Lipids

Lipids are heterogeneous group of organic compounds that are generally insoluble in water and are soluble in non-polar organic solvents (diethyl ether, acetone, hexane and carbon tetrachloride). They are the components of living systems consisting of basically carbon, hydrogen and oxygen; in addition some have nitrogen and phosphorus. Lipids include fats, oils, steroids, waxes, and related compounds.

Lipids are divided into several classes based on structural similarities.

1- Simple lipids: These are esters of fatty acids with alcohols. Simple lipids are sub-classified into two types, depending on the type of alcohols:

a. **Neutral lipids:** Esters of fatty acid with glycerol like triglyceride.
b. **Waxes:** Waxes are esters of long-chain saturated and unsaturated fatty acids with long-chain alcohols.

2- Compound or complex lipids: These are esters of fatty acids containing other groups in addition to alcohol and fatty acids; such as phospholipids and glycolipids.

3- Derived lipids: These are derived from the hydrolysis of above two classes of lipids; such as fatty acids, sterols, terpenes and fat soluble vitamins.

Fatty acids

Fatty acid is an aliphatic monocarboxylic acids consisting of an alkyl hydrophobic tail (hydrocarbon) and a terminal hydrophilic (polar head i.e., carboxyl group) group with a basic formula.

$$CH_3\text{-}(CH_2)_n - COOH$$

Hydrocarbon Carboxyl group

Most lipids contain fatty acids which may be obtained from complete hydrolysis of simple and compound lipids. Natural fatty acids may be saturated or unsaturated,

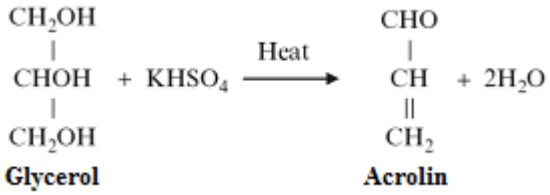
and the saturated acids have higher melting points than unsaturated acids of corresponding size.

Saturated	Unsaturated
Lauric acid: $CH_3(CH_2)_{10}CO_2H$	Arachidonic acid: $CH_3(CH_2)_4(CH{=}CHCH_2)_4(CH_2)_2CO_2H$
Myristic acid: $CH_3(CH_2)_{12}CO_2H$	Palmitoleic acid: $CH_3(CH_2)_5CH{=}CH(CH_2)_7CO_2H$
Palmitic acid: $CH_3(CH_2)_{14}CO_2H$	Oleic acid: $CH_3(CH_2)_7CH{=}CH(CH_2)_7CO_2H$
Stearic acid: $CH_3(CH_2)_{16}CO_2H$	Linoleic acid: $CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7CO_2H$
Arachidic acid: $CH_3(CH_2)_{18}CO_2H$	Linolenic acid: $CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_7CO_2H$

Tests for lipids

Test one: Acrolein Test for free and esterified glycerol

The Acrolein test is a general test for the presence of **glycerol** (**in free form or in esterified form**) in a molecule. Potassium bisulfate is both a strong acid, and strong dehydrating reagent. When potassium bisulfate is heated with a fat, hydrolysis occurs, and the glycerol produced is dehydrated to form acrolein (CH2= CHCHO). Acrolein has a characteristic sharp irritating odor.



Reagents:

1- Lipid or fat

2-Potassium bisulfate

Procedure:

1- Take a clean dry test tube and put 2 drops of oil or fat to it.

2- Then place 0.5 grams of potassium bisulfate (potassium hydrogen sulfate) in it. (Make sure the oil or fat is in physical contact with the potassium bisulfate).

3- Heat the test tube slowly on Bunsen burner flame till fumes appear. Note the odor of the fumes and cautiously smell the fumes of gas by fanning. Acrolein should produce a characteristic sharp pungent, unpleasant, and irritating odor. Both the pungent smell and the black color indicate the presence of glycerol and fat.

