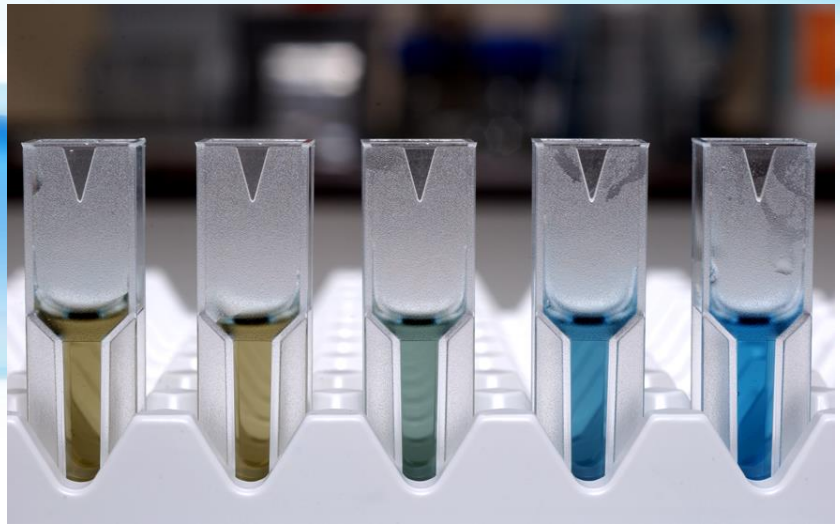
Spectrophotometric Methods For Determination Of Proteins



Objectives

To Learn Different Method Of proteins determination

In this Lab you will using the following spectrophotometric methods:

1. **Bradford Method.**

Chemical reagents are added to the protein solutions to develop a color whose intensity is measured in a spectrophotometer.

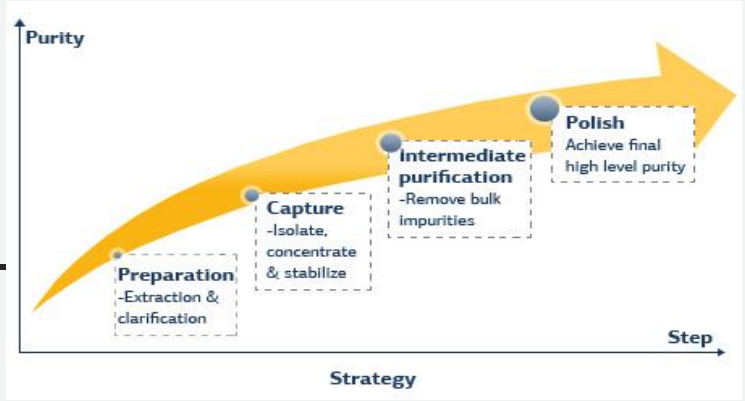
2. Warburg-Christian Method (A280/ A260 Method).

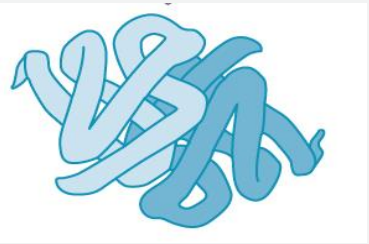
Relies on direct spectrophotometric measurement

Importance of determining concentration of protein

- Protein assays are one of the most widely used methods in life science research.
- Estimation of protein concentration is necessary cell biology, molecular biology and other research applications.

- Is necessary before processing protein samples for isolation, protein purification, separation and analysis.





Important Terms

Assays

Qualitative assays

Determine if specific substance is there or not, by color or some other quality

Quantitative assays
Determine the concentration of a substance

Specifity and sensivity

Sensitivity of an assay is a measure of how little of the analyte the method can detect

Specificity of an assay relates to how good the assay is in discriminating between the requested analyte and interfering substances

