THE NEOTIA UNIVERSITY

ज्ञानम् आत्म प्रदीपाय

Department of Biotechnology
School of life Sciences

Practical Manual

Course: **Biochemistry**

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Experiment 1: Preparation of percent, normal and molar solutions

Aim: To prepare solutions of desired percent, normal and molar concentration.

Introduction/principle/method:

Molar solution: Preparing and measuring solutions in **molarity (M)** is one of the ways to measure the concentration of an element, ion, or compound in solution.

This unit of concentration is based on the chemical concept of a "**mole**" ("mol"). A **mol** is the gram-atomic mass of an element (or molecule) and is a measure of the number of atoms (or molecules). Gram atomic mass = **mass**, in **grams**, of one mole of **atoms**, equal to atomic weight in grams.

A Mole is: 6.023 x 10^{23} of anything. Formula mass in grams of a ***substance*** contains one mole of particles. N_a = Avogadro's Number = 6.02 x 10^{23}

Example: from the periodic chart, the gram-atomic mass of hydrogen is 1.0. One gram of hydrogen is one mol and contains 6.0 x 10^{23} hydrogen atoms. The gram-atomic mass of oxygen is 16. Sixteen grams of oxygen is one mol and also contains 6.0 x 10^{23} oxygen atoms.

Even though the masses are different, one mol of each element contains the same number of atoms i.e. Avogadro's number of atoms. Number of moles = weight of that substance/gm mol wt

This same concept is also used to determine the mol of a **compound**. The gram-molecular mass of water (H_2O)= 18 (two hydrogen atoms at 1 and one oxygen at 16 = 18). Eighteen grams of water is one mol and contains 6.0 x 10^{23} water molecules.

Using the mol as a measurement tool is especially helpful when determining how much of one compound or element reacts with another.

When the mol unit of measurement is used with compounds in solution, it is called **molarity**. It is defined as the gram-molecular mass of a compound per liter of solution.

Molarity: No of moles of solute present in 1 lt of solution (no of moles / lt of solution)

One mol of KCl is equal to 74.6 grams (from the periodic chart, the gram-atomic mass of potassium is 39.0 and that of chlorine is 35.5. Add together for the gram-molecular mass of 74.6). If you took 74.6 grams of KCl and diluted this to one liter with water, you would have a 1.00 M KCl solution. Double the grams to 149 and dilute to one liter and you would have a 2.00 M KCl solution.

Formula for determining how many grams of a compound we need for a specific molarity.

Grams of the compound needed = (M) x (gram-molecular weight) x (litre)
An example: A laboratory procedure requires that you standardize a meter with a 0.010 M KCl solution. You don't want to make a whole liter; 250 ml would be enough. How many grams of KCl do I need to dilute to 250 mL to obtain a 0.010 M KCl solution?
Grams of the compound needed = (0.010 M)(74.6)(0.250 liters) = 0.187 grams KCl.

Normality (N) is a way to express concentration of a solution and almost exclusively used to measure the concentrations of **acids and bases.**

Normality Definition: *Normality is a measure of concentration equal to the gram equivalent weight per liter of solution* or *Normality is the number of gram equivalent of the solute present per liter of its solution.*

[***Gram equivalent weight**: weight of substance which combines with 1 gram of Hydrogen (or) 8 grams of oxygen (or) 35.5 gram of chlorine*]

Eq weight: Mol. wt / total valence of cation or anion

Normality is similar in concept to molarity. Molarity (M) represents the concentration of an ion or compound in solution, Normality (N) goes one step further and represents the molar concentration only of the acid component (usually the H^+ ion in an acid solution) or only the base component (usually the OH^- ion in a base solution).

1. Relationships of Normal acid and Normal base solutions:

A 1N solution of the acid H_2SO_4 will completely neutralize an equal volume of a 1N solution of the base NaOH. Even though the H_2SO4 provides two (acid) H+ ions per molecule verses only one (base) OH- ion per NaOH molecule, the calculations of Normality take into account these differences and puts it all into an equivalent scale.

2. Conversion from Molarity to Normality

If we know the Molarity of an acid or base solution, you can easily convert it to Normality by multiplying Molarity by the number of hydrogen (or hydroxide) ions in the acid (or base).

N = (M) X (number of hydrogen or hydroxide ions)

For example, a 2 M H_2SO_4 solution will have a Normality of 4N (2 M x 2 hydrogen ions).

A 2 M H3PO4, solution will have a Normality of 6N (2M X 3 hydrogen ions)

3. How to make a solution of a **predetermined normality** ?

*How many grams of sodium hydroxide would you need to prepare a liter of **1N NaOH** solution?*

Steps:

First, we must determine the compound's **equivalent mass**. This is done by taking the compound's gram-molecular mass (**Gram equivalent weight**) and dividing by the number of hydrogen ions or hydroxide ions.

Equivalent mass = gram equivalent weight / no. of hydrogen or hydroxide ions

NaOH, sodium hydroxide. The gram-molecular mass is 40. The number of hydroxide ions (OH-) is 1. Equivalent mass for NaOH is 40/1 = **40**.

H_2SO_4, Sulfuric acid.
The gram-molecular mass is 98 {1x2}+32+{16x4}=98).

The number of acid hydrogen ions (H+) is 2. Equivalent mass for H_2SO_4 is 98/2 = **49**.

H_3PO_4, phosphoric acid.
The gram-molecular mass is also 98. The number of hydrogen ions (H+) is 3. Equivalent mass for H3PO4 is 98/3 = 32.6.

Second: Once the equivalent mass of an acid or base is determined, you can then calculate the amount of grams needed per volume of water for N.

The formula to calculate this is:

Grams of compound needed = (N desired) (equivalent mass) (volume in liters desired).

The equivalent mass (weight) of NaOH is 40
Grams of NaOH needed = (1N)(40 eq. mass)(1 liter) = 40 grams of NaOH.

Similarly, *how many grams of* potassium hydrogen phthalate ($KHC_8H_4O_4$) *would you need to prepare a 250 ml of 0.05 N* potassium hydrogen phthalate *solution (acid solution)?*

first determine the equivalent mass.
From the periodic chart, K=39, H=1, C=12, O=16. Its gram-molecular mass is 39+1+(12x8)+(1x4)+(16x4) = 204.

The number of hydrogen ions it can produce is 1 (*The acid hydrogens are usually on the left side of a chemical formula. Hydrogens listed anywhere else usually don't contribute to the "acid" part of the compound. In the case of $KHC_8H_4O_4$, only the left most hydrogen is an "acid" hydrogen.*) Its equivalent mass is 204/1 = 204.
To find the amount of potassium hydrogen phthalate ($KHC_8H_4O_4$) needed to make 0.25 liters of a 0.05N solution:
Grams of $KHC_8H_4O_4$ needed = (0.05N)(204 eq. mass)(0.25 liters) = 2.6 grams of $KHC_8H_4O_4$.

Both of the chemicals in the examples above, sodium hydroxide and potassium hydrogen phthalate, are considered dry chemicals, which makes it relatively straightforward to calculate their Normalities.

For liquid chemicals where the main compound is only a fraction of the total volume, such as the concentrated forms of hydrochloric (HCl), sulfuric (H_2SO_4), and phosphoric (H_3PO_4) acids, a few additional calculations must be performed to make a solution of a particular Normality.

Normal solutions:

Making Normal solutions from concentrated mineral acids like sulfuric acid, nitric acid, and hydrochloric acid.

First, **Purity**. None of the stock solutions of acids found in the lab are one hundred percent pure. Sulfuric acid is only about 97% pure, nitric is about 69.5%, and hydrochloric acid is about 37.5% pure.

Second, their **specific gravities**. The specific gravity of a liquid is, in most cases, synonymous with the more familiar term of density. **Water has a specific gravity of 1**. If the specific gravity of a liquid is greater than 1, then the liquid is heavier than water. Less than 1, and the liquid is lighter than water. The specific gravity for concentrated sulfuric acid is about 1.84, or 1.84 times heavier than an equal volume of water. The specific gravity of concentrated nitric acid is about 1.42 and that of concentrated hydrochloric acid is about 1.19.

Both the **percent concentration** and **specific gravity** values of the acid are required to determine the amount of concentrated acid needed when making a Normal solution.

This information is usually printed on a label attached to the bottle of acid. To make a solution of a predetermined Normality, we must

1. determine the equivalent mass of the chemical

2. determine the grams needed of that chemical. (As described befor).

3. Convert the number of grams into its volume equivalent.

4. Once this volume is determined, do a simple dilution after that.

Example:

We want to make only **250 mL of 1 N H2SO4** solution. How many milliliters of concentrated sulfuric acid do we need to make 250 ml of a 1N solution?
1. To determine **how many grams of sulfuric acid** you will need, you will first need to calculate the equivalent mass of H_2SO_4. This is the gram-formula weight divided by the number of acid hydrogens in the compound. It is 98/2 = 49.
2. Then you can calculate the amount of grams of H_2SO_4 that are needed.

The formula to calculate this is:
Grams of compound needed = (N desired)(equivalent mass)(volume in liters desired).

Substituting the above numbers into the equation, we get:

grams of compound needed = (1 N)(49)(0.250 liters) = 12.25 grams.

