2

CHAPTER

Biochemistry

2.1 DETERMINATION OF pK_a VALUE

Objective

To determine the pK_a value of glacial acetic acid.

Principle

pK_a or ionization constant is defined as the negative logarithm of the dissociation constant of a weak acid. pK_a determination helps in calculating the amount of neutral and charged species present at any pH. It is a quantitative measurement of strength of an acid. The larger the pK_a value, the smaller the extent of its dissociation at any particular pH, i.e., the weaker the acid.

$$pK_a = -\log_{10}(K_a)$$

The relation between pH and pK_a is given by **Henderson-Hasselbalch** equation that can be expressed as follows:

$$pH = pK_a + \log \frac{[\text{conjugate base}]}{[\text{acid}]}$$

when, $[\text{base}] = [\text{acid}]$,

then, $pH = pK_a + \log 1$,

or $pH = pK_a$ [as $\log 1 = 0$]

In other words, pK_a is the pH at which the concentrations of the acid and its conjugate base are equal.

Requirements

Glassware and Chemicals

1. Beakers
2. Pipettes

Table 2.2 Preparation of mixture of salt and acid solution

Sl. No.	Acetate (M)	Acetic Acid (N)	Ratio Acetate/Acetic Acid	pH
1	0	0.2	0	
2	0.05	0.2	0.05/0.2	
3	0.10	0.2	0.10/0.2	
4	0.15	0.2	0.15/0.2	
5	0.20	0.2	0.20/0.2	
6	0.30	0.2	0.30/0.2	
7	0.40	0.2	0.40/0.2	

Observation

Calculate the pH and pK_a using Henderson-Hasselbalch equation.

Conclusion

The pK_a value of glacial acetic acid was found to be _____.

2.2 VALIDATION OF BEER-LAMBERT LAW

Objective

To validate the Beer-Lambert law.

Principle

Quantitative measurement of a solution in spectrophotometer is evaluated by following the Beer-Lambert law. This law states that when a monochromatic light of intensity 'I_0' passes through a solution of path length 'l' the intensity 'I' drops along the pathway in an exponential manner. The relationship between I and I_0 depends on the path length (l) of the absorbing medium and the concentration (c) of the absorbing solution. It is given as follows:

$$A = k.l.c$$

where

A = absorbance or $-\log (I/I_0)$

k = absorption coefficient or absorptivity

l = path length of light through the sample, or thickness of the cell in cm

c = molar concentration of absorbing material in the sample

However, the Beer-Lambert law is a combination of Beer's law and Lambert's law.

Beer's Law

Beer's law states that the amount of light absorbed is inversely proportional to the concentration of the absorbing material in solution:

So, $$I = I_0 e_1^{-kc} \quad \text{(i)}$$

Lambert's Law

When a ray of monochromatic light passes through an absorbing medium, its intensity decreases exponentially as the length of the absorbing medium increases.

So, $$I = I_0 e_2^{-kl} \quad \text{(ii)}$$

These two laws are combined together in the Beer-Lambert law

$$I = I_0 e_1^{-kc} \times I_0 e_2^{-kl}$$

$$I = I_0 e^{[-k_1 + -k_2]^{-cl}}$$

$$I = I_0 e_3^{-kcl} \quad \text{(iii)}$$

where $$k_3 = k_1 + k_2$$

The ratio of intensities is known as the transmittance (T) and this is usually expressed as a percentage.

$$\text{Percentage } T = I/I_0 \times 100 = e_3^{-kcl}$$

$$-\log I/I_0 = k_3 lc = k.l.c$$

$$\log I_0/I = k.l.c$$

The Beer-Lambert law is valid for low concentrations only. At higher concentration, the molecules associate amongst themselves leading to deviations from the ideal behaviour.

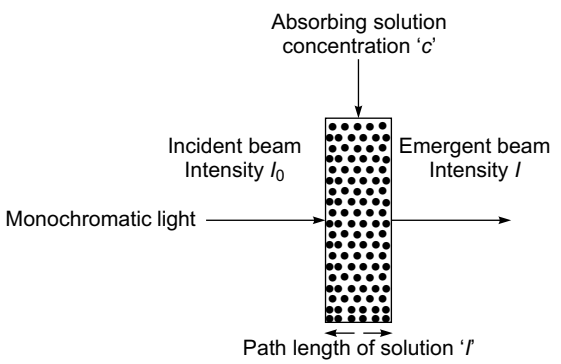

Figure 2.1 Relationship between I and I_0 with path length and concentration of absorbing solution

Requirements

Glassware

1. Test tubes
2. Test tube stand
3. Pipette
4. Beaker
5. Spectrophotometer

Reagents

Stock solution I, bromophenol blue solution (0.1%, v/v)

Dissolve 0.1 g bromophenol blue in 100 ml of distilled water.

Stock solution II, bromophenol blue solution (0.01%, v/v)

Take 1 ml of stock solution-I of the dye in a test tube and add 9 ml of distilled water.

Procedure

1. Prepare various concentrations of bromophenol blue as mentioned in Table 2.3 given below.
2. Take absorbance of solutions against distilled water blank in ascending order. Note the absorbance readings in respective columns against respective concentration at 600 nm.

Table 2.3

Test Tube No.	Stock Sol II (ml)	Distilled Water (ml)	Total Volume (ml)	Concentration (lg/ml)	Absorbance (A)
1	1	9	10	10	
2	2	8	10	20	
3	3	7	10	30	
4	4	6	10	40	
5	5	5	10	50	
6	6	4	10	60	
7	7	3	10	70	
8	8	2	10	80	
9	9	1	10	90	
10	10	0	10	100	

Observation

Plot a graph between absorbance against the concentration for different concentrations of bromophenol blue.

2.3 DETERMINATION OF ABSORPTION MAXIMA

Objective

To determine the absorption maxima of bromophenol blue dye solution.

Principle

Absorption maxima is the wavelength at which a compound absorbs maximum light, which is a characteristic of that compound. Each coloured compound absorbs maximum light at a particular wavelength of visible spectrum. It is normally measured to determine the optimal wavelength (A_{max}) of absorbance for a given solution. The optimal wavelength (A_{max}) is the wavelength that is maximally absorbed by the solutes in solution. Based on this characteristic, the concentration of a substance in solution can be determined.

Requirements

Chemicals and Glassware

1. 0.1% (v/v) solution of bromophenol blue
2. Test tubes
3. Pipettes (1 ml and 10 ml)
4. Spectrophotometer

Reagents

Dissolve 0.1 g bromophenol blue in 100 ml of distilled water. Dilute 100 times before use.

Procedure

1. Switch on the spectrophotometer and allow it to stabilize for 10 min.
2. Adjust zero extinction with distilled water at 400 nm.
3. Note the absorbance readings of the dye by taking 4 ml of 0.1% bromophenol blue solution in a glass cuvette.
4. Repeat steps 2 and 3 at wavelengths of 425, 475, 500, 525, 550, 575, 600, 625 and 650 nm and record the readings in table.
5. Plot absorbance in *Y*-axis against wavelength in *X*-axis.
6. From the graph, determine the A_{max} for bromophenol blue.

Result

Table 2.4 Tabulation for absorbance of bromophenol blue at different wavelengths

Wavelength (nm)	Absorbance (A)
400	
425	
450	
475	
500	
525	
550	
575	
600	
625	
650	

Observation

The absorption maxima (A_{max}) of bromophenol blue is found to be _____ nm.

2.4 CALIBRATION CURVE OF STARCH

Objective

To make a calibration curve for starch.

Principle

Starch is composed of amylose and amylopectin. Amylose is a non-branched polymer of glucose. When starch reacts with iodine-potassium iodide, iodine is adsorbed within the helical coils of amylose to produce a blue-coloured complex which has an absorption maximum at 610 nm. The absorbance (A) of the complex is proportional to the concentration of the starch solution. The colour can be detected visually with concentrations of iodine as low as 20 μm at 20°C. However, the intensity of the colour decreases with increasing temperature and in the presence of water-miscible organic solvents.

