



SPECTROPHOTOMETRIC METHODS FOR  
DETERMINATION OF PROTEINS  
CONCENTRATION

Lab#3

**BCH 333**



## PROTEIN QUANTIFICATION METHODS:

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- Depending on the **accuracy** required and the **amount** and **purity** of the protein available, different methods are appropriate for determining protein concentration.
- *Two types of protein determination methods:*
  1. Simple and direct assay method (old method):  
Warburg-Christian Method (  $A_{280}$  /  $A_{260}$  Method).
  2. Indirect reagent-based protein assay techniques :  
Biuret test, Bradford test, bicinchoninic acid assay (BCA assay).

## 1. BICINCHONINIC ACID (BCA, SMITH) METHOD:

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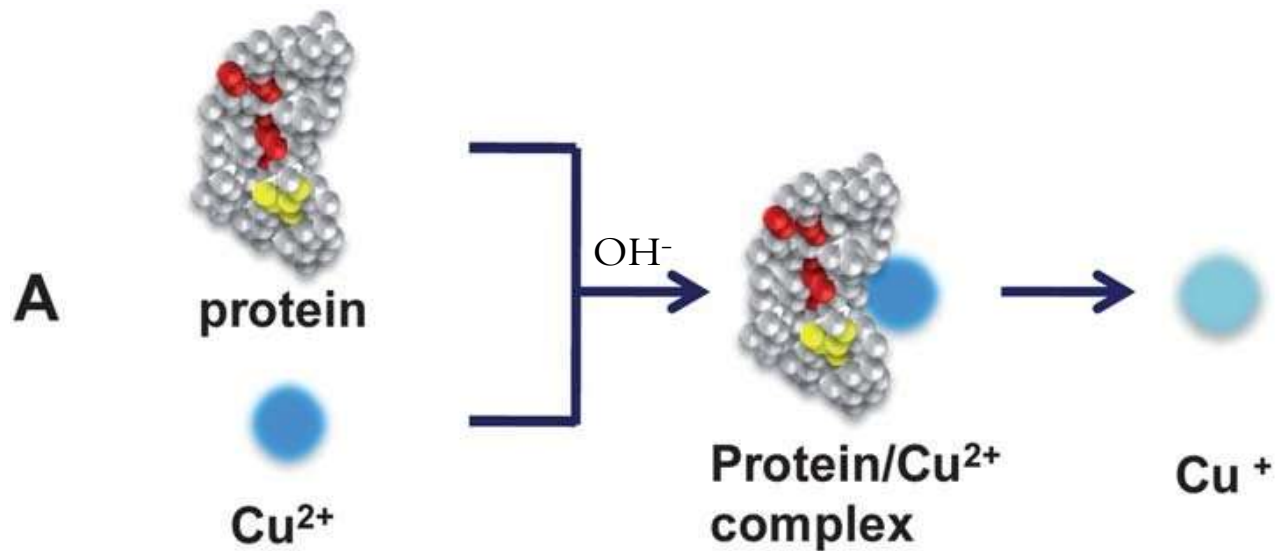
- **Sensitivity:** high, 1  $\mu\text{g}$  of protein can be detected.
- **Time:** Slow (60 min).
- **Reagent:**  $\text{Cu}^{2+}$ , bicinchoninic acid.
- **Medium:** alkaline.
- **Principle:**

The purple color resulting from this method is due to two reactions:

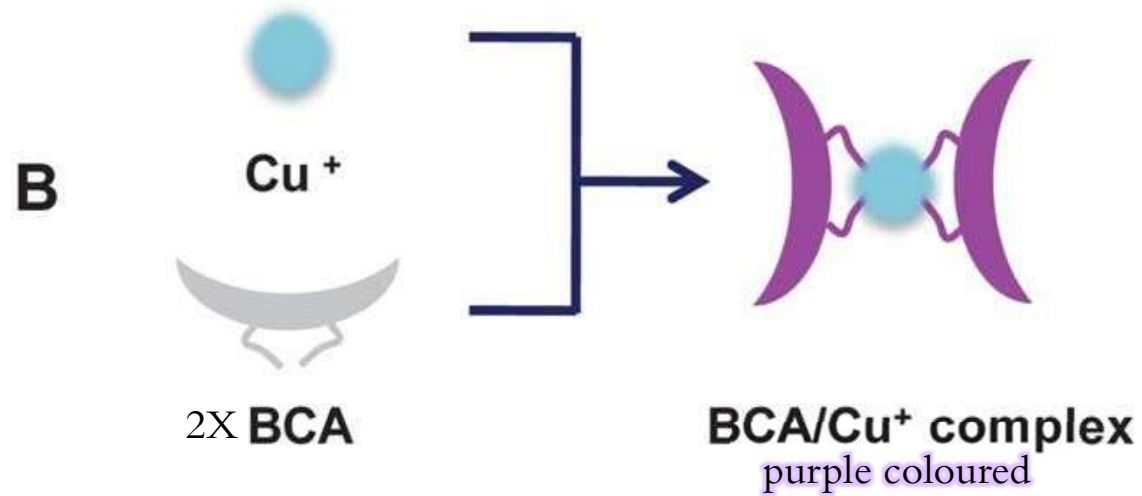
A. The nitrogen in peptide bonds of protein reduce cupric ions  $\text{Cu}^{2+}$  to cuprous  $\text{Cu}^{+}$  [reduction reaction].

→ a temperature dependent reaction under alkaline conditions.

B. The cuprous  $\text{Cu}^{+}$  ions chelated by two molecules of BCA to produce a purple coloured (copper-BCA complex) with maximum absorption ( $\lambda_{\text{max}}$ ) of 562 nm.



The amount of reduced copper is proportional to the amount of protein in the sample.



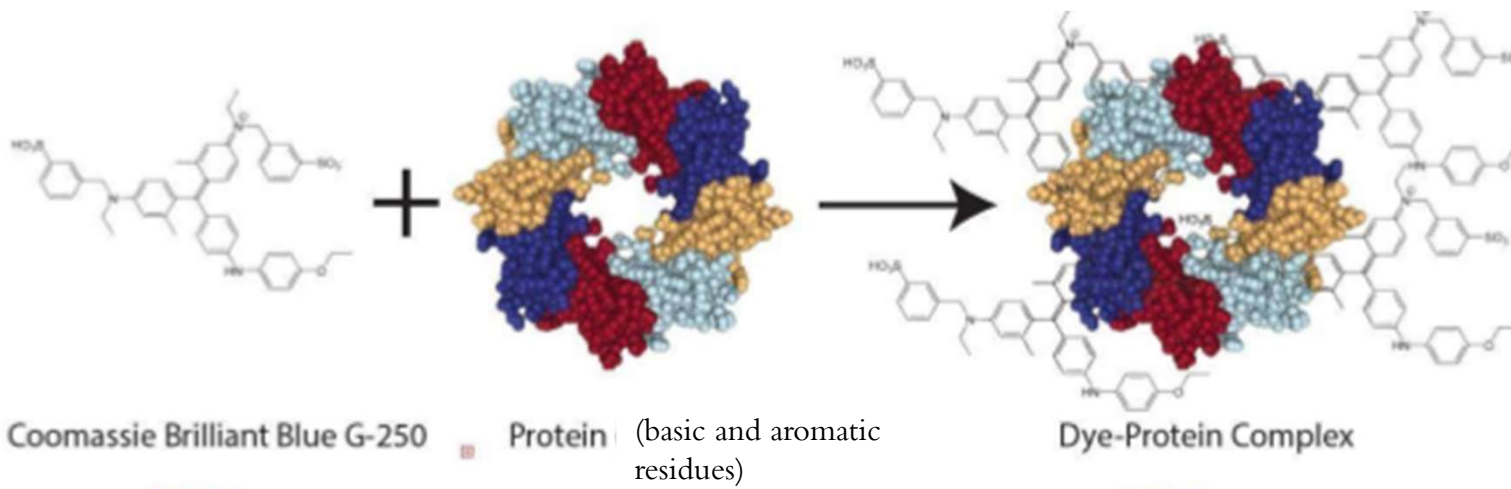
The second reaction comprises the highly sensitive and selective colorimetric detection of the cuprous cations by the Bicinchoninic acid.