3. A 1 N solution requires 12.25 g of a pure sulfuric acid powder (if one existed) diluted to 250 ml.

But the acid is a liquid and it is not one hundred percent pure active sulfuric acid.

We will need to calculate what volume of the concentrated acid that contains 12.25 grams of sulfuric acid. The formula for this is:

Volume of concentrated acid needed = (grams of acid needed)/(percent concentration x specific gravity)

For this example, we are using those values previously mentioned here:

Volume of concentrated acid needed = (12.25 grams)/(0.97 x 1.84) = 6.9 mL

If we took 6.9 mL of concentrated sulfuric acid and diluted it to 250 mL, we would have a 1 N H_2SO_4 solution.

Materials required:
Basic laboratory glass wares and salts

Experiment-2: Making solutions by dilutions

Aim: To prepare solution of desired concentration from stock solutions of higher comcentrations

Introduction/Principle: Many solutions used in biochemistry are prepared by the dilution of a more concentrated stock solution. In preparing to make a dilution (or series of dilutions), you need to consider the goal of the procedure. This means that you need to consider both the desired final concentration and required volume of the diluted material.

A. Simple dilution: A simple equation allows the dilution to be calculated readily:

$$C1V1 = C2V2$$

where **C1** is the concentration of the initial solution;

V1 is the volume of the initial solution available to be used for dilution (this may be a small fraction of the initial solution),

C2 is the desired final concentration,

and **V2** is the desired final volume.

In most cases, the initial concentration and the final concentration are either known or are chosen in order to work correctly in the experiment being planned. The final volume is usually an amount that is chosen based on the amount required for a given experiment. This means that at least **three** of the required terms are either known or can be chosen by the experimenter.

Example:

You have a stock solution of 1000 mg/ml BSA, and you want 200 ml of 20 mg/ml BSA solution.

In this case, C1 = 1000 mg/ml; C2 = 20 mg/ml, and V2 = 200 µl.

This leaves V1 as the unknown value (*i.e.* how much of the stock solution must be diluted to 200 ml final volume to yield the desired concentration). Rearranging the dilution equation gives:

$$V_1 = \frac{C_2 V_2}{C_1}$$

Therefore, V1 = (20 * 200) / 1000 = 4 ml

Thus, you need to dilute 4 ml of the stock solution to a final volume of 200 ml (*i.e.* by adding 196 ml water).

B. Step dilution: If, in the example, you wished to make a solution of 0.1 mg/ml for 200 ml volume from a 1000 mg/ml stock, the same equation would indicate that you need 0.02 ml of the 1000 mg/ml stock solution for 200 ml of the final diluted sample.

This is a problem: 0.02 ml could be very difficult to measure accurately.

You have two choices: **change the final volume** (*i.e.* if V2 is larger, then V1 must also increase),

or perform **step dilutions** (*i.e.* instead of diluting the stock solution by a factor of 1000 in one step, dilute the stock solution, and then make a further dilution of the diluted stock).

In many cases, while the final *concentration* is important, the final *volume* is not. In these cases, use a **convenient** dilution: a dilution that involves volumes that are easily pipetted.

Prepare 10 mg/ml solution for 200 ml and then from 10 mg/ml solution prepare 0.1 mg/ml solution.

Pipetting 1.3333 µl is usually less accurate than pipetting 4 µl, both because 4 µl is a larger volume, and because it is difficult to set the pipet for 1.3333 µl. In this case, 4 µl is a convenient volume, while 1.3333 µl is not.

C. When starting concentration is not known: In some cases, you may not know the actual starting concentration. If, for example, you need to measure the enzyme activity in a sample, and you find that the activity is too high to measure accurately, you will need to dilute the starting material. Since you don't know the actual starting concentration, all you know is the concentration **ratio** between starting and final solutions. As long as you keep track of the concentration ratio in all of your dilutions, you can easily determine the enzyme activity in the initial solution, even though you cannot measure it directly.

Example: Measuring protein concentration of a sample.

1. Dilute the sample 10 times with a buffer with certain pH.
2. Measure the absorbance at certain wavelength (280 nm).
3. The protein concentration in the original sample can be calculated by using following formula: Protein concentration in the original sample : =

$$\frac{A_{280} \times \text{Dilution factor}}{0.06} \text{ mg/ml}$$

Where Dilution factor = 1 ent of lysozyme i.e A280

D. Stock concentration: Concentration ratios are frequently of considerable value. For example, you have a stock solution of buffer that contains 450 mM Tris-HCl, 10 mM EDTA, and 500 mM NaCl. You actually wish to use a final concentration of 45 mM Tris-HCl, 1 mM EDTA, and 50 mM NaCl. In each case the concentration of the final buffer is one tenth that of the original. Simply performing a 1:10 dilution of the stock solution then gives the appropriate final concentration of each component. The stock solution of buffer is typically called a 10x stock, because it is ten-times more concentrated than the final, useful buffer.

The 1:10 dilution mentioned is performed by taking one part of the initial solution, and adding nine parts of solvent (usually water). This results in a final concentration that is ten-fold lower than the original.

E. Serial dilution:

A dilution series is a succession of step dilutions, each with the same dilution factor, where the diluted material of the previous step is used to make the subsequent dilution.

To make a dilution series, use the following formulas:

Move Volume = Final Volume / (DF -1)

Diluent Volume = Final Volume – Move Volume

Total Mixing Volume = Diluent Volume + Move Volume

Example 1: Make a 7-point 1:3 standard curve, starting with **Neat**, such that you can pipette duplicates of 50 uL per well.

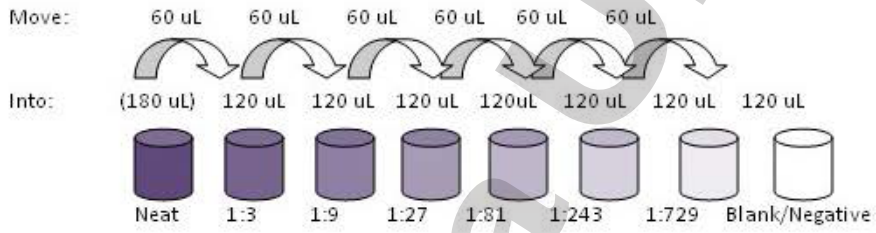
1. Calculate the minimum diluent volume per step: 50 uL per well * 2 for duplicates = 100 uL minimum. Add extra volume to compensate for pipetting error, for example, 20 uL, which brings our desired **Diluent Volume** to 120 uL.

2. Calculate **Move Volume**: Move Volume = 120 uL / (3-1) = 60 uL

3. Calculate Total Mixing Volume: Total Mixing Volume = 120 uL + 60 uL = 180 uL

Answer:

i. Prepare the first point of the standard curve, which is 180 uL of Neat standard.
ii. Prepare the diluent for the rest of the points, or six aliquots of 120 uL of diluent.
iii. Move 60 uL of the first point into the second and mix thoroughly, move 60uL of that into the next, and so on.



Materials Required:

Pipetmen

Pipet tips

Parafilm

Water

1.0 M $CuSO_4$ solution

Unknown $CuSO_4$ solution

Simple Dilutions-1

Prepare 1 ml of the following dilutions of the $CuSO_4$ solution using water: 1:2, 1:5, 1:10, 1:50, and 1:100.

Simple Dilutions-2

Assuming the $CuSO_4$ solution is a 5X stock, prepare the following solutions: 0.5X, 1X, 2X.

Problem 1: Calculate the amount of 1 M NaOH aqueous solution needed to make 100 mL of 0.5 M NaOH aqueous solution.

Problem 2: How many milliliters of 5.0 M copper(II) sulfate solution must be added to 160 mL of water to achieve a 0.30 M copper(II) sulfate solution?

Problem 3: Prepare 100 mL of a 0.10 M solution of $NiCl_2$, from a 0.20 M stock.

Problem 4. Make a 7-point 1:2 standard curve, starting at a 1:5, such that you can pipette duplicates of 50 uL per well.

Problem 5: 50.0 mL of 12.0 N HCl is diluted to a total volume of 250.0 mL. What is the concentration of the diluted solution?

Serial Dilutions

Prepare the following solutions of the $CuSO_4$ solution using serial dilutions: 1:5, 1:25, 1:125, and 1:625

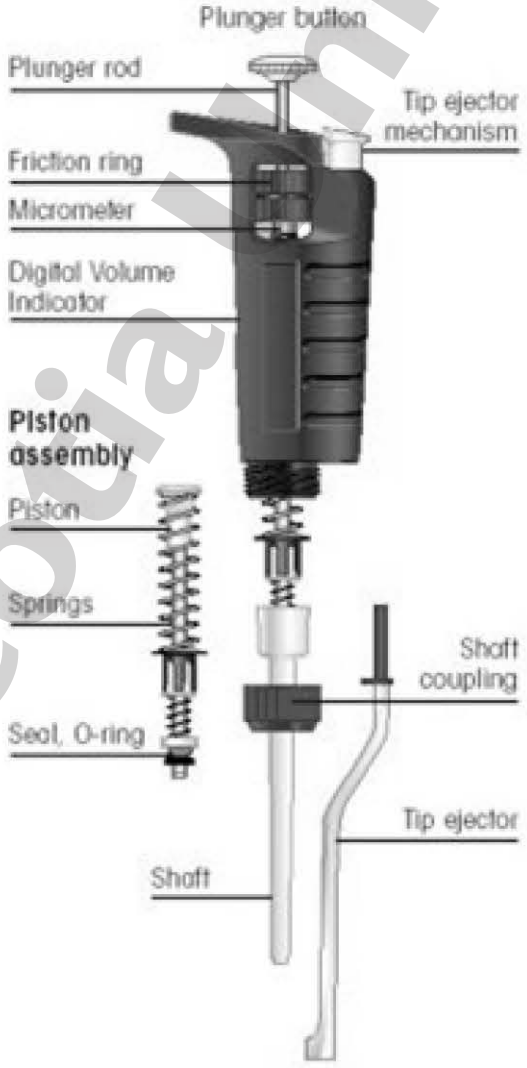
1. Describe how each serial dilution was prepared.

