CHAPTER III

MATERIALS AND METHODS
### MATERIALS AND METHODS

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3. MATERIALS AND METHODS

3.1. Ghee Samples

3.1.1. Laboratory samples: Buffaloes' and Cows' milk used for the preparation of ghee was taken from the Institute's farm. Cows' milk was a mixture of the milk of Red Sindhi, Sahiwal and Tharparkar breeds. Buffaloes' milk used was the herd milk from Murrah buffaloes. Cream was separated in a laboratory separator and used in the preparation of ghee samples.

Six samples each of cow and buffalo ghee were prepared by direct cream method during winter, summer and rainy seasons.

A few ghee samples were also prepared by the creamery butter method and by the desi (indigenous) method.

Clarification of Cream: Fresh cream was heated over a burner in a stainless steel vessel to 119°-120°C and kept at this temperature for about 5 min. It was then strained through a double fold muslin cloth, filled in bottles, cooled and kept in the refrigerator at a temperature of 4°-5°C for analysis. The moisture content in the samples studied varied from 0.15 to 0.25 per cent.

3.1.2. Ghee from different regions: Pure buffalo ghee samples were procured from different States through the courtesy of the Directorate of Marketing and Inspection (Government of India). One sample in each season was received for analysis. The samples were prepared from buffaloes' milk as per the instructions given. A record of the feed given to the animals was kept. Ghee was obtained
by the desi method by clarifying at 100°-105°C.

3.1.3. Ghee samples from Delhi Market: Ghee sold in sealed containers were procured from the market in different seasons. 3 samples of different brands were purchased and kept in refrigerator at 4°-5°C till analysed. Five samples packed in 4 kg tins were procured to study the crystallization behaviour (separation into layers) of ghee. In all 14 samples were analysed.

3.2. Animal Body Fats:

Buffalo, goat, pig and sheep body fat tissues were procured from slaughter houses and fat extracted by directly heating the tissues in a stainless steel vessel over low flame. Heating was continued till all the fat melted out of the tissues and the latter became brown. By this time the temperature of the melted fat was 120°-125°C. Body fat samples were also prepared during different seasons of the year. The moisture content of the samples was 0.1 - 0.2 per cent. In all 12 samples from each of the 4 species were analysed in course of 2 years.

3.3. Sterculia foetida Oil:

This was procured through the courtsey of United States Department of Agriculture, New Orleans, Louisiana, (U.S.A.) as a gift sample.

3.4. Cottonseed Oil:

Cottonseed oil was extracted from cottonseeds (obtained from the local market) by Soxhelet extraction method
using hexane, was refined by the procedure described by Williams (1966).

3.5. **Determination of Common Characteristics:**

3.5.1. **Analytical Constants:** Reichert, Polenske, iodine (Wijs) and saponification values, B.R. index and melting point (slip point) were determined by the method specified in IS:3508. All the analysis were conducted in duplicate.

3.5.2. **Estimation of Free Fatty Acids:** Ten g of the sample was weighed in a 250 ml conical flask. In a second flask 50 ml of ethyl alcohol was boiled and neutralised to phenolphthalein with 0.1N NaOH. The neutralized ethyl alcohol was poured on the fat sample in the flask, the contents of the flask mixed, and boiled again. The hot material was titrated with 0.1N NaOH solution with vigorous mixing during the titration. The end point of the titration was reached when the addition of a single drop of 0.1N NaOH solution produced a slight but definite colour change persisting for at least 15 sec. The percentage of free fatty acids was expressed as oleic acid.

3.5.3. **Estimation of Peroxide Value:** Approximately 1 g of the sample was weighed accurately into a test tube (150x25 mm) and 1 g of freshly powdered KI was added while the fat was still in the liquid form. To this 20 ml of the solvent mixture (glacial acetic acid : chloroform, 2:1) was added and the contents of the tube quickly boiled on a water bath so that it boiled within 30 sec and vigorous boiling
continued for another 30 sec. The tube was closed with a rubber bung, cooled under tap water and quickly transferred to a 250 ml conical flask containing 20 ml of 5 per cent aqueous solution of KI. The test tube was washed three times with 25-30 ml of distilled water. The solution was then titrated with 0.002 N sodium thiosulphate solution using 1 per cent starch as indicator. A blank test was carried out under similar conditions. Peroxide value was expressed as ml of 0.002 N sodium thiosulphate solution required for 1 g of fat.

