STUDIES ON UNSAPONIFIABLE MATER OF GHEE (CLARIFIED BUTTERFAT) AND ANIMAL BODY FATS WITH A VIEW TO DETECT ADULTERATION

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN DAIRY CHEMISTRY

BY
SURESH KUMAR SHARMA

DIVISION OF DAIRY CHEMISTRY
NATIONAL DAIRY RESEARCH INSTITUTE
(I.C.A.R.)
KARNAL (HARYANA) INDIA
1989

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Dedicated

to

My Father

Late Sh. C. D. Sharma

and

Sister Parveen

That Science is the greatest as age demands which makes us to know the external secret of Nature, changeful, evanescent, the world of death, of woe, of misery but the science of Him who changes not, the Blissfull one, where alone is peace, where alone is life eternal, where alone is perfection, where alone all misery ceases - that according to our ancestors was the sublimest science of all. **Even Gita says, "It is I whom four Vedas seek to know".**

The inspiration of science atleast to me has been from the very spirit of science, **Be pure, and, believe and uphold truth, you will have the power to stand up a giant.** On par excellence, it is the science that challenges and wrecks the hidden mystery of Nature that surrounds a man as a volcano of trasure of knowledge for him more to think, insight, search, wreck and progress to a high realm. The assimilation of a bit of science has upholded belief in me, that for a better Science none can prevent it from seeking the inherent truth.

Arise, awake, and stop not till the desired result is reached is the line of life in Science. That fruits of the result spreads throughout the universe, it decays and rots and out of that decay sprouts the root and the future tree (Young Scientist), perhaps mightier than the first one.

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(SURESH KUMAR SHARMA)

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November 20 , 1989

CERTIFICATE

This is to certify that the thesis entitled **"Studies on unsaponifiable matter of ghee (clarified butterfat) and animal body fats with a view to detect adulteration"** submitted by Mr. **Suresh Kumar Sharma** in partial fulfilment of the requirements for the award of Degree of **Doctor of Philosophy** in Dairy Chemistry of the National Dairy Research Institute (Deemed University), Karnal (Haryana), India is a bonafide research work carried out by him under my supervision and guidance, and no part of the thesis has been submitted for any other degree or diploma.

(O.P. SINGHAL)
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C O N T E N T S

contd.....

Chapter **Page**

contd....

contd....

Chapter Page

contd.....

Chapter **Page**

contd....

Chapter **Page**

contd....

Chapter **Page**

contd.....

LIST OF TABLES

contd...

LIST OF FIGURES

contd....

contd....

ABBREVIATIONS

BR	:	Butyro-refractometer reading
$CaCl_2$	:	Calcium chloride
$CaCO_3$	:	Calcium carbonate
CTD	:	Critical temperature of dissolution
DTA	:	Differential thermal analysis
Fig.	:	Figure
GLC	:	Gas liquid chromatography
HCl	:	Hydrochloric acid
HMDS	:	Hexamethyl disilazane
H_2SO_4	:	Sulphuric acid
IDF	:	International Dairy Federation
IR	:	Infra-red
ISO	:	International Organization for Standardization
KOH	:	Potassium hydroxide
PFA	:	Prevention of Food Adulteration Act
PV	:	Polenske value
RM	:	Reichert-Meissl
TLC	:	Thin layer chromatography
TMCS	:	Trimethyl chlorosilane
TMS	:	Trimethyl silyl

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 Ghee (clarified butterfat) is by far the most important indigenous milk product and has an important place in Indian dietary from the ancient days. It is considered superior over other fats nutritionally because of rich source of lipid nutrients such as fat soluble vitamins and essential fatty acids. It contributes good flavour, pleasant aroma, efficient energy and comfortable feeling through normal intervals between the meals. The rich pleasing flavour of milkfat is not easily duplicated by any other fat. Butterfat as a cooking media has advantages over other cooking fats, particularly for the persons suffering from digestive disorders. Ghee is the only animal fat universally acceptable by both "vegetarian" and "non-vegetarian" population in India for culinary purposes. Several medicines under Ayurvedic and Unani systems are made or served with ghee as the base. It is indeed prized as the most important article of diet by Indians and no Indian kitchen is considered perfect without a tin of ghee.

1.2 MANUFACTURE AND MARKETING OF GHEE

Ghee manufacture is the most convenient method of preserving the surplus milk as it has long shelf life compared to other milk constituents. Different methods of manufacture and different temperatures of clarification

of cream/butter into ghee are used. Depending upon the regional preferences, the temperatures of clarification may vary from 80° to 130°C (Srinivasan, 1945; Rangappa and Achaya, 1948). The method used is generally adaptable for small or large scale manufacture. Most of the ghee coming to the market is prepared on a small scale essentially by the desi method under village conditions by converting raw or boiled milk into curd, churning the curd to obtain butter and converting butter into ghee. During the last two decades, some of the ghee about 15-20 per cent is being manufactured on a large scale by milk plants and the dairy industries from the surplus milk either by direct cream or cream butter method.

Generally buffalo milk which is rich in fat is used. With the rising demand for liquid milk and improved marketing facilities provided by the milk schemes under private and public sector enterprises, the quantity of milk used for the preparation of ghee is decreasing year by year. In the year 1951, an estimated amount of 39.3 per cent of the total milk produced was utilized in making ghee whereas in 1987-88 nearly 28 per cent (about 13 million tonnes of milk) is reported to be utilized in making ghee (Dairy India, 1987).

1.3 ADULTERATION OF GHEE

The supply of ghee is far short of demand. This coupled with high price (current average price about Rs.65/ kg) led to several malpractices. To extend the available supply in order to contain the increasing demand and to

earn more profit, ghee is adulterated with cheaper fats known as adulterants. The commonly used adulterants are (i) vegetable oils and fats, and (ii) animal body fats. Besides these adulterants, refined fish oils are also used for adulteration of ghee. The problem of adulteration is unique not only in India but all throughout the world. The adulteration of ghee in India bears a close analogy to the adulteration of butter with margarine in several European countries. Of the several adulterants, the vegetable fats and oils provide the most convenient source for adulteration of ghee. The introduction of present day refining and hydrogenation of vegetable fats/oils have provided such artifically blended ghee substitute not easily distinguished from the normal ghee.

1.4 STANDARDS FOR GHEE

The detection of adulteration in ghee under Indian conditions is complicated by the fact that there are considerable variations in its composition due to diverse feeding practices. For this reason different standards have been prescribed for ghee to meet the regional requirements. Apart from the standards prescribed for each state, under the Prevention of Food Adulteration Rules (1955), there are Agmark Standards prescribed by the Directorate of Marketing and Inspection, Government of India. Ghee packed under Agmark is marketed throughout the country irrespective of the local standards. These standards are shown in Tables 1 to 4.

Table 1. Standards for ghee for different States/Union Territories (Prevention of Food Adulteration Rules, 1955) Amended upto 1976

Ghee means the pure clarified fat derived solely from milk or from curd or from *desi* (cooking) butter or from cream to which no colouring matter or preservative has been added

Sl. No.	State/Union Territory	Butyro-Refractometer reading at 40°C	RM value (minimum)	Percent	
				Free fatty acids (as oleic acid) (maximum)	Moisture content (maximum)
1	2	3	4	5	6
1.	Andhra Pradesh	40.0 - 43.0	24	3.0	0.5
2.	Arunachal Pradesh	40.0 - 43.0	26	3.0	0.5
3.	Assam	40.0 - 43.0	26	3.0	0.5
4.	Bihar	40.0 - 43.0	28	3.0	0.5
5.	Goa	40.0 - 43.0	26	3.0	0.5
6.	Gujarat:				
	(a) Cotton-tract areas	41.5 - 45.0	21	3.0	0.5
	(b) Areas other than cotton-tract areas	40.0 - 43.5	24	3.0	0.5
7.	Haryana:				
	(a) Cotton-tract areas	40.0 - 43.0	26	3.0	0.5
	(b) Areas other than cotton-tract areas	40.0 - 43.0	28	3.0	0.5
8.	Himachal Pradesh	40.0 - 43.0	26	3.0	0.5
9.	Jammu & Kashmir	40.0 - 43.0	26	3.0	0.5
10.	Karnataka:				
	(a) Belgaum district	40.0 - 44.0	26	3.0	0.5
	(b) Other areas	40.0 - 43.0	24	3.0	0.5

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1	2	3	4	5	6
11.	Kerala	40.0 - 43.0	26	3.0	0.5
12.	Madhya Pradesh:				
	(a) Cotton-tract areas	41.5 - 45.0	21	3.0	0.5
	(b) Areas other than cotton-tract areas	40.0 - 43.0	26	3.0	0.5
13.	Manipur	40.0 - 43.0	26	3.0	0.5
14.	Meghalaya	40.0 - 43.0	26	3.0	0.5
15.	Mizoram	40.0 - 43.0	26	3.0	0.5
16.	Nagaland	40.0 - 43.0	26	3.0	0.5
17.	Orissa	40.0 - 43.0	26	3.0	0.5
18.	Punjab	40.0 - 43.0	28	3.0	0.5
19.	Rajasthan:				
	(a) Jodhpur Division	41.5 - 45.0	21	3.0	0.5
	(b) Other areas	40.0 - 43.0	26	3.0	0.5
20.	Sikkim	40.0 - 43.0	28	3.0	0.5
21.	Tamil Nadu	41.0 - 44.0	24	3.0	0.5
22.	Tripura	40.0 - 43.0	26	3.0	0.5
23.	Uttar Pradesh	40.0 - 43.0	26	3.0	0.5
24.	West Bengal:				
	(a) Bishnupur sub-division	41.5 - 45.0	21	3.0	0.5
	(b) Other areas	40.0 - 43.0	28	3.0	0.5
Union Territories					
25.	Andaman & Nicobar	41.0 - 44.0	24	3.0	0.5
26.	Chandigarh	40.0 - 43.0	28	3.0	0.5

contd.....

contd..... table 1

1	2	3	4	5	6
27.	Dadra & Nagar Haveli	40.0 - 43.0	24	3.0	0.5
28.	Delhi	40.0 - 43.0	28	3.0	0.5
29.	Daman & Diu	40.0 - 43.5	24	3.0	0.5
30.	Lakshadeep	40.0 - 43.0	26	3.0	0.5
31.	Pondicherry	40.0 - 44.0	26	3.0	0.5
		Baudouin test shall be negative			

Explanation: By cotton tract is meant the areas in the States where cottonseed is fed extensively to the milch animals and so notified by the State Government concerned.

Table 2. Agmark Standards for ghee produced in areas other than cotton tracts (Directorate of Marketing and Inspection, Government of India), Ghee Grading and Marking(Amendment Rules, 1969)

	Grade designation		
	Special	General	Standard
Baudouin test	Negative	Negative	Negative
Butyro-Refractometer reading at 40°C	40.0-43.0	40.0-43.0	40.0-43.0
Reichert-Meissl value (minimum)	28.0	28.0	28.0
Polenske value	1.0- 2.0	1.0- 2.0	1.0- 2.0
Moisture content (maximum)	0.3%	0.3%	0.3%
Percentage of free fatty acids (as oleic acid) (maximum)	1.4	2.5	3.0

Table 3. Agmark Standards for ghee prescribed in cotton-tract areas (of Saurashtra and Madhya Pradesh)

	Grade designation					
	Special		General		Standard	
	Winter Sept-Feb	Summer March-Aug	Winter Sept-Feb	Summer March-Aug	Winter Sept-Feb	Summer March-Aug
Baudouin test	Negative	Negative	Negative	Negative	Negative	Negative
Butyro-Refractometer reading at 40°C	41.5-44.0	42.5-45.0	41.5-44.0	42.5-45.0	41.5-44.0	42.5-45.0
Reichert-Meissl value (minimum)	23.0	21.0*	23.0	21.0*	23.0	21.0*
Polenske value	0.5- 1.2	0.5- 1.0	0.5- 1.2	0.5- 1.0	0.5- 1.2	0.5- 1.0
Moisture content (maximum)	0.3%	0.3%	0.3%	0.3%	0.3%	0.3%
Percentage of free fatty aids (as oleic acid (maximum)	1.4	1.4	2.5	2.5	3.0	3.0

Explanation: By cotton-tract is meant the areas where cottonseed is extensively fed to milch animals

* Ghee with RM value between 19-21 shall be graded only after phytosteryl acetate test has been performed and the result thereof found to be negative

Table 4. Agmark standards for ghee prescribed in areas given below:

Sl. No.	Place	Baudouin test	Butyro-Refractometer reading at 40°C	Reichert-Meissl value (minimum)	Polenske value	Moisture content (max.) %	Percentage of free fatty acids (as oleic acid)		
							Special (max.)	General (max.)	Standard (max.)
1	2	3	4	5	6	7	8	9	10
1.	Andhra Pradesh Tamil Nadu & Maharashtra State	Negative	40.0-43.0	26.0	1.0-2.0	0.3	1.4	2.5	3.0
2.	Haryana Narnaul tract of Mahendergarh district	Negative	40.0-43.0	26.0	0.5-1.5	0.3	1.4	2.5	3.0
3.	Himachal Pradesh (Chamba dist.)	Negative	41.0-44.5	24.0	0.5-1.5	0.3	1.4	2.5	3.0
4.	Maharashtra State (Wardha & Amravati districts)	Negative	41.5-45.0	21.0	0.5-1.5	0.3	1.4	2.5	3.0
5.	North Gujarat (Patan area)	Negative	40.0-43.0	26.0	0.5-1.5	0.3	1.4	2.5	3.0

contd.....

contd..... table 4

1	2	3	4	5	6	7	8	9	10
6.	Round about Anand in Gujarat State	Negative	40.0-43.0	26.0	0.5-1.5	0.3	1.4	2.5	3.0
7.	Rajasthan (except Jodhpur Division)	Negative	40.0-43.0	26.0	1.0-2.5	0.3	1.4	2.5	3.0
8.	Rajasthan (Jodhpur Division)	Negative	41.5-45.0	24.0	0.5-2.0	0.3	1.4	2.5	3.0
9.	West Bengal (Bishnupur Sub-division	Negative	41.5-45.0	24.0 (For standard grade, not less than 21.0)	0.7-1.4	0.3	1.4	2.5	3.0

Note: Subject to the condition that when PV is above 1.5, the lot should be graded only after performing the phytosteryl acetate test

1.5 EFFECT OF COTTONSEED FEEDING ON THE COMPOSITION OF GHEE

It has been observed that milk fat obtained from animals fed extensively with cottonseeds acquire characteristics similar to ghee adulterated with animal body fats. There is a common belief among the farmers that feeding of cottonseeds to milch animals is advantageous for increasing the fat percentage of milk. Thus the milch animals are commonly fed with whole cottonseeds and cottonseed cakes in the areas where cotton crop is grown. Ingestion of cottonseeds alters the composition of milk fat secreted markedly. Bailey (1948) has reported that feeding of cottonseeds increases the melting point of ghee and indirectly enhances its shelf life. Achaya and Banerjee (1946) reported that ghee obtained from buffaloes fed normal ration had RM value of 37.4 whereas ghee obtained from buffaloes fed with liberal quantities of cottonseeds (especially Saurastra in Gujarat) in summer season when fodder is not easily available had RM value of 20.7. Cottonseed feeding results a considerable decrease in the molar percentage of lower chain fatty acids (especially butyric acid) and marked increase in the molar percentage of higher saturated fatty acids. It is apparent that extensive cottonseed feeding to milch animals results in the lowering of RM value and increasing of BR reading. The RM value of ghee obtained from cottonseed fed milch animals normally varies from 19 to 28. However, under the PFA Rules (1955), a provision has been made whereby low value of RM and high value of BR reading have been prescribed as legal standards for ghee produced in cotton-tract areas.

1.6 DETECTION OF VEGETABLE FATS/OILS IN GHEE

To prevent the use of hydrogenated vegetable oils (vanaspati) in ghee, a provision has been made under Vegetable Oil Products Control Order (1947) for compulsory addition of 5 per cent sesame oil (Sesamum indicum) to vanaspati. In several European countries, the compulsory addition of sesame oil as a tracer in margarine is recommended to make detection of margarine in buttereasy by the Baudouin test. Sensitive methods like phytosteryl acetate test and Baudouin test are available for the detectioon of vegetable fats and oils, and vanaspati in ghee. However, the phytosteryl acetate test fails to differentiate between ghee and animal body fats as both ghee and animal body fats contain cholesterol.

1.7 DETECTION OF ANIMAL BODY FATS IN GHEE

The detection of animal body fats in ghee is a difficult problem as the resultant mass has physical and chemical properties similar to pure ghee. Further, the ghee obtained from the milk of cottonseed fed animals acquire properties similar to ghee adulterated with animal body fats. Woodman (1952) and Cocks and VanRede (1966) reported that depot fat and milkfat obtained from animal fed with cottonseeds often show a positive Halphen test but this test too has the disadvantage of being not always in all cases. Singhal, Ganguli and Dastur (1970) developed an opacity test for the detection of animal body fats in

ghee but the method failed in the presence of cotton-tract ghee. Later on Singhal et al. (1971) developed a methylene blue reduction test to distinguish normal ghee from the ghee obtained from cottonseed fed milch animals.

1.8 SCOPE OF THE STUDY

Considerable efforts have been made in the past to develop simple methods for the detection of animal body fats in ghee but yet no reliable test is available which could give undisputed results when both animal body fats and vegetable fats/oils are added to ghee. With the objective of removing this lacuna, the present study was planned and carried out on the detailed analysis of unsaponifiable matter of ghee and animal body fats. Unsaponifiable matter of ghee and animal body fats consists of a major portion of sterols besides other constituents such as tocopherol, vitamins, hydrocarbons etc. Though encouraging results have been reported by several workers in studies with unsaponifiable matter of milkfat, beef, lard, refined fish oils and vegetable fats/oils yet a detailed study of unsaponifiable matter of ghee and animal body fats with a view to detect adulteration has not been attempted so far. It was, therefore, decided to search for differences in the constituents of unsaponifiable matter of ghee and animal body fats using modern techniques like TLC, GLC and infra-red spectroscopy. Detection of animal body fats in ghee in the presence of vegetable oils, viz., vanaspati (hydrogenated vegetable oils), coconut oil and

refined grondnut oil is difficult. Attempts have been made to detect vanaspati, coconut oil and refined groundnut oil using P-hydroxy benzaldehyde test, TLC and turbidity test respectively. There are distinct differences in the constituents of free and bound sterols of ghee and animal body fats. The digitonides of free and bound sterols of the two fats will thus differ. Adopting such an approach, the present studies were planned, and the results obtained have been recorded. Before presenting the methodology and the results of the present study, it would be pertinent to give a review of the relevant literature to understand the approach adopted by earlier workers and the degree of success achieved.

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 METHODS USED FOR THE DETECTION OF ADULTERATION

Methods used for the detection of adulteration in ghee (butterfat) are based on the differences in the nature and content of major and/or minor components of ghee and adulterant fats. Methods generally used are based on the determination of physical and chemical constants,differences in the nature and content of specific fatty acids, fatty acid composition, glyceride structure, variation in the content of the components of unsaponifiable matter, differences in the nature and content of sterols, spectroscopic characteristics of fat and colour reactions. Butterfat on account of its rich pleasing flavour has a special appeal to the consumer and for this reason, it commands high price. These factors lend to the frequent adulteration of butterfat with cheaper fats from animal and vegetable sources. The problem is further complicated by the fact that a marked difference exists in the composition of milkfat from the cow and the buffalo, and under the prevailing conditions in India, the two species are generally maintained side by side by the farmers. The composition of butterfat also varies with the diet given to the animals and the season. The manufacture of substituted fats which resemble in their superficial properties to the natural properties of butterfat has further complicated the matter and encouraged the adulteration.

Extensive studies have established that vegetable fats could be detected unambiguously by the phytosteryl acetate test. However, the differentiation between milkfat and adulterant animal body fats is difficult as both contain cholesterol and often over 25 per cent of animal body fats could be masked. Due to the extensive literature available on the subject of detection of adulteration in milkfat, the following review of literature is discussed specially to the work dealing with the detection of animal body fats and vegetable fats/oils.

2.2 SPECIFIC METHODS

2.2.1 BAUDOUIN TEST FOR THE DETECTION OF VANASPATI IN GHEE

Under the provisions of Vegetable Oil Products Control Order (1947), addition of 5 per cent sesame oil to vanaspati has been made compulsory to detect the presence of vanaspati in ghee by Baudouin test. This method involves the development of a permanent pink colour with furfural in in the presence of concentrated hydrochloric acid in ghee adulterated with vanaspati. Kapur, Srinivasan and Subramanyan (1960a) used hydrofuramide and concentrated hydrochloric acid dissolved in alcohol to detect adulteration of ghee with vanaspati at 10 per cent level.

2.2.2 PHYTOSTERYL ACETATE TEST FOR VEGETABLE OILS AND FATS

The method was developed by Bomer (1901) based on the earlier work of Salkowski (1887) for the detection of vegetable fats in milkfat. The method is based on the differences between cholesterol present in animal fats and

phytosterols present in vegetable fats and oils. The melting point of cholesteryl acetate varies between 114-115°C while the melting point of phytosteryl acetates varies between 125-137°C depending upon the character of fat. The melting point of steryl acetates more than 117°C indicates the adulteration of ghee with vegetable fats. Further, the microscopic examination of sterols revealed an elongated hexagonal form with an apical angle of 108° for phytosterols and a re-entry angle for cholesterol-phytosterol mixture. This test could detect 5 per cent phytosterols in cholesterol-phytosterol mixture. The test has been included in National (BIS: 3508, 1966) and International (FIL, IDF, 32, 1965; ISO, 3595); standards. The phytosteryl acetate test for the detection of vegetable fat has been studied by German Federal Republic (1978), Den Herder (1955) and Cannon (1953,1956,1957).

2.3 METHODS BASED ON THE NATURE AND CONTENT OF COMPONENTS OF UNSAPONIFIABLE MATTER

The unsaponifiable matter of fats from vegetable and animal sources contain among other constituents, sterols and tocopherols. Several methods have been suggested for the detection of vegetable fats in ghee based on the examination of the nature and content of sterols and/or tocopherols isolated from the unsaponifiable matter. The advantage of the analysis of sterols is to detect not only the adulteration but also the nature of the adulterant fats. This has attracted the attention of analysts to study the constituents of unsaponifiable matter especially the sterols of milkfat and other edible fats.

.3.1 CHROMATOGRAPHY OF UNSAPONIFIABLE MATTER

Chromatographic methods being simple, precise and quick are being increasingly used for the study of unsaponifiable matter and especially the identification of sterols to detect adulteration in milkfat.

2.3.1.1 Paper chromatography

A Circular paper chromatographic method for the detection of vanaspati and animal body fats in ghee using a solvent mixture of methyl alcohol:petroleum ether:water (80:10:10, v/v) was developed by Ramachandra and Dastur (1959). The spot of unsaponifiable matter of ghee moved as a whole alongwith the solvent front while that of ghee adulterated with animal body fats at 5 per cent level or vanaspati at 10 per cent level did not move at all. Nath et al. (1959) used circular paper chromatography and a solvent system, butanol:acetic acid:water (4:1:5) for the detection of corn and groundnut oils in ghee. Unsaponifiable matter of corn and groundnut oils showed a spot with Rf value similar to 1,2-dienoglucose. Sulser (1959) and, Harke and Vogel (1963) using paper chromatography detected 5 per cent animal body fat in butter fat. Tetradecane impregnated paper and a solvent system of acetic acid-water-ethyl acetate (6:2:2) was also found to be equally sensitive for the detection of animal body fat in butter fat (Vitagliano, 1957). Descending paper chromatographic technique for direct resolution of sterols on paraffin impregnated paper and 84 per cent acetic acid as a solvent

was used by Peereboom and Roos (1960,1963) for the detection of 5 per cent animal body fats in butterfat and, by Gallego (1966) for the detection of margarine in butter based on the presence of ergosterol, stigmasterol and sitosterol in the unsaponifiable matter of margarine. Zotti et al. (1959) used this technique for the separation of unsaponifiable matter and sterol fractions isolated from unsaponifiable matter of vegetable oils and observed that the sterols from vegetable oils mainly consisted of B-sitosterol.

2.3.1.2 Thin Layer Chromatography (TLC)

Considerable work has been carried out using Thin Layer Chromatographic technique for the detection of adulteration. The use of TLC for the detection of adulteration is advantageous for quick and effective separation of sterol components. A TLC technique to detect adulteration of ghee at 10 per cent level with vegetable oils or animal body fats using silicic acid containing plaster of Paris as a binder and 5 per cent ethyl acetate in n-hexane as a developer was used by McGugan (1959). Coconut oil at 20 per cent in milkfat escaped detection by this method. Peereboom (1963) reported the separation, identification and estimation of sterols present in milkfat and other oils/fats by reverse phase TLC. TLC analysis of steryl acetates for the detection of vegetable fats in milkfat using reverse phase system and undecane/acetic acid-acetonitrile saturated with undecane as solvent system has been reported in International Standard (FIL, IDF, 1966). A technique similar to IDF (1966) was used by Roos (1966) to

detect vegetable fats in milkfat. The detection was based on the identification of phytosteryl acetate band at low Rf compared to cholesterol. Ramamurthy et al. (1967) used $CaCO_3$ + soluble starch (10 g + 4 g) plate impregnated with liquid paraffin and solvent system consisting of methyl alcohol:acetic acid:water (20:5:1, v/v) for detecting the presence of cottonseed oil, groundnut oil, sesame oil and hydrogenated fats at 10 to 13 per cent level and coconut oil at 25 per cent level in ghee on the basis of separation of cholesterol and phytosterols from the unsaponifiable residue. Butter/butterfat adulterated with substituted fat containing 2.5 to 5 per cent inter-esterified fat was detected by TLC of unsaponifiable matter (Huyghebaert, 1967; Huyghebaert and Hendrickx, 1968b). Unsaponifiable matter of butterfat/butter adulteratd with substituted fat gave a special spot which was absent in pure butterfat or butter.

Reverse phase TLC separation of unsaponifiable matter on Kieselgel HF_{254} has been described by Bachman (1969) to detect 5 per cent vegetable fats in milkfat. Kuzdzal-Savoei and Kuzdzal, 1969b) reported the detection of vegetable fats/oils in butter by TLC analysis of sterols, steryl acetates or trimethylsilyethers on silica gel G. Maglitto et al. (1970) employed TLC analysis of unsaponifiable matter to detect synthetic fat in butter on the basis of certain hydrocarbons. Parodi (1972) distinguished synthetic milkfat (a mixture of beef tallow, coconut oil and tributyrin with subsequent inter-esterification) from butterfat by a simple

procedure using silica gel G and solvent mixture of hexane: di-isopropyl ether, 80:20 and 93:7 v/v, respectively for examining free and esterified sterols. The synthetic milkfat contained mainly sterol esters of high molecular weight-fatty acids. Argentation TLC was used to detect 5 per cent phulwara butter in ghee and 10 per cent palm oil in vanaspati by Gupta and Sil (1983) and Sengupta et al.(1984), respectively.

2.3.1.3 Gas Liquid Chromatography (GLC)

The GLC technique has been employed in the recent past for the detection of adulteration of milk fat with vegetable fats especially based on the identification of sterols. A marked difference in the sterols of fats from animal and vegetable origin exists. Cholesterol is an entity of animal fats and phytosterols that of vegetable oils/fats. The two types of sterols differ in their nature, content and composition. Based on these differences, a number of publications appeared during 1960s and 1970s on GLC analysis of sterols for the detection of vegetable fats and substituted fats containing small proportion of vegetable fats/oils in butter and butterfat. Among phyto-sterols, mainly B-sitosterol has been used as an index of adulteration (Eisner et al., 1962; Eisner and Firestone, 1963; Guyot, 1967,1968a, Iwaida et al., 1967; Parodi, 1967, 1969; Lacroix, 1969; Roos,1967; Roos et al., 1969; Hendrickx and Huyghebaert, 1970b; Huyghebaert and Hendrickx, 1968a; Thorpe et al., 1969; Thorpe, 1970; Homberg and

Bielefeld, 1979; Zurcher et al., 1976, Colombini et al., 1978; Nakazawa, 1981).

Hendrickx and Huyghebaert (1970a) reported the presence in substitute fat of aliphatic ketones absent in butterfat.

The use of GLC technique for the analysis of sterols obtained from sterol digitonides for the detection of vegetable fats/oils in butterfat has been adopted by IDF (1970), British Standards (1977) and Taiwan National Bureau of Standards (1986). Martin et al. (1981) analysed unsaponifiable matter of milkfat and adulterant trans-esterified fat by GLC technique. The unsaponifiable matter of trans-esterified fats showed the presence of monoketones as major components which were absent in unsaponifiable matter of milkfat. The results were used to devise a method for detecting trans-esterified fats in milkfat.