Requirements

Chemicals and Glassware

1. Starch
2. Iodine (I_2) and potassium iodide (KI)
3. HCl (1 N)
4. Test tubes
5. Beaker
6. Conical flask
7. Colorimeter
8. Distilled water (DW)

Reagents

0.1%, w/v solution of starch

Dissolve 100 mg of starch in 100 ml distilled water by boiling.

1%, w/v KI solution

Dissolve 1 g of KI in 100 ml distilled water.

Procedure

1. Prepare blank by taking mixture of 7.8 ml of DW + 2 ml of 1 N HCl + 0.2 ml of iodine solution.
2. Take 5 different dilutions of starch solution ranging between 100-1000 µg/ml as per the table given below.
3. To the starch solutions add 2 ml of 1 N HCl and 0.2 ml of iodine solution. Adjust the volume to 10 ml by adding DW. A blue colour will develop.
4. Measure the absorbance at 610 nm.

Result

Plot the graph by taking concentration of starch and absorbance.

Table 2.5 Tabulation

Sl. No.	Starch Solution (ml)	HCl (ml)	I_2-KI (ml)	DW(ml)	Absorbance (A)	Mean A
Blank	–	2	0.2	7.8		
1	0.2 (200 µg)	2	0.2	7.6		
1′	0.2 (200 µg)	2	0.2	7.6		

Contd.

Table 2.5 *Contd.*

2	0.4 (400 µg)	2	0.2	7.4		
2′	0.4 (400 µg)	2	0.2	7.4		
3	0.6 (600 µg)	2	0.2	7.2		
3′	0.6 (600 µg)	2	0.2	7.2		
4	0.8 (800 µg)	2	0.2	7.0		
4′	0.8 (800 µg)	2	0.2	7.0		
5	1.0 (1000 µg)	2	0.2	6.8		
5′	1.0 (1000 µg)	2	0.2	6.8		

Observation

Use the graph to calculate concentration from absorbance readings gained during an investigation.

A typical calibration curve of starch taking different concentration of starch is given below for standard calculations.

Table 2.6

Starch Concentration	Absorbance (610 nm)
0	0
0.3	0.708
0.05	0.436
0.1	0.517
0.5	0.91
0.7	0.966
1	1.078

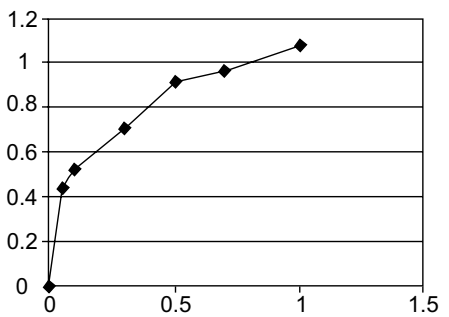

Figure 2.2 Calibration curve of starch

2.5 CARBOHYDRATES

Theoretical Background

Carbohydrates (also known as sugar) are carbon compounds that contain hydrogen and oxygen. Chemically, carbohydrates are compounds of aldehyde or ketone derivatives of polyhydric alcohol with empirical formula $C_n(H_2O)_n$. These are known as aldoses and ketoses. Carbohydrates are widely present in plant kingdom. These are often referred to as saccharides and broadly classified into three groups—monosaccharides, oligosaccharides and polysaccharides—based on the number of sugar units present in them. The basic unit of carbohydrates is monosaccharides. Common monosaccharides are glucose, fructose, galactose, ribose, etc. The joining of two hexoses by the glycosidic bond results in the formation of disaccharide (e.g., sucrose, lactose and maltose). Longer chains composed of 3–10 monosaccharide units are called oligosaccharides. Polysaccharides usually contain hundreds or thousands of monosaccharide units. Starch and cellulose are polysaccharides consisting of many monosaccharide residues. These polysaccharides are divided into homopolysaccharides (homoglycans), composed of equal units of sugar (starch, glycogen, cellulose), and heteropolysaccharides (heteroglycans), composed of various sugar and non-sugar units (glycosaminoglycans).

Carbohydrates can be analyzed by both qualitative and quantitative methods. In case of the qualitative analysis, the presence or absence of a carbohydrate in the given sample is determined, whereas in the quantitative analysis, the concentration of the carbohydrate in the given sample is determined or compared with a suitable reference standard compound.

Qualitative Analysis of Carbohydrates

While analyzing a sample containing a mixture of carbohydrates, particularly the sugars, several difficulties are encountered in their qualitative as well as quantitative analysis. These difficulties are attributed to their structural and chemical similarity and also with respect to their stereoisomerism. Several rapid tests are available to establish the presence or absence of a sugar or a carbohydrate in a sample. These tests are based on specific colour reactions typical for each group.

Objective

To identify the presence of carbohydrates in the given sample by qualitative methods.

Principle

Sugars can be defined as polyhydroxy aldehydes or ketones. Aldehydes (–CHO) and ketones (=CO) are active groups in carbohydrates. Carbohydrates contain many hydroxyl groups, and the number of hydroxyl groups varies with the number of carbon atoms. The presence of the hydroxyl groups allow carbohydrates to interact with the aqueous environment and to participate in the hydrogen bonding, both within and between the chains. The chemical properties of saccharides vary depending on the number of hydroxyl groups and the presence or absence of –CHO/= CO groups. These variations are the basis in the development of coloured reactions to identify the saccharides. Simple tests, as given in Table 2.7, are used to determine the presence/absence of certain saccharides in the sample.

Requirements

Preparation of reagents for qualitative analysis of carbohydrates

1. Molisch's reagent: 5%, w/v α naphthol in alcohol.
2. Anthrone reagent: Anthrone (2 g/l in conc. H_2SO_4).
3. Iodine solution: 0.005 N in 3 % KI, i.e., 3 g of KI dissolved in 100 ml water and then 63.5 mg of iodine is dissolved.
4. Fehling's reagent: Fehling's solution A: Dissolve 35 g of $CuSO_4 \cdot 5H_2O$ in water and make the volume to 500 ml. Fehling's solution B: Dissolve 120 g of KOH and 173 g Na-K tartrate (Rochelle salt) in water and make the volume to 500 ml. Mix equal volumes of Fehling's solution A and B prior to use.
5. Benedict's solution: 17.3 g of sodium citrate and 10 g of sodium carbonate are dissolved in 75 ml of water. 1.73 g of $CuSO_4 \cdot 5H_2O$ is dissolved in 20 ml of water. The $CuSO_4$ solution is mixed with the alkaline citrate solution with continuous stirring, and finally the whole volume is made up to 100 ml with distilled water.
6. Barfoed's reagent: 13.3 g of copper acetate in 200 ml of water and add 2 ml of glacial acetic acid.
7. Picric acid: Dissolve 13 g of picric acid in distilled water, boil and cool.
8. Seliwanoff's reagent: Dissolve 50 g of resorcinol in 100 ml of conc. HCl in the ratio of 1:2.
9. Bial's reagent: Dissolve 300 mg of orcinol in 100 ml of concentrated HCl.
10. Concentrated HNO_3
11. Concentrated HCl
12. Concentrated H_2SO_4
13. Osazone reagent: Phenyl hydrazine hydrochloride, sodium acetate and acetic acid.
14. Fearons test: Take methylamine hydrochloride (MH) 5% in H_2O and NaOH (20%, w/v) in 100 ml distilled water.

Table 2.7 Qualitative analysis (tests) to detect the presence/absence of different forms of carbohydrates

Sl. No.	Test/Principle	Procedure	Observation	Remark
1	**A. General tests for carbohydrates**			
	Molisch's test It is a group test for all carbohydrates, whether free or in combined form.	Add 2 drops of Molisch's reagent to about 2 ml of test solution and mix well. Then add con. H_2SO_4 carefully along the sides of the inclined test tube.	Red-cum-violet coloured ring is formed at the junction of the two layers. Presence of carbohydrate is confirmed	The colour formed is due to the reaction of α-naphthol with furfural and/or its derivatives formed by the dehydration of sugars by concentrated H_2SO_4.

