CHAPTER 12

Analysis of Fixed Oils, Fats, and Waxes

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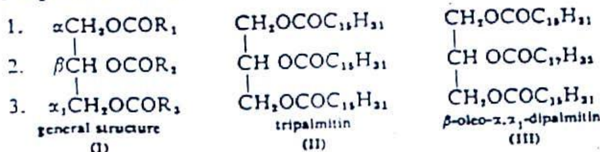
12.1 INTRODUCTION

The substances to be dealt with in this chapter are members of the general class of compounds known as *lipids*. They are compounds of carbon, hydrogen, and oxygen which are seen as derivatives of fatty acids through ester linkages. Fixed oils and fats are glyceryl esters of fatty acids, differentiated only on the basis of physical form at room temperature, that is, liquid or solid. Waxes are esters of a fatty acid and a high molecular weight monohydroxy alcohol, and generally have a harder, more brittle physical form than the fats.

An exhaustive treatment of the analysis of fixed oils, fats, and waxes is beyond the scope of a book of this nature, and for a more comprehensive presentation the reader is referred to texts and compendia dealing extensively with this subject.[1–4]

A general discussion of the chemical structure of the lipids of interest here may be advantageously confined to the glyceryl esters. The esters of glycerin are named as *glycerides*, or more specifically, in the case of fixed oils and fats, as *triglycerides*. Structural formulas for *triglyceride* structures are (I) through (III). The carbons of glycerin are identified by the use of α, β, α_1; or 1, 2, 3; and the situation where $R_1 = R_2 = R_3$ produces a "simple" glyceride as in tripalmitin (II). When the R's are not equal we have a "mixed" glyceride as in β-oleo-α,α_1-dipalmitin (III).



Naturally occurring fixed oils and fats are best described as mixtures of mixed triglycerides. Very few natural fats contain only two or three different fatty acids esterified with glycerin, but more often have six to eight or more in certain instances. As an example, milk fat contains 16 identifiable major and minor component acids.[5] The presence of unsaturated fatty acids results in a decreased melting point until, if a sufficient percentage is present, the fat is a liquid at room temperature and is designated as a fixed oil.

Fixed oils, fats, and waxes have a number of properties in common. They are greasy or waxy to the touch, and with few exceptions are lighter than

water. They are insoluble in water, slightly soluble in alcohol, and soluble in the so-called fat solvents such as ether, chloroform, and benzene. They cannot be distilled, and upon heating strongly, the glycerides decompose with the characteristic odor of burnt fat (owing to the formation of acrolein). Numerous fixed oils and fats turn rancid upon oxidation, with the accompanying development of a disagreeable odor and an acid reaction.

As esters, fixed oils, and fats are hydrolyzed to a slight extent in the presence of water, and for this reason natural fixed oils and fats contain traces of free fatty acids. The hydrolysis reaction shown in Eq. (12.1) is accelerated by

$$\underset{\text{tripalmitin}}{C_3H_5(OCOC_{15}H_{31})_3} + H_2O \rightleftharpoons \underset{\text{glycerol}}{C_3H_5(OH)_3} + \underset{\text{palmitic acid}}{3C_{15}H_{31}COOH} \qquad (12.1)$$

acids, bases, or enzymes. Hydrolysis in the presence of base forms the corresponding salts of the fatty acids, and since this removes the acids from the right of Eq. (12.1), the equilibrium is shifted strongly in favor of hydrolysis. The metallic salts of the fatty acids are called *soaps*, and the process of alkaline hydrolysis is known as *saponification*.

Because they are natural products, fixed oils, fats, and waxes are not definite chemical compounds, but are of a variable composition. This variability is seen even when the products are obtained from the same natural source. Since the fixed oils and fats are commercial products of considerable economic importance, there is always a possibility of their being subject to adulteration by cheaper varieties. However, owing to the natural variation mentioned above, the failure of the crude products to agree with official specifications is not necessarily evidence of adulteration.

The analysis of fixed oils and fats is concerned with the determination of physical and chemical properties as a means of establishing the purity of a given sample rather than the percentage composition as in other analytical procedures. The physical and chemical properties are usually expressed in terms of analytical constants for the test performed, and in pure oils, the variation of the constants will normally be slight. Mixtures of fixed oils or fats will be characterized by a "mixed constant," whose value will reflect the composition of the mixture. Some of the physical and chemical constants of use in pharmaceutical analysis are the refractive index, specific gravity, melting point, moisture content, iodine value, acid value, saponification value, Reichert-Meissel value, Polenske value, acetyl or hydroxyl value, and unsaponifiable matter. Table 12.1 lists physical and chemical characteristics of a select group of fixed oils, fats, and waxes.

The physical and chemical constants are utilized by official compendia such as the *U.S. Pharmacopeia*[6] and the *British Pharmacopoeia*[7] as a means of standardizing the fixed oils, fats, and waxes used as medicinal agents or in pharmaceutical preparations. Both compendia have official methods for the determination of many of the previously mentioned constants, and in general they will be seen to be similar in theory but differing sometimes in the

TABLE 12.1: Physical and Chemical Characteristics of Some Oils, Fats, and Waxes

Oil, fat, or wax	Iodine value	Saponification value	Acid value	Refractive index, 25°C
Almond	93–103	183–207	0.5–3.5	1.4593–1.4646[a]
Beef tallow	35–42	196–200	0.25	
Beeswax	8–11	88–100	17–21	1.4445–1.4473[b]
Butter fat	26–28	210–230	0.45–35.4	1.4555–1.4578[a]
Castor	81–91	175–183	0.12–8.0	1.4733–1.4771
Coconut	6–10	253–262	2.5–10	1.4477–1.4495[a]
Cocoa butter	33–42	193–195	1.1–1.9	1.4537–1.4580[a]
Cod liver	137–166	171–189	5.6	1.4758–1.4783
Corn	111–128	187–193	1.4–2.0	1.4733–1.4773
Cotton seed	103–111	194–196	0.6–0.9	1.4743–1.4752[c]
Laurel (bayberry)	68–80	198–199	26.3	1.4783
Linseed	175–202	188–195	1–3.5	1.4797–1.4802
Mutton tallow	48–61	195–196	1.7–14	1.4545–1.4585[a]
Myrtle wax	4–9	205–211	3–4.4	1.4511[a]
Olive	79–88	185–196	0.3–1.0	1.4657–1.4667
Peanut	88–98	186–194	0.8	1.4620–1.4653[a]
Safflower	122–141	188–203	0.6	1.4731–1.4769[a]
Soya	122–134	189–193	0.3–1.8	1.4723–1.4756
Spermaceti	2–8	121–135	0.5–3.0	
Sunflower	129–136	188–193	11.2	1.4659–1.4721[a]
Wool fat	17–29	82–130	59.8	1.4784–1.4822[a]

[a] At 40°C.
[b] At 65°C.
[c] At 15°C.

techniques used for the determination. For example, the USP gives stepwise directions for the determination of specific gravity, whereas the BP defines it exactly, but speaks of the actual determination in more general terms.

The determination of the *acid value* is carried out in the same manner in both compendia with the exception that the USP uses sodium hydroxide and the BP uses potassium hydroxide in the titration. When sodium hydroxide is used in the titration it must be remembered that the definition and calculation of the acid value is in terms of milligrams of potassium hydroxide.

Another comparison can be made concerning the *iodine value*, where the USP utilizes iodine monobromide, and the BP adds iodine to the existing double bonds with iodine monochloride solution. Both compendia direct that the residual iodine be titrated with standard sodium thiosulfate solution.

In their definitions of *saponification value* the BP and USP differ in that the USP considers the neutralization of any free fatty acids present in the sample, whereas the BP includes these acids as arising from saponification. In practice the value would be the same, because in both cases a sample is weighed, saponified with excess alkali, and the residual alkali titrated with standard acid.

A number of procedures are given in the following section which are found satisfactory for the determination of the commonly encountered physical and chemical constants.

12.2 PROCEDURES FOR THE DETERMINATION OF PHYSICAL AND CHEMICAL CONSTANTS

A. PREPARATION OF SAMPLES OF FIXED OILS, FATS, AND WAXES

In the sampling of fixed oils and fats the necessity of obtaining a representative sample must be emphasized. Sampling is best done when the material is completely liquefied and thoroughly mixed. The product should be warmed to dissolve any suspended matter, such as stearin, and if insoluble material is present in the warmed sample, it should be clarified by filtration using a heated funnel support to maintain the fluid state.

The devices used for sampling should be such that a representative sample is obtained, with particular care being taken where large quantities are being tested. Tank cars, drums, and dispensing containers all require their own devices and techniques. A metal tube 2 in. in diameter with a closure valve at the bottom, and of sufficient length that it can reach to within ¼ in. of the bottom of the tank, is used for sampling tank cars. Attention must be given to the design of the tank in considering the samples taken at a given level; for example, if the bottom 1-ft section is ¼ the capacity of the middle 1-ft section, then one 1-ft sectional sample should be drawn from the bottom and four 1-ft samples should be taken at the middle. The layer samples are then mixed to constitute the analytical sample.

An oil thief of glass tubing up to ½ in. in diameter, tapered at the bottom to about a ¼-in. orifice, such that the top opening may be closed with the finger as in the operation of a pipette, is used for sampling drums. The oil thief may be used for sampling dispensing containers, or simple pouring after mixing may suffice.