Test two: Dunsten's test for free glycerol

The Denstan test is specific for **free glycerol** and it is a general test used for differentiates free glycerol and esterified glycerol. This test is depended on the reaction between free glycerol and borax in the presence of phenolphthalein, which leads to disappear the pink color of phenolphthalein due to formation of glyceroboric acid, and dissociated by heating and the pink color reappear on heating.

Reagents:

1- Lipid or fat

2- Glycerol

3- Phenolphthalein

4- Borax solution (0.5%).

Procedure:

1- Take 2 ml of borax solution in test tube. Add two drops of phenolphthaleine indicator, the pink color appear.

2- Add glycerol solution drop by drop till the pink color disappears because the PH is changed to acidic medium.

3- Heat the test tube, the pink color reappears.

Test three: Cupric acetate test for free fatty acids

Cupric acetate test depends upon the presence of **free fatty acids**, so this test is used to differentiate between free fatty acids and esterified fatty acids (neutral lipids). In this test free fatty acid react with cupric acetate to form cupric salts. Depending upon the presence of fatty substance, the two layers are obtained.

Reagents:

1- Esterified fatty acids (neutral lipid).

2- Saturated fatty acids

3- Unsaturated fatty acids

4- Cupric acetate (10%)

5- Petroleum ether.

Procedure:

1- Dissolve 0.5 gm of fatty substances in 4 ml of petroleum ether.

2- Add 3 ml of cupric acetate, and mix well.

3- Allow the solution to stand for five minutes. The solutions are separated into two layers:

a- If the two layers remain colorless, this means the presence of esterified fatty acids (neutral lipids).

b- If the upper layer (petroleum ether layer) is green, this means the presence of unsaturated fatty acids.

c- If the upper layer remains colorless and the lower layer is precipitate as a blue color, this means the presence of saturated fatty acids.

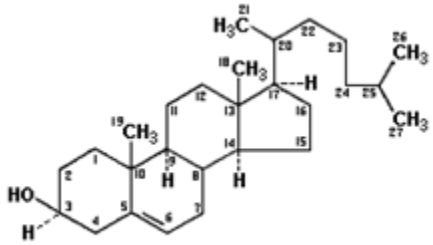
Caution

-Phenolphthalein is a carcinogen and irritant. Handle with care. Wear safety glasses and gloves.

-Potassium Hydroxide is corrosive. Wear safety glasses and gloves.

Test four: Tests for Cholesterol

Cholesterol is the major sterol in animal tissues, it occurs only rarely in higher plants. Cholesterol is a major structural constituent of the cell membranes and plasma lipoproteins. Cholesterol is a precursor in the biosynthesis of all steroid hormones (like Testosterone), vitamin D and bile salts.



Cholesterol

a- Salkowski test

In **Salkowski test**, chloroform solution of the cholesterol when shaken with concentrated sulphuric acid and on standing yields red color.

Reagents:

1- Cholesterol

2- Concentrated H2SO4

3- Chloroform

Procedure:

1- Take a perfectly clean and dry test tube and add 2 ml cholesterol to it.

2- Then add 2 ml of concentrated H2SO4 dropwise along the side of the test tube.

3- Two layers are formed. The upper brown one is formed by chloroform CHCl3, and the lower one yellow in color formed by concentrated H2SO4. This layer of concentrated H2SO4 gives fluorescence.

b- Liebermann- Burchard test

The **Lieberman-Burchard reaction** uses acetic anhydride and sulfuric acid as reagents, which gives a characteristic green color in the presence of cholesterol. This

color is due to the OH group of cholesterol and the unsaturation found in the adjacent fused ring. The color change is gradual: first it appears as a pink coloration, changing later to violet, and finally to deep green.

Reagents:

1- Cholesterol.

2- Concentrated H_2SO_4.

3- Acetic anhydride.

4- Chloroform.

