SPECTROPHOTOMETRIC METHODS FOR DETERMINATION OF PROTEINS CONCENTRATION

Lab#3

BCH 333

PROTEIN QUANTIFICATION METHODS:

- 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:

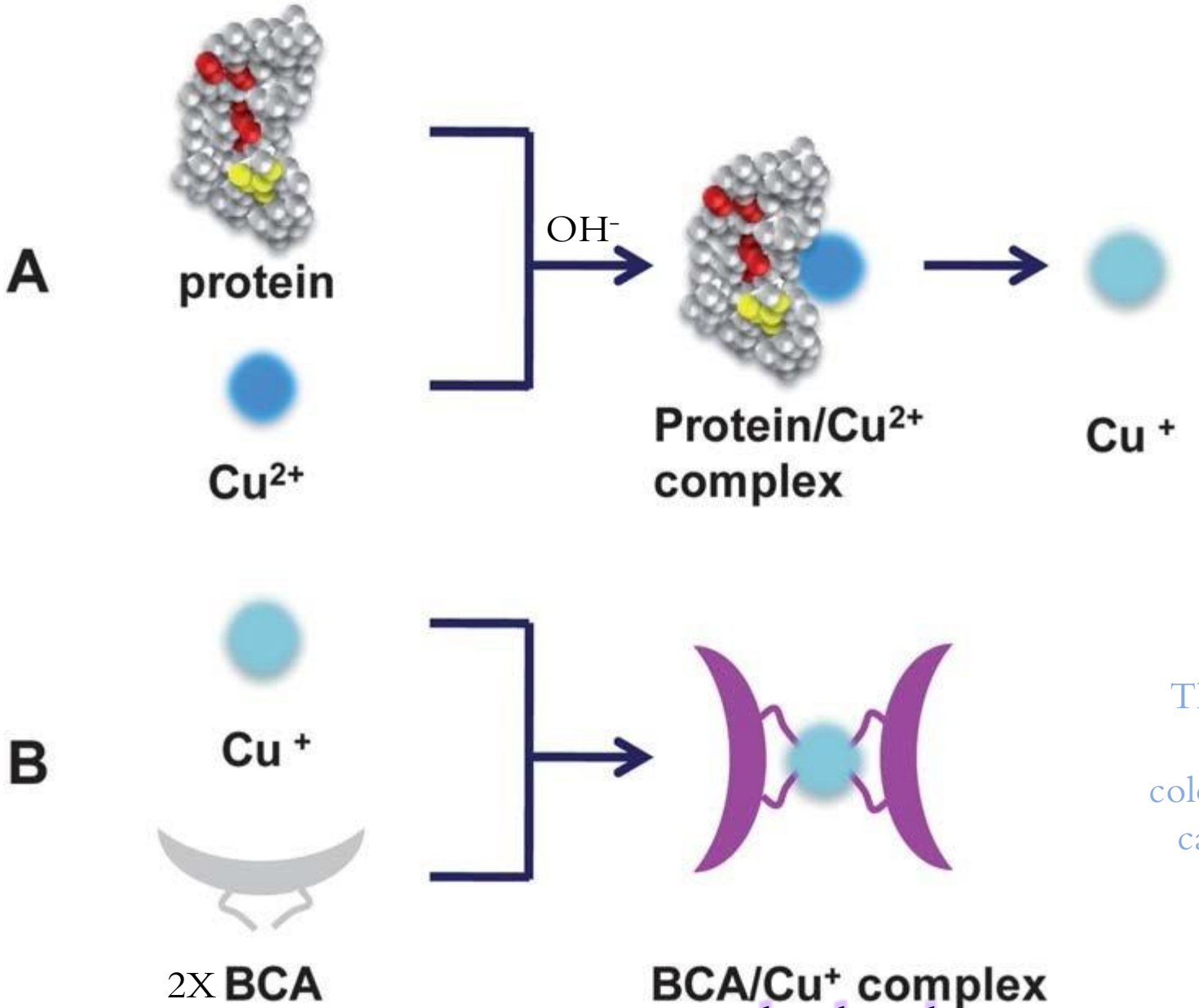
- **Sensitivity:** high, 1 µg of protein can be detected.
- **Time:** Slow (60 min).
- **Reagent:** Cu^{2+} , bicinchoninic acid.
- **Medium:** alkaline.
- **Principle:**

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

1. The nitrogen in peptide bonds of protein reduces cupric ions Cu^{2+} to cuprous Cu^{+}[reduction reaction].

➔a temperature-dependent reaction under alkaline conditions.

