5. QUALITATIVE AND QUANTITATIVE ANALIYSIS OF AMINO ACIDS AND PROTEINS

Amino acids are colorless biomolecules that are insoluble in organic solvents except water. Their melting points are very high and they are not volatile. For this reason, if they are heated above their melting points, they will be degraded. Amino acids are present as ionized form in aqueous solutions known as zwitterion ions.

There are about 300 different amino acids in nature. 20 of the amino acids known as "standard amino acids "are encoded by the DNA and form the proteins. There are also amino acids that do not exist in the structure of proteins but have various biological functions in the cell. Standard amino acids consist an amino group and a carboxyl group, which bond to the same carbon atom. They are represented by three-letter abbreviations and one-letter symbols. The amino acids can be classified according to the polarity of the R side chains or their interaction with water at the biological pH (acidic, basic).

"Non-standard amino acids" are the amino acids formed as a result of changes in the structure of the standard amino acids after entering the protein structure.

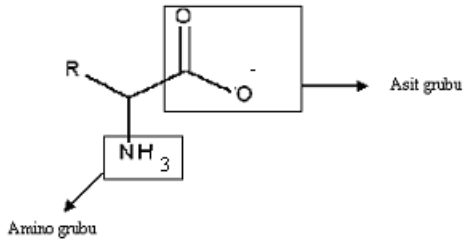
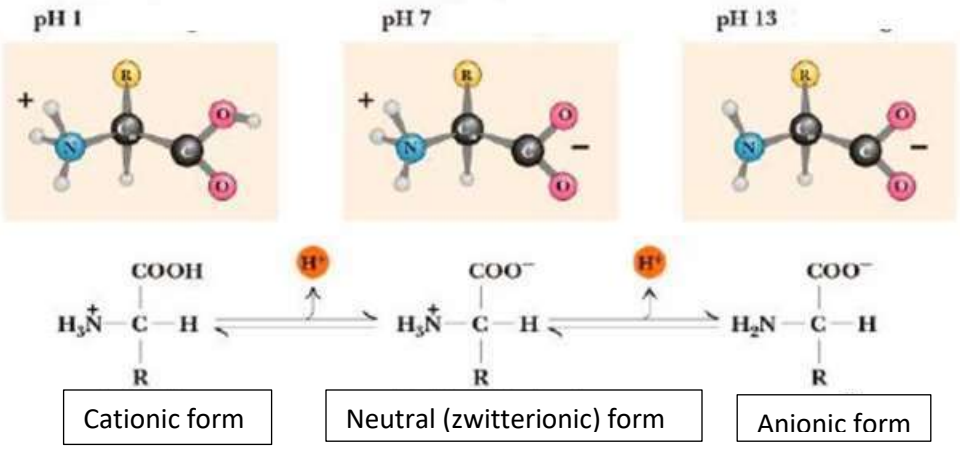


Figure 5.1. Basic formula of an amino acid

5.1 . Isoelectric Point in Amino Acids

Amino acids have both acid and base properties due to amino (-NH 2) and carboxyl (- COOH) groups in their structure. In other words, they are called as amphoteric compounds since they act as base in an acidic environment, and acts as an acid in basic environment. The pH value of an amino acid molecule in the solution where the net charge of that amino acid is zero is called the isoelectric point (pI) and it is a specific value for each amino acid.



Amino acids give characteristic titration curves due to the ionization of both the carboxyl group and the amino group by giving a proton. If an ionizable, side chain free amino acid is titrated, two pKa values are observed; the first value (pKa1) belongs to the α-carboxyl group, the second value (pKa2) belongs to the α-amino group: pKa1 value of the amino acids (pKa of the COOH group) ranges from 1.8 to 2.4; The pKa 2 value (pK of the 'NH$_3$ group) also varies between 8.8 and 11.0. Acid-base titration comprise gradually addition or breaking of the protons.