3.5.4. Density and Specific Gravity of Fat Samples: The fat sample was dried in an oven (150°C) till constant weight. Anhydrous sodium sulphate was added and filtered through a previously dried filter paper. Dried but weighed specific gravity bottle was filled with the fat sample and closed with a standard joint thermometer. The whole apparatus was now kept in an oven maintained at 40 ± 1°C for an hr or until the temperature of the fat was constant at 40°C. The specific gravity bottle was cleaned from outside and weighed rapidly. The process was repeated three times using the same sample. Similar readings were taken with boiled and cooled distilled water using the same sp.gr. bottle. The specific gravity of the fat samples at 40°C was calculated as

$$\text{Sp.gr.} = \frac{\text{Wt. of the fat at } 40^\circ\text{C}}{\text{Wt. of an equal volume of water at } 40^\circ\text{C}}$$

Density of the fat was calculated by dividing the mass of the fat contained in the sp.gr. bottle over the volume of
the specific gravity bottle calibrated earlier at 40°C.

3.6. Estimation of Unsaponifiable Matter:

After saponification of a known amount of the fat sample (2 to 2.5 g), the unsaponifiable matter was extracted with three 50 ml portions of peroxide free ether. The combined ether extract was washed three times with 20 ml portions of distilled water (avoiding violent shaking). The ether extract was then washed three times alternately with 20 ml of 0.5 N aq. KOH and distilled water, shaking vigorously each time. Finally the ether extract was washed successively with 20 ml portions of distilled water until washings were no longer alkaline to phenolphthalein.

The ether solution was transferred to a 250 ml Erlenmeyer flask, rinsing separately funnel and its pouring edge with ether into the main solution. Ether was evaporated to about 5 ml and transferred quantitatively using several small portions of ether to a 50 ml previously dried and weighed Erlenmeyer flask. Ether was almost evaporated, 2-3 ml acetone was added and the solvent was completely removed on a steam bath with the help of a gentle current of air. Finally the flask containing the unsaponifiable matter was dried at 100°C till constant weight. The results were checked by dissolving the unsaponifiable matter in 2 ml ether, adding 10 ml neutralized alcohol and titrating with 0.1N alcoholic KOH to ensure that not more than 0.1 ml was required.
3.7. **Estimation of total Cholesterol:**

The method recommended by Bindal and Jain (1973) was adopted. Liebermann Burchard reagent was prepared by chilling A.R. concn. H$_2$SO$_4$ and acetic anhydride before use. One ml of the chilled H$_2$SO$_4$ was mixed to 20 ml of the chilled acetic anhydride in cold, using ice for cooling. The mixture was then kept in the refrigerator (4°-5°C) for 27 min. The sterols and sterol esters gave greenish colour with this reagent. The reagent was freshly prepared before use.

3.7.1. **Estimation of total Cholesterol directly in Fat:**

0.20 to 0.25 g fat was dissolved in 2.5 ml A.R. choloroform and the volume was made to 3 ml with chloroform. To this solution 4 ml of Liebermann Burchard reagent was added and the colour allowed to develop for 15 min at 25°C. The optical density (O.D) was recorded at 650 nm in the Lumitron colorimeter, along with the unknown samples a blank and a standard cholesterol sample was also developed. In the case of standard, 0.2 ml of standard cholesterol solution (containing 4 mg cholesterol/ml) was added to 2.3 ml chloroform and 4 ml reagent. A standard curve (Fig.1-A) was prepared using samples containing known amounts of cholesterol. However, as a check, standard containing known amount of cholesterol was always prepared whenever cholesterol was estimated.

3.7.2. **Estimation of total Cholesterol in Unsaponifiable matter:** Unsaponifiable matter extracted from 5 g fat was
FIG. 1 STANDARD CURVE FOR CHOLESTEROL

OPTICAL DENSITY (650 nm)

CHOLESTEROL (µg)

A

B
dissolved in 25 ml A.R. glacial acetic acid. To 2 ml of this solution 4 ml of Liebermann Burchard reagent was added and the colour developed at 25°C for 35 min. Optical density was then recorded at 650 nm. A blank and a standard cholesterol sample was always prepared along with the unknown samples. A separate standard curve using the same solvents and under similar conditions was also prepared (Fig.1-B).

3.8. Spectroscopic Measurements:

The method used by Rego, et al, (1964) was adopted. Carl Zeiss spectrophotometer, model DU2, equipped with U.V. and visible range was used. A.R. quality n-hexane before use was redistilled over anhydrous calcium chloride and kept in a glass stoppered bottle over fine sodium wire for subsequent use.

