Welcome to Biochemistry's laboratory

If you have any questions,

please contact me with this number: 68759795 (O)

Requirements

1. **Lab safety and keep Lab clean and orderly：**

   Always wear Lab-coat

   Eating, drinking, smoking in the Lab is prohibited.

   Use and dispose of hazardous chemicals properly.

2. **Attendance policy (15%)**

   Attend every time, arrive on time and well prepared.

3. **Lab reports (15%)**
4. **Middle Examination (15%)**
5. **Final Examination (55%)**

Contents

- SPECTROPHOTOMETRY (qualitative and quantitative analysis)
- ELECTROPHORESIS (identify and isolation)
- CHROMATOGRAPHY (identify and isolation)
- TECHNIQUES OF MOLECULAR BIOLOGY

SPECTROPHOTOMETRY

- **Definition:**

A quantitative measurement depends on light reflection or transmission properties of a material.

- **Application:**

1. *To identify unknown compounds by their characteristic* ***absorption spectra.*** (qualitative analysis).
2. *To determine the concentrations of analytes in solutions.* (quantitative analysis).

SPECTRUM OF LIGHT USED IN SPECTROPHOTOMETRY

λ (nm) 100 200 400 700 1000 2000 4000 7000 10,000 20,000 40,000

spectral region: VAC UV | UV | VISIBLE | NEAR IR | IR | FAR IR

sources

continuous:

Argon lamp

Xenon lamp

H_2 or D_2 lamp

Tungsten lamp

Nernst glower (ZrO_2 + Y_2O_3)

Nichrome wire (Ni + Cr)

Globar (SiC)

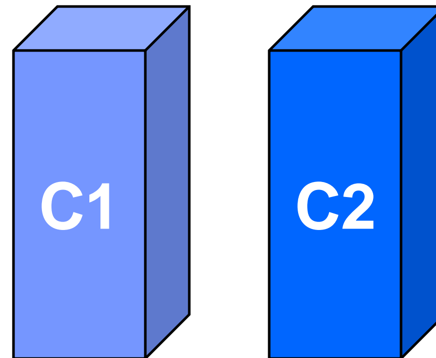
discontinuous:

Hollow cathode lamps

* VAC UV: Vacuum UV

SPECTROPHOTOMETRY

- *Spectrophotometric measurement often use the absorption of light in the* ***visible*** *(between 380 and 760 nm) and* ***ultraviolet*** *regions (200~ 380 nm).*
- *Measurement of intensity of colored solutions (absorption of visible light) is also called* ***Colorimetry****.*



Part one:

Applications of Spectrophotometry

---- **Identify unknown compounds**

(Qualitative measurement)

Identify unknown compounds

Principle

Because the extent to which a sample absorbs light depends strongly on the wavelength of light (λ), spectrophotometry is performed using *monochromatic light*.

For a new unknown material in a solution,

measures the absorbance (A) of the solution for each wavelength (λ) and construct the *absorption spectrum*.

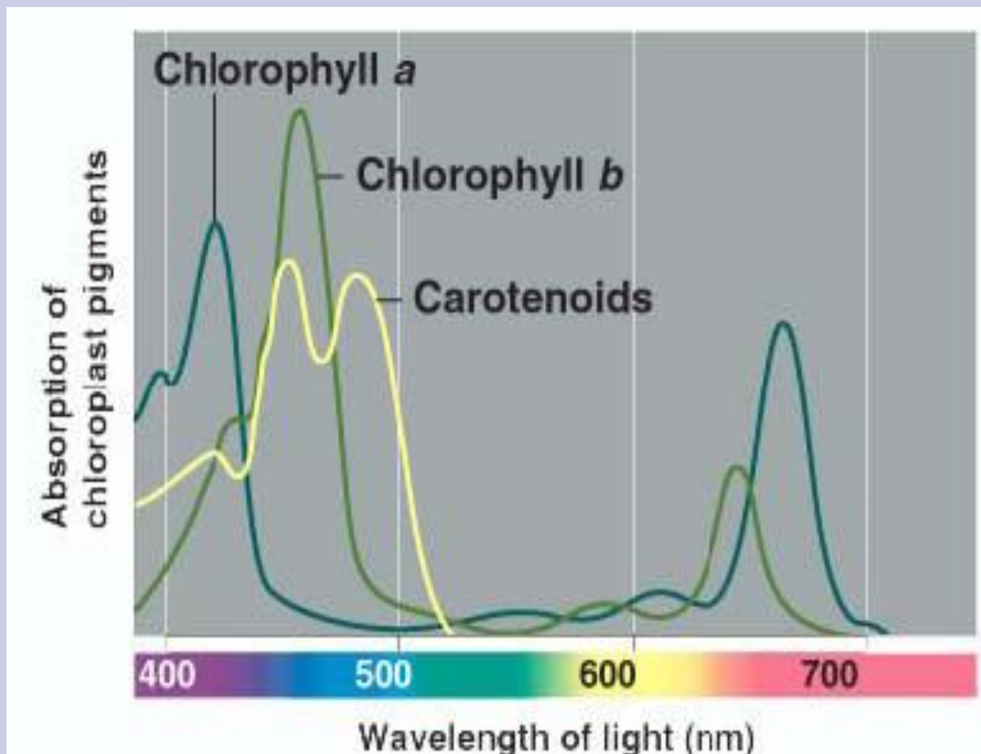
a ray of monochromatic light means a light of the same wavelength.