$$pH = pI = \frac{pK_{a1} + pK_{a2}}{2}$$

Glycine is an apolar amino acid with the formula (Gly or G) NH_2CH_2COOH. It is the simplest of the 20 amino acids found structurally in proteins. It has only one hydrogen atom in its side chain. Since the α-carbon atom in glycine is also hydrogen bonded, glycine is not optically active, ie the optical isomer is absent. Figure 5.2 shows the titration curve of the diprotic form of glycine. At a very low pH, the dominant ionic form of glycine + H_3N-CH_2-COOH is the fully protonated form. The COOH group of glycine at the midpoint of the first stage of titration loses proton. At any titration, the midpoint is reached where the pKa value of the protonated group, which starts to vibrate, is equal to pH. The pH for the glycine is 2.34 in the midpoint. pKa measures the tendency of a group to lose protons, and when this trend decreases by 10 times, pKa increases by one. Another important point in glycine titration is the point at which the pH reaches 5.97. At this point, the separation of the first proton was completed and the separation of the second began. The second phase of titration loses the

proton of the 2NH_{3+} group of glycine and the pH of the medium is equal to the pKa2 value of the NH_{3+} group.

The titration is completed at about pH 12 and at this point the dominant form of glycine is H2NCH2-COO-.

The first important information from the glycine titration curve yields quantitative, pKa measurement values of two ionizable groups of 2.34 for -COOH and 9.60 for 9.NH_{3+}. This effect is due, in part, to electronegative oxygen atoms in carboxyl groups that attract electrons regardless of the charge of the carboxyl group and increase the tendency of amino groups to proton. Therefore, the pKa value of the α-amino group is lower than the value of aliphatic amines such as methylamine.

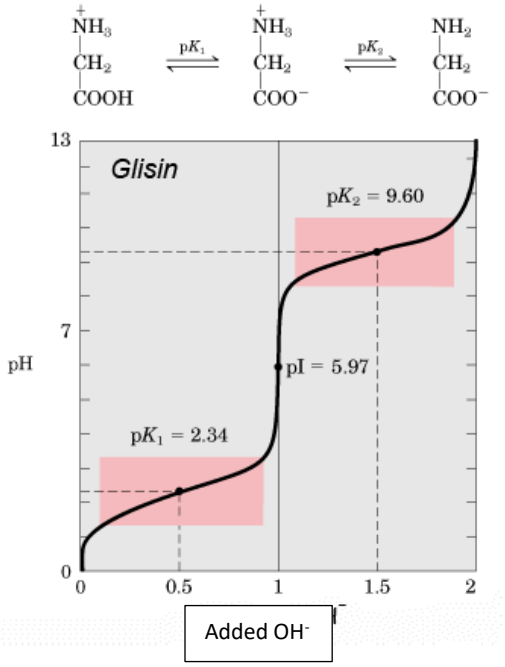


Figure 5.2. Titration curve of Glycine

5.2. Proteins

Peptides are formed via peptide bonds between the α-carboxyl group of an amino acid and the α-amino group of the other amino acid with forming a molecule H_2O. The dipeptide consists of two amino acids, the tripeptide consists of three amino acids, the oligopeptide consists of up to 10 amino acid, and the polypeptide forms with more amino acids.

Proteins are polypeptides that are formed via binding of amino acids by covalent and non-covalent bonds to several characteristic straight chains during a given number of sequences. Covalent bonds in the structure of proteins are peptide bonds and disulfide bonds; non-covalent bonds are hydrogen bonds, ionic bonds, hydrophobic interactions, and Van der Waals interactions.

Proteins have four structures: primary, secondary, tertiary and quaternary.

The primary structure is formed by peptide bonds between amino acids, defined by amino acid sequences. The secondary structure is formed with H bonds between the carbonyl groups and the amide groups in the main chain. The tertiary structure is formed via H bonds in the amino acid side chains, ionic bonds, hydrophobic interactions, disulfide bonds and Van der Waals interactions.

The roles of proteins in metabolism can be listed as follows;

(1) Proteins are the structural component of organs and soft tissues,

(2) They are active in the formation of new tissues in growth in adolescence.

(3) They have the function of repairing damaged tissues,

(4) They exist in the structure of enzymes and hormones,

(5) They play a role in the conduction of nerve stimuli,

(6) Take part in the movement and support for life.

(7) They are used for the protecting of the body against diseases

(8) They are involved in the vascular transport of oxygen and other substances,

(9) They play a role in blood clotting,

(10) Has direct or indirect roles in the keeping water- electrolyte balance.

Under physiological conditions, daily protein excretion in urine is below 150 mg. Detection of protein excretion above this value (proteinuria) should not be ignored in repeated measurements. Protein excretion above this level is generally an important indicator of kidney damage.