A 0.1 per cent solution of fat sample was prepared in the purified n-hexane and the absorbancy was measured in a 1 cm cell from 200-320 nm. Similarly, unsaponifiable matter extracted from various fat samples was dissolved in purified n-hexane and screened through U.V. spectrophotometer between 200 and 320 nm using a 1 cm cell.

3.9. Chromatography Studies:

3.9.1. Circular Paper Chromatography: The solvent system comprising of ethyl alcohol-amyl alcohol-carbon tetrachloride (35:55:10) and Whatman No.3 filter paper used by Ramchandra and Dastur (1959) did not give good
result under the conditions of working. From trials with 25 solvent systems, one consisting of ethyl alcohol (95 per cent) and iso-amyl alcohol (b.p. 131°C) in 1:1 ratio and Whatman filter paper No.1 was selected as giving the best results. Ghee, body fats and adulterated samples were dissolved in equal volume of carbon tetrachloride (W/V), 0.05 ml of the fat solution was spotted on the filter paper, allowed to dry at room temperature for about an hr, placed between 2 glass plates, and irrigated at a temperature of 4°-5°C. The solvent was allowed to run up to a distance of about 6 cm from the origin. The paper was then removed and air dried. The spots on the filter paper were located by exposing the paper in a desiccator to iodine vapours.

3.9.2. Thin Layer Chromatography: Glass plates (20x10 cm) with 0.3 mm thick adsorbent layer, were prepared. About 3 g of silica gel G (Stahl, for thin layer chromatography) and 6 ml of distilled water for each plate sufficed. The glass plates after application of slurry were air dried, activated at 110°C for 1½ hr, cooled in a desiccating cabinet over silica gel, and retained in the same cabinet till use.

3.9.2.1. Study of Ghee and Body Fats: From trials with 30 solvent systems, one consisting of A.R. acetone:methanol:glacial acetic acid (70:30:2) was selected as giving the best results. Sixty to 70 ml of the solvent was poured into a specimen jar (22x15 cm) and covered with the lid of the jar making the system almost air tight. The atmos-
phere within the jar was allowed to get saturated within 30 min. The plates before spotting and after activating were placed slowly into the jar and were allowed to over-run so that any impurity in the silica gel was washed out. These plates were now removed from the jar, air dried for 5 min, dried in an oven at 100°C for 10 min and cooled in the desiccating cabinet. The chromatoplates were now spotted with the test material. A 50 per cent solution (W/V) of the fat samples in carbon tetrachloride was prepared and 0.02 ml was spotted, equi-distance from each other, about 1.5 cm apart and 2 cm above the bottom edge of the plates with the help of a fine calibrated capillary tube or a graduated micro-pipette. The plates were then air dried to remove the solvent and gently placed in the same specimen jar containing the same solvent mixture which was used for purifying the plates, taking necessary precautions so that the spots might not be washed away by the solvent. The jar was immediately covered making the system air tight. The chromatoplates were allowed to develop for 1 hr within which period the solvent had moved about 15 to 16 cm. The plates were then taken out of the jar, air dried and sprayed with 50 per cent sulphuric acid. The plates were again air dried and then charred in an oven at 160°C for 1½ hr when the spots on the chromatoplates were clearly visible.

3.9.2.2. Study of the Saturated Triglycerides: Saturated triglycerides from ghee, body fats and adulterated ghee
samples were extracted by diethyl ether method, recommended by the International Organisation for standardisation-ISO (1969) for pork fat for the determination of Bömer value. A 1 per cent solution of the saturated triglycerides was prepared in chloroform. 20 µl of the triglyceride solution (0.2 mg of the material) was used for spotting. The solvent system used in this case was acetone:chloroform:glacial acetic acid (70:30:2).

3.9.3. Gas Liquid Chromatography (GLC): A.R. methyl alcohol was further purified by the method described by Vogel (1971). Five g of clean dry magnesium turnings and 0.5 g of resublimed iodine were placed in a 2 litre round bottom Pyrex flask, fitted with a double surface reflux condenser. About 75 ml of MeOH were added through the condenser and the mixture was warmed on a water bath until the iodine disappeared. Then 900 ml MeOH was added, the mixture refluxed for 30 min and distilled with exclusion of moisture using a guard tube containing silica gel, and stored in glass stoppered brown bottle in dark.