## 2. BRADFORD METHOD:

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- **Sensitivity:** high, 1  $\mu\text{g}$  of protein can be detected.
- **Time:** Rapid (15 min).
- **Reagent:** Coomassie Brilliant Blue G-250
- **Medium:** Acidic.
- **Note:** The method is recommended for general use, especially for determining protein content of cell fractions and assessing protein concentrations for gel electrophoresis, about 1- 20  $\mu\text{g}$  protein for micro assay or 20-200 $\mu\text{g}$  protein for macro assay.
- **Principle:**

The Bradford reagent consists of the dye Brilliant Blue G in phosphoric acid and methanol or ethanol. This method relies on forming a complex by the binding of the dye Coomassie Brilliant Blue G-250 to the proteins in acidic condition resulting in a shift in the absorption maximum of the dye from 465 to 595 nm. The absorption at 595 nm is proportional to the amount of protein present in the sample. The dye reagent reacts primarily with arginine residues and less so with histidine, lysine, tyrosine, tryptophan, and phenylalanine residues. The resulted blue color is stable for one hour.



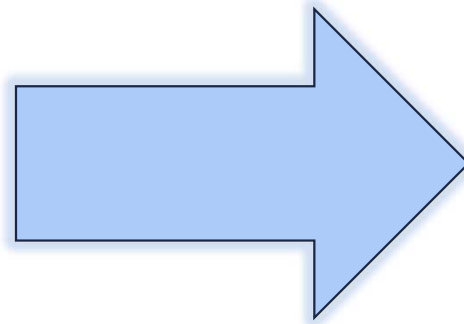
absorbance max @ 465nm

absorbance max @ 595nm

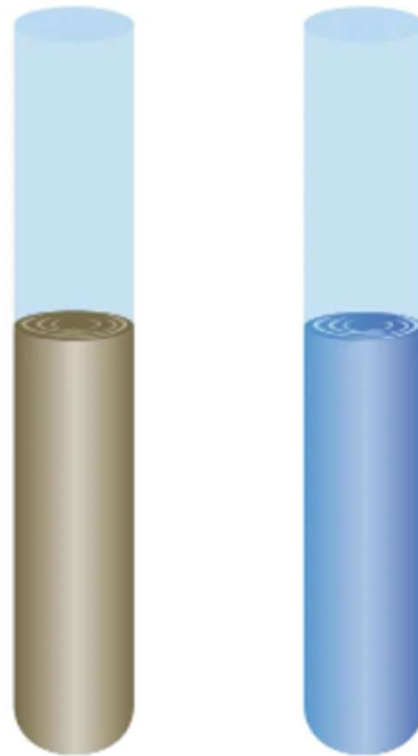




Test tube containing:  
Bradford reagent alone.  
 $\lambda_{\max}$  of the dye 465nm



Test tubes containing:  
Bradford reagent with protein added.  
 $\lambda_{\max}$  is shifted to 595nm.