In pathological conditions, the most common protein type in urine is albumin, so the term proteinuria that refers the presence of protein in the urine is often used instead of the term "albuminuria".

5.2.1. Blood Proteins

Blood plasma consists of 91% water, 8% organic substances and 1% inorganic substances. Almost all the organic components are protein. The three basic proteins of plasma are albumin, globulin and fibrinogen. There are 4.5 g albumin, 2.5 g globulin and 0.3 g fibrinogen in 100 milliliters plasma.

Albumin: It derives from "albumen" (egg white) sprout out from a Latin word "albus". At the time of the first definition of proteins, the class of proteins that are soluble in water and coagulated in the present of heat is named as it is. As it has similar properties, the most common protein found in the serum is called "serum albumin". Similarly, "ovalbumin in eggs and "lactalbumin" in milk are likewise named. However, they do not have any other similarities with serum albumin. It is the protein that has the lowest molecular weight. Three quarters of the osmotic pressure of the blood is provided by albumin. The osmotic pressure maintains the blood-plasma ratio. Albumin is produced in the liver. Liver produces 60% of blood proteins. In addition, It is found in large amount in tissue fluids, especially muscle and skin, small amounts in tears, sweat, stomach juices and bile. 30-40% of total albumin in the body is in the blood. In addition to carrying fatty acids and various other substances in the blood, the most important function is to make a water compensation between blood and tissue fluids.

Globulin: There are many types of globulins. Globulins can be divided into $-and-\gamma$ globulins via electrophoresis. $-\alpha$ and $-\beta$ globulins bind to various proteins and deliver them where they are needed. In disease, to provide an immunity, substances for defense are synthesized from $-\gamma$ globulins.

Fibrinogen: It has a role in the last step of the blood clotting mechanism. Fibrinogen molecules turn into fibrin fibers and clotting occurs.

5.3. Methods Used for Protein Analysis

Electrophoresis

Chromatography

Protein fingerprint

ELISA "Enzyme-linked immunosorbent assay"

X-Ray Crystallography (XRD)

Nuclear Magnetic Resonance (NMR)

Mass spectrometry (MS)

5.3.1. Chromatographic Methods

Chromatography is widely used in the separation processes of substances that are very similar to their physical and chemical properties.

The basic principle of the chromatography is based on the passing various substances in a mixture through a stationary phase with the help of a mobile phase along with moving at different speeds during this transition.

- Stationary phase: "Solid" or "liquid layer impregnated on the solid support".
- Mobile Phase: Comprise from “a liquid” or “a gas”

There are various chromatographic methods. Some of those are column chromatography, thin layer chromatography (TLC), paper chromatography, gas chromatography, ion exchange chromatography, gel permeation chromatography, high-pressure liquid chromatography and affinity chromatography.

Paper Chromatography: In this method, a thick filter paper acts as a support; and the water in its pores acts as "liquid phase". The mobile phase is a convenient liquid or liquid mixture placed in a running tank.

The solvent running on the paper drags the components in the mixture at different speeds in relation to their interest in the stationary phase and separates them from each other. On a paper or plate, the ratio between the distance traveled by the component and the distance of the solvent reaches after a certain time is known as the Rf value and is used in the qualitative analysis.

5.4. Experimental Methods

Qualitative Determination of Amino Acids

Experiment 5.4.1. Ninhydrin Reaction

Principle of the experiment: It is based on the principle that amino acids form purple complex with ninhydrine.

Ninhydrin is a strong organic oxidant. Amino acids get oxidative deamination and decarboxylation by ninhydrin; aldehyde, ammonia and carbon dioxide are formed from amino acids while the ninhydrin is reduced. The resulting reduced ninhydrin also reacts with nonreduced ninhydrin and ammonia to form a purple complex;