Solid materials in flake or granule form are best sampled by grab sampling from several containers. The samples are mixed, and appropriate treatment such as quartering is used to obtain an analytical sample. When the solid is in the form of large lumps, these should be broken up before taking grab samples.

B. DETERMINATION OF WATER

The solubility of water in fixed oils and fats is very slight, and only small amounts are generally present. When water is present in excess of its solubility, it is usually found as a separate phase, and since the rate of separation is fairly rapid, this presents a problem in obtaining an homogenous sample. Vigorous agitation with a stirrer is helpful during removal of the sample.

Generally, fixed oils and fats contain small amounts of materials that are volatile in nature. When evaporation by heat is used to determine the water content, a certain percentage of the loss in weight will be due to volatile materials, and this fact is generally recognized by the method of reporting the results of evaporation, which should always be as "moisture and volatile matter."

A number of methods are used to determine the moisture content of fixed oils and fats, varying from different oven-drying techniques, distillation with an immiscible liquid, or titration as in the procedure of Karl Fischer.

1. Vacuum Oven Method

A 5-g sample of the well-mixed material is weighed into an aluminum moisture dish of about 2-in. diameter and 1-in. depth. The sample is dried to constant weight in a vacuum oven at a temperature 20 to 25° above the boiling point of water at the operating pressure, which is held below 100 mm Hg. The sample is cooled in a desiccator and weighed. Constant weight is considered reached when the loss from successive 1-hr drying periods does not exceed 0.05%:

$$\% \text{ moisture and volatile matter} = \frac{\text{wt. of sample} - \text{wt. of residue} \times 100}{\text{wt. of sample}}$$

2. Immiscible Solvent Distillation Method

This method is somewhat less accurate, but more specific for water, than the previous drying technique. The modified Dean and Stark apparatus (Fig. 12.1) consists of a 500-ml flask connected to a distilling tube receiver, which is graduated to contain 5 ml and subdivided into 0.1-ml divisions, with a straight Liebig condenser attached to the receiver. The entire apparatus is cleaned with chromic acid cleaning solution just prior to use, and thoroughly dried in an oven after efficient rinsing.

Aromatic hydrocarbons, because of their mutual solubility with water, often prevent a clear separation. Paraffinic hydrocarbons do not suffer this disadvantage, and if heptane boiling near 100° is used, results closely approach those obtained by the oven method.

A sample of the fat or oil of sufficient size to yield 2 to 4 ml of water is weighed into the flask, about 200 ml of heptane is added, and an ebulliator or several glass beads are added to retard bumping during distillation. The apparatus is assembled and the side arm filled with heptane. Distillation is carried out by heating with an oil bath or heating mantle at such a rate as to cause about 2 drops per sec to come over. The rate is increased to 4 drops per sec as the water content decreases. Any water adhering to the condenser walls is brushed down using a heptane-saturated brush attached to a copper wire. Distillation is continued for an additional short period, and the whole allowed to cool. The volume of water in the side arm is observed, and the percentage of water in the sample calculated.

3. The Karl Fischer Titrimetric Method

The determination of the water content of many substances may be carried out by some modification of the Karl Fischer titration. This method depends upon the fact that a solution of sufur dioxide and iodine in pyridine and methanol reacts with water in such a manner that the reagent may be standardized against a known water content of a stable hydrate such as sodium

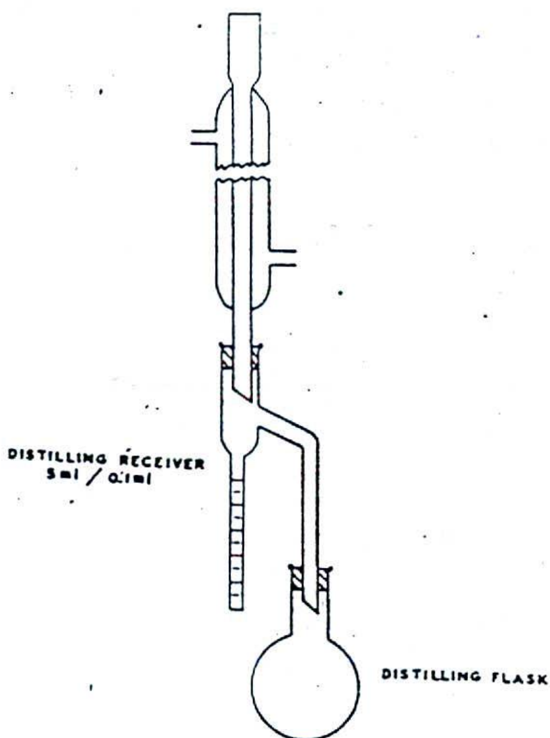


FIGURE 12.1: Moisture-determination apparatus.

tartrate dihydrate or sodium acetate trihydrate. The water equivalent for the reagent is thus established, but frequent standardization of the solution is necessary, owing to a certain degree of instability of the reagent. A full discussion of the reagent, standardization, and end-point detection has been discussed in Chapter 9.

A 5- to 10-g sample is weighed into a dry flask equipped for sealing out air, or for the passage of dry nitrogen. Because of the presence of moisture in the air, exclusion of moist air from the titration flask and burette is necessary. This is best accomplished by using drying tubes on the burette, and the passage of dry nitrogen over the reaction surface in the flask. Fifty milliliters of

chloroform are added and the sample dissolved by swirling or by the use of a magnetic stirring device. The resulting solution is titrated with the Karl Fischer reagent with vigorous stirring before each addition. When platinum electrodes and a suitable galvanometer are used, the endpoint is taken when the galvanometer current suddenly increases and remains up for at least 30 sec. Titrate a 50-ml blank of chloroform only, and calculate the percentage of water present.

$$\% \text{ water} = \frac{F(S - B)}{\text{wt. of sample} \times 10}$$

Here F is the water equivalent of the reagent in mg/ml, S is the sample titration in milliliters, and B is the blank titration in milliliters.

C. MELTING POINT

When heated, solid fats pass through a gradual softening before becoming liquid. The transformation is not definite nor sharp as in the case of a pure substance; so that at best the melting point is an arbitrary value depending upon the technique used in its determination.

1. Closed-Capillary-Tube Method

The sample is melted and filtered as described in the section discussing sampling, and is drawn up into several capillary tubes. The tubes are sealed by fusion at the end containing the fat, taking care not to burn the fat in the tube. The sealed capillaries are maintained at ice temperature for at least 12 to 16 hr. The tubes are attached to a thermometer by a small rubber band in such a manner that the fat is close to the bulb of the thermometer, and the thermometer and bulb are immersed in a beaker containing water. The water bath is agitated gently and heated at such a rate that the temperature increases at 0.5° per min. The melting point is that temperature at which the contents of the tube become clear. The mean of several determinations should be calculated as the final value.

2. Wiley's Method

The Wiley method[2] depends upon the observation that a flat disc of solidified fat suspended in an hydro-alcoholic solution will lose its flat shape and become spherical when it melts. The rate of heating at the melting point must be slow in order that the decision involved in determining when the fat globule has reached a constant shape does not introduce a significant error. One or more preliminary trials are best carried out; followed by two determinations agreeing closely with each other.

The fat discs are prepared by allowing the melted fat to drop 15 to 20 cm onto a smooth piece of ice floating in distilled water. Thin discs about 1 to 1.5 cm should be formed, and they may be released by forcing the ice below

the surface of the water and then picking up the free floating discs on a spatula blade. The discs should be well chilled in a refrigerator for several hours before use.

A wide test tube is filled 2 to 3 in. with hot, recently boiled distilled water, and an equal volume of hot, recently boiled alcohol is carefully layered over the water by slowly pouring into the test tube held at an angle. The tube is suspended in a 1000-ml beaker (Fig. 12.2) containing cold water until it cools to at least 10° below the melting point of the fat. Drop a disc of fat into the test tube, and it will sink to the level where the density of the hydro-alcoholic solution matches its own. Suspend an accurate thermometer, capable of reading to 0.1°, just above the disc. Slowly heat the water bath while stirring the bath and slowly rotating the thermometer suspended above the disc.

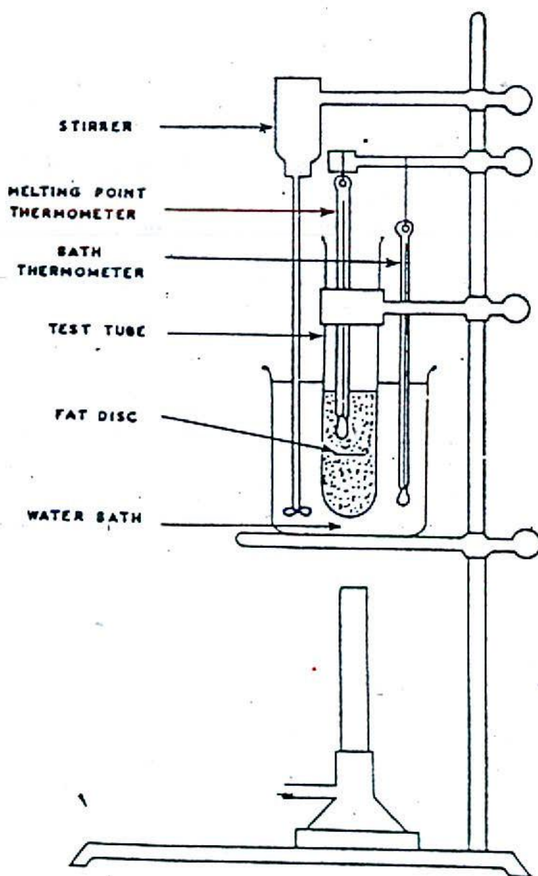


FIGURE 12.2: Modified Wiley melting-point apparatus.