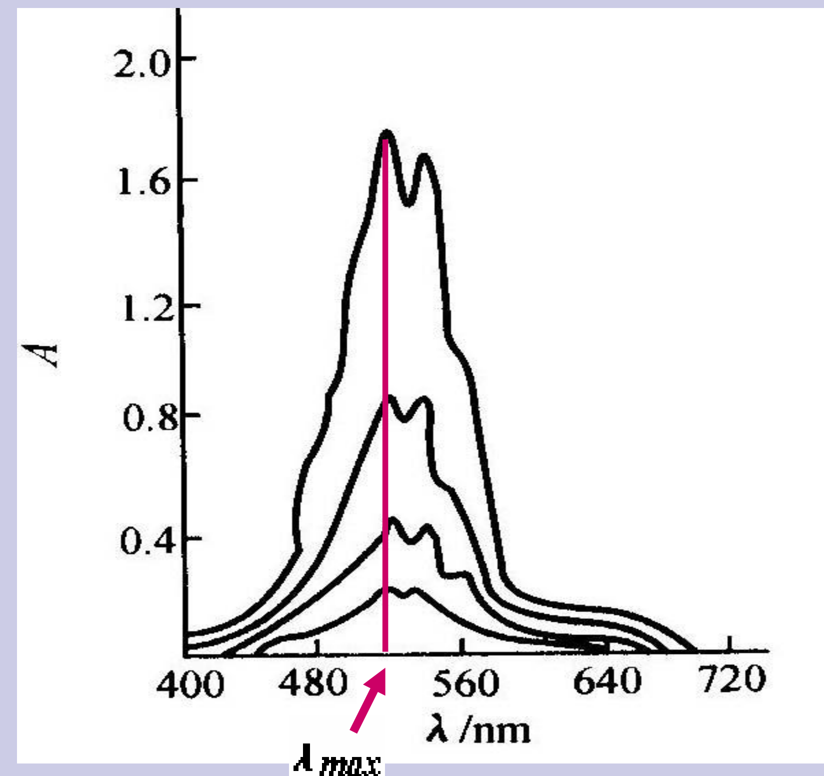
Absorption Spectrum

a plot of **A** vs **λ**,

show how the A of light depends on the λ

and is characterized by the greatest wavelength (*λmax*)





λmax

- *is **characteristic** of each compound and provides information on the electronic structure of a analyte.*
- *obtain the highest sensitivity and to minimize deviations in **quantitative measurement***

The shape of the spectra and λmax

can be used to identify the property of an unknown compounds.

Part two:

Applications of Spectrophotometry ——

determine the Concentration Unknown

(Quantitative measurement)

Laws of Absorption of light

■ Lambert-Beer's Law:

cell path length

L

$A = -\lg T$

monochromatic light
(incident light)

I_0

I_t

Transmitted light

Absorbance

A

Transmittance

$$A = k \times C \times L$$

uniform

$$T = \frac{I_t}{I_0}$$

When a ray of monochromatic light passes through an uniform absorbing solution , Absorbance of the solution (also know as Optical Density or Extinction) is directly proportional to the concentration of the substance and the depth of the solution through which the light passes (cell path length).

