## 2. REVIEW OF LITERATURE

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2. REVIEW OF LITERATURE

2.1. Methods used in the Detection of Foreign Fats in Milkfat.

2.1.1. The methods used for detection of adulteration of ghee (butterfat) and other fats are based on the differences in the nature and composition of the major and/or minor components of the adulterants and those of pure fats. The methods generally depend on either the determination of physical and chemical constants, determination of specific fatty acids and glycerides, spectrophotometric characteristics, examination of unsaponifiable matter, and development of colour, fluorescence and turbidity under certain conditions. Butterfat on account of its characteristic flavour has a special appeal to the consumer and for this reason it commands high price. These factors also lend to the frequent adulteration of butterfat with fats of animal and vegetable origin which are cheaper. The problem is complicated by the fact that butterfat from the cow and the buffalo differ markedly in composition and under the conditions in India both these species are often maintained side by side. The composition of butterfat also varies with the diet given to the animals and the season. Butterfat takes up certain characteristics of the fat ingested in the diet within 36 hr of feeding. For this reason there is a marked seasonal variation in the composition and characteristics of butterfat (Rangappa and Achaya, 1948). Extensive studies have established that the presence of vegetable fats in butterfat could be detected unambiguously by the phytosteryl acetate test which depends on the determination
of the melting point of sterols present. As phytosteryl acetates have a higher melting point than cholesteryl acetate, repeated crystallisation of the isolated steryl acetates ordinarily enables detection of the presence of less than 5 per cent of vegetable fat in butterfat. However, differentiation between butterfat and commonly used animal body fats is not simple and often over 25 per cent adulteration could be masked.

2.1.2. Due to extensive literature on the subject of detection of adulteration in butterfat, the following review of published work is confined mainly to work dealing with detection of animal body fats. To list all the methods which have been studied for the detection of adulteration with vegetable and animal body fats itself will be lengthy. The following list gives an indication of the wide field covered with respect to physical, chemical and other miscellaneous methods:

2.1.2.1. **Physical methods:**

a. Refractive index and refractive dispersion (Godbole & Sadgopal, 1936; Achaya & Banerjee, 1946-b; Bhalerao & Kummerow, 1954);

b. Melting point, Solidification point, and Titre value (Mohr, 1924; Venkatachalam, 1937-a);

c. Microscopic examination (Litterschied, 1925; Butcher, 1934);

d. Cryoscopic studies (Pathak, et al, 1948);

e. Critical temperature of dissolution (Atkinson, 1926; Sanyal, 1929; Felman & Lepper, 1950);

f. Examination of fluorescence and luminescence under light of different wave lengths (Achaya & Banerjee, 1945; Bramnsdorf, 1932-a);
g. Differential thermal analysis (Yoncoskie, 1967; Roos & Tuinstra, 1969);

h. Spectroscopic analysis, including ultra-violet (U.V.) and infra-red spectroscopy (Bramnsdorf, 1932-b; O'Connor, 1960; de Ruig, 1968);

2.1.2.2. Chemical Methods:

i. Identification of principal fatty acids - Reichert, Polenske, Kirschner, 'A' & 'B', Baryata values etc. (Winton & Winton, 1958);

j. Measurement of unsaturated fatty acids (Venkitasubramanian & Banerjee, 1949; Bartlet & Chapman, 1961);

k. Enzymic hydrolysis (Giri & Bhargava, 1937);

l. Complexes with urea (Shipe, 1955) and hydroxamic acid (Bassatte & Keeny, 1956);

m. Chromatography-paper, column, thin layer and gas chromatography (Ratz, 1952; Ramachandra & Dastur, 1959, 1960; Freeman & West, 1966; Thorpe, 1970);

n. Fractionation of glycerides and glyceride structure (Hilditch & Williams, 1964; Rangappa & Achaya, 1948);

o. Unsaponifiable matter and study of sterols (Nazir & Nagar, 1959; Eiser & Firestone, 1963; Peerboom & Bukes, 1965; Roos et al., 1969; La Croix, 1970);

2.1.2.3. Other Methods:

p. Correlation between various constants - Reichert & iodine value, Reichert & saponification value, etc. (Achaya & Banerjee, 1946-a);

q. Detection of added tracer substances - Baudouin test, detection of phenolphthalein, etc. (Subrahmanyan et al., 1952; Fitolson, 1954).

The utility of these methods in detecting adulteration in butterfat have been critically reviewed by Woodman (1952), Bhalerao & Kummerow (1954), Roos (1963), Pruthi & Sachday (1968).
2.2. Examination of Triglycerides: Methods based on the differences in the properties of triglycerides of butterfat from those of other edible fats have been suggested to detect adulteration of butterfat. Besides direct estimation, several empirical methods like solubility in various solvents at a specified temperature have been evolved.