Contd.

Table 2.7 *Contd.*

	Principle Concentrated H_2SO_4 dehydrates carbohydrates to form furfural and its derivatives. This product combines with sulphonated α-naphthal to give violet-purple colour.			
	Anthrone test It is another general test for detection of carbohydrates. **Principle** Its principle is same as that of *Molisch's test*, except that the furfurals and hydroxylmethyl furfurals give condensation products with anthrone that are bluish-green in colour.	To 5 drops of sugar solution add 2 ml anthrone reagent.	Blue-green colour complex is formed, indicating the presence of carbohydrate.	Concentrated H_2SO_4 dehydrates carbohydrates to form furfural and its derivatives. The furfural reacts with anthrone (10-keto-9, 10-dihydro anthracene) to give a blue-green complex.
2	**B. Specific test**			
	(*a*) *Iodine test* This test is performed to distinguish polysaccharides from mono- and disaccharides. **Principle** Iodine forms a coloured absorption complex with polysaccharides due to the formation of micellae aggregate. Iodine will form a polysaccharide inclusion complex.	Add a few drops of iodine solution to about 1 ml of the test solution and observe the colour change.	(i) Appearance of deep blue colour indicates the presence of starch in solution. (ii) Appearance of dark brown colour indicates the presence of polysaccharide (glycogen) in solution.	The blue colour is due to the formation of starch-iodine complex, and dark brown colour indicates the presence of polysaccharide.
3	***(b) Test based on reducing property of carbohydrates***			
	(*i*) *Fehling's Test* It distinguishes reducing sugar from non-reducing sugar.	To 1 ml of Fehling's solution 'A', add 1 ml of Fehling's solution 'B' and a few drops of the test solution. Boil for a few minutes.	Formation of yellow or brownish red precipitate indicates the presence of reducing sugars in the test solution.	The blue alkaline cupric bydroxide present in Fehling's solution, when heated in the presence of reducing sugars, gets reduced to yellow or red cuprous oxide and it gets precipitated.

Contd.

Table 2.7 *Contd.*

	(*iv*) *Picric acid test* It is another test for the detection of reducing sugar. **Principle** The reducing sugar reacts with picric acid to form a red colour picramic acid.	Add 1 ml of picric acid solution to the 1 ml of test solution, followed by 0.5 ml 10% of Na_2CO_3 solution, and the contents are boiled.	Appearance of red colour indicates the presence of reducing sugar in the solution.	Red colour confirms the presence of reducing sugar.
	(*c*) *Seliwanoff's test* This test distinguishes aldoses from ketoses. **Principle** Ketoses are dehydrated more rapidly than aldoses to give furfural derivatives, which then condense with resorcinol to form a red colour complex. It is a timed colour reaction specific for ketones.	To 1 ml of the test solution, 3 ml of Seliwanoff's reagent is added and the contents are boiled in water bath for 2 min.	Cherry red colour is obtained indicating ketoses (e.g., sucrose).	In concentrated HCl, ketoses undergo dehydration to yield furfural derivatives more rapidly than do aldoses. These derivatives form complexes with resorcinol to yield deep red colour.
	(*d*) *Bial's test* This test is specific for pentoses sugar. **Principle** Pentose is heated with conc. HCl, furfural, which condenses with orcinol in the presence of ferric ion to give a blue-green colour.	To 2–3 ml of the test solution, 5 ml of Bial's reagent is added. The contents are heated gently. When bubbles rise to the surface, it is cooled under the tap water.	Appearance of blue or green colour precipitate indicates the presence of pentose sugar.	In the presence of ferric ion orcinol and furfural condense to yield a coloured product. Formation of green solution and precipitate indicates the presence of a pentose sugar.
	(*e*) *Test for sucrose* **Principle** Sucrose present in the unknown solution is hydrolyzed by acid to glucose and fructose. The resulting fructose formed in this solution is then tested by Seliwanoff's reagent.	To about 2-3 ml of test solution, add 1-2 drops of conc. HCl and boil in a water bath for 8-10 min. Then add about 5 ml of Seliwanoff's reagent and again keep it in a water bath for 1 min.	Appearance of red colour indicates the presence of fructose which is the hydrolytic product of sucrose.	In concentrated HCl, fructose undergoes dehydration to yield furfural derivatives. These derivatives form complexes with resorcinol to yield deep red colour.

Contd.

Table 2.7 *Contd.*

	(*f*) *Mucic acid test* This test is specific for galactose. **Principle** Galactose forms mucic acid in the presence of HNO_3. Mucic acid (Galactaric acid) is formed from galactose due to the oxidation of both aldehyde and primary alcoholic group at C1 and C6.	Add a few drops of conc. HNO_3 to the concentrated test solution or substance directly and evaporate it over a boiling water bath till the acid fumes are expelled. Add a few drops of water and leave it for overnight.	Formation of colourless needle-like crystals will indicate the presence of galactose.	Oxidation of galactose produces insoluble and mucic acid.
	(*g*) Phenylhydrazine test/ *Osazone test* This test is used to differentiate maltose and lactose. **Principle** Compounds containing aldehyde and keto groups form crystalline osazone with phenyl hydrazine hydrochloride. Osazone crystals have characteristic shape and melting point which helps in the identification of reducing sugar.	To 0.5 g of phenylhydrazine hydrochloride add 0.1 g of sodium acetate and 10 drops of glacial acetic acid. To this mixture add 5 ml of test solution and heat on a boiling water bath for about half an hour. Allow the tubes to cool slowly and examine the crystals under a microscope.	Yellow colour crystals are formed as observed under a microscope. Different osazones show crystals of different shapes. Glucose, fructose and mannose produce needle-shaped yellow osazone crystals; lactosazone is mushroom-shaped. Maltose produces flower-shaped crystals.	The ketoses and aldoses react with phenylhydrazine to produce a phenylhydrazone, which in turn reacts with another two molecules of phenylhydrazine to form the osazone.
4	***Fearon's test*** **Principle** The disaccharides, lactose and maltose, under alkaline condition are hydrolyzed to form enediol. This endiol reacts with methylamine HCl to form a red coloured complex.	To 1 ml of the test solution, 2 ml of Fearon's reagent is added and the content is heated. Then NaOH is added to the cold mixture.	(i) Red coloration appears.	Presence of reducing disaccharide.
			(ii) No colour change.	Absence of reducing disaccharide.

Notes

- For osazone test, the pH of the reaction mixture should be between 5 and 6. Fructose takes 2 min. to form the osazone, whereas for glucose it is 5 min. The disaccharides take a longer time to form osazones. Dissacharides form crystals only on cooling.
- When a mixture of carbohydrates is present in the test sample, chromatographic methods should be employed to identify the individual sugars.

2.6 QUANTITATIVE ANALYSIS OF CARBOHYDRATES

2.6.1 Estimation of Total Carbohydrates by Anthrone Method

Objective

To estimate the concentration of total carbohydrates in a given sample by Anthrone method.

Principle

Carbohydrates are first hydrolysed into simple sugars using sulphuric acid. In hot acidic medium, glucose is dehydrated to hydroxymethyl furfural. Anthrone reagent is used as a colouring agent that reacts with furfural derivative to form a blue-green complex. Absorbance of these compounds is measured by a spectrophotometer at 630 nm.

Glucose + H_2SO_4 | Hydroxymethyl furfural + Anthrone reagent | Blue-green complex

Requirements

Reagents

1. Test sample
2. 2.5 N HCl: Prepare the solution fresh
3. Anthrone reagent
   Dissolve 200 mg anthrone in 100 ml of ice cold 95% H_2SO_4. Prepare fresh before use.
4. Standard glucose
   Stock: Dissolve 100 mg in 100 ml water.
   Working standard: 10 ml of stock diluted to 100 ml with distilled water. Store at 4°C after adding a few drops of toluene.