- The effect is measured either as Transmittance (T, the percentage of light that goes through the sample) or as the Absorbance (A, the amount of light absorbed by the sample):

$$T = I_t / I_o$$

$$A = -\lg T = \lg 1/T$$

Equation for Lambert-Beer's Law

$$A = \lg\frac{I_0}{I} = \lg\frac{1}{T} = k \times C \times L$$

I_0 is the intensity of the incident light

I is the intensity of the transmitted light

k is the constant that depends on the absorbing substance and wavelength of light

L is **cell path length**

T is the transmittance of the light

ε

- **Molar extinction coefficient**

When length 'L' is in 1 centimeter and concentration 'C' = 1mol/L, the absorbance is equal to 'ε'

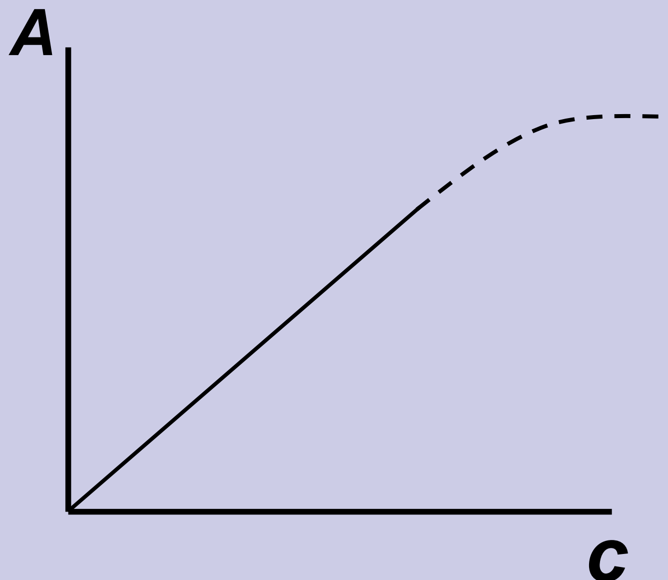
usually written as $\varepsilon^{1mol/L}1cm$ and dimension of $1mol^{-1}cm^{-1}$.

Prerequisite of Lambert-Beer's Law

- Monochromatic light
- λ_{max}
- Solution is uniform and stable
- solution concentration is not too high （A=0.15 ~ 0.7）

Deviation of Lambert-Beer's Law

If the condition of Lambert-Beer's Law is not obeyed



Deviation of calibration curve

a non-linear plot is showed

How to determine Concentration Unknown?

- ***The assays are most sensitive at the extinction peak (absorption) of the substance.***
- ***The absorption produced is due to the test substances (specific absorbance), not by all the solvent and compounds in the reagents (nonspecific absorbance).***

Requirements of Absorptiometric Assay

- **Blank solution** : contains all other chemicals in the standard and test solution and undergoes the same stages but **without the assayed substance.** This will help to exclude the absorption due to reagents.
- **Standard solution :** contains all the reagents of test or blank but it also includes a solution of **known concentration of the substance** which is going to be determined in the test tub.
- **Test solution :** contains almost all the reagents as present in the blank and standard and undergoes the same steps, but it **contains an unknown quantity of the substance.**

Two Methods of Quantitation

1 -----Standard addition method

The A of the **standard solution** and **test solution** are measured .

According to **Lambert-Beer's Law** ,

$$\left.\begin{array}{l} A_S = k_S \times C_S \times L_S \\ A_t = k_t \times C_t \times L_t \end{array}\right\} \frac{A_t}{A_S} = \frac{C_t}{C_S}$$