2.2.1. According to Roos (1963), as early as 1849, Arzbacher made use of the ether insoluble triglycerides for the examination of beef suet and mutton fat. Using this technique Hoorn in 1872 was able to detect foreign fats and especially beef suet, in milk fat. In 1913, Bömer published a method for detecting the presence of beef suet in lard on the basis that ether insoluble triglycerides of lard contained beta-palmitodistearin, and those of beef suet contained alpha-palmitodistearin. These two forms of ether insoluble triglycerides differed in their melting points. On the same basis in 1916, Amberger designed a method to detect adulteration of beef suet in milk fat. The amount of ether insoluble triglycerides was used as an analytical characteristic. Pure milk fat yielded practically no insoluble triglycerides on crystallisation. The above method was modified by Seidenberg (1918). Instead of determining the quantity of ether insoluble triglycerides, the volume of ether-ethyl alcohol mixture just sufficient for dissolving 10 g of fat was determined. That the adulteration of beef tallow in milk fat could be detected on the basis of saponification value and melting points of
fully saturated triglycerides (GS₃) was pointed out by Christian & Hilditch (1930). Fractionation by selective solidification for detecting butterfat adulteration was utilized by Krienke (1953). The sample was fractionated by a process of partial solidification and filtered at successive temperatures at 95°, 80°, 70° and 60°F. (35.0°-15.5°C) to yield various fractions. The differences in the Reichert value of fractions was claimed to indicate positive identification of adulteration at levels as low as 2 per cent of foreign fat. A method depending on the solubility of triglycerides in absolute alcohol was developed by Bhalariao & Kummerow (1954). The fat was separated into alcohol-soluble and alcohol-insoluble triglycerides fractions at 20°C, and the refractive index of the fractions determined. The refractive index of alcohol-soluble fraction was lowered with the addition of 10 per cent coconut oil, while the refractive index of the insoluble fraction was increased with the addition of other vegetable fats. The same authors further refined the technique and the percentage of GS₃ content of the alcohol-soluble fraction was estimated (Bhalariao & Kummerow, 1954). Recently, Latif & Mazloum (1969-a) suggested fractionation of cow and buffalo butterfat (samm) from dry acetone at room temperature, 10°, and 0°C to form groups of simpler glycerides than in the original sample. It was observed that Reichert, Polenske, Kirschner, iodine and saponification values of the fractions differed in the case of cow and buffalo butter-
fats. The same authors (Latif & Mazloum, 1969-b) applied
the above technique for the identification of animal and
vegetable fats in shortenings and also for the detection
of adulteration of butterfat by foreign fats at 10 per cent
level. They observed that in all the fractions Reichert
and Kirschner values were depressed by the presence of
foreign fats.

2.2.2. Extensive work has been done by Hilditch & Lea
(1927), Kartha (1953), and Hilditch & Williams (1964) on
the transformation of unsaturated fatty acids by oxidation
into azeleic acid, and azelao-glycerides. It was observed
that the addition of a substitute fat to butterfat caused
differences in the proportions of azelao-glycerides formed
during the oxidation.

2.2.3. Paper, thin-layer and gas liquid chromatography
techniques have been helpful in finding out the triglyceride
structure of fats. These techniques have been utilized for
the detection of adulteration of butterfat with foreign
fats. Paraffin impregnated paper was used by Chakrabarty,
et al., (1963, 1966) for differentiating natural fats from
rearranged fats on the basis of difference in the composi-
tion of their triglycerides. 

The TLC technique has been
favoured for the detection of adulteration in ghee because
of higher sensitivity and sharper separation (Mangold &
Malins, 1960; Mangold, 1961). A TLC method was developed
by Cerbulis & Zittle (1965) for the identification of milk
fats from the cow, the goat, the ewe and the cow’s colos-
trum in other fats of animal and vegetable origin using
a silica gel G plate and a solvent mixture of petroleum ether, ethyl ether and acetic acid. All the milk fats gave only one triglyceride spot. Admixture of 1 to 2 per cent milk fat was detected. Another TLC method was suggested by Hendrickx & Huyghebaert (1968) who concluded that as little as 2.5 per cent substitution fats containing interesterified fats in butter of normal acidity could be detected by the appearance of a mono-glyceride band on a silica gel G plate using petroleum ether:diethyl ether:formic acid (60:40:1.5) as the developer. These monoglycerides were formed during the preparation of the substitution fats. TLC of GS3 after randomizing the fatty acid radicals was employed for the detection of 5-10 per cent hydrogenated groundnut oil, tallow and mahua oil in butterfat by Chakrabarty et al. (1968). The GS3 from each sample were reduced further into components by reverse phase chromatography. Each individual component of GS3 thus separated was later subjected to micro-saponification and the fatty acids identified. Differences in the concentration of GS3 components between pure and adulterated butterfats were clearer after randomization.

2.2.4. A gas liquid chromatography (GLC) technique was used by Kuksis & McCarthy (1964) for the detection of adulteration of butterfat with lard and vegetable fats. It was shown that by quantitative analysis of the triglycerides, adulteration of butterfat with 3 per cent lard and 1 per cent vegetable fats could be detected from the increase in
the $c^{52}$ and $c^{54}$ peaks, respectively, when the triglyceride composition of the unadulterated fat was known. However, detection of a mixture of lard and coconut oil was not possible by this technique even at fairly high levels.