Procedure

1. Take clean and dry test tubes and mark all the tubes as per the protocol.
2. Pipette out 0.1-0.5 ml of glucose standard solution in duplicate test tubes.
3. In one test tube take only 1 ml of distilled water and mark it as blank.
4. Make up the volume to 1 ml in each test tube by adding distilled water.
5. Then add 3 ml of anthrone reagent to each test tube and mix thoroughly.

6. Heat the test tubes for 8 min. in a boiling water bath.
7. Cool rapidly and read the green to dark green colour at 630 nm.
8. Draw a standard graph by plotting concentration of the standard on the *X*-axis versus absorbance on the *Y*-axis.
9. From the graph calculate the amount of carbohydrate present in the sample tube.

Result

Table 2.9 Determination of glucose standard curve by taking the absorbance at 630 nm

SI. No.	Glucose (ml)	Concentration of Glucose (lg/ml)	Distilled Water (ml)	Anthrone Reagent (ml)		Absorbance (630 nm)	Mean
Blank	–	–	1	3		0.000	0.000
1	0.1	10	0.9	3			
1′	0.1	10	0.9	3			
2	0.2	20	0.8	3			
2′	0.2	20	0.8	3			
3	0.3	30	0.7	3			
3′	0.3	30	0.7	3			
4	0.4	40	0.6	3			
4′	0.4	40	0.6	3			
5	0.5	50	0.5	3			
5′	0.5	50	0.5	3			
Unknown				3			

Calculation

$$\text{Amount of carbohydrate present in 100 mg of the sample} = \frac{\text{mg of glucose} \times 100}{\text{Volume of test sample}}$$

Precaution

Cool the contents of all the tubes on ice before adding ice-cold anthrone reagent.

2.6.2 Estimation of Reducing Sugars by Nelson-Somogyi Method

Objective

To estimate the concentration of reducing sugar in the given sample by Nelson-Somogyi method.

8. Add 1 ml of alkaline copper tartarate reagent to each tube.
9. Place the tubes in boiling water for 10 min.
10. Cool the tubes and add 1 ml of arsenomolybolic acid reagent to all the tubes.
11. Make up the volume in each tube to 10 ml with water.
12. Read the absorbance of blue colour at 620 nm after 10 min.
13. Plot a graph by taking concentration of sugar on *X*-axis and absorbance on *Y*-axis.
14. From the graph drawn, calculate the amount of reducing sugars present in the sample.

Calculation

Absorbance corresponds to 0.1 ml of test = x mg of glucose

10 ml contains = $(x / 0.1) \times 10$ mg of glucose = % of reducing sugars

Observation

The concentration of reducing sugar in the given sample is _____

2.6.3 Estimation of Reducing Sugars by Dinitrosalicylic Acid (DNSA) Method

Objective

To estimate the concentration of reducing sugar in a given sample by dinitrosalicylic acid (DNSA) method.

Principle

For estimation of reducing sugar, dinitrosalicylic acid method is an alternative to Nelson-Somogyi method. This method is simple, sensitive and adoptable for handling a large number of samples at a time. This method tests for the presence of free carbonyl group (C=O), present in the so-called reducing sugars. This involves the conversion of reducing sugar to furfural under alkaline conditions, which reduces one of the nitro group ($–NO_2$) of DNSA to amino group ($–NH_2$) to produce orange brown colour 3-amino-5-nitrosalicylic acid, with absorbance maxima at 540 nm.

Aldehyde group $\xrightarrow{\text{oxidation}}$ **Carboxyl group**

3,5-dinitrosalicylic acid $\xrightarrow{\text{reduction}}$ **3-amino-5-nitrosalicylic acid**

Requirements

1. Test sample
2. Standard glucose solution (1 mg/ml): Dissolve 100 mg of glucose and make the final volume up to 100 ml with distilled water.
3. Dinitrosalicylic acid reagent (DNS Reagent)
   Dissolve by stirring 1 g dinitrosalicylic acid, 200 mg crystalline phenol and 50 mg sodium sulphite in 100 ml 1% NaOH. Store at 4°C. Since the reagent deteriorates due to sodium sulphite, long storage is required, sodium sulphite may be added at the time of use.

4. 40% Rochelle salt solution (potassium sodium tartrate)
5. Glassware: Test tubes, conical flask, glass pipette, beaker

Procedure

1. Take clean and dry test tubes and mark all the tubes as per the protocol.
2. Weigh 100 mg of the sample and extract the sugars with hot 80% ethanol twice (5 ml each time).
3. Collect the supernatant and evaporate it by keeping it on a water bath at 80°C.
4. Add 10 ml water and dissolve the sugars.
5. Take out 0.5 to 3 ml of the extract in test tubes and equalize the volume to 3 ml with water in all the tubes.
6. Add 3 ml of DNS reagent.
7. Heat the contents in a boiling water bath for 5 min.
8. Add 1 ml of 40% Rochelle salt solution when the contents of the tubes are warm.
9. Cool and read the intensity of dark red colour at 510 nm.
10. Run a series of standards using glucose (0–500 µg) and plot a graph, of concentration of glucose v/s absorbance at 630 nm.

Result

Calculate the amount of reducing sugars present in the sample using the standard graph.

Table 2.10 Tabulation for determination of standard curve for glucose by taking the absorbance at 630 nm

SI. No.	Glucose (ml)	Concentration of Glucose (lg/ml)	Distilled Water (ml)	DNSA Reagent (ml)		Absorbance (630 nm)	Mean
Blank	–	–	1	3	Boil in a water bath at 100°C	0.000	0.000
1	0.1	10	0.9	3			
1′	0.1	10	0.9	3			
2	0.2	20	0.8	3			
2′	0.2	20	0.8	3			
3	0.3	30	0.7	3			
3′	0.3	30	0.7	3			
4	0.4	40	0.6	3			
4′	0.4	40	0.6	3			
5	0.5	50	0.5	3			
5′	0.5	50	0.5	3			
Unknown				3			

Observation

Concentration of unknown reducing sugar is _____.

2.6.4 Estimation of Cellulose

Objective

To estimate the cellulose in a given sample by anthrone method.

Principle

Cellulose, a major structural polysaccharide in plants, is the most abundant organic compound in nature. It is composed of glucose units joined together in the form of repeating units of the disaccharide cellobiose with numerous cross linkages. It is also a major component in many of the farm wastes. Cellulose undergoes acetolysis with acetic/nitric reagent forming acetylated cellodextrins, which get dissolved and hydrolyzed to form glucose molecules on treatment with 67% H_2SO_4. This glucose molecule is dehydrated to form hydroxymethyl furfural, which forms a green colour product with anthrone, and the colour intensity is measured at 630 nm.

Glucose + H_2SO_4 | Hydroxymethyl furfural + Anthrone reagent | Blue-green complex

Requirements

1. Acetic/nitric reagent
   Mix 150 ml of 80% acetic acid and 15 ml of concentrated nitric acid.
2. Anthrone Reagent
   Dissolve 200 mg anthrone in 100 ml concentrated sulphuric acid. Prepare fresh and chill for 2 h before use.
3. Sulphuric acid (67%)

Procedure

1. Add 3 ml acetic/nitric reagent to a known amount (0.5 g or 1 g) of the sample in a test tube and mix properly.
2. Place the tube in a water bath at 100°C for 30 min.
3. Cool and then centrifuge the contents for 15–20 min.
4. Discard the supernatant.
5. Wash the residue with distilled water.
6. Add 10 ml of 67% sulphuric acid and allow it to stand for 1 h.
7. Dilute 1 ml of the above solution to 100 ml.
8. To 1 ml of this diluted solution, add 10 ml of anthrone reagent and mix well.
9. Heat the tubes in a boiling water bath for 10 min.
10. Cool the content in test tube and measure the colour at 630 nm.

- If solution heated in step 9 is insufficiently acidic, a white precipitate forms, preventing the collection of absorbance data. If the solution is heated much longer than 5 min, it will turn brown, and the absorbance at 620 nm will no longer be linear with glucose concentration.

2.6.5 Estimation of Hemicellulose

Method-I

Objective

To estimate the concentration of hemicellulose by p-bromoaniline method.

