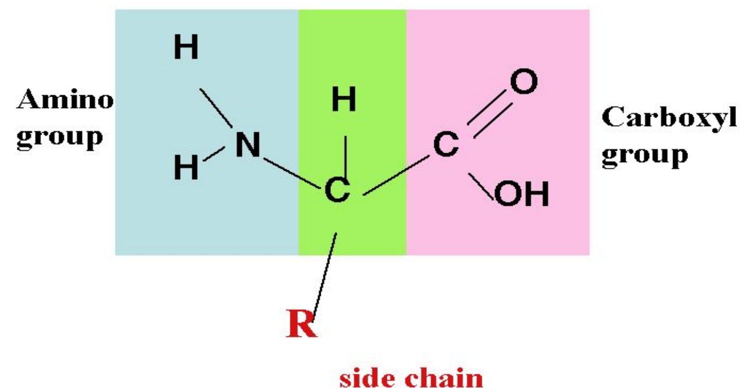
QUANTITATIVE DETERMINATIONS

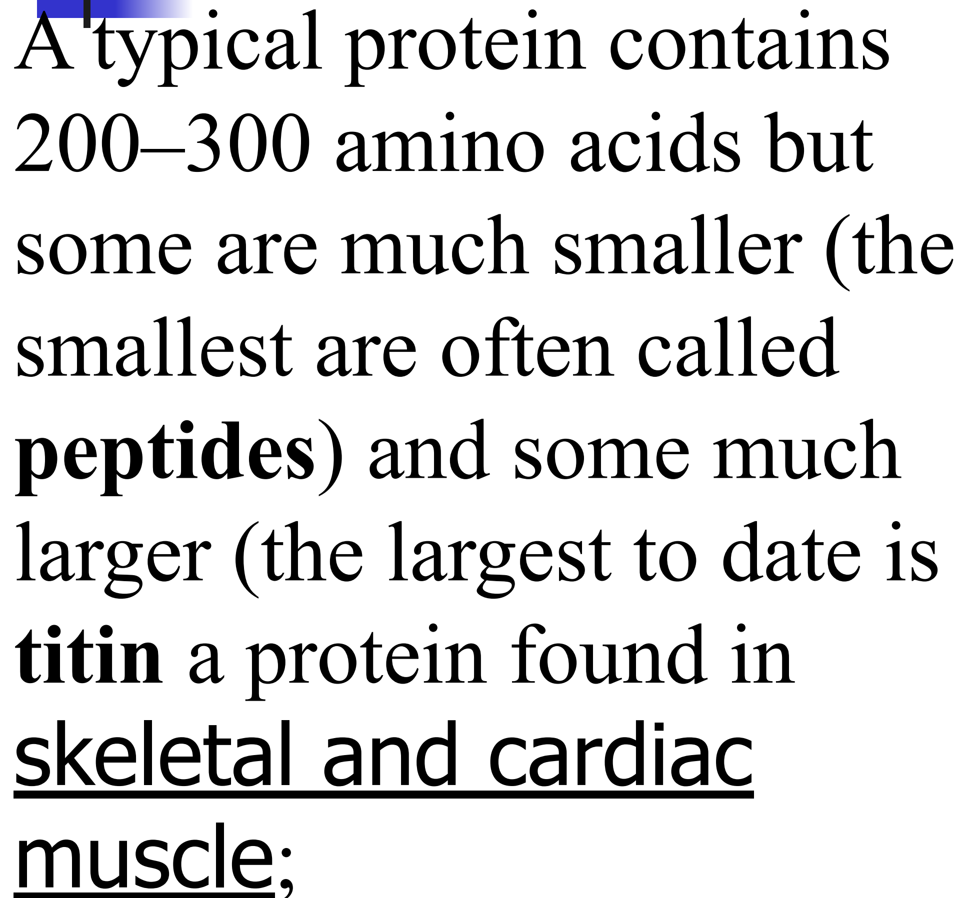
- INTRODUCTION
  - PROTEIN TYPES
  - PROPERTIES
  - QUALITATIVE TESTS
- ESTIMATION METHODS
  - COLORIMETRIC METHODS
  - SPECTROSCOPIC METHODS
  - CHROMATOGRAPHIC METHODS

