LABORATORY MANUAL
ON
TECHNIQUES IN
BIOCHEMISTRY

DEPARTMENT OF VETERINARY BIOCHEMISTRY
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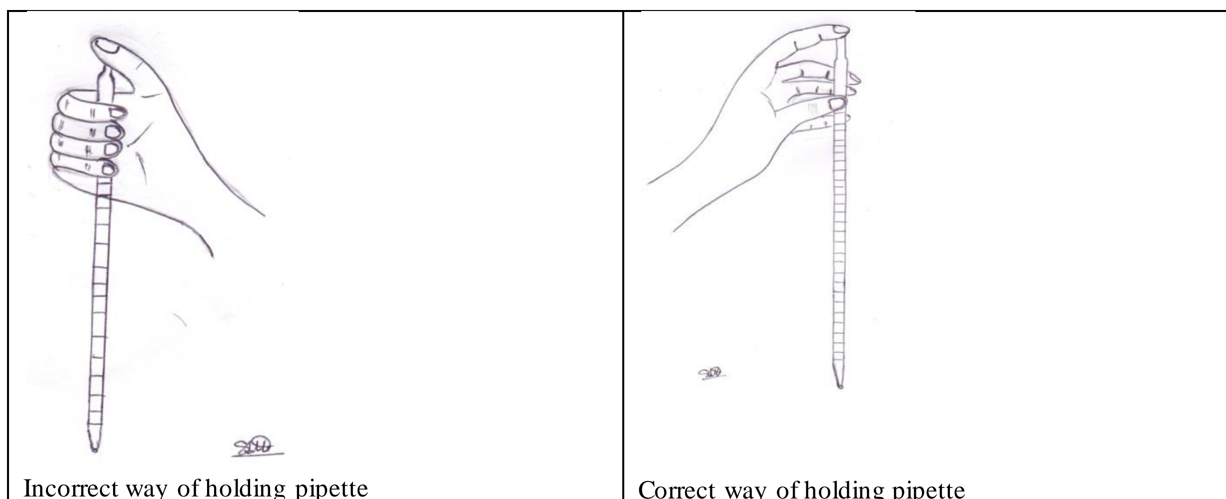
SAFETY RULES IN THE BIOCHEMISTRY LABORATORY

The following is a list of general safety rules to be followed by all the students!

1. Eating, drinking, and/or smoking in the laboratory is strictly forbidden.
2. Laboratory chemicals are not to be tasted since many are toxic. When instructed to smell REAGENTS, do so with great caution, and NEVER put your nose over the bottle! Avoid looking into the mouth of any reaction vessel in which a reaction is in progress. Never point a test tube that contains a heated liquid at anyone.
3. Proper attire must be worn at all times such as apron. THE OPEN-TOED OR SANDALS ARE STRICTLY PROHIBITED.
4. No one will perform any unauthorized experiments, nor will anyone work in the lab alone, or outside of regularly scheduled hours.
5. Report ALL injuries, allergies and/or medical problems to the lab instructor.
6. Firepolish all glass tubing and stirring rods; protect your hands with a towel and lubricate the glass tubing or thermometer when inserting into stoppers.
7. When pouring something out of a reagent bottle, always READ THE LABEL TWICE to be certain that you are using the correct material; always hold the bottle by placing your hand over the label.
8. Label every chemical container to avoid mix-ups.
9. If you spill something, clean it up (GET HELP WITH HAZARDOUS MATERIALS)! Wash your hands immediately after skin contact with any chemical reagent. Also wash them after lab. If liquids drip down the side of the bottle, while pouring, wash the bottle off.
10. NEVER return excess chemicals to the reagent bottle.
11. Dispose of the excess chemicals in the proper waste container, as indicated by the lab instructor.
12. The MOBILE PHONES are strictly prohibited in the lab.
13. DO NOT DRAW THE STRONG ACIDS USING MOUTH PIPETTING.



Incorrect way of holding pipette | Correct way of holding pipette

POWER PREFIXES

Prefix	Symbol	Factor
yotta	Y	10^{24}
zetta	Z	10^{21}
exa	E	10^{18}
peta	P	10^{15}
tera	T	10^{12}
giga	G	10^{9}
mega	M	10^{6}
kilo	k	10^{3}
hecto	h	10^{2}
deka	da	10^{1}
deci	d	10^{-1}
centi	c	10^{-2}
milli	m	10^{-3}
micro	µ	10^{-6}
nano	n	10^{-9}
pico	p	10^{-12}
femto	f	10^{-15}
atto	a	10^{-18}
zepto	z	10^{-21}
yocto	y	10^{-24}

IMPORTANT CONVERSIONS:

$1\ cm^3 = 1$ ml	1 deci liter (dl)= 100 ml	$C^o = \frac{(F^o - 32) \times 5}{9}$	{ C°=degree Celsius F°= degree Fahrenheit}
1 mole= 6.02×10^{23} (Avagadro's Number)			

METHODS FOR EXPRESSION OF CONCENTRATION

A solution is a soluble solid substance dissolved in a liquid. The former is called the solute and the latter the solvent. There are different methods of expressing concentration of solution -

1. In percent (%):

   a) % w/v or w/v ratio - The concentration of a solution is expressed by the weight of a solute in the total volume of the solvent. It is the most commonly used method of expressing the concentration of solutions. It can be defined gram of solute in 100ml of solution. It can be expressed in different way also e.g. %, g/dl, g/100ml

   b) % v/v or v/v ratio

2. Molar or molal: The concentration of a solution is expressed by volume of solute in the total volume of the solvent. It is commonly used for expressing the concentration of liquid solute such as ethanol. It can be defined as ml of solute in total 100 ml of solution. Eg. 100 ml of 70% ethanol indicates 70 ml of ethanol added to 30 ml of water. To prepare 'V' ml of X % of a solute→

$$\text{ml of liquid solute to be taken} = \frac{X}{100} \times V$$

According to system international units (SI units) solute concentrations are expressed in mole or millimole per litre.

One mole of a substance is defined as the atomic or molecular weight of that substance. A gram symbol or a formula weight of any substance contains 6.02×10^{23} (Avogrado number of particles). When one mol of substance dissolved in 1 kg of water the concentration is defined as 1 molal (1m) solution. When 1 mol of substance dissolved in 1 litre of a final solution the concentration is defined as 1 molar (1M or 1 mol/ L) solution.

Molar solution is a part of metric system, hence M = g MW/l, mM = mg MW/L, μM = μg MW/L etc.

Problem:

3) Normality: used to express the concentration of acid, base, oxidizing and reducing solution. Equivalent weight in grams in 1000 ml gives one normal. A normal solution of an acid contains the weight of acid per litre of solution, which donates formula weight of H^+ (6.02×10^{23} H^+).

A normal oxidizing solution contains sufficient oxidizing agent liter to increase the positive valence or decrease the negative valence of a gram-formulae weight of reducing agent by one unit and vice-versa.

Problem:
Relationship between molarity and normality: -

Molarity and normality is equal when valence is one. But one molar substance is two normal when the valency of the substance is 2 and so on.

Problem:

Relationship between millimole, milliosmol and milliequivalent: -

The term millimole is used to express the concentration of solute in solution. One millimole means atomic weight or molecular weight of that substance in 1000 ml solution.

The osmole proportion of a solute in solutions is related to the number of particles in solution and not to its weight or its charge. One osmole is equal to molecular weight in grams, which gives 6.02×10^{23} particles in 1000 ml of solution. In case of non-electrolyte millimole and milliosmol is same but in case of electrolyte, it depends on number of dissociable ions.