Care must be taken that the disc does not touch the sides of the tube. If this occurs the trial must be repeated. At a point about 5 to 6° below the melting point of the fat the disc will begin to shrivel. At this point lower the thermometer until the bulb is level with the fat globule, and decrease the rate of heating so about 10 min is required for the last 2°. Continue to rotate the thermometer slowly around the fat globule; the point at which the globule becomes spherical is the Wiley melting point.

D. REFRACTIVE INDEX

When a light ray passes from air into a denser medium it is bent or refracted toward the normal. The refractive index of a substance is the ratio of the sine of the angle of incidence to the sine of the angle of refraction. The refractive index of a substance decreases with increasing wavelength of the illuminating light and with increasing temperature. Monochromatic light of the D line of sodium is ordinarily used, and the temperature is maintained constant by some type of circulating bath liquid passing through the instrument. The notation used for the designation of refractive index is n_D^t, with t being the temperature of the determination and D indicating the D light of sodium (5893 A).

The refractive index of oils is generally taken at 25° and solid fats at 40°, or higher if necessary, as is seen in Table 12.1.

The Butyro-refractometer and the Abbe refractometer are both used to determine the refractive indices of oils and fats. The Abbe refractometer has greater general utility, and its use will be discussed in this section. Butyro-reading to refractive index conversion tables may be seen in previously mentioned references.[2,3]

The Abbe refractometer is charged by opening the double prisms by means of the screw clamp, and placing a few drops of the oil or melted fat on the lower prism. The prisms are closed by tightening the screw clamp, which spreads the oil out into a thin layer between the prisms. After standing for a few minutes in order that the temperature of the sample may come to that of the prisms and bath, the border line is brought within the field of the ocular by moving the arm on the left attached to the prisms, and the mirror is adjusted for maximum transmission of light. The border line is adjusted until the cross hairs of the ocular are bisected, and the refractive index read from the scale by means of the attached magnifier. If the border line is not sharp it may be adjusted to this condition by turning the compensator knob on the barrel of the telescope.

E. SPECIFIC GRAVITY

The specific gravity of fixed oils or fats is determined by means of standard pycnometers or specific gravity bottles and reported at 25°.

A carefully cleaned and dried pycnometer is filled with recently boiled and cooled, distilled water at about 20°, and placed in a constant-temperature bath at 25°. After 30 min the water level is adjusted, the cap placed on the vessel, and the pyconmeter wiped dry and weighed. The pycnometer is emptied, rinsed several times with alcohol, and finally with ether. The rinsed pycnometer is then dried throughly and weighed.

The clean dry pycnometer is then filled with the oil sample which was previously cooled to 20°, placed in a constant-temperature bath held at 25°, and allowed to come to equilibrium over a period of 30 min. The oil level is adjusted, the cap placed upon the pycnometer. and the whole dried thoroughly. The pycnometer and oil are then weighed. The weight of oil contained in the pycnometer is determined by subtraction of the weight of the pycnometer empty from that when full of oil, and the weight of water is determined in the same manner. The specific gravity at 25°/25° is then determined.

$$\text{specific gravity } 25°/25° = \frac{\text{wt. of volume of oil}}{\text{wt. of equal volume of water}}$$

If the specific gravity is to be determined at an elevated temperature, as for solid fats, the procedure is the same except the temperatures are adjusted upward accordingly. The initial temperature of the fat is set at 56°, and that of the bath at 60°. The approximate specific gravity at 25° may be calculated from the formula $G = G' + 0.00064(T - 25°)$. Here G is specific gravity at 25°, G' is specific gravity at $T/25°$, T is temperature at which the specific gravity was determined, and 0.00064 is mean correction per degree.

F. TITER TEST

The titer test, as a measure of the solidification temperature of the mixed fatty acids, is much more characteristic than the melting point of a fat. However, the time and effort involved in performing the test are much greater than that for the melting-point determination. The titer test is routinely used for the grading of hard fats such as hydrogenated oils as well as those fats in soap production.

1. Apparatus

The apparatus is illustrated in Fig. 12.3, and consists of a 2-liter beaker, wide-mouth bottle (450 ml, 190 mm height, and 38 mm ID neck), test tube (25 × 100 mm), and a wire stirrer with a 19-mm loop. The bath thermometer is an accurate 0 to 150° thermometer, whereas the titer thermometer is a −2° to 68°, 45-mm immersion, special-design thermometer, designated AOAC Titer Test.

2. Determination

Heat 110 g of 5:1 glycerin/potassium hydroxide to 150° in an 800-ml beaker. To this solution add 50 ml of the previously filtered fat or oil, and continue heating with stirring at 150° for 15 min. Complete saponification is usually indicated by an homogenous appearance and the presence of soap bubbles rising to the surface. The 150° temperature should not be exceeded at any time. Allow the solution to cool somewhat, carefully add 50 ml of 30% sulfuric acid. and continue stirring and heating with addition of water if necessary until the layer of fatty acids has completely separated and is clear and liquid. Syphon off the acidic aqueous layer, add water, and heat for 2 to 3 min. Repeat this washing procedure two or three times until the water is neutral to methyl orange. Collect and filter the fatty acids while melted through rapid paper to exclude any water. Heat the melted, filtered, fatty acids on a hot plate at 130° for 1 to 2 min to remove any traces of moisture, and pour them into the titer test tube to a height of 57 mm. from the bottom.

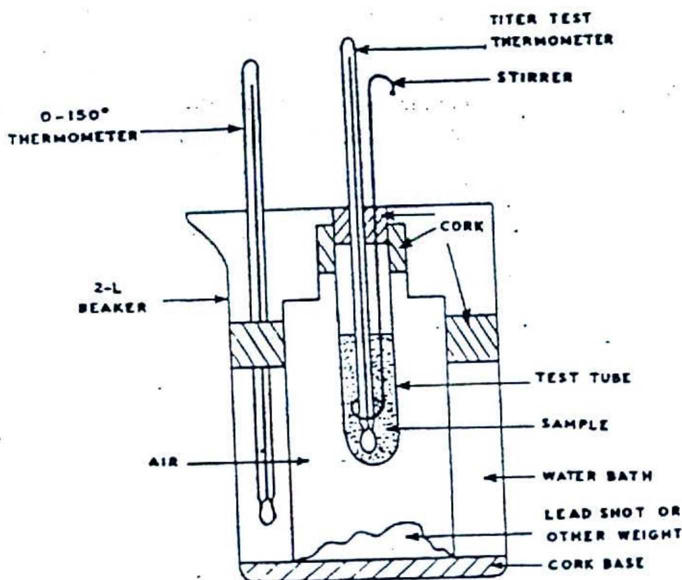


FIGURE 12.3: Titer-test apparatus.

The test tube is placed in the bottle, which provides a water-cooled air bath in which the fatty acids are solidified. Lead shot or other weight adds stability to the apparatus. The water bath is filled to 1 cm above the level of the sample, and the bath is held at 15 to 20° below the titer point at all times by means of ice in the water. The titer thermometer is immersed to the

mark in the sample, and stirring is initiated at the rate of 100 strokes per min. This rate of stirring is maintained until the temperature remains constant for 30 sec or begins to rise in less than 30 sec. Cease stirring at the constant-temperature point, remove the stirrer, and observe the rise in temperature. Report the highest point reached by the thermometer as the titer point. Normally, duplicate determinations should agree within 0.2°.

G. ACID VALUE

The acid value of a fixed fat or oil is defined as the milligrams of potassium hydroxide required to neutralize the free fatty acids in 1 g of the sample. By the use of appropriate factors in the calculations, this value may be converted to give the percentage of a free fatty acid such as oleic, palmitic, or lauric.

The presence of free fatty acids is normally due to hydrolysis of the glycerides as shown in Eq. (12.1). Bacterial action, chemicals, light, and heat accelerate this reaction. The hydrolysis reaction, accompanied by the production of shorter-chained fatty acids through oxidation of double bonds, can produce a high acid value as in the case of rancidity in the fat or oil.

A 10-g sample is dissolved in 50 ml of a previously neutralized mixture of equal quantities of alcohol and ether, and the solution warmed slightly. The warm solution is then titrated with 0.1 *N* potassium hydroxide solution using phenolphthalein indicator. The titration is continued until a pink color persists for at least 15 to 30 sec. Continuous, vigorous agitation should be maintained during the titration.

$$\text{acid value} = \frac{\text{titration} \times N \times 0.05611 \times 1000}{\text{wt. of sample}}$$

The conversion factors for expression of the results as percentage of three common fatty acids are that each milliliter of 0.1 *N* alkali is equivalent to: (a) 0.0200 g lauric acid; (b) 0.0256 g palmitic acid; or (c) 0.0282 g oleic acid.