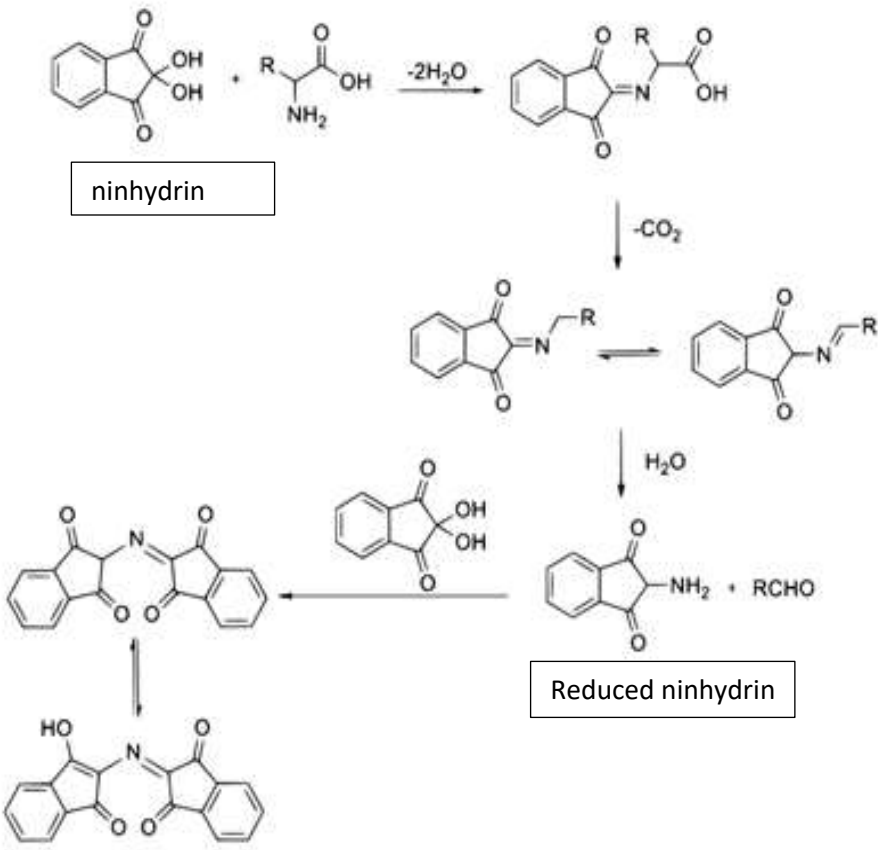


Figure 5.3 Ninhydrin reaction

Experimental Method:

Phase I (Preparation of egg-albumin): Take 1 egg whites into a 250 mL beaker and add 100 mL of distilled water and boil it in very low flame. In the meantime, avimycin aggregate in the egg white. After cooling, the upper solution is used.

Phase II: 2 mL of egg albumin solution is added to a test tube, 0.5 mL of ninhydrin solution is added afterwards. Let it in boiling water bath for 10 minutes. The tube is rest for cooling and the formation of blue-violet color is observed.

Solutions:

Ninhydrin solution: Prepare 0.1 % Ninhydrin solution in 100 mL ethyl alcohol.

Experiment 5.4.2. Xanthoprotein Assay

Principle of the experiment: It is generally based on the yellow colored formation of the amino acids containing aromatic rings such as phenylalanine, tyrosine, tryptophan, and proteins via nitrosylation with nitric acid and the conversion of the yellow colored derivative into an orange color with the addition of base.

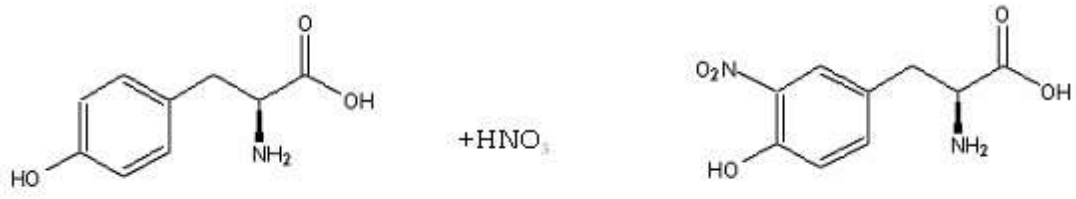


Figure 5.4 The reaction of tyrosine with nitric acid

Experimental Procedure: 2 mL of egg albumin solution is put into a test tube, then 1 mL of concentrated nitric acid is added carefully, and mix them, as a result white precipitate is observed. The tube is heated in a water bath until a yellow precipitate is formed. It is then cooled under tap water. Finally by adding dropwise of 33% NaOH, the color of the sediment changes from yellow to orange.

Experiment 5.4.3. Biuret Experiment

Principle of the experiment: It is based on formation of characteristic violet complexes with proteins and Cu^{2} + ions in alkaline environment due to their peptide bonds. Free amino acids do not give complex with the biuret solution because they do not have peptide bonds.

