a Repipetter. The instructor will demonstrate how to use this device.

6. Cover each tube with part of a square of Parafilm and invert it several times. This is better than vortexing the samples because it does not generate a lot of foam.

7. Allow the tubes to sit at room temperature for 10 minutes.

8. Use the solution in tube # 1 to set the instrument to zero absorbance since this "blank" contains only water and Bradford Reagent.

9. Measure the absorbance of each tube at 595 nm using the chart shown below. The tubes will fit directly into the cuvette holder of the Genesys 20 spectrophotometers.

Enter your raw data for the absorbance measurements of the samples from the bovine serum albumin (BSA) standard curve

Table ____ Title: _____

Tube	Volume BSA (µl)	A(595 nm)	Average A(595 nm)	µg of protein
1	0			0
2	5			
3	5			
4	10			
5	10			
6	15			
7	15			
8	20			
9	20			
10	30			
11	30			
12	40			
13	40			
14	50			
15	50			
16	60			
17	60			

10. Calculate the average absorbance value for each of the duplicate samples

11. Then determine the total amount of protein in each of the pairs of tubes**. Remember that the stock solution is 1.0 mg/mL or 1.0 µg/µL. Example: if tube 2 contains 5 µL of solution you would conclude that it also contains 5 µg of protein since it contains 1.0 µg/µL of BSA.**

12. Using a piece of linear graph paper, plot the average absorbance values as a function of the amount of BSA in each pair of tubes. Draw a "best fit" straight line through data points with a ruler. **This line should go through the origin since 0 BSA = 0 Absorbance.** The line should pass through or come close to most of the data points. You might find, however, that the standard curve becomes nonlinear at high protein concentrations. (Insert a copy of this graph into your lab manual after this page) Note: non-linearity is not slight deviations; instead it is the beginning of a slight curve and should not appear in these data since you are using fairly small concentrations.

13. Discuss the graph with the instructor. If it looks good, you can proceed to the next part of the experiment**. If some of the points deviate badly from the straight line, set up new tubes for those amounts of protein and repeat the assay.**

14. Once you get a good standard curve, make up a conversion factor relating the absorbance at 595 nm to the amount of protein (_____ A_{595}/µg). This conversion factor is the slope of the line through the linear region of the standard curve and can be calculated from any convenient set of points within the linear region. **Record your conversion factor in your lab notebook.**

C. Protein Concentration of the Food Source Extract

The purpose of this part of the experiment is to determine the protein concentration of your food source extract. Since you do not know the concentration of the unknown solution, you will need to make several dilutions so that some of your protein samples will fall within the range of the standard curve.

1. Make 3 serial 1/10 dilutions of your unknown solution in the following way. Add 900 µL of pH 7.0 phosphate buffer to each of three 1.5 ml microcentrifuge tubes. Mix the extract you prepared earlier and then and add 100 µL of it to the first tube. Close the cap and invert several times to mix. Then add 100 µL of the 1/10 dilution to the second tube to make a 1/100 dilution. Again, close the cap of the second tube and invert to mix. Finally add 100 µL of the 1/100 dilution to the third tube to make a 1/1000 dilution. Close the cap and invert to mix. It may be a good idea to vortex these before proceeding to the next step. Then using these diluted solutions set up an assay as described in #2 below.

2. Set up a new protein assay as shown in the following table.

Table ____ Volumes of liquid used to set up an assay of _____protein extract.

Tube	Water (µl)	Sample	Volume (µl)	Bradford Reagent (ml)
1	100	None	0	3.0
2	90	Undiluted	10	3.0
3	70	Undiluted	30	3.0
4	30	Undiluted	70	3.0
5	90	1/10 Dilution	10	3.0
6	70	1/10 Dilution	30	3.0
7	30	1/10 Dilution	70	3.0
8	90	1/100 Dilution	10	3.0
9	70	1/100 Dilution	30	3.0
10	30	1/100 Dilution	70	3.0
11	90	1/1000 Dilution	10	3.0
12	70	1/1000 Dilution	30	3.0
13	30	1/1000 Dilution	70	3.0

Note that by following this protocol, you will test 10 µL, 30 µL, and 70 µL volumes of each of the dilutions. Again, the total volume in each tube before adding the Bradford Reagent will be 100 µL.

3. Using micropipetters, add the water to the tubes first. Then add the extract you just made whose protein content is theoretically unknown. Again, it will help the accuracy if you use a new tip for each sample, wipe the outside of the tip quickly with a Kim-Wipe, and draw the liquid up and down in the water to rinse the inside of the tip and to mix the water and proteins together. (go only to the first stop on your pipette when mixing)

4. When all of the samples have been prepared, add 3.0 mL of Bradford Reagent to each tube using a Repipetter provided.

5. Cover each tube with part of a square of Parafilm and invert several times. This is better than vortexing the samples because it does not generate a lot of foam.