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



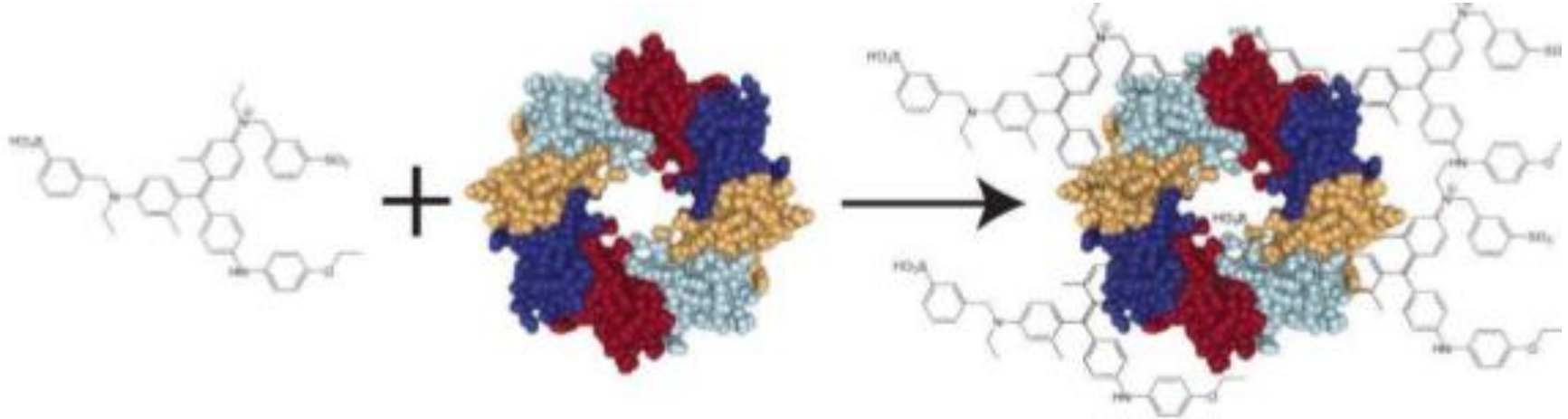
The amount of reduced copper is **proportional** to the amount of protein in the sample.

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

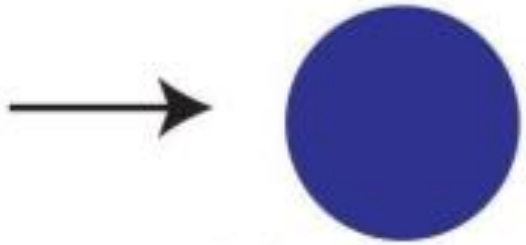
2. BRADFORD METHOD:

- **Sensitivity:** high, 1 µg 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 µg protein for micro assay or 20-200µg 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