INTRODUCTION

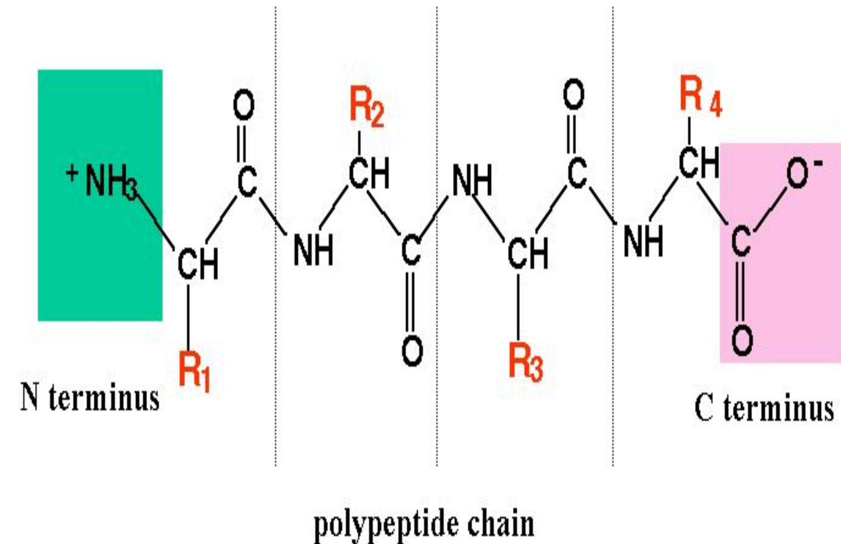
Proteins are highly complex natural compounds composed of large number of different alpha amino acids.

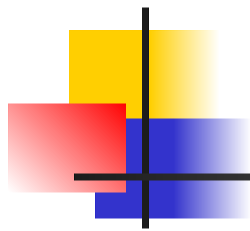
Proteins are large molecules and can be split into smaller units by hydrolysis-amino acids.





- A typical protein contains 200–300 amino acids but some are much smaller (the smallest are often called **peptides**) and some much larger (the largest to date is **titin** a protein found in skeletal and cardiac muscle;





- The protein consists of two polypeptide chains, a long one on the left of 346 amino acids — it is called the **heavy chain** — and a short one on the right of 99 amino acids.