6. Allow the tubes to sit at room temperature for 10 minutes.

7. Measure the absorbance of the solution in each tube at 595 nm and record the value. You will probably find that some of the solutions are very dark and give absorbance values beyond the range of the standard curve. You may also find that some of the solutions are very light and give absorbance values that are too low (<0.05) to be meaningful or very accurate.

   Enter the raw data for the unknown protein sample here

   Table _____ Raw data giving absorbance of _____ prior to any calculation of protein content.

Tube	Water (µl)	Sample	Sample Volume (µl)	A (595 nm)
1	100	None	0	
2	95	Undiluted	10	
3	95	Undiluted	30	
4	90	Undiluted	70	
5	90	1/10 Dilution	10	
6	85	1/10 Dilution	30	
7	85	1/10 Dilution	70	
8	80	1/100 Dilution	10	
9	80	1/100 Dilution	30	
10	70	1/100 Dilution	70	
11	70	1/1000 Dilution	10	
12	60	1/1000 Dilution	30	
13	60	1/1000 Dilution	70	

8. For analysis of protein content **use only those samples whose absorbances fell within the linear range of your BSA standard curve**. You can either interpolate directly along the line of the standard curve or use the simple conversion factor (slope of the line) derived from it. **Calculate the amount of protein in µg in each of the usable sample**. (Absorbance measured from your unknown protein) = Slope of the line (from your graph) multiplied by (x).+ 0 since our y intercept in this case is zero. See the introduction for more specifics on how to do this calculation.

Enter the amount of protein in each **USABLE** sample in the following table:

Tube	Sample	Sample Volume (µl)	Amount of Protein (µg)
1	None	0	
2	Undiluted	10	
3	Undiluted	30	
4	Undiluted	70	
5	1/10 Dilution	10	
6	1/10 Dilution	30	
7	1/10 Dilution	70	
8	1/100 Dilution	10	
9	1/100 Dilution	30	
10	1/100 Dilution	70	
11	1/1000 Dilution	10	
12	1/1000 Dilution	30	
13	1/1000 Dilution	70	

**Enter N/A for samples that are not usable

9. Then, correct for the volume used in each sample and the dilution factor to calculate the protein concentration of the original protein suspension in mg/mL. For example, suppose that 30 µl of a 1/10 dilution turns out to contain 13.7 µg of protein. The protein concentration is then:

$$\frac{13.7\ \mu g}{30\ \mu l} \times 10 \times \frac{1000\ \mu l}{ml} \times \frac{1\ mg}{1000\ \mu g} = \frac{4.57\ mg}{ml}$$

Include the calculations in your lab notebook.

10. If you have several samples that give absorbance values within the range of the standards, calculate the protein concentration for each sample separately. Then average the values to get a single protein concentration for the original solution. **Record that value here or in your lab notebook.**

Final Average protein concentration in _____
(food tested)

_____mg/mL

D. Final Calculations

The purpose of this part of the experiment is to complete the calculations necessary to determine if the nutritional label on your animal or plant food source is accurate.

1. After completing Section C, you should have a value for the protein concentration of your food source extract in mg/ml. Multiply this value by the total volume of the extract that you measured in Section A to get the total amount of protein from your sample.

   For example, if the protein concentration was 9.42 mg/ml and the total volume of the extract was 53.5 ml, the total amount of protein can be calculated as:

   $$\frac{9.42\ \text{mg}}{\text{ml}} \times 53.5\ \text{ml} = 504\ \text{mg} = 0.504\ \text{g}$$

   Record this calculation in your lab notebook.

2. All of this protein came from the initial amount of material you added to the liquid in the blender as measured in weight or volume. Depending on whether you used a full serving size, one-half, or one-fourth of a serving size, calculate the total amount of protein per serving size**. Compare the value you found with the value on the nutritional label. Record your final values and include a brief comment regarding your protein source and how it relates to the amount of protein you expected to find.**

   Expected (from label): _____g protein/serving

   Observed (from your calculations): _____ g protein/serving

V. Post Lab Analysis

In addition to a data sheet for Lab 4, you will **write up the Methods section for Lab 4**.