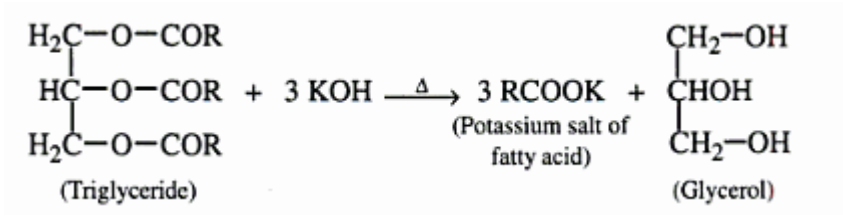
Procedure:

1- Take 2 ml of chloroform in a test tube and dissolve a pinch of cholesterol in it.

2- Then add 10 drops of acetic anhydride and mix well. Then add 2 drops of concentrated H_2SO_4 from the sides of the test tube. A deep green colored solution is obtained. This indicates the presence of cholesterol.

Test five: Saponification Number

The saponification number is a measure of the average molecular weight of the triglycerides (triacylglycerols) in a sample. Saponification is the process of breaking down a neutral fat into glycerol and fatty acids by treatment with alkali:



The saponification number is defined as the number of mg of KOH required to saponify completely one gram of fat. The lipid is first extracted and then dissolved in an ethanol solution which contains a known excess of KOH. This solution is then heated so that the reaction goes to completion. The unreacted KOH is then determined by adding an indicator and titrating the sample with HCl. The

saponification number is then calculated from knowledge of the weight of sample and the amount of KOH which reacted. The smaller the saponification numbers the larger the average molecular weight of the triglycerides (triacylglycerols) present.

Reagents:

1- Oil or Fat.

2- Hydrochloric acid HCl (0.5 N).

3- Alcoholic KOH (10%) (Dissolve 10gm KOH in 100 ml of alcohol).

4- Fat solvent (a 1:1 mixture of ethanol (95%) and ether)

5- Phenolphthalein indicator 1% (in alcohol).

Procedure:

1- Dissolve 1 gm of fat or 1 ml of oil in 5 ml Fat solvent in a conical flask.

2- Add 25 ml of alcoholic KOH into the flask containing the fat or oil. Mix the contents well.

3- Connect reflux condenser and boil them gently for about half an hour until all the oil globules disappear and a yellow cake is formed by potassium salts of fatty acids.

4- After half an hour, take out the conical flask, cool it to room temperature.

5- Add about 5 drops of phenolphthalein indicator and titrate against 0.5 N HCl until the pink color just disappears. Record the volume of HCl (T).

6- Run a blank titration, without using oil under similar conditions and record the volume of HCl (B).

Note

The saponification number gives an idea about the type of fatty acid present in fat or oil. For a fat or oil saponification number is a constant, hence it can be used as an index in finding out rancidity in fat or oil.

Cautions

- Phenolphthalein is a carcinogen and irritant. Handle with care. Wear safety glasses and gloves.
- Potassium Hydroxide is corrosive. Wear safety glasses and gloves.
- Alcohol is inflammable, so use electrical heating.

Calculations:

Volume of HCl required for saponified solution = T ml.

Volume of HCl required for blank titration = B ml.

Molecular weight of 0.5 N KOH contains 28.05 mg of KOH. Therefore, to calculate the saponification number, multiply the titre value by 28.05.

This means that:

1 ml of HCl (0.5 N) = 28.05 mg of KOH (0.5 N).

$$\text{Saponification number of 1 gm of oil} = \frac{\left[\text{V. of HCl for blank}\right] - \left[\text{V. of HCl for sample}\right]}{\text{Weight of sample}} \times N_{NaOH} \times \text{M.wt}_{NaOH} \times (56.1)$$

Multiply this value by 3 for triglycerides, as 3 molecules of fatty acids are released from triglyceride.