Experiment 3: **Use of Micropipette and lab report preparation**

Aim: To learn how to precisely use calibrated variable volume micropipettors and to create a lab report using computer.

Introduction: Automatic pipettes are used to accurately transfer small liquid volumes. Glass pipettes are not highly accurate for volumes less than 1 milliliter (1 ml), but the automatic pipettes are both accurate (less than 1% error) and precise (less than 0.5%).

Parts of Micropipet:



Principle:

Materials Required:

- Various variable volume micropipettes (**10 µL, 100 µL, 200 µL , 1000 µL**) and tips
- Beakers
- Balance
- weigh boats
- Water
- Concentrated or partially-diluted HCl
- NaOH pellets
- Volumetric flasks

Procedure:

1. Obtain some deionized water with a beaker. Pipet exactly 1.000 mL using a P1000 pipet. Obtain a weigh boat and place it on the balance and press tare. Pipet the water into the weighboat. Record the mass. Is it 1.0000 mL?

Comment on the accuracy. If not, make adjustments to your technique until you are close to the expected value.

2. Weight out 1000 µL of water ten times using a P1000 and record your results in a table. Comment on your precision

3. Use a P1000 pipettor to dispense volumes of 0.200, 0.400, 0.600, 0.800 and 1.000 mL. Record the values used and the mass determined for each point. Graph your data.

4. Using a computer, use a spreadsheet to make a plot of "volume dispensed" vs. the "Pipettor Setting" on a line graph.

Calculate the "best" line (***trendline***) for this data using linear regression and add the "fit" to the line graph and include the R^2 value and line equation on the graph.

Label your graph with a title (y vs. x), and x-axis and y-axis labels with units.

5. Did the micropipet deliver the mass/volume of water expected? If you needed to adjust your technique, was the delivery as expected? Why or why not? Was the pipet accurate?

6. Compute the mean, median, mode, range of the water delivered 10 times and calculate the standard deviation.

Observation and Result:

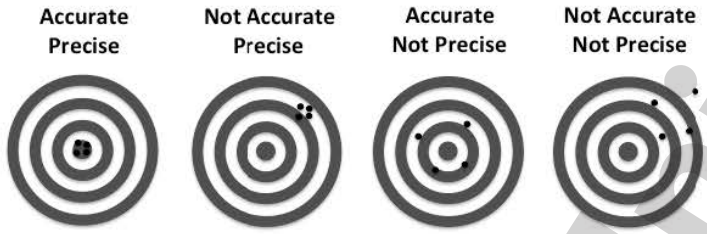
1. was the pipet precise?

2. Which of the pipettors that you used was the most accurate?

Interpretation:

Accuracy is how close a measured value is to the actual (true) value (even after averaging).

Precision is how close the measured values are to each other.



Experiment 4: Use of pH meter, calibration of pH meter and determination of pH of a solution

Aim: To learn how to use a pH meter, how to calibrate the machine and how to determine pH of solution

Introduction: pH = "Power of hydrogen"

pH is a logarithmic measure of molar concentration of hydrogen present in a solvent.

pH = -log[H^+], Where **log** is a base-10 logarithm and **[H^+]** is the concentration of hydrogen ions in moles per liter of solution.

pH of 7 is considered "neutral", because the concentration of hydrogen ions is exactly equal to the concentration of hydroxide (OH^-) ions produced by dissociation of the water.

Here concentration of H^+ ion = 1.0×10^{-7} *M* / lt; **pH = - log [1.0×10^{-7}] = 7**

Increasing the concentration of hydrogen ions above 1.0×10^{-7} *M* produces a solution with a pH of less than 7, and the solution is considered "**acidic**". Decreasing the concentration below 1.0×10^{-7} *M* produces a solution with a pH above 7, and the solution is considered "**alkaline**" or "basic"

pH = - log [1.0×10^{-6}] = 6 (acidic)

pH = - log [1.0×10^{-8}] = 8 (alkaline)

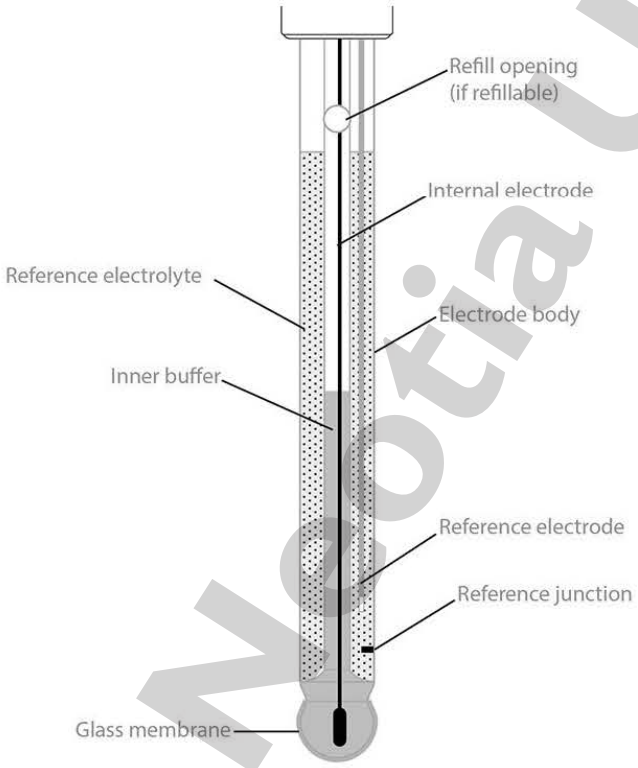
This is correct as long as the solutions being compared both use the same solvent. You can't use pH to compare the acidities in different solvents because the neutral pH is different for each solvent. For example, the concentration of hydrogen ions in pure ethanol is about 1.58 $\times 10^{-10}$ *M, so ethanol is neutral at pH 9.8. A solution with a pH of 8 would be considered acidic in ethanol, but basic in water.*

Principle: pH meter:

When a metal is brought in contact with a solution of salts or acids, an electric potential is caused, which has led to the invention of batteries. Similarly, an electric potential develops when one liquid is brought in contact with another one, but a membrane is needed to keep such liquids apart.

A pH meter measures essentially the electro-chemical potential between a known liquid inside the **glass electrode** (membrane) and an unknown liquid outside. To complete the electrical circuit, also a **reference electrode** is needed.

The normal hydrogen electrode is the glass electrode, and the reference electrode is a calomel electrode in which calomel ($HgCl_2$) is bathed in a solution of potassium chloride.



The glass electrode consists of a thin glass bulb containing dilute HCl, into which is inserted an Ag–AgCl wire, serving as the electrode with a fixed voltage. The HCl solution is separated from the test solution by a **membrane** of special glass, usually a lithium silicate of particular composition.

When the glass bulb is immersed in a solution, a electrical potential difference develops across this membrane between the solution in the bulb and the solution outside the bulb which can be measured by a potentiometer.

The electrical potentials developed by this electrode are the membrane potential, plus the potential of the Ag–AgCl–HCl reaction inside the electrode: AgCl----→ Ag + + Cl-. This reaction is reversible and the Ag–AgCl potential is likely to remain constant at definite temperature. This potential is cancelled out when the electrode is standardized against a standard pH buffer solution.

The glass electrode develops a second potential at the membrane separating the standard HCl and the test solutions. The minute current flow required by the pH meter causes ion exchange at the inner and outer surfaces, and causes diffusion of ions across the glass membrane. The potentials across the glass membrane can be closely calibrated to the approximate value of the H^+ activity.

A typical reference electrode consists for a calomel (HgCl2) wire bathed in KCl which makes electrical contact with the pH meter. Current flows from the electrode to the solution phase through the reversible reaction: HgCl2 ---→ 2Hg+ + 2Cl-. The Cl^- activity is fixed by the KCl concentration (usually saturated KCl). As a result this potential also cancels out when the pH electrode system is standardized in a standard pH buffer.

KCl diffusion through the orifice makes the electrical contact between the reference cell and the test solution. This KCl connection forms a 'salt bridge' between the test solution and the reference electrode. The reference electrode is often built in the same body as the glass electrode to form a combination electrode.

When measuring pH, the potential (E) in the glass electrode is formulated by the Nernst equation: **E=(RT/nF)log(K/H+)**

where R is the gas constant, T the absolute temperature, n the valence, F the Faraday constant, K a constant, and H^+ the activity of H^+ ions. E is called the 'half-cell potential' and cannot be measured alone. If the glass electrode is placed against a reference calomel electrode, the potential difference between the two ($E - E_{ref}$) is measurable.