H. SAPONIFICATION VALUE

The saponification value is the number of milligrams of potassium hydroxide needed to neutralize the free fatty acids, and to saponify the esters in 1 g of the fixed oil, fat, or wax. This value includes the free fatty acids, and thus is a measure of both free and combined fatty acids. When the acid value is subtracted from the saponification value, the result is the so-called ester value. The saponification value is inversely proportional to the mean molecular weights of the fatty acids of the oil; thus rancidity, which leads to the formation of low molecular weight acids, would be indicated by an abnormally high value, and adulteration with mineral oil by a low value. The saponification value for most edible oils and fats ranges from 180 to 200, with most waxes falling around 100.

1. Reagent

Alcoholic potassium hydroxide solution is prepared by refluxing 10 g of potassium hydroxide with about 6 g of granulated or foil aluminum in 1200 ml of alcohol for 30 min. At the completion of the reflux period, the equipment is arranged for distillation, and 1000 ml of alcohol is collected after discarding the first 50 to 75 ml of distillate. The freshly distilled alcohol is cooled to below 15°, and 40 g of potassium hydroxide are dissolved while maintaining the temperature at or below 15°. The solution is allowed to stand with occasional shaking until the potassium hydroxide has dissolved, and after standing overnight, it is filtered into a clean, dry, glass-stoppered bottle for storage.

2. Determination

Weigh accurately a 4- to 5-g sample of the filtered material into a 250- to 300-ml Erlenmeyer flask. This size sample should result in about a 50% excess of the reagent during the determination. Pipette 50 ml of the alcoholic alkali into the flask, connect the flask to an air condenser, and reflux until the oil or fat is completely saponified. Saponification usually requires about 1 hr, but wool fat and waxes such as beeswax require longer periods of reflux—up to 2 hr. Carry out a blank determination at the same time with omission of the oil sample. During the reflux period care must be taken that the refluxing alcohol vapor does not rise out of the condenser.

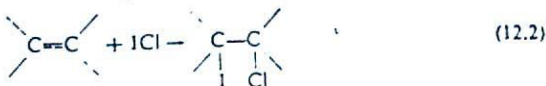
When saponification is complete, wash down the condensers with a little water, allow the solutions to cool, and disconnect the condensers. Add about 1 ml of phenolphthalein test solution to each flask and titrate each with 0.5 N hydrochloric acid to the first disappearance of the pink color.

$$\text{saponification value} = \frac{(B - S) \times N \times 0.05611 \times 1000}{\text{wt. of sample}}$$

Here B is the milliliters required for blank titration, S is the milliliters required for sample titration, and N is the normality of acid.

I. IODINE VALUE

The iodine value is a measure of the degree of unsaturation of the fatty acids composing the glycerides of the fixed oil or fat and is expressed as the grams of iodine absorbed by 100 g of the oil or fat. In reality, depending upon the method used, iodine monochloride or monobromide is the agent absorbed as seen in Eq. (12.2). However, in either method the results are still reported



$$\text{>C=C<} + \text{ICl} \rightarrow \text{>C(I)—C(Cl)<} \qquad (12.2)$$

in terms of iodine absorbed. The iodine value is reliable in the case of isolated double bonds, but, where conjugated bonds are present, the absorption is not complete. Efforts to improve this determination have resulted in the suggestion of other solutions such as the chloroform-dioxane-bromine solution of Said et al.[8]

The two most widespread methods are those of Wijs and of Hanus, utilizing iodine monochloride and iodine monobromide, respectively. Iodine monochloride and monobromide are available commercially, or they may be formed in situ as the reagent is prepared. There is some difference in the results produced by the use of the Wijs or the Hanus method, but if the iodine value is below 100, the difference is no greater than the variation of the iodine value of the oils and fats themselves.

The iodine values fall into three general categories. Solid fats are generally seen to have values of less than 100, so-called nondrying oils have values ranging from 100 to 140, and semidrying and drying oils have values of 150 or greater (Table 12.1).

1. Reagent (Hanus Method)

Dissolve 13.61 g of iodine in 825 ml of glacial acetic acid with the aid of the heat of a steam bath. (The glacial acetic acid should show no green color when warmed on a water bath with potassium dichromate and sulfuric acid). Pipette 25 ml of this iodine solution into a flask, add 20 ml of 15% potassium iodide solution, 100 ml of distilled water, and titrate with 0.1 N sodium thiosulfate solution to a starch-indicator end point (determination of Section 12.I, 2).

To a 200-ml portion of glacial acetic acid add 3 ml of bromine and mix well. Pipette 5 ml of this solution into 10 ml of 15% potassium iodide in water as before, and titrate with 0.1 N sodium thiosulfate solution to a starch end point. Calculate the volume of bromine solution to be added to the remaining 800 ml of iodine solution as

$$A = \frac{I \times 800}{B}$$

Here A is the milliliters of bromine solution to be added, I is the thiosulfate equivalent of 1 ml of iodine solution, and B is the thiosulfate equivalent of 1 ml of bromine solution.

2. Determination

It is important that a 100 to 150% excess of iodine be present in the reaction. For this reason, a sample size must be selected which allows this condition to be realized when using a fixed volume of reagent in the reaction. In general, the procedure of dividing 20 by the expected iodine value will give a sample size within range of meeting the requirement of an excess of reagent.

Weigh an appropriate size sample into a 500-ml iodine flask, and dissolve in 10 ml of chloroform. Pipette 25 ml of the Hanus iodine solution into the flask, stopper it, swirl it, and allow it to stand for 30 min in a dark place with occasional swirling. At the end of the time period, add in order 10 ml of 15% potassium iodide solution and 100 ml of cooled, freshly boiled, distilled water, washing down any adhering iodine on the stopper or walls of the flask. Titrate the resulting solution with 0.1 N sodium thiosulfate solution until a pale-yellow color is produced in the solution as the iodine color fades. Add several drops (about 1 ml) of starch test solution, and titrate to the discharge of the blue color. Near the end of the titration vigorous agitation with the stopper in the flask should be used to get the last traces of iodine from the chloroform layer. Conduct a blank determination using the same volumes of the reagents for the same time, but omitting the sample:

$$\text{iodine value} = \frac{(B - S) \times N \times 0.1269 \times 100}{\text{wt. of sample}}$$

Here B is the blank titration, S is the sample titration, and N is the normality of sodium thiosulfate solution.

In the analysis of fixed oils for their iodine value, where samples of 0.2 to 0.3 g are routinely used, it may be found expedient to weigh out a number of samples using glass weighing cups* on a microscope slide as seen in Fig. 12.4.

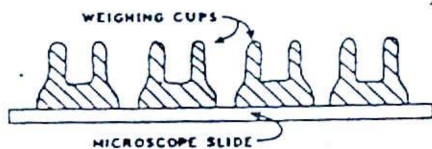


FIGURE 12.4: Iodine-value sample-weighing cups.

In this procedure several cups may be placed on a microscope slide and all tare weighed on a balance. The individual samples may be added using a dropper, and following each sample addition the whole is weighed again. In this manner several samples are obtained with a minimum number of weighings. After all have been weighed and recorded, the cups may be picked up individually and slid through the neck of the iodine flask as an efficient transfer of the sample for solution in the chloroform.

J. REICHERT–MEISSEL AND POLENSKE VALUES

The Reichert–Meissel and Polenske values are a measure of the volatile fatty acids occurring in fixed oils and fats—principally in milk fat, coconut,

* The cups are prepared from 6- or 8-mm tubing by closing one end in a flame; the base is formed by pressing onto a flat surface while still molten. After cooling they are cut from the tubing at a height of about ½ in., and the edges are fire polished.

and palm kernel oils. By definition the *Reichert–Meissel value* is the number of milliliters of 0.1 *N* alkali needed to neutralize the volatile, water soluble fatty acids of 5 g of the oil or fat. The *Polenske value* is the number of milliliters of 0.1 *N* alkali needed to neutralize the volatile, water insoluble fatty acids from 5 g of the oil or fat.

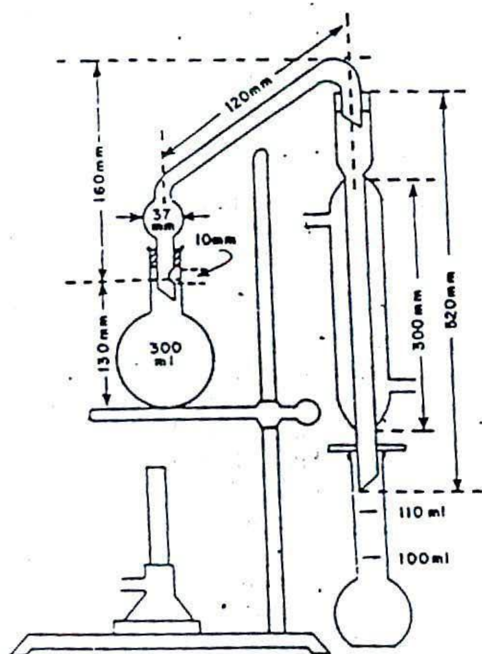


FIGURE 12.5: Reichert–Meissel or Polenske apparatus.