The term milliequivalent is used to express the concentration of ions (electrolytes) in biological fluids. Equivalent weight in grams in 1000 ml gives 1Equivalent weight. For univalent ions, 1 mole equals to 1 equivalent (Eq). For multivalent ions, 1 Equivalent weight is equal to the molecular weight in grams (i.e. 1 mole) divided by charge of particle

IMPORTANT FORMULAE

1. Preparation of a solution of concentration of 'M' Molar of 'V' volume:

$$\textbf{Grams of solute to be dissolved in V ml of water} = \frac{\textbf{Molecular Weight} \times \textbf{M} \times \textbf{V}}{\textbf{1000}}$$

2. Preparation of a solution of concentration of 'N' Normal of 'V' volume:

$$\text{Grams } \textbf{of solute to be dissolved in V ml of water} = \frac{\textbf{Molecular Weight} \times \textbf{N} \times \textbf{V}}{\textbf{Valency} \times \textbf{1000}}$$

3. Preparation of Solution of concentration of 'Os' Osmolar of 'V' volume:

$$\text{Grams of solute to be dissolved in V ml of water} = \frac{\text{Molecular Weight} \times \text{Os} \times \text{V}}{\text{n} \times 1000}$$

n = number of dissociated particles of a ionizable compound

OBJECTIVE: PREPARATION OF NORMAL SOLUTION OF ACID (APPROXIMATE)

METHOD: GRAVIMETRIC ANALYSIS

PRINCIPLE:

Many acids are liquid at the ordinary temperature, hygroscopic and not pure, it is not possible to weigh the exact amount of acid. An approximate strength of acid can be prepared by following formula:

$$V1 = \frac{\text{Molecular Weight of Acid} \times N1 \times (V2)}{10 \times \text{Specific Gravity} \times \text{Purity} \times (\text{Valency})}$$

V1= the volume of concentrated acid to be taken for dissolving in water.

V2= the total of given volume of dilute acid of normality N1 to be prepared.

Problem:

TITRATION

Titration is a volumetric technique by which concentration of an unknown solution (usually an acid or base) is determined by reacting with a corresponding neutralizing solution of known strength (called standard solution). The end point of neutralization is marked by the change of colour of an indicator added to the titrating system.

TEST PROPER:

(i) Prepare the burette for the solution. The burette should be clean, free from chips or cracks and stopcock lightly. To grease a clean stopcock, apply a bit of grease with the fingertip down the two sides of the stopcock away from the capillary bore. Then insert the stopcock in the burette and rotate it until a smooth covering of the whole stopcock is obtained. If the burette is equipped with a Teflon plug (plastic), the stopcock need not be lubricated. Rinse the burette with a little amount of the titrant, which will later be used to fill the burette. Rinsing is done by rolling the titrant over the inside wall and discarding the rinsed solution through the stopcock. During rinsing, watch if the burette is clean. A clean burette will drain without any solution clinging to its sides; if the burette is dirty, there will be droplets of liquid clinging to the sides.

(ii) Fasten the burette clamp to the burette stand.

(iii) Fill the burette slowly and carefully with the titrant (unknown). Do not allow air bubbles to form inside the burette. A small beaker may be used during pouring and allow the titrant to flow down the inside wall of the burette. Fill the titrant past the zero mark of the burette, then bring the miniscus exactly to the zero mark by draining. Use the stopcock to control the flow of the titrant. Collect the drained titrant in the beaker used for filling the burette.

(iv) Measure the second solution (standard) into a Erlenmeyer flask (or beaker) with a volumetric pipette. Take extreme care to measure the volume accurately. Add the required amount of the indicator solution into the flask. If the volume is low, you can add 5 to 10 ml of the distilled water to dilute the volume of the fluid in the flask. The volume of diluent is not critical, since it does not enter into the reaction or affect the volumes of the solutions that are being titrated.

(v) Before starting the titration, inspect the burette carefully for any air bubble trapped within the burette column or inside the stopcock or on the tip. Presence of air bubbles adds air-volume to the volume of the titrant as an error. Keep the tip of the burette well within the Erlenmeyer flask, which contains the fluid to be titrated (standard); clamp the burette at this position. Put one hand at the stopcock and the other is used to swirl the flask.

Titration is done by adding the solution in the burette to the Erlenmeyer flask by rotating the stopcock carefully. For accurate reading, titration is done at least three times. The titration readings of three replicates must be close; the second and the third readings should be almost identical.

Clean up the burette following titration by thoroughly rinsing the burette several times with tap water followed by rinsing with distilled water. Dry the burette by holding it on the burette stand.

OBJECTIVE: DETERMINANTION OF CONCENTRATION OF A GIVEN SOLUTION OF SODIUM HYDROXIDE USING A STANDARD SOLUTION OF 0.1 N OXALIC ACID.

PRINCIPLE: The concentration of a given solution of an alkali can be determined by titrating it against a standard solution of acid. For example, if the given solution is NaOH, it is required to be titrated against standard solution of oxalic acid.

For this, the formulae $\boxed{N_B V_B = N_A V_A}$ is to be used. Where N_B and N_A are the strength of alkali and acid respectively and V_B and V_A are the volume of alkali and acid respectively.

MATERIALS REQUIRED:

a. GLASSWARES: Funnel, Burette, Burette stand, Beaker (250 ml) and conical flask (50 ml).
b. SOLUTIONS AND CHEMICALS: 0.1 N Oxalic Acid, given solution of NaOH, Phenolphthalein.

TEST PROPER:

1. Take the given solution of NaOH in beaker.
2. Place the funnel over the top of burette. And fill the burette with the given solution of NaOH using beaker. Remove the air gap inside the tip of burette. Bring the lower meniscus of NaOH solution to the mark of "0".
3. Take 10 ml of 0.1 N Oxalic acid along with two drops of phenolphthalein in 50 ml conical flask.
4. Place the flask below the tip of burette. Allow the NaOH solution to fall into the oxalic acid drop by drop in very slow speed. Once the pink color appears, the flask is required to be shaken and the persistence of the pink color is to be observed. If the pink color fades away, the process is to be continued else to be stopped.
5. The point of burette reading where the oxalic acid solution develops the persistent pink color is called the end-point. That point is to be considered equal to V_B.
6. The titration is to be repeated thrice. The average value of second and third reading is to be considered for the calculation.

RESULT:

Initial Burette Reading (A)	Final Burette Reading (B)	V_B (B—A)	Average V_B

CALCULATIONS:

THE CONCENTRATION OF GIVEN SOLUTION OF NaOH IS: …………………….

OBJECTIVE: **PREPARATION OF 250 ml N / 10 NaOH SOLUTION**

DATE:

PRINCIPLE:

Equivalent weight in grams in 1000 ml gives 1N solution. One normal acid solution is that solution which donates 6.02 X 10^{23} number of H^+ per litre.

To prepare N/10 250 ml NaOH solution: 1g sodium hydroxide to be dissolved in 250 ml water. It is not possible to weigh sodium hydroxide accurately because it is a hygroscopic substance. Therefore, we can prepare N/10 sodium hydroxide by volumetric analysis (titration) using the following formula.

$N_BV_B = N_AV_A$ where N_B and N_A are the strength of alkali and acid respectively and V_B and V_A are the volume of alkali and acid respectively.

Apparatus:

i) Burette
ii) Conical flask
iii) Measuring cylinder
iv) Volumetric flask (250ml)
v) Beaker
vi) Pipette volumetric 10 ml and graduated 10 ml

REAGENTS:

i) Preparation of carbonate free saturated solution of sodium hydroxide: In a one litre beaker or wide mouth flask dissolve approximately 110 g sodium hydroxide (pellets) in 100ml g distilled water. Stir until all pallets have dissolved (the solution will be hot). The solution will be almost saturated and is approximately 27 N. Use a high-fighting cover and allow to stand for 2 to 3 days until sodium carbonate settles out, leaving a clear solution of sodium hydroxide. Carefully separate the clear solution by decanting or by passing through a fine sintered glass filter. Transfer the carbonate-free solution of sodium hydroxide to a polythene bottle.

Dilute 5 to 7 ml of the strong solution of sodium hydroxide in about 1 litre of freshly boiled and cooled distilled water (to remove dissolve carbon dioxide)

ii) Primary standard: N/10 oxalic acid - dissolve .... g (calculation is shown on page ....) oxalic acid in 1000 ml of distilled water.

iii) Indicator: 1% phenolphthalein in alcohol.

TEST PROPER:

1) Fill the burette with diluted sodium hydroxide solution (not too much concentrated NaOH).

2) Take accurately 10 ml N/10 oxalic acid in conical flask. Add 1 to 2 drops phenolphthalein. Mix. Three replicates should be done for better accuracy.

3) Titrate the sodium hydroxide solution to the end point, a pink colour persists 15 seconds. The end point is one when one more drop of alkali solution will turn the acid solution to a red colour.

4) Record the amount of alkali solution required to titrate the acid solution.

5) Repeat the titration for each replicate of the replicate (Table ....) and average is taken.

6) Calculate the normality (shown on page ....).