Before any pH measurement, the two electrodes have to be laced first in a solution of known pH. This is called 'standardizing' the electrodes and the pH meter. The overall potential of the total cell E_0 equals $E - E_{ref}$:

Theoretical calculation of pH:

pH of strong acid can be calculated as

pH = −log (f × [H^+])

For HCl: [H+]= 1 X [HCl]

For H_2SO_4: [H+]= 2 × [H_2SO_4]

Calculate the pH of 0.06 M HCl: pH = − log0.06 = 1.22

Calculate the pH of 0.02 mol/L H_2SO_4 , pH = − log0.04 = 1.4

Strong Base:

E.g. NaOH, KOH,

pH of strong base can be calculated as

pOH = −log (f × [OH-])

pH = 14 − pOH = 14 −(−log (f × [OH-])

Calculate the pH of NaOH 0.5 M.

pH = 14−(−log0.5) = ~13.7

Materials Required:

1. pH meter
2. standard buffer
3. tissue paper
4. basic laboratory glassware

Storage of pH probe:

How should pH probes be stored best?

All pH probes are delivered with a plastic protection cap that can also be used for storage. Pour a few drops of saturated KCl solution into the cap to ensure that the glass membrane is kept hydrated and ready to use. If an electrode dries out during storage, a regeneration procedure is required to restore the hydrated glass layer and the reference junction in order to make the electrode operable.

For short-term storage, the pH probe can be placed in a solution of 3.5 molar KCl or pH 4.0 or pH 7.0 pH buffer. Always rinse before use.

As a general rule, pH probes should never be stored in deionised water. as this will cause ions to leach out of the glass membrane and reference electrolyte, causing a slow and sluggish response.

Safety precautions: Because pH buffer solutions are always the reference of our pH measurements, the pH result can only be as good as the pH buffers used for calibration. If the buffers are contaminated or used improperly, the calibration will be false and all following measurements will be wrong. Therefore proper handling, storage and use of buffers is important.

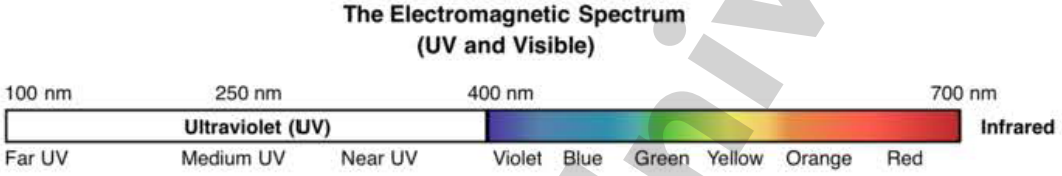
1. Always use pH buffers which bracket the pH of your sample. If the sample has a pH of 6 use buffers pH 4 and pH 7. If the sample has a pH of 7, use buffers with pH 4 and pH 10. If an alkaline sample is measured, calibrate with buffer pH 7 and pH 12.45.
2. To shorten the stabilization time and to achieve an accurate calibration, make sure the sensor and the buffer are at the same temperature.
3. For greatest accuracy measure samples at close to the same temperature as your calibration buffers.
4. Buffers have limited shelf lives. Do not use a buffer if the expiration date has passed. Store buffers at controlled room temperature and if possible always at the same place.
5. Never return used buffer to the buffer bottle. Discard it.
6. Do not let the buffer bottle open for a longer time (exposure to air). The atmospheric carbon dioxide lowers the pH of alkaline buffers.
7. If a calibration with alkaline buffer gives suspect results first try changing the alkaline buffer. Alkaline buffers are susceptible to contamination by CO2.
8. Rinse the sensor with deionised water before placing it in the buffer. Remove excess water from the sensor by gently wiping it with a clean tissue.

Experiment-5: Principle and operation of spectrophotometer

Aim: To learn how to operate a sprectophotometer and to verify Lambert – beer's law for using KMnO4 solution.

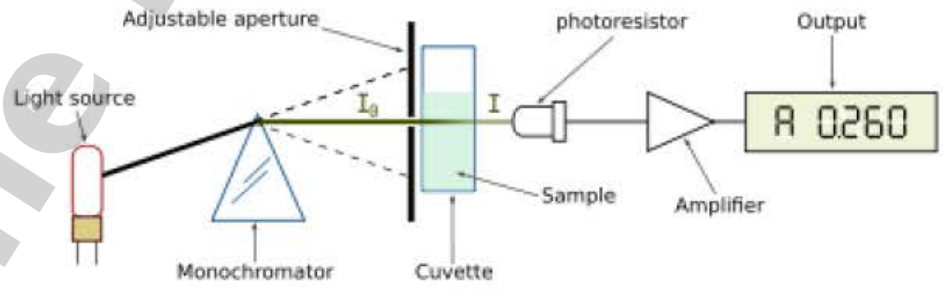
Principle:

Spectrophotometers are standard laboratory equipment. They usually contain two light sources: a deuterium lamp, which emits light in the UV region and a tungsten–halogen lamp for the visible region.



Wavelength, nm	Colour	Complementary colour
400-430	Violet	Yellow-green
430-490	Blue	Yellow
490-550	Green	Purple
550-590	Yellow	Blue
590-630	Orange	Green-blue
630-700	Red	Blue-green

After passing through a monochromator (or through optical filters) the light is focused into the cuvette and the amount of light that passes through the sample is detected by a photomultiplier or a photodiode. In double-beam instruments a cuvette with buffer is placed in the reference beam, and its absorbance is subtracted from the absorbance measured for the sample.



Verification of Lambert-Beers law

According to Lambert, for parallel, monochromatic radiation that passes through an absorber of constant concentration, the radiant intensity decreases logarithmically as the path length, **l**, increases arithmetically (*Lamberts law*).

According to Beer, the transmittance of a stable solution is an exponential function of the concentration, **c**, of the absorbing solute (Beers law).

When both path length and concentration are variable, the combined **Beer-Lambert law** is given as follows: $\mathbf{I_t = Io_{exp}\ (-\ k\ c\ l)}$ or, $\mathbf{log_e(\ I_o\ /\ I_t) = k\ c\ l}$

where $\mathbf{I_o}$ and $\mathbf{I_t}$ are the incident and transmitted intensities, respectively and **k** is a constant but is a *function of wavelength*. Converting to the base 10 logarithm, the equation becomes: $\log\ (\ I_o\ /\ I_t) = \mathbf{A = \varepsilon\ c\ l,}$ where **A** = **absorbance** and **ε**, another constant= **absorptivity** (formerly called the **extinction coefficient**).

The absorptivity depends on the wavelength of light as well as on the identity of the absorbing substance and the identity of the solvent. If the concentration is measured in $mol.L^{-1}$, the absorptivity is called the molar absorptivity.

Spectra are plotted either as absorbance, A, or as the transmittance, T, against wavelength, wavenumber or frequency, where $T = (I_t/I_o)$ or sometimes as percentage transmittance= 100 T. Absorbance (A) can have any value from 0 to infinity. T must be between 0 and 1.

One can find from the above mathematical form of the Beer-Lambert law that it is a straight line equation with zero intercept. That is, a plot of absorbance versus concentration should be a straight line passing through the origin whose slope is (ε. l) when c is in gL-1. If the path length of the sample is 1 unit (say, 1 cm), the slope equals the numerical value of ε.

Therefore, in order to verify the validity of Beer-Lambert Law, a number of absorbance concentration data points should be obtained for a sample that are measured in a given sample holder of unit path length.

Calibration graphs of A against c may be plotted to verify that the linearity of Beer-Lambert law. *It is worth noting that a certain degree of data scatter usually occurs due to personal errors in measurements.* Therefore, it is necessary to obtain a best fit straight line. In addition to these small scatters in data points, sometimes deviations from linearity occur though there are no known exceptions to the Beer-Lambert law. The deviations from linearity may arise from high analyte concentrations, chemical associations, and instrumental origin. Real deviations arise from changes in the refractive index of the analytical system; these changes

will be significant only in high-absorbance differential measurements. The Beer-Lambert law is well obeyed by many substances at low to moderate concentrations. *Dilute solutions will give better linearity of results*. Deviations occur at higher concentrations where absorptivity depends on the concentration. Specific chemical effects such as association of the molecules of the substance affects the analyte species which changes the nature and hence of the absorbing species. When the incident radiation is polychromatic (or measured in a part of the spectrum other than at an absorbance maximum), the Beer-Lambert plot shows a negative deviation. Readout scales are often calibrated to read absorbance as well as transmittance.

Materials required:

1. Spectrophotometer
2. Weighing balance
3. KMnO4 solution
4. Test tubes
5. Conical flask
6. Tissue paper
7. Graph paper/computer with graph ploting software like MS-Excel

Procedure:

1. Prepare a standard 0.1 M aqueous KMnO4 solution. This is used as stock solution.

2. From the above stock solution, prepare a 0.0001M KMnO4 solution.

3. Measure the absorbance of the solution either in 340-400 or 400-600 nm range for each 20 nm increment.

4. Thus determine the λ max of KMNO4 solution.

5. Prepare series of different concentration of KMNO4 solution. Say 0.0002M, 0.0004M, 0.0006M, 0.0008M, 0.001M, 0.002M, 0.004M, 0.006M, 0.008M, 0.01M from the stock solution.