2.2.5. There is a marked difference in the GS$_3$ fractions of butterfat and animal body fats. None of the studies noted above have gone into sufficient details with a view to evolve a simple method for detecting adulteration in butterfat, especially in relation to the behaviour of butterfat itself when animals ingest cottonseeds (Achaya & Banerjee, 1946; Anantakrishnan, et al, 1947). This aspect has, therefore, been further examined in the course of the present studies and will be discussed later. Various empirical methods based on the solubility of triglycerides in different solvents to detect adulteration are discussed in section 2.5.3 (p.37).


2.3.1. Commonly Used Constants as a Measure of Fatty Acids: Amongst the commonly used fats in India, ghee is the only fat which contains appreciable amount of butyric acid generally represented by the Kirschner and Reichert values. A minimum standard for the Reichert value of ghee has been prescribed for different States and for different regions to check adulteration of ghee (cf. Table 2, page 6). Ghee with high Reichert value offers scope to mix cheaper vegetable and animal body fats to a percentage which still leaves the butyric acid content well above the required
legal limit for the Reichert value and similar other constants. Amongst the many studies on this subject, recently the effect of adding different adulterants like hydrogenated oils, refined cottonseed oil, beef tallow and coconut oil separately, and in pairs, to ghee on the various fat constants was studied by Ali & Nemazi (1966). It was observed that the fat constants were not altered in the same direction by the contaminants, except for the Reichert value which was always lowered. Adulteration up to 10 per cent was difficult to detect. Empirical estimations like the Reichert value though employed universally are of no specific value for demonstrating adulteration of butterfat on account of the variation in the composition of butterfat itself (Murthy, 1955). The practice of feeding cottonseeds to milch animals further complicates the matter and it is known that the normal Reichert value of 30-32 comes down to well below 18 in the same animal. Achaya & Banerjee (1946) in a study of the fatty acid composition of ghee samples of high and low Reichert values found that in a sample from cottonseed fed animals the Reichert value was only 20.7. The sum of butyric + caproic acid amounted to 11.5 molar per cent, compared to a value of 16.5 molar per cent in ghee of high Reichert value (37.4), and 13.9 molar per cent in ghee of medium Reichert value (30.8). Decrease in lower fatty acids was accompanied by an increase in C₁₈ acids.
2.3.2. **Newer Techniques for the Estimation of**

**Characteristics of Fatty Acids:** More refined techniques for the estimation of characteristic fatty acids of butterfat are now available. A method for estimating fatty acids by directly saponifying dairy products without extracting the fat was used by Harper & Armstrong (1954). Butyric acid was separated by partition chromatography with silicic acid column and butanol-chloroform as the flowing solvent. The molar concentration of butyric acid in the fat from dairy products was found to vary from 9.5 to 10.0 per cent and this index was used for detecting fat substitution in dairy products. Keeney (1953) used the detergent method of extracting fat from ice cream samples. The fatty acids were obtained by saponifying the fat with KOH in isopropyl alcohol. Partition chromatography of the fatty acids between ethylene glycol and hexane yielded a pure butyric acid fraction and another fraction containing fatty acids of longer chain length. The butyric acid estimated by titration against alcoholic KOH was found to vary within narrow limits so as to permit detection of more than 10 per cent adulteration of butterfat. The author further surveyed the butyric acid content of butterfat from different sources for more than a year and recorded the extreme range of butyric acid content as 9.6 to 11.3 molar per cent (Keeney, 1955). A new analytical method for detecting adulteration by comparing the ratios between two fractions obtained in the Reichert value distillation was suggested by Curli (1955). The favourable results reported above...
with butyric acid estimation are in the main due to the limited area covered, limited period of study, and uniformity of feeding practices. The rations of dairy cattle undergo radical changes under Indian conditions with the changes in season where the small farmer relies on whatever byproduct that is available from the field. In areas where cottonseed is grown, feeding of cottonseeds ad lib is a common practice resulting in a marked alteration in ghee composition.