A_S

A_t

*A_t is the **specific absorbance** of the test substance

*A_S is the **specific absorbance** of the same substance

$$C_t = \frac{A_t}{A_S} \times C_S$$

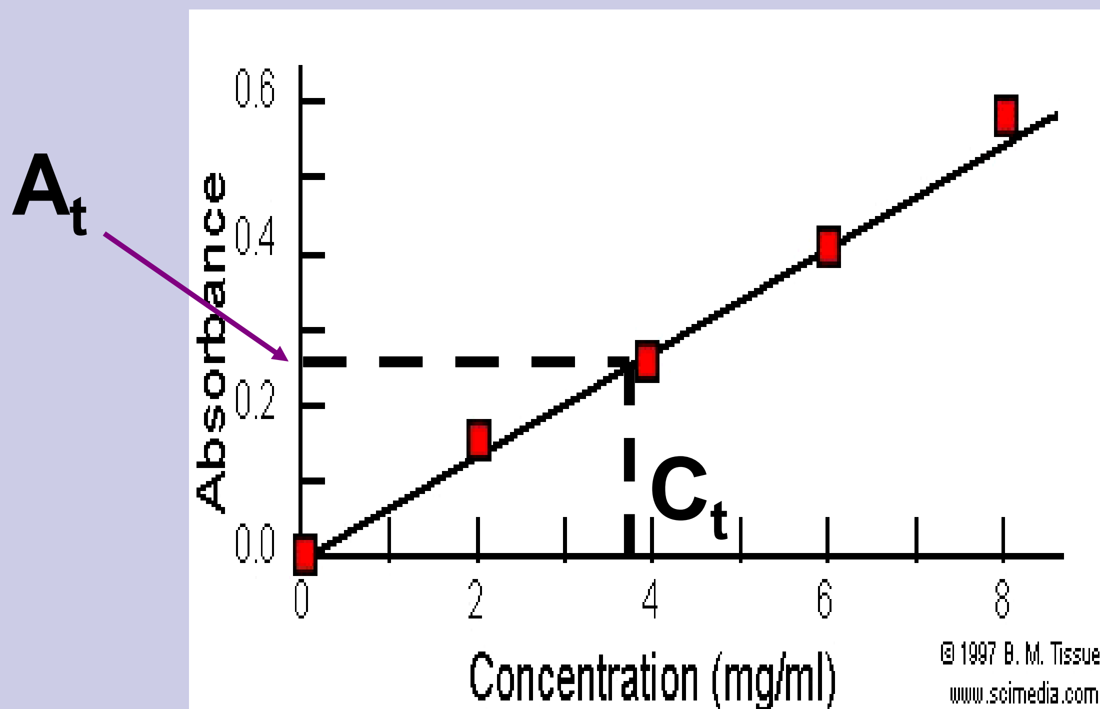
2 -----calibration curve method

Calibration curve (also called working curve) shows how A changes with the C of a solution.

The curves are obtained by measuring the signal from a series of standards of known concentration.

* The curves are used to determine the C unknown or to calibrate the linearity of an assay.

- Prepare a series of standard solutions
- Measure A of the standard solutions
- Prepare a calibration curve (a plot of A vs C).
- The A of the unknown solution is used in conjunction with the calibration curve to determine the C of the analyte.



A perfect calibration curve

- Assays should normally be performed in **duplicate**.
- There must be **at least five points**.
- It must **pass the origin**.
- It must be **beeline**, and **through the points**.
- Calibration curves should never be extrapolated beyond the highest A measured;
- **A=0.05~0.8**
- The reproducibility of the method is important.

Measurement of Light Absorption

Components of Spectrophotometer

- *Source of Light*
- *Monochromator – a device for selection of a band (wave-length) of light*
- *Sample Container - Cuvette*
- *Detector for unabsorbed radiant energy*
- *Associated read out meters*

STRUCTURE OF SPECTROPHOTOMETER

QUANTITATIVE DETERMINATION OF PROTEINS

(Lowry Method , Folin-Phenol Method)

Quantitative Determination of Proteins

- *There is no completely satisfactory single method to determine the concentration of protein in any given sample*
- *The choice of the method depends on the nature of the protein, the nature of the other components in the protein sample, desired speed, accuracy and sensitivity of assay*

The quantitative analysis of proteins includes absorption measurements in the ultraviolet and in the visible range.

- **Absorption measurement at 280 nm**
- **Colorimetric determination at 550 to 600nm**

Methods Used for Protein Determination

- *The Kjeldahl method*
- *UV Spectrophotometry*
- *Biuret Test*
- *BCA (Bicinchoninic Acid) Assay*
- *Bradford (Dye-Binding) Assay*
- *Lowry (Folin-Phenol Method) Assay*

Absorption measurement at 280 nm (A_{280})

- Absorption of UV *(A_{280})* by proteins depends on the **Tyrosine** and **Tryptophan** content (and a very small extent on the amount of Phe and disulfide bonds).

Therefore A_{280} varies greatly between different proteins.

- Advantages : simple; rapid; the sample is recoverable.
- Disadvantages:

may be disturbed by non-proteins (e.g. DNA/RNA). So should be pure protein solutions

less sensitive (concentrations > 4 mg/ml) and requires higher protein concentrations.