6. Measure the OD value of those concentrations at the λ max wavelength.

7. Prepare a standard curve for concentration Vs OD value. It should be a straight line through the origin, confirming Lambert beers law.

8. Measure OD of the provided KMNO4 solution of unknown concentration.

Experiment 6: Buffer preparation and determination of buffer capacity

Aim: To learn how to prepare buffer of deisred strength and to determine the buffer capacity of the prepared buffer.

Introduction: A buffer solution is one that is resistant to change in pH when small amounts of strong acid or base are added. For example, when 0.01 mole of strong acid or base are added to distilled water, the pH drops to 2 with the acid and rises to 12 with the base. If the same amount of acid or base is added to an acetic acid – sodium acetate buffer, the pH may only change a fraction of a unit.

Buffers are important in many areas of chemistry. When the pH must be controlled during the course of a reaction, the solutions are often buffered. This is often the case in biochemistry when enzymes or proteins are being studied. Our blood is buffered to a pH of 7.4. Variations of a few tenths of a pH unit can cause illness or death. Acidosis is the condition when pH drops too low. Alkalosis results when the pH is higher than normal.

Principle:

Two species are required in a buffer solution. One is capable of reacting with OH^- and the other will react with H + or H_3O^+. The two species must not react with each other. Many buffers are prepared by combining a weak acid and its conjugate base (acetic acid and sodium acetate) or a weak base and its conjugate acid (ammonia and ammonium chloride). *In general, the pH range in which a buffer solution is effective is +/- one pH unit on either side of the pKa.*

The Henderson–Hasselbalch provides the information needed to prepare a buffer.

$$pH = pKa + \log \frac{[conjugatebase]}{[weakacid]}$$

Buffer capacity: There is a limit to the amount of acid or base that can be added to a buffer solution before one of the components is used up. This limit is called the **buffer capacity** and is defined as the *moles of acid or base necessary to change the pH of one liter of solution by one unit.*

Buffer Capacity = (number of moles of OH^- or H_3O^+ added) / (pH change) (volume of buffer in L)

Problem: Show the calculations for the preparation of **100 mL of an acetic acid – sodium acetate pH 5.0 buffer** using:

1. 0.30 M acetic acid
2. 0.50 M acetic acid

Before preparing the buffer solutions, we must determine the amount of acetic acid and sodium acetate required.

Given: pK_a of acetic acid is 4.756.

In this experiment, the Henderson-Hasselbalch equation will be used to determine the amount of acetic acid and sodium acetate required to prepare a series of acetic acid buffer solutions.

Note the relationship between the acid and its conjugate base in the equilibrium:

$HC_2H_3O_2(aq) + H_2O(l) \rightleftharpoons C_2H_3O_2^-(aq) + H_3O^+(aq)$

Once the buffer solutions have been prepared, their buffer capacity will be determined.

Materials required:

1. acetic acid
2. NaOH
3. sodium acetate
4. Buret
5. buret clamp
6. pH Meter
7. standard buffer solution (pH 4 & 7)
8. volumetric flasks

9. Beaker

10. Magnetic stirrer

11. stir bar

Procedure:

First, we will make 100 mL of acetic acid buffer with pH = 5.0 using 5 mL of a **0.3M acetic acid solution**.

Using the Henderson-Hesselbalch equation, the mass of sodium acetate needed to make the buffer is calculated as follows:

Calculation:

Step 1. Calculate the concentration of acetic acid in the final 100 mL:

(5.00 mL × 0.300 M) / (100.0 mL) = **0.0150 M**

Step 2: Plug the values into Henderson-Hesselbalch equation and solve for unknown:

$$pH = pKa + \log \frac{[conjugatebase]}{[weakacid]}$$

5.00 = 4.756 + log [A^-] /0.0150M

$10^{0.244}$ = [A^-]/ 0.0150M

[A-] = **0.0263 M**

Step 3. Calculate the moles of A^- (conjugate base) needed to obtain this concentration in 100 mL (0.1 L):

0.0263 M × 0.100 L = **0.00263 moles**

Step 4: Calculate the mass (in gm) of Na-acetate ($C_2H_3NaO_2$) (mol wt 82) needed to obtain this number of moles:

0.00263 moles × 82.04 g/mol = **0.216 g**.

Determining buffer capacity:

A. pH = 5.0 Buffer A (100 ml) (with 0.3 M, 5 ml acetic acid)

B. pH = 5.0 Buffer B (100 ml) (with 0.5 M, 5 ml acetic acid)

C. Testing the Buffer Capacity:

1. Calibrate a pH meter.

2. Once the pH has been calibrated, measure the pH of the buffer solution that your group prepared. Record the value in your data table.

3. Using a volumetric pipette, transfer 25.0 mL of Buffer A into a 125 mL Erlenmeyer flask

4. Load a 50 mL buret with 0.1M NaOH solution. Make sure that the tips of the burets are filled and that the level of the liquids is at or below the 0.00 mL line.

5. Begin adding the NaOH to the buffer solution in small increments. After each addition, record the total volume of NaOH added and the pH of the solution in the data table. Continue adding the NaOH solution until the pH has risen **at least one pH unit**.

Run two titrations: a quick one and a careful one. For the quick one, add NaOH in 1 mL increments. For the careful one, use your best judgment in adding titrant.

6. Record total volume of NaOH required to change the pH for 1 unit.

7. Repeat all steps for Buffer B.

8. Compare the buffer capacity based on the base required to change pH for one unit and comment.

Safety: If a magnetic stirrer is to be used, keep the tip of the electrode above the stir bar

Observation/results:

Trial 1		Trial 2		Trial 3	
mL NaOH	pH	mL NaOH	pH	mL NaOH	pH

Calculations:

1. Summarize the data for your titrations in the table below.

	Trial 1	Trial 2	Trial 3
Vol of buffer, mL			
Vol of buffer, L			
Change in pH			
Vol 0.100 M NaOH			
Moles of NaOH			
Buffer Capacity (in mols)			

2. Show the calculations for one of the trials above.

3. What was the average buffer capacity for the buffer that your group prepared?

Experiment 7: Qualitative test for amino acids-1

Aim: To detect presence of Amino acid in a given sample by Ninhydrin

Principle:

Ninhydrin (1,2,3-Indantrione monohydrate, or triketohydrindene hydrate), a powerful oxidizing agent is often used to detect α amino acids and also free amino and carboxylic acid groups on proteins and peptides. This chemical detects ammonia or primary and secondary amines. When reacting with these free amines, (in a pH range of 4-8) a deep blue or purple color compound known as **Ruhemann's purple** (discovered by Siegfried Ruhemann in 1910) is produced. When ninhydrin reacts with amino acids, the reaction also releases CO_2. The carbon in this CO_2 originates from the carboxyl carbon of the amino acid.

In presence of Ninhydrin, amino acids undergo ***oxidative deamination*** *liberating* ***ammonia, carbon dioxide****, a corresponding* ***aldehyde*** *and* ***reduced form of ninhydrin****.* The ammonia formed from α-amino group reacts ninhydrin and its reduced product to give a blue substance diketohydrin (Ruhemann's purple). However, in case of imino acids like proline, a different product having a bright yellow colour is formed. Asparagine which has a free amide group reacts to give a brown coloured product.

In the presence of concentrated nitric acid phenyl ring of aromatic amino acid gets nitrated to give yellow coloured nitro derivatives. These derivatives forms orange colour in presence of an alkali solution. The salts of these derivatives are orange in colour. Proteins containing these amino acids also give a positive response to this test.

Alpha amino acids react with Ninhydrin involved in the development of color which is explained by the following five steps.

1. **α-amino acid + Ninhydrin ---> Reduced ninhydrin +α imino acid +H_2O**

This is an oxidative deamination reaction that elicit two hydrogen from the alpha amino acid to produce an alpha – imino acid. Also the ninhydrin reduced and loses an oxygen atom with the formation of water molecule.

2. **α-imino acid + H_2O ---> α-keto acid +NH_3**

The rapid hydrolysis of NH group in the alpha – imino acid will cause the formation of an alpha-keto acid with an ammonia molecule. This alpha-keto acid further involved in the decarboxylation reaction of step.

3. α-keto acid + NH_3 ---> aldehyde + CO_2

Under a heated condition to form an aldehyde that has one less carbon atom than the original amino acid. A carbon dioxide molecule is produced along with aldehyde. These first three steps produce the reduced ninhydrin and ammonia that are required for the production of color. The overall reaction for the above reactions is as follows:

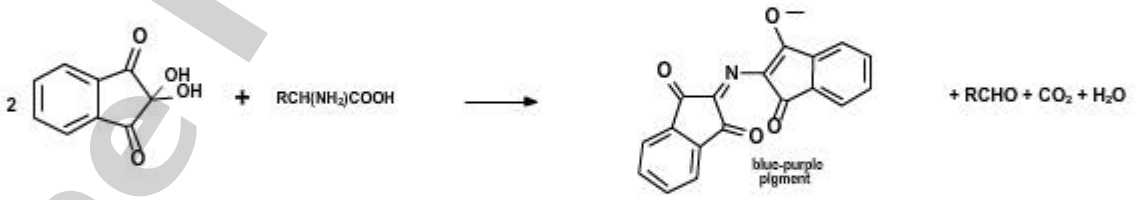
4. α-amino acid + 2 ninhydrin ---> CO_2 + aldehyde + final complex (BLUE) + $3H_2O$

In summary, ninhydrin, which is originally yellow, reacts with amino acid and turns deep purple. It is this purple color that is detected in this method. *Ninhydrin will react with a free alpha-amino group, NH_2-C-COOH. This group is present in all amino acids, proteins or peptides. Whereas, the decarboxylation reaction will proceed for a free amino acid, it will not happen for peptides and proteins.* Theoretically only amino acids produce color with ninhydrin reagent.