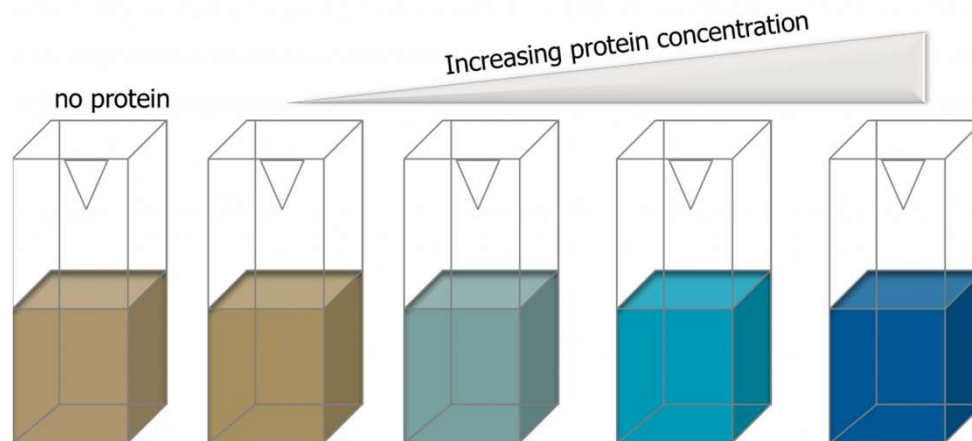
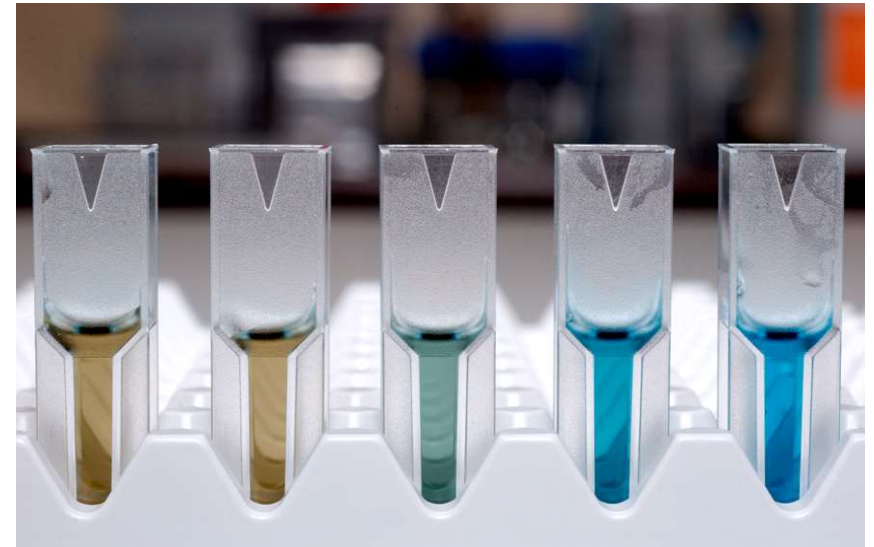
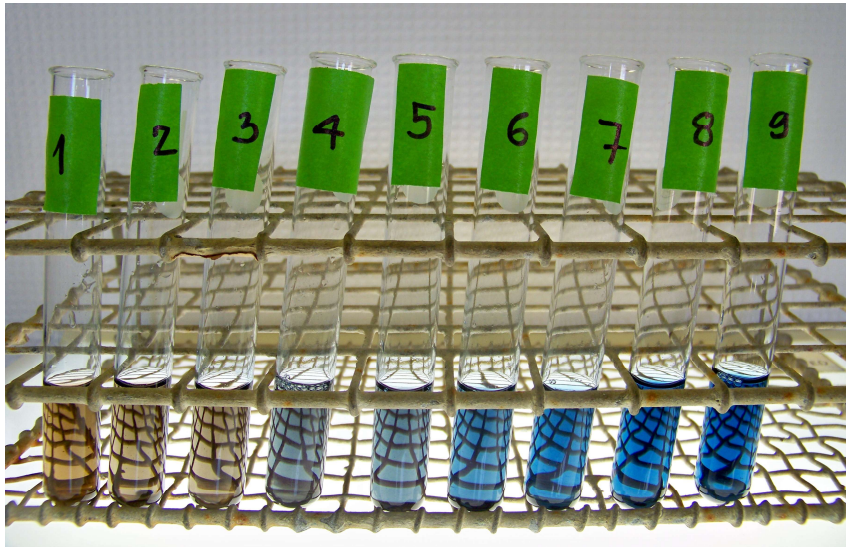


**Figure 3.10** Bradford Protein Assay

*The acidic form (left) has a brown-green hue; the basic form (right), which is created by interactions with proteins in solution, has a brilliant blue hue.*



From lower to higher concentration



There is a **linear relationship** between **blue color developed** → **concentration and absorbance at 595 nm.**

### 3. BIURET ASSAY OF PROTEIN:

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- ***Sensitivity:*** low, 1-20 mg of protein can be detected.
- ***Time:*** Moderate (20-30 min).
- ***Reagent:*** Alkaline copper sulphate.
- ***Medium:*** Alkaline.
- ***Principle:***

Biuret method is based on copper ions  $\text{Cu}^{2+}$  binding to peptide bonds of protein under alkaline condition to give a **violet colour** that have a maximum absorbance at 540 nm. The intensity of the color, and hence the absorption at 540 nm, is directly proportional to the protein concentration.