The determination of protein concentration

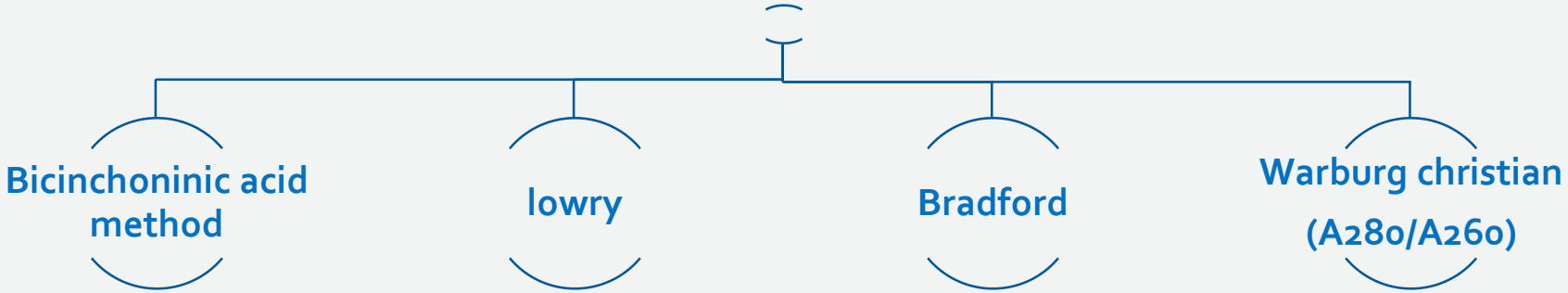
Spectrophotometric and colorimetric methods

- Used fror routine estimation, most of them are **colometric**
- Where a portion of the protein solution is reacted with a reagent that produces a coloured product.
- **However, none of these methods is absolute,**

Acid hydrolyse a portion of the sample

- And then carry out amino acid analysis on the hydrolysate
- A truly accurate method
- **However, this is expensive and relatively time-consuming, particularly if multiple samples are to be analysed.**

Spectrophotometric method for determining the protein concentration



There are a wide variety of protein assays available. but each assay has its own advantages and limitations

The factors that you should consider :

- Sensitivity
- The presence of interfering substance
- Time available of the assay

1-Bicinchoninic acid method

- The mechanism of color formation for the **BCA** assay is similar to that of the **Lowry** protein assay.

- Both BCA assay and Lowry assay are based on the conversion of Cu^{2+} to Cu^{1+} under alkaline conditions.

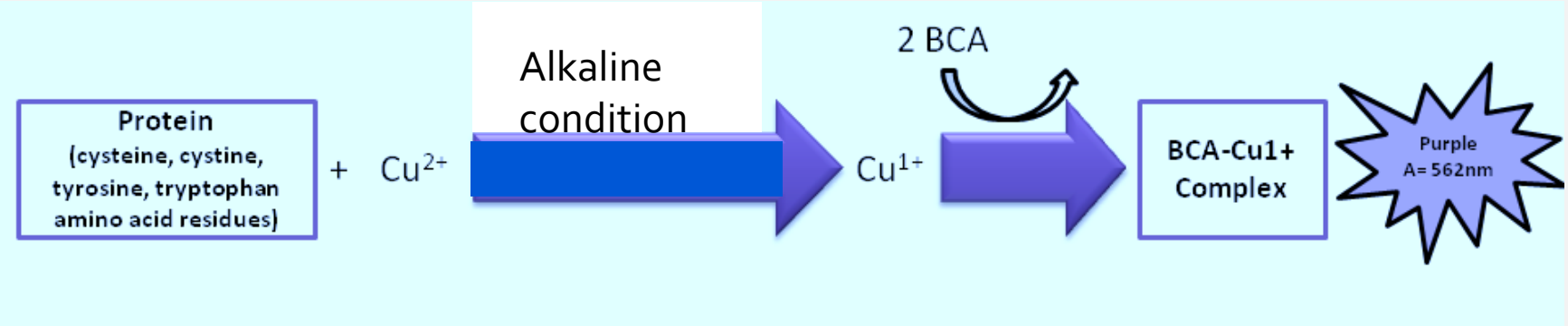
- In BCA the color develops in a single step but Lowry method in two steps

- In general, this method has a high sensitivity (**1 µg**)