Principle

Hemicelluloses are non-cellulosic, non-pectic cell wall polysaccharides. They are composed of xylans, mannans, glucomannans, galactans and arabinogalactans. Hemicelluloses are categorized under 'unavailable carbohydrates' since they are not split by the digestive enzymes of the human system. The p-bromoaniline method for the estimation of hemicellulose is based on the formation of furfural from pentoses in acetic acid containing thiourea at 75°C, and the reaction of furfural with p-bromoaniline acetate to form a pink coloured product.

Requirements

Reagents

1. p-bromoaniline reagent
2. H_2SO_4 3%, w/v
3. 10 N NaOH
4. Standard xylose solution (0.1%, w/v)
5. Dissolve 0.1 gm xylose (pre-dried in oven at 105°C for 1 h) in 100 ml distilled water.

Procedure

1. Take sample with H_2SO_4 (3%, w/v) at a ratio of 1 : 10 in a conical flask.
2. Autoclave the samples at 121°C, 15 psi.
3. Cool the samples and add distilled water to make the volume to 100 ml and adjust the pH to 7.0-7.5.
4. Add 1 ml of distilled water in each of the test tubes.
5. Add 5 ml of p-bromoaniline to 1 ml of the samples and incubate it for 10 min at 70°C.
6. Then incubate the samples at room temperature in dark for 70 min.
7. Measure the absorbance at 540 nm.
8. For standard curve preparation:
   Take 100 mg xylose in a test tube and proceed from Step No. 4. Take a series of volumes (say 0.1–1 ml corresponding to 10–100 µg of xylose, and to that add 0.9, 0.8 … 0 ml of distilled water to make up the volume to 1 ml) and develop the colour.

2.6.6 Determination of Acid Detergent Fibre by Refluxing

Objective

To determine acid detergent fibre (ADF) in all types of forages.

Principle

An acidified quaternary detergent solution is used to dissolve cell solubles, hemicellulose and soluble minerals leaving a residue of cellulose, lignin, and heat damaged protein and a portion of cell wall protein and minerals (ash). ADF is determined gravimetrically as the residue remaining after extraction.

Requirements

Equipment

Refluxing apparatus
Berzelius beakers (600 ml)
Fritted glass (Gooch) crucibles (coarse porosity, 50 ml)
Analytical electronic balance, accurate to 0.1 mg
Suction filtering device with trap in line and valve to break vacuum
Forced-air drying oven set at 100°C

Reagents

Acid detergent solution
Sulphuric acid (1 N), 1000 ml
Cetyltrimethylammonium bromide (CTAB), 0.005 N. 20 g
Acetone

Procedure

1. Dry the samples at 55°C to 85% dry matter and then ground to pass a 1 mm screen.
2. Dry 50 ml fritted glass crucibles overnight at 100°C and hot weigh (W1), recording weight to nearest 0.1 mg.
3. Thoroughly mix and weigh sample (W2) (approximately 0.9 to 1.1 g, record weight accurate to 0.1 mg) into Berzelius beaker.
4. Weigh a second subsample for laboratory dry matter determination.
5. Add 100 ml acid-detergent solution at room temperature and place the beaker on a heater.
6. Heat to boiling for 5-10 min; reduce heat to avoid foaming as boiling begins.
7. Reflux 60 min from onset of boil, adjusting boiling to slow, even level.
8. After about 30 min, wash down sides of the beaker with minimal amount of acid detergent solution. A wash bottle is convenient for dispensing solution.
9. Remove beaker, swirl, and filter through fritted glass crucible (step-2) using minimal vacuum.

10. Rinse the Berzelius beaker with boiling water while inverted over the crucible to ensure quantitative transfer of all fibre particles into the crucible.
11. Soak twice with boiling (95-100°C) water by breaking up mat and filling crucible each time with vacuum off and allowing to soak for a minimum of 15 to 30 sec (2 min recommended) after each wash. While filling the crucible with hot water or acetone, rinse the top edge and sides to remove residual acid detergent.
12. Rinse twice with 30-40 ml acetone by filling crucible each time with vacuum off, allowing a minimum of 15 to 30 sec (2 min recommended) before vacuuming dry.
13. Dry 3 hr or overnight in forced-air oven (100°C) and weigh hot, recording weight (W3) to nearest 0.1 mg.

Result

Percent Acid Detergent Fibre (ADF)

Calculation

% ADF (DM basis) = (W3 – W1/W2 | Lab DM/100) | 100

where, W1 = tare weight of crucible in grams
W2 = initial sample weight in grams
W3 = dry weight of crucible and dry fibre in grams

Precautions

- Sulphuric acid for acid detergent fibre solution must be standardized between 0.995 and 1.005 N. Variation in normality outside of this range can result in low or high ADF values.
- Timing of refluxing is critical and should not vary more than 5 min from the 60 min described by the method.
- Acid must be thoroughly washed from the sample because it will become concentrated when water is removed during drying. The combination of strong sulphuric acid and high temperature can char the sample and result in low ADF values. If black discoloration occurs during drying, repeat the analysis.
- Boiling water must be used for washing the samples. This is particularly true of samples containing pectic substances, mucilages or glycoproteins.

2.6.7 Estimation of Lignin

Introduction

Lignin is typically present at the concentration of 15-20% in lignocellulosics. It is the second most abundant source of renewable and sustainable carbon after cellulose. It is an aromatic heteropolymer, predominantly composed of coniferyl alcohol ($C_{10}H_{12}O_3$). The quantification of lignin is difficult because of its varying monomeric composition as well as its covalent linkage with cell wall carbohydrates, proteins, phenolics or other compounds. These compounds interfere with determination of lignin leading to over- or underestimation. Usually, two different principles — galvanometric method and spectrophotometric method— have commonly been employed for determination of lignin. The latter is based on decomposition of lignin into soluble degradation

Analysis of Sample for Acid Insoluble Lignin

1. Vacuum filter the autoclaved hydrolysis solution in a filtering flask and collect the filtrate.
2. Transfer an aliquot, approximately 50 ml, into a sample storage bottle. This sample will be used to determine acid soluble lignin as well as carbohydrates, and acetyl if necessary. (Acid soluble lignin determination must be done within 6 hr of hydrolysis. If the hydrolysis liquor must be stored, it should be stored at 4°C for a maximum of two weeks.)
3. Use deionized water to quantitatively transfer all remaining solids out of the pressure tube into the filtering crucible of the vacuum filter.
4. Dry the crucible and acid insoluble residue at 105°C until a constant weight is achieved, usually a minimum of 4 hr.
5. Remove the samples from the oven and cool in a desiccator.
6. Record the weight of the crucible and dry residue to the nearest 0.1 mg.
7. Place the crucibles and residue in the muffle furnace at 575°C for 24 hr.

Analysis of Sample for Acid Soluble Lignin

1. Take the blank as deionized water.
2. Measure the absorbance of the vacuum filtered sample at a wavelength of 240 nm on a UV-visible spectrophotometer.
3. Dilute the sample as necessary to bring the absorbance into the range of 0.7–1.0, recording the dilution.
4. Deionized water can be used to dilute the sample, but the same solvent should be used as a blank. Record the absorbance to three decimal places.
5. Analyze each sample in duplicate, at minimum. (This step must be done within 6 hr of hydrolysis.)
6. Calculate the amount of acid soluble lignin present in the sample.

Result

Calculate the oven dry weight (ODW) of the extractives free sample, using the average total solids content as determined by the LAP "Standard Method for the Determination of Total Solids in Biomass".