For reproducible and meaningful results very close adherence to the standard procedures must be observed.

1. Reichert–Meissel Value

A 5-g sample of the clarified oil is weighed into the distillation flask (Fig. 12.5). Mix 20 ml of a 1:1 solution of sodium hydroxide with 180 ml of glycerin, and add 20 ml of the glycerin-sodium hydroxide solution to the sample. Heat until saponification is complete, as shown by a clear solution. Also, an indication of complete saponification is that the walls of the flask should be uniformly wet by the solution. The flask is allowed to cool to about 100°, 135 ml of recently boiled distilled water is added in increasing portions, and this is followed by the addition of 6 ml of 20% (w/v) sulfuric acid. A few pieces of pumice are added, and the solution distilled. The distillation rate

should be such that 110 ml of distillate is collected in 30 (±2) min. The temperature of the distillate must not exceed 20°, and when 110 ml have been collected, the receiving flask is replaced by a 25-ml graduated cylinder, the flame is removed, and the distilling head disconnected.

The contents of the receiving flask are mixed well, and the flask is immersed for 15 min in a constant-temperature bath held at 15°. The distillate is filtered through a rapid paper (do not discard the paper if the Polenske value is to be determined), and 100 ml of the filtrate are titrated with 0.1 *N* sodium hydroxide solution to a phenolphthalein end point that persists for 2 min. Conduct a blank determination using the same reagents as before, but omitting the sample.

$$\text{Reichert–Meissel value} = 1.1(B - S)$$

Here B is the blank titration and S is the sample titration.

2. Polenske Value

The residue remaining on the filter paper after the filtration in the Reichert–Meissel value determination is the water-insoluble fatty acids of the sample. This residue is washed with three successive 15-ml portions of distilled water, each having been passed down through the condenser into the 25-ml graduated cylinder beneath and through the 110-ml receiving flask. The water washings are discarded. The insoluble fatty acids are then dissolved by washing with three 15-ml portions of previously neutralized alcohol using the same sequence as with the three water washes. The combined alcohol washes are then titrated with 0.1 *N* sodium hydroxide using phenolphthalein as the indicator. Conduct a blank determination identical in all respects to the analysis except the sample has been omitted.

$$\text{Polenske value} = S - B$$

Here S is the sample titration and B the blank titration.

K. HYDROXYL AND ACETYL VALUES

The hydroxyl and acetyl values are a measure of the hydroxylated fatty acids in a fixed oil or fat, and of the free alcohols present in a wax. Castor oil is the only oil of commercial interest having a significant acetyl value, 150, and carnauba wax, 54–56, is representative of waxes with higher values. The acetyl values of most fixed oils and fats range up to 20. Older oils, having some mono- or diglycerides present, have hydroxyl groups which will react, and for this reason they tend to have high values.

The hydroxyl value and acetyl value are determined through acetylation techniques, but are expressed in different manners. The distinction between the two values is seen in their definitions, which also shows the hydroxyl value to be the higher of the two.

The *hydroxyl value* is the number of milligrams of potassium hydroxide equivalent to the hydroxyl content of 1 g of the sample.

The *acetyl value* is the number of milligrams of potassium hydroxide required to neutralize the acetic acid resulting from the saponification of 1 g of the acetylated oil or fat.

In the past, the acetyl value was determined by acetylation of the fat using acetic anhydride, and the hydroxyl value was then calculated from the saponification values of the acetylated and unacetylated portions of the sample. More recent refinements have resulted in a simple method using acetic anhydride and pyridine for the determination of the hydroxyl value.[1] In this method the oil is acetylated, the excess anhydride is hydrolyzed, and the acetic acid is titrated.

A sample of oil is taken, whose weight is governed by the expected hydroxyl value, ranging from 10 g for 0 to 20 values, 5 g for 20 to 50 values, 3 g for 50 to 100 values, and 2 g for 100 to 200 values. The sample is weighed into a 250-ml standard taper flask, and 5 ml of pyridine/acetic anhydride solution (3:1 v/v) are added by means of a burette. For 10-g samples an additional 5 ml of pyridine may be added. Connect the flask to a reflux condenser after gentle swirling to mix the contents.

The mixture is heated for 1 hr on a steam bath, 10 ml. of water are added through the condenser, and the heating continued for an additional 10 min.

After cooling, the condenser and sides of the flask are washed down with several portions of *n*-butyl alcohol totaling 50 ml, and the solution is titrated with 0.5 *N* alcoholic potassium hydroxide solution (Section 12.2, H, 1), using phenolphthalein as the indicator.

Perform two control determinations; omitting only the oil from one, and only the acetic anhydride from the other, but they should be identical in all other respects.

$$\text{hydroxyl value} = \frac{(B + A - S) \times N \times 0.0561 \times 1000}{\text{wt. of sample}}$$

Here B is the milliliters of standard alkali for blank without oil, A the milliliters of standard alkali for blank without anhydride, S the milliliters of standard alkali for sample, and N the normality of standard alcoholic potassium hydroxide solution.

The acetyl value (A) may be determined from the hydroxyl value (H) using

$$A = \frac{H}{1 + (0.00075H)}$$

L. UNSAPONIFIABLE MATTER

As fats and oils are encountered in commerce, they do not consist entirely of glycerides, but also contain some small percentage of substances unaffected by the saponification reaction. *Unsaponifiable matter* is defined as those

substances which are not saponified by alkali, and which are soluble in petroleum ether or ether. In general, if a fixed oil or fat has unsaponifiable matter present in excess of about 2%, there is reason to suspect adulteration. Sterols are the most common naturally occurring substances making up the unsaponifiable fraction of an oil or fat. Other substances such as vitamins A and D, unsaturated hydrocarbons such as squalene, pigments, and high molecular weight monohydroxy alcohols in the case of waxes, are seen in the unsaponifiable fraction.

At present there are two general methods for determination of the unsaponifiable matter in animal and vegetable fixed oils and fats. These are the extraction of the unsaponifiable matter with petroleum ether, or extraction with ethyl ether. Both methods give comparable results in the analysis of ordinary oils and fats containing a normally small unsaponifiable fraction. When a high unsaponifiable content as seen in fish oils is present, the petroleum ether method is less satisfactory than the ethyl ether method. It should be noted however, that ethyl ether is generally more prone to the formation of emulsions during the extraction procedures. An excellent discussion of manipulative techniques in dealing with lipid extraction, separatory funnels, stopcocks, and emulsion breaking is given by Sperry.[9]

There is a slight tendency for soaps to hydrolyze in solution, and the fatty acids thus formed are withdrawn from the aqueous layer into the ether layer during extraction. This removal of fatty acids from the aqueous layer tends to shift the equilibrium of the hydrolysis reaction in favor of hydrolysis, and increasing amounts of free fatty acids are extracted. Although washing the ethereal extracts with several portions of alkali will remove most of these acids, a small percentage remains, and the presence of these residual fatty acids is corrected by means of a final titration with standard alkali.

1. Petroleum Ether Method

A 5-g sample of the clarified oil is weighed into a 200-ml Erlenmeyer flask; 30 ml of 95% alcohol and 5 ml of 50% aqueous potassium hydroxide are added. The mixture is heated under reflux for 1 hr, or until the sample is saponified.

The soap solution is transferred to an extraction cylinder having graduations at 40, 80, 130 ml, with a total capacity of at least 150 ml, and of about 35 mm diameter and 300 mm height. The flask is washed with 95% alcohol, and the washings added to the cylinder until the 40-ml mark is reached. Complete the transfer with warm and then cold water until the 80-ml mark is reached. Finally, rinse the flask with 50 ml of petroleum ether, and add this to the contents of the cylinder after cooling the cylinder and contents to 20 to 25°.

The cylinder is stoppered, shaken vigorously for at least 1 min, and allowed to stand until the layers have separated cleanly. The upper layer is removed

by siphoning with a thin tube into a 500-ml separatory funnel. The petroleum ether extraction is repeated at least six times.

The combined extracts are washed three times in the separatory funnel with 25-ml portions of 10% alcohol-water. Care should be taken that none of the petroleum ether layer is removed when the hydro-alcoholic layers are drained off.

The washed petroleum ether extract is then transferred to a tared beaker and evaporated under a stream of clean dry air. Final drying to constant weight is carried out in an oven at temperatures not exceeding 100°, and the weight of the residue is determined after cooling in a desiccator.

After the weight of the residue has been determined, the residue is dissolved in 50 ml of warm, previously neutralized 95% alcohol, and the solution titrated with 0.1 *N* alkali to a phenolphthalein end point. The weight of residue is corrected for the fatty acids present using the relationship (1 ml of 0.1 *N* alkali = 0.0282 g of oleic acid)

$$\% \text{ unsaponifiable matter} = \frac{(\text{wt. residue} - \text{wt. fatty acids}) \times 100}{\text{wt. of sample}}$$

2. Ethyl Ether Method

A sample of the oil or fat ranging from 2.0 to 2.5 g is weighed into a 250-ml Erlenmeyer flask, and saponified by refluxing for 30 min with 25 ml of 95% alcohol and 1.5 ml of potassium hydroxide solution (50% w/w).