The molecular weight of oil can be calculated by:

$$\text{Molecular weight of oil} = \frac{3 \times \text{M.wt of alkai}}{\text{Saponification number}} \times 1000$$

Test six: Iodine Value

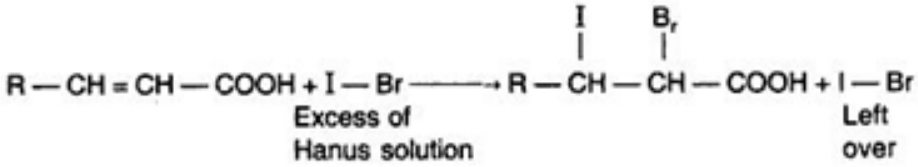
This is a test to measure the amount of unsaturation in fat and oil. The iodine value (IV) gives a measure of the **average degree of unsaturation of a lipid**: the higher the iodine value, the greater the number of C=C double bonds. By definition the iodine value is expressed as the grams of iodine absorbed per 100g of lipid. Iodine

number is a useful characteristic for assessment of both purity and nutritive value of the fat. The iodine numbers of some important fats are mentioned below:

Fats	Iodine numbers
Butter fat	26-28
Human fat	65-70
Peanut oil	80-90
Corn oil	110-125
Soybean oil	137-143
Linseed oil	170-200

The lipid to be analyzed is weighed and dissolved in a suitable organic solvent, to which a known excess of iodine chloride is added. Some of the IBr reacts with the double bonds in the unsaturated lipids, while the rest remains:

Fatty acids react with a halogen (iodine) resulting in the addition of the halogen at the C=C double bond site. In this reaction, iodine monochloride reacts with the unsaturated bonds to produce a di-halogenated single bond, of which one carbon has bound an atom of iodine.



After the reaction is complete, the amount of iodine that has reacted is determined by adding a solution of potassium iodide to the reaction product.

$$IBr + KI \longrightarrow I_2 + KBr$$

The amount of IBr remaining is determined by adding excess potassium iodide to the solution to liberate iodine, and then titrating with a sodium thiosulfate ($Na_2S_2O_3$) solution in the presence of starch to determine the concentration of iodine released:

$$I_2 + 2Na_2S_2O_3 \longrightarrow 2NaI + Na_2S_4O_6$$

Iodine itself has a reddish brown color, but this is often not intense enough to be used as a good indication of the end-point of the reaction. For this reason, starch is usually used as an indicator because it forms a molecular complex with the iodine that has a deep blue color. Initially, starch is added to the solution that contains the iodine and the solution goes a dark blue. Then, the solution is titrated with a sodium thiosulfate solution of known molarity. While there is any I_2 remaining in the solution it stays blue, but once all of the I_2 has been converted to NaI it turns colorless.

The concentration of C=C in the original sample can therefore be calculated by measuring the amount of sodium thiosulfate needed to complete the titration.

Reagents:

1- Oil or Fat.

2- Hanus solution IBr (dissolves 13.2 gm of I_2 in 1 litter of glacial acetic acid, then add 3 ml of Br_2.

3- Sodium thiosulfate (0.1 M).

4- Potassium iodide KI (10%).

5- Chloroform or CCl_4

6- Starch (1%).

Procedure:

1- In a 250 ml conical flask, dissolve 0.25 ml of oil or 0.5 gm of fat in 10 ml chloroform or CCl_4. Then add 25 ml of Hanus solution. Mix well, cover the mouth of the flask with a paper and keep it in dark place for 30 minutes for reaction to take place.

2- After 30 minutes, add about 50 ml of warm distilled water to wash down all the IBr into the solution. The colors of the solution turn blue-black. Mix well, and then add 10 ml of KI solution into it.

3- Titrate the contents of the flask with standard sodium thiosulfate till the color changes from blue-black to a pale strew color, add 10 drops of starch solution as indicator (blue color) and titrate again till there is no free iodine to change the blue color to colorless. Note down the titre value which is X ml.

4- To prepare a blank, use only 10 ml of chloroform or CCl_4 only instead of oil or fat sample and repeat the same procedure as in the test. Note down the titre value which is Y ml.

Calculation:

The volume obtained for test titration = x ml

The volume obtained for blank titration = y ml

The difference between the two (i.e. blank-test) indicates the volume of sodium thiosulfate (0.1M) required to react with an equivalent volume of iodine. To convert this volume into grams of iodine, multiply (Blank- Test) by 12.7/1000, as 1litter of 0.1 M iodine contains 12.7 gm of iodine.