2.3.3. **Detection of Iso-Valeric Acid**: A number of techniques designed for the detection of dolphin oil and hydrogenated dolphin oil in butterfat by the detection of characteristic iso-valeric acid have been described by workers in Italy. Paper chromatography technique for the identification of iso-valeric acid in butter have been detailed by Priori (1955) and Canuti (1958) who claim that 5 per cent adulteration with dolphin oil could be detected. Chioffi (1956) showed that acetic acid and iso-valeric acid in dolphin oil could be distinguished from the volatile fatty acids natural to butterfat on the basis of their partition coefficients between water and carbon tetrachloride. Fabris & Vitagliano (1954) and Bottini & Campanella (1955) using similar technique were able to detect less than 3 per cent adulteration with hydrogenated dolphin oil. These results though interesting are not of direct interest as adulteration of butterfat with marine fats has not been reported in the absence of use of marine oils for culinary purposes in India.
2.3.4. Chromatography Techniques in the Examination of Fats
2.3.4.1. Paper chromatography, Thin layer chromatography (TLC), Adsorption chromatography and Gas liquid chromatography (GLC), have been employed in the examination of fatty acids and glycerides, as well as, in the study of the unsaponifiable matter. Paper chromatography for the analysis of fatty acids was suggested by Kaufmann and collaborators (1958). A technique for the differentiation of fats commonly used as adulterants for ghee was standardized by Ramchandra & Dastur (1960). Fifty per cent solutions of the fat in carbon tetrachloride were spotted on the paper chromatogram and solvent system ethyl alcohol, iso-amyl alcohol and carbon tetrachloride (35:65:10) was used. The movement of the spotted samples was visible in ordinary light. It was possible to detect adulteration of ghee with 10 per cent Vanaspati and 5 per cent body fats by this technique. Rego & Garcia-Olmedo (1963) studied samples of butter, margarine, their mixtures and samples of commercial butter by one dimensional paper chromatography with a view to detect adulteration. It was observed that adulteration of butter with margarine could be detected from the size of the spot due to butyric acid on the chromatogram when fatty acids of hydrolysed fats were examined. Adsorption chromatography has been applied successfully in the separation of fatty acids and especially the C18 group of acids (Riemensneider, et al, 1949; Kurtz, 1952). The application of TLC has been summarised by Roos (1963) and
it has been subsequently used for the detection of adulteration in butterfat by Ramamurthy, et al., (1967) and Chakrabarty, et al., (1968).

2.3.4.2. The GLC analysis of fatty acids has attracted considerable interest after the publication of the article by James & Martin (1952) and has substantially contributed to the knowledge of the fatty acid composition of fats. The method was used for detecting adulteration of butterfat with coconut oil, tallow and pig fat transesterified with butyric acid by de Francisco & Avancini (1961). Butterfat with a ratio of $C_{12}:C_{10} > 1.6$, or $C_4: C_6+C_8 > 1.8$ was considered to be adulterated. From an examination of fatty acid composition of genuine butter of various origin, Provvedi & Gallela (1961) found the fatty acid ratios $C_{14}:C_{12} > 2$, $C_{18}:C_{18:0} > 2$, and $C_{12}:C_{10} > 1.2$. Deviation from these ratios was considered an indication of adulteration. The fatty acid composition of Belgium butter samples over a period of one year, along with the principal dietary fats, was studied by the GLC method by Guyot & Piraux (1966). The range of values, average values and relative proportions of the various fatty acids were determined and their utility in the detection of foreign fats in butter have been discussed. Twenty to 30 per cent horseback fat was detected in butter by means of fat characteristics and values for constituent fatty acids observed in GLC according to Lorenzola & Torazzo (1966). On the basis of analysis of 40 butter samples and 9 brands of margarine for fatty acids by GLC, Jamoschek & Martin (1968) concluded that
lower fatty acid ratios like \( \frac{C_6}{C_8} \), \( \frac{C_{12}}{C_{10}} \) and \( \frac{C_{14}}{C_{12}} \) could be used for the detection of margarine in butter.

A method for the detection of interesterified fats (made mainly from beef fat) in butter was suggested by Huyghebaert (1967). Such fats were difficult to detect by means of fat constants like Reichert, Polenske, Xylol and 'A+B' values, because these fats had been so prepared that their analytical characteristics resembled those of butterfat.

The fatty acid fractions isolated from butterfat and different types of interesterified fats after the Reichert, Polenske, Xylol and 'A+B' values determinations were examined by the GLC and the ratio \( \frac{C_4}{C_6} \) acids was determined. This ratio for the Reichert fraction from butterfat was found to be 2, while for interesterified fats it varied from 24 to 97. Detection of adulteration of transesterified lard in butter on the basis of deficiency in percentage composition of \( C_6 \), \( C_8 \) and \( C_{10:1} \) fatty acids was suggested by Kufferath (1968).

A commercial sample labelled as pure butter was analysed by Parodi (1969) to determine its authenticity. The chemical constants of the sample such as the Reichert, Polenske, Kirschner, saponification and iodine values, together with the refractive index, were all within the range of natural milkfat. However, on the basis of sterol analysis and fatty acids composition by the GLC, it was concluded that the sample contained vegetable fat. The same author has also studied determination of modified milkfats and interesterified fats (Parodi, 1972). Recently it has been observed by Hendrickx & Huyghebaert (1970) that to detect substitute fat in butterfat on the basis of classical tests for
butterfat was difficult. In the GLC, methyl esters and unsaponifiable fractions show differences and establish the presence of substitute fat in butterfat. In a later review these authors (Hendrickx and Huyghebaert, 1971) suggest use of more sophisticated technique like infrared spectroscopy.