2.3.2 TESTS BASED ON THE NATURE AND CONTENT OF TOCOPHEROL

Tocopherols are important constituents of unsaponifiable matter of vegetable and animal fats. It is known that tocopherol content of several vegetable fats exceeds that of milkfat (Lange, 1950). Accordingly, an elevated tocopherol content could be an index for the presence of added vegetable fats in milkfat. The content of tocopherol as an index of adulteration has been suggested by several workers. The method, however, is not specific for the detection of animal body fats as the content of tocopherol in animal body fats has been reported almost similar to milk fat. Reinart

(1949), Handwerk and Bird (1951), Anglin et al. (1955), Mahon et al. (1955a) and Narayanan et al. (1956) reported that the tocopherol content of butterfat lay between 10 ug/g and 45 ug/g of fat. The authors reported that vegetable fats and oils in ghee could be detected on the basis of tocopherol content. Mahon and Chapman (1954) developed a method (modification of Emmieri and Englers Colorimetric test) for the estimation of tocopherol content in vegetable oils and reported that tocopherol content of several vegetable oils (300-1600 ppm) exceeded that of ghee (maximum of 55 ppm). It was reported that ghee samples with tocopherol content more than 60 ppm could be suspected as adulterated with vegetable oils.

A colorimetric method to determine the concentration of non-alpha tocopherols was developed by Shipe (1955a). Alpha-tocopherol is the principal form present in milkfat whereas vegetable fats contain considerable quantities of non-alpha tocopherols. The presence of non-alpha tocopherols in dairy products was presumed to be an evidence of adulteration of milkfat with vegetable fats. Several other workers have also tried to exploit this technique for the detection of adulteration in butterfat (ghee) (Bird et al., 1954; Nazir and Magar, 1959; Markuze-Zofia, 1962).

2.4 METHODS BASED ON THE NATURE AND CONTENT OF THE 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 in butterfat.

Besides direct estimation, several emperical methods have been evolved. According to Roos (1963), Arzbacher, as early as 1849, made use of ether insoluble triglycerides for the examination of beef suet and mutton fat. Bomer (1913) published a method for detecting the presence of beef suet in lard on the basis that the ether insoluble triglycerides of lard contained B-palmitodistearin and those of beef suet contained ∝ -palmitodistearin. These two forms of ether insoluble triglycerides differed in their melting points. 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 different temperatures, 95°, 80°, 70° and 60°F(35-15.5°C) to yield various fractions. The differences in the Reichert value of these fractions showed the positive indication of adulteration at levels as low as 2% of foreign fats. A method based on the solubility of triglycerides in absolute alcohol was developed by Bhalerao and Kummerow (1954). The fat was separated into alcohol soluble and alcohol insoluble triglyceride fractions at 20°C and the refractive indices of these fractions were determined. The refractive index of alcohol soluble fraction was lowered with the addition of 10 per cent coconut oil while that of the insoluble fraction increased with the addition of vegetable fats. The same authors (1956a) refined this technique and the percentage of trisaturated glycerides in alcohol soluble fraction was estimated. Ten per cent foreign fat added in

butterfat was detectable by this technique. Latif and Mazloum (1969) fractionated fat samples in dry acetone at 20°, 0° and -10°C and the fat constants, viz., saponification, iodine, Reichert, Polenske and Kirschner values were determined for each fraction. The iodine value of butterfat was increased by the addition of 10 per cent vegetable fat whereas it was not altered by the addition of animal body fats. The presence of animal body fats in butterfat could be detected by the depression in Reichert and Kirschner values of various fractions.

Extensive work has been done by Hilditch and Lea (1927), Kartha (1953) and Hilditch and William (1964) on the transformation of unsaturated fatty acids by oxidation into azelaic acid and azeleo glycerides. It was observed that the addition of substituted fats to butterfat caused differences in the properties of azeleo-glycerides formed during oxidation. Movia and Remoli (1977) applied pancreatic lipase hydrolysis for detecting lard, beef and horse fat in butter from 2 monoglyceride composition. The detection was based on the ratios (per cent in 2 monoglyceride to per cent in fat) for myristic, palmitic, stearic and oleic acids.

2.4.1 CHROMATOGRAPHY OF WHOLE FATS

Chromatographic methods like paper, thin layer and gas liquid have been useful in the determination of triglyceride structure of fats to detect adulteration of foreign fats in butterfat.

2.4.1.1 Paper chromatography

The paper chromatographic analysis of triglycerides has been described by Jaky(1961) and Kaufmann and Schurbusch (1959). Kaufmann and collaborators (1959) separated triglycerides on paraffin impregnated paper using acetone: acetonitrile (3:1) as a solvent. A Circular paper chromatographic technique for the detection of common ghee adulterants was standardized by Ramachandra and Dastur (1960). Fifty per cent solutions of fats in Carbontetra chloride were spotted on the paper chromatogram and a solvent system, ethyl alcohol:isoamyl alcohol:Carbontetra chloride (35:65:10) was used. Normal ghee samples moved well away from the origin whereas cottonseed fed buffalo ghee did not move and behaved like adulterated ghee. It was possible to detect 10 per cent vanaspati and 5 per cent animal body fats in ghee by this technique. Paraffin impregnated paper was used by Chakrabarty et al. (1963,1966) to distinguish neutral fats from re-arranged fats on the basis of differences in the composition of their triglycerides. Pruthi and Gupta (1969) detected tallow at a level greater than 5 per cent in ghee by paper chromatography. However, detection in the presence of cotton-tract ghee was not possible.

2.4.1.2 Thin Layer Chromatography (TLC)

Thin Layer Chromatographic technique for the detection of hydrogenated groundnut oil, tallow and mohua oil was developed by Chakrabarty et al. (1968,1969). A solution of

of fat in hexane after randomization of fatty acid radicals was resolved into trisaturated glycerides by argentation TLC in a solvent mixture of petroleum ether:diethyl ether: formic acid (60:40: 1.5, v/v). The trisaturated glycerides were further resolved in component glycerides on paraffin impregnated Kieselguhr G plates and after microsaponification, the obtained fatty acids were again isolated on paraffin impregnated Kieselguhr G plates. The solvent systems used in the two cases were acetone:methanol:acetic acid (60:40:0.5, v/v) and 90% acetic acid, respectively. Detection of adulteration upto 5 per cent level was possible by this method. The detection was based on the differences in triglycerides and their fatty acids especially (C12-C16). A TLC method based on the identification of monoglycerides resulting from incomplete inter-esterification of the substituted fats has been described by Hendrickx and Huyghebaert (1968) and Huyghebaert and Hendrickx (1968b). Carisano and Riva (1976) detected as low as 5 per cent beef tallow in butter by TLC. A solution of fat in carbon tetrachloride was spotted on silica gel G plates and separated into short and long chain triglycerides followed by enzymic hydrolysis to determine the fatty acids ratio (C18/C16) at position-2 of triglycerides of each band. The ratio of long and short chain fractions was found greater than or equal to 5 units in pure butter, 10-15 units in butter containing 5 per cent tallow and 20-27 units in butter containing 10 per cent tallow. A technique similar to Carisano and Riva (1976) was used by Soliman and Younes

(1986) to detect 2 to 6 per cent of beef tallow or cotton-seed oil in butterfat.

2.4.1.3 Gas Liquid Chromatography (GLC)

Kuksis and McCarthy (1964) analysed triglycerides by GLC for the detection of vegetable fats or lard in butterfat and observed that the lower limit for the detection of adulteration of an unknown butterfat with lard or vegetable fats was 5 to 10 per cent. The detection of adulteration was based on the increase in content of high molecular weight triglycerides, C52 and C54 peaks, respectively. Parodi and Dunstan (1969) using the earlier method of Kerkhoven and deMan (1966) determined the triglyceride composition of 5 samples of Australian butterfat collected during 3 periods of the year and considered that the tri-saturated glycerides (GS3) analysis would enable detection of beef tallow at 5 to 10 per cent level in butterfat. Parodi (1971) analysed by GLC 112 samples of Australian butterfat adulterated at various levels with beef tallow and reported that of the samples analysed, 59.8 per cent at 10 per cent level, 35.7 per cent at 15 per cent level, 9 per cent at 20 per cent level and 2.7 per cent at 25 per cent level of adulteration were not detectable based on triglycerides and fatty acid ratios. Later on the same author (1973) using the similar technique observed that all butterfat samples adulterated with beef tallow (20 per cent level) and vegetable oils (5 per cent) were detectable. Guyot (1977a,1977b,1978) examined Belgian Commercial butters for triglycerides distribution by GLC. It was found that

triglycerides were in order of C36 > C38 > C40 > C42 > C44, C50 > C52 for commercial butter, C50 ≤ C52 was found occasionally in farm butter. In beef tallow and lard, the main triglyceride C52 varied respectively from 42 to 61 per cent and from 54 to 65 per cent. The C52/C50 ratio was found < 1 in pure butter and between 2 and 3-4 respectively in beef tallow and lard. Adulteration with beef tallow at 5, 10 and 15 per cent levels indicated that 37, 66 and 93 per cent of Belgian butters respectively showed C52/C50 > 1. Adulteration with 5 and 10 per cent lard could be detected respectively in 72 and 99 per cent of the butter samples. Relative proportions of C36, C38, C40 and C52 triglycerides and especially the C36/C40, C38/C50 and C50/C52 ratios could be helpful for detection of adulteration in butter/milkfat.

Vanoni et al.(1979) used GLC for fatty acids analysis in triglycerides and 2 monoglycerides isolated from the liquid and solid fractions of fats crystallized from acetone at 0°C. The authors reported that the ratio of C16/C18:2 fatty acids in 2 monoglycerides of the liquid fraction could detect beef suet in butter at 10 per cent level. Adulteration of milkfat with margarine at 5-10 per cent level was detected on the basis of triglycerides as margarine had more triglycerides with 48-54 acyl carbon atoms than milk fat (Marjanovic et al., 1984). Stock (1985, 1986) detected 5-10 per cent of cattle tallow, vegetable oils in cow milkfat using Timms method (1980). Luf et al. (1987) examined by GLC, 276 butter samples adulterated

at various levels and reported that detection of adulteration in butter with beef tallow and lard at 5 and 10 per cent levels was possible based on the ratio of C52 and C40 triglycerides and of vegetable fats/oils at 5-10 per cent level on the basis of ratio of long chain triglycerides (C50-C54) to medium chain triglycerides (C40 or C38-C40).

2.5 METHODS BASED ON THE NATURE AND CONTENT OF FATTY ACIDS

The natural variation in the contents of the individual fatty acids and the possibility of preparing by inter-esterification fats with characteristics within the limits for milk fat poses a problem. Milkfat containing higher percentages of lower fatty acids offers a good scope to the adulterator to mix foreign fats to a percentage which still leaves the characteristics for the content of lower fatty acids at just about the required limit. Therefore, the fatty acid contents are of hardly any value for demonstrating adulteration with foreign fats.

2.5.1 TESTS BASED ON ANALYTICAL CONSTANTS AS A MEASURE OF FATTY ACIDS

Among animal fats, milkfat is the only fat which contains an appreciable amount of butyric acid generally represented by Reichert and Kirschner 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. Ghee with high Reichert value offers good scope to mix cheaper fats (vegetable and animal body fats) to a percentage which keep the butyric acid content

well above the required limit for the Reichert value and other fat constants. Considerable work on the examination of the purity of milkfat by means of the contents of volatile fatty acids has been reported by Karim (1953) and Velu (1971). The effect of adding different adulterants like hydrogenated oils, refined cottonseed oil, beef tallow and coconut oil separately or in mixture to ghee on various fat constants was studied by Ali and Tremazi (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 upto 10 per cent level was diffiult to detect. Emperical estimations 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). These characteristics enable the detection of only gross adulteration. The practice of feeding cottonseeds to milch animals further complicates the matter and it is known that normal Reichert value of 30-32 comes down below 18 in the same animal when fed with cottonseeds. Achaya and 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 + capric 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 fatty acids.

2.5.2 TESTS BASED ON THE CONTENT OF SPECIFIC FATTY ACIDS

Milkfat differs markedly from the adulterant fats in nature and content of certain specific fatty acids. Such specific fatty acids are either characteristic or absent in milkfat or present in low content as compared to adulterant fats. Taking advantage of this, several workers have used certain specific fatty acids, viz., butyric, iso-oleic, iso-valeric etc. as an index for the detection of foreign fat in milkfat.

2.5.2.1 *Butyric acid*

Harper and Armstrong (1954) estimated fatty acids by direct saponification method. The molar concentration of butyric acid was used as an index for detecting adulteration of milkfat with vegetable fats. Decrease in the butyric acid content below 9.6 mole per cent suspected adulteration of ghee with vegetable fats. Keeney (1953,1956) and Eckizen and Deki (1975,1976) detected the adulteration of butterfat with vegetable fats/oils using butyric acid content as an index of adulteration.

2.5.2.2 Iso-valeric acid

The detection of iso-valeric acid in dolphin oil has been used by several Italian workers as the basis for the detection of dolphin oil and hydrogenated dolphin oil in butter (Fabris and Vitagliano, 1954; Bottino and Campanello, 1955; Priori, 1955; Canuti, 1958). Chioffi (1956) demonstrated that acetic acid and iso-valeric acid in dolphin oil

could be distinguished from volatile fatty acids natural to butter on the basis of their partition co-efficients between water and carbon tetrachloride. The use of partition chromatography for the detection of hydrogenated dolphin oil on the basis of iso-valeric acid has also been reported by Antonani and Ceruti (1954), Parrozziani and Mancinelli (1954) and Arrigo (1955).

2.5.3 GAS LIQUID CHROMATOGRAPHY OF FATTY ACIDS

Various workers have analysed fats by Gas Liquid Chromatography and used different fatty acid ratios for the detection of adulteration in butter/butterfat. The GLC analysis of fatty acids attracted considerable interest after the publication of an article by James and Martin (1952). Wolff (1960) and Boniforti (1962) used C12/C10 fatty acid ratio for the detection of vegetable oils or margarine in milkfat. Francesco and Avancini (1961) reported that butterfat with a ratio of C12/C10 fatty acids > 1.6 or C4/C6+C8 fatty acids > 1.8 was considered to be adulterated with coconut oil or tallow or pig fat trans-esterified with butyric acid. The ratios of fatty acids, C14/C12 > 3,C18:1/ C18:0 > 2 and C12/C10 > 1 were suggested for the detection of vegetable oils in butterfat (Provvedi and Cialella, 1961; and Vallusi and Cefleri, 1962). The ratios of fatty acids such as C14/C12 by Timmen (1963) and C6/C8, C12/C10 and C14/C12 by Jamoschek and Metin (1968) were used for the detection of margarine in butter/butterfat. To detect adulteration of beef tallow, lard, margarine and synthetic

butterfat in butterfat (Guyot and Piraux, 1964,1968; Guyot, 1967,1968b; Parodi, 1969; Kuzdzal-Savoie and Kuzdzal, 1969a; Goursand and Luquet, 1970) and substitute butterfat in butter (Hendrickx and Huyghebaert, 1970b), the ratios of various fatty acids were suggested. The ratios of various fatty acids and their composition (Vanoni et al., 1978), and C18:1/C14:0 (Trieger and Bordnikova, 1979) were suggested for the detection of beef suet and lard, and hydrogenated vegetable oils and lard in butterfat respectively. The ratios of fatty acids, C16/C14, C14/C12, C18/C14, C18:1/C16 and group of fatty acids (Parodi, 1970,1971) and C18:1/C18:0, C18:0/C8, C14: 0/C18:0 and C6-C12/C18:0 fatty acids (Toppino et al., 1980) were used for the detection of animal and vegetable fats/oils, and beef suet in butterfat respectively. Faraq et al. (1983) determined the fatty acids profiles of 3 fractions separated by fractional crystallization from cow and buffalo ghee adulterated at various levels with lard and margarine. The detection of adulteration was based on the correlation of concentration of C18:0 fatty acid in ghee adulterated with lard at $\geq$ 10 per cent level and C18:1 fatty acid for adulteration of margarine at $\geq$ 15 per cent level in cow ghee and $\geq$ 20 per cent level in buffalo ghee.

2.6 METHODS BASED ON THE PHYSICAL CHARACTERISTICS OF FATS

2.6.1 SPECTROSCOPIC METHODS

Spectroscopic methods have been used by several workers for the characterisation of fats and oils. It is

known that animal body fats contain isolated trans-unsaturated fatty acids. Such characteristics of body fats have been exploited to detect the presence of these fats in butterfat by spectroscopic analysis.

2.6.1.1 Visible and ultra-violet spectroscopy

The use of visible spectroscopy has been reported for the detection of lard and cheuri fat in ghee by Faraq et al. (1980) and Jha (1988), respectively.

Based on the differences in absorption of margarine and butter in UV region at 268 and 302 mu, Lembke, Kaufmann and Farfoletti-Casali (1953) reported that UV spectroscopy could be used to detect the presence of hydrogenated fat and margarine in butterfat and butter, respectively. Elsaid and El-Mangouri (1958) and Rego et al. (1964) using UV spectroscopy estimated linoleic acid content for the detection of unsaturated vegetable oils in butterfat. Cerutti (1955) and Provvedi (1957) distinguished between butter and margarine on the basis of fluorescence observed in ultra-violet light. UV spectroscopy was used by Franzke (1964) for detecting fish oil in edible fats. The $E^{1\%}_{1cm}$ value at 315 nm after alkali isomerization for animal body and vegetable fats was less than 1.0 and for fish oil was between 12-64. The adulteration at 2 to 8 per cent level of fish oil in edible fats could be detected. Colombini, Amelotti and Vanoni (1978) investigated butter samples by UV spectroscopic technique and observed the ratio of extinction value at 301 nm and 315 nm between 1.106-1.442 in

in butter, 1.230-1.267 in butter + 10-20 per cent suet and 1.211-1.260 in butter + 10-20 per cent lard. The UV spectrum between 220 nm and 420 nm could detect adulteration of butter with lard at 10 per cent level.

2.6.1.2 Infra-red spectroscopy

Firestone and Luz (1961) and Kaufmann et al. (1959, 1961) reported that the absorption in IR region, 10.36 u could be used for the detection of hydrogenated fats in butterfat. Bartlet and Chapman (1961) demonstrated the presence of hardened fats in milkfat by recording IR Spectra of 4 per cent solution of fat dissolved in carbontetrachloride in the region of 10 u (967 cm^{-1} and 948 cm^{-1}). It was observed that the presence of hardened fats increased the absorption at 967 cm^{-1}. Luck and Kohn (1963) detected 20 per cent inter-esterified fat in butter by a comparison of IR spectra and dielectric constant. It was found that inter-esterified fat showed the presence of free-OH groups of mono- and di-glycerides with an hydroxyl value of 29. Differential IR spectroscopy was used by deRuig (1968,1971) for the detection of adulteration in Dutch butterfat and observed that in solid state, butterfat and body fats showed an absorption band at 920 cm^{-1}. At 32.5°C, this band was absent from the differential spectrum of pure butterfat but present in butterfat adulterated with lard at 10 per cent level. Based on the differences in IR absorption of polyene fraction isolated from unsaponifiable matter of synthetic fats and butterfat over alumina column, Francesco and Co-workers (1971) detected synthetic fats at 10 per cent level

in butterfat. Faraq et al (1980) reported the use of this technique for the detection of adulteration of animal fats (lard and shortening) in milkfat at 5 per cent level. Konevets et al. (1987) using IR spectroscopy estimated cis and trans unsaturation to detect the adulteration of milkfat with animal fats, vegetable and hardened fats at 10 per cent level. This part of the study has been studied in detail both for whole fat and unsaponifiable matter of milkfat, adulterant body fats and their admixtures to differentiate between ghee and animal body fats.

2.6.2 SOME PHYSICAL CHARACTERISTICS OF GHEE

Physical properties are additive in nature, it is a common practice to admix such synthetic fats which will have the superficial properties resembling those of pure butterfat. Several workers have carried out studies to devise simple physical and physico-chemical tests for detecting adulteration on the basis of physical properties of ghee and adulterant fats.

2.6.2.1 Opacity test

The opacity test developed by Singhal et al. (1970) to detect the adulteration of ghee with body fats is based on the solidification time of melted fat samples at 23°C. The time taken to show noticeable opacity (O.D. 0.5) by buffalo, goat and sheep body fats was 10, 15 and 20 seconds, respectively, whereas normal ghee took more than 35 minutes. The cotton-tract ghee and pig body fat took 2 minutes each to become opaque. It was found that the differences in

opacity time between pure ghee and ghee samples adulterated with pig, sheep, goat and buffalo body fats at 5, 10 and 20 per cent levels, each was 7-8, 9-10 and 14-16 minutes; 18-20, 26-29 and 33-34 minutes; 18-22, 28-31 and 34 minutes and 22-26, 31-33 and 35-36 minutes. A detection limit for pig body fat at 20 per cent level and buffalo, goat and sheep body fats at 5 per cent level has been reported.

2.6.2.2 Bomer value

Bomer (1913) developed this test for the detection of lard in tallow. Taking advantage of the test, Singhal (1973,1980) determined Bomer values of ghee, adulterant body fats and ghee samples adulterated at 5, 10 and 20 per cent levels with different body fats and observed that pig body fat (lard) could be detected at 5 per cent level in ghee without any ambiguity even in the presence of cotton-tract ghee. Other body fats like buffalo, goat and sheep were difficult to be detected in the presence of cotton-tract ghee. The differences between the Bomer value of lard, beef suet and, pork fat, beef fat and mutton fat have been reported by International Organization for Standardization (ISO), Sub-committee on Meat and Meat Products (1969).

2.6.2.3 Critical Temperature of Dissolution (CTD)

A number of investigations based on the critical temperature of dissolution with different solvents to detect adulteration of ghee with vegetable fats, mineral oils and body fats have been reported in the literature after the

publication of paper by Crook (1879) who observed two distinct layers in milkfat using a solvent mixture of carbolic acid and water (10:1, v/v). Sanyal (1929) detected the presence of beef tallow in normal ghee at a temperature of 30°C using a solvent mixture of diethyl ether and 95 per cent ethyl alcohol (3:4, v/v). Venkatachalam (1937) used a solvent mixture of dry acetone and absolute alcohol (65:35, v/v) at 30°C to detect hydrogenated fats, mutton and body fats in ghee. Fryer and Weston (1918) and Felman and Lepper (1950) used a solvent mixture of 95 per cent ethyl alcohol and iso-amyl alcohol in the ratio of 2:1,v/v to detect margarine in butter. Bhide and Kane (1952) found CTD for genuine ghee ranged from 39-45°C while for vanaspati from 62-72°C. Deviation in CTD value of ghee above the normal range indicated adulteration with vegetable fats. Prakash et al. (1956) using the same solvent (Felman and Lepper, 1950) reported for the pure ghee, a CTD value between 49.5-53.5°C. Ten to fifteen per cent of beef tallow in ghee could be detected from increased CTD value. The use of critical temperature of dissolution for the detection of adulteration of ghee with mineral oils was also carried out by Kane and Ranadive (1951) and Mahon et al. (1955b). The CTD is considerably affected by casual impurities like water, free fatty acids and peroxides. Consequently the CTD values become less characteristics unless the necessary corrections are employed.

2.6.2.4 Differential Thermal Analysis (DTA)

The transition of butterfat from a solid to a liquid

state has been studied with the help of differential thermal analysis (DTA). Antila et al. (1965) detected 5 per cent coconut fat, cocoa fat and hardened vegetable fat in butterfat by differential scanning calorimetry based on the differences in the shape of melting curves. However, tallow, lard and vegetable oil added at 5 per cent level in butterfat escaped detection by this method. Roos and Tunistra (1969) using DTA of Dutch butterfat reported that addition of 5 or 10 per cent of beef tallow in butterfat showed changes in solidification curve. The solidification in butterfat adulterated with tallow started at higher temperature and took place in two steps. Lamblet et al. (1980) detected goat body fat in ghee by DTA technique using the Mettler and Co Model TA-2000 B at temperature range from -170 to 55°C. The differences in melting and crystallization patterns of goat body fat and ghee provided a basis for the detection and determination of adulteration in cow or buffalo ghee. Ten per cent goat body fat in ghee could be detected qualitatively with the help of melting diagram and estimated quantitatively from crystallization diagram. Lamblet and Ganguli (1983) using differential scanning calorimetry detected pig and buffalo body fats in ghee on the basis of an extra peak observed at high temperature in the melting and crystallization curves. Adulteration of pig fat in ghee could be detected qualitatively and buffalo body fat in ghee qualitatively as well as quantitatively from the crystallization curve. The method, however, failed to detect coconut oil, cottonseed fed ghee and ghee

adulterated with other animal body fats. Differential scanning calorimetric technique for the detection of beef suet at 5 per cent level in ghee on the basis of differences in the exothermic peak from the crystallization thermograms was used by Amelotti et al. (1983).

2.6.2.5 Fractionation of ghee

This method is based on the crystallization of glycerides as such or by dissolving the fat in a suitable solvent at different temperatures. The fractions obtained contain specific glycerides and fatty acids which gave each fraction a distinct character different from those of original butterfat and from fractions isolated from other fats under similar conditions. Bhalerao and Kummerow (1954, 1956a,b) fractionated 10 per cent solution of fats by first dissolving in hot absolute alcohol and then allowing the solution to stand for 2 hours 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 percentage of tri-saturated glycerides in alcohol soluble fraction was again fractionated from acetone at 0°C and the iodinated acetone soluble fraction was then used for refractive index determination. The author detected 10 per cent of vegetable fats in butterfat by this method. Detection of butterfat adulteration by separation of fatty acids into urea stet adducts was first suggested by Holasek and Ibrahim (1953). A method based on the above technique was used by Shipe

(1955b) to detect vegetable and animal fats in butterfat. The fatty acids from butterfat were fractionated using 10 and 20 per cent methanolic solution of urea to form urea fatty acid adducts. The selectivity of the fractionation procedure was demonstrated by measuring the refractive indices of the fractions to ascertain adulteration. Krienke (1953) and Bassette and Keeney (1956) used the method of fractionation by selective solidification as an aid in detecting butterfat adulteration. Pruthi and Gupta (1969) fractionated ghee, animal body fats and their admixtures in ethyl alcohol at 20°C and each fraction was subjected to refractive index determination to detect ghee adulteration with body fats.

2.7 HALPHEN AND METHYLENE BLUE REDUCTION TESTS FOR COTTONTRACT GHEE

The halphen test was originally developed as an emperical method of testing the adulteration of various vegetable oils with cottonseed oil (Gunstone, 1969). It is known that the tissues or eggs of hens that have been fed on cottonseed oil showed positive Halphen test (Lorenz and Almquist, 1934; Shenstone and Vickery, 1959). Singhal (1973) used the original method developed hy Halphen (1897) and described in A.O.A.C. (1970) for distinguishing cottontract ghee from normal ghee. The test was based on the development of a crimson colour in ghee with Halphen reagent (carbon disulphide containing 1 per cent sulphur in isoamyl alcohol) after incubation for an hour in a boiling bath of saturated sodium chloride solution.

A methylene blue reduction test for identification of cotton-tract ghee was suggested by Singhal et al. (1971). 0.1 ml of 0.1 per cent methylene blue solution in chloroform:methanol mixture (1:1) was added to 5 g of the clear fat sample and mixed by gentle shaking. The methylene blue colour was discharged by cottonseed fed ghee.

2.8 TESTS BASED ON THE NATURE AND CONTENT OF TRACERS

With a view to provide a rapid and reliable tool to the analyst and the consumer to identify the added foreign fat in butterfat, the addition of tracer has been suggested. A tracer can be latent which is specifically identified by its reaction with certain chemicals or it may impart coloration distinct from that of the natural colour of butterfat. Tracers thus can be classified either as colouring matter both natural and synthetic or as latent colours.

F-Vonsoxlhet (1887) first suggested the addition of sesame oil as tracer to margarine at 10 per cent level for the detection of margarine in butter. Later on several countries including India introduced the addition of tracer into national legislation for making adulteration more easily detectable. Among the tracers, sesame oil is commonly added to vanaspati for its detection in ghee. Several workers have suggested the addition of colours to vanaspati or margarine for their easy detection in ghee/ butter. The addition of Ratanjot (Dastur et al., 1947; Mukherji et al., 1960), turmeric root or curcumin (Kapur

et al., 1960b) in vanaspati have been suggested for the detection in ghee. Recently Technicean (1973) suggested the addition of chlorophyll E140 and carotenoides in vanaspati/margarine for its detection in ghee/butter. The addition of vegetable colouring matter such as chlorophyll, carotene, alkanaet, Kamala, ratanjot and turmeric to hydrogenated fats has been recommended (Subramanian et al.,1952).

2.9 SUMMARY AND OBJECTS OF THE PRESENT STUDY

From the above summary of the literature, it is evident that as yet no simple and reliable test is available that could give undisputed results when ghee is adulterated with animal body fats and vegetable fats/oils simultaneously. Cottonseed feeding further complicates the problem. Cottonseed fed ghee acquires many of the physico-chemical properties which resemble those of ghee adulterated with animal body fats. Under the conditions prevailing in India, some of the units producing ghee on large scale are located in the cotton-tract areas where cottonseeds and cottonseed concentrates are frequently fed to the animals. There is a common belief among the farmers that cottonseed feeding increases the fat percentage of milk. As milk is priced on the basis of its fat content, it is difficult to convince the farmers to give up the practice of feeding cottonseeds entirely.