High protein concentration → Depth blue color → High absorbance at 595 nm

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

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

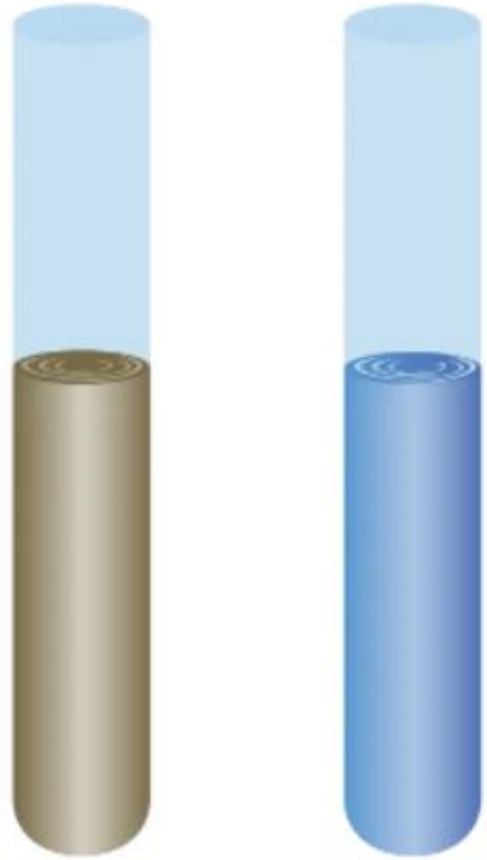
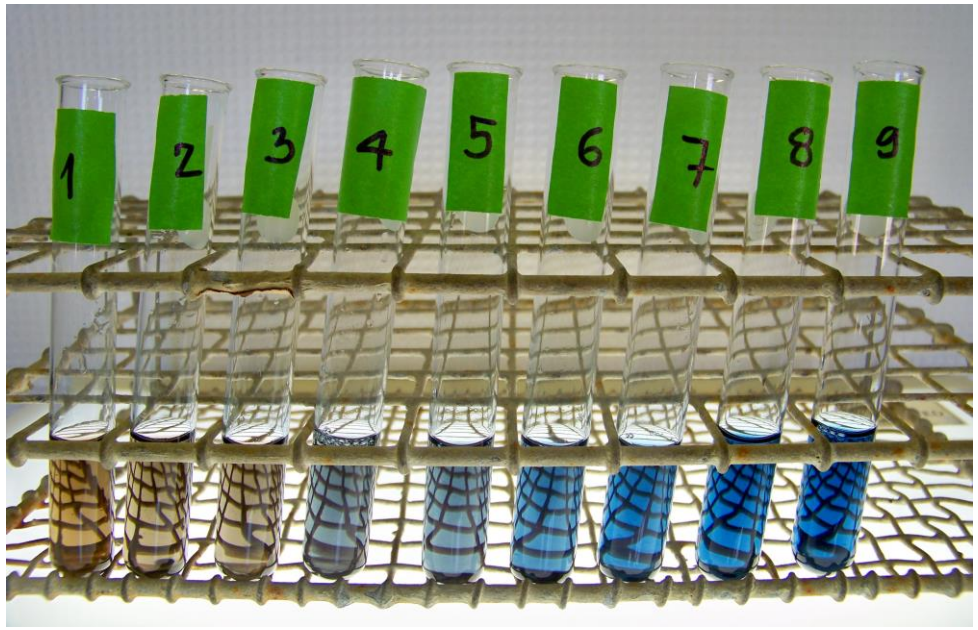
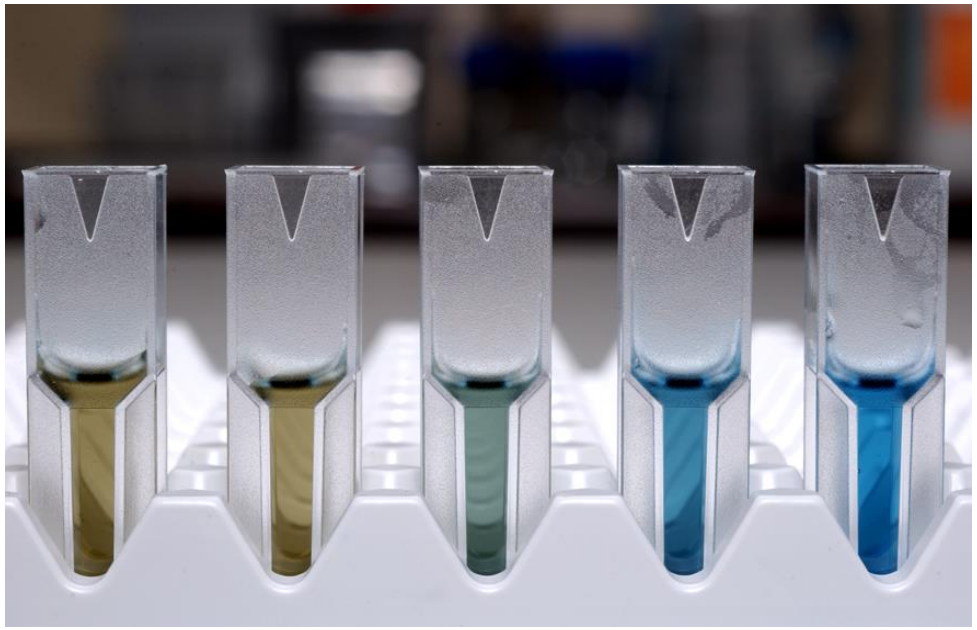
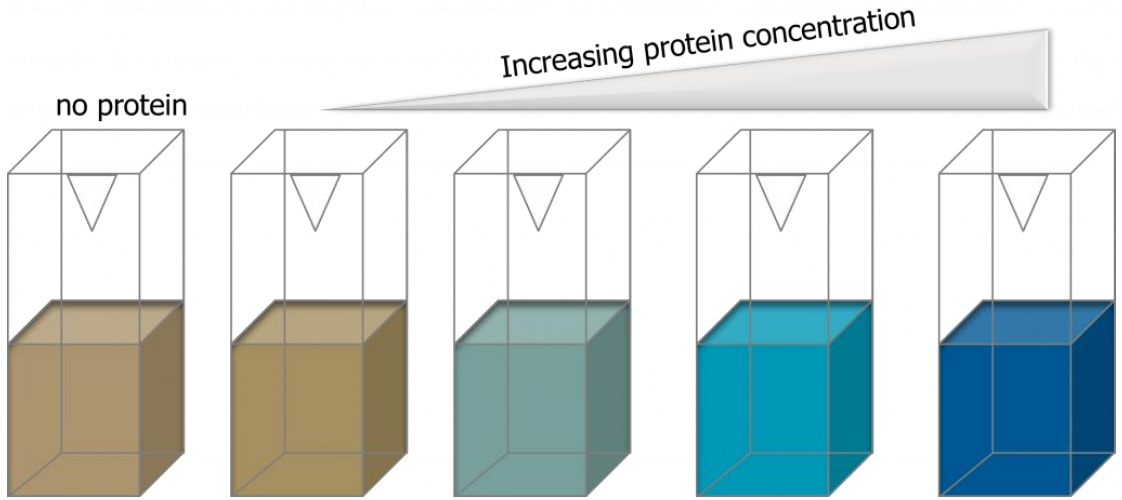