Calculations

$$\text{ODW} = (\text{Weight}_{\text{air dry sample}} \times \% \text{ of total solids})/100$$

Calculate and record the weight percent acid insoluble residue (AIR) and acid insoluble lignin (AIL) on an extractives free basis

$$\% \text{ AIR} = [(\text{Weight}_{\text{crucible plus AIR}} - \text{Weight}_{\text{crucible}})/ \text{ODW}_{\text{sample}}] \times 100$$

$$\% \text{ AIL} = \{[(\text{Weight}_{\text{crucible plus AIR}} - \text{Weight}_{\text{crucible}}) - (\text{Weight}_{\text{crucible plus ash}} - \text{Weight}_{\text{Crucible}}) - \text{Weight}_{\text{Protein}}/\text{ODW}_{\text{sample}}\} \times 100$$

where,

UV abs = average UV-V is absorbance for the sample at appropriate wavelength (refer table below)

Volume hydrolysis liquor = volume of filtrate, 86.73 ml
Dilution = volume of sample + volume of diluting solvent
μ = absorptivity of biomass at specific wavelength (refer table below)
ODW sample = weight of sample in milligrams
Path length = path length of UV-Vis cell in cm

Reference table for acid soluble lignin calculation
Absorptivity constants for acid soluble lignin measurement for select biomass types

Biomass Type	Lambda max (nm)	Absorptivity at Lambda max (L/g cm)	Recommended wavelength (nm)	Absorptivity at recommended wavelength (L/g cm)
Pinus Radiata-NIST SRM 8493	198	25	240	12
Bagasse-NIST SRM 8491	198	40	240	25
Corn Stover-NREL supplied feedstock	198	55	320	30
Populus deltiodes-NIST SRM 8492	197	60	240	25

The total amount of lignin on an extractives free basis

% ASL + % AIL = % Lignin free extract

Note

The wavelength to be taken depends on the substrate used for the experiment.

2.7 ESTIMATION OF PROTEINS

Theoretical Background

Proteins are large and complex macromolecules that are present in all tissues of the body and contribute maximum to the structure of the cell. They perform a vast array of functions within living organisms, including catalyzing metabolic reactions, replicating DNA, responding to stimuli, and transporting molecules from one location to another. Proteins consist of one or more long chains of amino acid residues. Twenty different types of amino acids can constitute a protein. The amino acid sequence determines each protein's unique 3-dimensional structure and its specific function. Thus, proteins perform a large range of functions in the body as antibody, enzymes, messenger proteins, structural proteins, transport proteins, etc.

In molecular biology experiments, to analyse proteins, or to determine the specific activity, total protein quantification assays are used. Many methods are employed to determine protein content in biological samples. These are (1) ultraviolet absorption method (optical density), (2) colorimetric assays such as Biuret method, Lowry method, Bradford protein assay and Kjeldahl analysis method (indirect method using nitrogen content). Besides, electrophoresis methods are also employed for detecting proteins and their concentration. It is shown that each assay has advantages and disadvantages relative to sensitivity, ease of performance, accuracy and reproducibility.

Table 2.12 *Contd.*

Bradford method	0.2–20 µg	Spectroscopic analytical procedure. Speed, convenience, stability of reagents and a few interfering substances.	100 µl of protein sample (containing 1-100 µg of protein), add 0.9 ml (for 1-20 µg) or 5 ml (for 20-100 µg) of reagent + A_{595}	Blue	Few substrates; except detergents. Linearizes by measuring the ratio of the absorbances, 595 over 450 nm

2.7.1 Biuret Method

Objective

To estimate the concentration of protein in a given sample by Biuret method.

Principle

The Biuret reaction for protein determination is one of the first colorimetric protein assays developed and is still used widely. It is mostly used in applications requiring fast but not highly accurate, determinations. The Biuret reaction occurs with all compounds that contain two or more peptide bonds. The reagent consists of a solution of dilute copper sulphate in strong alkali. The purple-blue colour produced is attributed to the formation of a coordination complex between the Cu^{2+} and four nitrogen atoms, two from each of two adjacent peptide chains. The failure of ammonium sulphate to interfere with colour formation makes it advantageous for determination during the early steps of purifying a protein.

Requirements

Chemicals

1. Bovine serum albumin (BSA)
2. Copper sulphate
3. Sodium-potassium tartrate
4. Sodium hydroxide
5. Potassium iodide

Glassware

Test tubes, conical flasks, glass pipette, beakers

Equipment

Hot water bath, stirrer with magnetic stirring bar, glass test tubes, spectrophotometer, weighing balance.

Table 2.13 *Contd.*

6	0.8	–	0.2	3	
7	1.0	–	–	3	
8	–	1	–	3	
9	–	1	–	3	

Calculation

$$\text{Protein conc. (mg \%)} = \frac{\text{OD (test)}}{\text{OD (std)}} \times \frac{\text{Conc (std)}}{\text{Volume (test)}} \times 100$$

The concentration of protein in a given sample estimated with Biuret method is…… mg/ml.

2.7.2 Lowery's Assay

Objective

To estimate the concentration of protein in a given sample by Lowry's method.

Principle

The Lowry method is sensitive to low concentrations of protein. Using this method, concentrations ranging from 0.002 – 0.1 mg of protein per ml can be estimated. The major disadvantage of this method is the narrow pH range within which it is accurate. As the method is sensitive to pH changes, the pH of assay solution should be maintained at 10 – 10.5.

The principle behind the Lowry method of determining protein concentrations lies in the reactivity of aromatic amino acids of proteins with the copper [II] ions under alkaline conditions. The reduction of Folin-Ciocalteau phosphomolybdic phosphotungstic acid forms a coloured complex, which is spectrophotometrically measured at 660 nm. The intensity of the colour depends on the amount of aromatic acids (tyrosine, tryptophan) present in the protein.

Requirements

Chemicals

BSA
Folin-Ciocalteau reagent
Na_2CO_3
NaOH
$CuSO_4$
Potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$)

Equipment

Weighing balance
Spectrophotometer

Glassware

Test tubes
Conical flasks
Glass pipette
Beaker

Solutions

Preparation of Folin–Ciocalteau reagent

(i) Prepare fresh and dilute 1:1 proportion of Folin–Ciocalteau reagent with DW.
(ii) Keep in amber colour bottle away from light source.

Preparation of reagent A

2% Na_2CO_3 in 0.1 N NaOH.

Preparation of reagent B

0.5% $CuSO_4$ and 1% $KNaC_4H_4O_6 \cdot 4H_2O$ in 100 ml D/W.

Preparation of reagent C (alkaline reagent)

Add 50 ml of Reagent A to 1 ml of Reagent B and mix well. It should be fresh solution.

Standard Protein

0.25 mg/ml solution of BSA

Procedure

1. Warm up spectrophotometer 15 min. before use.
2. Take 9 clean glass test tubes. Into each test tube, carefully pipette one of the following volumes from a 250 µg/ml solution of BSA: 0.0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 ml. In the last two test tubes put 1 ml of test samples each as duplicates.
3. Bring the total volume of liquid in all tubes to 4.0 ml by adding an appropriate amount of distilled water.
4. Add 5.5 ml of Reagent C (alkaline reagent) in all tubes, vortex thoroughly and incubate at room temperature for 10 min.
5. Add 0.2 ml of Folin–Ciocalteau reagent in all test tubes, vortex well and incubate for another 20 min at 37°C.
6. Determine the OD at 660 nm of each sample. Plot the standard curve using concentration of std. tubes of BSA (µg/ml) against the absorbance.
7. Calculate the mean of absorbance of the duplicate sample and find out the protein concentration present in the given sample from standard. Graph and formula (given below) and express in mg/ml.

will also bind with dye and give spurious positive results. In addition, many proteins will not dissolve properly in an acidic reaction medium.