The present day system of hydrogenation and refining of vegetable fats and oils, and inter-esterification of milkfat with animal body fats and vegetable fats/oils have

provided such ghee substitutes which are difficult to be distinguished from the normal milkfat. Further the marketing of ghee all over the country without any regional specification has made the problem of checking the purity of ghee all the more difficult.

In course of the present investigations, attempts have been made to study the characteristics of the body fats from buffalo, goat, sheep and pig, and vegetable fats and oils along with those of ghee and their admixtures. Simultaneously market samples of ghee and vegetable oils, and ghee procured from the cotton-tract area of Indore have been examined. Amongst the analytical constants, the Reichert and Polenske values have been examined to study the nature of the fat samples used. Techniques like GLC, TLC and paper chromatography, and visible, UV and IR spectroscopy have been used to identify the specific characters which could be employed to detect adulteration with the objective that the method should be able to detect animal body fats even in the presence of vegetable fats/oils if added simultaneously. Also the method should not give any false indication in case of ghee from cotton-tract areas. It is known that the constituents of unsaponifiable matter of animal body fats (free and bound sterols) show marked differences from those of ghee. With this objective, an extensive study has been made on sterols of ghee and body fats to develop a test that could detect the adulteration of animal body fats in ghee at low levels. TLC examination of sterol digitonides (free and bound)

cleaved by pyridine showed marked differences between ghee and animal body fats. The interesting results obtained have been recorded. In course of studies, it was observed that p-hydroxy benzaldehyde in the presence of concentrated hydrochloric acid gave characteristic red colour with vanaspati and sesame oil. This led to the development of a simple method for the detection of vanaspati in ghee. The method seems to be simpler than Baudouin test as the furfural used in the Baudouin test being unstable, has to be redistilled, from time to time. The estimation of insoluble unsaponifiable matter based on the technique described by Roos et al.(1969) was attempted. An interesting observation emerged that all the animal body fat samples studied showed thick crystals on overnight storage in a refrigerator. Normal ghee samples showed negligible crystallization. This observation was exploited to develop a simple method to detect animal body fats in ghee at low levels. The crystallization behaviour of saponified fat samples fractionated in a mixture of water and alcohol (1:1 v/v) cooled under controlled conditions showed marked differences between ghee and animal body fats. The results obtained during the course of study are delineated and discussed in the following chapters.

CHAPTER 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 PREPARATION OF SAMPLES

3.1.1 GHEE

Samples of ghee were prepared by direct cream method using fresh cow and buffalo cream collected from Experimental Dairy of the Institute. Fresh cream was heated in a stainless steel vessel (degchi) over a hot plate to a temperature not exceding 120°C. Ghee so obtained was allowed to settle and filtered through a double fold of muslin cloth. The samples were filled in clean and dry glass stoppered bottles, cooled to room temperature and thereafter stored at 4 ± 1°C.

Three sets of samples each of cow and buffalo ghee were prepared during winter, summer and rainy seasons.

3.1.2 ANIMAL BODY FATS

Goat, sheep and pig body fat tissues were procured from Karnal slaughter house and the buffalo body fat tissues were procured from Delhi slaughter house. The fat tissues were collected from different parts of the slaughtered animals to have a representative sample. The fat tissues were heated in a stainless steel vessel containing some water over a hot plate and heating was continued to a final temperature of 120-125°C till all the fat melted out of the tissues and the tissues turned light brown indicating that

the fat was free of moisture. The fat was then filtered through a double fold of muslin cloth, cooled and filled in clean and dry glass bottles.

Three sets of samples, each of buffalo, goat, sheep and pig body fats were prepared during different seasons of the year. The body fat samples were stored at 4 ± 1°C.

3.2 VANASPATI AND OTHER VEGETABLE OILS

Samples of hydrogenated vegetable oils (vanaspati) and other edible vegetable oils were procured in sealed containers from the local market. Samples of vegetable oils like sesame (raw), ricebran, cottonseed, soyabean, and vanaspati were obtained from M/s.K.C. Vanaspati (J&K). The samples were stored at 4 ± 1°C.

3.3 SOLVENTS

A.R. grade reagents were used. The unspecified grade solvents were purified as per prescribed procedures.

3.4 DETERMINATION OF REICHERT-MEISSL AND POLENSKE VALUES

IS:3508 method was used for determination of Reichert-Meissl and Polenske values of samples.

3.5 UNSAPONIFIABLE MATTER AND THEIR DERIVATIVES

3.5.1 ESTIMATION OF UNSAPONIFIABLE MATTER

IS:3508 method was employed for extracting unsaponifiable matter from fat samples. About 5 g of fat was saponified under reflux using 50 ml of 0.5 N alcoholic potassium hydroxide. The unsaponifiable matter was extracted

with three 50 ml portions of peroxide free ether. The combined ether extract was washed thrice with 20 ml of distilled water followed by gentle shakings. The etheral extract was washed three times alternately with 20 ml portions of 0.5 N aqueous KOH and distilled water, shaking each time vigorously. The ether extract was finally washed with distilled water till the washings were no longer alkaline to phenolphthalein. The ether extract was evaporated to about 5 ml and transferred quantitatively (using small portions of ether) to a 50 ml dried and weighed Erlenmeyer flask. Ether was then evaporated completely and acetone (2-3 ml) was added and then it was removed completely on a steam bath. The flask containing unsaponifiable matter was dried at 80-85°C to a constant weight. The results were checked by dissolving unsaponifiable matter in 2 ml of neutral alcohol and titrating with 0.1 N KOH to ensure that no more than 0.2 ml of alkaline solution was used.

3.5.2 ESTIMATION OF UNSAPONIFIABLE MATTER FROM FAT FRACTIONS IN DIETHYL ETHER

About 30 g of fat sample was melted at 45°C and filtered. Melted and filtered sample (10 ± 0.1 g) was taken in a 100 ml glass stoppered flask and 40 ml of diethyl ether was added to it. Stoppered the flask and dissolved the fat by gentle warming and swirling over a water bath. Kept the flask at 4-5°C for 3 hours and shaked the flask intermittently at an interval of half an hour. The crystallized fat was filtered through filter paper soaked in

ether and washed the fat on the paper twice with ether (2 x 5 ml) pre-chilled to 4°C. Scrapped the crystallized fat and transferred quantitatively into a flat bottom flask (250 ml) with the help of 0.5 normal alcoholic KOH followed by a washing with 96 per cent alcohol (2 x 5 ml). Fat was saponified with 0.5 normal alcoholic KOH, cooled and added 50 ml of distilled water and transferred to a separating funnel. Unsaponifiable matter was extracted with ether and evaporated the solvent on water bath and dried the residue in an oven at 80°C. The filterate and washings collected were heated on a water bath to remove the solvent completely and transferred the melted fat into a 250 ml flat bottom flask with the help of 0.5 normal KOH (2 x 10 ml) and alcohol (2 x 5 ml). Saponified the fat with 0.5 N alcoholic KOH and extracted unsaponifiable matter as described above.

3.6 ESTIMATION OF TOTAL CHOLESTEROL

The method of Bindal and Jain (1973) for the estimation of total cholesterol in ghee was employed.

3.6.1 WHOLE FAT

About 0.2-0.25 g of fat sample was taken in a glass stoppered test tube and dissolved in chloroform (3 ml). Freshly prepared Lieberman-Burchard reagent (4 ml) was added and the mixture was kept at 25°C for 15 minutes for colour development. The optical density readings were recorded at 650 nm on Spectron 21. Blank was also run simultaneously. A standard curve was prepared using 50, 100, 150, 200,

250, 300 and 350 ul aliquots of a 0.4 per cent solution of cholesterol in chloroform and developing colour under identical conditions (Fig. 1).

3.6.2 UNSAPONIFIABLE MATTER

Unsaponifiable matter isolated from 5 g of fat was dissolved in chloroform and the volume was made upto 3 ml. To this solution, 4 ml of Lieberman-Burchard reagent was added and the colour was developed as per procedure described above. Trials were also made for the estimation of cholesterol from unsaponifiable matter of ghee and animal body fats by the method proposed by Bindal and Jain(1973) using glacial acetic acid. A standard curve was prepared as per procedure (Fig. 2).

3.7 ESTIMATION OF FREE AND ESTERIFIED STEROLS

The method proposed by Den Herder (1955) with slight modification was used. Fifteen grams of fat was dissolved in chloroform (20 ml) by warming gently at 35°C. Added 40 ml of digitonin solution (1% in 96% ethyl alcohol) and kept the solution overnight at 4-5°C. The precipitated free sterol digitonides were filtered through Buchner funnel lined with a disc of Whatman No.1 filter paper. The precipitate was washed twice with 5 ml portion of a mixture of chloroform:ethyl alcohol (1:1) and finally washed with chloroform to make the digitonides free from fat. Collected the filterate and the washings, and evaporated on a water bath to obtain the residue containing fat and esterified sterols. Saponified the residue with 10 ml of aqueous

KOH solution (40%) and 20 ml of ethyl alcohol (96%). The unsaponifiable matter was again extracted with ether as above. The solvent was evaporated and the residue was dried in an oven at 80°C. The residue was dissolved in 5 ml alcohol (96%) and 10 ml of 1 per cent alcoholic solution of digitonin was added to it and kept overnight at 4-5°C. The precipitate was filtered through a gena filter (10 ml), washed alternately with 2-3 ml of 96 per cent ethyl alcohol and chloroform and finally dried in an oven at 100°C for 10-15 minutes to a constant weight.

Calculation

$$\text{\% sterol in terms of sterol digitonide} \quad S = 0.24 \times \frac{W_1}{W} \times 100 + 0.02\%$$

where, S = Percentage of sterol

W_1 = Weight in gram of the digitonide

W = Weight in gram of fat used

0.02 = The loss factor (standard) of sterol caused by the solubility of the digitonides in the soap solution

3.8 ESTIMATION OF INSOLUBLE UNSAPONIFIABLE MATTER AND CHOLESTEROL AS DIGITONIDE

Method of Roos et al. (1969) was employed for the estimation of insoluble unsaponifiable matter and cholesterol as digitonide.

Fat (5 ± 0.1 g) was refluxed with 3.5 ml of aqueous potassium hydroxide (4 g in 6 g distilled water, w/w) and

7 ml of 96 per cent alcohol. Added 65 ml of 96 per cent alcohol and 120 ml of distilled water. Kept the flask at 4-5°C for 12 hours and filtered the precipitated insoluble unsaponifiable matter (soapy in nature) through a gena filter (10 ml). Washed the insoluble matter first with 10 ml 96 per cent alcohol and then distilled water till foaming stopped. Transferred the insoluble matter on a watch glass and dried in an oven at 40°C. Extracted the insoluble unsaponifiable matter from the dried matter in 15 ml of n-pentane. The solvent was evaporated completely and the insoluble matter was dried and weighed. The pooled filterate obtained after extraction of insoluble unsaponifiable matter was transferred to a 500 ml separating funnel and 50 ml of distilled water was added to it. The soluble unsaponifiable matter was extracted with ether. The ether extract was washed with distilled water till it was free from alkali. The solvent was evaporated on a steam bath and the soluble unsaponifiable matter was dried at 80°C. The digitonides were obtained by using 10 ml of 1 per cent alcoholic solution of digitonin.

Calculation

$$\% \text{ insoluble unsaponifiable matter} = \frac{W_1 \times 100}{W}$$

where W_1 = Weight in gram of the insoluble unsaponifiable matter

W = Weight in gram of the fat taken

$$\text{Total sterol digitonide} = 0.25 \times X$$

where, X = Wt of sterol digitonides in gram

$$\% \text{ sterol as sterol digitonide} = 0.25 \times \frac{X}{W} \times 100$$

3.9 TURBIDITY METHODS

Method of Roos et al. (1969) for the estimation of insoluble unsaponifiable matter in synthetic butterfat was employed to detect the presence of animal body fats in ghee with modifications as under:

One gram of molten and filtered fat was saponified using 2 ml of aqueous potassium hydroxide (4 g in 10 ml water) and 2 ml of 96 per cent ethyl alcohol. The contents were cooled and 10 ml of each of distilled water and 96 per cent ethyl alcohol were added and mixed gently. Aliquots (2-3 ml) of the saponified matter were taken in small test tubes and chilled to 0°C in an ice bath over varying time intervals. The absorbance of the turbid matter was measured at 550 nm using Spectron 21. The absorbance readings were plotted against the time interval to obtain the curve related to the nature of fat samples. Blank consisting of a mixture of distilled water and 96 per cent ethyl alcohol (1:1) was also run.

Turbidity method for ghee samples containing added standard fatty acids:

To ascertain the nature and kind of fatty acids responsible for inducing turbidity in ghee samples adulterated with body fats, standard fatty acids in quantitative amount were added to normal ghee samples to study their effect on turbidity of normal ghee samples by the procedure given above. The added fatty acid combinations were (i) C16:0, 100 mg, (ii) C18:0, 100 mg, (iii) C16:0 + C18:0,

40 and 60 mg, and (iv) C16:0 + C18:0 + C18:1 (oleic acid), 20, 30 and 50 mg each.

3.10 CHROMATOGRAPHIC METHODS

3.10.1 PAPER CHROMATOGRAPHY

3.10.1.1 Whole fat

About 0.5 g of fat sample was dissolved in 1 ml of chloroform and different aliquots (0.01 ml) were applied on a Whatman No.1 filter paper sheet(15 x 20 cm) equidistant (about 2 cm) from each other. The paper was punched at two corners about 2 cm below its upper edge and mounted on a glass stand (15 x 10.5 x 15.5 cm). Placed the glass stand in the chromatography tank (20 x 16 cm) previously saturated with solvent system, dichloromethane/iso-propanol/glacial acetic acid (80/30/20, v/v), and allowed to irrigate till the solvent had travelled a distance of 12 cm from the base line. The paper was thereafter taken out and dried in air to remove the solvent and then sprayed with 10 per cent alcoholic solution of phosphomolybdic acid. The paper was dried in the oven at 80°C and then washed with dilute solution of glacial acetic acid. A distinct greyish yellow streaking appeared on the paper. The paper was finally dried in air at room temperature.

3.10.1.2 Unsaponifiable matter

Unsaponifiable matter extracted from 5 g of fat by IS:3508 method (1966) was dissolved in 2 ml of chloroform and different aliquots (0.01 ml) were spotted on Whatman

No.1 paper sheet (15 x 20cm) about 2 cm apart from each other. Dried the paper in air, punched on two corners each about 2 cm below the upper edge of the paper and mounted it on the glass stand. Placed the stand in the chromatography tank (20 x 16 cm) previously saturated with solvent system, dichloromethane/iso-propanol/glacial acetic acid (80/30/20, v/v), and allowed to develop the chromatogram to a distance 2 cm below the punched holes. The stand was taken out from the tank, the paper was dried in air to remove solvent completely and sprayed with 10 per cent alcoholic solution of phosphomolybdic acid. The paper was first dried in air and then gently warmed in an oven taking care that paper should not char. Distinct blue spots were observed on the paper.

Unsaponifiable matter of fat samples was applied separately on Whatman No.1 filter paper sheet (20 x 18 cm) and first developed vertically in solvent system, 96 per cent ethyl alcohol:iso-amyl alcohol:carbon tetrachloride (35/35/1) and then after drying was again developed horizontally in another solvent mixture, viz. with 15 per cent ethyl acetate in cyclohexane to the same distance as run in the previous solvent system. The paper was dried in air and sprayed with 10 per cent alcoholic solution of phosphomolybdic acid and then warmed in an oven to make the blue spots visible.

3.10.1.3 Unsaponifiable matter from ether soluble and ether insoluble fractions

About 0.01 ml of unsaponifiable matter extracted from ether fractions dissolved in 1 ml of ether was fractionated

by paper chromatography using solvent system, petroleum ether 40/60:ether:glacial acetic acid (80:20:1, v/v). Unsaponifiable matter components from ether fractions were identified as per procedure given before.

3.10.2 THIN LAYER CHROMATOGRAPHY

Glass plates (20 x 20) or (20 x 10 cm) with 0.3 mm thickness adsorbent layer were prepared by applying silica gel G slurry in water (1:2.2, w/v), The plates were air dried, activated at 110°C for 1½ hour, cooled and kept in a dessicating cabient.

3.10.2.1 Estimation of free and esterified cholesterol by thin layer chromatography

Free and esterified cholesterol was estimated as per method given by Bindal and Jain (1972). Glass plates (20 x 20 or 20 x 10 cm) were coated with silica gel G slurry in water (1:2.2, w/v) to a 0.5 mm thickness. The plates were then air dried, activated at 110°C for 2 hours and kept in a dessicated cabinet.

Sample of fat (0.1 to 0.15 g) dissolved in chloroform (0.2 ml) was carefully spotted on the plates in the form of a streak about 2 cm above the bottom edge of the plate with the help of a micropipette. After drying in air, the plates were developed in a chromatographic tank (25 x 11.5 cm) pre-saturated with the solvent system, petroleum ether (60/80) : petroleum ether(40/60) : ether : chloroform: acetic acid (25:12.5:15:5:0.5). The plates were removed from the tank, when the solvent had travelled a distance of

about 16 cm from the base line and thereafter air dried to remove solvent. The lower zone (Rf value, 0.45-0.65) consisting mainly of free cholesterol and the upper zone (Rf value higher than 0.65) mainly consisting of triglycerides, esterified cholesterol were scrapped off and the extruded material was eluted with three portions of 15 ml of chloroform. The elute was evaporated slowly on water bath to a small volume and transferred quantitatively using small portions of chloroform to a glass stoppered test tube and the volume was made upto 3 ml by chloroform. Lieberman Burchard reagent (4 ml) was then added and kept the mixture at 25°C for 15 minutes for colour development. The optical density was measured at 650 nm on Spectron 21. Blank was also run simultaneously under identical conditions. A standard curve was prepared as per procedure (Fig. 3).

3.10.2.2 Whole fat

Aliquots (0.02 ml) of the fat samples(0.5 g dissolved in 2 ml of chloroform) were applied on the TLC plate, equi-distant from each other. The plates were dried in air to remove the solvent and placed in the tank previously saturated with solvent system, hexane:ether:glacial acetic acid:96 per cent ethyl alcohol (25:20:0.5:1, v/v). The plates were allowed to develop for 110 minutes or till the solvent travelled a distance of about 15 to 16 cm from the base line. Removed the plates from the tank, air dried and sprayed with 10 per cent alcoholic solution of phospho-molybdic acid. Allowed the plates to dry at room temperature for 5 to 10 minutes and then kept in the oven at 150°C

for 10-15 minutes to make the spots on the chromatoplates clearly visible.

3.10.2.3 Unsaponifiable matter

Pre-activated TLC plate coated with silica gel G (0.3 mm thickness) was applied with 0.02 ml of unsaponifiable matter from fat dissolved in 3 ml of chloroform. The plate was dried and developed in a solvent system consisting of hexane:ether:96 per cent ethyl alcohol:glacial acetic acid (25:20:1:0.5) till the solvent had travelled a distance of about 15-16 cm in 110 minutes. The plate was removed from the tank, air dried and sprayed with 10 per cent alcoholic solution of phosphomolybdic acid and thereafter heated in an oven at 120°C for 10-15 minutes to make the spots visible. Standard sterols, viz. cholesterol, dihydrocholesterol, 7-dehydrocholesterol, Lanosterol and B-Sitosterol were also run alongwith the samples.

3.10.2.4 Total sterol digitonides

About 0.01 ml of total sterol digitonides (5 mg) free of insoluble unsaponifiable matter dissolved in 1 ml of dimethyl formamide by heating over a flame with constant shaking was applied on pre-activated TLC plate (0.3 mm thickness). Allowed the plate to dry at room temperature for ½ hour and then developed in a solvent mixture, chloroform: benzene (3:1, v/v). Removed the plate from the tank after the solvent had moved to a distance of about 13-15 cm, dried at room temperature and then sprayed with 10 per cent solution of phosphomolybdic acid. Warmed the plate in the

oven at 115-120°C for 15 minutes to make spots on the chromatoplate visible.

3.10.2.5 Free and bound sterol digitonides

Cleavage of free and bound sterol digitonides:

About 10-15 mg of free sterol-digitonide was weighed in a test tube (20 ml) and heated with 2 ml of pyridine in a boiling water bath until digitonides dissolved completely. In another set, about 2-3 mg of bound sterol-digitonide was taken in another test tube and dissolved in 0.5 ml of pyridine. Heated the contents on boiling water bath until digitonides dissolved completely and then cooled.

Aliquots (about 10 ul) of cleaved digitonides were applied on pre-activated TLC plate (20 x 20 cm) coated with silica gel G of 0.25 mm thickness. Dried the plates in air and developed them using a solvent system consisting of petroleum ether (40/60) /ether/glacial acetic acid (80/ 20/1) for 2 hours. After drying, the plates were sprayed with 10 per cent alcoholic solution of phosphomolybdic acid and heated in the oven at 150°C for 15 minutes to develop spots.

3.10.2.6 Steryl acetate

Preparation of steryl acetates of sterol-digitonides:

About 10-15 mg of free sterol digitonides was transferred quantitatively to Stock's tube , added 1 ml of acetic anhydride and heated the tube in a glycerol bath at 145°C. After all of the digitonides were dissolved, heating was

continued for another 2-3 minutes. Cooled the contents and added 4 ml of ethyl alcohol (96%). Filtered the liquid while still hot through a Whatman No.1 filter paper previously soaked in alcohol (96%). Heated the filterate to boiling and added distilled water drop by drop (7 ml) till the steryl acetate precipitate just appeared and crystallized out on cooling. Cooled the contents to 4-5°C for half an hour and filtered through Watman No.1 filter paper to separate the steryl acetate precipitate. Washed the precipitate with alcohol (2 x 0.5 ml),dried by squeezing and finally dried at room temperature. Scrapped the precipitate, transferred to a watch glass and dried in an oven at 100°C.

About 0.5 mg of steryl acetates was dissolved in 1 ml of dimethyl formamide by heating over a water bath, cooled and applied (0.01 ml) on an activated thin layer plate coated with silica gel G of 0.25 mm thickness. The plate was dried in air and developed in the solvent system, hexane:ethyl acetate (85:15) for 110 minutes. Removed the plate from the tank and dried in air to remove solvent completely. Sprayed with 10 per cent alcoholic solution of phosphomolybdic acid and then heated in an oven at 120°C for 10-15 minutes to develop spots.

3.10.2.7 Trimethyl silyl ethers (TMS)

Preparation of trimethyl silyl ethers of unsaponifiable matter

The method proposed by Oil and Oilseeds Sectional Committee, CAFDCS (1986) Shri Ram Test House, New Delhi for

the preparation of trimethyl silyl ethers of sterols was employed. Unsaponifiable matter was taken in a glass stoppered test tube and dissolved in 1 ml of dimethyl formamide. To this was added 0.5 ml of hexamethyl disilazane (HMDS). The contents were mixed well and 0.2 ml of trimethyl chlorosilane (TMCS) was added to it. Closed the test tube immediately with the glass stopper, shaked the contents vigorously and allowed to stand at room temperature for 15 minutes. Added 1 ml of heptane and 10 ml of ice cold distilled water. Shaked the test tube gently for 5 minutes and allowed the layers to separate. The silane derivatives of sterols were extracted by heptane. Pipetted out the upper layer of trimethyl silyl ether derivatives into another test tube. The TMS ethers of standard sterols (cholesterol and dihydrochloesterol) were also prepared alongwith the samples. About 5 mg of standard sterol was weighed in a test tube and dissolved in 1 ml of dimethyl formamide. Trimethyl silyl ether derivatives were prepared with HMDS and TMCS under similar conditions.

Aliquots (Ca 20-25 ug of silane derivatives in heptane) were spotted on TLC plate coated with silica gel G, about 2 cm apart from each other. The plate was developed in the solvent system, petroleum ether (40/60)/ether/glacial acetic acid (80/20/1) till the solvent had travelled a distance of 13-14 cm. Air dried the plate, sprayed with 10% alcoholic solution of phosphomolbdic acid and finally heated at 120°C for 10-15 minutes to make the spots visible.

3.10.3 GAS LIQUID CHROMATOGRAPHY

3.10.3.1 Extraction of fatty acids

The insoluble unsaponifiable matter was dissolved in 20 ml of distilled water. Dilute H_2SO_4 (N/10) was added to it drop by drop till no further precipitation appeared. Transferred the contents to a 250 ml separating funnel and the fatty acids were extracted with ether. The ether was evaporated on a steam bath and dried the residue at room temperature. Fatty acids were also extracted from turbid material obtained by turbidity test from animal body fats.

3.10.3.2 Preparation of methyl esters of fatty acids

Dried and powdered fatty acids mixture (Ca 50-75 mg) was weighed in a 2 ml teflon screw cap tube. Dichloromethane (0.1 ml), methyl urea (270-300 mg) and 14 per cent BF_3-methanol reagent (1 ml) were added to it. The tube was closed tightly to make leak proof and vortexed until methyl urea was dissolved. Kept the tube in a water bath maintained at 90-95°C for 5 minutes and thereafter cooled to room temperature. Transferred the contents quantitatively to a separating funnel with the help of petroleum ether (40/60°C). Saturated solution of sodium chloride (4 ml) was added to the separating funnel, mixed gently and allowed to separate the layers for 4 minutes. Drained off the lower aqueous layer, and transferred to another separating funnel and repeated extraction with petroleum ether (2 ml). The combined etheral extract was dried over anhydrous sodium

sulphate for 1 hour and filtered through Whatman filter paper No. 4 previously soaked in petroleum ether. Washed the filter paper twice with petroleum ether (0.5 ml) and transferred the etheral solution to a dried 50 ml Erlenmeyer flask. Evaporated ether on water bath and dissolved the residue in 50 ul of dichloromethane. The material was then fractionated by GLC under specified conditions as below:

G.L.C. Unit, Nucon Model 5700, equipped with flame ionization detector was employed for fractionation. A glass column (6' x ¼" O.D.) packed with 15 per cent diethylene glycol succinate (DEGS) on 80-100 mesh chromosorb, WAW DMCS was employed. The column temperature was first maintained at 85°C for 5 minutes and then increased at the rate of 10°C per minute to 150°C, and finally maintained at 198°C. The injector port and the detector were kept at 210° and 220°C, respectively. Pure nitrogen at 20 p.s.i. was used as carrier gas. Six ul sample was injected into the column. Full scale (omniscribe recorder) with 0.01 mv, at 1,000 sensitivity with an attenuation of 16 and the chart sheet maintained at ¼" per minute were adjusted. The fatty acids in methyl esters of recorded samples were identified with respect to their retention times on comparison with standard fatty acid methylates. Each peak area was measured by triangulation method.

3.11 SPECTROSCOPIC METHODS

3.11.1 ULTRAVIOLET STUDY

Method of Rego et al. (1964) was employed. Spectron 21 Spectrophotometer, Bausch and Lomb model equipped with UV and visible range was used. AR quality n-hexane before use was drained through Celite 545 column and then dried over anhydrous $CaCl_2$ and distilled.

3.11.1.1 Whole fat

0.1% solution of fat samples in pure n-hexane was prepared and scanned through UV range 200 to 320 nm on Spectron 21.

3.11.1.2 Unsaponifiable matter

The unsaponifiable matter (ghee and animal body fats) was dissolved in 20 ml of purified n-hexane and scanned through UV range 200 to 320 nm on Spectron 21.

3.11.2 INFRA-RED SPECTROSCOPY

IR spectra of whole fat and unsaponifiable matter were recorded in double beam IR Spectrophotometer in the region 4000-600 cm^{-1} at Chemistry Department of Kurukshetra University, Kurukshetra. Sodium chloride disc (0.1 mm thickness) was used.

3.11.2.1 Whole fat

Fat samples melted at 35°C were scanned neatly through IR range 4000 - 600 cm^{-1}.

3.11.2.2 Unsaponifiable matter

Unsaponifiable matter of fat samples was scanned (neat) in IR range 4000-600 cm^{-1}.

3.12 MELTING POINT OF THE FATTY ACIDS

The melting point of dried and powdered fatty acids mixtures obtained from insoluble unsaponifiable matter and turbid material was determined in concentrated H_2SO_4 bath by capillary method.

3.13 MODIFIED COLOUR TEST FOR VANASPATI

During the course of study while trying to develop a colour test for the detection of vegetable oils and animal body fats in ghee using different reagents like phenol, benzaldehyde, ferric chloride, potassium permagnate, thymol blue, Rhodamine 6B and p-hydroxy benzaldehyde, it was observed that p-hydroxy benzaldehyde produced colour with sesame oil like furfuraldehyde.

Five gram of molten fat with an equal quantity of concentrated hydrochloric acid was taken in a test tube and thereafter 9-10 drops of 5 per cent alcoholic solution or 10-15 mg of solid p-hydroxy benzaldehyde was added to it. The contents were shaken vigorously for 40-50 seconds taking care that fat should not solidify. The development of red colouration in lower layer indicated the presence of vanaspati containing sesame oil.