While warm, the soap solution is transferred to an extraction cylinder of at least 150-ml capacity by washing first with a total of 50 ml of water followed by 50 ml of ethyl ether. The cylinder and contents are then cooled to 20 to 25°.

The contents of the cylinder are shaken vigorously, allowing any pressure build-up to be released through occasional release of the stopper, and the cylinder is allowed to stand in order that the layers might separate cleanly. The upper layer is removed by siphoning through a thin glass tube into a 250-ml separatory funnel containing 20 ml of water. The extraction is repeated two more times using 50-ml portions of ether, and the washings combined in the separatory funnel. The funnel is rotated gently to wash the extract with the 20 ml of water present in the funnel; the water is allowed to separate completely, and is then drawn off. The process of washing with 20 ml of water is repeated two more times, using increasingly vigorous agitation.

The ether extract is washed three times with 20-ml portions of approximately 0.5 *N* potassium hydroxide solution. If emulsions form at this stage, allow the layers to separate as clearly as possible, and drain off the water layer leaving any emulsion still unbroken in the funnel. After the third alkaline wash, the ether extract is washed with successive 20-ml portions of water until the wash water no longer reacts alkaline to phenolphthalein solution.

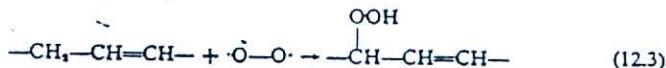
The ether extract is transferred to a 250-ml beaker and the funnel rinsed with ether. The ether is evaporated on a steam bath to a volume of 5 to 10 ml, and the residue transferred to a tared 50-ml Erlenmeyer flask with the aid of several ether washes. Remove the ether on a steam bath until almost all has evaporated; then to the last small amount of ether add 2 to 3 ml of acetone, and evaporate to dryness on the steam bath under a stream of clean dry air. Dry the residue to constant weight in an oven at a temperature not exceeding 100°.

After the residue weight has been determined, the contents of the flask are dissolved in 2 ml of ether and 10 ml of neutral 95% alcohol, and titrated with 0.1 N alkali to a phenolphthalein end point. Correct the residue weight using the relationship 1 ml of 0.1 N alkali = 0.0282 g of oleic acid. Correct the weight of the residue for a blank conducted in the same manner, but without the sample.

$$\%\text{ unsaponifiable matter} = \frac{(\text{wt. of residue} - \text{wt. of acid} - \text{wt. of blank}) \times 100}{\text{wt. of sample}}$$

M. PEROXIDE VALUE

Fixed oils and fats absorb oxygen from the air in the autoxidation of the double bonds present in the component fatty acids. The reaction is thought to be as shown in Eq. (12.3), where the oxygen adds to the double bond form-



$$-CH_2-CH=CH- + \cdot\dot{O}-O\cdot \rightarrow -\overset{OOH}{\overset{|}{\dot{C}}}H-CH=CH- \qquad (12.3)$$

ing unstable hydroperoxides. The reaction proceeds more rapidly in the presence of conjugated double bonds because of the greater resonance stability of the intermediate radicals. The hydroperoxides thus formed decompose, or are further oxidized to a variety of products such as aldehydes, ketones, and ketoacids of lower molecular weight. These lower molecular weight products contribute to the odor produced during the rancidity process.

The *peroxide value* is the determination of the amount of iodine liberated from potassium iodide by the peroxides of the oil or fat in terms of milliequivalents per kilogram.

A 5-g sample of the oil or fat is weighed into a 250-ml Erlenmeyer flask, and 30 ml of acetic acid/chloroform (3:2) is added. The contents of the flask are swirled to dissolve the sample, and 0.5 ml of saturated potassium iodide solution is added by means of a measuring pipette. The solution is allowed to stand 1 min with occasional shaking, and 30 ml of distilled water is added. The resulting solution is titrated with 0.1 N sodium thiosulfate solution to a starch indicator end point (Section 12.I, 2). If the titration is less than 0.5 ml, repeat using 0.01 N sodium thiosulfate.

$$\text{peroxide value} = \frac{\text{titration} \times N \times 1000}{\text{wt. of sample}}$$

A modification of this method, utilizing benzene and water, and applicable to oil or fat containing pharmaceutical preparations, has been reported by Kovacs.[10]

With respect to the use of the peroxide value as a means of indicating the development of rancidity, it should be pointed out that oils with a high iodine number have a high peroxide value at the beginning of rancidity. Also, oils and fats with a low iodine number will have a low peroxide value at the start of rancidity, but those oils and fats with a high antioxidant content will always have a high peroxide value at the beginning of rancidity.

Other tests of a qualitative nature may be used as an indication of the degree of rancidity of an oil or fat, and one of these is the Kreis test. In the Kreis test a sample of the oil or fat is treated with an ethereal solution of phloroglucinol in the presence of hydrochloric acid, and a pink color, due to the reaction of β-hydroxyacrolein with phloroglucinol, is an indication of rancidity. The intensity of color is roughly an indication of the degree of rancidity. It should be pointed out that certain crude vegetable oils, notably cottonseed, give a positive Kreis test even though they are free of rancidity. Also, care should be taken in the interpretation of the results because the test will indicate rancidity well before it may be detected by the senses.

12.3 IDENTIFICATION

The identification of fixed oils or fats is of interest in pharmaceutical analysis, and is generally based upon certain specific tests, in addition to the physical and chemical constants discussed previously. It is possible, in certain instances, to identify an oil or fat through the determination of its physical and chemical constants and comparison with reported values. However, the constants overlap in so many cases, and can be adjusted through adulteration, so that this method alone is not entirely satisfactory.

Various identification tests of a qualitative nature have been developed for the detection of oils and fats. In most instances the tests should be performed on the unprocessed materials, because they frequently rely upon the presence of minor constituents which are often altered during the processing procedures. Processing and admixture often produce products that are difficult to identify as to the constituent oils, and a combination of the use of physical and chemical constants along with the identification test will be found to be most helpful.

The procedures to be discussed here will be limited to a few of the tests and procedures of pharmaceutical interest from the standpoint of purity or the detection of adulterants.

A. COTTONSEED OIL (HALPHEN TEST)

This test is carried out by mixing in a test tube 3 ml of the oil and 3 ml of the reagent, which is prepared from equal volumes of carbon disulfide and

amyl alcohol, and contains 1% sulfur. The tube is heated in a bath maintained at 120° for 1 to 2 hr, and a red color at the end of this time indicates the presence of cottonseed oil. The intensity of color is roughly proportional to the amount of cottonseed oil present. However, strong heating, or hydrogenation of the cottonseed oil, will prevent the formation of the color. Also, kapok oil gives an equal or stronger reaction to this test.

B. PEANUT OIL (BELLIER TEST)

This test is limited to the detection of peanut oil in cottonseed, olive, corn, and soybean oils.

One gram of oil is weighed into an Erlenmeyer flask, and saponified under reflux by 5 ml of 1.5 *N* alcoholic potassium hydroxide solution. After 5 min of reflux, 50 ml of 70% alcohol and 0.8 ml of hydrochloric acid (sp. gr., 1.16) are added, and the solution heated to dissolve any precipitate that may be formed. Stir with a thermometer, cooling the solution at such a rate that the temperature falls 1° per minute; the presence of peanut oil in olive oil is indicated if turbidity appears above 9°C.

C. SESAME OIL (VILLAVECCHIA TEST)

A sample of 5 ml of the oil is placed in a test tube along with 5 ml of hydrochloric acid. A few drops (2 to 3) of a reagent prepared from 2% furfural in alcohol are added, and the mixture shaken vigorously for 30 sec. The test is allowed to stand until the emulsion breaks; a deep-red color in the acid layer is an indication of the presence of sesame oil in the sample.

It should be pointed out that with this test as well as the Halphen test, if animals are fed upon the oil cake, there may be a positive test in the animal fat, for example, butter fat of the cow or lard of the hog.

D. TEASEED OIL

The chemical compositions of olive and teaseed oil are very similar with respect to the glycerides. The major difference in the oils is in the sterol content of the unsaponifiable matter.

The reagent is prepared in a test tube by adding 0.8 ml of acetic anhydride, 1.5 ml of chloroform, and 0.2 ml of sulfuric acid (sp. gr., 1.84). Mix and cool the contents of the tube to 5°, then add 7 drops of the sample (equivalent to about 0.22 g), mix again, and maintain the temperature at 5° for 5 min. If turbidity appears, add acetic anhydride and shake until the solution is clear. Add 10 ml of anhydrous ethyl ether and stopper the test tube. Invert the tube several times and return the tube to the bath. Observe the color, and, if teaseed oil is present, an intense red color will develop within 1 min, fading away upon longer standing (5 min). Mixtures of teaseed and olive oils will

show colors in proportion to their teaseed content. If corn oil is present during this test, a blue-violet color develops 5 to 10 sec after the initial mixing of acetic anhydride, chloroform, sulfuric acid, and the oil. If soya oil is present, a light green color develops after 2 min.

E. MARINE OIL (FISH OIL)

Unhydrogenated fish oils as adulterants may be detected by means of the insolubility of the bromides of the unsaturated fatty acids characteristically present in these oils.