2.4. Studies with the Unsaponifiable Matter.

2.4.1. The Phytosteryl Acetate Test: In addition to the triglycerides and free fatty acids, fats from vegetable and animal sources contain unsaponifiable matter which amongst other constituents, includes sterols and tocopherols. Methods have been suggested to detect vegetable fats in ghee by isolating the unsaponifiable fractions and examining these for the nature of sterols, or an excess of tocopherols. The phytosteryl acetate test described by Bömer as early as 1901 based on the earlier work of Salkowski (1887) is still used for detecting the adulteration of butterfat with vegetable fats (A.O.A.C., 1970). The test is based on the differences between the cholesterol occurring in animal fats and phytosterols occurring in vegetable fats. Further, the crystalline form of cholesterol differs from that of phytosterols when examined under the microscope. The melting point of cholesteryl acetate is 118°C, while that of phytosteryl acetates depends on the character of the fat from which these originate and generally lies between 125°C and 137°C. As cholesteryl acetate is more soluble in alcohol than phytosteryl acetates, repeated crystallisation from 96 per cent ethyl alcohol raises the m.p. of the steryl acetates if vegetable fats are present in the mixture. The phytos-
teryl acetate test is the most reliable test for detecting the presence of vegetable fats in ghee. Adulterations as low as 3 per cent could be detected. Whilst several methods are available for this determination, the method most commonly used by the Public Analysts in India is the one standardised by Hawley (1933). Phytosteril acetate determination has been included in the national (IS:3508) and international standards (FIL-IDF:32). Modern methods such as the TLC (FIL-IDF:38) and GLC (FIL-IDF:54) have also been standardised. The latter technique has been useful in detecting synthetic butterfat (Roos, et al, 1969). Neither the quantitative distribution of the unsaponifiable matter, nor the differences in the nature and form of sterols present in butterfat and animal body fats have received much attention from research workers.

2.4.2. Chromatography Technique for the Examination of Unsaponifiable Matter: Chromatography techniques are being increasingly used for the study of the unsaponifiable matter and especially for the identification of sterols.

2.4.2.1. A paper chromatography technique for the characterisation of unsaponifiable matter of ghee was suggested by Ramchandra & Dastur (1959). It was shown that it was possible to detect the presence of Vanaspati and 5 per cent animal body fats in ghee, using a solvent system methyl alcohol, petroleum ether and water (80:10:10) by measuring the distance cleared from the place of spotting the samples. The spots were visible under U.V. light, or when exposed to iodine vapours. Ghee from animals fed 4 kg or more of cotton-
seeds behaved like adulterated ghee on the chromatogram.

2.4.2.2. A TLC technique for the unsaponifiable matter to detect foreign fats in butterfat was suggested by McGugan (1959). The glass plates coated with a mixture of silicic acid containing plaster of Paris were spotted with 25 μg of unsaponifiable matter solution. The spots were developed with 5 per cent ethyl acetate in n-hexane. The spots were made visible by spraying with 10 per cent nitric acid in concentrated sulphuric acid and charring the plates by heating. It was shown that adulteration at 10 per cent level could be detected. A TLC method for detecting cholesterol and phytosterols using the unsaponifiable residue from the fats has also been developed by Ramamurthy, et al., (1967). Adulteration of milkfat with cottonseed oil, groundnut oil, sesame oil, hydrogenated fats at 10-13 per cent level, and of coconut oil at 25 per cent level, could be detected by the method. The TLC has been used for the detection of 5 per cent interesterified substitute fats in butterfat (Huyghebaert & Hendrickx, 1968, 1970). The unsaponifiable fraction of the butterfat samples containing substitute fat gave special spot on the chromatogram which was absent in the case of pure butterfat.

2.4.2.3. The GLC technique has been increasingly used during recent years in the detection of adulteration of butterfat, especially in the identification of sterols (Copius Peereboom, 1963; Cannon, 1964; LaCroix, 1970; Thorpe, 1969, 1970). Boniforti (1962) noted that the C_{12}:C_{10} ratio was 1.0-1.2 for genuine butter but higher
values were obtained for adulterated samples. Detection of adulteration of butter with lard at 5 per cent level, containing small proportion of vegetable fat, has been reported by Guyot (1960) by GLC analysis of sterols. Hendrickx & Huyghebaert (1979) utilised the detection of stigma sterol in vegetable fats to detect adulteration in butter using GLC.

2.4.3. Tocopherol Estimation: Tocopherols are important constituents of unsaponifiable matter obtained from butter-fat and vegetable fats. The tocopherol contents of several vegetable fats exceed those of milk fats. Accordingly, an elevated tocopherol content should point to the presence of added vegetable fat. Several workers have successfully exploited the possibility of using this method for the detection of adulteration of ghee (Mahon, et al., 1955; Nazir & Magar, 1959). The method is, however, not so specific as the phytosteryl acetate test and cannot be used for establishing the presence of animal body fats. It is thus only of passing interest in context of the present studies. Alpha-tocopherol is the principal form present in milkfat whereas vegetable fats contain considerable quantities of non-alpha forms. A colorimetric method to determine the concentration of the non-alpha forms was developed by Shipe (1955-b). The presence of a detectable amount of the non-alpha tocopherol in dairy products was claimed to be an evidence of adulteration with vegetable fats.
2.5. **Some Physical Characteristics of Ghee:**