CHAPTER 4

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 THE REICHERT AND POLENSKE VALUES OF GHEE, ANIMAL BODY FATS AND ADULTERATED GHEE SAMPLES

All the ghee and body fat samples used in the study were examined for their Reichert and Polenske values to assess their initial quality. The values for ghee samples were well within the range and body fat samples had almost negligible Reichert and Polenske values. The cow and buffalo ghee samples were then adulterated with different body fats, viz., buffalo, goat, sheep and pig at 10 per cent level and analysed for Reichert and Polenske values. It was observed that the Reichert and Polenske values of the adulterated ghee samples were not significantly affected at 10 per cent level of adulteration and remained well within the acceptable standards. The per cent decrease in Reichert-Meissl value of the adulterated ghee samples varied from 7 to 9.

4.2 UNSAPONIFIABLE MATTER CONTENT OF GHEE AND ANIMAL BODY FAT SAMPLES

Unsaponifiable matter which contains sterols as a major portion of its content was estimated in ghee and animal body fats to find differences, if any, in the content of total unsaponifiable matter of ghee and animal body fats. The results illustrated in Table 5 reveal that cow ghee contained slightly higher level of unsaponifiable

matter (0.458 - 0.562%; average, 0.505%) compared to buffalo ghee (0.426 - 0.526%; average, 0.472%). The unsaponifiable matter content in cottonseed fed buffalo ghee (0.406 - 0.438%; average 0.429%) was lower than normal cow and buffalo ghee and was in the range for the buffalo, goat and sheep body fats. Among body fats, pig body fat contained the lowest amount of unsaponifiable matter (0.269-0.347%; average, 0.302%). It is apparent that total unsaponifiable matter content was relatively higher in ghee as compared to animal body fats.

Marked difference in the unsaponifiable matter content of ghee and animal body fats was not observed. Feeding cottonseeds did not effect the unsaponifiable matter content of ghee. These results were in agreement with the earlier studies. Sankhla and Yadava (1981) reported the total unsaponifiable matter, 0.416 ± 0.02 per cent in cow ghee and 0.392 ± 0.07 per cent in buffalo ghee. Singhal (1973) reported higher content of unsaponifiable matter in ghee as compared to animal body fats. Ghee samples exhibited significant variation in unsaponifiable matter content. Animal body fats did not show much variations in content of unsaponifiable matter.

4.3 UNSAPONIFIABLE MATTER CONTENT OF ADULTERATED GHEE SAMPLES

With a view to ascertain difference in the unsaponifiable matter content on adulteration, ghee samples were mixed in different proportion with animal body fats. The results are given in Table 6. It was noticed that content

of total unsaponifiable matter decreased in ghee samples adulterated with animal body fats and the decrease in the content of unsaponifiable matter depended on the amount of adulterant body fat present in ghee. Similar results were reported by Singhal (1973). The content of unsaponifiable matter of adulterated ghee samples was on the higher side than the lower limits in pure ghee. On average,the values of unsaponifiable matter content in adulterated ghee samples were within the range of pure ghee samples and thus evidenced that the unsaponifiable matter content of fat samples did not show any encouraging results which could be utilized for the detection of adulteration.

4.4 SEASONAL VARIATIONS IN THE CONTENT OF UNSAPONIFIABLE MATTER OF GHEE AND ANIMAL BODY FAT SAMPLES

Seasonal variations in unsaponifiable matter content are depicted in Table 7. It was noticed that ghee and animal body fat samples contained higher content of unsaponifiable matter in winter in comparison to summer. Cow ghee contained higher level of unsaponifiable matter in winter (0.444 - 0.574%; average, 0.507%) and summer (0.422 - 0.548%; average, 0.475%) as compared to buffalo ghee (0.438-0.550%; average, 0.483 in winter and 0.418 - 0.510%; average, 0.451% in summer). Amongst animal body fats, buffalo body fat contained higher content of unsaponifiable matter in winter and summer as compared to other body fats. The pig body fat contained low content of unsaponifiable matter. The variations in unsaponifiable matter content were significant in ghee samples compared to animal body fats. It was

observed that total unsaponifiable matter content of ghee and animal body fat samples in winter and summer did not show much difference except that pig body fat contained low content of unsaponifiable matter.

Higher values of unsaponifiable matter in cow ghee during winter and summer in comparison with buffalo ghee were in conformity with the results of Bindal and Jain (1973) and Pantulu et al. (1972). Bindal and Jain (1973) reported higher content of unsaponifiable matter in cow ghee in winter (average value, 463 mg %) as compared to summer (average value, 428 mg %). In case of buffalo ghee, unsaponifiable matter was higher in winter (average value, 399 mg %) than summer (average value, 392 mg %). Among animal body fats, buffalo body fat exhibited higher value of unsaponifiable matter as compared to other body fats. On comparison, the content of unsaponifiable matter was observed low in animal body fats compared to ghee. Pig body fat showed low value of unsaponifiable matter among ghee and body fat samples examined. The seasonal variations among animal body fats were more marked in pig body fat. Data in regard to seasonal variations in content of unsaponifiable matter in animal body fats are not available for comparison.

4.5 UNSAPONIFIABLE MATTER CONTENT IN ETHER SOLUBLE AND ETHER INSOLUBLE FRACTIONS OF GHEE AND ANIMAL BODY FATS

In view of difference in the composition of ether soluble and ether insoluble fractions of ghee and animal body fats,it was expected that the difference in the content

of unsaponifiable matter in these fractions of ghee and animal body fats might exist. Unsaponifiable matter was estimated from ether soluble and ether insoluble fractions obtained from ghee and animal body fat samples fractioned in ether at 4 to 5°C. The results are presented in Table 8. Unsaponifiable matter content in ether soluble fractions of buffalo ghee and cow ghee was observed, 411 and 362 mg per cent, respectively, while ether soluble fractions of buffalo and pig body fats contained 221 and 144 mg per cent of unsaponifiable matter. The content of unsaponifiable matter in ether insoluble fractions of buffalo ghee and cow ghee was 72 and 142 mg per cent, respectively, whereas ether insoluble fractions of buffalo and pig body fats showed 185 and 86 mg per cent content of unsaponifiable matter.

It was observed from the results that unsaponifiable matter content in ether insoluble fraction of buffalo body fat showed higher value than in ether insoluble fractions of ghee samples. Unsaponifiable matter content in ether insoluble fraction of pig body fat was observed higher compared to that of buffalo ghee.

These differences led to further studies on estimation of unsaponifiable matter content in ether soluble and ether insoluble fractions of adulterated ghee samples.

4.6 UNSAPONIFIABLE MATTER CONTENT IN ETHER SOLUBLE AND ETHER INSOLUBLE FRACTIONS OF ADULTERATED GHEE SAMPLES

The results for the unsaponifiable matter content of ether soluble and ether insoluble fractions of ghee samples

adulterated with buffalo and pig body fats at 10 per cent level are given in Table 9. Unsaponifiable matter content in ether soluble fractions of adulterated ghee samples showed decrease on adulteration while ether insoluble fractions of cow ghee adulterated with buffalo body fat and buffalo ghee adulterated with buffalo and pig body fats showed increase in unsaponifiable matter content. The differences in the value of unsaponifiable matter of ether insoluble fractions of adulterated ghee samples from those of pure ghee samples were not significant and could be varied due to experimental errors. The method, therefore, could not be useful for the detection of animal body fats in ghee.

4.7 CHOLESTEROL CONTENT

4.7.1 CHOLESTEROL CONTENT OF GHEE AND ANIMAL BODY FATS

In view of differences observed in unsaponifiable matter content of ghee and animal body fats, total cholesterol was estimated by colorimetric methods to ascertain the differences in the content of cholesterol in ghee and animal body fats. The results for cholesterol content of ghee and animal body fats are recorded in Table 10. It was observed that cholesterol content of animal body fats was low as compared to ghee samples. Among ghee samples, cholesterol content was low in cottonseed fed buffalo ghee than that in normal ghee samples. On comparing the colorimetric methods, it was noticed that the direct colorimetric method (Method Ia) gave higher content of cholesterol in ghee samples (average, 333 mg % in cow ghee; average,

300 mg % in buffalo ghee and average, 282 mg % in cotton-tract buffalo ghee) compared to unsaponifiable matter technique using acetic acid as solvent (average, 263 mg% in cow ghee; average, 249 mg % in buffalo ghee and average, 246 mg % in cotton-tract buffalo ghee). The unsaponifiable matter technique using chloroform as solvent showed higher content of cholesterol compared to unsaponifiable matter technique using acetic acid as solvent in ghee samples (average, 308 mg % in cow ghee; average, 266 mg % in buffalo ghee and average, 258 mg % in cottonseed fed buffalo ghee). The content was, however, less than that of direct colorimetric method. Similar pattern of cholesterol content in animal body fats was observed by these colorimetric methods. It was observed that cow ghee contained highest level of cholesterol and pig body fat, the lowest level of cholesterol among all ghee and animal body fat samples examined. The cholesterol content was observed low in animal body fats compared to ghee samples. The variations in cholesterol content were more marked in ghee samples than those in animal body fats.

The methods used for the estimation of cholesterol are generally based on colorimetric estimation of cholesterol/cholesterol digitonides (Schoenheimer and Sperry, 1937; Sperry and Webb, 1950) or involve saponification of fat samples followed by gravimetric estimation of cholesterol as digitonide (Peereboom, 1963). The cholesterol content of fat samples can be estimated by direct (fat) and indirect (unsaponifiable matter) methods. However, fats have

limited solubility in acetic acid and this is reduced still further by subsequent addition of Lieberman-Burchard reagent. This limitation was overcome when acetic acid was replaced by chloroform. Further during estimation of cholesterol by direct colorimetric method in animal body fats, some turbidity was observed. Thus, the unsaponifiable matter technique for estimation of cholesterol was applied to all fat samples in chloroform and acetic acid as solvents. The coloured complex of cholesterol with Lieberman-Burchard reagent showed max at 645 nm (Fig. 4) and did not affect cholesterol measurements made at 650 nm. The results were in agreement with earlier reports. Pantulu et al. (1972) reported that cow milk fat (386 mg %) contained higher level of cholesterol than buffalo milk fat (334 mg %). Higher value of cholesterol has also been reported (640 mg%) in milk fat(Dam,1934).The low content of cholesterol in ghee and animal body fat samples estimated by direct colorimetric method compared to unsaponifiable matter techniques was also in coincidence with reports of Singhal (1973). On comparison of the colorimetric methods, it was clear that direct fat technique showed better results and being simpler and quick was adopted for cholesterol estimation in ghee samples on adulteration.

4.7.2 CHOLESTEROL CONTENT OF ADULTERATED GHEE SAMPLES

Based on the differences in the content of cholesterol between ghee and animal body fats, ghee samples adulterated with different animal body fats at 10 per cent

level were examined for cholesterol to evolve a simple test which could detect adulteration of ghee with animal body fats on the basis of differences in cholesterol content. The results are recorded in Table 11. The cholesterol content of ghee samples on adulteration showed decrease. At 10 per cent adulteration, the cholesterol content of ghee samples adulterated with animal body fats was on the higher scale than the lower limit observed in pure ghee. These results were in agreement to the report of Singhal (1973). On average, the content of cholesterol in *adulterated ghee samples was observed well within the range* of normal ghee. This estimation was, therefore, of no practical use in the detection of adulteration of ghee.

4.7.3 CHOLESTEROL CONTENT OF GHEE AND ANIMAL BODY FATS ESTIMATED BY GRAVIMETRIC METHODS (DIRECT AND INDIRECT METHODS)

Unsaponifiable matter among sterols, contains other constituents also which may react wtih digitonin and co-precipitate with sterol digitonides (Roos et al., 1969). To overcome this limitation in order to estimate true amount of cholesterol as cholesterol digitonide, the insoluble unsaponifiable matter was extracted from ghee and animal body fats after saponification. The cholesterol content *was, therefore, estimated from* unsaponifiable matter as whole and unsaponifiable matter free from insoluble unsaponifiable matter by gravimetric methods. The results for cholesterol content of ghee and animal body fats estimated by direct and indirect gravimetric methods are given in Table 12. Cow ghee contained higher content of

cholesterol (Method IIa, average, 285 mg % and Method IIIb, average, 261 mg %) compared to buffalo ghee (Method IIa, average, 265 mg % and method IIIb, average, 247 mg %). In case of animal body fats, the buffalo body fat contained higher content of cholesterol (Method IIa, average, 217 mg % and Method IIIb, average 199 mg %) than in other animal body fats. Among ghee samples, the cholesterol content was low in cotton-tract buffalo ghee (Method IIa, average, 256 mg % and Method IIIb, average, 238 mg %) than in normal ghee. The pig body fat exhibited low content of cholesterol (average, 106 and 93 mg %) among all ghee and animal body fat samples. On comparison, it was noticed that both, direct and indirect gravimetric methods gave comparable results except that the direct gravimetric method gave a little higher value of cholesterol than the indirect gravimetric method.

It was apparent from the results that marked variation in the content of cholesterol in ghee and animal body fat except pig body fat was observed (Table 12). The results of cholesterol in ghee samples estimated by direct gravimetric method were in agreement with those reported by Bindal and Jain (1972). Data on the cholesterol content of animal body fats estimated by gravimetric methods are not available for comparison. However, the values were in coincidence to the results evaluated by direct and indirect colorimetric methods. On comparison of the two methods, it was observed that direct gravimetric method show better results and obviates the unusual loss of unsaponifiable

constituents in extraction of insoluble unsaponifiable matter in case of indirect gravimetric method. The low content of cholesterol in animal body fats compared to ghee samples led to the further studies of ghee samples on adulteration.

4.7.4 CHANGES IN CHOLESTEROL CONTENT OF ADULTERATED GHEE SAMPLES (GRAVIMETRIC METHODS)

The results for cholesterol content of adulterated ghee samples are presented in Table 13. It was observed that cholesterol content of ghee samples decreased on adulteration. At 10 per cent adulteration, cholesterol content of adulterated ghee samples was found on the higher scale than the lower limit in pure ghee samples. The results were observed to show similar pattern in the decrease of cholesterol content in adulterated ghee samples as in direct and indirect colorimetric methods. At 10 per cent adulteration, on average the values of cholesterol in adulterated ghee samples were observed within the range of pure ghee samples and, therefore, did not give useful clue for the detection of animal body fats in ghee. However, the precipitation of a white flocculent residue called insoluble unsaponifiable matter in animal body fats in course of cholesterol estimation by indirect gravimetric method gave encouraging preliminary incentive for further studies for the detection of adulteration in ghee.

4.7.5 COMPARATIVE STUDY OF CHOLESTEROL CONTENT ESTIMATED BY *COLORIMETRIC AND GRAVIMETRIC METHODS IN GHEE AND* ANIMAL BODY FATS

The results for cholesterol estimated by colorimetric

and gravimetric methods for comparative study in ghee and animal body fats are presented in Table 14. It was noticed that direct colorimetric method (Method Ia) showed higher content of cholesterol in ghee and animal body fats compared to indirect colorimetric (Method IIa and Method IIIb) methods. On an average, the values of cholesterol estimated by direct gravimetric method (Method IIa) were almost close to direct colorimetric method. The indirect colorimetric methods and indirect gravimetric method (Method IIIb) showed low values of cholesterol in ghee and animal body fats compared to methods Ia and IIa, respectively. The results of cholesterol estimated by direct colorimetric method were observed in better conformity than other methods to the earlier reports on ghee and animal body fats. Moreover, the direct fat technique being simple, quick and accurate is better than gravimetric methods since the later methods are complicated and involve the use of expensive digitonin reagent. Hence, the direct fat technique is the suitable method for cholesterol estimation and could be adopted for cholesterol estimation in ghee and animal body fats.

4.8 FREE AND ESTERIFIED CHOLESTEROL CONTENT IN GHEE AND ANIMAL BODY FATS

It is known that there are distinct differences between free and bound sterols present in ghee and animal body fats. In view of such expected difference, free and esterified sterols were estimated by TLC and gravimetric methods. The precipitation of cholesterol digitonide, before and after saponification of the fat gave a measure

of free and total cholesterol, respectively. Esterified cholesterol was generally estimated indirectly by subtracting free cholesterol from total cholesterol. The precipitation of cholesterol as digitonide before and after saponification of the fat devoid of free cholesterol gave direct measurement of free and esterified cholesterol. The results for the estimation of free and esterified cholesterol by TLC and gravimetric methods in ghee and animal body fats are presented in Table 15. The level of free cholesterol estimated by TLC method in cow ghee varied between 235 to 251 mg per cent (average value, 244 mg %) and that of buffalo ghee between 229 to 246 mg per cent (average value, 236 mg %). The level of free cholesterol estimated by gravimetric method in cow ghee varied between 221 to 241 mg per cent (average value, 231 mg %) and that of buffalo ghee between 207 to 227 mg per cent (average value, 217 mg %). Calculations revealed that on average, the percentage of free cholesterol in case of TLC method (Method IIIa) was higher in cow ghee (86.6%) compared to buffalo ghee (83.5%). A similar distribution pattern of free cholesterol by gravimetric method (Method IVb) was observed in cow ghee (89.57%) and buffalo ghee (85.56%). Amongst animal body fats, the level of free cholesterol was low in pig body fat (Method IIIa; average value, 87 mg% and Method IVb; average value, 86 mg %) compared to other body fats. Buffalo body fat exhibited higher content of free cholesterol than other animal body fats. However, animal body fats showed low content of free cholesterol compared to ghee samples.

Calculations showed that on average, the percentage of free cholesterol in case of TLC method was higher in buffalo body fat (86.43%) followed by sheep (85.54%), goat (84.83%) and pig (75.85%) body fats. The average percentage of free cholesterol by gravimetric method was, however,observed higher in sheep (88.26%) followed by buffalo (88.06%), goat (87.56%) and pig (83.94%) body fats.

Unlike free cholesterol, cow ghee contained low level of esterified cholesterol compared to buffalo ghee. The level of esterified cholesterol estimated by TLC method varied in cow ghee between 35.79 to 42.89 mg %, average, 37.7 mg % and 42.46 to 50.12 mg %, average, 46.73 mg % in bufalo ghee. In case of gravimetric method, the level of esterified cholesterol varied in cow ghee between 22.4 to 31.4 mg %, average, 26.9 mg % and 34.8 to 38.67 mg %, average, 36.63 mg % in buffalo ghee. Calculations of results showed that the proportion of per cent esterified cholesterol on average was of the order of 13.38 per cent (Method IIIa) and 10.43 per cent (Method IVb) in cow ghee and 16.51 per cent (Method IIIa) and 14.44 per cent (Method IVb) in buffalo ghee, respectively. The per cent level of esterified cholesterol in animal body fats was in order of pig (Method IIIa, 24.14% and Method IVb, 16.04%), goat (Method IIIa, 15.16% and Method IVb, 12.44%), sheep (Method IIIa, 14.45% and Method IVb, 11.74%) and buffalo (Method IIIa, 13.56% and Method IVb, 11.74%) body fats, respectively.

It is apparent from the results that cow ghee exhibited higher value of free cholesterol whereas buffalo

ghee showed higher value of esterified cholesterol among all ghee and animal body fat samples examined. Body fats showed low values of free and esterified cholesterol compared to ghee samples. Among animal body fats, pig body fat showed low level of free and esterified cholesterol. Buffalo body fat exhibited higher level of free and esterified cholesterol among all animal body fats except that sheep body fat showed a little higher value of free and esterified cholesterol in case of gravimetric method. The variations in level of free cholesterol were marked in both ghee and animal body fats while the variations in level of esterified cholesterol were more marked in ghee samples compared to animal body fats. Our results were in broad agreement with the results of earlier workers. Kritchevsky and Tepper (1961) reported 319 mg per cent of free cholesterol in cow ghee. Higher level of free cholesterol, viz., 391 mg per cent (Hardy and Mackie,1969), 360 mg per cent (Steger, 1962) and 330 mg per cent (deMan, 1969) have also been reported in cow ghee. Several workers have reported the occurrence of only free cholesterol in milkfat (Dam, 1934; Laskowski, 1962). Bindal and Jain (1972) reported that on average, the level of free cholesterol was 283 mg per cent in cow ghee and 212 mg per cent in buffalo ghee. The results of esterified cholesterol were also in conformity with those of Bindal and Jain (1972) who reported the level of esterified cholesterol in the order of about 14 per cent in cow ghee and 23 per cent (higher value) in buffalo ghee, while Den

Herder (1955) and Nieman et al. (1951) observed 10 and 9 per cent, respectively, the proportion of esterified cholesterol in cow ghee. Higher values of esterified cholesterol such as 36 per cent have also been reported (Fox and Gardner, 1923). The results for the level of free and esterified cholesterol in animal body fats are not available for comparison. On comparison of the two methods, it was noticed that values of free and esterified cholesterol estimated by TLC method in ghee and animal body fats were higher compared to gravimetric method. Further the results of TLC method were in close conformity with the colorimetric methods and direct gravimetric method. Further, TLC method enabled the direct and independent estimation of free and esterified cholesterol. The rather drastic saponification step for the estimation of esterified cholesterol in case of gravimetric method was avoided. The TLC mehod being less time consuming and obviates the use of expensive reagent, digitonin is better method for estimation of free and esterified cholesterol in fats. The differences observed in the level of free and esterified cholesterol between ghee and animal body fats led to further analysis of ghee samples on adulteration.

4.9 FREE AND ESTERIFIED CHOLESTEROL CONTENT OF ADULTERATED GHEE SAMPLES

Only the gravimetric method was tried for the estimation of free and esterified cholesterol in ghee samples adulterated with different animal body fats at 10 per cent level. The results are illustrated in Table 16.

As expected, the content of free and esterified cholesterol of ghee samples decreased on adulteration and the decrease in the value was dependent on the amount of animal body fat added to ghee. At 10 per cent adulteration, the levels of free and esterified cholesterol were observed within the range of free and esterified cholesterol of pure ghee samples. The results showed no significant variations in the level of free and esterified cholesterol of adulterated ghee samples from pure ghee samples and, therefore, this study did not give any useful clue for the detection of body fats in ghee.

4.10 INSOLUBLE UNSAPONIFIABLE MATTER CONTENT OF GHEE AND ANIMAL BODY FATS

In course of estimation of cholesterol by gravimetric method (Method IIIb), it was observed that animal body fats showed the precipitation of a white flocculent residue latently called as insoluble unsaponifiable matter. Taking advantage of this observation, the quantitative estimation of insoluble unsaponifiable matter in ghee and animal body fats was tried to see the possibilities of detecting animal body fats in ghee. The results are presented in Table 17. Among ghee samples, the cotton-tract buffalo ghee showed a difference having higher content of insoluble unsaponifiable matter (0.112 - 0.148 %) compared to cow ghee (0.07%) and buffalo ghee (0.08%). In case of animal body fats, the content of insoluble unsaponifiable matter was higher in buffalo body fat (33.89 - 36.42%; average, 35.15%) than in goat (15.96 - 20.48%; average, 18.22%) and sheep (12.92 -

18.52%; average, 15.72%) body fats, respectively. Pig body fat showed low content of insoluble unsaponifiable matter (0.176 - 0.198%; average, 0.187%) and behaved almost similar to cotton-tract buffalo ghee.

It appeared from the results (Table 17) that ghee samples showed negligible amount of insoluble unsaponifiable matter compared to all animal body fats except that pig body behaved similar to cotton-tract buffalo ghee. Roos et al. (1969) also reported the content of insoluble unsaponifiable matter (0.06%) in synthetic butterfat. A wide variation in insoluble unsaponifiable matter content was observed in animal body fats except pig body fat. The precipitation of higher amount of insoluble unsaponifiable matter in animal body fats compared to ghee samples was due to the presence of higher content of long chain saturated fatty acids in animal body fats compared to ghee samples (Para 4.13). The low content in pig body fat was apparent from its soft nature due to presence of higher content of unsaturated fatty acids compared to other animal body fats. Such wide differences in the content of insoluble unsaponifiable matter between ghee and animal body fats encouraged to investigate the content of insoluble unsaponifiable matter in ghee samples on adulteration to evaluate differences, if any, in the content of insoluble unsaponifiable matter of adulterated ghee samples from pure ghee samples.

4.11 INSOLUBLE UNSAPONIFIABLE MATTER CONTENT OF ADULTERATED GHEE SAMPLES

The results for insoluble unsaponifiable matter content of ghee samples adulterated at 10,15 and 20 per cent levels with different animal body fats are presented in Tables 18 and 19. At 10 and 15 per cent adulterations, the content of insoluble unsaponifiable matter was observed negligible in adulterated normal ghee samples (Table 18). The cotton-tract buffalo ghee at 15 per cent adulteration, however, showed quantitative amount of insoluble unsaponifiable matter (Table 19). Only at 20 per cent adulteration, the quantitative amount of insoluble unsaponifiable matter was observed in adulterated ghee samples. The content of insoluble matter was noticed higher in ghee samples adulterated at 20 per cent level with buffalo body fat compared to ghee samples adulterated with other animal body fats. The adulterated cotton-tract buffalo ghee, however, showed a little higher content of insoluble unsaponifiable matter than in adulterated normal ghee samples.

It was clear from the results that the precipitation of insoluble unsaponifiable matter was dependent on the nature and amount of body fat added to ghee. Ghee samples adulterated at 10 and 15 per cent levels did not show quantitative precipitation of insoluble unsaponifiable matter which could detect adulteration. At 15 per cent adulteration, the adulterated normal ghee samples, however, showed visual precipitation of insoluble unsaponifiable

matter and the quantitative recovery of insoluble matter was not possible at this level of adulteration because the insoluble unsaponifiable matter precipitated though observed visually still remained in solution. The cotton-tract buffalo ghee adulterated with animal body fats except pig body fat at 15 per cent level, however, showed quantitative precipitation of insoluble unsaponifiable matter which could detect body fats adulterated in cotton-tract ghee. Only at 20 per cent adulteration, the quantitative amount of insoluble unsaponifiable matter in adultered ghee samples was recovered and detection of body fats in ghee samples was possible at this level. The precipitation of insoluble unsaponifiable matter in ghee samples adulterated at 20 per cent level was due to the presence of higher content of long chain saturated fatty acids in animal body fats that showed precipitation in ghee samples on adulteration with animal body fats as their corresponding soap. The absence of quantitative precipitation of insoluble unsaponifiable matter in ghee samples adulterated at 10 and 15 per cent levels might be due to the low content of higher saturated fatty acids which could not precipitate out from solution of adulterated ghee samples. The presence of higher content of insoluble unsaponifiable matter in ghee samples adulterated with buffalo body fat compared to ghee samples adulterated with other animal body fats was due to the presence of comparatively higher content of higher saturated fatty acids in buffalo body fat compared to other body fats (Para 4.13). This method can be used for the

detection of buffalo, goat, sheep and pig body fats at 20 per cent level in ghee samples and at 15 per cent level except in pig body fat in cotton-tract ghee samples. The method, however, could not detect adulteration of body fats in ghee below 20 per cent. Since the object of the study was to devise a test that could detect adulteration of animal body fats in ghee at 10 per cent level or below. Therefore, this method can not be used for detection of animal body fats in ghee at low level. This aspect of study has given promising clues for further studies to evolve a simple test for detecting animal body fats at 10 per cent level.

4.12 MELTING POINT OF FATTY ACIDS

Based on the soapy nature of insoluble unsaponifiable matter, fatty acids were extracted from insoluble unsaponifiable matter of different body fats and their melting points were determined to know the nature and type of fatty acids. The results are shown in Table 20. It was observed that the melting point of fatty acids mixture in pig, sheep, buffalo and goat body fats was 59, 61, 62 and 63°C, respectively. The melting point showed low value in pig body fat and higher value in goat body fat compared to other body fats. The melting points of fatty acids mixture did not show much variation in body fats and their values were observed near to the range value of melting point of standard fatty acids, viz., palmitic, stearic and arachidic. Roos et al. (1969) reported a little higher value of melting point (68°C) of insoluble unsaponifiable matter in synthetic butterfat. The low value of melting points of fatty acids of insoluble unsaponifiable matter in

animal body fats may be due to the presence of some medium chain low melting fatty acids alongwith higher chain fatty acids in the fatty acid mixture. The low value of melting point of fatty acids in pig body fat was due to low content of saturated fatty acids compared to other body fats as evidenced from its soft nature and higher iodine value. The close conformity of the results of melting point of fatty acids to the range of melting point of standard fatty acids, palmitic, stearic etc. suggested the nature of insoluble unsaponifiable matter of body fats as the soap of higher saturated fatty acids especially, palmitic, stearic, arachidic etc.