Prepare a test tube containing 12 ml of equal volumes of chloroform and glacial acetic acid. To this tube add 5 g of the oil. Maintain the solution in a bath at 20°, and add bromine dropwise until a slight excess is present. Allow the tube to stand in the bath for 15 min more, and then place the tube into a boiling water bath. If only vegetable oils are present, the solution remains perfectly clear. If fish oils are present, the solution will be cloudy, or a precipitate will appear, due to the insoluble bromides.

12.4 INDIVIDUAL CONSTITUENTS AND STRUCTURE DETERMINATION

The identification, quantitative determination, and structure of the constituent parts of fixed oils and fats represent an area of analytical chemistry of wide diversity. The techniques employed may range from the simple titration of a fatty acid using standard alkali to the NMR determination of unsaturation. It is in this area of lipid analysis that the more recently developed analytical techniques have been applied with significant results. This section is to be devoted to a short discussion of the application of such techniques of separation and determination as infrared and ultraviolet spectrophotometry, NMR spectroscopy, column chromatography, and gas-liquid chromatography to the analytical problems of oil and fat chemistry.

In the past, the approach to the problem of the separation and identification of the constituent fatty acids of a fat or oil was to effect a partial separation as to the general class of acids, and then to distill fractionally the methyl esters of the acid mixtures to obtain the acids present and their percentage composition. For example, the general class separation might be into volatile and nonvolatile acids by means of steam distillation, or by precipitation of the lead salts.

A. LEAD SALT SEPARATION

Saturated fatty acids are separated from unsaturated fatty acids by means of conversion to their lead salts and precipitation from alcohol as suggested by Twitchell.[11] After filtration, the lead salts of the solid fatty acids are

acidified and separated from the aqueous solution of lead chloride by means of ether. The mixed saturated fatty acids are obtained by evaporation of the ether and drying of the residue. The lead salts of the unsaturated fatty acids are obtained by evaporation of the filtrate from the original precipitation. Acidification and extraction of the residue with ether, followed by evaporation of the ether and drying, yields the unsaturated fatty acids.

B. ESTER DISTILLATION

The fatty acid mixtures obtained as described previously are converted into their methyl esters by boiling with four times their weight of methyl alcohol in the presence of 1 % sulfuric acid (sp. gr., 1.84). The conversion to esters is usually of the order of magnitude of 97 to 98 %. A discussion of the esterification procedures has been given by James[12] in connection with gas-chromatographic separation of fatty acids. Recently, Mason and Waller[13] have reported on the use of dimethoxypropane for the transesterification of the glycerides of fats and oils. After conversion into their methyl esters, the fractionation is carried out under vacuum on an efficient fractionating column.

Details concerning the experimental techniques, columns, conditions, as well as tabulation and interpretation of typical ester fractionation data, are given by Hilditch.[14]

C. COLUMN CHROMATOGRAPHY

Selective adsorption onto materials such as silicic acid in the technique of column chromatography has been used extensively as a means of separating mixtures of fatty acids. The different affinities of the fatty acids for a solid adsorbent supported in column form is utilized to effect a separation as a mixture of fatty acids is washed or eluted down the column by a suitable developing solvent. As the elution of the column is carried out, the individual acids separate according to their affinity for the adsorbent, and fractions are collected at the base of the column. The fatty acid content of each fraction may be determined by appropriate means such as titration. A convenient form of the apparatus for this procedure is shown in Fig. 12.6.

The mole per cent of butyric acid in fat is determined chromatographically.[1,2] In this procedure the fat under consideration is saponified. and the resulting soap hydrolyzed by means of acid. The freed fatty acids are then extracted from the hydrolysis mixture with hexane-butanol, and the resulting solution is chromatographed on a column of silicic acid using hexane-butanol to develop the separation. If the sample contained butter fat, the butyric acid is usually seen as a yellow band moving down the column. The longer-chain fatty acids precede this band, and do not form colored bands. Just before the yellow band reaches the bottom of the column, the receiver is exchanged, and the band collected for titration. The elution is continued until all the band has passed into the receiver, and the acid content of this

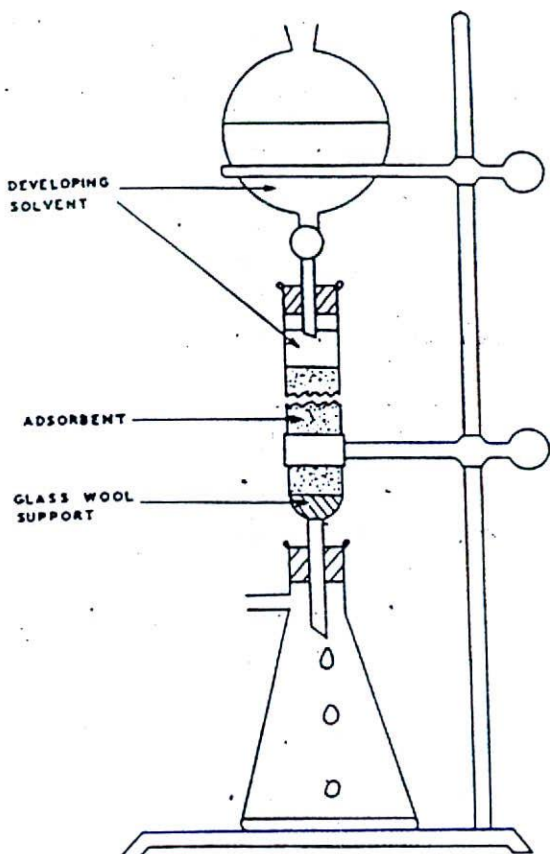


FIGURE 12.6: Apparatus for column chromatography.

fraction is then determined, using a standard solution of potassium hydroxide in isopropanol with thymol blue as the indicator.

D. GAS-LIQUID CHROMATOGRAPHY (GLC)

Separations by gas-liquid chromatography are based upon the partitioning effect of a liquid phase held in place on an inert support in a column while a gas phase flows over the liquid phase. The components of the mixture under consideration are resolved on the column and eluted by the inert gas. The operation is carried out in an isothermal chamber, or programmed heating of the column may be used. An excellent discussion of the theory of gas-liquid chromatography is given in a monograph by Purnell.[16]

The technique of GLC is well-adapted to analytical problems encountered in lipid analysis. Procedures for the separation of monofunctional aliphatic acids up to about C_{21} are well-established, and the separation of mixtures of fatty acids up to C_{18} may be carried out directly without the preparation of derivatives if an acid is incorporated into the liquid phase. Most procedures

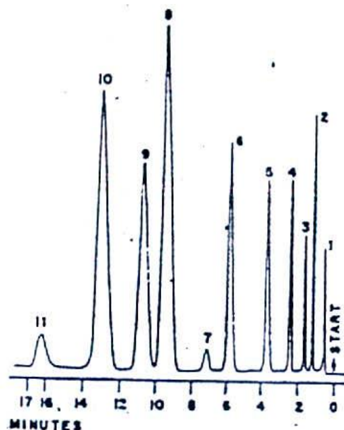


FIGURE 12.7: Gas-chromatographic separation of fatty acids as methyl esters. (Courtesy of Perkin–Elmer Corp.) Peaks: 1, air; 2, methyl caprylate; 3, caprate; 4, laurate, 5, myristate, 6, palmitate, 7, palmitoleate, 8, stearate, 9, oleate, 10, linoleate, 11, linolenate.

utilize the methyl esters, or butyl esters of the short-chain acids, for the separation.

Reviews of the application of GLC to the separation of fatty acids are given by James[12] and Horning and VandenHeuvel.[16] A typical chromatogram of the separation of a series of methyl esters of fatty acids may be seen in Fig. 12.7. Mason et al.[17] have recently reported a procedure for the simultaneous quantitative determination of glycerol and the fatty acid content of oils and fats.

E. INFRARED AND ULTRAVIOLET SPECTROPHOTOMETRY

When molecules are exposed to radiation ranging from the ultraviolet through the visible to the infrared region, transitions occur within the molecular energy levels depending upon the wavelength of the incident radiation. Energy changes resulting from electronic, vibrational, rotational, and translational motions may be observed. A large amount of energy is needed to bring about electronic shifts, and lesser energy is needed to change vibrational and rotational levels. Ultraviolet radiation thus is seen to facilitate transitions between vibrational and rotational energy levels of different electronic levels, whereas infrared radiation promotes transitions in a molecule between

rotational and vibrational energy levels of the ground, or lowest, electronic energy state.

Instruments capable of measuring the differences of ultraviolet or infrared radiation, before and after passing through a sample, are called spectrophotometers. The extent of absorption, may be calculated using Beer's law, expressed as

$$A = \log \frac{I_0}{I} = abc,$$

where I_0 is the intensity of incident light, I the intensity of transmitted light after passing through b cm of solution of concentration c in grams per liter. A is known as absorbance, and a is a proportionality constant known as absorptivity.

The application of ultraviolet spectrophotometry to the analysis of fixed oils and fats is seen in the determination of polyunsaturated fatty acids.[1,2] In this procedure the conjugated acids are determined by measurement of ultraviolet absorption at specific wavelengths of 233 mμ for dienoic, 262, 268, 274 mμ for trienoic, tetrenoic at 308, 315, 322 mμ, and pentaenoic at 346 mμ. Absorptivity for the various regions are then determined. Nonconjugated polyunsaturated constituents are partially conjugated by heating with potassium hydroxide-glycol solution, and the absorptions redetermined. The percentage of conjugated diene, triene, tetraene, and pentaene acids are then calculated by simultaneous equations.