Ghee has several physical properties which distinguish it from fats of vegetable and animal origins commonly used as adulterants. However, due to the change in the composition of ghee with diet, season, breed, nutrition status of the animals, and stage of lactation, the common physical properties like the density and specific gravity, refractive index, melting point, solidifying point, etc., show a wide range and often merge with the values for other fats (Doctor, et al., 1940). As physical properties are mostly additive in nature, it is possible to prepare a mixture with two or more natural or processed fats which will have the superficial properties of butterfat. For this reason physical characteristics like texture, grain structure, refractive index are only used as rapid sorting tests. Ghee could be fractionated by allowing to stand at different temperatures after melting, crystallization from pure solvents or mixtures of solvents at different temperature, and fractionated with chemicals like urea, etc. Such fractions contain selected glycerides and fatty acids which gives each fraction a distinct character differing from those of the original butterfat and from fractions isolated from other fats under identical conditions in their yield, solubility, refractive index, melting point, and such other properties. Thus a number of studies have been carried out to devise simple physical and physico-chemical tests for detecting adulteration by taking advantage of the physical properties. Some of these properties are discussed below.
2.5.1. Melting Point, Titre Value, Clouding Temperature and Differential Thermal Analysis (DTA) of Butterfat.

2.5.1.1. Melting Point: The m.p. of butterfat is known to vary over a wide range extending from less than 28° to 43.5°C (Godbole & Sadgopal, 1933). The range for cows' ghee was from less than 28° to 41.0°C and for buffaloes' ghee it was 32.0° to 43.5°C. Amongst the 200 samples examined, ghee from cottonseed feeding tracts were not included, else the upper limit would have been further extended. Due to such variations the value is of little use in detecting adulteration. As no data on the m.p. of ghee from cottonseed fed animals were available a few preliminary trials were carried out in the course of the present studies to confirm that this characteristic was of no value for judging the quality of ghee. The temperature at which melted butterfat begins to become opaque has also been studied. Zurnini (1933) found the clouding temperature for pure butterfat to lie between 19° to 20°C. The solidification temperature, i.e., the point where fat shows first signs of solid phase, of milkfat very much depends on the cooling procedure employed as discussed in detail by Webb & Johnson (1965).

The turbidity technique was used by Venkatachalam & Sundaram (1957) to detect the presence of mineral oils in butterfat. Some work on this aspect has been included in the present studies as the results of opacity of ghee under specified conditions showed interesting results when animal body fats were present.

2.5.1.2. The Titre Test: Several workers have used the
solidifying point of the fatty acids of ghee to judge its purity. In a survey carried out by the Directorate of Marketing & Inspection, Government of India, the titre value of 57 samples of ghee from the cottonseed feeding area was noticed to vary from 40.4° to 44.6°C. The Reichert value of these samples ranged from 21.3 to 26.3. At one time the Titre Value was included as a test for judging the quality of ghee produced in cottonseed feeding tracts but later dispensed with as it was not of much value. Venkatachalam (1937) proposed a relationship between the Titre Value (T) of the residual fatty acids in the Reichert value estimation and its refractive index (R) at 45°C represented as:

\[ T + (R-1.4000) \times 1000 + (R-1.4440) \times 1000. \]

For ghee the value found was between 84 and 86. Any value above 86.4 was regarded as an indication of adulteration. It was possible to detect 10 per cent adulteration with animal body fats. However, no trials were carried out with ghee from cottonseed fed animals.

2.5.1.3. DTA Technique: The transition of butterfat from a solid to a liquid state has been studied with the help of the sensitive DTA technique. The latest work of Roos & Tuinstra (1969) with Dutch butterfat showed that addition of 5 to 10 per cent beef tallow in butter changed the solidification curve. The solidification curves showed that on the addition of beef tallow solidifications started at higher temperature and the solidification took place in two steps.

2.5.1.4. Cryoscopic and Heat of Dissolution Studies: A cryoscopic method was used by Pathak, et al, (1943) to study
the molecular weights of ghee to judge its purity. A 4 per cent solution of fat in benzene gave a range of 665-682 for buffaloes' ghee and 660-675 for cows' ghee. Fats from vegetable sources and body fats gave higher values, with the exception of coconut oil which had a range of 605-610. Lard gave molecular weight of 775-778 and tallow 785. Development of free fatty acids and rancidity lowered the molecular weight of ghee. The authors suggest that the method could be used to detect adulteration over 10 per cent. With a similar object Puri, et al, (1964) studied the heat of dissolution of ghee and other fats in benzene and found the values to range from 4.52 to 5.40 cal./g with an average of 5.05 cal./g. Hydrogenated vegetable fats gave a value of 12.87, goat tallow 15.31, Lard 3.14. Vegetable oils had values ranging from 1.50 to 3.04.

2.5.2. Density and Specific Gravity of Butterfat: Studies by Jenness, et al, (1942) showed that fat extracted from milk with acetone had an average density of 0.8892 g/ml at 60°C, and the change in density in the range 30º - 60ºC was 0.00070 g/ml per ºC. The authors observe from these studies that the density of purified butterfat was relatively constant and was not affected to a marked extent by breed, season, or feed. This determination, therefore, was included in the current studies to see how far sp.gr. could be applied to detect adulteration, or to distinguish ghee from cottonseed fed animals.