4.13 GLC OF METHYL ESTERS OF FATTY ACIDS OF INSOLUBLE UNSAPONIFIABLE MATTER OF ANIMAL BODY FATS

In view of the expected nature of fatty acids of insoluble unsaponifiable as the mixture of long chain fatty acids from the melting point, it was desired to study the composition of fatty acids of insoluble unsaponifiable matter of body fats from buffalo, goat and sheep as their methyl esters by GLC (Fig. 5). The results are illustrated in Table 21. As expected, the body fats of all the three species of animal (buffalo, goat and sheep) were devoid of short chain fatty acids (C4-C8). The content of medium chain fatty acids, C10:0 and C12:0 was low and almost similar in all body fats except that sheep body fat showed higher content of fatty acid, C12:0. Goat body fat contained higher content of fatty acids, C14:0 (2.67%) and C16:0 (46.82%) compared to sheep (C14:0, 1.85% and C16:0, 31.98%)

and buffalo (C14:0, 1.35% and C16:0, 29.48%) body fats. The level of fatty acid, C18:0 was observed higher in buffalo body fat (68.72%) compared to sheep (63.94%) and goat (49.75%) body fats.

The absence of short chain fatty acids and the presence of higher content of long chain saturated fatty acids (especially, C16:0 and C18:0) in animal body fats (buffalo, goat and sheep) were in agreement with the results of earlier workers (Tandon and Ganguli, 1972; Singhal, 1973). The presence of higher level of saturated fatty acids, C14:0 and especially, C16:0 and C18:0 in insoluble unsaponifiable matter of animal body fats was the possible reason of increased precipitation of insoluble unsaponifiable matter in animal body fats and ghee samples adulterated with animal body fats compared to pure ghee samples. The higher level of fatty acid, C18:0 and higher value of fatty acid ratio, C18:0/C16:0 in buffalo body fat (2.33) compared to sheep (C18:0/C16:0, 1.99) and goat (C18:0/C16:0, 0.99) body fats was mainly responsible for higher extent of precipitation of insoluble unsaponifiable matter in buffalo body fat and ghee samples adulterated with buffalo body fat compared to sheep and goat body fats and ghee samples adulterated with these body fats.

4.14 TURBIDITY TEST

The phenomenon of turbidity which many workers have found more effective for the detection of adulterants in ghee is the solidification of molten fat by allowing to

stand at different temperatures or solidification of fat dissolved in a suitable solvent or a mixture of solvents at different temperatures or crystallization of fat after saponification in suitable solvents. The difference between ghee and animal body fats in the content of insoluble unsaponifiable matter (Table 17) gave useful clues to evolve a test that could detect adulteration of animal body fats in ghee at low level. The solution of saponifiable fat in a solvent mixture of 96 per cent ethyl alcohol and water in different proportions was transparent in appearance and became turbid on cooling. Hence, the turbidity test was developed.

4.14.1 TURBIDITY BEHAVIOUR OF GHEE AND ANIMAL BODY FATS IN DIFFERENT SOLVENT COMBINATIONS AT DIFFERENT TEMPERATURES

The turbidity behaviour of ghee and animal body fats was tried in different combinations of a solvent mixture of 96 per cent ethyl alcohol and water at different temperatures to see the differences in turbidity behaviour of animal body fats from ghee samples which could be helpful for the detection of body fats in ghee at a suitable temperature using a better combination of solvent mixture. It was observed that ghee samples started showing turbidity in the solvent mixture of 96 per cent ethyl alcohol and water in two combinations (15:20 and 10:10 ml each) at temperatures ranged from -4 to -6°C after 30 to 45 minutes. On the other hand, body fats from buffalo, goat and sheep showed noticeable turbidity in all combinations of a solvent

mixture, 96 per cent ethyl alcohol and water (25:25, 15:25, 15:20, 15:15 and 10:10 v/v) at temperatures from -2 to 5°C and each fat took time from seconds to minutes to show turbidity depending upon the temperature of cooling and solvent combination. The pig body fat, however, exhibited turbidity only at temperature, ranged from -2 to 0°C in the last two combinations of solvent mixture (15:15 and 10:10, v/v). Ghee samples did not show turbidity at 0°C in a mixture of 96 per cent alcohol and water (1:1, v/v) even upto 2 hours whereas all animal body fats and cotton-tract ghee showed noticeable turbidity at 0°C in this solvent combination. This combination showed marked differences in the turbidity time of animal body fats from ghee samples. Cotton-tract ghee also showed marked difference from animal body fats in turbidity behaviour. Difference in the turbidity behaviour among animal body fats were more marked in this combination at 0°C. Differences in the crystallization behaviour of ghee and animal body fats except pig body fat and cotton-tract ghee at 23°C have also been reported by Singhal (1970, 1980).

The studies conducted in solvent mixture of 96 per cent ethyl alcohol and water (1:1,v/v) at 0°C has the following advantages over the opacity test, (i) firstly, ghee samples except cotton-tract ghee did not show turbidity, (ii) cotton-tract ghee differed markedly from pig body fat in turbidity behaviour, (iii) the differences in the time interval of turbidity between ghee and animal body fats were many fold and (iv) temperature of 0°C can be maintained easily.

In view of these observations,the turbidity behaviour of ghee and animal body fats were, therefore, studied at 0°C using 96 per cent alcohol and water (1:1,v/v) to develop a turbidity test for the detection of animal body fats in ghee.

4.14.1.1 Turbidity behaviour of ghee and animal body fats at 0°C

The absorbance profiles of ghee and animal body fats are illustrated in Figures 6 and 7. The time taken to show start of turbidity in goat, sheep, buffalo and pig body fats was 20, 20, 30 and 210 seconds, respectively. Ghee samples did not show turbidity (absorbance, 0.1) in more than 2 hours (Fig. 6) whereas animal body fats, viz., goat, sheep, buffalo and pig became turbid (absorbance, 0.6) in 30, 40, 50 and 240 seconds (Fig.7). Cotton-tract buffalo ghee took 15 minutes to show turbidity (absorbance, 0.45, Fig. 8). Like normal ghee samples, vanaspati and coconut oil neither showed turbidity nor marked absorbance at 0°C in two hours (Fig. 6) whereas groundnut oil behaved similar to animal body fats (Fig. 7) and took 40 seconds to show noticeable turbidity (absorbance, 0.55).

A marked difference in the turbidity behaviour of ghee and animal body fats was observed. There was complete absence of turbidity in ghee samples for more than 2 hours while animal body fats had the property of showing turbidity in seconds. Among ghee samples, only the cotton-tract buffalo ghee showed difference having the property to exhibit turbidity. The turbidity behaviour of cotton-tract

ghee unlike in opacity test (Singhal, 1970, 1980) was different from pig body fat and 4 to 5 fold difference in the turbidity time between pig body fat and cotton-tract buffalo ghee was observed. The difference in the turbidity time of animal body fats from ghee samples was many fold. An hundred time fold difference in the turbidity time to show noticeable turbidity between groundnut oil and, vanaspati and ghee samples gave encouraging clue for detection of groundnut oil in ghee and vanaspati. The marked difference in turbidity behaviour of animal body fats from ghee samples led to the further study of ghee samples on adulteration.

4.14.1.2 Turbidity behaviour of adulterated ghee samples at 0°C

The results for the turbidity behaviour of ghee samples adulterated with different animal body fats at 10 per cent level are illustrated in Figures 9 and 10. The time taken to show significant turbidity (absorbance, 0.4) by cow ghee adulterated with buffalo, sheep and goat body fats was 10, 20 and 50 minutes (Fig. 9) whereas buffalo ghee adulterated with buffalo, sheep and goat body fats showed turbidity (absorbance, 0.4) in 5, 8 and 40 minutes (Fig.10). Ghee samples from buffalo and cow on 10 per cent adulteration with pig body fat took 60 and 90 minutes respectively to show turbidity (absorbance, 0.2) as shown in Figures 9 and 10. Cotton-tract buffalo ghee adulterated with buffalo, sheep, goat and pig body fats became turbid (absorbance, 0.6) in 5, 5 , 6 and 10 minutes respectively

(Fig. 8). Ghee samples adulterated with animal body fats, except pig body fat, at 5 per cent level showed start of turbidity after 1½ hour.

A marked difference in the turbidity profile of refined groundnut oil from that of ghee and vanaspati led to analyse ghee samples and vanaspati adulterated with refined groundnut oil at different levels. The results are illustrated in Fig. 6. Only at 30 per cent adulteration, ghee samples adulterated with groundnut oil showed turbidity (absorbance, 0.4) in 5 minutes. The time taken to show noticeable turbidity by vanaspati samples adulterated at 15, 20, 25 and 30 per cent levels with groundnut oil was 60, 45, 6 and 5 minutes, respectively (Fig.6). Adulteration of coconut oil in ghee upto 20 per cent level did not show marked difference in the turbidity from normal ghee (Fig.6).

It was apparent from the results that adulterated cow ghee samples took more time to show noticeable turbidity compared to adulterated buffalo ghee samples. Cotton-tract buffalo ghee samples adulterated with buffalo, sheep and goat body fats at 10 per cent level took 8,35 and 40 minutes less than cow ghee samples adulterated with buffalo, sheep and goat body fats and, 6, 30 and 35 minutes lesser than buffalo ghee adulterated with buffalo, sheep and goat body fats to show turbidity (absorbance, 0.4-0.6). Buffalo and cow ghee samples adulterated with pig body fat at 10 per cent level took 80 to 90 minutes more compared to cotton-tract buffalo ghee adulterated with pig body fat to show turbidity (absorbance, 0.25). The presence of higher content

of long chain saturated fatty acids (especially, C16-C18) in animal body fats(Para 4.14.3) resulted in inducing turbidity in ghee samples on adulteration with diffeent animal body fats. The property of ghee samples adulterated with buffalo body fat to show turbidity more quickly than ghee samples adulterated with other animal body fats was due to presence of higher amount of saturated fatty acids, myristic, palmitic, stearic as compared to other animal body fats. The lesser time taken by adulterated cotton-tract buffalo ghee than adulterated normal ghee samples may be due to the presence of a little higher quantity of higher saturated fatty acids in cotton-tract ghee than normal ghee samples. The differences in turbidity time between cow and buffalo ghee samples adulterated with animal body fats were because of their difference in the composition. The presence of higher content of saturated fatty acids (C16-C18) in animal body fats compared to ghee samples and differences in the fatty acid composition of buffalo ghee and cow ghee have also been reported by Singhal (1973) and Tandon and Ganguli (1972). Such marked differences between pure ghee and ghee samples adulterated with animal body fats were helpful in the detection of animal body fats at 10 per cent level in normal ghee samples and cotton-tract ghee safely. The property of ghee samples adulterated with buffalo, sheep and goat body fats at 5 per cent level to show turbidity in 90 minutes at 0°C could detect all the three body fats in ghee samples. Ghee samples adulterated with pig body fat at 5 per cent level, however, failed to show turbidity and could not be detected at 0°C.

Marked difference in the turbidity behaviour and absorbance profiles of groundnut oil and, vanaspati and ghee samples was observed. Ghee samples adulterated with groundnut oil at 30 per cent level behaved similar to vanaspati adulterated with groundnut oil at 25 per cent level. Vanaspati adulterated with groundnut oil at 15 and 20 per cent level did not show much difference in turbidity time to show turbidity (absorbance, 0.4). Ghee samples adulterated with groundnut oil at 25 per cent level did not show noticeable turbidity whereas vanaspati adulterated at this level showed marked turbidity (absorbance, 0.4) just in 6 minutes. Since groundnut oil behaved similar to animal body fats in turbidity behaviour and was expected that it might induce turbidity on adulteration in ghee at low level. Absence of turbidity in ghee samples adulterated with groundnut oil below 25 per cent level may be due to the presence of higher content of unsaturated fatty acids alongwith a good quantity of higher fatty acids, particularly behenic acid (1-3%) in groundnut oil. The presence of behenic acid in higher amount alongwith other higher fatty acids may be responsible for inducing turbidity in pure groundnut oil and in ghee samples adulterated with groundnut oil below 30 per cent level. The content of this higher fatty acid may be too low to influence the crystallization behaviour of other fatty acids as well to show turbidity.

Hence, turbidity test can be used for the detection of all animal body fats at 10 per cent level and buffalo,

sheep and goat body fats at 5 per cent level in ghee and groundnut oil at 15 per cent level in vanaspati and at 30 per cent level in ghee.

4.14.2 MELTING POINT OF FATTY ACIDS OF TURBID MATTER OF ANIMAL BODY FATS

The melting point of fatty acid mixture of turbid matter in animal body fats are presented in Table 20. It was observed that melting point of fatty acid mixture in pig, goat, sheep and buffalo body fat was 57, 59, 61 and 62°C, respectively. Among animal body fats, the pig body fat showed low value of melting point. The values of melting point of fatty acid mixtures were observed in the range of melting point of standard fatty acids, palmitic, stearic. The low melting point in pig body fat was expected due to higher content of C18:1 (oleic acid) fatty acid as reported by Singhal (1973). The coincidence of melting point of fatty acid mixture of turbid matter to those of palmitic and stearic fatty acids revealed the presence of higher saturated fatty acids in turbid matter of animal body fats.

4.14.3 GLC OF METHYL ESTERS OF FATTY ACIDS IN TURBID MATTER OF ANIMAL BODY FATS

The results for the fatty acid composition of turbid matter of animal body fats obtained by GLC fractionation of methyl esters of fatty acids (Fig. 11) are presented in Table 22. Goat body fat contained higher content of fatty acids, C10:0 (0.598%) and C12:0 (2.04%) compared to buffalo (C10:0, 0.231%; and C12:0, 1.12%) and sheep (C10:0, 0.33% and C12:0, 1.02%) body

fats. Buffalo and sheep body fats contained similar level of fatty acids, C14:0, C16:0 and C18:0. The content of fatty acids, C18:0, was higher (59.9 %) and C14:0 (4.21%) and C16:0 (32.83%) lower in goat body fat compared to buffalo (C14:0, 5.46%; C16, 39.4% and C18 53.66%) and sheep (C14, 5.4%; C16, 39.02% and C18, 53.0%) body fats. It was clear from the results that turbid matter of animal body fats was devoid of short chain fatty acids (C4-C8). The presence of higher content of saturated fatty acid (especially,C16 to C18) observed in turbid matter of animal body fats was responsible for inducing turbidity in body fats and ghee samples adulterated with animal body fats. The property of ghee sample adulterated with buffalo body fat to show turbidity in lesser time compared to ghee samples adulterated with goat body fat was due to the presence of higher content of saturated fatty acids, C14 to C18 in buffalo body fat (98.51 %) than in goat body fat (95.9 %). Inspite of levels of fatty acids, C14:0, C16:0 and C18:0 in turbid matter of sheep body fat almost similar to that of buffalo body fat, ghee samples adulterated with sheep body fat became turbid in longer time than ghee samples adulterated with buffalo body fat. This effect might be due to structural differences in the composition of fats. It was thus deduced by GLC of esters of fatty acids of turbid matter that the presence of higher saturated fatty acids (especially, C16-C18) was mainly responsible for exhibiting turbidity in animal body fats and ghee samples adulterated with animal body fats.

4.14.4 EFFECT OF ADDITION OF STANDARD FATTY ACIDS ON TURBIDITY OF GHEE

Ghee samples containing added fatty acids in different proportions were examined by turbidity test at 0°C in a solvent mixture of 96 per cent alcohol and water (1:1) to see the effect of their addition on the turbidity of ghee sample. The results are illustrated in Fig. 12. Ghee sample containing stearic and palmitic acids showed turbidity (absorbance, 0.3) in 20 and 45 minutes. Ghee sample containing added fatty acid mixture (C16:0 + C18:0) took 30 minutes to show turbidity whereas ghee sample with added fatty acid mixture of C16:0 + C18:0 + C18:1 did not show turbidity even in 90 minutes.

The disappearance of turbidity in ghee sample on addition of oleic acid (C18:1) alongwith palmitic and stearic acids may be due to the low melting point of oleic acid that caused a decrease in the crystallization of soap of fatty acids in ghee and hence, remained in solution. The property of ghee samples to show noticeable turbidity on the addition of palmitic or stearic acid or a combination (C16 + C18) was expected due to their higher melting points and got precipitated as their corresponding soap on cooling at 0°C. Among fatty acids, stearic acid and, stearic and palmitic acids in combination produced more pronounced effect in inducing turbidity than palmitic acid. These observations thus confirmed that ghee samples on adulteration with animal body fats showed turbidity mainly due to the presence of higher content of palmitic and

stearic acids and the extent of turbidity was dependent on the level of these fatty acids present in animal body fats.

4.15 DETECTION OF VANASPATI IN GHEE

Several tests like Baudouin test for the detection of sesame oil added as a tracer, i.e. hydrogenated vegetable oil (vanaspati) are available. Attempts were made with different compounds to search for a simple colour test that could detect vanaspati in ghee at low level. An interesting observation emerged that sesame oil (raw) and vanaspati had the property of exhibiting red colour with p-hydroxy benzaldehyde in presence of concentrated hydrochloric acid. Neither ghee samples nor animal body fats showed this property. Other vegetable oils, viz. palmolein, palm, groundnut, coconut, rapeseed, mustard, soyabean, rice bran and maize corn oils, respectively did not show red colouration. These observations expected to be useful for the detection of vanaspati in ghee led to develop a p-hydroxy benzaldehyde test for detecting vanaspati in ghee at *low level*.

4.15.1 p-HYDROXY BENZALDEHYDE TEST FOR VANASPATI

On the basis of observations (Para 4.15), trials were made with different concentrations of p-hydroxy benzaldehyde for vanaspati and sesame oil to evolve a test for detecting vanaspati easily in ghee. It was observed that both vanaspati and sesame oil showed red colouration with 5 per cent alcoholic solution or solid p-hydroxy benzaldehyde in presence of concentrated hydrochloric acid.

Neither ghee samples nor other vegetable oils showed this property. Ghee samples adulterated with vanaspati upto 1 per cent level and sesame oil at 0.1 per cent level produced noticeable red colour. It was clear from the results that p-hydroxy benzaldehyde produced red colouration in vanaspati and sesame oil and ghee samples adulterated with vanaspati and sesame oil. The test could, therefore, be used for the detection of vanaspati in ghee upto 1 per cent level with 5 per cent alcoholic solution or solid p-hydroxy benzaldehyde in presence of concentrated hydrochloric acid. Addition of several tracers to vanaspati for its easy detection in ghee has been reported by several workers (Dastur et al., 1947; Subramanian et al., 1952; Kapoor et al., 1960; Guyot, 1971). In India, under vegetable oil products control order, 1947, the addition of sesame oil to vanaspati by 5 per cent by weight has been made compulsory for the detection of vanaspati in ghee by Baudouin test. The test reported is, however, advantageous due to the following observations: (i) very stable nature of p-hydroxy benzaldehyde, (ii) a stable colour in vanaspati with alcoholic or solid p-hydroxy benzaldehyde, (iii) obviates the distillation and redistillation of furfural each time in case of Baudouin test, and (iv) stable solution of p-hydroxy benzaldehyde in alcohol for 3 months.

4.15.2 IDENTIFICATION OF COMPONENTS RESPONSIBLE FOR COLOUR REACTION IN VANASPATI WITH p-HYDROXY BENZALDEHYDE

Unsaponifiable matter extracted by IS:3508 from sesame oil and vanaspati was tried with p-hydroxy benzaldehyde in concentrated hydrochloric acid.

Unsaponifiable matter of both sesame oil and vanaspati showed red colouration. The appearance of red colouration in unsaponifiable matter revealed that the constituent of unsaponifiable matter of vanaspati and sesame oil was responsible for producing red colouration with p-hydroxy benzaldehyde in presence of concentrated hydrochloric acid.

In view of these observations, it was decided to fractionate unsaponifiable matter of vanaspati and sesame oil and ghee samples adulterated with vanaspati and sesame oil using chloroform:benzene (94:6, v/v) and 50 per cent solution of p-hydroxy benzaldehyde in concentrated hydrochloric acid as solvent system and spray reagent, respectively. The results are illustrated in Figures 13 and 14. Unsaponifiable matter showed three spots in sesame oil (Rf 0.63, 0.76 and 0.98) and two spots in vanaspati (Rf 0.75 and 0.98) as shown in Fig. 13. The spot at Rf 0.98 was red in colour while other spots were blue in colour. Unsaponifiable matter of ghee samples adulterated with vanaspati at 10 per cent level and sesame oil at 5 per cent level gave two coloured spots, red (Rf 0.98) and blue (Rf 0.76) as illustrated in Fig. 14.

It appeared from the results that the presence of a red coloured spot at solvent front in sesame oil/vanaspati might be the unsaponifiable component responsible for producing red colouration with p-hydroxy benzaldehyde in presence of concentrated HCl. It was observed during the study that neither the whole fat of vanaspati and sesame oil

nor their unsaponifiable matter showed red colour in absence of concentrated HCl with p-hydroxy benzaldehyde.

This evidenced that the unsaponifiable matter of sesame oil/vanaspati contains a constituent that is released only after acidic hydrolysis and then reacts with p-hydroxy benzaldehyde. This property is also reported in Baudouin test that involves the hydrolysis of fat with concentrated HCl to release the sesamol component bound to sesamolin for its reaction with furfural to produce crimson colouration. It is known that sesame oil contains sesamol (free and bound form), sesamolin and sesamin. Out of three spots observed in the unsaponifiable matter, the red coloured spot at solvent front is possibly due to sesamol component of sesame oil responsible for giving red colour spot in unsaponifiable matter of vanaspati and sesame oil on spray with 50 per cent p-hydroxy benzaldehyde solution in concentrated HCl. The fractionation of unsaponifiable matter showed extra spots in vanaspati and ghee sample adulterated with vanaspati at 10 per cent level. This study was, therefore, also useful for the detection of vanaspati in *ghee at 10 per cent level.*

4.15.3 *REACTION MECHANISM OF* p-HYDROXY BENZALDEHYDE WITH THE COMPONENT PRESENT IN SESAME OIL RESPONSIBLE FOR COLOUR REACTION

It is clear from the discussion that the component sesamol present in sesame oil is responsible for colour reaction with p-hydroxy benzaldehyde in presence of concentrated HCl. The negative p-hydroxy benzaldehyde reaction

with unsaponifiable matter of sesame oil/vanaspati without concentrated HCl revealed that sesamolin present in sesame oil generated sesamol component on hydrolysis with concentrated HCl and then sesamol reacted with p-hydroxy benzaldehyde producing red coloured complex. As expected that aldehydic group of p-hydroxy benzaldehyde might react with sesamol component of sesame oil. The negative reaction of sesame oil with benzaldehyde, phenol and salicyaldehyde in presence of concentrated HCl apparently convinced that neither aldehydic nor phenolic group of p-hydroxy benzaldehyde alone shows reaction in sesame oil. It is probably the hydroxy group at para position of p-hydroxy benzaldehyde that catalyzes the reactivity of aldehyde group (-CHO⟶ -CHOH) to react with methylene group of oxirane ring of sesamol by transfer of proton and itself get converted into ketone group. The intermediate complex formed after the loss of water molecule is very unstable and undergoes immediate transfer of proton to regain the lost aromatic character and thereby ketonic group is converted into hydroxyl group. The transfer of proton to regain the aromatic character of benzene nucleus is considered to be the driving force for p-hydroxy benzaldehyde reaction to go to completion to give a stable complex (Fig. 15).

4.16 CHROMATOGRAPHIC METHODS

Chromatographic techniques have been extensively employed in separation and characterization of physico-chemical nature of fats and oils. The composition of fats is so complex and their physico-chemical properties are

so similar in character that attempts made so far in separation of whole fat could not be helpful for the detection of adulteration in ghee. It is known that both ghee and animal body fats contain cholesterol and other related sterols which are so similar in character that past attempts in separation of unsaponifiable matter of fats for detection of body fats was a major problem and such attempts were rarely undertaken. The advent of chromatography has resulted a revolutionary breakthrough in the separation of closely related sterols from fats and have recently made possible not only the detection of adulteration but the identification of adulterants fats added to ghee too.

4.16.1 PAPER CHROMATOGRAPHY

4.16.1.1 Whole fat

Paper chromatography has been employed by several workers for detection of foreign fats in ghee. Among different solvent systems tried, one consisting of dichloromethane:isopropanol:glacial acetic acid (80/30/20, v/v) was selected for giving the best results (Figs. 16, 17 and 18). It was observed that ghee samples showed a complete mobility alongwith solvent giving a single spot at solvent front whilst buffalo and goat body fats were completely immobile and showed a distinct band shaped streaking (Fig. 16).

Pig body fat, however, moved half way alongwith the solvent and showed a streaking about 5-6 cm above the point

of origin. Cotton-tract buffalo ghee behaved similar to normal ghee and showed mobility alongwith the solvent (Fig. 16).

Ghee samples adulterated with buffalo and goat body fats at 10 per cent level behaved almost similar to pure body fats and showed distinct streakings about 2.5 cm above the point of sample application whereas adulteration of ghee samples with pig body fat at 10 per cent level showed streaking about 7 cm from the origin (Figs. 17 and 18).

Ramchandra and Dastur (1959) and Singhal (1973) also reported the detection of body fats from goat, sheep and buffalo upto 5 per cent level in the absence of cotton-tract ghee. They also reported that detection of pig body fat at 10 per cent level in ghee samples was not possible. The method reported here has the advantage in the detection of buffalo, goat and pig body fats at 10 per cent level even in the presence of cotton-tract ghee which behaved similar to normal ghee.

4.16.1.2 Unsaponifiable matter

4.16.1.2.1 Single dimensional: The technique was used to resolve unsaponifiable matter of ghee and animal body fats using a solvent mixture of dichloromethane:isopropanol: glacial acetic acid (80/30/20, v/v). It was observed that cow ghee gave one spot (Rf 0.96) whereas buffalo ghee exhibited two spots at almost same Rf, 0.96. In case of animal body fats, pig and buffalo fats showed two spots

(Rf 0.94 and 0.96). Cow and buffalo ghee samples on 10 per cent adulteration with buffalo and pig body fats gave two spots.

It was clear from the results that separation of unsaponifiable matter by single dimensional paper chromatography showed an extra spot in cow ghee adulterated with pig and buffalo body fats at 10 per cent level whereas unsaponifiable matter of adulterated buffalo ghee samples did not show any differential behaviour from pure buffalo ghee and hence the method could not be used for the detection of adulteration in ghee.

4.16.1.2.2 Double dimensional: In view of poor resolution of unsaponifiable matter of ghee and body fats by single dimension paper chromatography, it was decided to resolve unsaponifiable matter of fat samples by two dimensional technique. It was noticed that unsaponifiable matter of body fats from sheep, buffalo, goat and pig showed only one spot at solvent front. Buffalo ghee showed two spots at almost similar Rf whereas cow ghee gave two spots at different Rf at solvent front in solvent system, 15 per cent ethyl acetate in cyclohexane. Unsaponifiable matter of ghee and body fats did not show much mobility when developed vertically in solvent system, ethyl alcohol: iso-amyl alcohol carbon tetrachloride (35:55:1 , v/v) whereas complete mobility of unsaponifiable matter of fat sample alongwith solvent front was observed on horizontal development in solvent system (15% ethyl acetate in cyclohexane). The results did not show extra spots in unsaponifiable matter of

body fats which could be helpful for the detection of adulteration and, therefore, further studies of ghee on adulteration were not made.

4.16.1.2.3 Unsaponifiable matter from ether soluble and ether insoluble fractions of fat samples: Based on difference in the unsaponifiable matter content in ether soluble and ether insoluble fractions of ghee and animal body fats (Table 8), chromatographic analysis of unsaponifiable matter from these fractions was carried out to see possibilities of any difference in the constituents of unsaponifiable matter in ether soluble and ether insoluble fractions of ghee and animal body fats. The results are illustrated in Figures 19 and 20. Unsaponifiable matter from ether soluble and ether insoluble fractions of ghee samples and ether soluble fraction of pig body fat showed only one spot at solvent front (Rf, 0.968) whereas unsaponifiable matter from ether soluble and ether insoluble fractions of buffalo body fat gave two spots (Rf, 0.84 and 0.97). Unsaponifiable matter from ether insoluble fraction of pig body fat gave two spots (Rf, 0.84 and 0.97). At 10 per cent adulteration, only unsaponifiable from ether soluble fractions of ghee samples adulterated with buffalo body fat showed two spots (Rf, 0.84 and 0.97) while unsaponifiable matter from ether insoluble fractions of ghee samples adulterated with buffalo body fat and ether soluble and ether insoluble fractions of ghee samples adulterated with pig body fat did not show any different behaviour from pure ghee samples and gave only one spot (Rf, 0.97). It is apparent from the results

that the unsaponifiable matter from ether soluble fractions of ghee samples adulterated with buffalo body fat showed an extra spot which could detect buffalo body fat at 10 per cent level in ghee samples. Unsaponifiable matter from ether insoluble fractions of ghee samples adulterated with pig body fat did not show any difference from pure ghee samples and showed only one spot. This might be due to the low content of unsaponifiable matter constituent in unsaponifiable matter of ether insoluble fraction of pig body fat and could not become visible on adulteration of ghee samples with pig body fat. The coincidence of extra spot (Rf, 0.84) to standard dihydro cholesterol revealed the presence of this sterol in pig and buffalo body fats. This study could not detect pig body fat in ghee sample and hence can not be useful for the detection of body fats in ghee.