Experimental Procedure: 3 mL of egg albumin solution is put into a test tube. Add 1 mL of 2.5 N NaOH to the mixture. 2-3 drops of $CuSO_4$ solution are added dropwise. The formation of violet color is then observed.

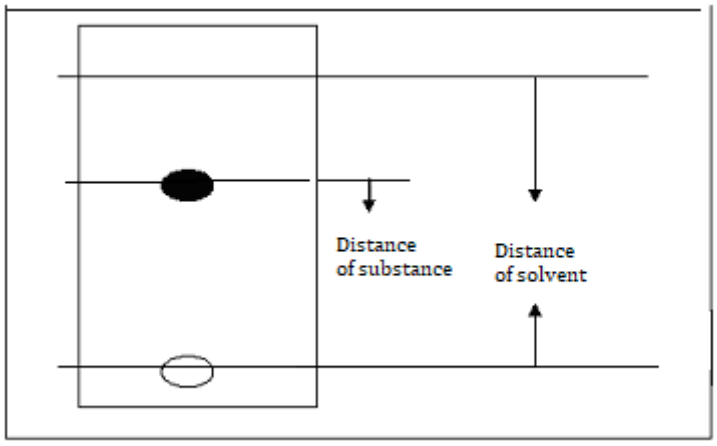
Solutions:

2.5 N NaOH: 10 g NaOH / 100 ml distilled water

0.01 M $CuSO_4$: 0.16 g $CuSO_4$ / 100ml distilled water

Experiment 5.4.4. Qualitative Determination of Amino Acids by Paper Chromatography

Principle of the experiment: It is based on the moving of various materials at different speeds on a stationary phase with the aid of a mobile phase.



$$Rf = \frac{Distance\ of\ substance}{Distance\ of\ solvent}$$

Figure 5.6 Calculation of Rf value.

Experimental Procedure:

A neat line (starting line) is drawn with the pencil 2 cm from the short edge on Whatman chromatography paper. A certain amount of the samples containing the amino acid mixture and the unknown amino acid mixture at intervals of 2 cm each is loaded and dried. The solution used in this process should not exceed an area of 0.5 cm diameter. The dried chromatography paper is then immersed in the solvent system so that the starting line is out. The solvent is removed from the system and then dried again, after the solvent system has been carried out until a distance of 2 cm is on the top of the paper. After the drying process, ninhydrin is sprayed on it, and it is placed in oven at 70-80 ° C until the separated amino acid stains are formed. Stains are marked and the Rf value is calculated for each item. The amino acids in known solution is compared with the stains in the unknown solution and, its Rf values to determine the amino acids in the unknown solution.

Solutions:

Arginine solution: 5 mg / mL solution

Proline solution: 5 mg / mL solution

Phenylalanine solution: 5 mg / mL solution

Solvent: n-butanol / acetic acid / water (16: 4: 20)

Ninhydrin: 0.3% solution in acetone

Experiment 5.4.5. Determination of Isoelectric Point of Glycine

Experimental Procedure: 10 ml of 20 mL of 0.1 N Glycine solution is added to beaker, and its pH is measured by pH meter. Then, it is titrated with 0.1 N HCl. During this process, pH is measured in every addition of 1 mL of acid. When pH relatively remains constant, this addition process is finished. After that, the electrode of the pH meter is washed with distilled water. When it is dried, another 10 mL of the prepared 0.1 N Glycine solution is added and the pH is measured again by pH meter. This time, it is titrated with 0.1 N NaOH. During this process, pH is again measured in every addition of 1 mL of alkaline. The process is continued until the pH remains relatively constant.

The resulting values are saved. Graphs are drawn by using the millimeter graph paper to add the acid and the added alkaline volume to the x axis and the measured pH values to the y axis.

Solutions:

0.1 N Glycine solution: 0.710 g glycine /100ml distilled water

0.1 N NaOH: 0.4 g NaOH / 100 ml distilled water

0.1 N HCl: Add 0.8 mL of a 37% HCl solution to 99.2 mL distilled water

Quantitative Determination of Amino Acids

Experiment 5.4.6. Lowry (Folin-Phenol) Method

Principle of the experiment: Cu^{+2} ions in alkaline medium forms a complex with peptide bonds in proteins and Cu^{+2} ions are reduced to Cu^{+}. The amino acids Tyrosine, Tryptophan and Cysteine, which are in the side chain of the reduced copper and proteins reduce the Folin-Phenol reactive and cause color formation. The intensity of the resulting color is directly proportional to the protein concentration and is measured spectrophotometrically at 750 nm.