Requirements

Chemicals

BSA, distilled water, Coomassie brilliant blue, ethanol, orthophosphoric acid, monobasic sodium phosphate, dibasic sodium phosphate

Equipment

Weighing balance, pH meter, spectrophotometer

Glassware

Test tubes, conical flasks, glass pipette, beaker

Solutions

Bradford Reagent

1. Take 100 mg Coomassie brilliant blue in an airtight bottle and dissolve in 50 ml ethanol (95 %). Incubate for an hour at room temperature.
2. After incubation, slowly add 100 ml of orthophosphoric acid (85%) and adjust the final volume to 1000 ml using DW.
3. Filter the reagent using Whatman filter paper no. 1 and store it in an amber coloured bottle, which is stable for maximum of 3 weeks.
   0.2 M Phosphate buffer (pH 7.5)

Standard Protein: 0.02 mg/ml solution of BSA

Procedure

1. Warm up spectrophotometer 15 min before use.
2. Take 9 clean glass test tubes. Into each test tube, carefully pipette one of the following volumes of a 20 µg/ml solution of BSA: 0.0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 ml. In the last two test tubes put 1 ml of test samples each as duplicates.
3. Bring the total volume of liquid in all tubes to 2.0 ml by adding an appropriate amount of phosphate buffer.
4. Add 2 ml of Bradford reagent in all tubes, vortex thoroughly and incubate at room temperature for 15 min at room temp.
5. Determine the OD of each sample at 595 nm. Plot the standard curve using concentration of standard tubes of BSA (µg/ml) against the absorbance.
6. Calculate the mean of absorbance of the duplicate sample and find out the protein concentration present in the given sample from standard graph and formula (given below) and express in mg/ml.

Result

Table 2.15 Concentration of standard: 20 μg/ml.

Sl. No.	Std. BSA (ml)	Sample (ml)	Phosphate Buffer (ml)	Bradford's Reagent (ml)	OD at 595 nm
1	–	–	2.0	2	
2	0.1	–	1.9	2	
3	0.2	–	1.8	2	
4	0.4	–	1.6	2	
5	0.6	–	1.4	2	
6	0.8	–	1.2	2	
7	1.0	–	1.0	2	
8	–	1	1.0	2	
9	–	1	1.0	2	

Calculations

The concentration of protein (% mg) in the given sample is:

$$\frac{\text{OD (test)}}{\text{OD (std)}} \times \frac{\text{Conc (std)}}{\text{Volume (test)}} \times 100$$

Observation

The concentration of protein in the given sample estimated with Bradford's method is found to be _____ mg/ml.

Precautions

1. Protein dye complex binds to quartz cuvettes but not to glass or plastic. Therefore, polystyrene cuvettes are recommended.
2. Bound dye has a broad absorption peak. Readings may be taken up to 620 nm.
3. Reference protein concentrations should be determined by OD at 280 nm. For BSA, the extinction coefficient for a 1 mg/ml solution is 0.66.
4. The serva Blue G has greater dye content than Coomassie Blue G and produces less difference in colour yield from protein to protein.

2.8 ESTIMATION OF AMINO ACIDS

Objective

To estimate the amount of amino acids by Ninhydrin reagent.

Table 2.16 Tabulation

Sl. No.	Test Tube No.	SAAS (ml)	DW (ml)	Conc. (uM)	NR (ml)		Ethanol (ml)	A	Mean A
1	B	0	2.0	0	2.0	Boil for 15 min	3.0		
2	1	0.25	1.75	12.5	2.0		3.0		
3	1′	0.25	1.75	12.5	2.0		3.0		
4	2	0.5	1.5	25.0	2.0		3.0		
5	2′	0.5	1.5	25.0	2.0		3.0		
6	3	1.0	1.0	50.0	2.0		3.0		
7	3′	1.0	1.0	50.0	2.0		3.0		
8	4	1.5	0.5	75.0	2.0		3.0		
9	4′	1.5	0.5	75.0	2.0		3.0		
10	5	2.0	0	100.0	2.0		3.0		
11	5′	2.0	0	100.0	2.0		3.0		

Precautions

1. Store ninhydrin reagent solution in a brown bottle. Always use fresh solution.
2. Before adding ethanol, cool the tubes to room temperature.

Note

Almost all the amino acids form purple coloured complex when they are heated with ninhydrin reagent. However, intensity of the colour varies from one amino acid to other. Proline and hydroxyproline give yellow colour with ninhydrin. The complex, unlike others, absorbs light at 440 nm.

2.9 ESTIMATION OF LIPID

Theoretical Background

Lipids are substances of biological origin that are soluble in organic solvents but relatively insoluble in water. They serve as major structural components of the membranes and also form a protective coating on many organisms. They serve diverse functions in biological systems.

The lipids are classified on the basis of their structure as (i) simple lipids, (ii) complex lipids, (iii) derived lipids and (iv) miscellaneous lipids.

Simple lipids are the esters of fatty acids with alcohols. They include fats, oil and waxes.

Complex lipids are the esters of fatty acids. The fatty acids are covalently joined by an ester linkage to a trihydroxy alcohol, glycerol, or its derivative. Examples of complex lipids are

acylglycerides, phospholipids, sphingolipids, lipoproteins, etc. Derived lipids are the derivatives of lipids obtained upon hydrolysis. Examples of such lipids include glycerol, mono- and di-acylglycerols, lipid-soluble vitamins, steroid hormones, etc. Miscellaneous lipids include a large number of compounds possessing the characteristics of lipids, for example, carotenoids, squalene, etc.

Objective

To estimate lipid by vanilline-phosphoric acid reagent.

Principle

Hot sulphuric acid converts unsaturated lipids to water-soluble sulphuric acid derivatives, which give colour complexes (reddish) with vanilline-phosphoric acid reagent, and it has absorption maxima at 525 nm.

Requirements

1. Conc. H_2SO_4
2. Vanillin-phosphoric acid reagent
   300 mg of vanillin is dissolved in 50 ml of hot water, and 200 ml of 85% phosphoric acid is added to it.
3. Cholesterol stock solution
   Dissolve 1 mg cholesterol in 1 ml chloroform. Dilute 5 times for use (200 µg/ml). Close the mouth of the vial tightly and keep in cold.
4. Unknown solution

Procedure

1. Take clean and dried test tubes and mark them as per the protocol tabulation.
2. Take 0.1, 0.2, 0.3, 0.4, 0.5 ml (20, 40, 60, 80, 100 µg) of cholesterol stock solution and the unknown solution (T) in test tubes in duplicates.
3. In blank tube take 0.1 ml of chloroform.
4. Evaporate the solution tubes at 60°C.
5. Add 0.2 ml of conc. H_2SO_4 to each test tube and incubate for 10 min at 60°C.
6. Cool the tubes to room temperature and add 4.8 ml of vanillin-phosphoric acid reagent to each tube.
7. Mix thoroughly and allow to stand for 10 min at room temperature.
8. Record absorbance at 525 nm against blank.
9. Draw the standard curve for concentration of lipid of cholesterol v/s absorbance.
10. Put the absorbance of the unknown solution in standard graph and calculate the concentration of lipid present in it.

5. Keep the test tubes in 100°C water bath for 10 min.
6. Cool down to room temperature and take absorbance at 595 nm.
7. Construct a standard graph A_{595} (ordinate) vs. quantity of DNA (abscissa) and then calculate the concentration of DNA dissolved in the saline citrate solution.

Result

Table 2.18 Tabulation

Tube No.	DNA Stock Solution (ml)	DNA Conc. (lg)	Saline citrate Solution (ml)	DPA Reagent (ml)	Optical Density	Mean
Blank			2	4		
1	0.5	100	1.5	4		
1′	0.5	100	1.5	4		
2	0.6	120	1.4	4		
2′	0.6	120	1.4	4		
3	0.7	140	1.3	4		
3′	0.7	140	1.3	4		
4	0.8	160	1.2	4		
4′	0.8	160	1.2	4		
S1	0.5		1.5	4		
S2	1.0		1	4		

Calculation

The graph was obtained by taking concentration of DNA stock solution along *X*-axis and respective absorbance along *Y*-axis.

1. Determine the slope ("Absorbance/" DNA amount) of the regression line.
2. The concentration of the unknown DNA is given by
   DNA concentration (µg/ml) = Absorbance × Dilution factor slope (1/µg × Volume of diluted DNA sample used for the assay (ml)

Observation

The concentration of the given unknown DNA sample is found to be _____ mg/ml

2.11 ESTIMATION OF RNA

Theoretical Background

Ribonucleic acid (RNA) is a polymer of ribonucleotides held together by 3′-5′-phosphodiester bridges. Most RNA is present in cytoplasm as soluble and ribosomal RNA, but about 10 per

Dissolve 1 g of ferric chloride ($FeCl_3.6H_2O$) in 1 litre of concentrated HCl and add 35 ml of 6%, (w/v) orcinol in alcohol.