Traditionally, the **Methods** section follows the introduction but in some journals it is now placed after the Discussion. This section should provide enough detail that you could duplicate the experiments conducted in the paper. However, in reality there is often not enough detail to do so. Sometimes, this section often contains references to other papers that describe protocols in more detail. This may mean that you have to pursue a long chain of papers until the complete method is described. For the more general reader, the Methods section is sometimes helpful for putting data presented in the Results section in context. Some papers put "background" data here that justifies the use of a technique or procedure. Sometimes there are assumptions or statements of limitation included in the Methods section. These can be very important to the performance of the experiment and the validity of the data. For clarity the Materials and Method section is often divided into clearly labeled subsections.

Methods Section Checklist

- Divide the methods section into clearly labeled subsections
- Write in full sentences and paragraphs; do not use a numbered list
- Use past tense
- Provide enough detail to enable the reader to repeat the experiment
- Do not list materials separately
- Do not refer to the containers (e.g., tubes, beakers, etc.)
- Do not explain routine procedures (e.g., do not explain how to use a micropipetter)
- Do not say that you will graph and interpret the data

The following results section illustrates several key points to remember when writing a methods section. The methods are from the following article:

> Field, A., and Field, J. 2010. Melamine and cyanuric acid do not interfere with Bradford and Ninhydrin assays for protein determination. Food Chemistry. 121(3): 912-917.

Methods are divided into clearly labeled subsections

Condense, where possible, instead of describing treatments for each sample separately.

Provides enough detail to repeat experiment (e.g. times, temperatures, concentrations, wavelengths, etc.)

Refer to samples rather than tubes.

*In general, there are **NO** tables or figures included in the methods section*

Methods are written in past tense.

2. Materials and methods

Melamine was purchased from Fluka and cyanuric acid was purchased from Aldrich; both were prepared as 25 mM solutions, 3.15 mg/ml of melamine or 3.23 mg/ml of cyanuric acid dissolved in water. A 5% cat food solution in water was prepared by grinding and sonicating Royal Canon Urinary SO cat food pellets (kibble). Carnation milk powder was purchased from a local supermarket and suspended at a concentration of 11.5 g in 240 ml of water according to the procedure recommended for reconstitution. The sample was diluted 20-fold in water for assays.

2.1. Sample hydrolysis/digestion

Some samples were treated with acid to promote the release of nitrogen. Where indicated, melamine, cyanuric acid, and cat food were digested as follows: 100 μl of 97% H_2SO_4 or 37% HCl, as indicated, was added to 900 μl of each sample solution and then incubated at 100 °C for 1 h. To neutralise the acid, 100 μl of a 10 M solution of NaOH was added to each sample. To digest samples with proteinase, where indicated (see figures), 1 ml of the 5% cat food solution was combined with 50 μl of a 20 mg/ml proteinase K solution and then incubated at 37 °C in a water bath for 1 h.

2.2. Ninhydrin assay

To run a Ninhydrin test, 2 μl of each sample was spotted on a Flexible Thin layer Chromatography (TLC) Plate. After the samples dried, the plate was sprayed with a 0.2% ninhydrin solution prepared by dissolving 0.2 g of ninhydrin in 100 ml of methanol. The plate was air dried and then heated in an oven at 70 °C for 10 min.

2.3. Bradford protein assay

To perform a Bradford assay, test samples were added to microcentrifuge tubes and brought to a volume of 800 μl with water. Next, 200 μl of 5× Bradford reagent (Bio-Rad laboratories catalogue number 500-0006) was added to each sample to bring it to a volume of 1 ml. The samples were then analysed in a Beckman spectrophotometer to determine their absorbance at 595 nm. A standard curve was prepared with bovine serum albumin (BSA) as a control for all experiments. To do this, 0, 2, 5, 10 or 20 μl of BSA at a concentration of 1.4 mg/ml or in some assays 2.0 mg/ml, was added to 800 μl of water and then used in the assay.

2.4. Ammonia assay

Ammonia assays were performed using a kit from Sigma (catalogue number AA0100), in which ammonia reacts with α-ketoglutaric acid and NADPH in the presence of L-glutamate dehydrogenase to form L-glutamate and NAD+. The oxidation of NAD+ is read as a decrease in absorbance at 340 nm. Where indicated, the samples were treated with H_2SO_4, HCl or proteinase K. For the assay, samples were brought to 100 μl with water and then 1 ml of the ammonia assay reagent was added. The samples were incubated for 5 min at 18–35 °C and their absorbance was then measured at 340 nm. Next, 10 μl of L-glutamate Dehydrogenase solution was added to each sample and after 5 min, the absorbance was measured again at 340 nm. A standard curve was generated using an ammonia standard and 1 ml of the ammonia assay reagent. The blank was 100 μl of water and 1 ml of the reagent. The data are presented as the change in the absorbance compared to the standard curve with ammonia. Experiments in this study were repeated 2–3 times each.