The ninhydrin reaction, one of the most important method of detecting amino acids, both technically and historically, has been conventionally used to detect their microgram amounts. When amino acids with a free alpha amino groups are treated with an excess of ninhydrin, they yield a purple colored product. Under appropriate conditions, the color intensity produced is proportional to the amino acid concentration.

Imino acids like proline, the guanidino group of arginine, the amide groups of asparagine, the indole ring of tryptophan, the sulfhydryl group of cysteine, amino groups of cytosine and guanine, and cyanide ions also react with ninhydrin to form various chromophores that can be analyzed.

The overall reaction can be written as follows:



In the quantitative estimation of amino acid using Ninhydrin reagent, the absorbance of the Ruhemann's purple formed by the reaction at 570nm is measured. For imino acids, the absorption happens at 440nm.

Materials required:

1. 0.2 % Ninhydrin solution in Acetone

2. Amino acid solution: 150 μg/ml each in isopropanol

3. Amino acid solution: Tyrosine, Tryptophan, Phenylalanine, Glycine, Lysine, Cysteine, Leucine, Proline, Hydroxyproline,

4. Concentrated Nitric acid

5. NaOH solution 40% (10M)

Procedure:

1) Add 2-5 drops of ninhydrin solution to 1 ml of test solution

2) Mix and keep for 5 min in boiling water bath and observe the development of pink, purple or violet – blue colour.

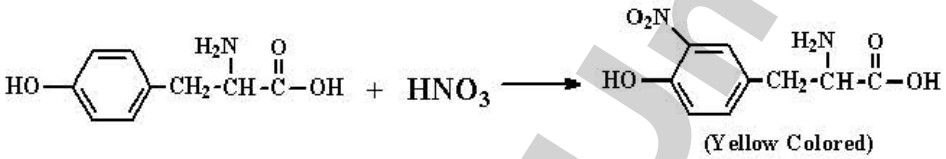
3) Imino acids like proline and hydroxyproline give a yellow coloured complex

Experiment 7: Qualitative test for amino acids-2

A. Xanthoproteic Test

Aim: To detect the presence of aromatic amino acids in a given sample.

Principle: Amino acids containing an aromatic nucleus (tyrosin, tryptophan, phenylalanine) form yellow nitro derivatives on heating with conc. HNO_3. The salts of these derivatives are orange in colour. Proteins containing these amino acids also give a positive response to this test.



Materials require:

1) Conc . HNO3 (Nitric acid).

2) NaOH solution (40% w/v):

3) Test solutions: separate solutions containing 50 µg/ml of amino acids like tyrosine, glycine, tryptophan, phenylalanine, lysine, cysteine, leucine etc.

Procedure:

1) Add 1 ml conc . HNO_3 into 1ml of test solution.

2) Mix the contents and keep in boiling water bath for 1 min.

3) Cool it and then slowly pipette NaOH till the solution becomes alkaline

4) Appearance of orange red colour denotes presence of aromatic amino acids.

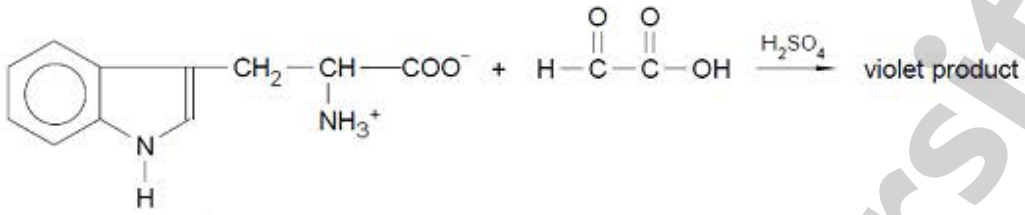
These reactions are characteristics of benzene ring and benzene derivatives. Nitration of benzene ring with Nitric acid.

B. Hopkin's Cole Test:

Objective: To detect indole containing amino acid tryptophan

Principle: The indole group of tryptophan reacts with glyoxylic acid (glacial acetic acid, which has been exposed to light, always contains glyoxylic acid CHOCOOH as an impurity)

in the presence of concentrated H_2SO_4 to give a purple ring between two layers. Apply this test to glycine, tryptophan and tyrosine.



Materials required:

1) Conc . H2SO4.

2) Glacial acetic acid containing glyoxalic acid

3) Test solutions: prepare separate solutions containing 50 µg/ml of amino acids glycine, tyrosine and tryptophan.

Procedure:

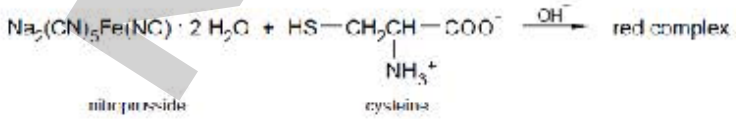
- To a few mL of glacial acetic acid containing glyoxylic acid, add 1-2 drops of the amino acid solution.

- Pour 1-2 mL H2SO4 down the side of the sloping test tube to form a layer underneath the acetic acid.

C. Nitroprusside Test:

Objective: To detect aromatic amino acid cysteine

Principle: The nitroprusside test is specific for **cysteine**, the only amino acid containing sulfhydryl group (-SH). This group reacts with nitroprusside in the presence of excess ammonia. Apply this test **cysteine, cystine and methionin.**



Materials required:

1) Nitroprusside solution

2) Ammonium hydroxide

3) Test solutions: prepare separate solutions containing 50 µg/ml of amino acids cystine, cysteine and methonine

Procedure:

- Put 2 mL amino acid solution into the test tube.

- Add 0.5 mL nitroprusside solution and shake thoroughly.

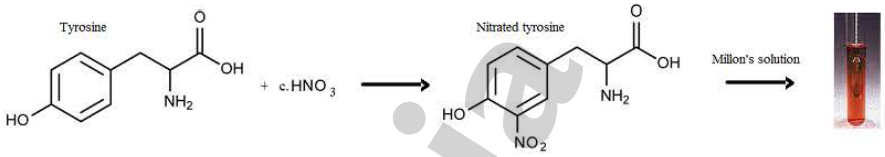
- Add 0.5 mL ammonium hydroxide.

- Observe the color change. (Orange colour)

D. Millon's Test:

Objective: To detect aromatic amino acid tyrosine

Principle: Millon's test is specific to **phenol containing structures** (***tyrosine*** *is the only common phenolic amino acid*). Millon's reagent is concentrated HNO3, in which mercury is dissolved. As a result of the reaction a red precipitate is considered as positive test. *A yellow precipitate of HgO is NOT a positive reaction but usually indicates that the solution is too alkaline.*



Materials required:

1) Millon's reagent

2) Na-nitrite

3) Test solutions: prepare separate solutions containing 50 µg/ml of amino acids tyrosine, phenylalanine and glycine

4) Water bath

Procedure: - To 2 mL amino acid solution in a test tube, add 1-2 drops of Millons reagent.

- Warm the tube in a boiling water bath for 10 min.

-Cool the contents under running tap water and add few drops of sodium nitrite solution.

- A brick red color is a positive reaction.

E. Lead-Sulfide Test:

Objective: To detect sulfur containing amino acid cysteine and cystine

Principle: When cystine is boiled with 40% NaOH, some of sulfur in its structure is converted to sodium sulfide (Na2S). The Na2S can be detected by using sodium plumbate solution which causes the precipitation of PbS from an alkaline solution. In order to apply this test, first the sodium plumbate solution should be prepared. Apply this test to **cysteine** and **cystine.**

Materials required:

1) Lead cetate

2) NaOH

3) Test solutions: prepare separate solutions containing 50 µg/ml of amino acids cysteine and cystine

4) Water bath

Procedure:

- Sodium Plumbate Solution Preparation:

o Add 5 mL dilute NaOH to 2 mL dilute **lead acetate**.

o A white precipitate of lead hydroxide forms.

o Boil until the precipitate dissolves with the formation of sodium plumbate.

- Boil 2 mL amino acid solution with a few drops of 40% NaOH for 2 min.

- Cool and add a few drops of the sodium plumbate solution.

- A brown color or precipitate is a positive test for sulfides.

F. Sakaguchi Test:

Objective: To detect basic amino acid arginine

Principle: The Sakaguchi reagent is used to test for a certain amino acid and proteins. The amino acid that is detected in this test is arginine. Since arginine has a guanidine group in its

side chain, it gives a red color with α-naphthol in the presence of an oxidizing agent like bromine solution. Apply this test to **arginine.**

Materials required:

1) alpha-napthol

2) NaOH

3) Bromine solution

4) Test solutions: prepare separate solutions containing 50 µg/ml of amino acids Arginine and glycine

Procedure:

- 1 mL NaOH and 3 mL arginine solution is mixed and 2 drops of α-naphthol is added.