Serine + Valine + Tyrosine + Cysteine

Peptide bond

Polypeptide

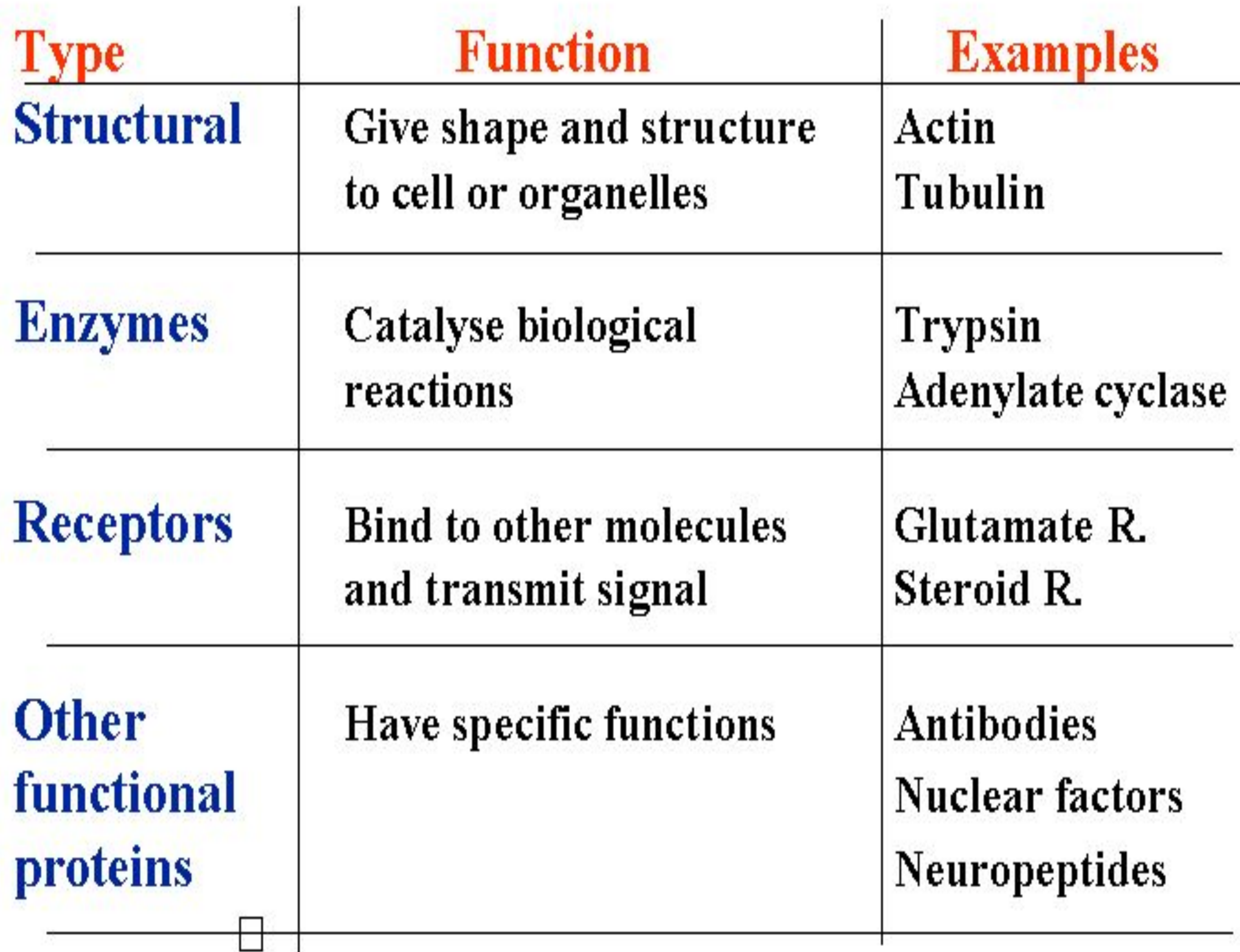
DEHYDRATION SYNTHESIS

Peptide bond

Serine Valine Tyrosine Cysteine

HYDROLYSIS

Protein types

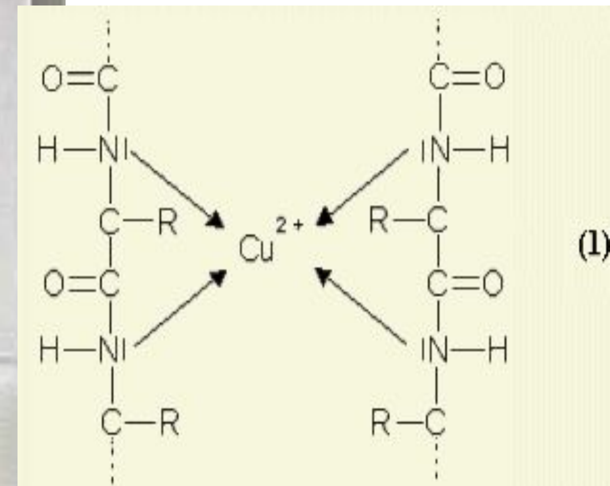


Type	Function	Examples
Structural	Give shape and structure to cell or organelles	Actin Tubulin
Enzymes	Catalyse biological reactions	Trypsin Adenylate cyclase
Receptors	Bind to other molecules and transmit signal	Glutamate R. Steroid R.
Other functional proteins	Have specific functions	Antibodies Nuclear factors Neuropeptides