Buffer solution

0.15 M NaCl (2.2 gm) in 0.015 M sodium citrate (1.1 gm) in 250 ml distilled water, pH-7.0.

Procedure

1. Take clean and dry test tubes and mark them properly as per tabulation.
2. Take RNA standard solution with 20, 40, 60 up to 200 µg concentrations.
3. Make up the volume to 2 ml by adding buffer to each test tube.
4. Take one blank tube containing 2 ml of buffer solution.
5. Add 3 ml of orcinol reagent to each test tube.
6. Keep the test tubes in 100°C water bath for 20 min.
7. Cool down to room temperature and take absorbance at 660 nm.
8. Draw the standard curve for conc. of RNA v/s absorbance.
9. Calculate the concentration of the RNA present in the given sample from standard graph.

Table 2.19 Tabulation

Tube No.	RNA Stock Solution (ml)	RNA Conc. (lg)	Buffer (ml)	Oricinol Reagent (ml)	Optical Density
Blank			2	3	
1	0.04	20	1.96	3	
2	0.08	40	1.92	3	
3	0.12	60	1.88	3	
4	0.16	80	1.86	3	
5	0.2	100	1.8	3	
6	0.24	120	1.76	3	
7	0.28	140	1.72	3	
8	0.32	160	1.68	3	
9	0.36	180	1.64	3	
10	0.4	200	1.6	3	
S1	0.2		1.8	3	
S2	0.4		1.6	3	

Calculation

$$\text{Conc. of RNA} = [\text{Abs (test)}/\text{Abs (std)}] \times \text{Conc. (std)}$$

6. Bromine water
   Dissolve 1-2 drops of liquor bromine in approximately 100 ml cool distilled water.
7. Ascorbic acid stock solution
   Dissolve 100 mg ascorbic acid in 100 ml of 4% oxalic acid solution in a standard flask (1 mg/ml).
8. Ascorbic acid working solution
   Dilute 10 ml of the stock solution to 100 ml with 4% oxalic acid. The concentration of working standard is 100 µg/ml.
9. Distilled water

Procedure

Extraction

1. Grind 0.5-5 g of sample material, either mechanically or using a pestle and mortar, in 25–50 ml of 4% oxalic acid solution.
2. Centrifuge or filter and collect the liquid.
3. Transfer an aliquot (10 ml) to a conical flask and add bromine water dropwise with constant mixing. The enolic hydrogen atoms in ascorbic acids are removed by bromine.
4. When the extract turns orange-yellow due to excess bromine, expel it by blowing in air.
5. Make up to a known volume (25 or 50 ml) with 4% oxalic acid solution.
6. Similarly, convert 10 ml of stock ascorbic acid solution into dehydro form by bromination.

Estimation

1. Pipette out 10-100 µg standard dehydroascorbic solution into a series of tubes.
2. Similarly, pipette out different aliquots (0.1-2 ml) of brominated sample extract.
3. Make up the volume in each tube to 3 ml by adding distilled water.
4. Add 1 ml of DNPH reagent followed by 1-2 drops of thiourea to each tube.
5. Set a blank as above but with water in place of ascorbic acid solution.
6. Mix the contents of the tubes thoroughly and incubate at 37°C for 3 h.
7. After incubation dissolve the orange-red osazone crystals formed by adding 7 ml of 80%, v/v sulphuric acid.
8. Measure absorbance at 540 nm.
9. Plot a graph of ascorbic acid concentration versus absorbance and calculate the ascorbic acid content in the sample.

Result

The ascorbic acid content of the given sample is _____ µg/ml.

Precaution

Liquid bromine can cause burns. The ampoule containing bromine water must be chilled before opening the seal.

2.14 QUANTITATIVE ESTIMATION OF PHOSPHATE SOLUBILISATION IN CULTURE MEDIUM

Principle

Orthophosphate present in the sample reacts with molybdate to form phosphomolybdic acid which is reduced by ammonium metavandate to form a blue complex. This blue colour can be measured at 430 nm by spectrophotometer.

Requirements

Phosphate solubilising microorganism
Pikovskaya's broth medium
Whatman No.1 filter paper
Centrifuge tube
Conical flask
Measuring cylinder
Beaker
Volumetric flask
KH_2PO_4
Distilled water
Spectrophotometer
Centrifuge
Barton's Reagent
Solution A: Dissolve 25 g of ammonium molybdate in 400 ml of distilled water.
Solution B: Dissolve 1.25 g of ammonium metavandate in 300 ml of boiling water, cool and then add 250 ml of conc. HNO_3
Mix solutions A and B to make up the volume to 1 litre.

Preparation of standard curve

1. Dissolve 0.2195 g KH_2PO_4 in distilled water to make up the volume to 1 litre (1 ml = 59 ppm phosphorus).
2. Make further dilution of 10 ml into 250 ml so as to get 1 ml = 2 ppm phosphorus.
3. Take aliquots of 2, 3, 4, 5, 6, 8, 10, 15 and 20 ml of the 2 ppm stock solution in 50 ml volumetric flask, add 2.5 ml of Bartons reagent, and add water to make the final volume of 50 ml.
4. After 10 min interval, measure OD at 430 nm in a spectrophotometer and plot a graph between OD and concentration of phosphorus.

Procedure

1. Prepare Pikovskaya broth (100 ml) in 250 ml conical flask and autoclave it.
2. Inoculate fungal or bacterial culture aseptically in the conical flask and incubate it at 28°C for fungal or 37°C for bacterial culture for 6-17 days.
3. Centrifuge the microbial culture at 10,000 rpm for 15 min.

4. Take the supernatant and filter it through Whatman filter paper.
5. Add distilled water to the solution to make a known volume (50-100 ml).
6. Take 10 ml aliquots of the clear filtrate and add 25 ml of Barton's reagent and make the volume up to 50 ml.
7. After 10 min, measure O.D. of the resultant colour in a spectrophotometer at 430 nm.

Result

Calculate the amount of phosphate solubilised in culture medium by comparing it with the standard curve.

REVIEW QUESTIONS

1. The absorbance (A) of a 5×10^{-4} M solution of the amino acid tyrosine, at a wavelength of 280 nm is 0.75. The path length of the cuvette is 1 cm. What is the molar absorption coefficient, *k*?
2. What happens to glucose or galactose when the Cu^{2+} in Benedict's is reduced?
3. Would you expect fructose or glucose to form a red colour rapidly with Seliwanoff's reagent?
4. What happens to the carbon groups in mucic acid test?
5. How can the iodine test be used to distinguish between amylose and glycogen?
6. Which carbohydrate(s) would have the following test results?
   (a) Produces a reddish-orange solid with Benedict's and a red colour with Seliwanoff's reagent in 1 min.
   (b) Gives a colour change with Benedict's test and a light orange colour with Seliwanoff's reagent after 5 min.
   (c) Gives no colour change with Benedict's or Seliwanoff's test, but turns a blue-black colour with iodine reagent.
7. Classify carbohydrates.
8. What are 'd' and 'l' stereoisomers?
9. Why does sugar react with the anthrone reagent to yield a blue-green colour?
10. Give examples of monosaccharides and disaccharides.
11. What is muta rotation?
12. What is the function of anthrone in this experiment?
13. What is the role of copper tartrate in estimation of reducing sugar?
14. Why is ammonium molybdate used in this experiment?
15. What are reducing sugars? Give two examples.
16. What is the function of arsenomolybolic acid reagent?
17. What is the role of DNS in estimation of reducing sugar?
18. Why is sodium sulphite used in the estimation of reducing sugar experiment?
19. What is the function of Rochelle salt?
20. How is DNS method different from Nelson-Smyogi method for the estimation of reducing sugar?