3.9.3.1. Preparation of methyl Esters:

3.9.3.1.1. Methyl Esters of Ghee: The sealed tube method of deMan (1964) was used for the preparation of methyl esters of ghee except that temperature of incubation was 65°C instead of 60°C. Approximately 25 mg of the fat sample to be analysed was introduced into the glass tube with 0.3 ml of 0.025 N sodium methylate, prepared by dissolving 0.06 g sodium in 100 ml anhydrous methanol. The other end of the tube was also sealed. The sealed tube was
placed in an oven at 65°C and shaken at regular intervals of 10 min. The methylation was complete in an hr, as evident from the change of two-phase to one-phase system. The tube was then removed from the oven, cooled to room temperature and the esterified mixture was used without further treatment for injection into the GLC column.

3.9.3.1.2. Methyl Esters of Animal Body Fat Samples:
The animal body fats being different in composition than ghee, the sealed tube method of deMan (1964) was modified according to the method of Luddy et al. (1968) for the preparation of methyl esters in the presence of large amounts of FFA. Approximately 25 mg of the melted fat was introduced into a glass tube with the help of a disposable pipette. 0.1 ml of purified A.R. benzene was added and the vial warmed slightly to aid in solubilizing the fat. Approximately 0.25 ml of 0.1N sodium methylate was added and the vial was sealed at the other end. The sealed vial was then kept at 65°C in an oven for an hr. The rest of the procedure was the same as described for the preparation of methyl esters of ghee samples under 3.9.3.1.1.

3.9.3.1.3. Methyl Esters of Saturated Triglycerides:
The method used under para 3.9.3.1.1. above was used for the preparation of methyl esters of saturated triglycerides extracted from ghee, body fats and adulterated ghee samples except that 0.1N sodium methylate was used instead of 0.025N sodium methylate.

3.9.3.2. Fractionation of Methyl Esters by GLC: Methyl
esters of the fatty acids of ghee, body fats and their saturated triglycerides were analysed by gas-liquid partition chromatography on an F and M gas chromatograph model 609 equipped with flame ionization detector. The column used for fractionation of methyl esters consisted of 20 per cent Diethylene glycol succinate (DEGS) coated on 60-30 mesh Diatoport P packed in a 5'x 3/16" stainless steel column. The column temperature was kept at 80°C, till the emergence of butyric acid peak and then maintained at 195°C. The injection port and the detector were kept at 260°C and 250°C, respectively. Pure nitrogen at 20 P.S.I was used as the carrier gas. The chromatograms were recorded with a 1-mv full scale recorder (Honeywell Brown Electronik) and the chart speed was maintained at 1/4"/min.

Three µl samples were injected into the GLC column by means of a 10 µl Hamilton syringe. The peaks recorded as a result of recorder response to the sample were tentatively identified by comparison of their retention time with those of known fatty acid methylates, by co-chromatography. Each peak area was measured by triangulation method, by multiplying the base of the peak with 1/2 height. These areas were converted to relative percentages. Throughout the text of this study the fatty acids have been referred to in short hand designation according to the notation given by Farquhar et al, (1959).

3.10. Opacity Profiles of Fats:

3.10.1. Opacity Profiles at different Temperatures: The
fat sample was heated to 60°C in an oven and filtered through a fluted Whatman filter paper No.1 at the same temperature. The transparent sample was poured in the colorimeter tube (8 x 1.5 cm) to a height of about 7 cm. The tube was now placed in a water bath. The assembly was then kept for ½ hr in an oven maintained at 50°C ± 2°C so that the fat sample and the water bath attained the temperature of the oven. The water bath with the fat sample was allowed to cool gradually to 40°C. The photo-electric colorimeter was adjusted with the fat sample (40°C) to 100 per cent transmittance using 690 nm (yellow) filter. The temperature of the water bath was brought down by adding cold water and the sample was kept in the water bath at each temperature for a definite period of interval and optical density was recorded. This process was continued over a temperature range 40°C to 15°C till the sample became opaque (optical density about 0.5).

3.10.2. Opacity Profiles at 23°C: Studies showed that at a temperature of 23°C lowest melting fat sample started showing opacity. Time taken by the clear melted fat to become opaque at 23°C was then recorded. The fat sample in the colorimeter tube treated as mentioned above was transferred from 50°C water bath to another water bath maintained at 23°C and the opacity was noted after a definite interval of time, say every one or two min as the case may be.
3.11. Determination of Bömer Value:

The Bömer value is defined as the sum of melting point of the triglycerides (isolated by the procedure described) and twice the difference between this melting point and that of the fatty acids obtained after saponification of these triglycerides. The diethyl ether method recommended by the ISO (1969) for pork fat intended for human consumption was followed.