Colorimetric determination

- The dye complex was produced by the reactions of functional groups of the proteins and dye-forming reagents.
- The intensity of the dye complex correlates directly with the concentration of the reacting groups .

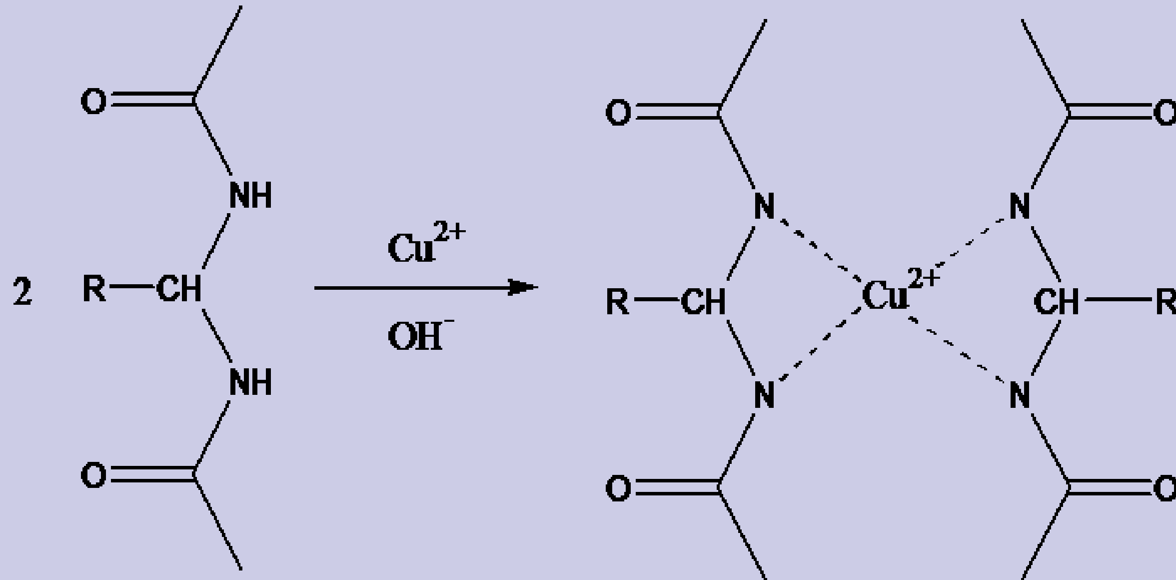
Colorimetric determination---1
Lowry assay (at 650 nm)

Principle:

- Under alkaline conditions ,copper complexes with protein ---Protein- Cu^{2+} compounds .
- The residues of tyrosine and tryptophan in protein-Cu^{2+} compounds can make phosphomolybdic acid-phosphoyungstic acid in phenol reagent lose one or two oxygen atom and let them reduce to blue compounds.
- The degree of blue has a direct proportion to content of the protein.

Lowry (Folin-Phenol Method) Assay

1.



2.

Tyrosine, Tryptophan & Cysteine → Molybdenum/Tungsten blue

Folin-Ciocalteu reagent
(phosphomolybdate and phosphotungstate)

- **Interfering substance**:

  barbital, CHAPS, cesium chloride, citrate, cysteine, diethanolamine, dithiothreitol, EDTA, EGTA, HEPES, mercaptoethanol, NP-40, phenol, polyvinyl pyrrolidone, sodium deoxycholate, sodium salicylate, thimerosol, Tricine, TRIS and Triton X-100.

- **Detectable range**: 10~1000 μg/ml

EXP. 1

Quantitative analysis of serum proteins

Lowry assay (650nm)

- **Folin-Phenol method is one of the sensitive and widely-used protein assays, being first described in 1951.**
- **Objective:**

Master how to determine the concentration of protein in serum

Understand the principles of Lowry method etc. and distinguish the advantage and disadvantage.

1. To treat serum sample

0.1ml Serum is accurately diluted to 50ml by 0.9% NaCl .

Times of dilution= ?