4.16.2 THIN LAYER CHROMATOGRAPHY (TLC)

4.16.2.1 TLC test for the detection of coconut oil in ghee

Thin layer chromatography finds extensive application in the identification and separation of substances often similar in their properties and offers better resolution than paper or column chromatography. Coconut oil, a relatively saturated oil resembles to ghee in most of its physico-chemical properties. It was observed from the results (Fig. 6) that coconut oil behaved similar to ghee in turbidity behaviour as well and escaped detection even upto 20 per cent level. In view of this, an attempt was

made to detect coconut oil in ghee by TLC. The results are illustrated in Figures 21 and 22. It was noticed that coconut oil showed difference from ghee samples in exhibiting an extra spot at Rf 0.12. This extra spot was noticeable in ghee samples adulterated with coconut oil at 10 per cent level. Co-chromatography of standard sterol, alongwith fat samples showed that this extra spot had Rf value equal to one of the 3 components of standard stigmasterol. The second spot with Rf 0.23 observed in all fats was more sharp in vanaspati and groundnut oil compared to coconut oil and ghee samples.

Vanaspati did not show this extra spot and on adulteration with coconut oil at 10 per cent level showed an extra spot (Rf, 0.12).

The presence of extra spot in coconut oil and, ghee samples and vanaspati adulterated with coconut oil at 10 per cent level and its absence in ghee samples or vanaspati adulterated below 10 per cent level with coconut evidenced that coconut oil could be detected in ghee/ vanaspati at 10 per cent level or above. The coincidence of extra spot to Rf of one of 3 components of stigmasterol revealed the presence of a component of a stigmasterol in coconut oil. The increased sharpness of second spot (Rf, 0.23) in vanaspati and groundnut oil compared to ghee samples showed that vanaspati and groundnut oil could be detected in ghee samples. Specific tests like phytosteryl acetate test could detect the presence of coconut oil in

ghee only above 25 per cent level. This method could, therefore, be adopted for detection of coconut oil in ghee or vanaspati at 10 per cent level or above.

4.16.2.2 TLC of unsaponifiable matter for the detection of animal body fats in ghee

In view of differences observed in the unsaponifiable matter and cholesterol contents of ghee and animal body fats (Tables 5 and 10), it was expected that TLC of unsaponifiable matter might show some differences in the resolution pattern between two classes of fats which could, therefore, be helpful for the detection of body fats in ghee. The results are shown in Figs. 23 to 25. It was observed that unsaponifiable matter of ghee samples gave eight spots whereas unsaponifiable matter of all animal body fats gave nine spots (Fig. 23). All animal body fats (buffalo, goat, sheep and pig) showed an extra spot (Rf, 0.79). Unsaponifiable matter of ghee samples adulterated at 10 per cent levels gave this extra spot at the same Rf 0.79 (Figs.24, and 25). The extra spot was observed more sharp in buffalo body fat followed by sheep, goat and pig body fats. Unsaponifiable matter of ghee samples adulterated with animal body fats at 5 per cent level also showed this extra spot.

It was obvious from the results that an extra spot (Rf, 0.79) was present in unsaponifiable matter of all body fats and ghee samples adulterated with body fats at 5 and 10 per cent levels. The coïncidence of Rf value of extra spot with Rf value of dihydro cholesterol (Fig.26) revealed the presence of dihydro cholesterol in all animal body fats. The identification of dihydro cholesterol in

body fats is advantageous to detect the presence of body fats in ghee. The method is, therefore, very useful for the detection of adulteration of animal body fats in ghee.

4.16.2.3 TLC of sterol digitonides

Sterol digitonides prepared from unsaponifiable matter free of insoluble matter were resolved by TLC using solvent mixture of chloroform:benzene (3:1,v/v). The results are presented in Figure 27. An examination of chromatogram revealed that sterol digitonides of ghee and animal body fats behaved in a similar fashion exhibiting only one spot (Rf, 0.6). The spot was found to match with Rf of cholesterol digitonide prepared from standard cholesterol. It is evident from the results that sterol digitonides of ghee and body fats did not show any different behaviour. Further studies on adulterated ghee samples were, therefore, not carried out.

4.16.2.4 TLC of steryl acetate

Steryl acetates prepared by the method (Den Herden, 1955) from free sterol digitonides were subjected to thin layer chromatography using a solvent system hexane:ethyl acetate (85:15, v/v). The results are depicted in Figure 28. It was observed that ghee and animal body fats gave two spots with Rf values, 0.81 and 0.98. The Rf value of first spot (Rf, 0.81) was found to match with Rf value of cholesterol acetate (Rf, 0.81) prepared from authentic cholesterol digitonide. The second spot at Rf 0.98 was less visible. The results showed that TLC of steryl acetate of free sterol

digitonides did not show any difference between ghee and body fats. The studies on adulterated ghee samples were, therefore, not made.

4.16.2.5 TLC of free and esterified sterol digitonides

In view of differences in the content of free and esterified cholesterol in ghee and animal body fats (Table 15), it was expected that TLC of free and esterified sterols might show differences between ghee and body fats to help in the detection of adulteration in ghee. Direct resolution of sterol digitonides on TLC plates revealed poor separation and showed no differences between two classes of fats (Fig. 28).

Taking into view these aspects, the free and esterified sterol digitonides of ghee and animal body fats were cleaved in pyridine before TLC analysis. The results for TLC analysis of free sterol digitonides are presented in Fig. 29. The results revealed that ghee sample gave only one spot (Rf, 0.45). Amongst animal body fats, buffalo body fat gave three spots (Rf, 0.45, 0.53 and 0.81) while body fats from goat, sheep and pig showed two spots (Rf, 0.45 and 0.81). The second spot (Rf, 0.53) was poorly visible in goat, sheep and pig body fats. At 10 per cent adulteration, ghee samples adulterated with goat, sheep and pig body fats gave two spots (Rf, 0.45 and 0.81) whilst ghee samples adulterated with buffalo body fat gave three spots (Rf, 0.45, 0.53 and 0.81) as shown in Fig. 30. The spot (Rf, 0.81) was observed more sharp in goat body fat

followed by buffalo, sheep and pig body fats. The spots with Rf values, 0.45, 0.53 and 0.81 showed coincidence in order of Rf values of digitnoides of standard cholesterol, dihydro cholesterol and one of the two spots of 7-dehydro cholesterol.

The results for esterified sterol digitonides of ghee and animal body fats are illustrated in Figure 31. It was observed that ghee samples gave only one spot (Rf, 0.45) while sheep and goat body fats showed four spots each and pig and buffalo body fats gave two and three spots, respectively. The spots in animal body fats in ascending order were observed at Rf 0.18, 0.28, 0.45 and 0.79. Esterified sterol digitonides of ghee samples adulterated with pig body fat gave two spots (Rf 0.45 and 0.79) while ghee samples adulterated with buffalo body fat and, sheep and goat body fats showed three spots at Rf 0.28, 0.48 and 0.79, and 0.18, 0.45 and 0.79, respectively (Figs. 32 and 33). The spots with Rf values, 0.45 and 0.79 showed correspondence to Rf values of digitonides of standard cholesterol and one of two spots of 7-dehydrocholesterol.

It was apparent from the results that TLC of free and esterified sterol digitonides cleaved in pyridine showed marked differences between ghee and animal body fats and, pure ghee samples and ghee samples adulterated with animal body fats at 10 per cent level. Free and esterified sterol digitonides showed the presence of an extra spot of one of two components of 7-dehydrocholesterol in all animal body fats and ghee sample adulterated with animal body

fats. A second extra spot of dihydrocholesterol was present in buffalo body fat (Rf, 0.53) and ghee samples adulterated with buffalo body fat at 10 per cent level. This spot being less visible in body fats of goat, sheep and pig could not be observed in ghee samples adulterated with these body fats.

Guyot (1969) has reported the presence of various sterols, viz., cholesterol, dihydrocholesterol, ergosterol and possibly some campesterol in animal fats (butter, beef, tallow, lard, horse fat, whole oil and cod liver oil). It is also known that ghee contains 7-dehydrocholesterol. The spot at Rf 0.79 observed in free and esterified sterol digitonides of animal body fats, therefore, could not be 7-dehydrocholesterol but a related component of 7-dehydrocholesterol. The presence of dihydrocholesterol (Rf, 0.53) in free sterol digitonides of animal body fats has also been confirmed by TLC of unsaponifiable matter of animal body fats (Fig. 23). The other two spots (Rf, 0.18 and 0.28) could not be identified. It is thus evidenced from these observations that animal body fats contain dihydrocholesterol, a related component of 7-dehydrocholesterol and two unidentified sterol components. This method was, therefore, useful not only in the detection of animal body fats at 10 per cent level but also helpful in identification of the nature of adulterant animal body fat added to ghee on the basis of sterols.

4.16.2.6 TLC of TMS ether derivatives of unsaponifiable matter of ghee and animal body fats

Trimethylsilyl ethers prepared from unsaponifiable

matter of ghee and animal body fats for GLC were subjected to TLC to see if any difference between these two classes of fats exists which could be useful for detecting animal body fats in ghee. An examination of chromatogram (Fig.34) revealed no differential behaviour in TLC separation pattern of TMS ether derivatives of animal body fats from ghee samples. Further studies on adulterated ghee samples were, therefore, not carried out.

4.17 SPECTROSCOPIC METHODS

4.17.1 UV SPECTROSCOPIC STUDIES

4.17.1.1 Whole fat

Ghee and animal body fats were dissolved in n-hexane and scanned on Spectron 21, spectrophotometer, Bausch and Lomb model between 200 to 320 nm. The results are shown in Figure 35. It was noticed that ghee and animal body fats showed an absorption with a maximum between 220 and 230 nm. However, cow ghee showed in addition another small maxima at 270 nm. The results obtained were similar to Singhal (1973) and no difference in ghee samples adulterated with animal body fats was observed.

4.17.1.2 Unsaponifiable matter

Unsaponifiable matter extracted from ghee and animal body fat samples by IS:3508 was dissolved in n-hexane and scanned in UV range 200 to 320 nm. The results are depicted in Figure 36. Ghee and animal body fats showed an absorption with a maxima between 215 to 220 nm. The ghee samples

showed a second maxima at 270 nm which was shifted from 270 to 280 nm in case of animal body fats. These differences in absorption between ghee and animal body fats were expected to show atleast three maxima at 220, 260 and 280 nm or a shift in the absorption from 270 nm to some other higher value on adulteration of ghee samples with animal body fats. The UV absorption of adulterated ghee samples behaved similar to that of pure ghee and thus did not give useful clue for detection of animal body fats in ghee.

4.17.2 INFRA-RED SPECTROSCOPIC STUDIES OF GHEE AND ANIMAL BODY FATS

In view of differences reported by deRuig (1968) between IR differential spectra of pure butterfat and tallow at 32.5°C, it was decided to analyse whole fat of ghee and animal body fat samples in molten state and their unsaponifiable matter through IR range 4000 to 600 cm^{-1} to see the differences, if any, in the absorption between these two classes of fats. The infra-red spectra that are presented in Figures 37 to 42 have been reduced in order to display more than one spectrum in each figure. Upward peaks may be considered as negative since they represented regions in which the absorbance is less than that of the reference while downward peaks were considered as positive.

4.17.2.1 Whole fat

The IR spectra of ghee and animal body fats are shown in Figures 37 and 38. Ghee samples showed similar spectra except an increased absorption at 970 cm^{-1} in buffalo

ghee compared to cow ghee (Fig. 37). A negative absorption band at 2320 to 2350 cm^{-1} was observed in ghee and animal body fats. Animal body fats showed different absorption from ghee samples especially in IR region 1420 to 700 cm^{-1} (Fig. 38). Animal body fats showed increased absorption compared to ghee samples at 720 to 725 cm^{-1} and the sharpness of peak was higher in buffalo body fat compared to sheep and goat body fats. Animals body fats showed increased absorption at 970 cm^{-1} compared to ghee samples. An absorption band at 920 cm^{-1} as reported by deRuig (1968) was observed in body fats. Animal body fats showed prominent differences in absorption from ghee samples in regions 1300 to 1100 cm^{-1} and 1120 to 1000 cm^{-1}. Ghee samples showed two bands at 1245 to 1240 cm^{-1} whereas sheep and goat body fats gave six bands at 1190, 1210, 1240, 1260, 1275 and 1300 cm^{-1} and 1180, 1200, 1225, 1240, 1260 and 1270 to 1290 cm^{-1}, respectively. Buffalo body fat, however, showed five bands at 1200, 1210, 1240, 1260 to 1270 and 1290 cm^{-1}. A normal absorption peak in buffalo body fat (1110 cm^{-1}) showed a minor split in sheep and goat body fats whereas this peak showed a clear split into two peaks at 1110 and 1100 cm^{-1} in ghee samples. The absorption band at 1385 cm^{-1} in ghee samples and buffalo body fat showed split into two bands at 1375 and 1355 cm^{-1} in sheep and goat body fats. Animal body fats showed other extra bands at 2680 to 2620, 2030 to 2020, 1580, 880 to 870 and 840 to 820 cm^{-1}. The shape of contour in the curves of animal body fats from 1100 to 800 cm^{-1} showed a marked difference

from that of ghee samples. Ghee and animal body fats showed similar absorption in other regions.

A marked difference in the IR spectra of ghee and animal body fats was observed. Higher absorption at 970 cm^{-1} due to isolated trans unsaturation in buffalo ghee compared to cow ghee showed the presence of higher content of unsaturated fatty acids with isolated trans unsaturation in buffalo ghee. The differences in absorption among animal body fats was observed. All animal body fats (buffalo, goat and sheep) showed increased absorption at 970 cm^{-1} and 980 cm^{-1} due to isolated trans unsaturation compared to ghee samples. This property was also evidenced due to extra band of trans unsaturation at 1580 cm^{-1} and increased absorption in goat body fat in this region compared to buffalo and sheep body fats. This revealed the presence of higher quantity of trans isolated unsaturated fatty acid in glycerides of body fats compared to ghee samples. Increased absorption in goat body fat (970-980,1580 cm^{-1}) compared to sheep and buffalo body fat shows the presence of more trans unsaturation in goat body fat than in sheep and buffalo body fats. Absorption peak at 720 to 725 cm^{-1} has been reported due to rocking mode of skeletal vibration of aliphatic chain [$(CH_2)_n$] 4 and higher absorption in this region in animal body fats compared to ghee indicates the presence of increased number of long chain aliphatic CH group in animal body fats than in ghee samples. This is also evidenced by GLC studies that showed the presence of higher content of long chain saturated fatty acids

(especially C16 and C18) in body fats compared to ghee samples. Among animal body fats, the higher absorption in buffalo body fat compared to sheep and goat body fats might be due to the more number of aliphatic CH_2 groups in buffalo body fat than in other two body fats. The absorption in the region 1250 cm^{-1} has been reported due to CH in plane wagging or rocking mode of CH_2 group while absorption at 1190 to 1180 cm^{-1} was due to C-O stretching vibration of -COOR group in triglyceride molecule. The presence of multiple bands in animal body fats (1250 - 1180 cm^{-1}) compared to two bands in ghee samples (1245 - 1240 cm^{-1}) might be due to the presence of increased long chain CH_2 or CH groups in body fats. It has been reported in the literature that the number of bands in the region 1250 cm^{-1} increases with increasing carbon chain length (Jones et al., 1952; Chapman, 1965). This reveals the presence of triglycerides with longer carbon chain length in animal body fats compared to ghee samples. The presence of an extra band in case of sheep and goat body fats compared to buffalo body fat in this region suggests that chain length of some glyceride molecules in sheep and goat body fats might be one unit higher than those of buffalo body fat. Reports are available that the number of bands in the region 1250 cm^{-1} increased per methylene unit in fatty acids, series from C16 to C21 (Jones et al., 1952). It has also been reported that sharpness and depth of normal peak at 1250 cm^{-1} decreases due to its multiple split at different wave lengths (1250 - 1180 cm^{-1}). This is reason of reduced

peak at 1250 cm^{-1} in body fats compared to its sharpness in ghee samples.

The presence of an extra band in animal body fats at 920 cm^{-1} has also been reported by deRuig (1968) in tallow at 32.5°C. The reason for the absence of this band in ghee samples might be due to continuous and random distribution of $[CH_2]_n$ chain in ghee samples.

It has been reported by Bartlet and Mahon (1958) that in highly saturated fats like coconut oil and palm/kernel, the sharpness of peak at 1100 cm^{-1} was highest. As unsaturation increases, the peak is either reduced or shows a split into two minor peaks at 1120 and 1100 cm^{-1}. It is for this reason that buffalo body fat showed a sharp peak at 1110 cm^{-1}, compared to ghee samples that showed split at 1110 and 1100 cm^{-1}. This suggests the presence of increased unsaturation in ghee samples compared to body fats. The absorption has been reported in this region due to unsaturated acids, viz., linoleic, linoleinic and oleic. The minor split of this peak at base in case of sheep and goat body fats reveals a little higher unsaturation in these body fats compared to buffalo body fat. The presence of extra bands in animal body fats at 2650 to 2620 cm^{-1} and 880 to 870 cm^{-1} due to stretching vibrations of CH_2 and CH_3 groups and skeletal vibration of $CH(CH_3)_2$ group or rocking mode of long aliphatic chain might be due to the presence of higher carbon number glyceride molecules in body fats than in ghee samples. This property has also been apparent in body fats in region 1250 cm^{-1}. It

can be deduced from these observations that extra bands at 880 to 870, 920, 1250 to 1180, 1580 and 2650 to 2620 cm^{-1} in animal body fats and difference in absorption at 970 and 1100 cm^{-1} alongwith different shape of contours from 1100 to 800 cm^{-1} in animal body fats compared to ghee samples gave encouraging results useful for the detection of body fats in ghee.

4.17.2.2 Infra-red spectroscopic measurements of adulterated ghee samples

The results for ghee samples adulterated with different animal body fats at 10 per cent level are illustrated in Figures 39 and 40. Absorption at 710 to 720 cm^{-1} showed an increase in sharpness of peak in ghee samples on adulteration with animal body fats and the increase was independent upon the nature and amount of animal body fat added to ghee. Among adulterated ghee sample, buffalo ghee adulterated with buffalo body fat showed more sharp peak at 720-710 cm^{-1} (Fig.40) compared to ghee samples adulterated with goat, sheep and pig body fats. A prominent difference in the IR spectra of pure and adulterated ghee samples was observed in the region 1250 cm^{-1}. Adulterated ghee samples showed multiple bands compared to two bands in ghee samples in this region. Buffalo ghee adulterated with goat body fat gave five bands at 1090, 1210, 1240, 1260 and 1300 cm^{-1} whereas buffalo ghee adulterated with buffalo body fat showed four bands at 1205, 1245, 1285 and 1300 cm^{-1}. Ghee samples adulterated with sheep body fat gave three bands at 1240, 1265 and 1300 cm^{-1}. Cow ghee adulterated with

sheep body fat and goat body fats showed extra bands at 900 and 880 cm^{-1} and 880 and 860 cm^{-1}, respectively, while buffalo ghee adulterated with buffalo body fat showed bands at 890 and 880 cm^{-1}. Ghee samples adulterated with sheep body fat gave extra absorption band at 2640 to 2630 cm^{-1}, while buffalo ghee adulterated with buffalo body fat showed extra absorption bands at 2640 and 2250 to 2015 cm^{-1}. The shape of contour of IR curves of adulterated ghee samples from 1100 to 800 cm^{-1} was markedly different from pure ghee samples. Ghee samples adulterated with pig body fat showed an extra band at 910 cm^{-1} and flat inflexions along the slope between 1061 to 1050 cm^{-1}.

IR spectra of adulterated ghee samples showed a marked difference from those of pure ghee samples. Adulterated ghee samples showed increased absorption at 720 to 710 cm^{-1} due to rocking mode of long aliphatic chain compared to pure ghee samples. This may show the presence of glyceride molecules with long CH_2 group chain in body fats which on adulteration in ghee samples showed increased absorption at this wave length. Increase in absorption in case of ghee samples adulterated with buffalo body fat compared to ghee samples adulterated with sheep, goat and pig body fats might be due to aliphatic chain with more CH_2 units in buffalo body fat compared to sheep and goat body fats (Fig. 40).

The presence of extra bands in adulterated ghee samples in the region 900 to 860 cm^{-1} has been reported due to rocking mode of long aliphatic CH_2 chain. The reason

for the appearance of bands at 900 and 880 cm^{-1} and 880 and 860 cm^{-1} in cow ghee adulterated with sheep and goat body fats and at 890 and 880 cm^{-1} in buffalo ghee adulterated with buffalo body fat are not known. The presence of two extra bands (1265 and 1300 cm^{-1}) in ghee samples adulterated with sheep body fat and three and four extra bands in buffalo ghee adulterated with buffalo (1265, 1285 and 1300 cm^{-1}) and goat (1090, 1210, 1260, 1300 cm^{-1}) body fats respectively clearly demonstrates the different behaviour of adulterated ghee samples from pure ghee samples. The presence of extra absorption bands in ghee samples adulterated with sheep body fat at 2640 to 2630 cm^{-1} and buffalo ghee adulterated with buffalo body fat at 2640 and 2250 cm^{-1} were due to stretching vibration of CH_2 and CH_3 groups. This extra absorption might be due to the presence of glyceride molecules with higher carbon number in body fats (Fig. 40). It is thus clear that the presence of extra bands in adulterated ghee samples in region 1250, 910, 900 to 860, 2650 and 2250 cm^{-1} and differences in absorption at 720 to 710 cm^{-1} and distinct shape of contours from 1100 to 800 cm^{-1} enabled the detection of animal body fats in ghee at 10 per cent level safely.

4.17.2.3 Unsaponifiable matter

The results of infra-red spectra of unsaponifiable matter of ghee samples and buffalo body fat are depicted in Figure 41. Ghee samples showed almost similar spectra. Absorption bands in ghee samples (at 950-960 and 980 cm^{-1})

and buffalo body fat (at 940 and 980 cm^{-1}) were observed. Multiple absorption bands were observed in buffalo (at 1610 - 1590, and 1560 - 1540 cm^{-1}) and cow (1585 and 1530 cm^{-1}) ghee and buffalo body fat (1580 - 1570 cm^{-1} and 1550 - 1540 cm^{-1}). Weak absorption bands were observed in cow and buffalo ghee and buffalo body fat at 830 and 840 cm^{-1} and 820 cm^{-1}, respectively. A normal absorption peak at 1130 to 1120 cm^{-1} in ghee samples showed a split into two peaks at 1120 and 1110 cm^{-1} in buffalo body fat. Similarly, normal peaks observed at 1460 cm^{-1} and 1390 to 1380 cm^{-1} in ghee samples revealed split at 1465 and 1440 cm^{-1} and 1380 and 1350 cm^{-1} in buffalo body fat. A prominent difference between ghee samples and buffalo body fat was observed in the region 1300 to 1100 cm^{-1}. Ghee samples gave two bands at 1280 to 1270 and 1120 cm^{-1} whereas buffalo body fat showed six bands in the region 1300 to 1190 cm^{-1}. Extra absorption bands at 2680 to 2620, 1415 and 940 cm^{-1} were also observed in buffalo body fat. Absorption at other regions in ghee sample and buffalo body fat was similar. The results showed a marked difference in the IR absorption of unsaponifiable matter of ghee samples from buffalo body fat. The absorption at 980, 960 and 940 cm^{-1} was reported due to trans double bond and a double bond in the side chain or in ring skeleton of sterols.

Rozenkrantz et al. (1952) reported the presence of bands at 950 and 940 cm^{-1} in cholesterol but had not given any definite assignment for these bands. Absorption in the region 10 to 13 u due to conjugated double bond

evidenced the presence of ergosterol skeleton in unsaponifiable matter of ghee and buffalo body fat. This is further supported by absorption due to conjugated double bond system (at 1600 - 1585 cm^{-1}) in ghee samples and buffalo body fat. Guyot (1969) also reported the presence of ergosterol in butterfat and tallow. Absorption at 840, 830 and 820 cm^{-1} due to bending vibration of CH linkage adjacent to 5, 6 double bond of sterol molecule showed the presence of a CH group adjacent to 5, 6 double bond in sterol molecules of unsaponifiable matter. The presence of normal peak in ghee samples (1130 - 1120 cm^{-1}) due to the presence of C-O moiety of alcoholic hydroxyl group of the sterol suggests the presence of carbon containing OH group with single bond linkage. The split of the peak in buffalo body fat might be expected due to the presence of hydroxyl group adjacent to a double bond or due to a ring structure. The multiple absorption in region 1300 to 1190 cm^{-1} in buffalo body fat (six bands) compared to two bands in ghee samples may be due to the presence of sterol or sterols with increased CH_2 chain units in side chain or skeleton of sterol molecule. The number of bands in IR region 1300-1190 cm^{-1} are reported proportional to the chain length of the compound (Chapman, 1965). Differences in absorption between ghee samples and buffalo body fat in the region 1470 to 1350 cm^{-1} were more marked.

The absorption at 1470 to 1465 and 1385 to 1350 cm^{-1} has been reported due to stretching and bending vibrations of CH bond of CH_2 and CH_3 groups and CH in plane wagging of cholesterol molecule. The presence of doublet at 1465

and 1440 cm^{-1} and 1380 and 1350 cm^{-1} in buffalo body fat has been attributed due to angular vibration of CH group and combined vibration of quarternary angular CH_3 group and the various tertiary substituted C - CH_3 groups of the side chain. These observations suggest the presence of either methyl or isopropyl groups in sterol skeleton or in side chain of sterol molecules in unsaponifiable matter of buffalo body fat with different side chain. The increased absorption at 1420 cm^{-1} in buffalo body fat compared to ghee samples suggests the presence of more saturated or unsaturated carbon skeleton in sterol molecule. It can be deduced that the presence of multiple bands at 1300 to 1190 cm^{-1}, extra bands at 2630 to 2620, 1415 and difference in absorption in regions 1470 to 1350 cm^{-1} and 1130 to 1120 cm^{-1} in buffalo body fat compared to ghee samples showed encouraging clues for further study of unsaponifiable matter of ghee samples on adulteration.

4.17.2.4 Unsaponifiable matter of adulterated ghee samples

The results of IR spectra of unsaponifiable matter of ghee samples adulterated with different body fats at 10 per cent level are presented in Figure 42. It was noticed that unsaponifiable matter of ghee samples adulterated with sheep, pig and goat body fats gave a very sharp doublet peak at 780 and 760 cm^{-1}. The absorption at these wavelengths was weak in unsaponifiable matter of pure ghee samples. A marked difference in the spectra of adulterated ghee samples from pure ghee samples was observed in the region 1250 cm^{-1}. Ghee samples adulterated with sheep and

goat body fats gave five bands at 1100, 1120, 1160, 1270 to 1250 and 1300 cm^{-1}, while buffalo ghee adulterated with pig body fat showed six bands at 1100, 1120, 1150, 1170, 1250 and 1280 cm^{-1}. Absorption at other regions in adulterated ghee samples was similar to pure ghee samples. The results revealed marked differences between adulterated ghee samples and pure ghee samples. The appearance of doublet in adulterated ghee samples at 7 0 and 760 cm^{-1} may be due to interaction of adjacent CH_2 groups or due to polymorphism in ghee samples on admixing with animal body fats. Adulterated ghee samples showed difference from pure ghee samples in exhibiting multiple bands in the region 1250 cm^{-1}. Buffalo ghee adulterated with sheep and pig body fats showed the presence of extra four bands at 1100, 1150, 1170 and 1250 cm^{-1} and three bands at 1110, 1160 and 1265 cm^{-1}, respectively. Cow ghee samples adulterated with sheep and goat body fats showed extra three bands at 1110, 1160 and 1265 cm^{-1} and 1110, 1150 and 1200 cm^{-1}, respectively. It is clear from the observations that a distinct doublet at 780 and 760 cm^{-1} and multiple bands in the region 1300 to 1100 cm^{-1} in adulterated ghee samples were encouraging for demonstrating adulteration of animal body fats in ghee samples. The IR spectroscopy could, therefore, be used to detect animal body fats in ghee at 10 per cent level.

CHAPTER 5

SUMMARY AND CONCLUSIONS

5. SUMMARY AND CONCLUSIONS

The Reichert and Polenske values of ghee and animal body fats were evaluated to examine whether these constants could be used to detect adulteration. In view of large variations of these constants and diverse feeding practices, these constants were found not very helpful in detection of adulteration.

No significant variations in unsaponifiable matter content of ghee and animal body fats were observed. The difference in unsaponifiable matter content of ghee and animal body fats except pig body fat was not large. Adulteration of ghee with animal body fats at 10 per cent level did not result significant changes in the unsaponifiable matter content.

Marked seasonal variations in unsaponifiable matter content of ghee and animal body fats were observed. Ghee and animal body fats samples showed higher content of unsaponifiable matter in winter and lower in summer. The unsaponifiable matter content in animal body fats was lower as compared to ghee samples in winter and summer.