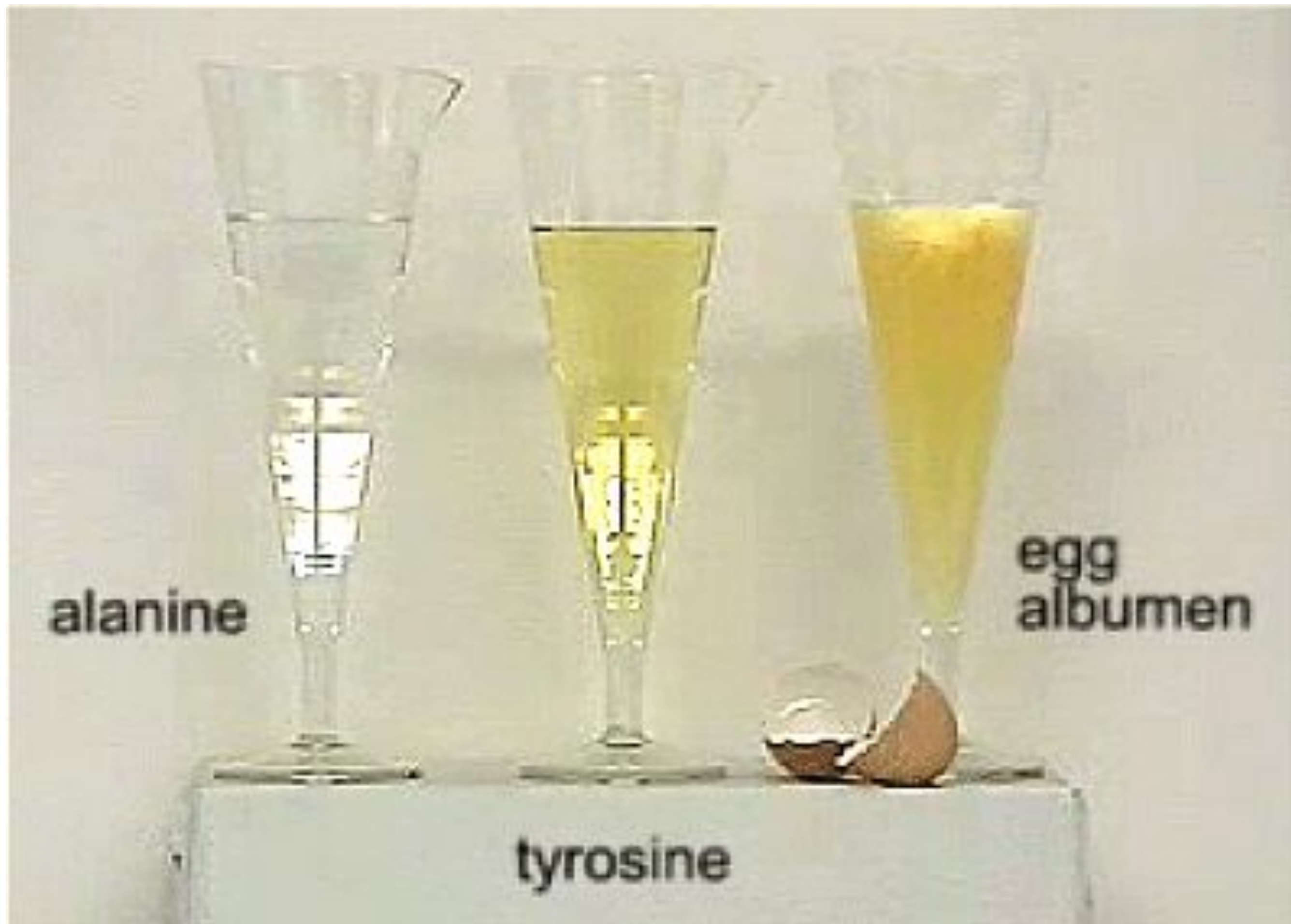
- Solubility
- Molecular weight
- Shape
- Isoelectric pH
- Denaturation of proteins

QUALITATIVE TESTS

Biuret Reagent + glycine + egg albumen

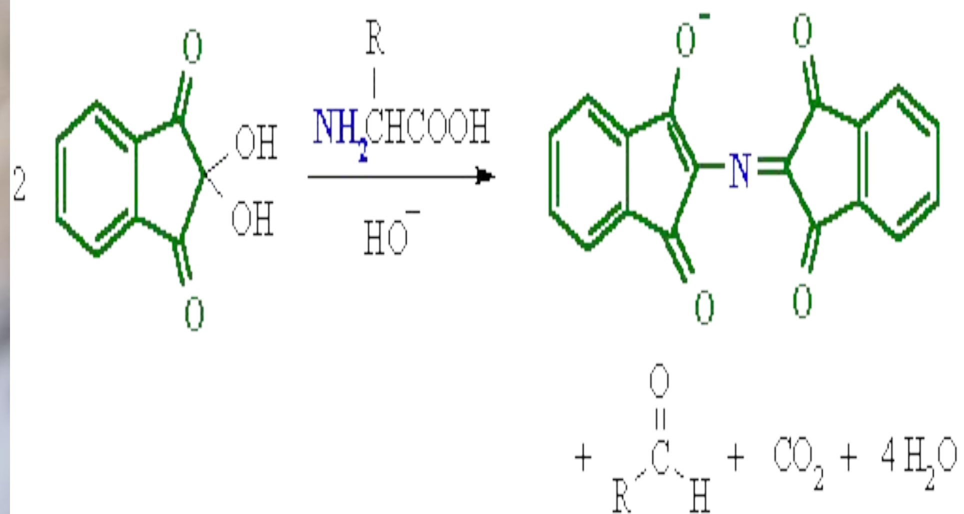


XANTHOPROTEIC TEST



MILLONS TEST

NINHYDRIN TEST



METHODS OF PROTEIN ESTIMATION

- Biuret method
- Bradford method
- Folin- Lowry method
- Kjeldahl method
- Bicinchoninic method
- UV method
- Flourimetric method
- Mass Spectrometry

BIURET METHOD

Principle:

Under alkaline conditions substances containing two or more peptide bonds form a purple complex with copper salts in the reagent.