7) Now dilute the alkali to get N/10 (Calculation is shown ....).

N.B.- Standard solution of sodium hydroxide can also be prepared using primary standard as N/10 HCI and methyl orange as indicator.

Initial Burette Reading (A)	Final Burette Reading (B)	V_B (B—A)	Average V_B

OBJECTIVE: DETERMINATION OF PK$_A$ VALUE OF PHOSPHATE BUFFER

What is pKa value of an acid?

The pKa value of an acid is that value of pH in which 50% of acid molecules are in ionized form and rest 50% in unionized form

$$HA \rightleftharpoons H^+ + A^-,$$

$$K_{eq} = \frac{[H^+][A^-]}{[HA]} = K_a$$

$$pK_a = \log \frac{1}{K_a} = -\log K_a$$

In case of strong acids, the K_a value is larger and pK_a is smaller. As per the Henderson-Hasselbalch equation,

$$pH = pK_a + \log \frac{[\text{proton acceptor}]}{[\text{proton donor}]}$$

$$pH = pK_a + \log \frac{[A^-]}{[HA]}$$

The $[A^-]$ is ionized form of acid and HA is the unioized form of acid. Thus, in the situation when the concentration of ionized form of acid is equal to unioized form of acid or

$[HA]$ equals $[A^-]$,

Equation mentioned above becomes

$$pH = pK_a + \log 1 = pK_a + 0 = pK_a$$

So, from the above equation, it can be said that pK_a is that value of pH when the concentration of ionized form of acid is equal to unioized form of acid.

So, for the calculation of pK_a of phosphate buffer, we need to take equal concentration of monobasic sodium phosphate (unionized acid) and di-basic sodium phosphate (conjugate base). The pH of the resulting solution would be the pK_a value of phosphate buffer.

Write the calculation here.

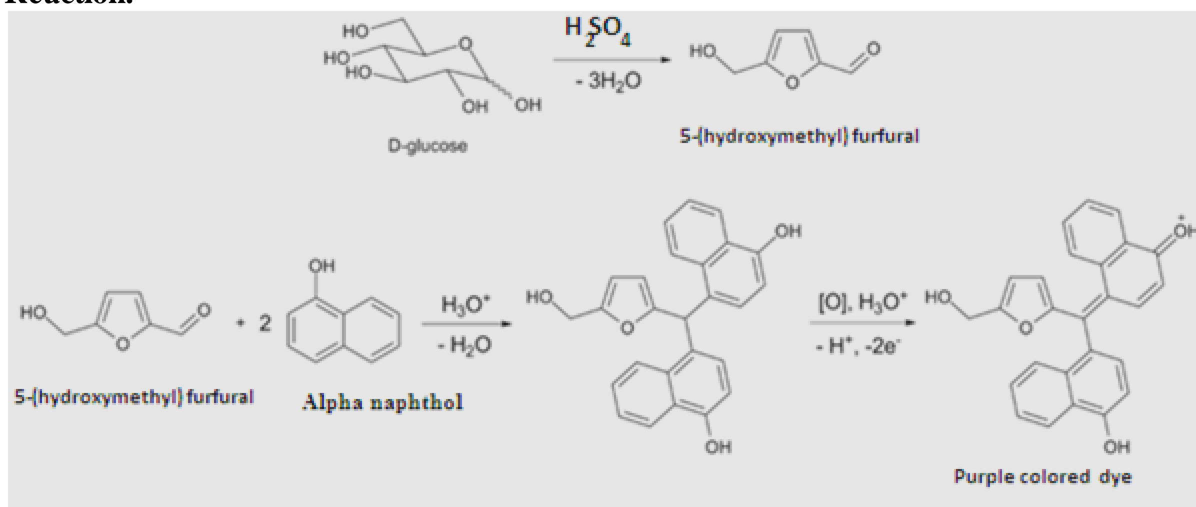
Find out what should be the ratio of dibasic sodium phosphate and monobasic sodium phosphate for maintaining the pH of 7.0, 7.2, 7.3 and 7.4 in phosphate buffer.

QUALITITAVE TESTS FOR CARBOHYDRATE

Sl. No: 1(a). MOLISCH'S TEST (named after Austrian botanist Hans Molisch)

PRINCIPLE: This is the general test for carbohydrate. Conc. sulphuric acid hydrolyses glycosidic bond(s) of disaccharide and polysaccharide to give the monosaccharides, which are then dehydrated to furfural and its derivatives. These products then combined with suphonated alpha-naphthol to give purple colour complex.

Reaction:



REAGENTS: 1. Conc. Sulphuric acid
2. Alpha naphthol (5% w/v in ethyl alcohol)

Sl. No. 1 (b) ANTHRONE TEST

PRINCIPLE: This is the general test for carbohydrate. Conc. sulphuric acid hydrolyses glycosidic bond(s) of disaccharide and polysaccharide to give the monosaccharides, which are then dehydrated to furfural and its derivatives. These products then combine with anthrone to give a blue green complex.

Reaction: See above.

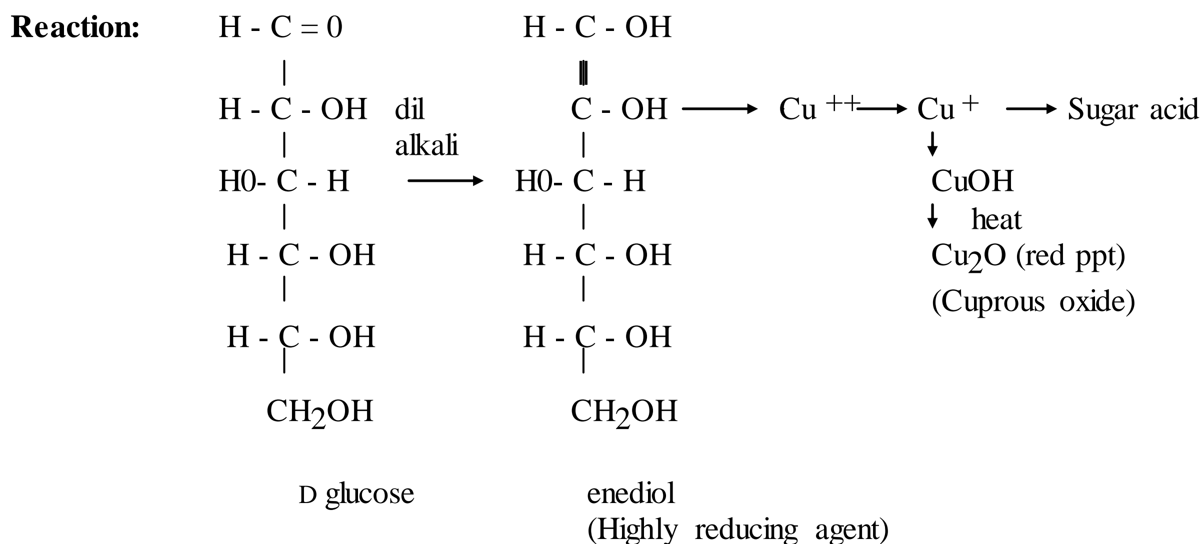
REAGENTS: 1. Conc. Sulphuric acid
2. Anthrone reagent (0.2 % in conc. Sulphuric acid)

Sl. No. 2 BENEDICT'S TEST

PRINCIPLE: This test is to detect reducing sugars. All monosaccharides contain free anomeric carbon atom, so they are reducing sugars. A disaccharide may be reducing or non-reducing. When both anomeric carbon atoms are involved in glycosidic bond formation then disaccharides are non-reducing e.g. sucrose trehalose. When anomeric carbon atom of any one of monosaccharides is free then disaccharide is reducing e.g. cellubiose, maltose, lactose etc.

In alkaline condition, there is a rearrangement of anomeric carbon atom forming enediol, which is highly reducing agent, reduces cupric ion to cuprous ion (cu^{++} to cu^{+}) forming cuprous hydroxide. On heating, it is converted to cuprous oxide, a red precipitate.

Reaction:



Reagent: 1. Benedict's reagent (Dissolve 173 g sodium citrate and 100 g sodium carbonate in about 800 ml warm water. Add 17.3 g copper sulphate, mix and make up the volume to 1 litre. Filter).