3.11.1. Preparation of Triglycerides: About 100 g of the fat sample was melted at about 50°C and filtered through a dry Whatman filter paper No.1. Fifty g of the filtered melted fat was weighed into a beaker of 150 ml capacity. Fifty ml of peroxide free diethyl ether was added, covered with watch glass, and the fat dissolved by swirling and gentle warming over a water bath. The solution was filtered if not quite clear. The covered beaker was then placed in a water bath at 12°C ± 1°C for 1 hr (at 15°C ± 1°C ghee samples did not give any crystal even in 2 to 3 hr), stirring the solution with a glass rod every quarter of an hr when the triglyceride crystals separated.

The crystals were filtered on a 5 cm diameter Buchner funnel using No.1 Whatman filter paper previously soaked in ether. The crystals on the filter were washed three times with 25 ml portions of ether cooled to 12°C. The crystals were removed from the filter and returned to the beaker used for the crystallization. The crystals were then dissolved in 50 ml ether and crystallization repeated.
Three such crystallization were done. The filter paper was then removed from the Buchner, placed on a watch glass, dried at a temperature of 35°C, weighed, ground in a small mortar to a fine smooth powder and stored in a glass stoppered bottle in a desiccator over sulphuric acid.

3.11.2. Preparation of Fatty Acids: About 0.2 g of the powdered triglyceride crystals were weighed into a 100 ml ground neck round bottom flask, 10 ml of ethyl alcohol (95-96 per cent V/V), and 0.4 g (2 pellets) of solid KOH added. The triglycerides were saponified by heating on a water bath and boiling gently for 15 min. The soap solution was then poured into a 100 ml beaker, alcohol allowed to evaporate until the soap solution had a pasty consistency. The soap was then dissolved in 50 ml of hot water and transferred to a separating funnel. Ten ml of 1N sulphuric acid was then added to the separating funnel. Shaken vigorously and cooled to room temperature. The fatty acids were extracted with 50 ml of ether and washed three times with 15 ml portions of distilled water. The ethereal solution of fatty acids was filtered through a dry filter paper into a beaker of 100 ml capacity. The ether was allowed to evaporate over a water bath. The beaker containing fatty acids was dried in an oven at 102°± 2°C for 15 to 20 min and allowed to cool in an inclined position. The fatty acid cake was then detached from the beaker and pulverised in a small mortar to a fine powder as the triglycerides and stored in a glass stoppered bottle in a desiccator over concentrated sulphuric acid.
3.11.3. Determination of Melting Points: A capillary glass tube 70-80 mm in length and 0.8 to 1.0 mm internal diameter with a wall thickness of 0.1 to 0.2 mm was sealed at one end and filled with the powdered fatty acids. The fatty acid powder was packed to a height of 5 mm by dropping the capillary tube through a glass tube of about 50 cm onto a glass plate. Another capillary tube was filled with the powdered triglyceride in a similar manner. Both the tubes and the thermometer were placed in the melting point apparatus. The temperature was raised at a rate of 2°C per min until 50°C and then at a rate of 0.5°C per min. The temperature at which the last solid particle disappeared was noted to within 0.1°C.

Bömer Value (B) = Tg + 2(Tg - Tf)

where Tg is the m.p. of the triglycerides and Tf is the m.p. of the fatty acids.

3.12. Methylene Blue Reduction Test for Cotton Tract Ghee:

3.12.1. Methylene Blue added directly to the Fat Samples: A 0.1 per cent methylene blue solution in chloroform:methanol mixture (1:1) was prepared using A.R. reagents. (This will be referred as dye solution). Five g of the clear fat sample was taken in a test tube, 0.1 ml of the dye solution was added and mixed by gentle shaking. Immediately after shaking (care was taken to avoid formation of air bubbles) the optical density was recorded in a colorimeter using 650 nm (red) filter. The samples were then incubated at 60°C and the intensity of blue colour
was measured at regular intervals till the colour was discharged. The blank used was the same fat sample without the dye.