The content of unsaponifiable matter extracted from ether soluble and ether insoluble fractions of ghee and animal body fats showed marked differences. The content of unsaponifiable matter from ether soluble fractions of

body fats was lower than from ghee. However, the differences in the unsaponifiable matter content of ether insoluble fraction of buffalo body fat from ghee samples was marked whereas the content of unsaponifiable matter from ether insoluble fraction of pig body fat was higher than that of buffalo ghee but lower than that of cow ghee. Adulteration at 10 per cent level showed differences only in unsaponifiable matter content from ether insoluble fractions of buffalo ghee adulterated with buffalo body fat. The method, therefore, could not be used to detect adulteration of body fats except buffalo body fat in buffalo ghee.

A marked difference in the content of total cholesterol in ghee and animal body fats was observed. However, the adulteration of ghee at 10 per cent level did not exhibit significant changes in content of total cholesterol which could demonstrate adulteration.

Free and esterified cholesterol content of ghee and animal body fats showed large variations. Adulteration at 10 per cent level, however, did not show much variations in the content of free and esterified cholesterol of adulterated ghee samples from pure ghee.

Animal body fats differed markedly from ghee samples by having higher content of insoluble unsaponifiable matter. The difference in the content of insoluble unsaponifiable matter in ghee samples adulterated at 10 per cent level with different animal body fats was not broad enough whilst ghee samples adulterated at 20 per cent level showed wide difference and it was possible to detect adulteration of

ghee with body fats at 20 per cent level without any ambiguity. The melting points of fatty acid mixture of insoluble unsaponifiable matter was higher in goat body fat followed by buffalo, goat and pig body fats and the melting points observed were in range of standard fatty acids, palmitic and stearic.

GLC of methyl esters of fatty acids of insoluble unsaponifiable matter of buffalo, sheep and goat body fats showed absence of short chain fatty acid (C4-C8) and the presence of higher content of long chain saturated fatty acids (C14,C16,C18) in all the three body fats. Buffalo body fat contained higher content of fatty acids (C14 - C18) compared to sheep and goat body fats.

Ascending paper chromatography of whole fat using dichloromethane:isopropanol:glacial acetic acid (80/30/20, v/v) as solvent system showed distinct difference between ghee and animal body fats. Adulteration with buffalo and goat body fats below 10 per cent level and pig body fat above 10 per cent level could be detected by this technique. This technique had the advantage over the existing methods in that the cotton-tract ghee behaved like normal ghee and adulteration of animal body fats at 10 per cent level can be detected in the presence of cotton-tract ghee.

Paper chromatography of unsaponifiable matter of ether soluble and ether insoluble fractions of ghee and animal body fats showed that unsaponifiable matter from ether soluble and ether insoluble fractions of buffalo

body fat, and ether insoluble fraction of pig body fat showed two spots compared to a single spot in ether soluble and ether insoluble fractions of ghee samples and ether soluble fraction of pig body fat. Adulteration at 10 per cent level exhibited two spots only in unsaponifiable matter from ether soluble fractions of ghee samples adulterated with buffalo body fat. The method, however, failed to detect pig body fat in ghee.

Ascending paper chromatography of unsaponifiable matter of ghee and animal body fats did not show any difference that could be used to ascertain adulteration.

Thin layer chromatography of whole fat using the solvent system, hexane:ether:glacial acetic acid:ethyl alcohol (25:20:0.5:1, v/v) showed distinct differences between ghee, vanaspati, refined groundnut oil and coconut oil. Only coconut oil showed an extra spot due to one of the components of stigmasterol. Adulteration of ghee or vanaspati with coconut oil at 10 per cent level and above could be detected by this technique.

Thin layer chromatography of unsaponifiable matter of ghee and animal body fats using hexane:ether:glacial acetic acid:ethyl alcohol (25:20:0.5:1, v/v) as the solvent system showed distinct differences between the two classes of fats. The presence of an extra spot due to dihydrocholesterol was observed in animal body fats and ghee samples adulterated at 10 per cent level with body fats. The method not only detected body fats but identified the presence of dihydrocholesterol in body fats too.

TLC of steryl acetates prepared from free sterol digitonides (Den Herder method, 1955) of animal body fats behaved similar to ghee samples.

Examination by TLC of free and esterified sterol digitonides cleaved in pyridine showed marked differences between ghee and animal body fats. Free sterol digitonides of all animal body fats exhibited an extra spot due to a related derivative of 7-dehydrocholesterol or other sterol. Free sterol digitonides of buffalo body fat, however, showed an additional distinct spot of dihydrocholesterol. This spot was very weak in appearance in sheep, goat and pig body fats. Esterified sterol digitonides of animal body fats showed differences from ghee in that, pig, buffalo, goat and sheep body fats exhibited one, two, three and three extra spots each compared to a single spot in ghee. The free sterol digitonides of ghee samples adulterated at 10 per cent level with pig, goat and sheep body fat, and buffalo body fat showed one and two extra spots while esterified sterol digitonides of ghee samples adulterated with pig body fat and buffalo, goat and sheep body fat, respectively gave one and two spots bands. The extra spots at Rf 0.53 and 0.79 or 0.81 were identified as dihydrocholesterol and a related component of 7-dehydrocholesterol. The other two extra spots in esterified sterol digitonide (Rf, 0.18 and 0.28) could not be identified. This method was very useful not only in the detection of adulteration but also in the identification of adulterant body fats on the basis of identified sterols.

Thin layer chromatography of trimethyl silyl ethers prepared from unsaponifiable matter of ghee, animal body fats and adulterated ghee samples did not show any difference which could be used to detect adulteration.

Turbidity behaviour and absorbance profiles of ghee and animal body fats clearly revealed the difference between the two classes of fats in solvent combination, 96 per cent ethyl alcohol:water (1:1, v/v) at 0°C. Normal ghee samples did not show turbidity upto 3 hours at 0°C while goat, sheep, buffalo and pig body fats showed turbidity (absorbance, 0.6) in 30, 40, 50 and 240 seconds. Cotton-tract buffalo ghee took 15 minutes to show turbidity (absorbance, 0.45). Cow ghee samples adulterated with buffalo, sheep and goat body fats took 10, 20 and 45 minutes whereas buffalo ghee adulterated with buffalo, sheep and goat body fats took 5, 8 and 40 minutes to show turbidity (absorbance, 0.4). Buffalo and cow samples adulterated with pig body fat showed turbidity (absorbance, 0.2) in 60 and 90 minutes, respectively. Cotton-tract buffalo ghee adulterated with buffalo, sheep, goat and pig body fats became turbid (absorbance, 0.6) in 5, 5, 6 and 10 minutes, respectively. Ghee samples adulterated with all body fats except pig body fat at 5 per cent level showed turbidity after 1½ hour.

A hundred fold difference in turbidity time between vanaspati and ghee, and refined groundnut oil was observed. Adulteration of ghee at 30 per cent level and vanaspati at 15 per cent level with refined groundnut oil respectively

showed significant differences in absorbance from pure ghee and vanaspati samples.

Turbidity method being simple and quick can be used as a practical test for the detection of all animal body fats at 10 per cent level in ghee and refined groundnut oil at 15 per cent level in vanaspati and 30 per cent level in ghee. The method could also detect animal body fats except pig body fat at 5 per cent level in ghee.

The melting point of fatty acids extracted from turbid matter (turbidity method) was higher in goat body fat compared to other animal body fats. The melting points observed were in correspondence to the range of melting points of standard fatty acids, palmitic and stearic.

GLC of fatty acid methyl esters of turbid matter of animal body fats showed a marked difference in the content of long chain fatty acids (C14:0 - C18:0). The content of fatty acids, C10:0 and C12:0 was higher in sheep and buffalo body fats compared to goat body fat and that of C18:0 higher in goat body fat compared to buffalo and sheep body fats. Buffalo and sheep body fats showed higher content of C14:0 and C16:0 compared to goat body fat. The presence of higher content of long chain fatty acids especially C16:0 and C18:0 alongwith C14:0 were responsible for inducing turbidity in animal body fats and ghee samples adulterated with animal body fats.

Vanaspati behaves alike to normal ghee in several physico-chemical properties. Colour test (Baudouin test) is

available for detection of vanaspati in ghee. The furfural reagent used in Baudouin test, being unstable, bears distillation and storage problems, a very stable reagent, p-hydroxy benzaldehyde was used to develop a simple test for detection of vanaspati in ghee at low level. Only vanaspati containing sesame oil showed immediate red colouration with solid as well as 5 per cent alcoholic solution of p-hydroxy benzaldehyde in the presence of concentrated HCl. Adulteration of vanaspati in ghee upto 1 per cent level was easily detectable by this test. The component responsible for red colouration in vanaspati was tentatively identified to be sesamol. The method being simple, quick and sensitive is better than Baudouin test and can be used in laboratory and field as well for the detection of vanaspati in ghee at low level.

The fractionation of unsaponifiable matter of vanaspati and sesame oil showed two and three extra spots and ghee samples adulterated with vanaspati at 10 per cent level showed two spots while unsaponifiable matter of ghee samples showed no spot. Thus, the TLC study could also be used to detect vanaspati in ghee at 10 per cent level using 50 per cent solution of p-hydroxy benzaldehyde in concentrated HCl.

UV spectrum of whole fat did not show any difference between ghee and animal body fats. A shift in second maxima from 270 to 280 nm was observed in spectrum of unsaponifiable matter of body fats. However, adulteration of ghee

with body fats at 10 per cent level did not show this shift in the maxima.

Infra-red spectroscopic study of whole fat showed distinct differences between ghee and animal body fats in IR regions 1300 - 1180 and 1120 - 1100 cm^{-1}. A prominent difference was observed in region 1300 - 1180 cm^{-1} in that buffalo body fat and goat and sheep body fats showed five and six bands compared to two bands in ghee samples. The sharpness of peak at 720 - 710 cm^{-1} was much pronounced in animal body fats than that of ghee. Body fats showed other extra bands at 2680 - 2600, 2030 - 2020, 1580, 920 - 910, 880 - 870 and 840 - 820 cm^{-1} respectively. A normal absorption peak in animal body fats showed a clear split into two peaks at 1110 and 1100 cm^{-1} in ghee samples. The shape of contours in the curves of animal body fats in region 1100 - 800 cm^{-1} was much distinct from ghee. Ghee samples adulterated with sheep, goat and buffalo body fats showed three, four and five extra bands (1300 - 1180 cm^{-1}), a broad and deep peak at 720 - 710 cm^{-1} and distinct shape of contours between 1100 - 800 cm^{-1} could be used for detection of animal body fats in ghee. Ghee samples adulterated with pig body fat showed an extra band at 910 cm^{-1}. Besides ghee samples adulterated with sheep and goat body fats exhibited extra bands at 2640 - 2630 cm^{-1} while buffalo ghee adulterated with buffalo body fat showed extra bands at 2640 and 2050 cm^{-1}. Ghee samples were devoid of these bands.

IR spectra of unsaponifiable matter of buffalo body

fat differed markedly from ghee in that buffalo body fat showed extra bands at 2880 - 2830, 1465 - 1440 (doublet), 1380 and 1350 (doublet), 1415 and 1300 - 1190 (sextet) cm^{-1} , respectively. Adulteration at 10 per cent level introduced significant changes in the IR spectra of unsaponifiable matter from adulterated ghee samples. A distinct doublet (780 and 760 cm^{-1}) and multiple bands (5-6 bands in IR region 1300 - 1110 cm^{-1}) observed in adulterated ghee samples clearly indicated adulteration of animal body fats. IR spectroscopic technique being quick and sensitive requires a very small quantity of fat samples and can be used for the demonstration of adulteration.

The critical studies on unsaponifiable matter in detail and whole fat for the detection of adulteration of ghee with animal body fats, the methods, viz., turbidity method, thin layer chromatography (unsaponifiable matter and free and esterified sterol digitonides), paper chromatography and IR spectroscopy are suggested. Detection of common vegetable fat adulterants like coconut oil, groundnut oil and vanaspati in ghee, the methods like TLC technique for coconut oil, p-hydroxy benzaldehyde test for vanaspati and turbidity method for refined groundnut oil are suggested. A combination of these tests for the detection of adulteration of ghee with animal body fats in presence of vegetable fats/oils would remove all lacuna encountered with the existing methods and make it possible to detect adulteration in ghee with body fats with or without vegetable fats/oils at 10 per cent level and below without any discrepancy.

Table 5. Unsaponifiable matter content of ghee and animal body fats

Nature of fat sample	No. of samples	Unsaponifiable matter (mg %)	
		Range	Average
Cow ghee	5	458-562	505
Buffalo ghee	5	426-526	472
Buffalo ghee (cotton-tract)	3	406-438	429
Buffalo body fat	3	416-438	426
Goat body fat	3	391-417	403
Sheep body fat	3	407-423	411
Pig body fat	3	269-347	302

Table 6. Unsaponifiable matter content of adulterated ghee samples

Ghee sample	Adulterant		Unsaponifiable matter (mg %) Average
	Animal body fat	Per cent added	
Cow ghee	-	-	504
	Buffalo	5	497
		10	492
	Goat	5	496
		10	489
	Pig	5	487
		10	483
	Sheep	5	496
		10	490
Buffalo ghee	-	-	473
	Buffalo	5	467
		10	459
	Goat	5	463
		10	456
	Pig	5	460
		10	452
	Sheep	5	465
		10	458

* No. of samples analysed were 3 in each case

Table 7. Seasonal variation in unsaponifiable matter content of ghee and animal body fats

Nature of fat sample	No. of samples	Unsaponifiable matter (mg %)			
		Winter		Summer	
		Range	Average	Range	Average
Cow ghee	6	444-574	507	422-548	475
Buffalo ghee	6	439-550	483	418-510	451
Buffalo body fat	6	438-449	437	413-424	417
Goat body fat	6	423-436	427	374-392	384
Sheep body fat	6	429-439	432	402-406	402
Pig body fat	6	294-398	338	279-341	308

Table 8. Unsaponifiable matter content of ether soluble and ether insoluble fractions of ghee and animal body fats

Nature of fat sample	No. of samples	Unsaponifiable matter (mg %)	
		Ether soluble fraction	Ether insoluble fraction
Cow ghee	3	362	142
Buffalo ghee	3	411	72
Buffalo body fat	3	221	185
Pig body fat	3	144	86

Table 9. Unsaponifiable matter content in ether soluble and ether insoluble fractions for adulterated ghee samples

Nature of ghee sample	Adulterant		Unsaponifiable matter (mg %)	
	Nature of animal body fat	Per cent added	Ether soluble fraction	Ether insoluble fraction
Cow ghee	-	-	362	142
	Buffalo	10	352	147
	Pig	10	342	135
Buffalo ghee	-	-	411	72
	Buffalo	10	402	83
	Pig	10	397	74

* Number of samples analysed were two

Table 10. Cholesterol content of ghee and animal body fats

Nature of fat sample	Cholesterol (mg %)		
	Method Ia	Method IIb	Method IIIc
Cow ghee	321-347 (333)	256-273 (263)	289-329 (308)
Buffalo ghee	268-326 (300)	232-261 (249)	245-288 (266)
Buffalo ghee (cotton-tract)	258-301 (282)	228-255 (246)	239-277 (258)
Buffalo body fat	217-237 (225)	201-222 (212)	211-222 (217)
Pig body fat	112-130 (121)	107-110 (108)	110-119 (117)
Goat body fat	186-204 (193)	168-188 (177)	176-194 (186)
Sheep body fat	206-216 (209)	181-197 (187)	186-211 (199)

Figures in parentheses indicate average value

No. of samples analysed were 3 in each case

Note: Method Ia is the direct fat technique for estimation of cholesterol

Method IIb is the unsaponifiable matter technique for cholesterol estimation using acetic acid

Method IIIc is the unsaponifiable matter technique for cholesterol estimation using chloroform

Table 11. Cholesterol content of adulterated ghee samples

Nature of ghee sample	Adulterant		Cholesterol (mg %)
	Nature of animal body fat	Per cent added	Method Ia (average)
Cow ghee	-	-	331
	Buffalo	10	317
	Pig	10	306
	Goat	10	313
	Sheep	10	307
Buffalo ghee	-	-	290
	Buffalo	10	282
	Pig	10	273
	Goat	10	279
	Sheep	10	280

* Number of samples analysed were 3

Table 12. Cholesterol content estimated by gravimetric methods in ghee and animal body fats

Nature of fat sample	No. of samples	Cholesterol (mg %)	
		Method IIa	Method IIIb
Cow ghee	4	261-311 (285)	243-283 (261)
Buffalo ghee	4	251-281 (265)	240-256 (247)
Buffalo ghee (cotton-tract)	4	242-269 (256)	227-254 (238)
Buffalo body fat	4	201-234 (217)	179-221 (199)
Pig body fat	4	91-119 (106)	85-100 (93)
Goat body fat	4	169-197 (182)	166-186 (174)
Sheep body fat	4	173-227 (200)	179-219 (198)

Figures in parentheses show average value

Note: Method IIa is the direct gravimetric method for cholesterol estimation as digitonide from unsaponifiable matter of fat sample

Method IIIb is the indirect gravimetric method for cholesterol estimation as digitonide from unsaponifiable matter after extraction of insoluble unsaponifiable matter of fat samples

Table 13. Cholesterol content of adulterated ghee samples (gravimetric methods)

Nature of ghee sample	Adulterant		Cholesterol (mg %)	
	Nature of animal body fat	Per cent added	Method IIa (average)	Method IIIb (average)
Cow ghee	-	-	286	263
	Buffalo	10	277	254
	Pig	10	266	244
	Goat	10	272	248
	Sheep	10	273	251
Buffalo ghee	-	-	266	248
	Buffalo	10	256	241
	Pig	10	244	229
	Goat	10	247	237
	Sheep	10	254	238

* No. of samples analysed were 2 in each case

Table 14. Comparative account of cholesterol content estimated by colorimetric and gravimetric methods in ghee and animal body fats

Nature of fat sample	Cholesterol (mg %, average value)				
	Method Ia	Method IIb	Method IIIc	Method IIa	Method IIIb
Cow ghee	333	263	308	285	267
Bufalo ghee	300	249	266	265	247
Buffalo ghee (cotton-tract)	282	246	258	256	238
Buffalo body fat	225	212	217	217	199
Sheep body fat	209	187	199	200	198
Goat body fat	193	177	186	182	174
Pig body fat	121	108	117	106	93

Table 15. Levels of free and esterified cholesterol in ghee and animal body fats

Nature of fat sample	No. of samples	Method IIIa (mg %)		No. of samples	Method IVb (mg %)	
		Free cholesterol	Esterified cholesterol		Free cholesterol	Esterified cholesterol
Cow ghee	6	235-251 (244)	35.79-42.89 (37.73)	2	221-241 (231)	22.4-31.4 (26.9)
Buffalo ghee	6	229-246 (236)	42.46-50.12 (46.7)	2	207-227 (217)	34.8-38.7 (36.63)
Buffalo body fat	6	189-211 (198)	28.91-33.10 (31.08)	2	181-196 (188)	22.2-28.4 (25.5)
Pig body fat	6	78-101 (87)	26.40-29.68 (27.7)	2	82- 91 (86)	11.8-20.6 (16.45)
Goat body fat	6	161-178 (170)	28.60-31.90 (30.4)	2	171-189 (180)	24.8-26.8 (25.58)
Sheep body fat	6	179-181 (184)	29.10-33.12 (31.1)	2	186-202 (194)	22.4-29.1 (25.75)

Figures in parentheses indicate average value

Note: Method IIIa is TLC-cum-colorimetric method for the estimation of free and esterified cholesterol from fat sample

Method IVb is the gravimetric method for estimation of free and esterified cholesterol before and after saponification of fat sample

Table 16. Levels of free and esterified cholesterol of adulterated ghee samples

Nature of ghee sample	Adulterants		Method IVb (mg %)	
	Nature of animal body fat	Per cent added	Free cholesterol (average)	Esterified cholesterol (average)
Cow ghee	-	-	230	26.9
	Buffalo	10	225	26.0
	Sheep	10	224	26.2
	Pig	10	214	25.2
	Goat	10	221	26.4
Buffalo ghee	-	-	218	36.7
	Buffalo	10	214	35.05
	Sheep	10	215	35.45
	Pig	10	204	33.7
	Goat	10	211	35.83

* Number of samples analysed were 2

Table 17. Levels of insoluble unsaponifiable matter in ghee and animal body fats

Nature of fat sample	No. of samples	Insoluble unsaponifiable matter (% by wt)
Cow ghee	6	0.070
Buffalo ghee	6	0.082
Buffalo ghee (cotton-tract)	6	0.112-0.148
Buffalo body fat	6	33.89 - 36.42
Pig body fat	6	0.176-0.198
Goat body fat	6	15.96 - 20.48
Sheep body fat	6	12.92 - 18.52

Table 18. Level of insoluble unsaponifiable matter in adulterated ghee samples

Nature of ghee sample	No. of samples	Adulterant		Insoluble unsaponifiable matter (mg/5 g)
		Nature of animal body fat	Per cent added	
Cow ghee	4	Buffalo	10	N.S.
			15	N.S.
			20	242-268
	4	Pig	10	N.S.
			15	N.S.
			20	117-121
	4	Goat	10	N.S.
			15	N.S.
			20	162-206
	4	Sheep	10	N.S.
			15	N.S.
			20	156-189
Buffalo ghee	4	Buffalo	10	N.S.
			15	N.S.
			20	269-279
	4	Pig	10	N.S.
			15	N.S.
			20	118-129
	4	Goat	10	N.S.
			15	N.S.
			20	197-210
	4	Sheep	10	N.S.
			15	N.S.
			20	176-201

N.S. = Not significant

Table 19. Level of insoluble unsaponifiable matter in adulterated ghee samples (cotton-tract)

Nature of ghee sample	No. of samples	Adulterant		Insoluble unsaponifiable matter (mg/5 g)
		Nature of animal body fat	Per cent added	
Buffalo ghee (cotton-tract)	4	Buffalo	10	N.S.
			15	127-133
			20	274-298
	4	Pig	10	N.S.
			15	N.S.
			20	143-158
	4	Goat	10	N.S.
			15	121-135
			20	241-289
	4	Sheep	10	N.S.
			15	124-131
			20	228-246

N.S. = Not significant

Table 20. Melting point (capillary method) of fatty acids of insoluble matter and turbid matter obtained from animal body fat

Nature of animal body fat	Melting point(°C)	
	Fatty acids (insoluble unsaponifiable matter)	Fatty acids (turbid matter)
Buffalo	62	62
Goat	63	59
Sheep	61	61
Pig	59	57

Table 21. Fatty acid composition (wt %) of insoluble unsaponifiable matter in animal body fats

Fatty acid	Animal body fat		
	Buffalo	Goat	Sheep
C10:0	0.273	0.09	0.30
C12:0	0.164	0.374	1.02
Unidentified	-	0.281	0.65
C14:0	1.35	2.67	1.85
C16:0	29.48	46.82	31.98
C18:0	68.72	49.75	63.94

Table 22. Fatty acid composition (wt %) of turbid matter in animal body fats

Fatty acid	Animal body fat		
	Buffalo	Goat	Sheep
C10:0	0.231	0.598	0.33
C12:0	1.12	2.04	1.02
Unidentified	0.241	0.399	0.402
C14:0	5.46	4.21	5.41
C16:0	39.49	32.83	39.02
C18:0	53.66	59.90	53.00