2.5.3. Critical Temperature of Dissolution (CTD):

2.5.3.1. A number of investigations based on the CTD
with different solvents to detect adulteration of ghee with vegetable fats, mineral oils and body fats have been reported in the literature beginning with the paper by Crook (1879) who used a mixture of carbolic acid and water (10:1) and found two distinct layers to occur in the presence of animal fat. Much later Sanyal (1929) found that when ghee was obtained from animals not fed on cottonseeds a mixture of dry ether and 95 per cent ethyl alcohol (3:4) was effective in detecting adulteration with beef tallow using a temperature of 30°C. When ghee was from cottonseed fed animals, the same solvent mixture in the proportion of 4:3 was useful. A mixture of dry acetone and absolute alcohol (65:35) at 30°C was used by Venkatachalam (1937) to detect hydrogenated fats, mutton and beef fats in ghee. A more systematic study was carried out by Felman & Lepper (1950) who found a mixture of 95 per cent (volume) ethyl alcohol and amyl alcohol (b.p.128°-132°C) in the ratio 2:1 very effective for distinguishing margarine from butter. The same solvent was found effective by Prakash et al, (1956) for detecting 10-15 per cent tallow in ghee. The CTD for genuine ghee was found to range from 29°- 45°C, that of Vanaspati from 62°- 72°C (Bhide & Kane, 1952). A market ghee sample showing CTD of 47°C was shown to contain sesame oil. In recent work, Delforno (1964,1965) has examined a large number of samples of butter and other fats using the critical temperature of solution in absolute alcohol (Crimser Index). For genuine butterfat the average critical temperature of solution was 54.07°C and the range
found was 50.0°- 58.0°C. Values outside the range indicated adulteration.

2.5.3.2. Fractionation of Ghee: A variation of the CTD technique which many workers have found more effective was the precipitation of glycerides at different temperatures by allowing the melted sample to stand, or precipitation at different temperatures after first dissolving in a suitable solvent, or precipitation with urea and other reagents. Bhalerao & Kummerow (1954) fractionated 10 per cent solution of fats by first dissolving in hot absolute alcohol and then allowing the solution to stand for 2 hr at 20°C. The soluble and insoluble fractions were used for refractive index determination. The percentage of soluble fraction in butterfat was 70 ± 4 per cent. The technique was successfully used by the authors to detect adulteration with vegetable fats. Detection of butterfat adulteration by separation of fatty acids in urea-complexes was first suggested by Holosek & Ibrahim (1953). A method based on the above technique was used by Shipe (1955-a) for the identification of vegetable and animal fats in butterfat. The fatty acids from butterfat were fractionated by using 10 per cent and 20 per cent solution of urea in methanol to form urea fatty acids complexes. The selectivity of the fractionation procedure was demonstrated by measuring the refractive indices of the fractions to enable their detection. A similar technique was adopted by Tawde & Nagar (1957) in their studies, using only the steam non-volatile fatty acids, combined with precipitation with urea at 5° to 7°C. Three
fractions were collected with different concentrations of urea. It was found that in the 3 butterfat samples from different areas, the analytical values for the fractions were different.

2.5.3.3. From the above review of the literature it appears that though CTD and allied methods have been examined by several workers no definite results have emerged that would enable a reliable test for detecting the presence of animal body fats, as distinct from butterfat secreted by animals fed cottonseeds.

2.5.4. **Ultra-Violet and Infra-Red Spectroscopy.**

2.5.4.1. U.V. and infra-red spectroscopy have been used by different workers for the detection of foreign fats in butterfat. U.V. spectrophotometry for the detection of fish oils in edible fats was used by Franzke (1964). The \( \frac{E_{1%}}{lc} \) value measured at 315 nm after alkali isomerization was found to be under 1.0 for animal and vegetable fats but 12-64 for fish oils. This difference was due to the content of tetraene acids. Between 2 to 8 per cent fish oil in fats was detected by the technique. Similar studies were carried out with butter, margarine and their mixtures by Rego, et al. (1964). The dried fats were dissolved in hexane and U.V. spectra at 220-330 nm examined. It was observed that genuine butter gave a peak with a maximum at about 232 nm. Using this peak, a new constant \( \Delta \) was defined as \( \Delta = \frac{1}{2}(a-220) \times (K_{\text{max}} - K_{220}) \), where \( K_{220} \) and \( K_{\text{max}} \) were extinction coefficients at 220 nm and at the maximum of the peak, and 'a'
the wave-length at which the extinction coefficient equals that at 220 nm. Values of $A$ for butterfat varied between 27.6 and 82.1 (average 55.3), and for margarine was zero. Adulteration of butterfat with margarine at 10 per cent level could be detected by this method.