3.12.2. Methylene Blue added to the Fat Solution in Iso-amyl Alcohol: Since it was not possible to dissolve the dye solution in a small amount of the fat samples (1 g of cottonseed oil and 0.5 g of Sterculia foetida oil), the dye was added to the fat solutions in iso-amyl alcohol. Five g of the liquified cotton tract ghee, 1 g of cottonseed oil and 0.5 g of Sterculia foetida oil were dissolved in 5 ml iso-amyl alcohol in a strong test tube and 0.1 ml of the dye solution was added. The contents of the tube were mixed and the tube was securely corked. The tube and its contents were then placed in a bath of boiling saturated NaCl solution and the disappearance of the methylene blue colour was noted. The conditions of the test were so adjusted that the maximum time of complete reduction was 30 min.

3.13. Halphen Test and Estimation of Cyclopropene Fatty Acids:

3.13.1. Halphen Test for Cotton Tract Ghee: The method originally developed by Halphen (1897) and described in A.O.A.C. (1979) was used. Carbon disulphide containing 1 percent sulphur in solution was mixed with equal volume of iso-amyl alcohol. To 5 ml of the mixture in a strong test tube, an equal volume of the liquified fat sample under examination was added and the tube was securely corked. The tube with its contents was then placed in a bath of boiling saturated NaCl solution for 1 hr. A characteristic crimson colour was produced in the presence of cotton tract ghee. Depth of
colour was proportional, to certain extent, to the quantity of cottonseeds fed to the animal. The component responsible for the colour reaction was determined by comparing the colour spectrum of the cotton tract ghee with that of standard *Sterculia foetida* oil and refined cottonseed oil containing cyclopropene ring (Nunn, 1952; MacFarlane *et al.*, 1957; Craven and Jeffery, 1959; Shenstone and Vickery, 1961), and responsible for colour reaction (Nordby *et al.*, 1962).

3.13.2. Estimation of Cyclopropene Fatty Acids in Cotton Tract Ghee: Halphen reaction can be used as a colorimetric method of estimating the total cyclopropene content of an oil (Deutschman and Klaus, 1960; Bailey, *et al.*, 1965). Total cyclopropene fatty acids in cotton tract ghee were determined by developing the Halphen colour as mentioned in para 3.13.1. and recording the optical density at 550 nm in Coleman Junior II spectrophotometer Model 6/20. Alongwith the unknown samples a blank and a standard *Sterculia foetida* oil sample was also developed. In the case of standard, 0.2 ml of the standard *Sterculia foetida* oil solution in iso-amyl alcohol (containing 2.5 mg cyclopropene fatty acid as sterculic acid per ml) was added to 4.8 ml of Halphen reagent. A standard curve (fig.2) was prepared using samples containing known amounts of cyclopropene fatty acids (sterculic acid). However, as a check, a standard containing known amount of cyclopropene fatty acids was always pre-
FIG. 2 STANDARD CURVE FOR CYCLOPROPENOID FATTY ACIDS
pared whenever cyclopropene fatty acids were estimated.

3.14. Crystallization Behaviour (Separation into Layers) of Ghee:

Buffalo and Cow ghee samples prepared in the laboratory and purchased from the market were stored in either a glass or metal container in the dark at room temperature (30° - 35°C) for a period of one and 6 months, respectively. The samples stratified into three layers, namely solid (hard clumps sticking by the sides and floating on the top), semi-solid (granular portion at the bottom), and a liquid layer. The different layers were removed carefully and analysed for the fat constants (13:3508), opacity profiles para (3.10), and fatty acid composition by GLC para (3.9.3).

3.14.1. Test for the Presence of Triacetin or similar other Material Contributing for the Reichert Value: Market samples were checked for the presence of triacetin, or similar other material by the method of Fincke (1903) as reported by Jacobs (1953). The method is based on the fact that triacetin is soluble in dilute alcohol. The Reichert value of a portion of the fat sample was determined in the usual manner. About 30 g of the fat sample was transferred to a standard joint 500 ml capacity F.B. flask. 150 ml of the distilled water, 150 ml 95 per cent ethyl alcohol and some pumice stones were added. The mixture was refluxed for an hr on a boiling water bath. The mixture, was then transferred to a separatory funnel, allowed to separate and cool. The water layer was drawn off and the fat was transferred to an evaporating dish,
and alcohol evaporated off. The fat was dried and the Reichert value of the treated fat was determined. If the Reichert value of the treated fat was lower than Reichert value of the original sample, triacetin or other material contributing to the Reichert value was present in the fat samples.