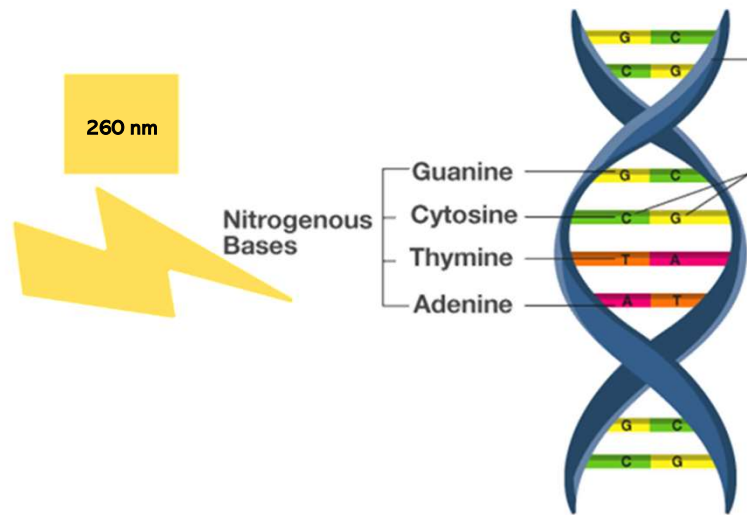
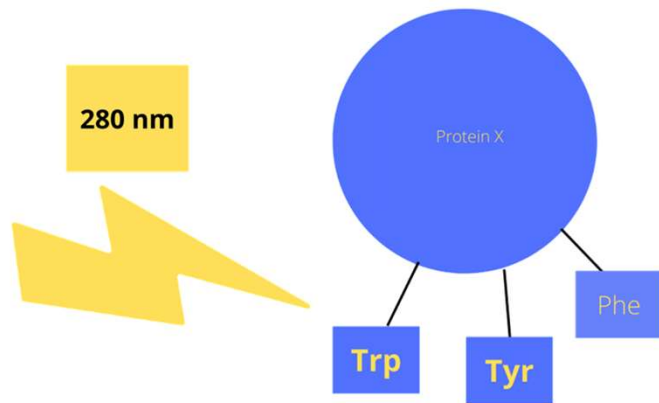


#### 4. WARBURG-CHRISTIAN METHOD ( $A_{280}/A_{260}$ METHOD):

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- **Sensitivity:** Moderate, 50–100  $\mu\text{g}$  of protein can be detected.
- **Time:** Rapid.
- **Reagent:** None.
- **Note:** Not accurate.
- **Principle:**

This method is based on the relative absorbance of **proteins** and **nucleic acids** at **280nm** and **260nm**, respectively. The aromatic rings of several amino acids (mainly tryptophan and tyrosine and to a lesser extent to phenylalanine) in a protein absorb in the ultraviolet at 280nm. Nucleic acids which contaminate samples interfere with this method. This problem is **overcome** by the fact that nucleic acids absorb more strongly at 260nm than at 280nm, while the reverse is true for proteins.



- A protein solution that has a **high**  $A_{280}/A_{260}$  ratio is **Less** contaminated by DNA.

[It shows a low absorbance at 260nm and high absorbance at 280nm].

- A protein solution that has a **Low**  $A_{280}/A_{260}$  ratio is **Highly** contaminated by DNA.

[It shows a high absorbance at 260nm and low absorbance at 280nm].

## 4. WARBURG-CHRISTIAN METHOD ( $A_{280}/A_{260}$ METHOD) CONT':

- *Calculating the unknown concentration of a protein sample, by two ways:*

1. [ $A_{280}$  x correction factor = \_\_\_\_\_ mg/ml protein].