According to Normality equation

1 ml of N/10 $Na_2S_2O_3$ solution = 1 ml of N/10 I-Br solution

1 ml of n/10 $Na_2S_2O_3$ solution = I ml of N/10 I-Br solution

= 1 ml of N/10 iodine solution

Normality of sodium $Na_2S_20_3$ = 0.1

Equivalent weight of iodine =127

1 ml of N/10 iodine = (127/1000) x (1/10) = 0.0127

Volume of Sodium thiosulphate used = [Blank- Test] ml

(Two molecules of $Na_2S_2O_3$ are equivalent to one molecules of iodine; thus one molecules of $Na_2S_2O_3$ is equivalent to one atom of iodine

1 ml of N/10 $Na_2S_2O_3$ solution = 0.0127 gm of iodine

Amount of iodine absorbed by given amount of oil or fat = (y-x) x 0.0127 gm of iodine

$$\text{Iodine number} = \frac{\left[\begin{array}{l}\text{V. of } Na_2S_2O_3 \\ \text{for blank}\end{array}\right] - \left[\begin{array}{l}\text{V. of } Na_2S_2O_3 \\ \text{for sample}\end{array}\right]}{\text{Weight of sample}} \times 0.0127 \times 100$$

Test seven: Determination of acid value of fats

Rancidity

The term "**rancidity**" is used to describe the development of bad flavors and odors in fats and oils when stored for long time.

There are two types of rancidity

1- **Hydrolytic rancidity**

   Hydrolytic rancidity is caused by the growth of microorganisms which secrete lipases and split triglycerides into glycerol and fatty acids. If fatty acids of low molecular weight are released, they impart unpleasant taste and odor.

2- **Oxidative rancidity**

   Oxidative rancidity occurs due to the auto-oxidation of the unsaturated fatty acids at their double bonds yielding short chain acids and aldehydes having rancid taste and odors. The amount of free fatty acids associated with fat gives a fair indication of its quality and age.

The **acid value** is defined as the number of milligrams of KOH required to neutralize the amount of free fatty acids present in 1 gm of the fat or oil.

Fat may become rancid after long storage. Fat or oil is hydrolyzed by different microorganisms with the formation of free fatty acids. The acid value is often a good measure of the breakdown of the triglycerides into free fatty acids, which has an adverse effect on the quality and the age of many lipids.

When fat or oil is dissolved in an ethanol solution containing an indicator and this solution is then titrated with alkali (KOH) until a pinkish color appears due to the

formation of free fatty acid. Thus the high acid number indicates a stale oil or fat and stored under improper conditions.

Reagents:

1- Oil or Fat.

2- Hydrochloric acid (0.5 N).

3- KOH solution (0.1 N).

4- Fat solvent (a 1:1 mixture of ethanol (95%) and ether)

5- Phenolphthalein indicator 1% (in alcohol).

Procedure:

1- Weigh 5 gm of fat or 5 ml of oil and transfer it into 250 ml conical flask.

2- Add 10 ml fat solvent to the oil solution.

3- Add 1 ml of phenolphthalein indicator and mix well.

3- Titrate this against the KOH solution until a faint pink color appears and persists.

Calculation:

Calculate the acid number (mg KOH/g) of the fats:

$$\text{Acid number} = \frac{\text{Volume of KOH}}{\text{Weight of sample}} \times N_{KOH} \times \text{M.wt (56.1)}_{KOH}$$

Test eight: Determination of peroxide value in fats and oils

Lipid oxidation (also called auto-oxidation) at fatty acid occurs by reaction of double bonds in fatty acids with oxygen present in the air, causing the formation of labile peroxides. The peroxides (ROOH) formed during auto-oxidation are unstable and decompose into free radicals .These initiate chain reactions which lead to eventually to decomposition of the fatty acid into various low molecular weight e.g., aldehydes, ketones, organic acids, and hydrocarbons.

The peroxide value is defined as the number of milliliters of sodium thiosulfate required to neutralize the peroxides contained in 1 gm of fat or oil.