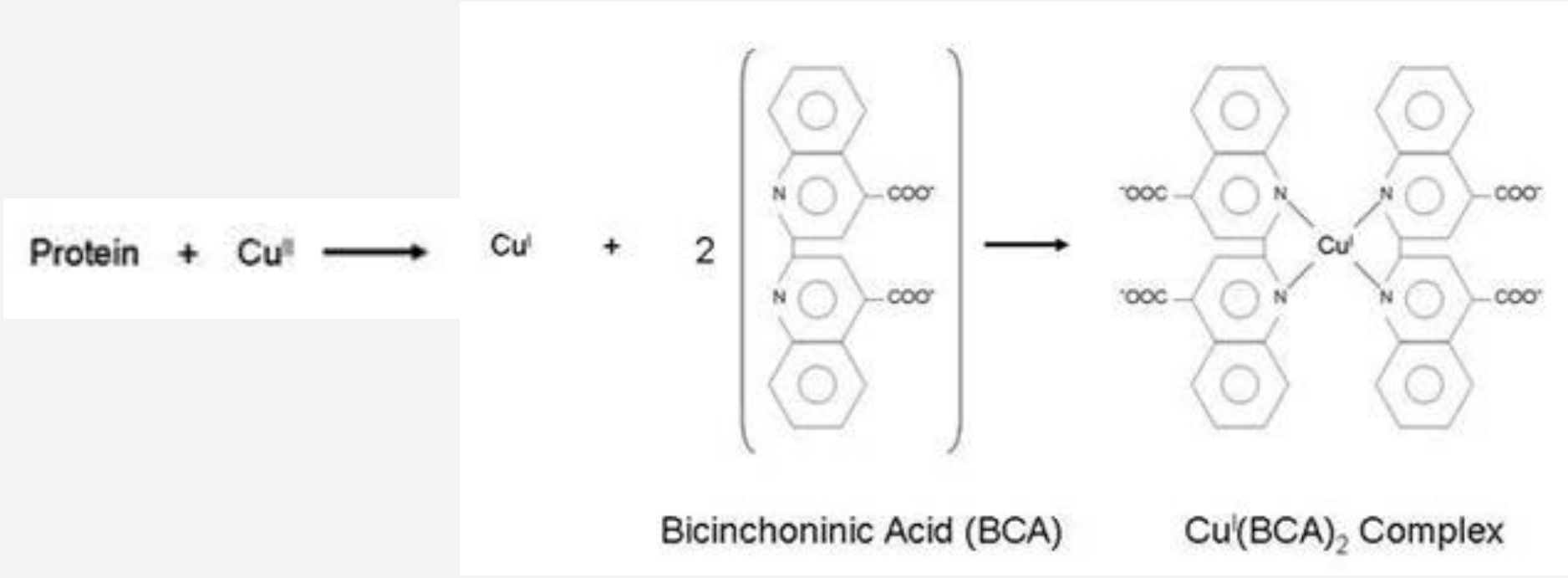
1-Bicinchoninic acid method

- **Principle:**
- Cu+2 form a complex with nitrogen of the peptide bond under alkaline conditions producing cu+(the Cu++ was reduced to Cu+)

- This cu+ will then chelated by BCA to produce a copper-BCA complex with maximum absorbance **562** nm