Sl. No. 3 BARFOED'S TEST

PRINCIPLE: This test is done to detect monosaccharides. Barfoed's reagent is weakly acidic and is reduced only by monosaccharides. The precipitate of cuprous oxide is less dense than Benedict's test.

Reagent: 1. Barfoed's reagent (Dissolve 13.3 g copper acetate in about 200 ml of water and add 1.8 ml of glacial acetic acid).

Sl. No. 4 BIAL'S TEST

PRINCIPLE: This test is to detect pentoses. When pentoses are heated with conc. HCl, furfural is formed which condenses with orcinol in the presence of ferric ions to give a blue-green colour. The reaction is not absolutely specific for pentoses since prolong heating of some hexoses yield hydroxy methyl furfural, which also reacts with orchinol to give coloured complexes.

REAGENTS:

1. Bial's orchinol reagent (Dissolve 1.5g of orchinol in 500 ml conc. HCl and add 20 drops of a 10% w/v solution of ferric chloride).
2. Amyl alcohol

Sl. No. 5 SELIWANOFF'S TEST

PRINCIPLE: This test is for detection of ketoses. Ketoses are dehydrated more rapidly than aldoses to give furfural derivatives, which then condense with resorchinol to form a red complex.

REAGENTS: Seliwanoff's reagent (50 mg of resorchinol in 100 ml 3N HCl).

Sl. No. 6 IODINE TEST

PRINCIPLE: This test is to detect polysaccharides. Iodine forms coloured absorption complex with polysaccharide.

REAGENTS: 1. Iodine solution (0.005N Iodine in 3% potassium iodide.)

QUALITATIVE TESTS FOR CARBOHYDRATE

Sl. No.	Test	Procedure	Observation	Remarks
1 (a).	Molisch's test	2 ml of test solution + 2 drops of α-naphthol solution. Mix. Carefully add 1 ml conc. H_2SO_4 down the side of the test tube so as to form two layers.	Purple color ring at the junction of two liquids	Carbohydrate present
1 (b).	Anthrone test	1 ml Anthrone reagent + 5 drops of test solution. Mix thoroughly.	Blue green complex	Carbohydrate present
2.	Benedict's test	2 ml Benedict's reagent in a clean test tube. Add 5 drops unknown solution. Mix. Boil and cool.	Red precipitate	Reducing sugar present
3.	Barfoed's test	2 ml Barfoed's reagent + 1 ml test solution, mix, boil for 1 minutes and cool (prolonged boiling should be avoided).	Red precipitate	Monosaccharide present
4.	Bial's test	5 ml Bial's reagent in a test tube. Boil and immediately add 2 ml test solution. Mix	Blue green complex	Pentose present

Sl. No.	Test	Procedure	Observation	Remarks
		thoroughly. Cool the tube. Add 2-3 ml of amyl alcohol and shake.		
5.	Seliwanoff's test	1 ml Seliwanoff's reagent + 2 drops unknown solution. Mix thoroughly, boil for 1 minute and cool.	Red complex	Ketose present
6.	Iodine test	2 ml test solution + 2 drops of dilute HCl. Mix thoroughly. Add 2 drops iodine solution and mix.	Coloured complex Blue- starch Red brown – glycogen Purple – dextrin	Polysaccharide present

QUALITATIVE TESTS FOR PROTEIN AND AMINO ACID

Sl. No. 1 BIURET TEST

PRINCIPLE: This is a both qualitative and quantitative test for protein. Cupric ions react with compounds in alkaline solution containing two or more peptide bones, to form a co-ordination complex, which is purple in colour. The depth of the colour obtain is a measure of the number of peptide bond present. This reaction is not absolutely specific for protein.

REAGENTS: 1. 40% Sodium hydroxide
1. 1% Copper sulphate

Sl. No. 2 NINHYDRIN TEST

PRINCIPLE: Ninhydrin (Triketohydrindene hydrate), a powerful oxidizing agent, reacts with all amino acids between pH 4 and 8 to give a purple compound. The reaction is also given by primary amines and ammonia but without the liberation of CO_2. The imino acids proline and hydroxiproline also react with ninhydrin, but in this case a yellow colour is obtained instead of the usual purple one.

The reaction is very sensitive and is ideal for the detection of amino acids.

REAGENTS: 1. Ninhydrin solution (0.2 % in water).

Sl. No. 3 XANTHOPROTEIC TEST

PRINCIPLE: Amino acids which contain aromatic nucleus form yellow nitro derivatives on heating with conc. nitric acid. The salts of these derivatives are orange.
REAGENTS: 1. Nitric acid (conc.)
2. Sodium hydroxide (40%).

Sl. No. 4 MILLON'S TEST

PRINCIPLE: Compounds containing the hydroxybenzene radical react with Millon's reagent to form red complexes. The only phenolic amino acid tyrosine and its derivatives give a positive reaction.

Reagent: 1. Millon's reagent (15% solution of mercuric sulphate in 15% sulphuric acid)
2. Sodium nitrite (1%).

QUALITATIVE TEST FOR PROTEIN AND AMINO ACIDS

Sl. No.	Test	Procedure	Observation	Remarks
1.	Biuret test	1 ml test solution + 1 ml of 40% NaOH. Mix thoroughly and add 3 drops of 1% $CuSO_4$ solution. Mix.	Violet coloured complex	Peptide (protein) present
2.	Ninhydrin test	1 ml test solution + 10 drops of ninhydrin solution. Mix and boil.	Purple colour	Protein or amino acid present
3.	Xanthoproteic test	1 ml test solution + 1 ml conc. HNO_3. Mix and cool. Observe the colour change. Add 2 ml of 40% NaOH solution.	Yellow colour in acid changed to bright orange colour	Aromatic amino acids present*
4.	Millon's test	1 ml test solution + 5 drops of Millon's reagent. Mix, boil and cool. Add 5 drops of sodium nitrite solution.	Brick-red colour	Phenolic amino acids present

* Phenylalanine gives a negative or weakly positive reaction.

** The sulphur of methionine is not affected by this reaction

OBJECTIVE: **TESTS FOR FATTY ACIDS (SAPONIFICATION)**

PRINCIPLE: When fat and oils are heated with alkali, free fatty acids and glycerol are liberated and this process is known as saponification.

The excess alkali present reacts with the liberated fatty acids to form the sodium or potassium salts, which give the solution a characteristics soapy appearance. Soaps are soluble in water but are precipitated on the addition of excess NaCl. The magnesium and calcium salts, on the other hand, are insoluble and give rise to the scum formed when soap is lathered in hard water.

TEST PROPER:

(vi) Take 4 ml of 2% Na_2CO_3 solution in a test tube and add 2 drops of mustard oil. Shake vigorously and boil. A clear soapy solution is formed. Cool and divide it up to 3 parts to study the properties of soap -

(a) In one test tube, add a few drops of conc. HCl and see that the fatty acid separates out and floats up. This is due to hydrolysis of soap by the acid.

(b) In another portion, dissolve an amount of finely powdered NaCl. White precipitates of soap separate out and float on the surface. This process is called "Salting out of soap".

(c) To the third portion, add a few drops of calcium chloride solution. A precipitate of insoluble calcium soap is obtained.

OBJECTIVE: PRECIPITATION OF PROTEIN BY HEAVY METALS.

PRINCIPLE:

At p^H 7 and above proteins are usually negatively charged. The positively charged metal ion neutralizes this charge and the protein comes out of solution. Precipitation by heavy metals is therefore more effective at neutral to slightly alkaline p^H values.

Materials:

1. Protein solution
2. Heavy metals (0.1 M $CuSO_4$, Pb-acetate and mercuric nitrate)

METHOD:

Add a few drops of the heavy metal solution to 2ml of protein solution. After sometime, there will be precipitation of protein.

OBJECTIVE: DETERMINATION OF ACID VALUE OF FAT

PRINCIPLE: During storage fat may become rancid due to peroxide formation at the double bond by atmospheric oxygen and hydrolysis by microorganism with the liberation of free acid. The amount of free acid present therefore gives an indication of the age and quality of the fat.

The acid number/value is the number of mg of KOH required to neutralize the free acid present in 1 g of fat.

Materials:

1. Olive oil.
2. Fat solvent (equal volumes of 95% alcohol and ether neutralized to phenolphthalein).
3. Phenolphthalein (1% in alcohol).
4. Potassium Hydroxide .01N, (5.6g/L)
5. Burette.