Equipment:

In addition to standard liquid handling supplies a visible light spectrophotometer is needed, with maximum transmission in the region of 450 nm. Glass or polystyrene (cheap) cuvettes may be used.

REAGENTS:

- Sodium potassium tartrate
- Copper sulfate
- Potassium iodide
- 0.2 M NaOH

PROCEDURE

Pipette out a series of tubes 0.1,0.2…1ml of protein solution

Make up to 4ml with water

6ml of biuret reagent added to each tube

Mix well

Heat the tubes at 37°c for 10 min

Purple color develops

Absorbance -520nm

Analysis

Prepare a standard curve of absorbance versus micrograms protein (or *vice versa*), and determine amounts from the curve.

BRADFORD METHOD

PRINCIPLE:

The assay is based on the ability of proteins to bind Coomassie Brilliant Blue G-250 and form a complex whose extinction coefficient is much greater than that of the free dye.

Equipment:

In addition to standard liquid handling supplies a visible light spectrophotometer is needed, with maximum transmission in the region of 595 nm, on the border of the visible spectrum. Disposable cuvettes may be used.

Reagents:

- Bradford reagent
- Dye concentrate
- Phosphate buffered saline

PROCEDURE:

Prepare a series of protein samples up to 100µl

Add 5ml of diluted dye

Mix well

Red dye turns blue within 5 min

Absorbance -595nm

PRINCIPLE:

The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteay phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids.

REAGENTS

A. 2% Na_2CO_3 in 0.1 N NaOH
B. 1% NaK Tartrate in H_2O
C. 0.5% $CuSO_4.5 H_2O$ in H_2O
D. 48 mL of A, 1 mL of B, 1 mL C
E. Phenol Reagent - 1 part Folin-Phenol [2 N] : 1 part water

[Reagents A, B and C may be stored indefinitely]

BSA Standard - 1 mg/ mL

Bovine Serum Albumin: 5 mg in 5 mL of water [1 µg / µl]. Freeze 1 mL aliquots.

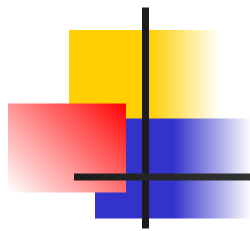
Pipette out 0.2,0.4…1ml of the working standard in to a series of test tubes.

Pipette out 0.1ml and 0.2ml of the sample extract in two other test tubes.

Add 2 mL of solution D to each test tube.

Incubate for 10 minutes at room temperature.

Add 0.2 mL of dilute Folin-phenol solution to each tube.



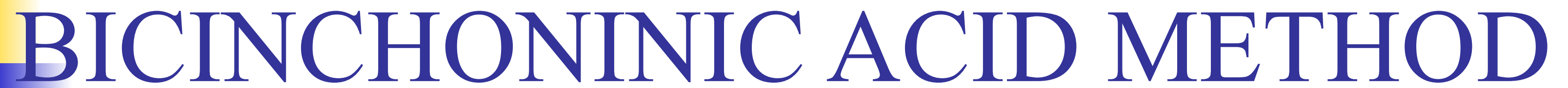
Vortex each tube immediately.

Incubate at room temperature for 30 minutes.

Determine absorbance of each sample at 600 nm.

Plot absorbance *vs* mg protein to obtain standard curve.

Set up triplicate assays for all "unknowns".



BICINCHONINIC ACID METHOD

PRINCIPLE:

It is based on reduction of cupric ion to cuprous ion by the proteins.

- BCA reagent
- Copper sulphate
- Copper-BCA reagent-mix

Pipette out a series of standard and test solutions.

Add 2ml of copper-BCA reagent.

Incubate at 37° for 30mins.

Cool at room temperature.

Absorbance-562nm.

Standard Curve:

r A562nm Standard = A562nm Std - A562nm Blank

Prepare a Standard curve by plotting the r A562nm of the Standard vs µg of protein.