- Mix thoroughly and add 4-5 drops of bromine solution **UNDER THE HOOD!!**

In alkaline solution, arginine react with α-naphthol and sodium hypobromite /chlorite as an oxidize agent, to form **red complexes** as a positive result

G. Pauly's test

Objective: To detect aromatic amino acid tyrosine or histidine

Principle: Diazotized sulphanilic acid couples with amines, phenols and imidazole to form highly colored azo compounds. This coupling reaction must be done in cold condition since diazonium compound is formed in cold. Amino acids tyrosine or histidine coupled with diazonium salt in alkaline condition to form red coloured azo dye.

Materials required:

- 1 % tyrosine, 1 % histidine, 1% glycine
- 1 % sulphanilic acid in 10 % HCI
- 5 % sodium nitrite
- 10 % sodium carbonate
- Ice bath

Procedures

1. Take 2ml test solution in dry test tube.
2. Similarly, take 2ml distilled water in another test tube as control.
3. Add 1ml of sulphanilic acid, mix well and keep in ice bath.
4. Now add 1ml sodium nitrite solution to all test tubes.
5. Leave in ice bath for 3 minutes.
6. Make the solution alkaline by adding 5ml of sodium carbonate.
7. Look for the development of red colored complex.

Experiment 8 : Estimation of amino acid by Ninhydrin

Aim: To estimate amount of amino acids present in a solution

Principle: Ninhydrin (1,2,3-Indantrione monohydrate, or triketohydrindene hydrate), a powerful oxidizing agent is often used to detect α amino acids and also free amino and carboxylic acid groups on proteins and peptides. This chemical detects ammonia or primary and secondary amines. When reacting with these free amines, (in a pH range of 4-8) a deep blue or purple color compound known as **Ruhemann's purple** (discovered by Siegfried Ruhemann in 1910) is produced. When ninhydrin reacts with amino acids, the reaction also releases CO_2. The carbon in this CO_2 originates from the carboxyl carbon of the amino acid.
In presence of Ninhydrin, amino acids undergo ***oxidative deamination*** *liberating* ***ammonia***, ***carbon dioxide***, *a corresponding* ***aldehyde*** *and* ***reduced form of ninhydrin***. The ammonia formed from α-amino group reacts ninhydrin and its reduced product to give a blue substance diketohydrin (Ruhemann's purple). However, in case of imino acids like proline, a different product having a bright yellow colour is formed. Asparagine which has a free amide group reacts to give a brown coloured product.
In the presence of concentrated nitric acid phenyl ring of aromatic amino acid gets nitrated to give yellow coloured nitro derivatives. These derivatives forms orange colour in presence of an alkali solution. The salts of these derivatives are orange in colour. Proteins containing these amino acids also give a positive response to this test.

Alpha amino acids react with Ninhydrin involved in the development of color which is explained by the following five steps.

1. **α-amino acid + Ninhydrin ---> Reduced ninhydrin +α imino acid +H_2O**

This is an oxidative deamination reaction that elicit two hydrogen from the alpha amino acid to produce an alpha – imino acid. Also the ninhydrin reduced and loses an oxygen atom with the formation of water molecule.

2. **α-imino acid + H_2O ---> α-keto acid +NH_3**

The rapid hydrolysis of NH group in the alpha – imino acid will cause the formation of an alpha-keto acid with an ammonia molecule. This alpha-keto acid further involved in the decarboxylation reaction of step.

3. **α-keto acid + NH_3 ---> aldehyde + CO_2**

Under a heated condition to form an aldehyde that has one less carbon atom than the original amino acid. A carbon dioxide molecule is produced along with aldehyde. These first three steps produce the reduced ninhydrin and ammonia that are required for the production of color. The overall reaction for the above reactions is as follows:

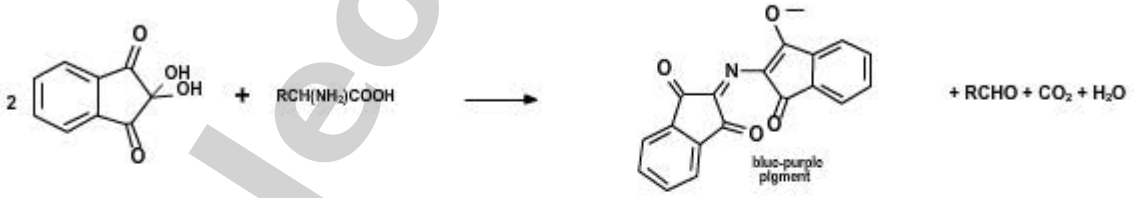
4. α-amino acid + 2 ninhydrin ---> CO_2 + aldehyde + final complex (BLUE) + $3H_2O$

In summary, ninhydrin, which is originally yellow, reacts with amino acid and turns deep purple. It is this purple color that is detected in this method. *Ninhydrin will react with a free alpha-amino group, NH_2-C-COOH. This group is present in all amino acids, proteins or peptides. Whereas, the decarboxylation reaction will proceed for a free amino acid, it will not happen for peptides and proteins.* Theoretically only amino acids produce color with ninhydrin reagent.

The ninhydrin reaction, one of the most important method of detecting amino acids, both technically and historically, has been conventionally used to detect their microgram amounts. When amino acids with a free alpha amino groups are treated with an excess of ninhydrin, they yield a purple colored product. Under appropriate conditions, the color intensity produced is proportional to the amino acid concentration.

Imino acids like proline, the guanidino group of arginine, the amide groups of asparagine, the indole ring of tryptophan, the sulfhydryl group of cysteine, amino groups of cytosine and guanine, and cyanide ions also react with ninhydrin to form various chromophores that can be analyzed.

The overall reaction can be written as follows:



In the quantitative estimation of amino acid using Ninhydrin reagent, the absorbance of the Ruhemann's purple formed by the reaction at 570nm is measured. For imino acids, the absorption happens at 440nm.

Materials required:

Standard amino acid stock solution: **1 mg / ml** (150 µg/ml)

Working solution: 0.1 mg/ml

Ninhydrin solution (freshly prepared): 0.2 % in Acetone (**2 %**)

Diluent solvent: Ethanol or n-propanol

Apparatus and Glass wares:

Test tubes, water bath, pipets, glass beads, micropipets, tips, Spectrophotometer etc

Procedure:

1. Pipet out 0.2, 0.4, 0.5, 0.6, 0.8 and 1.0 ml of standard amino acid solution to the respective labeled test tube

2. Add distilled water to all tubes to make the volume 4 .0 ml

3. Add 4 ml of water to the test tube labeled **BLANK**

4. Add 1 ml of ninhydrin (0.2% in acetone) to all the test tubes including blank and unknown

5. Mix by vortexing

5a. Put few glass beads into each tube and cover the tubes with Al-foil

6. Incubate in boiling water bath for 15 mins

7. Cool the tubes in tap water and add 1 ml of diluents solvent (Ethanol) to each tube and mix well

8. Record the absorbance at A 570 nm using a spectrophotometer

9. Draw a standard curve and determine the concentration of unknown sample

Observation and Calculations:

Vol. of amino acid (ml)	Vol. of distilled water (ml)	Conc. of amino acid (µg)	Vol of ninhydrin reagent (ml)		Volume of diuent (Ethanol) (ml)		Reading at 570 nm
00 (Blank)	4	0	1		1		
0.2	3.8	20	1	Incubation at boiling water bath for 15 min	1		
0.4	3.6	40	1		1		
0.5	3.5	50	1		1	Incubate at RT for 10 min	
0.6	3.4	60	1		1		
0.8	3.2	80	1		1		
1.0	3.0	100	1		1		

UK1	?	?	1		1		
UK2	?	?	1		1		

Draw a standard curve taking all the 6 points (20 µg- 100 µg) putting amino acid concentration on X axis and absorbance (A_{570}) on Y axis. *Ideally it should be a straight line and the line should touch at least 4-5 points.*

Now put the absorbance value of two unknown sample (UK1 & UK2) onto the curve and determine their amino acid concentrations in µg/ml.

Experiment 9 : Qualitative test for carbohydrates

A. Molisch Test

Aim: To distinguish between carbohydrate and non-carbohydrate

Principle: In the presence of conc. H2SO4 sugars are dehydrated forming furfuryl derivates. Furfural is derived from the dehydration of pentoses, while hydroxymethylfurfural is produced from hexoses. α-Naphthol reacts with the cyclic aldehydes to form purple colored condensation products (furfuryl-diphenyl-methane-dyes). Although this test will detect compounds other than carbohydrates (i.e. glycoproteins), a negative result indicates the absence of carbohydrates. Monosaccharides give a rapid positive test. Disaccharides and polysaccharides react slower.

Materials required

1. Molisch's reagent (by *dissolving 0.5-1 g reagent grade* ***α-naphthol*** *in10 mL of* ***95% ethanol***.)

2. Sugar test samples, 0.1 %

Procedure:

1 Add 0.02 mL of the reagent to 1 mL of 0.1% carbohydrate (1 mg/mL) solution in a small test tube.

2. After mixing, tilt the tube and carefully add without mixing, 0.5-1 mL of concentrated sulfuric acid by pouring it down the side of the tube. (Use a glass Pasteur pipette to add the H2SO4: do not use a mechanical pipettor with concentrated acids.)