METHOD: Accurately measured 10 g of the test compound and suspend the melted fat in 50 ml of fat solvent. Add 1 ml of phenolphthalein solution, mix thoroughly and titrate with 0.1M KOH until the faint pink colour persist for 20-30 sec. Note the number of ml of standard alkali required and calculate the acid value of the fat.

For eg. Initial reading of the burette = 2 ml,

Final reading of the burette =3 ml.

KOH used for the titration equal = 1 ml.

Since, 1 ml of KOH contain 5.6 mg. therefore 5.6 mg is required to neutralized 10g of olive oil. Hence acid number is 5.6/10 = 0.56.

OBJECTIVE: ESTIMATION OF LACTOSE IN MILK

PRINCIPLE: Lactose is determined on the protein free filtrate of milk. The protein free filtrate is heated with alkaline Cupper Sulphate using a special tube to prevent reoxidation. The Cuprous oxide form is treated with phosphomolybdic acid solution and a blue colour being obtain which is compared with that of a standard.

REAGENTS/CHEMICALS:

1. 10% sodium tungstate solution.
2. 2/3 N sulphuric acid.
3. Alkaline cupper sulphate solution (4g of pure anhydrous sodium carbonate + 0.750g sodium potassium tartrate + 0.45g crystalline cupper sulphate. Make the volume 100 ml with distilled water).
4. Lactose standard (30 mg of lactose in 100 ml of 0.2% Benzoic acid.)

TEST PROPER :

Introduce 0.1 ml of milk into a 10 ml of centrifuge tube; add 0.2 ml of 10% sodium tungstate. Add gradually 0.2 ml of 2/3N sulphuric acid. Make the volume 10 ml with water. Mix well, lets stand for 5 minutes and filtered/ centrifuge. Into a Folin- Wu sugar tube introduce 1 ml of filtrate and 1 ml of distilled water. Into another tube, place 2 ml of lactose standard solution. Add 2 ml of distilled water in another tube as blank. Add 2 ml of alkaline cupper solution to each tube and allow to boil in boiling water for 8 minutes. Cool and add 1 ml of acid molybdate reagent to each tube. After 1 minute dilute the content upto 12/25 ml mark if colour is dark. Take the optical density in a spectrophotometer at 420 mμ.

Calculation:

$$\frac{\text{Density of unknown}}{\text{Density of standard}} \times \text{Concentration of standard (0.06)} \times \frac{100}{\text{Volume of sample used ((0.01)}}$$

= mg of lactose per 100 ml of milk.

COLORIMETRY

The concentration of a biochemical compound can be determined by measuring the light absorbed by solution using a colorimeter. Complete isolation of the compound is not necessary and the constituents of such a complex mixture as blood can be determined after little treatment. Many compounds are not coloured but can be made to absorb in the visible region by specific and sensitive chemical reaction and quantified at mM concentration.

LAWS OF LIGHT ABSORPTION:

When monochromatic light passes through a solution, the intensity of light transmitted decreases exponentially with increasing path length (Lambert's law) and with increasing concentration of the absorbing substance (Beer's law). The combination is often termed as the Beer- Lambert law.

Measurement of extinction/ absorbance / transmittance:

There are different types of colorimeter to measure extinction/absorbance/ transmittance.

1) Visual colorimeter
2) Photoelectric colorimeter
3) Spectrophotometer

1) Light Source

For most purposes in clinical biochemistry only the ultraviolet, visible and near infrared parts of the spectrum 200 - 1200 nm, need to be covered. The light source is usually a tungsten lamp for the visible region (400 - 700 nm) and either a hydrogen or deuterium lamp for the ultraviolet (200 - 400). Infrared spectrophotometer use a silicon carbon rod heated to 1200°C.

2) Wave Length Selector / Optical System

Wavelength selection may be made by absorption or interference filter, prisms or diffraction grating incorporated in a monochromator.

(a) Filter: The filters consist either of tinted glass or of gelatin impregnated with organic dyes held between two plates of glass, and function by selective absorption of unwanted wavelengths, thus give narrow transmission bands approximate to monochromatic light. The colour of filter is complementary to the colour of the solution under investigation (Table).

Table: The two columns indicate complementary colours. For a given colour of solution the choice of filter is indicated in opposite column

Wave Length (nm)	*Colour*	*Wave length* (nm)	*Colour*

400 - 435	Violet	555 - 575	Yellow-green
435 - 480	Blue	575-600	Yellow
480-490	Green-blue	600-620	Orange
490-500	Blue-green	620-700	Red
500-555	Green	620-700	Purple
		400-480	Red + blue)

(b) Monochromator:

Monochromator are optical systems, which produce, from a multiwave length source of radiation, a parallel beam of monochromatic radiation (radiation of a single wave length) usually based upon refraction by a prism or diffraction by a grating.

3) Associated optical system and slits:

Instrument using filters as wavelength selectors required lenses to focus correctly the light from the source through the filter and cuvette to the detector. A variable slit or iris diagram is inserted into the optical pathway.

For instruments, which use a prism or diffraction, the optical requirements are stringent.

4) Sample holder, cells or cuvette:

Optically transparent cells are usually used to contain the material under study, which is normally dissolved in a suitable solvent. For visible wave lengths, glass or plastic cells are convenient but below 340 nm silica or quartz must be used. Rectangular cells are better than cylindrical cells.

In most instruments the light path in the cuvette is 1cm. Cuvette of longer path length are available for increased sensitivity. The volume of fluid needle to fill the 1 cm cuvette from a few micro litre to about 10 ml.

(5) Photosensitive detectors:

Convert quanta of radiation to electrical energy, which may be amplified, detected and recorded. They are different types: (a) Barrier larger cells, (b) photoconductive cells, (c) photoemissive tubes and (d) photomultiplier tubes.

(6) Output device:

Most modern instruments are of the direct reading type where the amplifical detector signal operates a galvanometer, servo-potentiometer recorded or digital display.

Differences between a photoelectric colorimeter and spectrophotometer:

Spectrometer is a sophisticated type of colorimeter where monochromate light is provided by a prism or grating. The beam width of the light passed by colorimeter filter is quite broad so that it may be difficult to distinguish between two compounds of closely related absorption with a colorimeter.

Absorption spectra:

Many compounds have characteristics absorption spectra in the ultraviolet and visible region so that identification of these materials in a mixture is possible and provides a sensitive and non-destructive form of assay.

Protein absorbs strongly at 280 nm according to these content of amino acids tyrosine and tryptophane.

Nucleic acid and the component bases show maximum absorption in the region of 260 nm.

P-nitrophenol in alkaline solution, gives a typical yellow colour with maximum absorption at 450 nm.

OBJECTIVE: EXPERIMENT ON BEER'S LAW. THE ABSORPTION SPECTRUM OF P-NITROPHENOL.

PRINCIPLE: Some compounds have characteristic absorption spectra in the UV and visible region.

REAGENTS:

1. Sodium hydroxide solution: 10 mM / litre.
2. P-nitrophenol:

Stock solution: 1mM/l, by dissolving 139.1 mg in 1000 ml

Working solution = 0.04 mM/litre (20 ml of solution to a 500 ml volumetric flask and make up the volume with sodium hydroxide solution.

TEST PROPER:

Read against a flask of sodium hydroxide solution from 395 to 420 nm

Test tube	Wave length (nm)	%T	Absorbance
1	395		
2	400		
3	405		
4	410		
5	420		

Result:

Interpretation:

OBJECTIVE: **EXPERIMENT ON BEER'S LAW. USING p-NITROPHENOL**

PRINCIPLE: When monochromatic light passes through a solution, the intensity of light transmitted decreases exponentially with increasing path length (Lambert's law) and with increasing concentration of the absorbing substance (Beer's law). The combination is often termed as the Beer- Lambert law. The Beer-Lambert law only applies for monochrometer light and providing there is no change in ionization, association, dissociation or solution of the solute with concentration.