The peroxide value is determined by measuring the amount of iodine which is formed by the reaction of peroxides (formed in fat or oil) with excess of KI.

$$\mathbf{ROOH + KI\ excess \rightarrow ROH + KOH + I_2}$$

The iodine liberated is titrated with sodium thiosulphate and a starch indicator:

$$\mathbf{I_2 + starch + 2Na_2S_2O_3\ (blue) \rightarrow 2NaI + starch + Na_2S_4O_6\ (colorless)}$$

The amount of sodium thiosulfate required to titrate the reaction is related to the concentration of peroxides in the original.

Peroxide values are generally less than **10 m.eq/kg** in fresh samples of oil. Due to temperature or storage, rancidity occur causing increase in peroxide value.

Reagents:

1- Solvent mixture (mix 2 ml of glacial acetic acid with 1 ml of chloroform).

2- Potassium iodide powder.

3- Potassium iodide KI (15%)

4- Sodium thiosulfate $Na_2S_2O_3$ (0.02 N or 0.02 M)

5-Starch indicator solution (0.5 %)

Procedure:

1- Weigh 1 gm oil (with precision of 0.001 g) into a 250 ml Erlenmeyer flask.

2- Add 1gm of powdered KI, and 20 ml of solvent mixture (glacial acetic acid with chloroform 2:1). Then place the Erlenmeyer flask in boiling water for 1 minute.

4- Cool and add 20 ml of KI solution. Then wash the flask by 20 ml distilled water and add 1 ml starch indicator.

5- Titrate the solution with sodium thiosulfate until blue color disappears.

6- Do the same procedure as in the test without using oil under similar conditions and record the volume of sodium thiosulfate.

$$\text{Peroxide number} = \frac{\left[\begin{array}{l}\text{V. of } Na_2S_2O_3 \\ \text{for sample}\end{array}\right] - \left[\begin{array}{l}\text{V. of } Na_2S_2O_3 \\ \text{for blank}\end{array}\right]}{\text{Weight of sample}}$$

Test nine: Kreis test for rancid fats or oils

Kreis test is a qualitative test used for indicating slight changes in the condition of a fat or oil to aldehydes and ketones, under various circumstances (created by its rancidity).

Rancid fats or oils contain the volatile and rather unstable aldehyde, epihydrin aldehyde. In the presence of hydrochloric acid, this substance produces a red color with phloroglucinol.

Reagents:

1- Fats or oils.

2- Solution of phloroglucinol in ether (0.1%).

3- Concentrated HCl.

Procedure:

1- 1 gm of fat or 1 ml of oil is mixed with 1 ml of hydrochloric acid in a test tube.

2- Then add 1 ml of phloroglucinol solution and mix thoroughly. A red color will develop if the oil is rancid.

Questions

Define

Lipid, Fatty acids, Acroline test, Denstan test, Cupric acetate test, saponification number, Iodine test, Rancidity, Hydrolytic rancidity, Oxidative rancidity, Acid number, Peroxide number, Salkawski test

Acroline test

By which test can you distinguish between lipid and other compounds?

Write the reaction between glycerol and potassium hydrogen sulfate?

What is the role of potassium hydrogen sulfate in acroline test?

Dunsten's test

By which test can you distinguish between esterified glycerol (lipid or fat) and free glycerol?

What is the role of borax in Denstan test?

The reaction between free glycerol and borax in the presence of phenolphthalein, leads to disappear the pink color of phenolphthalein, why? And the pink color reappear on heating, why?

Cupric acetate test

By which test can you distinguish between free fatty acids and esterified fatty acids (lipids, or fat)?

What is the role of cupric acetate in cupric acetate test?

How can you distinguish between saturated and unsaturated fatty acids in cupric acetate test?

Salkowski test

By which test can you distinguish between cholesterol and lipid?

What is the role of H2SO4 in Salkowski test?

Liebermann- Burchard test

By which test can you distinguish between cholesterol and lipid?

What is the role of acetic anhydride and H2SO4 in Lieberman-Burchard test?

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