Sample Determination :

r A562nm Sample = A562nm Test - A562nm Blank

Determine the mg of protein using the Standard Curve.

mg Protein = (mg of protein from standard curve) (df)

df = Dilution factor

$$\% \text{ Protein} = \frac{(\text{mg of protein})(100)}{\text{mg solid/ml Reagent A}}$$

KJELDAHL'S METHOD

PRINCIPLE:

The method consists of three basic steps:

1) Digestion of the sample in sulfuric acid with a catalyst, which results in conversion of nitrogen to ammonia;
2) Distillation of the ammonia into a trapping solution; and
3) Quantification of the ammonia by titration with a standard solution

EQUIPMENT:

- Kjeldahl flasks, 500 to 800 Ml
- Kjeldahl digestion unit with fume removal manifold
- Kjeldahl distillation apparatus –
- Kjeldahl flask connected to distillation trap by rubber stopper.
- Distillation trap is connected to condenser with low-sulfur tubing.
- Outlet of condenser should be less than 4 mm diameter. Erlenmeyer flask, 500 mL Analytical balance, sensitive to 0.1 mg

REAGENTS

- Standard HCl solution(0.01N)
- NaOH solution(40%w/v)
- Conc H_2SO_4
- Standard 1% ammonium sulphate solution
- Sodium thiosulphate solution
- $HClO_4$ solution-0.1M
- Methyl red
- Mercuric oxide

PROCEDURE

Sample having(0.03-0.04g N)

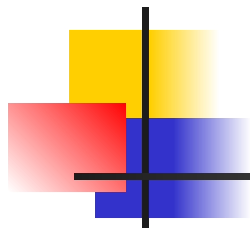
Add 0.7g mercuric oxide and 15g powdered sulphate

Add 40ml conc H2SO4

Heat and boil for 2 hrs

Cool

Add 200ml water and 25ml sodium thiosulphate solution



Mix

Add a piece of granulated zinc

Add suff NaoH solution

Connect to distillation flask

Add the acid

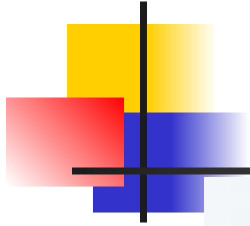
Mix

Add 5 drops of methyl red

- Blank also performed
- **CALCULATIONS:**

  1ml of 0.01N HCl=140µgN

  Protein=amount of nitrogen(s)x6.25



KDN-2C

ULTRAVIOLET SPECTROSCOPIC METHOD

<u>PRINCIPLE:</u>

Absorption of radiation in the near UV by proteins depends on the Tyr and Trp content (and to a very small extent on the amount of Phe and disulfide bonds). Therefore the A280 varies greatly between different proteins (for a 1 mg/mL solution, from 0 up to 4 [for some tyrosine-rich wool proteins], although most values are in the range 0.5-1.5 [**1**]).

PROCEDURE

- The experimental procedure is simple.
- The optical density of the test solution is measured at 260nm and 280nm by using the following formula:
- Protein concentration (mg/ml)=1.55x o.d at 280nm-0.76xo.d at 260nm

PRINCIPLE:

It is based on the derivitization of the protein with o-phthaldehyde(opa) which reacts with the primary amines of the protein.

The sensitivity of the test can be increased by hydrolyzing the protein before testing.

REAGENTS

- Borate buffer
- Stock opa reagent
  - 120mg opa-1.5ml methanol
  - Dissolve
  - Add ml of borate buffer-mix
  - Add 0.6ml of polyoxyethylene lauryl ether-mix
- OPA reagent
  - Add 15ml of 2-mercaptoethanol-5ml of stock opa reagent

PROCEDURE

Prepare a series of test and standard solutions

Take 10µl from above solutions

Add 100µl of opa reagent-mix

Stand for 15 min

Add 3ml of 0.5N NaoH-mix

Absorbance-340nm

High-Performance Liquid Chromatography

- Estimation of total soluble protein in must and wine by high-performance liquid chromatography is achieved with a size exclusion column.
- A protein standard, bovine serum albumin, is eluted from this column with linear response to a concentration of 1 g/L.
- Protein is separated from other UV absorbing components of must and wine and estimated at 280 nm on a Waters Protein Separation System with a 0.1-M ammonium acetate mobile phase containing 10% glycerol.

HPLC METHOD

- Category :food(must and wine)
- Column: size exclusion column
- Standard: bovine serum albumin
- Conc:1g/L
- Mobile phase:0.1M ammonium acetate
- Absorbance-280nm