If a suitable standard is prepared and the absorbance of this and the unknown solution are read, then provided beer's law is obeyed.

$$\frac{\text{Concentration of unknown}}{\text{Concentration of standard}} = \frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}}$$

$$\text{Therefore, conc. of unknown} = \frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \text{ X Conc. of standard}$$

REAGENTS:

1. p-nitrophenol standard

   (a) Stock standard (10 mM / L) - Dissolve 0.1391 g p-nitrophenol in water and diluted to 100 ml with water

   (b) Working standard: 0.05 mM / L - Dilute 5 ml stock standard to 1000 ml with water

2. Sodium hydroxide 0.2N - Dissolve 8 g NaOH in 1000 ml water

TEST PROPER:

Test tube No.	Dist. water (ml)	NaOH (ml)	Working standard (ml)	Concentration (mM/L)	OD at 415 nm
1	9	1	0	0	Adjusted to 0
2	8	1	1	0.00005	
3	7	1	2	0.00010	
4	5	1	4	0.00020	
5	1	1	8	0.00040	

CENTRIFUGATION

Centrifugation is a separation technique that is based on behaviour of particles in an applied centrifugal field and separate the molecules based on parameters such as relative molecular mass, shape and density. The centrifugation is of two types: preparative and analytical. The preparative centrifuges are concerned with isolation, separation, and purification of analytes. The analytical centrifugation is concerned with pure or nearly pure macromolecular structures rather than particles. They are used to determine the sedimentation coefficient and molecular weights of the molecules.

The velocity of centrifugation is directly propotional to difference in the density of medium and particles, radius of the particle and radius of the rotor of centrifuge.

$$v = \frac{2Rp^2 \times (\rho P - \rho M)\omega^2 r}{9\eta}$$

Where

Rp= Radius of particle (assuming the particle is spherical or almost spherical).

ρP= desnisty of particle

ρM= density of medium where the particle is suspended.

ω=angular velocity of rotor.

η= coefficient of medium where the particle is suspended.

r=radius of the rotor

The particles having density less than the density of the medium cannot be precipitated by any centrifugation method.

The speed of centrifugation is expressed in terms of revolutions per minute (RPM) or relative centrifugal force (RCF). We generally recommend the use of RCF as it is same in all centrifuge machines as it direct indication of actual force being exerted on the analyte or the particle in the medium. The RCF is expressed as times g (×g). The relationship between RPM and RCF is through the radius of rotor as below:

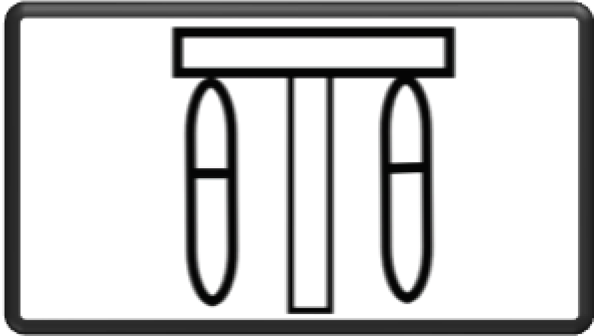
$$RCF = 1.12 \times r\frac{RPM^2}{10^5}$$

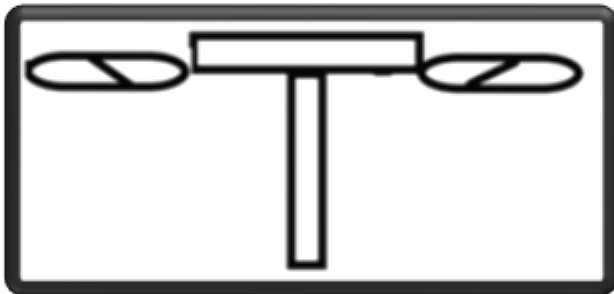
There are different types of centrifuge machines

1. **Small bench centrifuge**: these types of centrifuge machines are used to to precipitate the large particles such as cells e.g. erythrocytes, bacterial cells or yeast cells. The maximum relative centrifugal force used in these types of centrifuge machines are 5000×g. they can be used to handle small volumes of samples that is up to 2 ml volume.
2. **Large capacity centrifuges**: these types of centrifuges can handle samples up to 70 ml. The maxiumum relative centrifugal force in these types of centrifuge machines is 6,000×g.
3. **High Speed Refrigerated Centrifuges**: These types of centrifuge machines can have maximum relative centrifugal force up to 30,000×g and can maintain the temperature at 4°C. The refrigeration in these types of centrifuges is required because in high speed centrifugation, the samples get heated up that may ruin the samples especially the protein samples.
4. **Ultra Centrifuges**: The maximum relative centrifugal force that ultra centrifuges can handle is 600,000×g. These can be used to separate that have low density such as bacterial outer membrane proteins, eukaryotic cell organelles, etc.

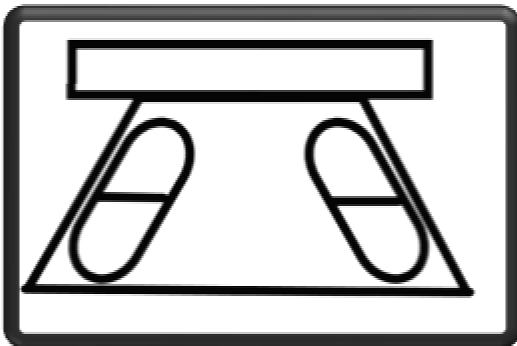
Different types of rotors in centrifuges:

1. **Swinging bucket rotors**: the buckets of the rotors that contain the samples are hung on the rotor when stationary but when the rotor makes the rotation they swing to horizontal position. This kind of rotors are good for density gradient centrifugation.





2. **Fixed angle rotors:**
   In this type of rotors the buckets are fixed at a particular angle (generally 45° angle). This type of rotors are good for precipitation of analytes.



OBJECTIVE: SEPARATION OF PLASMA AND SERUM FROM WHOLE BLOOD

LARGE VOLUME OF BLOOD

METHOD FOR PREPARATION OF SERUM (MAXI-PREP):

1. Collect the blood in a test tube without containing any anticoagulant.
2. Keep the tube in slanting position for about one hour.
3. Clear straw colored fluid appears after the blood has clotted. Collect this fluid in a clean test tube and store it in -20ºC till further use for biochemical analysis.

METHOD FOR PREPARATION OF PLASMA (MAXI-PREP):

1. Collect the blood in a centrifuge tube containing suitable anticoagulant.
2. Slowly mix the blood with the anticoagulant.
3. Centrifuge the blood at 2000 × g for 5 minutes.
4. The blood cells settle down forming the cell pellet and the supernatant fluid present in the tube is plasma.
5. Transfer the plasma into a fresh tube and store it in -20ºC till further use for biochemical analysis.

SMALL VOLUME OF BLOOD

METHOD FOR PREPARATION OF SERUM (MINI-PREP):

1. Collect the blood in a micro-centrifuge tube without containing any anticoagulant.
2. Keep the tube in room temparature for one hour.
3. Centrifuge the clotted blood @2000 to 3000×g for 5 minutes.
4. Clear straw colored fluid appears as supernatant.
5. Collect the fluid carefully with micropipette without disturbing the RBC pellet.
6. Collect this serum in a fresh tube and store it in -20ºC till further use for biochemical analysis.

METHOD FOR PREPARATION OF PLASMA (MINI-PREP):

1. Collect the blood in a micro-centrifuge tube containing suitable anticoagulant.
2. Slowly mix the blood with the anticoagulant by repeatedly inverting the tube gently.
3. Centrifuge the blood at 2000 × g for 5 minutes.
4. Remove the plasma that appears as supernatant using micropipette gently without disturbing the RBC pellet.
5. Transfer the plasma into a fresh tube and store it in -20ºC till further use for biochemical analysis.

ANTICOAGULANT:

Anticoagulant	Concentration	Remarks
EDTA	1 mg per ml blood	EDTA chelates calcium ions to form a soluble complex
Sodium Citrate	6 mg per ml blood	Removes calcium ions by forming soluble citrate complex
Heparin	0.2 mg per ml blood	Stops the formation of thrombin from pro thrombin therefore stopping the formation of fibrin from fibrinogen

Sodium Fluoride	10 mg per ml blood	Inhibit glycolysis. Suitable for estimation of blood glucose.
Potassium oxalate	1 mg per ml	Removes calcium ions by forming soluble citrate complex

OBJECTIVE: **ESTIMATION OF PLASMA/SERUM PROTEIN**

METHOD: Biuret method

PRINCIPLE:

Biuret is the most commonly used method for total plasma or serum protein estimation. Biuret reagent consists of alkaline copper sulphate solution containing sodium-potassium tartarate. The cupric ions form a co-ordination complex with four – NH groups present in peptide bonds giving an absorption maximum at 540 nm. The method is reliable, reproducible and useful in the clinical field. Its main disadvantage is its lack of sensitivity, being unsuitable for the assay of proteins at concentration much less than 1 mg/ml.