2.5.4.2. Infra-red spectroscopy was applied by different workers for the detection of hardened fats containing isoleic acids (trans-octadecenoic acid) that showed a characteristic absorption maximum at 10.36 $\mu$. This technique was used by Bartlet & Chapman (1961) for demonstrating the presence of hardened fats in milk fat. Absorption spectrum of a 4 per cent fat sample in carbon tetrachloride was recorded in the region of 10 $\mu$ (at 967 cm$^{-1}$ and 948 cm$^{-1}$). It was observed that hardened fats increased the absorption at 967 cm$^{-1}$. Butter and butter-like interesterified fats were compared by measuring infra-red spectra and dielectric constants by Luck & Kohn (1963) and a detection limit of 20 per cent level was suggested. Infra-red spectroscopy for the detection of foreign fats in milk fat was recently tried by de Ruig (1963). Butterfat was characterised using the isolated trans-absorption band at 10.34 $\mu$ (967 cm$^{-1}$) and the cis-trans conjugated band at 10.5 $\mu$ (943 cm$^{-1}$). In the case of tallow added to butterfat when in solid state, an absorption band at 10.35 $\mu$ (920 cm$^{-1}$) appeared.

2.5.4.3. The general indication thus is that spectroscopic methods unless some special constituent is involved, are not sensitive to detect small percentages of adulteration.
Such methods can be used only for confirmation under special conditions but not as routine tests.

2.6. Addition of Tracer Substances to Check Adulteration.

2.6.1. With a view to provide a rapid and reliable tool to the analyst and the consumer to identify added foreign fat to butterfat, addition of some tracer substance has been suggested. A tracer substance can be latent which is specifically identified by its reaction with certain chemicals, or it may impart direct colouration distinct from that of the natural colour of butterfat. Addition of sesame oil to hydrogenated fats has been successfully adopted in several countries (Baudouin test). Such an addition is feasible when the concerned fat is processed only at central places. In India there is no industry for processing edible animal fats for culinary purposes. Thus it is not feasible to implant any tracer substance or visible colour in animal body fats. The subject has been ably reviewed by Subrahmanyan, et al., (1952). As it was not relevant to the present study, it has not been detailed here once again.

2.7. Summary and Objects of the Present Study.

2.7.1. From the above summary of the literature, it is seen that as yet no simple reliable test is available that could be used in the routine analysis of ghee for checking adulteration with animal body fats. Many promising methods have been tried with samples collected from a small area. Under the conditions prevailing in India the problem is
complicated by the fact that some of the best ghee producing areas are located in cottonseed feeding tracts where cottonseeds are fed to the extent of 4 to 6 kg per day in place of concentrates. Apart from the ready availability of the seeds, there is a strong belief that cottonseed feeding increases the fat percentage of milk. As milk is purchased on the basis of its fat content, it is difficult to convince the farmers to give up feeding cottonseeds entirely. Even where balanced concentrates have been introduced, feeding of concentrates and cottonseeds is alternated. On feeding cottonseeds, the secreted milkfat readily takes up many of the physical and chemical properties resembling those of animal body fats. There is free movement of ghee all over the country for the purpose of marketing and the work of the Public Analyst for checking the purity of ghee is made all the more difficult.

2.7.2. In course of the present studies a fresh attempt has been made to study the characteristics of the body fats from the buffalo, the goat, the ewe and the swine, along with those of ghee, and their mixtures. Simultaneously, market ghee samples, and ghee produced in cottonseed feeding tracts have been examined, along with samples from animals fed different quantities of cottonseeds under controlled conditions. Under field conditions, feeding of cottonseeds is accompanied by much lower intake of green feeds. This dependance on dry feeds such as straw aggravates the change in the composition of ghee so that it closely resembles body
fats. Ghee from cottonseed fed animals gives larger grains on cooling, a characteristic preferred by certain class of consumers, has little of the liquid phase on the top when packed in tins, and is known to have better storage life. Such ghee has a lower content of vitamin A (Patel & Ray, 1949).

2.7.3. Besides the determination of the conventional analytical constants like the Reichert, Polenske, iodine and saponification values, the specific gravity and the melting point of the fat samples have been examined. Samples of ghee were stored for a year and examined at intervals. The optical density of the melted fat samples, cooled under controlled conditions, was studied in detail as the preliminary work showed promising results. The yields and melting points of isolated glycerides and their fatty acids (Bömer number) were determined. Technique of TLC, GLC and U.V. spectroscopy have been used to identify specific characters which could be employed to detect adulteration, the object being that the method should be able to detect animal body fats at a level of 5 to 10 per cent without giving a false indication in the case of fat secreted by cottonseed fed animals. In course of the studies, it was observed that cow ghee from cottonseed fed animals lost carotene faster. This led to the study of the behaviour of ghee when mixed with methylene blue, resazurin, triphenyl tetrazoleum chloride and 2,6 dichlorophenol indophenol. An interesting observation emerged that ghee from cottonseeds fed animals had the property of discharging rapidly the colour of methylene blue. Neither ghee
body fats, or other commonly used vegetable fats had this property. This observation is expected to provide a simple reliable technique for the preliminary sorting of samples to identify if these are from cottonseed fed animals or adulterated with animal body fats. After such a preliminary sorting, more precise tests like the TLC could be used to definitely establish the presence of animal body fats. These results are described and discussed in the following chapters.