The nature of infrared energy is such that absorption occurs only where a change of dipolar character of the molecule occurs. Total symmetry about certain bonds will eliminate certain absorption bands. For example the C═C stretching band is absent in *trans*-dichloroethylene, whereas the *cis*-isomer gives a band for C═C stretching.

Absorption in the infrared region may be used to calculate the percentage of a *trans*-isomer, as elaidic acid or its methyl ester, by measurement of the absorption at 10.36 μ, through comparison to standards of known *trans*-isomer content.[18] The absorptivity is calculated for the methyl ester at 10.36 μ by the baseline technique,[19] using 10.02 and 10.59 μ points for the baseline, and the percent of *trans*-isomer may be calculated from

$$\% \; trans = \frac{100 a_s}{a_k}$$

Here a_s and a_k are the absorptivities of the sample and standard, respectively.

F. NUCLEAR MAGNETIC RESONANCE (NMR)

Atomic nuclei may have a spin or magnetic momentum. Of the about 100 different kinds of nuclei that possess an intrinsic angular momentum perhaps the proton is the one of most general interest at the present time. Those isotopes which do not have a zero spin value have a magnetic moment and behave as tiny magnets. The ratio of the magnetic moment to the angular

momentum is known as the gyromagnetic ratio, which has a unique value for each nuclear species.

An NMR spectrometer is made up essentially of a powerful electromagnet, a source of a radiofrequency field (rf oscillator), and an rf detector. When a substance of the above-mentioned properties is placed in the magnetic field and subject to the rf field, a signal is observed at the rf detector for specific combinations of frequency and magnetic field strength.

The relationship between frequency and field strength is given by the Larmour Precession Theorem

$$\omega = \gamma H,$$

where ω is the frequency, γ is the gyromagnetic ratio, and H is the magnetic field strength. This relationship has been established for a bar magnet spinning on its long axis in a magnetic field. The spinning magnet (or nucleus) is observed to precess as a top around the static magnetic field. All nuclei of the same charge and mass number have the same gyromagnetic ratio. When the nuclei are subject to a changing rf field, some frequency will be reached which equals the precessional frequency of the nuclei, and a resonance phenomena occurs where a constant additional torque is applied to the nuclei causing them to flip to another angle with the absorption of energy. The receiver coil of the detector picks up this change as an NMR signal. The signal is fed into the y axis of an oscilloscope or graphic recorder to yield a plot of the detector signal versus magnetic field.

For example, in ethyl alcohol three field dependent groups of lines are observed: one for the protons of CH_3, one for protons of $—CH_2—$, and one for the proton of —OH. The relative strength of these signals are an indication of the abundance as seen from the ratio of 3:2:1. A calculation of the abundance, through integration under the curves produced, may thus be used to indicate structural features.

The abundance and types of proton signals have been used in the determination of the degree of unsaturation of natural glycerides by Johnson and Shoolery[20] and by Hopkins and Bernstein.[21]

The spectra of fixed oils are characterized by four sets of signals: the olefinic protons, the four glyceride methylene protons, methylene protons adjacent to the double bonds, and protons of the remaining saturated carbon atoms. The relative abundance of the protons may be calculated by integration of the various signals, and an iodine number calculated from an average molecular weight and the number of olefinic protons. The NMR iodine value agreed with established values in all cases except Tung oil, where conjugation causes low values in the chemical determination by the Wijs or the Hanus method.[20]

A characteristic spectrum of corn oil may be seen in Fig. 12.8. The assignment of signals has been indicated, and comparisons may be made to the four characteristic sets of signals enumerated previously.

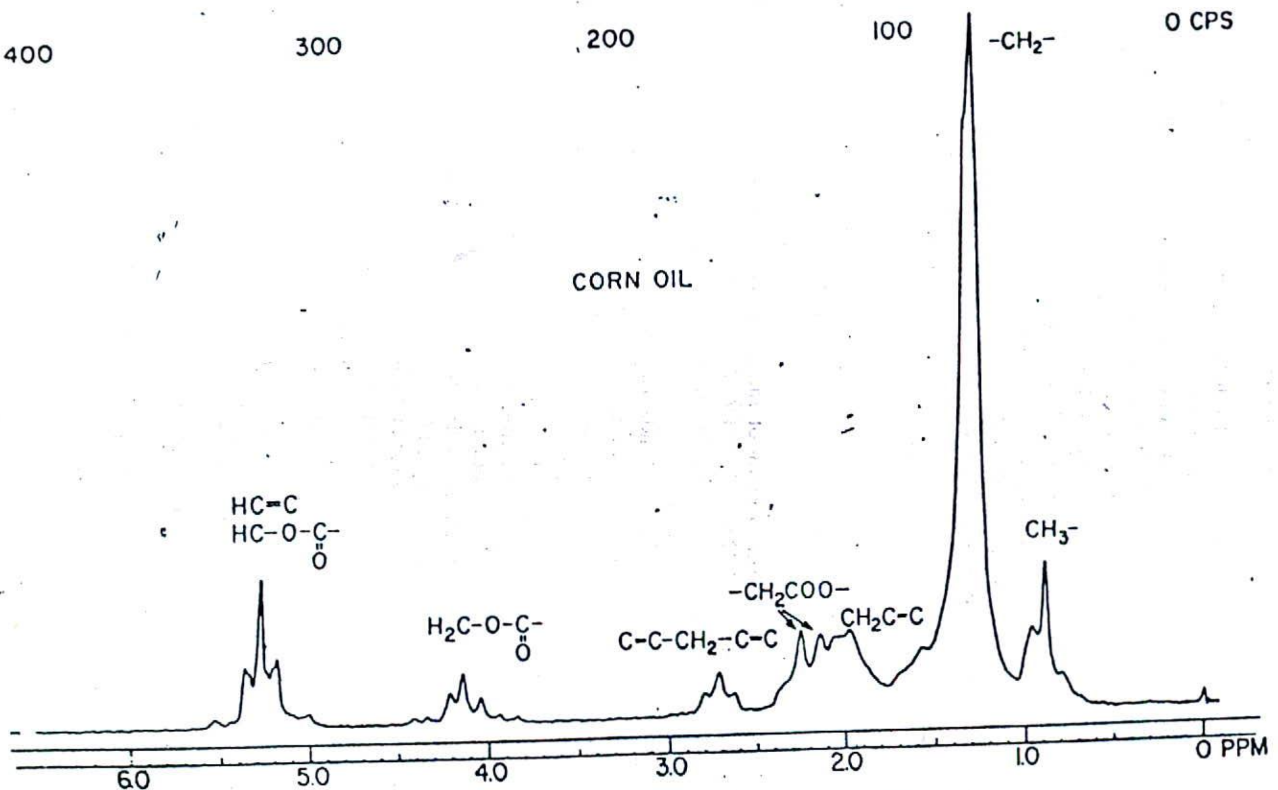


FIGURE 12.8: 60 Mc spectrum of corn oil. (Courtesy Varian Associates.)

QUESTIONS

Q12.1. Define or illustrate oil thief, acid value, hydroperoxides, and iodine value.

Q12.2. Explain why the residue in the determination of unsaponifiable matter must be titrated with standard alkali.

Q12.3. What information do the following tests or values give concerning the molecules of fixed oils and fats under consideration: iodine value, saponification value, Halphen test, and Bellier test?

Q12.4. What are some of the sources of the free acids found in fixed oils and fats?

Q12.5. If the water content of an oil or fat were determined by the oven method and immiscible solvent distillation, would you expect the results to be the same within the limits of experimental error? Why?

PROBLEMS

P12.1. A sample of cod liver oil was tested for its acid value with the following results. A sample weighing 11.3421 g required 11.06 ml of 0.01032 *N* potassium hydroxide to neutralize the free acids. What is the acid value?

P12.2. In the determination of the saponification value of olive oil the following data were collected. Weight of sample 5.2131 g, titration of the blank determination 105.12 ml of 0.5426 *N* hydrochloric acid, and titration of sample determination 72.57 ml of 0.5426 *N* hydrochloric acid. What was the saponification value of the oil?

P12.3. In the preparation of iodobromide reagent for use in the determination of an iodine value, it was determined, after dissolving 13.16 g of iodine in 825 ml of acetic acid, that a 25-ml portion required 31.50 ml of 0.1001 *N* sodium thiosulfate for determination of the iodine present. The bromine content of an additional 200 ml of acetic acid was also determined, after dissolving 3 ml of bromine in it, by titrating a 5-ml sample of the resulting solution with 29.16 ml of 0.1001 *N* sodium thiosulfate solution. How many milliliters of the bromine solution must be added to the remaining 800 ml of iodine solution to prepare the reagent?

P12.4. The iodine value of 0.2895 g of corn oil was determined using 25 ml of Hanus' solution as the iodinating reagent. After the reaction, it was determined that the residual iodine required 22.68 ml of 0.1039 *N* sodium thiosulfate solution, and a blank determination required 48.51 ml of the same sodium thiosulfate solution. What is the iodine number of the castor oil under examination?

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