REAGENTS:

1. Biuret reagent – Prepare 200 ml of 2% (w/v) of NaOH solution. Put this solution in 500 ml volumetric flask. Add 4.5 grams of sodium potassium tartarate and 15 grams of copper sulphate pentahydrate and 1 gram of potassium iodide. Mix the contents continuously until complete dissolution. Add distilled water till the mark of 500 ml in the volumetric flask.

2. Bovine serum albumin (BSA) standard – 6 g/100 ml NSS.

TEST PROPER:

	Test (a)	Standard (b)	Blank (c)
Distilled water (ml)	-	-	0.02
Serum/plasma (ml)	0.02	-	-
Standard (ml)	-	0.02	-
Biuret reagent (ml)	1	1	1

Incubate all the tubes at room temperature for 10 min. Read at 555 nm. Adjust reagent blank at 0.

Calculation:

$$\textbf{Serum total protein} \;\; \textbf{g} \;\; \textbf{dl} \;=\frac{\textbf{OD of test}}{\textbf{OD of Standard}}\times(\textbf{Concentration of standard})$$

OBJECTIVE: **ESTIMATION OF PROTEIN IN BIOLOGICAL FLUID**

METHOD: Lowry (Folin – Ciocalteau) method

PRINCIPLE:

The phenolic group of tyrosine residues in a protein will produce a blue/purple colur, with maximum absorption in the region of 660 nm with Folin and Ciocalteau reagent, which consist of sodium tungstate, molybdate and phosphate. The method is sensitive down to about 20 μg/ml and is probably the most widely used protein assay in spite of the fact that it is only a relative method.

REAGENTS:

1) Reagent A: Alkaline reagent – 2% sodium carbonate and 0.1N NaOH.
2) Reagent B: 0.5% Copper sulphate and 1% potassium sodium tartrate.
3) Reagent C: 50 ml Reagent A + 1 ml Reagent B
4) Reagent D (Folin – Ciocalteau reagent (2N)): Dilute it to 1 N.
5) Standard BSA (20, 40, 80, 160, 320 μg/ml)

TEST PROPER:

Test tube	Test (T)	Standard (S) (*5 nos of "Standard" tubes are to be taken*)	Blank (B)
Biological fluid	0.2 ml		
Standard		0.2 ml	
Diluent			0.2 ml
Reagent C	1 ml	1 ml	1 ml
Mix and incubate at room temperature for 10 min.			
1 N Folin-Ciocalteau reagent (ml)	0.1	0.1	0.1
Immediately mix and incubate at room temperature in DARK for 30 min. Read at 650 nm.			

Calculation: Calculate the amount of protein using regression analysis taking the OD of standard as y-variable and concentration as x-variable.

The R statistical program can be downloaded from CRAN website.

R codes for regression analysis:

```
y<- c(….., ……, ……., ……., ……..)

x<- c(….., ……, ……., ……., ……..)

z<-lm(y~x)

summary(z)
```

y= enter the OD values of standard solutions.

x= enter the concentration of standard solutions

The concentration of test solution will be equal to (Conc)

$$\boldsymbol{Conc = x\ coefficient \times OD\ of\ test\ solution + intercept}$$

The R-square value should be more than 0.9 and all three p values should be less than 0.05. If the desired results are not obtained, go for log transformation with the following commands:

```
logz<- lm(log10(y)~x)

summary(logz)
```

The concentration of test solution will be equal to

10^ $\boldsymbol{x\ coefficient \times OD\ of\ test\ solution + intercept}$

OBJECTIVE: ESTIMATION OF TOTAL LIPIDS

PRINCIPLE: In this method described by Frings and Dunn (1970) includes the sulpho-phospho-vanillin reaction in which lipids from pink dye stuff that is measured at 540 nm. The sulphuric acid reacts with double bond and gives cabonium ions that react with phosphate ester of vanillin to form a coloured complex.

REAGENTS:

1. Concentrated sulphuric acid.
2. 0.6% vanillin solution in distilled water.
3. Colouring reagent: 80 ml of orthophosphoric acid is mixed with 20 ml of vanillin solution at the time of use.
4. Standard lipid: 500 mg of olive oil is dissolved in 100 ml of absolute ethanol.

TEST PROPER:

1. Take 0.1 ml of test solution/ standard/ blank solution in a test tube.
2. Add 2 ml of concentrated sulphuric acid.
3. Keep the tubes in boiling water bath for 15 minutes.
4. Take out 0.1 ml of suspension from each tube and put it in a fresh tube.
5. Add 5 ml of the colouring reagent.
6. Mix the tube vigorously.
7. Keep the tubes in standing at room temperature for 40 minutes.
8. Read the absorbance at 540 nm against blank.
9. Calculate the concentration by intrapolating from the standard graph or by regression analysis.

Reference:

Fringe, C.S and Dunn, R.T. 1970. American Journal of clinical Pathology.

OBJECTIVE: ESTIMATION OF TOTAL CHOLESTEROL

PRINCIPLE: Based on reaction of both cholesterol and cholesterol esters, with solution of $FeCl_3$ in a mixture of glacial acetic acid and concentrated H_2SO_4. The $FeCl_3$-acetic acid reagent precipitates the serum protein and the cholesterol liberated remains in the supernatant. The cholesterol on reaction with concentrated sulphuric acid produces purple colour complex whose intensity is directly propotional to the concentration of cholesterol.

CHEMICALS:

1. Concentrated H_2SO_4.
2. Ferric cholide.
3. Glacial acetic acid.
4. Cholesterol.

REAGENT

Ferric chloride-Acetic acid reagent. Dissolve $FeCl_3$ in glacial acetic acid making final concentration of $FeCl_3$ of 1.85 mM in glacial acetic acid.

STANDARD

Dissolve 40 mg of cholesterol in 10 ml of Ferric chloride-Acetic acid reagent. Incubate the solution at 60°C for 10 minutes to dissolve cholesterol. Take 1 ml of the solution in fresh tube. Add upto 100 ml of ferric chloride acetic acid reagent.

TEST PROPER

1. Take 0.1 ml of test solution or blank solution in a test tube.
2. Add 9.9 ml of ferric chloride-Acetic acid reagent.
3. Mix thoroughly and let it stand at room temperature for 15 minutes.
4. Incubate it at 60°C water bath for 2 minutes with occasional shaking every 15 seconds.
5. Let the suspension cool at room temperature (or keeping the tubes in running water).
6. Centrifuge the suspension at 10,000×g for 10 minutes.
7. Take the supernatant in fresh tube.
8. Add 3 ml of sueprnatent in fresh tube.
9. Add 3 ml of concentrated H_2SO_4 for every 5 ml of supernatant. This will give purple color complex after thoroughly mixing. *The color develops after about 2-3 minutes.*
10. Read the absorbance at 560 nm by making the blank OD zero.
    For standard solution:
    Take 5ml of cholesterol standard for every 3 ml of H_2SO_4 to be added.

Calculation:

$$Total\ cholesterol\ (mg/100ml) = \frac{OD\ of\ the\ test}{OD\ of\ the\ standard} \times conc.\ of\ standard$$

OBJECTIVE: **ESTIMATION OF BLOOD/SERUM/PLASMA GLUCOSE LEVEL**

SAMPLE: **Anticoagulant:**

METHOD: **Folin - Wu method (Benedict's test for reducing sugars)**

PRINCIPLE:

The protein free filtrate is heated with alkaline copper solution using a special tube to prevent reoxidation. The cuprous oxide formed (Refer to VBC 111 Manual) is treated with a phosphomolybdic acid solution, a blue colour being obtained which is compared with that of standard.

REAGENTS:

1) 10% w/v sodium tungstate solution (10 g Na_2WO_4 in 100 ml distilled water).

2) 2/3 N H_2SO_4 (2 ml H_2SO_4 in 100 ml distilled water).

3) 0.25% Benzoic acid (250 mg benzoic acid in 100 ml distilled water).

4) Alkaline copper reagent (40 g pure anhydrous Na_2CO_3 in 1 L flask + 400 ml H_2O + 7.5 g tartaric acid. Shake to dissolve, add 4.5 g $CuSO_4$, 5 H_2O. Make the volume with distilled water).