To find the correction factor:

$A_{280}$  = \_\_\_\_\_

$A_{260}$  = \_\_\_\_\_

$A_{280}/A_{260}$  = \_\_\_\_\_ → form the table, the correction factor = \_\_\_\_\_

So, Unknown protein concentration = \_\_\_\_\_ mg/ml

$A_{280}/A_{260}$  Method

$A_{280}/A_{260}$	Correction factor	Nucleic acid (%)
1.75	1.12	0.00
1.63	1.08	0.25
1.52	1.05	0.50
1.40	1.02	0.75
1.36	0.99	1.00
1.30	0.97	1.25
1.25	0.94	1.50
1.16	0.90	2.00
1.09	0.85	2.50
1.03	0.81	3.00
0.98	0.78	3.50
0.94	0.74	4.00
0.87	0.68	5.00
0.85	0.66	5.50
0.82	0.63	6.00
0.80	0.61	6.50

Or

2. *Grove's formula:*

Protein concentration [mg/ml] = [1.55 X  $A_{280}$ ] - [0.76 X  $A_{260}$ ]

The percentage of DNA contamination, compared to the ratio ( $A_{280}/A_{260}$ ) in the sample

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1.03	0.81	3.00
0.98	0.78	3.50
0.94	0.74	4.00
0.87	0.68	5.00
0.85	0.66	5.50
0.82	0.63	6.00
0.80	0.61	6.50



# Practical part

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## OBJECTIVES:

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- To determine the concentration of extracted proteins by [Bradford method](#).
- Using [Warburg-Christian method](#) to estimate the concentration of a protein sample.



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*Estimation of protein concentration by  
Bradford method*

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## METHOD:

1. Set up 9 tubes and label them as follows:

<i>Tube</i>	<i>Bovine Serum Albumin (BSA) (150<math>\mu</math>g/ml) (ml)</i>	<i>Distilled Water (ml)</i>	<i>Sample with Unknown Concentration (ml)</i>	<i>Bradford reagent</i>
Blank	-	1	-	<i>5 ml</i>
A	0.07	0.93	-	
B	0.13	0.87	-	
C	0.26	0.74	-	
D	0.4	0.6	-	
E	0.66	0.34	-	
F	1	-	-	
Dialyzed sample	-	-	1	

2. Mix and Incubate at room temperature for 5 min.
3. Measure the absorbance at 595 nm.

# RESULTS:

Tube	Concentration ( $\mu\text{g/ml}$ )	Absorbance at 595 nm
A		
B		
C		
D		
E		
F		
Dialyzed sample	From the standard curve	

Standard Solutions  
 $C1 \times V1 = C2 \times V2$

- Plot a standard curve of absorbance at 595 nm against BSA protein concentration ( $\mu\text{g/ml}$ ).
- From the curve determine the concentration of protein with the unknown concentration (Dialyzed sample). (using TREND formula).

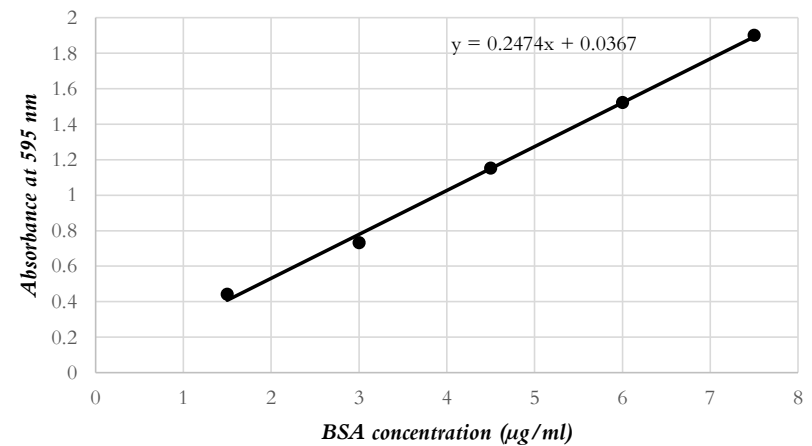


Figure 1. Determination of protein concentration using Bradford method.



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*Estimation of protein concentration by  
Warburg-Christian Method*

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## METHOD AND RESULTS:

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1. Read the absorbance of (protein sample A) at 280nm, then read the same sample at 260nm.
2. Record your results:
  - A280= \_\_\_\_\_
  - A260= \_\_\_\_\_
  - A280/ A260 ratio = \_\_\_\_\_
  - Correction factor from the table= \_\_\_\_\_
  - *Unknown concentration of protein sample* = \_\_\_\_\_ mg/ml.