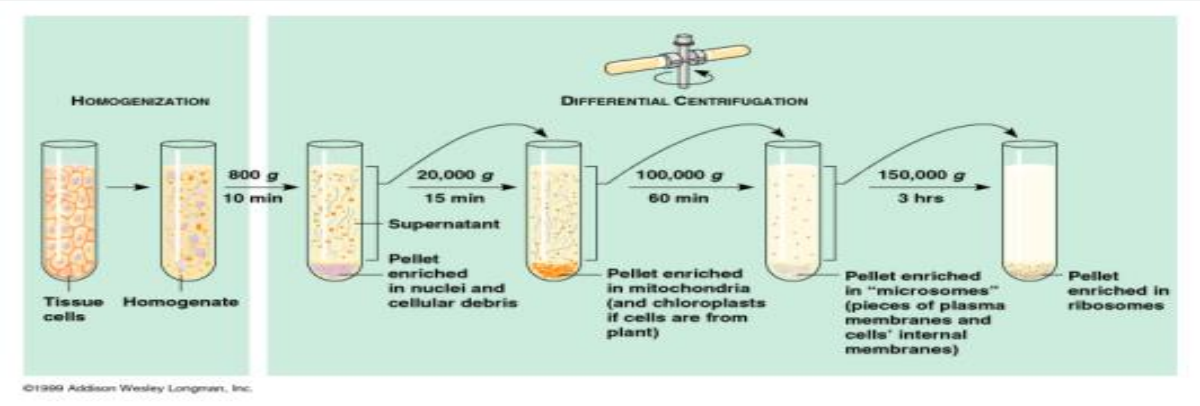


1-Bicinchoninic acid method



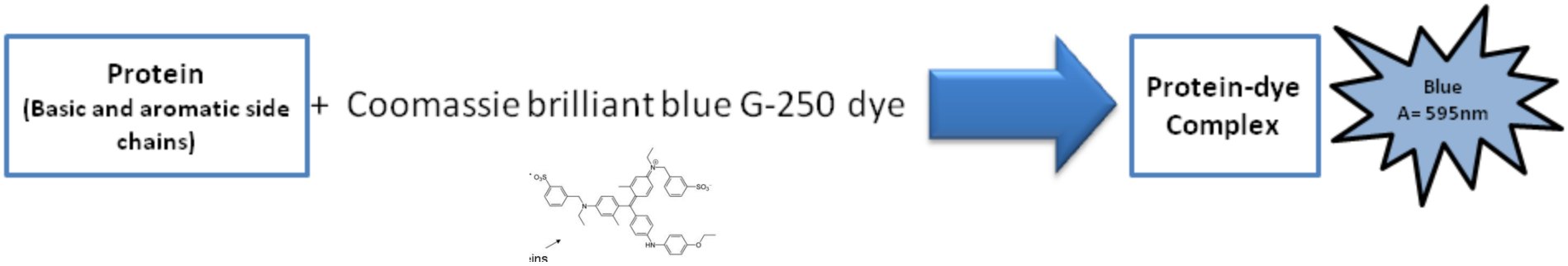
2-Bradford assay

- Very fast(15 min)
- Accurate
- Highly sensitive(1 µg protein can be detected)
- The amount of this coloured product is then measured spectrophotometricallyand the amount of colourrelatedto the amountof protein present by appropriate calibration.
- Disadvantages: Coomassie (Bradford) Protein Assay produces a nonlinearstandard curve. Why?



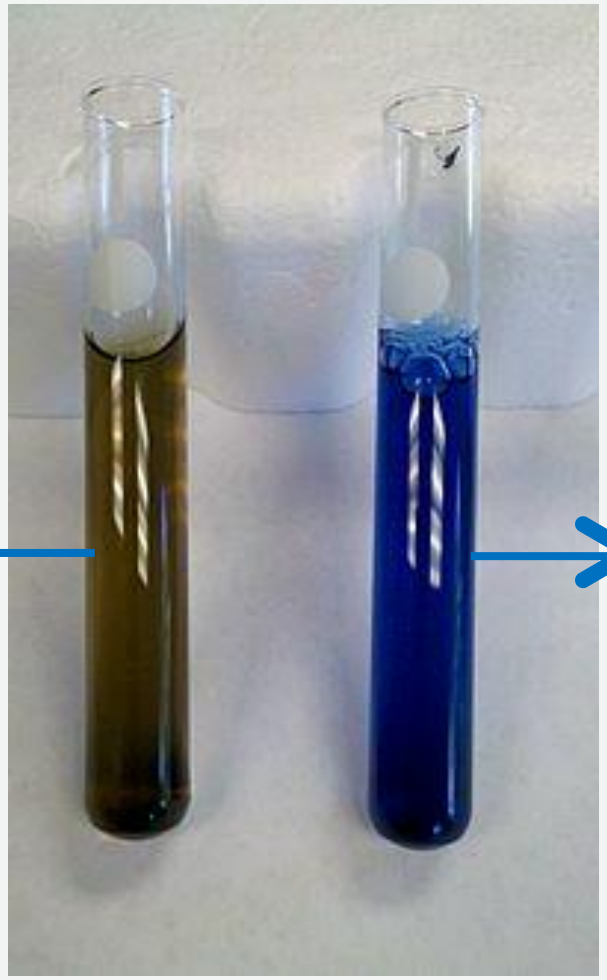
2-Bradford assay

- Principle:
- Coomessie brilliant blue G-250 bind to protein (**binds particularly to basic and aromatic amino acids residues**) in **acidic solution**
- Make a complex which will shift the wavelength of maximum absorbance **465** to **595** nm.
- This complex stabilized by hydrophobic and ionic interaction



Bradford assay

Bradford reagent alone – maximum absorbance at (465 nm)



Bradford reagent and protein maximum absorbance at (595 nm)

Bradford assay-Method

A- Set up 9 tubes and label them as follows:

Tube	Bovine Serum Albumin(BSA) (150µg/ml)	Distilled Water	Unknown	Concentration (µg/ml)
(blank)	-	1 ml	-	**Blank**
A	0.07 ml	0.93 ml	-	**10.5**
B	0.13 ml	0.87 ml	-	**19.5**
C	0.26 ml	0.74 ml	-	**39**
D	0.4 ml	0.6 ml	-	**60**
E	0.66 ml	0.34 ml	-	**99**
F	1 ml	-	-	**150**
G	-	-	1 ml	**?**
H	-	-	1 ml	**?**

Add 5ml of Bradford reagent to each tube [blank – H].