Experimental Procedure: The standard solutions are prepared according to the table with BSA (Bovine serum albumin) and rested for 30 minutes. The intensity of the blue color is measured at 750 nm. The sensitivity this method is approximately 10-200 μg protein / mL. The concentration of the unknown sample (μg / mL) is plotted against the A_{750} values of the standards.

Preparing standard calibration graph

Tube	**Standard protein μL**	**H_2O μL**	**Low. Reak. (C) mL**	**Low. Reak. (D) mL**
Blank	0.00	500	2.5	0.25
Standard 1	100	400	2.5	0.25
Standard 2	250	350	2.5	0.25
Standard 3	500	300	2.5	0.25
Standard 4	750	250	2.5	0.25

Determination of the concentration of the sample

Tube	**Sample μL**	**H_2O μL**	**Low. Reak. (C) mL**	**Low. Reak. (D) mL**
Sample	200	300	2.5	0.25

Solutions:

Standard protein: 1 mg BSA / mL

Lowry reactive (A): Dissolve 20 g Na_2CO_3 in 1 L 0.1 N NaOH (4 g NaOH / L). (Store at 4 ^{0}C in a polyethylene bottle)

Lowry reactive (B1): 2 g of $CuSO_4.5H_2O$ in 100 mL H_2O (Store at 4 ^{0}C)

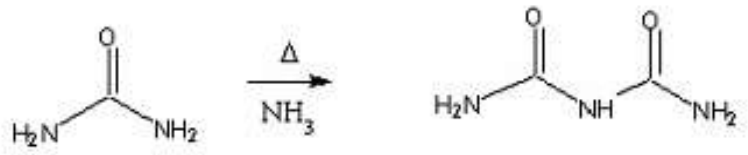
Lowry reactive (B2): 2 g $NaKC_4H_4O_6.4H_2O$ in 100 mL H_2O (Store at 4 ^{0}C)

Lowry reactive (C): B_2: B_1: A is mixed in a ratio of 1: 1: 100 (should be prepared fresh).

Lowry reactive (D): 2N Folin reactive is diluted with water to 1N (stored in a dark colored bottle).

5.4.7. Biuret's Test

Principle of the experiment: It is based on the interaction of Cu^{2+} ions with ammonia compounds (amino acids, peptides, proteins and biuret) to form a blue-purple complex in basic environment. The protein and peptides form a colored complex of Cu2 + from the *biure* reagent with 4 nitrogen atoms involved in the formation of two peptide bonds.



Urea Biuret

Figure 5.7. Biuret formation reaction

The resulting complex gives maximum absorbance at the wavelength of 540-560 nm.

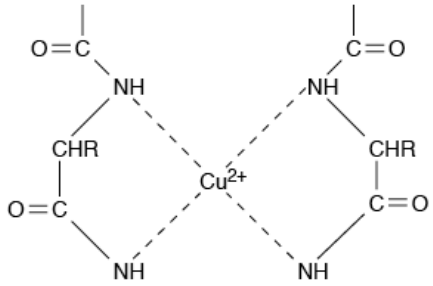


Figure 5.8. Biuret complex formation (pink-violet color)

Experimental Procedure: Three test tubes are taken and marked as blank, standard and sample

Solutions	**Blank**	**Standard Tube**	**Sample Tube**
Standard Solution	-	0.1 mL	-
Study solution	5 mL	5 mL	5 mL
Serum	-	-	0.1 mL

The tubes are mixed separately, and incubated at room temperature for 30 min and the optical density is measured at 545 nm at the spectrophotometer (Stain stills for 30min.)

Calculation:

$$\text{g/L protein} = \frac{\text{OD sample}}{\text{OD standard}} \text{ X 100}$$

Solutions:

Standard solution: Casein sodium salt (100 g/L)

Base reagent: 9 g sodium potassium tartrate, 0.2 g sodium hydroxide and 5 g potassium iodide in 1L distilled water

Color reagent: Copper sulphate solution (150 g/L)

Working solution: Mix 5mL color reactive to 1L of base reagent.