2. Quantitative analysis of serum proteins

① **According to A of samples, C of serum protein can be looked up in the Calibration curve.**

② **Serum protein concentration can also be calculated according to the following formula.**

Serum protein concentration (g/L)

$$= At/As_3 \times Cs_3 \times \text{Times of dilution}$$

Procedure

7 test tubes are used and the operation is done according to the following table

Reagents (ml)	Blank	1	2	3	4	5	diluted sample
Protein standard solution (250μg/ml)	----	0.1	0.2	0.3	0.4	0.5	0.5
0.9%NaCl solution	0.5	0.4	0.3	0.2	0.1	----	----
Alkaline copper sulfate reagent	2.5	2.5	2.5	2.5	2.5	2.5	2.5

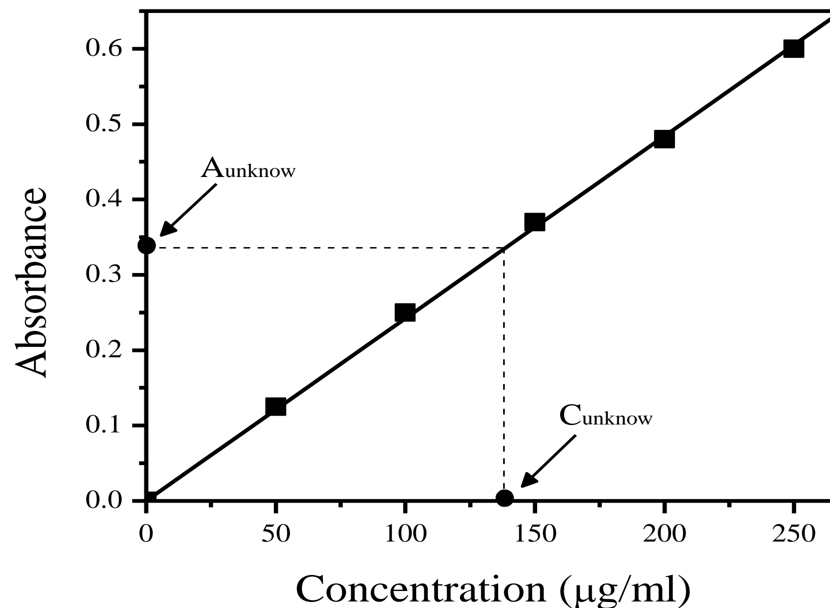
Mix the contents of tubes, incubate 20 min at room temperature

Phenol reagent	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Equal protein concentration (ug/mL)	0	50	100	150	200	250	?

After Folin-phenol reagent is added into the tubes for 30 min, A is read at wavelength 650 nm on condition that A of blank tube is adjusted to zero.

Quantitative analysis 1-- Calibration curve

- draw a **Calibration curve** shows how absorbance changes with the concentration of a solution .
- According to absorbance of samples, concentrations of serum protein can be looked up in the standard curve.



Quantitative analysis 2--**Standard addition method**

According to **Lambert-Beer's Law** ,

$$\left.\begin{aligned} A_S &= K_S \times C_S \times L_S \\ A_t &= K_t \times C_t \times L_t \end{aligned}\right\} \frac{A_t}{A_S} = \frac{C_t}{C_S}$$

$$C_t = \frac{A_t}{A_S} \times C_S$$

How to handle a pipette and rubber bulb

- **Classified according to the accuracy and scales, read the volume marks on the stem**
- **One (pipette/tip) to one (solution)**
- **Immerse the pipette into the fluid and fill it by suction using rubber bulb, holding it vertically while adjusting the lowest liquid level to the calibrating line when it is sighted at eye level.**
- **When dispensing the fluid, the tip should be touched to the inclined surface of the receiving container.**
- **Blow out to deliver (slow) when the total volume of the pipette is less than 1.0 mL.**

Colorimetric determination----2

Biuret assay

- **Principle:** The reactivity of the peptide bonds with Cu^{2+} under alkaline conditions to form purple biuret complex.
- **Interfering substance:** Ammonium sulfate, Tris, etc.
- **Sensitivity:** >mg/L

Biuret Test

2 R—CH (peptide chains) $\xrightarrow[OH^-]{Cu^{2+}}$ Biuret complex

Peptide Chains

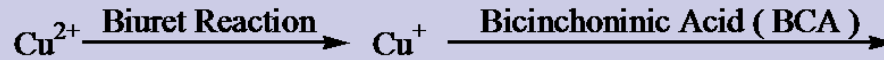
Biuret Complexes (purple color)

The Biuret Method is the most widely used method for protein determination , relies on the complexation of Cu^{2+} by the function groups involved with the peptide bond. Upon complexation, a purple color is observed. The absorbance of the Cu^{2+}-protein complex is measured at 540 nm.