C- Mix and Incubate at room temperature for 5 min.

D- Measure the absorbance at 595 nm.

Bradford assay-Results

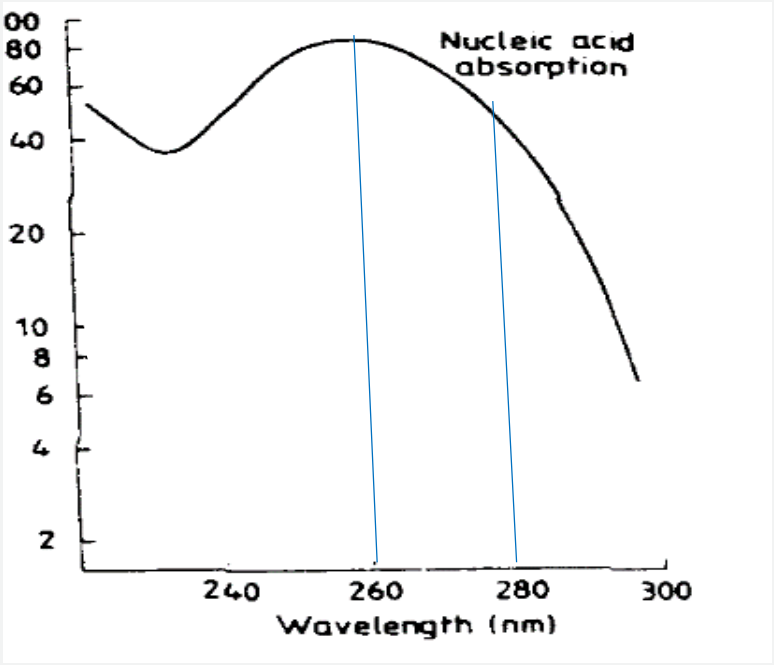
Tube	Concentration (µg/ml)	Absorbance at 595 nm
(blank)	Blank	
A	10.5	
B	19.5	
C	39	
D	60	
E	99	
F	150	
G	=.....	
H	=.....	

3-Warburg christian (A280/A260)

- **Relies on direct spectrophotometric measurement.**
- **Fast**
- **Semiquantitative analysis**

- **Principle:**
- **Proteins can absorb light at 280 ultraviolet**
- **This is because proteins contains aromatic amino acids tyrosine and tryptophan give proteins .**
- **The amount of these residues vary greatly from protein to protein so this method is semiquantitave**

Warburg christian (A280/A260)



- **Nucleic acid interfere with this method.**
- **So to solve this problem, we will measure the absorbance at 280 then we measure at 260**
- **Calculate A280/A260 ratio,**
- **then from a specific table we can get the correction factor**
- **A280 x correction factor**
- **A protein solution that has a high A280/A260 ratio: Less contaminated by DNA**
- **Or by another way:**
- **[groves formula]:**
- **Protein concentration [mg/ml]=[1.55 X A280]-[0.76 X A260]**

Warburg christian (A280/A260)

-Calculate the protein concentration in the unknown from the following equations:

A280 =……
A260 =……
A280/ A260 =……
Correction factor =……
A280 x correction factor =…… mg/ml protein
Unknown concentration =…… mg/ml

2-or [groves formula]:

Protein concentration [mg/ml]=[1.55 X A280]-[0.76 X A260]

Warburg christian (A280/A260)

-A protein solution that has a high A280/A260 ratio: Less contaminated by DNA.

[It shows a lower absorbance at 260nm comparing to absorbance at 280nm].

-A protein solution that has a low A280/A260 ratio: Highly contaminated by DNA.

[It shows a higher absorbance at 260nm comparing to absorbance at 280nm].

Summary

- **Protein assay is important in many aspects**
- **There are Many Methods for protein determination, each had it own advantages and disadvantages**

ASSAY	ABSORPTION	MECHANISM	reagent
UV absorption	280 nm	Tyrosine and tryptophan absorption	No reagent
Bicinchoninic acid	562 nm	copper reduction (Cu^{2+} to Cu^{1+}), BCA reaction with Cu^{1+}	BCA
Bradford or Coomassie brilliant blue	595 nm	complex formation between Coomassie brilliant blue dye and proteins	Coomassie brilliant blue