Observation: A red-violet layer at the interface between the acid (bottom) and aqueous (upper) layers is a positive test for carbohydrates.

B. Iodine/KI Test

Aim: To distinguish between simple carbohydrate and starch

Principle: Iodine test is an indicator for the presence of starch. Strach contain α-amylose, a helical polysachharide and amylo pectin. Iodine forms a large complex when reacts with α-amylose. Simple mono or even oligo sachharides do not form this complex hence no coloration.

Materials required:

1. Iodine solution: (Lugols iodine): *Dissolve potassium iodide (KI) in about 200 ml distilled water and then add iodine crystals.* Make the solution up to 1 litre with distilled water. It is essential to prepare it 24 hours before it is required, as iodine is slow to dissolve

Dissolve 10 gm of KI in about 20-30 ml of distilled water. Add 5 gm of iodine and heat gently with constant mixing until iodine is dissolved. Dilute to 100 ml with distilled water. Store in amber glass-stoppered bottle in the dark.

2. .1% solutions of monosaccharide and polysaccharide (starch) solution

Procedure: - To 2-3 mL of polysaccharide (starch) solution, add 1-2 drops of iodine solution.

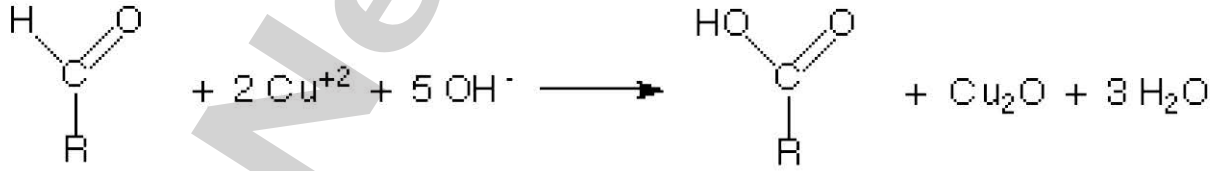
Observation: Starch solution will produce deep blue colour

C. Benedict's test

Aim: To distinguish between reducing and non reducing sugar

Principle: Some sugars such as glucose are called reducing sugars because they are capable of transferring hydrogens (electrons) to other compounds, a process called reduction. When reducing sugars are mixed with Benedicts reagent and heated, a reduction reaction causes the Benedicts reagent to change color. The color varies from green to dark red (brick) or rusty-brown, depending on the amount of and type of sugar.

The **Benedict's test** identifies reducing sugars (monosaccharide's and some disaccharides), which have free ketone or aldehyde functional groups.



Materials required:

1. **Benedict's solution**

Anhydrous	sodium	carbonate	=	100	gm
Sodium	citrate	=		173	gm
Copper(II) sulfate pentahydrate =				17.3 gm	

One litre of Benedict's solution can be prepared from 100 g of anhydrous sodium carbonate, 173 g of sodium citrate and 17.3 g of copper(II) sulfate pentahydrate.

Procedure:

1. Approximately 1 ml of sample is placed into a clean test tube.
2. 2 ml (10 drops) of Benedict's reagent ($CuSO_4$) is placed in the test tube.
3. The solution is then heated in a boiling water bath for 3-5 minutes.

Observation: Observe for color change in the solution of test tubes or precipitate formation

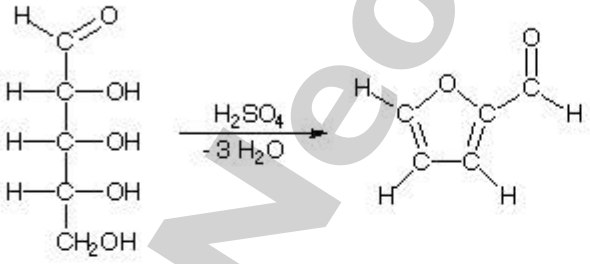
Positive Benedict's Test: Formation of a reddish precipitate within three minutes. Reducing sugars present. Example: Glucose

Negative Benedict's Test: No color change (Remains Blue). Reducing sugars absent. Example: Sucrose.

D. Bial's Test:

Aim: To distinguish between pentoses and hexoses sugar

Principle: The test reagent dehydrates pentoses to form furfural. Furfural further reacts with orcinol and the iron ion present in the test reagent to produce a bluish product



Materials required:

1. Bials reagent Bial's reagent consists of 0.4g Orcinol in 200 ml of concentrated hydrochloric acid and 0.5 ml of a 10% solution of ferric chloride

2. Sugar samples

Procedure: To 3 mL of Bial's reagent add 2–3 mL of test solution and warm gently in a hot water bath for 1-2 minutes .

Observation: A positive test is indicated by:
The formation of a bluish green product. All other colors indicate a negative result for pentoses. Hexoses generally react to form green, red, or muddy brown products.

E. Barfoed's Test:

Aim: To detect reducing sugars that are monosachharides

Materials required:

1. Barfoed's Reagent: This looks like Benedict's but differs somewhat. The reagent is prepared by dissolving 70 g copper acetate monohydrate and 9 mL glacial acetic acid in water to a final volume of one liter. The reagent is stable for years.When 1 mL of reagent is heated with sample in a boiling water bath, a positive test for monosaccharides is formation of a brick-red precipitate within five minutes. Disaccharides generally don't give any reaction even for ten minutes. The precipitate isn't nearly as voluminous as that seen with Benedict's test and tends to adhere to the walls of the test tube.

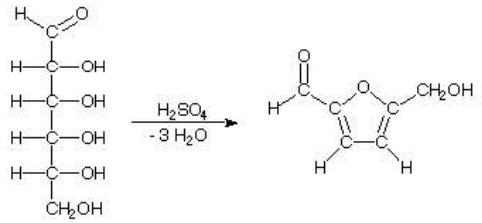
Perform this test with **glucose, fructose, maltose, lactose**

Procedure: - To 1-2 mL of Barfoed's reagent, add an equal volume of sugar solution. - Boil for 5 min. in a water bath and allow to stand. You will observe a brick-red cuprous oxide precipitate if reduction has taken place.

F. Seliwanoff's Test:

Aim: To distinguishes between aldose and ketose sugar

Reactions: The test reagent dehydrates ketohexoses to form 5-hydroxymethylfurfural. 5-hydroxymethylfurfural further reacts with resorcinol present in the test reagent to produce a red product within two minutes (reaction not shown). Aldohexoses react to form the same product, but do so more slowly.



Materials required:

Seliwanoff's reagent: Dissolve 1 g resorcinol in 330 mL concentrated HCl, dilute to one liter (approx. 4 M HCl final). This reagent seems to be stable for more than a year

Procedure: 1. One half ml of a sample solution is placed in a test tube. Two ml of Seliwanoff's reagent is added

2. The solution is then heated in a boiling water bath for two minutes.

Observation: A positive test is indicated by the formation of a red product.

G. Fehling's Test:

Aim: To detect reducing sugar

Principle: In this test the presence of aldehydes but not ketones is detected by reduction of the deep blue solution of copper(II) to a red precipitate of insoluble copper oxide. The test is commonly used for reducing sugars but is known to be NOT specific for aldehydes. For example, fructose gives a positive test with Fehling's solution as does acetoin.

Materials required:

1. Fehling's "A" uses 6.5 g $CuSO_4.5H_2O$ dissolved in 100 ml distilled water containing 2 drops of dilute sulfuric acid.
2. Fehling's "B" uses 35g of Na-K tartrate and 12g of NaOH in 100 ml of distilled water.

Procedure::
1. Mix 15 ml of solution-"A" with 15 ml of solution-"B"
2. Add 2 ml of this mixture to an empty test tube.
3. Add 3 drops of the compound to be tested to the tube.
4. Place the tube in a water-bath at 60° C for few minutes

Observation: A positive test is indicated by the production of yellow 'or brownish-red precipitate of cuprous oxide indicates the presence of reducing sugars in the given sample

H. Osazone Test

Aim: To confirm presences of certain carbohydrates in test sample

Principle: Osazone formation involves hydrazone formation at C1 of an aldose (or C2 of a ketose) and oxidation of C2 (or C1) of an alcohol froup to a ketone (or aldehyde). The new carbonyl group is converted to hydrazone.

Materials required:

1. Phenylhydrazine hydrochloride

2. Na-acetate

3. Water bath

Procedure:

1. To 0.5 g of phenylhydrazine hydrochloride add 0.1 gram of sodium acetate and ten drops of glacial acetic acid.

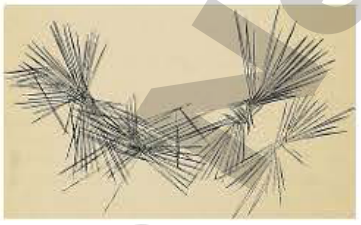
2. Add 5 mL of test solution to this mixture, shake well and heat under boiling water bath for about half an hour or till ppt forms.

3. Cool the solution slowly and examine the crystals under a microscope with low power objective.

Observation: Needle-shaped yellow osazone crystals will be observed for glucose and fructose, whereas lactosazone shows mushroom shaped and maltose produces flower-shaped crystals. *Identical osazones are obtained from D-glucose, D-fructose and D-Mannose.*

Osazone crystals:

Needle shaped crystals: Glucosazone, Fructosazone, Mannosone



Sunflower shaped: Maltose confirmed

Powder puff shaped: lactose confirmed