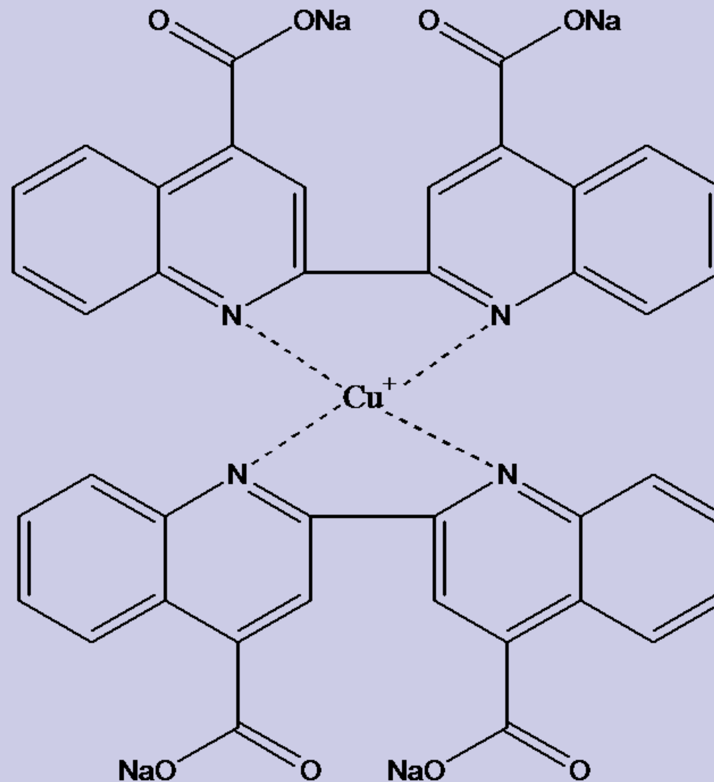
Colorimetric determination----3
Bicinchoninine acid assay 562nm (BCA)

- Very sensitive and rapid if you use elevated temperatures （60°C for 15 min）
- Compatible with many detergents
- Working reagent is stable
- Very little variation in response between different proteins
- Broad linear working range

BCA (Bicinchoninic Acid) Assay



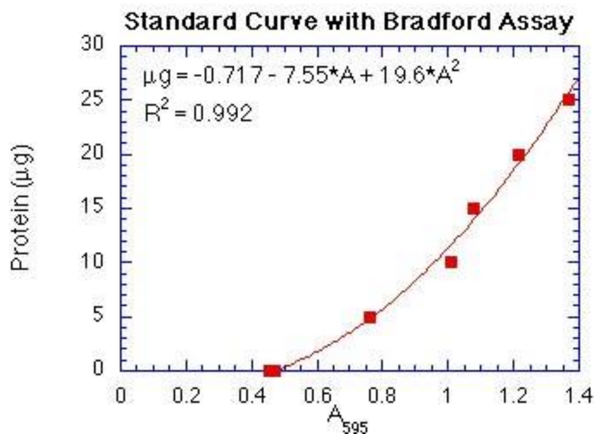
incubate the tubes at 60°C for 15 min.

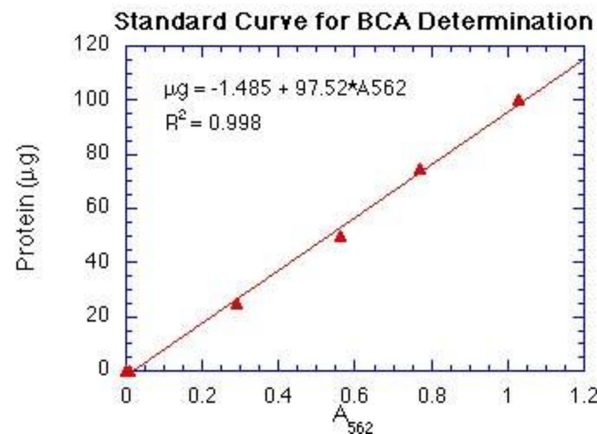


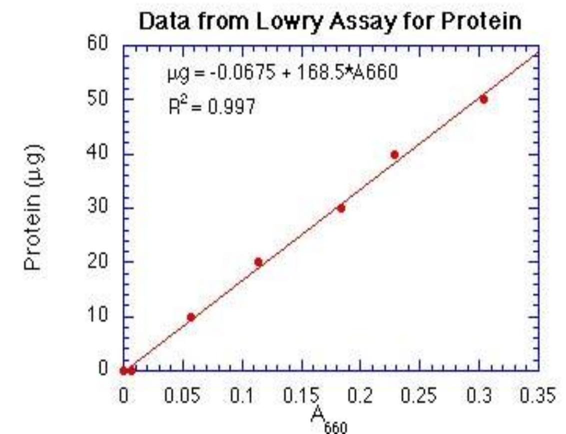
cool to room temperature and determine A_{562} .