Figure 3.10 Bradford Protein Assay
The acidic form (left) has a brown-green hue; the basic form (right), which is created by interactions with proteins in solution, has a brilliant blue hue.

From lower to higher concentration





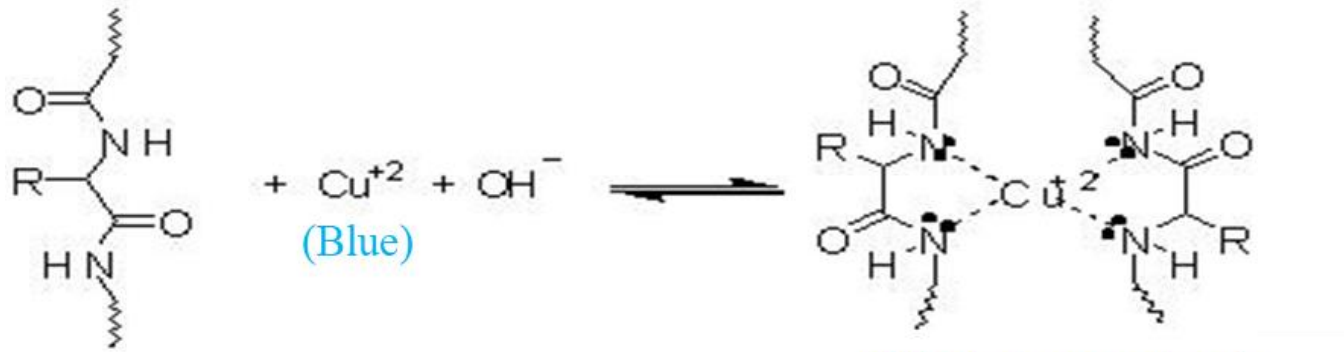


There is a linear relationship between **blue color developed ➔ concentration and absorbance at 595 nm.**

3. BIURET ASSAY OF PROTEIN:

- **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 Cu^{2+} binding to peptide bonds of protein under alkaline conditions to give a violet colour that has 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.



Peptide bonds of protein

(Violet complex)

Absorbance at 540 nm

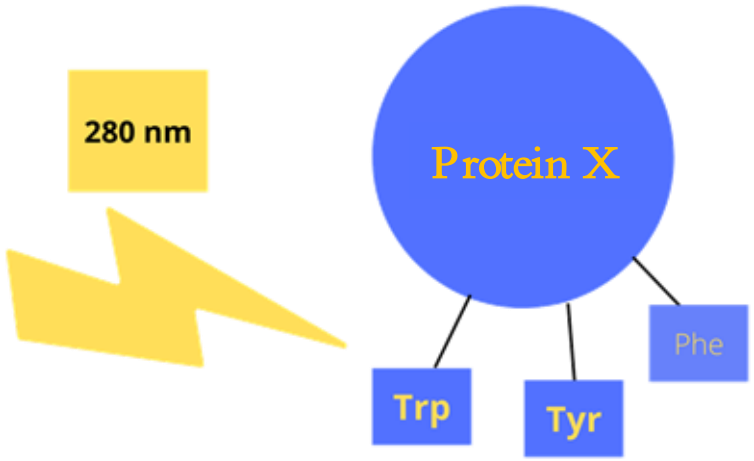
From lower to higher concentration

There is a linear relationship between purple color developed ➔ concentration and absorbance at 540 nm.