5) Phosphomolybdic acid solution (35 g molybdic acid + 5 g $NaWO_4$ + 200 ml 10% NaOH + 200 ml H_2O. Boil for 20 to 40 minutes to remove NH_3. Cool, dilute to about 350 ml and then add 125 ml conc. H_3PO_4 (85%), dilute to 500 ml with H_2O.

6) Stock standard: (1% w/v glucose – 1 g highest purity dextrose in 100 ml 0.25% benzoic acid)

7) Working standard: (0.1 mg/ml – 1 ml of above stock standard diluted to 100 ml with H_2O.

Preparation of protein-free filtrate:

For each ml of sample taken, add 7 volume of distilled water and mix. Add 1 volume of 10% sodium tungstate solution and mix. Finally add slowly with continuous shaking 1 ml of 2/3 N H_2SO_4. Let stand for 10 minutes. Filter through Whatman No. 1 filter paper.

PROCEDURE:

Folin-Wu tube no.	**Blank 1**	**Standard 2**	**Test 3**
Distilled water (ml)	2	0	0

Protein free filtrate	0	0	2
Working standard	0	2	0
Alkaline copper reagent	2	2	2
Mix, place rapidly in a boiling water bath for 8 min. Cool in running water without shaking.			
Phosphomolybdic acid reagent	2	2	2
Wait for 1 min. Dilute it to mark. Mix properly.			
OD at 420 nm	Blank adjusted to 0		

Calculation:

$$\frac{\text{OD of test}}{\text{OD of standard}} \times \text{Concentration of standard} \times \frac{100}{0.2}$$

= mg of glucose/100 ml of blood.

OBJECTIVE: **ESTIMATION OF BLOOD UREA OR BLOOD UREA NITROGEN (BUN)**

METHOD: **Diacetyl monoxime method**

PRINCIPLE:

When urea is heated with substances such as diacetyl, $CH_3COCOCH_3$, containing two adjacent carboxyl groups, coloured compounds are formed. Techniques employing this reaction have been devised for determining urea. Both diacetyl and its monoxime, $CH_3.CO.C = NOH.CH_3$ have been used.

REAGENTS:

(a) Folin Wu filtrate of blood or serum (See page No.).

(b) 0.667 N H_2SO_4: 2 ml conc. H_2SO_4 in 100 ml H_2O.

(c) Na_2WO_4 – 10% w/v (10 g Na_2WO_4 in 100 ml distilled water).

(d) Diacetyl monoxine solution – Dissolve 1 g diacetyl monoxime in approximately 100 ml of H_2O in 200 ml volumetric flask. Add 30 g NaCl dissolved by adding water. Finally dilute up to 200 ml with H_2O. Filter. It is stable indefinitely.

(e) Ferric-alum-acid reagent – Dissolve 1 g ferric alum [$FeNH_4$ $(SO_4)_2$. 12 H_2O] in 100 ml of H_2O and carefully add 100 ml of 85% phosphoric acid and 100 ml of concentrated H_2SO_4 with cooling. The reagent is stanble indefinitely.

(f) Standard stock urea – 200 mg/100 ml diluting solution.

(g) Working standard urea – 0.02 mg/0.1 ml

(h) Diluting solution – 1 ml Na_2WO_4 + 1 ml 2/3 N H_2SO_4 + 18 ml H_2O

(i) Reagent mixture – Mix equal volume of DAM (d) + Ferric alum acid reagent (e).

PROCEDURE:

	Test	Blank	Standard
Folin – Wu filtrate of serum/blood	0.4 ml	-	-
Standard urea (0.02 mg)	-	-	0.1 ml
Diluting fluid (H_2O)	-	0.4 ml	0.3 ml
Reagent mixture	5.1 ml	5.1 ml	5.1 ml

Place in boiling water bath exactly for 15 min in dark. Then cool it in dark (do not expose to direct light during boiling or cooling).

Read at 475 nm colour. Stable for 1 hour if not exposed to direct light.

Calculation:

$$\frac{\text{OD of unknown}}{\text{OD of standard}} \times \text{concentration of standard} \times 2500$$

= mg of urea/100 ml of blood, serum or plasma

(mg of urea N/100 ml x 2.14 = mg of urea/100 ml

or mg of urea/100 ml x 0.467 = mg of urea N per 100 ml).

OBJECTIVE: **ESTIMATION OF SERUM BILIRUBIN**

METHOD: **Malloy and Evelyn**

PRINCIPLE:

Bilirubin couples with diazotized sulphanilic acid to form a purple coloured azobilirubin complex (Van den Bergh reaction). The intensity of the purple colour that is formed is proportional to the bilirubin concentration in the serum. Direct bilirubin (conjugated) reacts with the diazo reagent in aqueous solution to form a coloured diazo compound within 1 minute. The subsequent addition of methanol accelerates the reaction of unconjugated bilirubin in serum, and a value for total bilirubin is obtained after letting the specimen stand for 30 minute. Absorbance values of the coloured solutions are taken at 540 nm (range 530 to 550 nm).

Specimen:

Serum is used in the quantitative determination of bilirubin in blood. A haemolysed specimen is unfit for bilirubin analysis. Serum must be kept away from bright light, since bilirubin is destroyed in ultraviolet light.

Reagents:

1) Absolute methanol

2) Hydrochloric acid

3) Diazo-reagent – Prepare freshly before use by adding 0.3 ml of solution B to 10 ml of solution A.

    a. Solution A: Dissolve 1 g of sulphanilic acid in 15 ml of concentrated hydrochloric acid and make up to 1 L with water.
    b. Solution B: Dissolve 0.5 g of sodium nitrite in water and make up to 100 ml

4) Standard solution of bilirubin: Prepare a solution containing 10 mg per 100 ml chloroform

5) Working standard (2 mg/dl): Dilute the above standard 1 in 5 with methanol.

Procedure:

	Blank (B)	Total bilirubin (TT)	Conjugated bilirubin (TD)	Standard (S)
Serum (ml)	0.2	0.2	0.2	-
Distilled water (ml)	1.8	1.8	4.3	1.5
Standard (ml)	-	-	-	0.5
1.5% HCl (ml)	0.5	-	-	-
Diazo reagent (ml)	-	0.5	0.5	0.5
Methanol (ml)	2.5	2.5	-	2.5

Calculation:

$$\text{Total bilirubin (mg/dl)} = \frac{\text{OD of TT}}{\text{OD of S}} \times 2$$

$$\text{Conjugated (Direct) bilirubin (mg/dl)} = \frac{\text{OD of TD}}{\text{OD of S}} \times 2$$

Unconjugated (Indirect) bilirubin (mg/dl) = Total bilirubin – Conjugated bilirubin

Conversion to International Units (SI):

mg bilirubin/dl x 17.1 = μmol bilirubin/L

NORMAL VALUES OF SOME OF THE SERUM BIOCHEMICALS IN DOMESTIC ANIMALS.

Analyte	Unit	Horse	Cow	Sheep	Goat	Pig
Glucose: S, P, HP	mg/dl	75-115 (95.6 ± 8.5)	45-75 (57.4 ± 6.8)	50-80 (68.4 ± 6.0)	50-75 (62.8 ± 7.1)	85-150 (119 ± 17)
Total cholesterol	mg/dl	75-150 (111 ± 18)	80-120	52-76 (64 ± 12)	80-130	36-54
Total proteins	g/L	52.0-79.0 (63.5 ± 5.9)	67.4-74.6 (71.0 ± 1.8)	60.0-79.0 (72.0 ± 5.2)	64.0-70.0 (69.0 ± 4.8)	79.0-89.0 (84.0 ± 5.0)
Urea nitrogen S, P, HP	mg/dl	10-24	20-30	8-20	10-20	10-30
Bilirubin: S, P, HP						
Conjugated	mg/dl	0-0.4 (0.1)	0.04-0.44 (0.18)	0-0.27 (0.12)		0-0.3 (0.1 ± 0.1)
Unconjugated	mg/dl	0.2-2.0 (1.0)	0.03	0-0.12		0-0.3
Total	mg/dl	1-2.0 (1.0)	0.01-0.5 (0.2)	0.1-0.5 (0.23 ± 0.1)	0-0.1	0-1.0 (0.2 ± 0.2)