Colorimetric determination----4
Bradford assay 595 nm

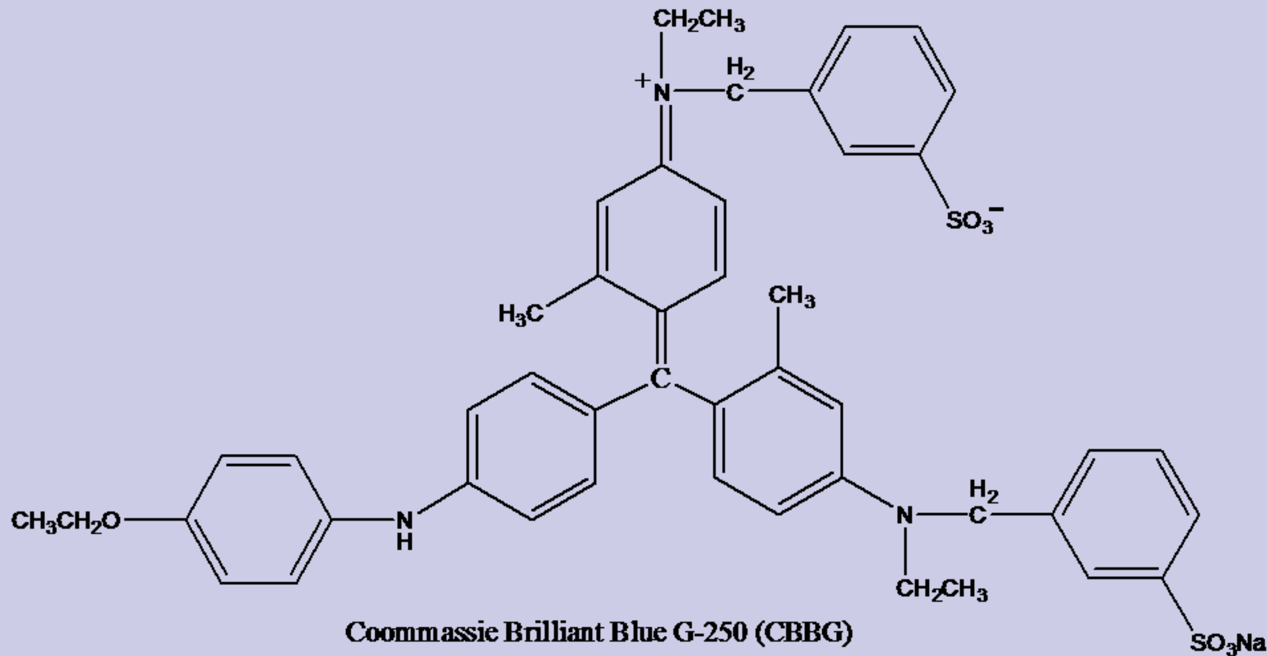
- Absorption spectra of anionic and cationic forms of the dye overlap.
- The assay performs linearly over short concentration stretches.



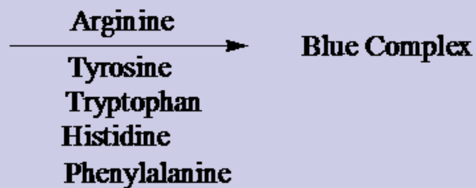




Bradford (Dye-Binding) Assay



Coommassie Brilliant Blue G-250 (CBBG)



- CBBG primarily responds to arginine residues (eight times as much as the other residues)

5 Special Binding Groups (heme)

4 Coomassie Blue G

Arg

Lys

Fe

(metal)

Active Site

Cu^{2+}

Tyr

206 nm (carbonyl)

3 280 nm (aromatic)

UV Absorbance

Biuret Method (carbonyl) 1

phsophomolybdic-phosphotungstate

Lowry Method 2