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

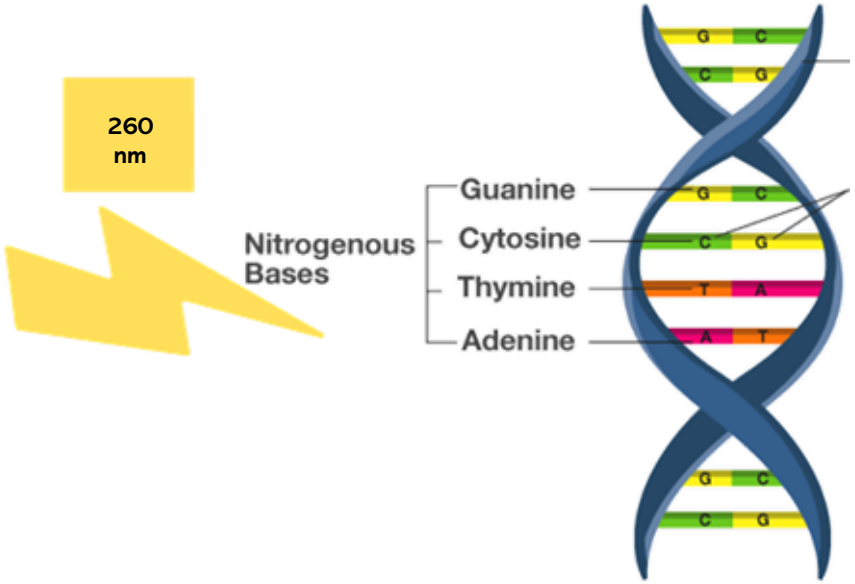
- **Sensitivity:** Moderate, 50-100 µg of protein can be detected.
- **Time:** Rapid.
- **Reagent:** None.
- **Note:** Not accurate.
- **Principle:**

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 A280/A260 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

A_{280}/A_{260} Method

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1.75	1.12	0.00
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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

Practical part

OBJECTIVES:

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

1
Estimation of protein concentration by Bradford method

METHOD:

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

Tube	Bovine Serum Albumin (BSA) (150µg/ml) (ml)	Distilled Water (ml)	Sample with Unknown Concentration (ml)	Bradford reagent
Blank	-	1	-	5 ml
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	
	-	0.5	0.5	

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

RESULTS:

Tube	Concentration (µg/ml)	Absorbance at 595 nm
A		
B		
C		
D		
E		
F		
Dialyzed sample	From the standard curve	

Standard Solutions
C1 x V1 = C2 x V2

- Plot a standard curve of absorbance at 595 nm against BSA protein concentration (µg/ml).
- From the curve determine the concentration of protein with the unknown concentration (Dialyzed sample). (using TREND formula).

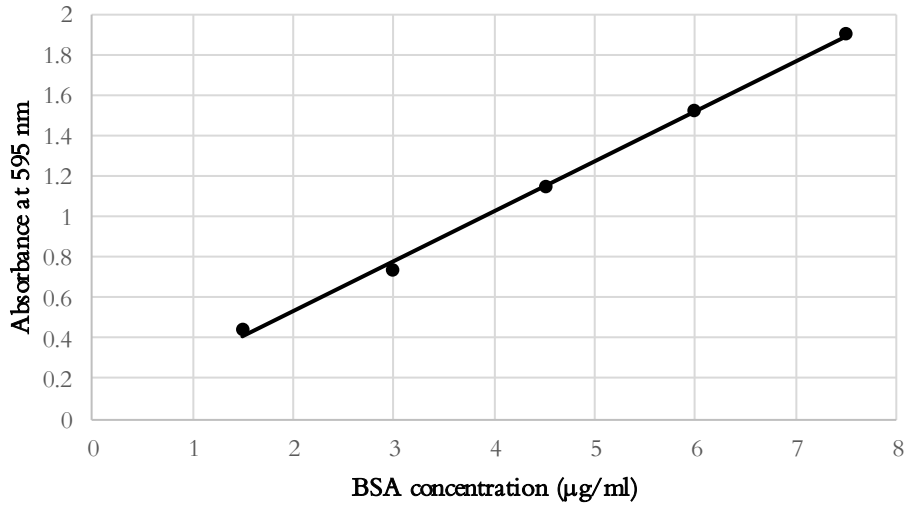

Figure 1. Determination of protein concentration using Bradford method.

2
Estimation of protein concentration by Warburg-Christian Method

METHOD AND RESULTS:

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.