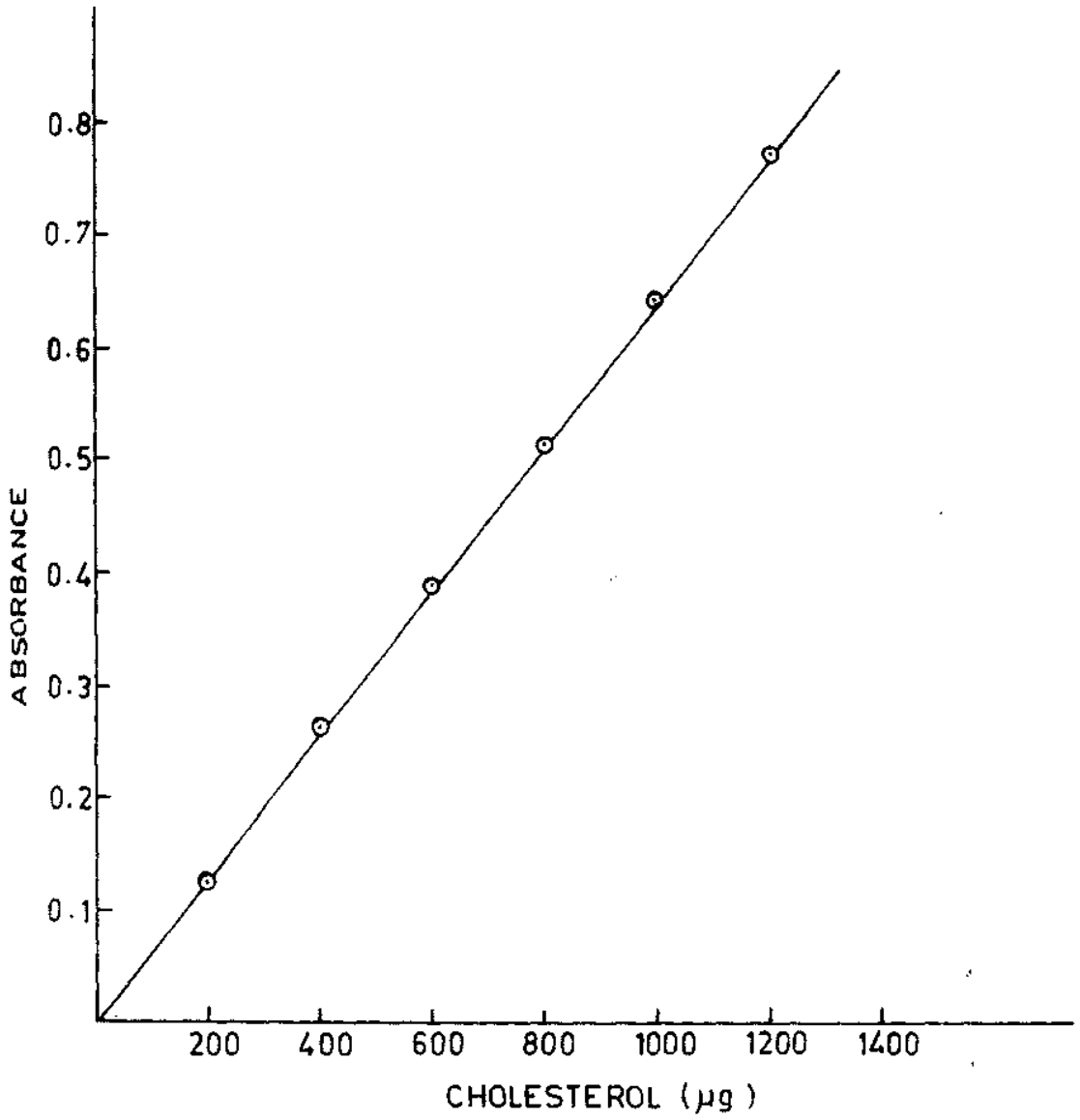


FIG.1 STANDARD CURVE FOR CHOLESTEROL IN CHLOROFORM

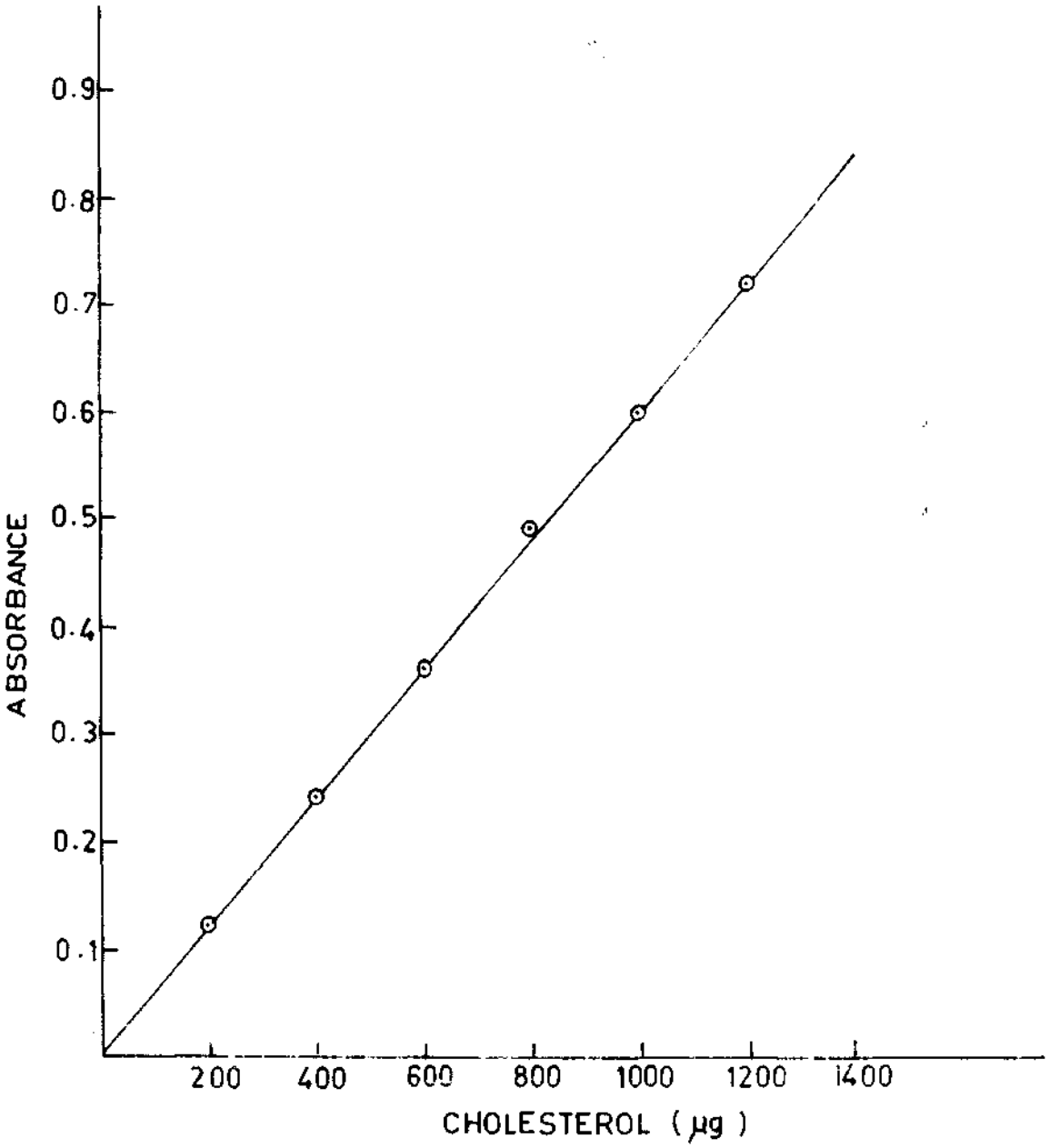


FIG. 2 STANDARD CURVE FOR CHOLESTEROL IN ACETIC ACID

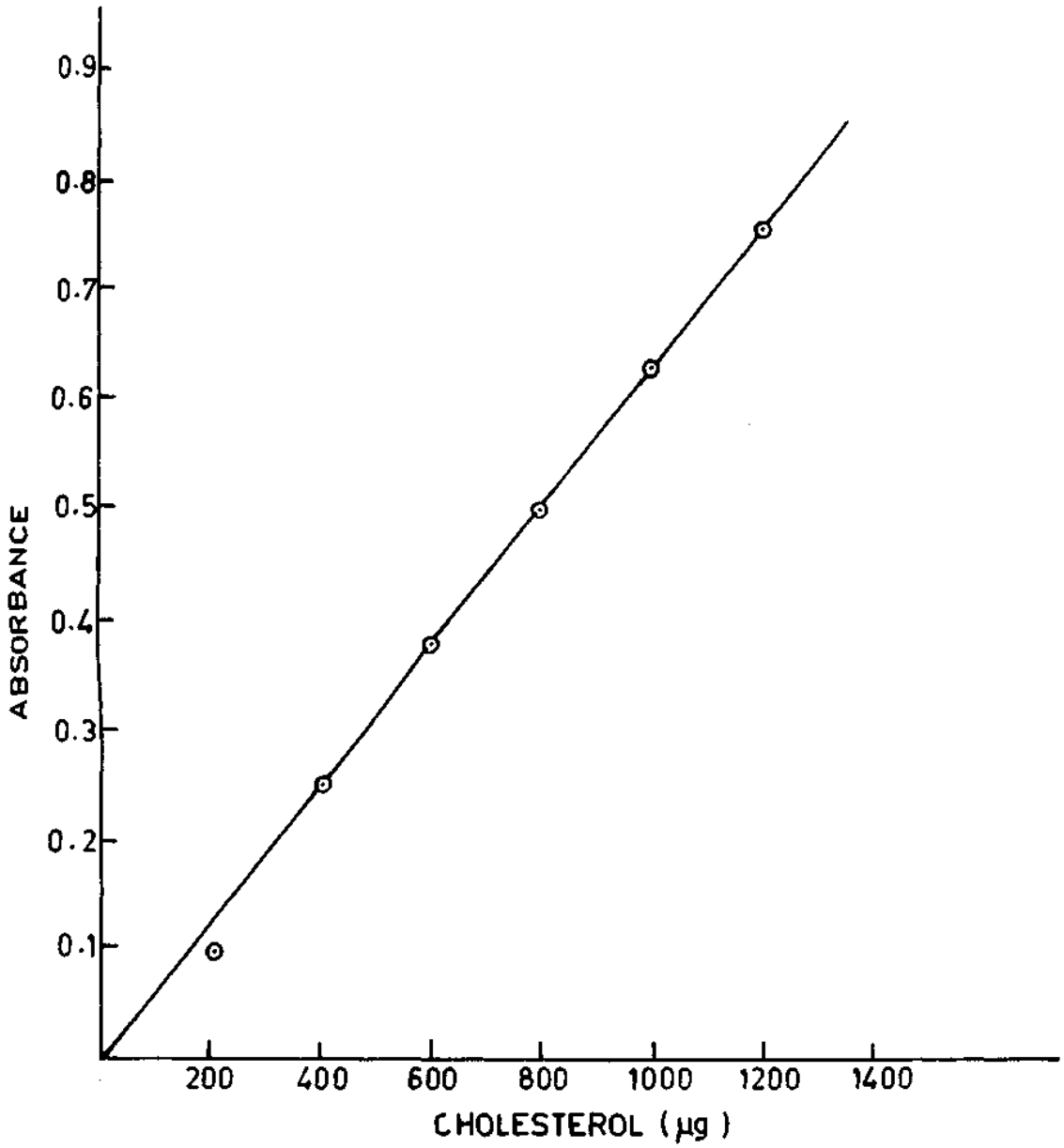


FIG. 3 STANDARD CURVE FOR ESTIMATION OF FREE AND BOUND CHOLESTEROL BY TLC METHOD

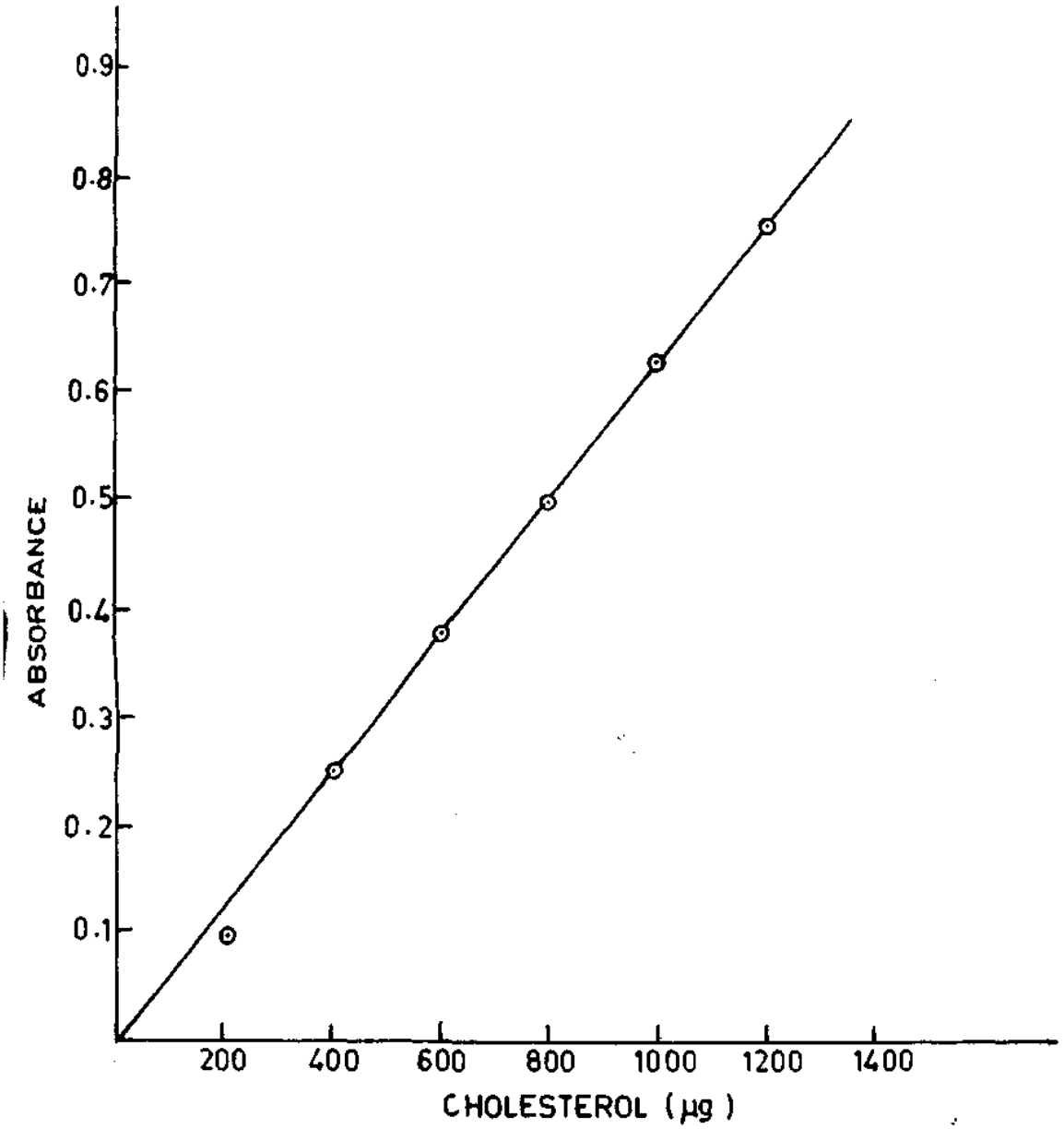


FIG. 3 STANDARD CURVE FOR ESTIMATION OF FREE AND BOUND CHOLESTEROL BY TLC METHOD

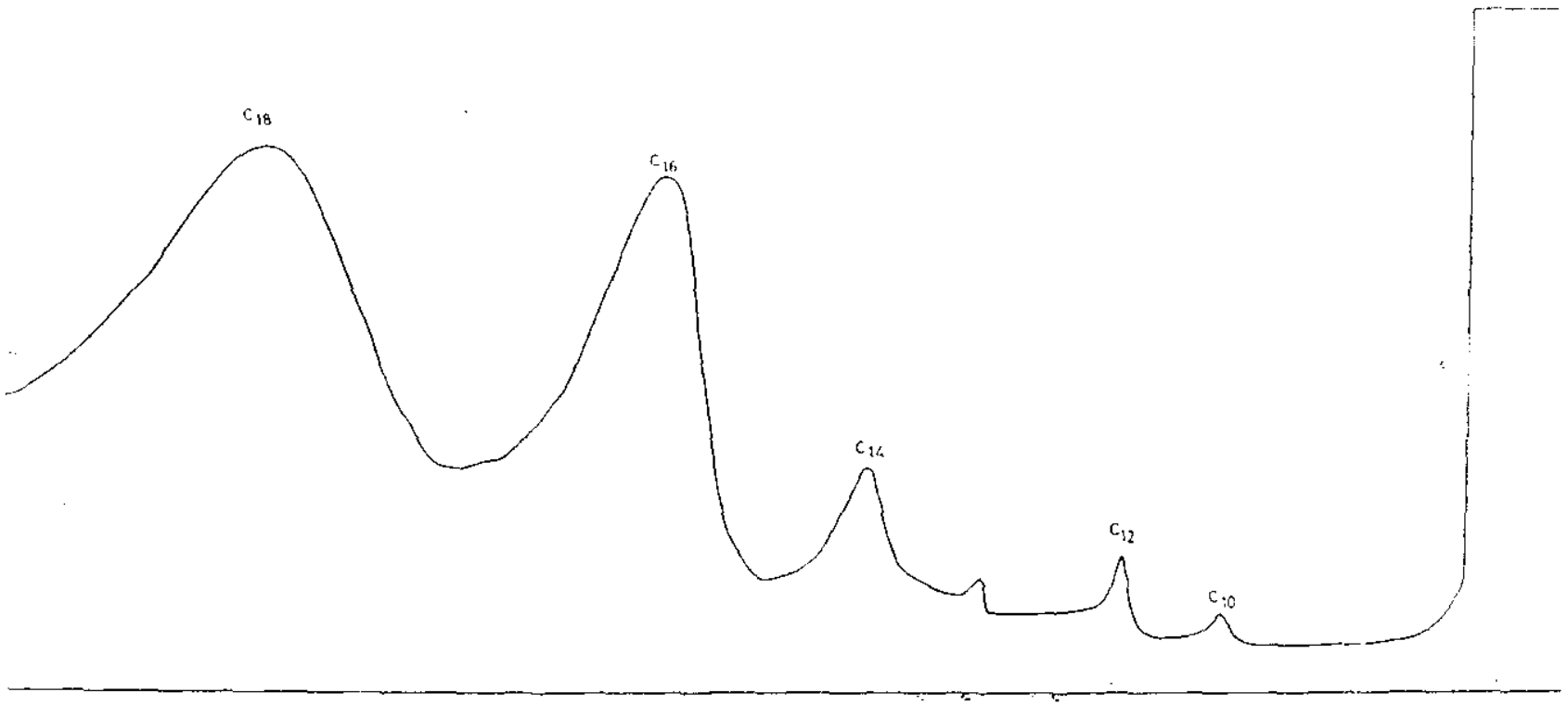


FIG. 5 GLC PATTERN OF METHYL ESTERS OF INSOLUBLE UNSAPONIFIABLE MATTER OF ANIMAL BODY FATS

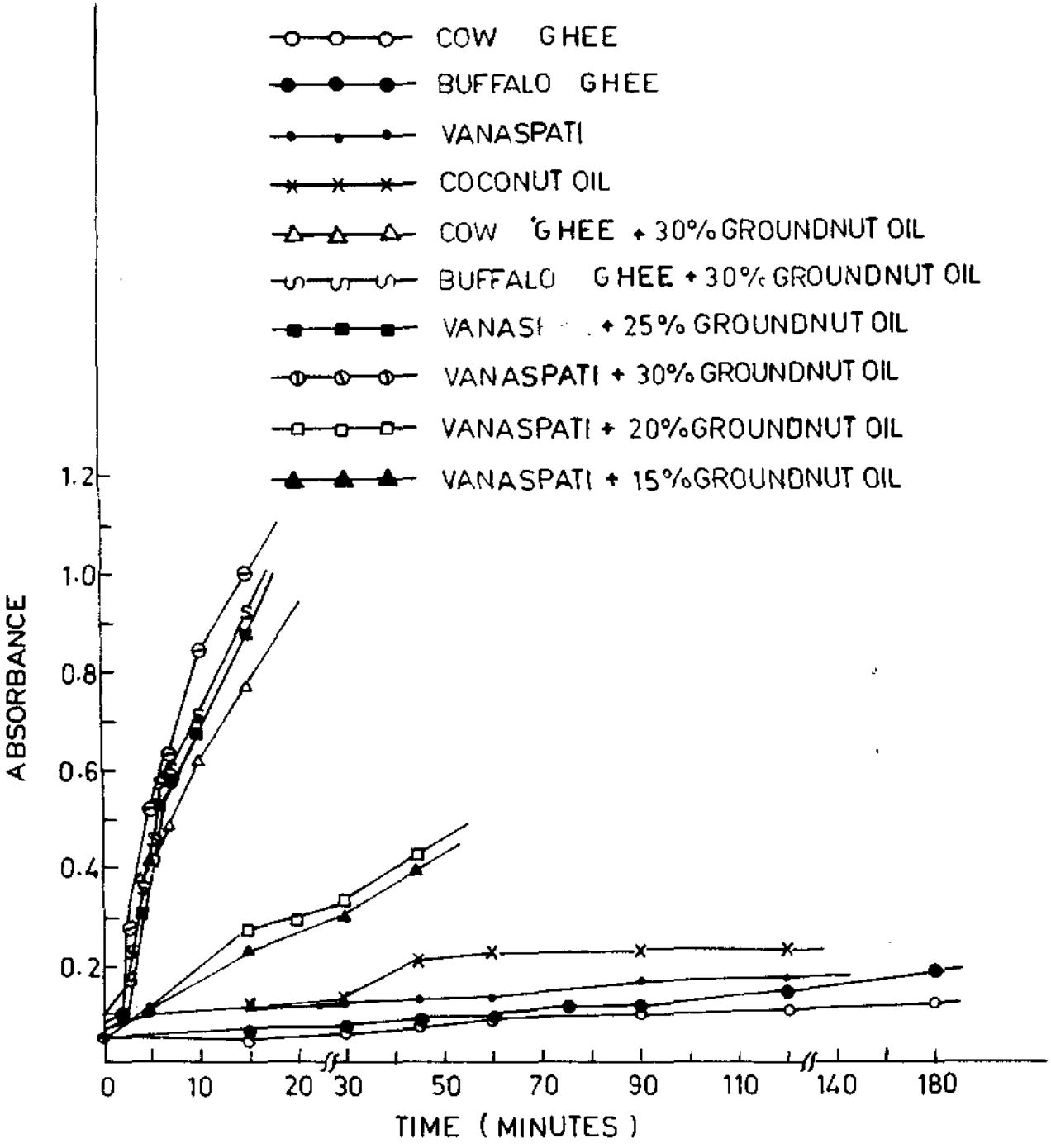


FIG. 6 ABSORBANCE (AT 550 nm) OF DIFFERENT PURE AND ADULTERATED FAT SAMPLES

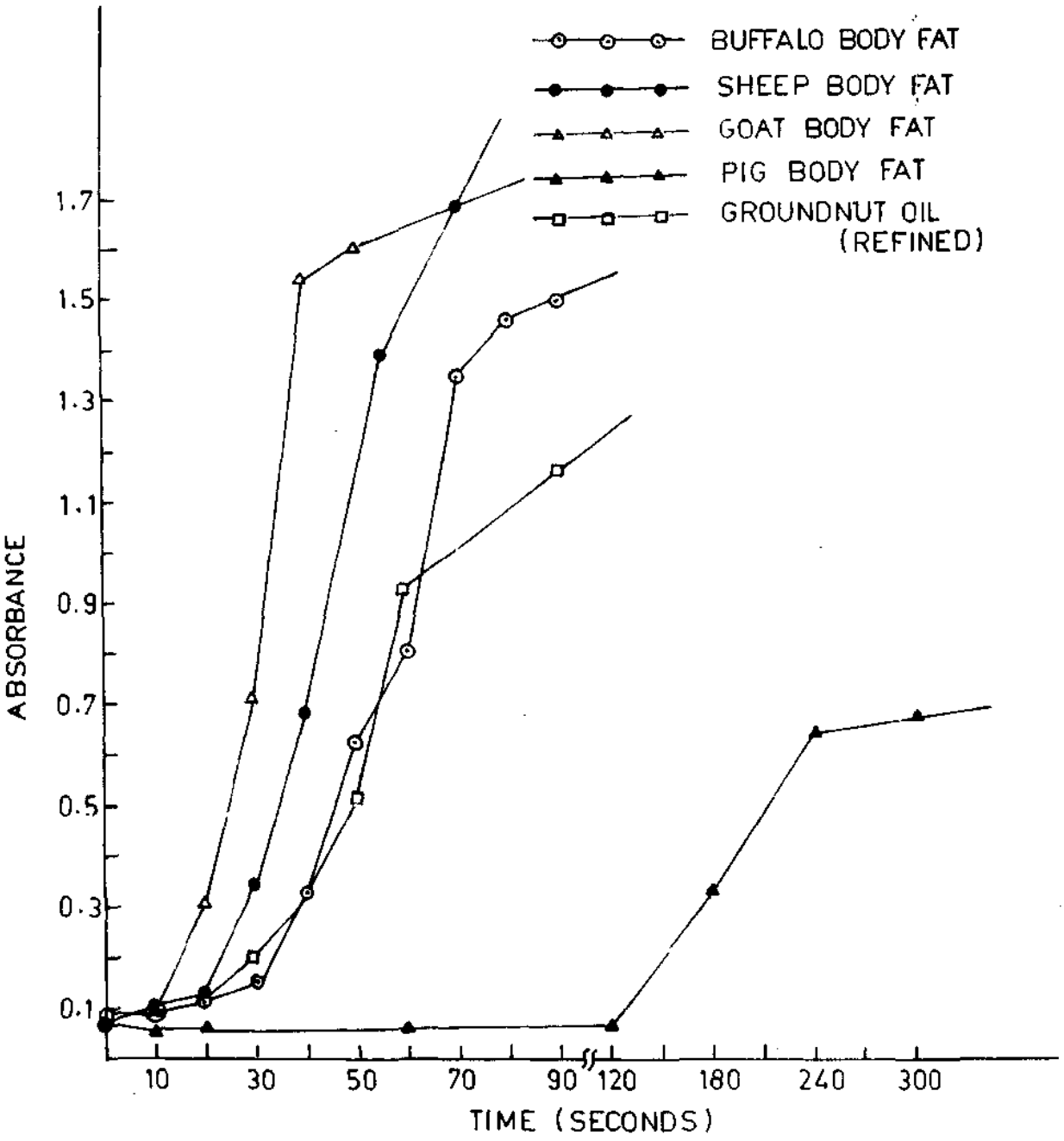


FIG. 7 ABSORBANCE (AT 550 nm) OF ANIMAL BODY FAT AND GROUNDNUT OIL

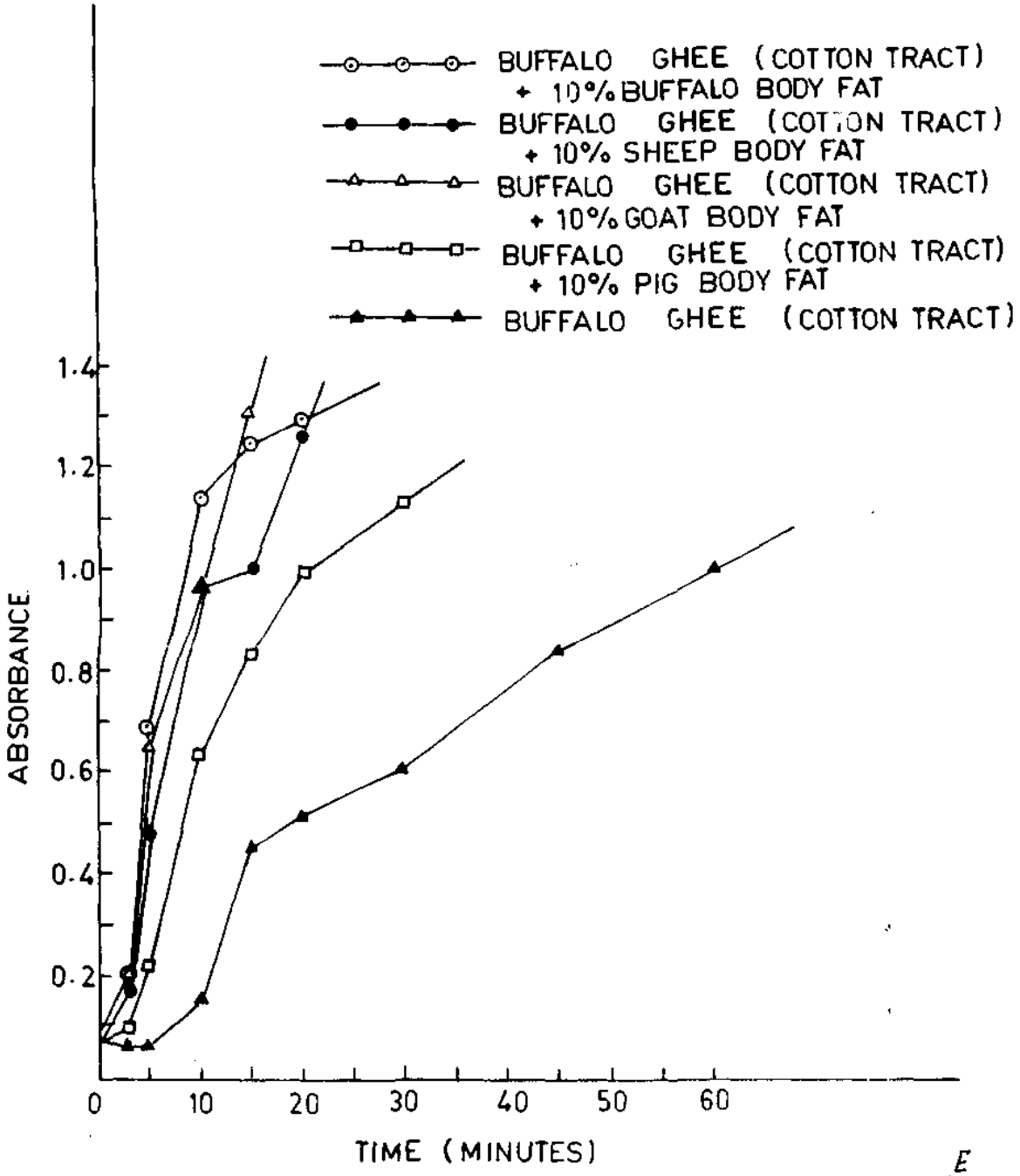


FIG. 8 ABSORBANCE (AT 550 nm) OF PURE AND ADULTERATED BUFFALO GHEE (COTTON TRACT)

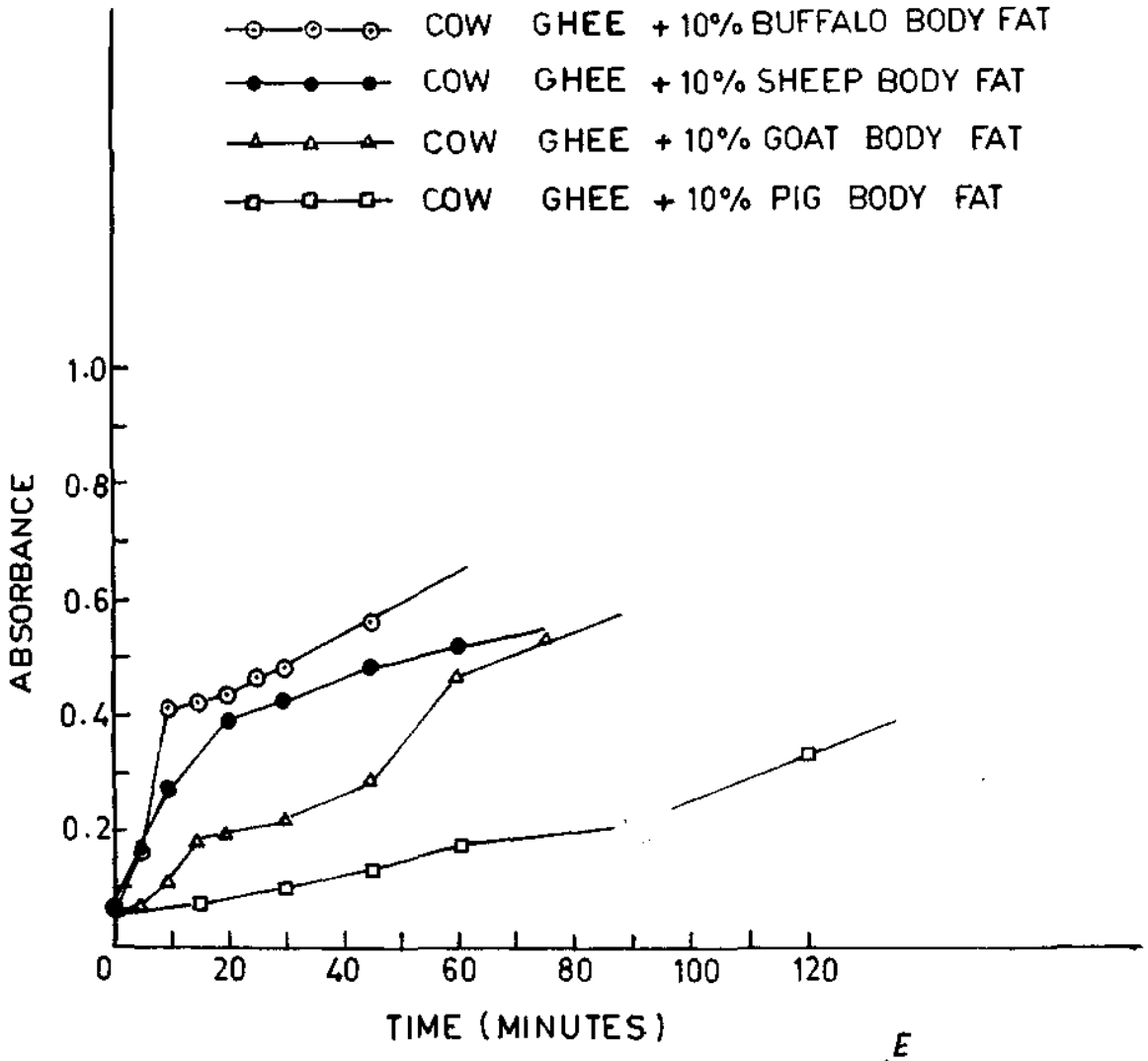


FIG.9 ABSORBANCE (AT 550 nm) OF ADULTERATED COW GHEE

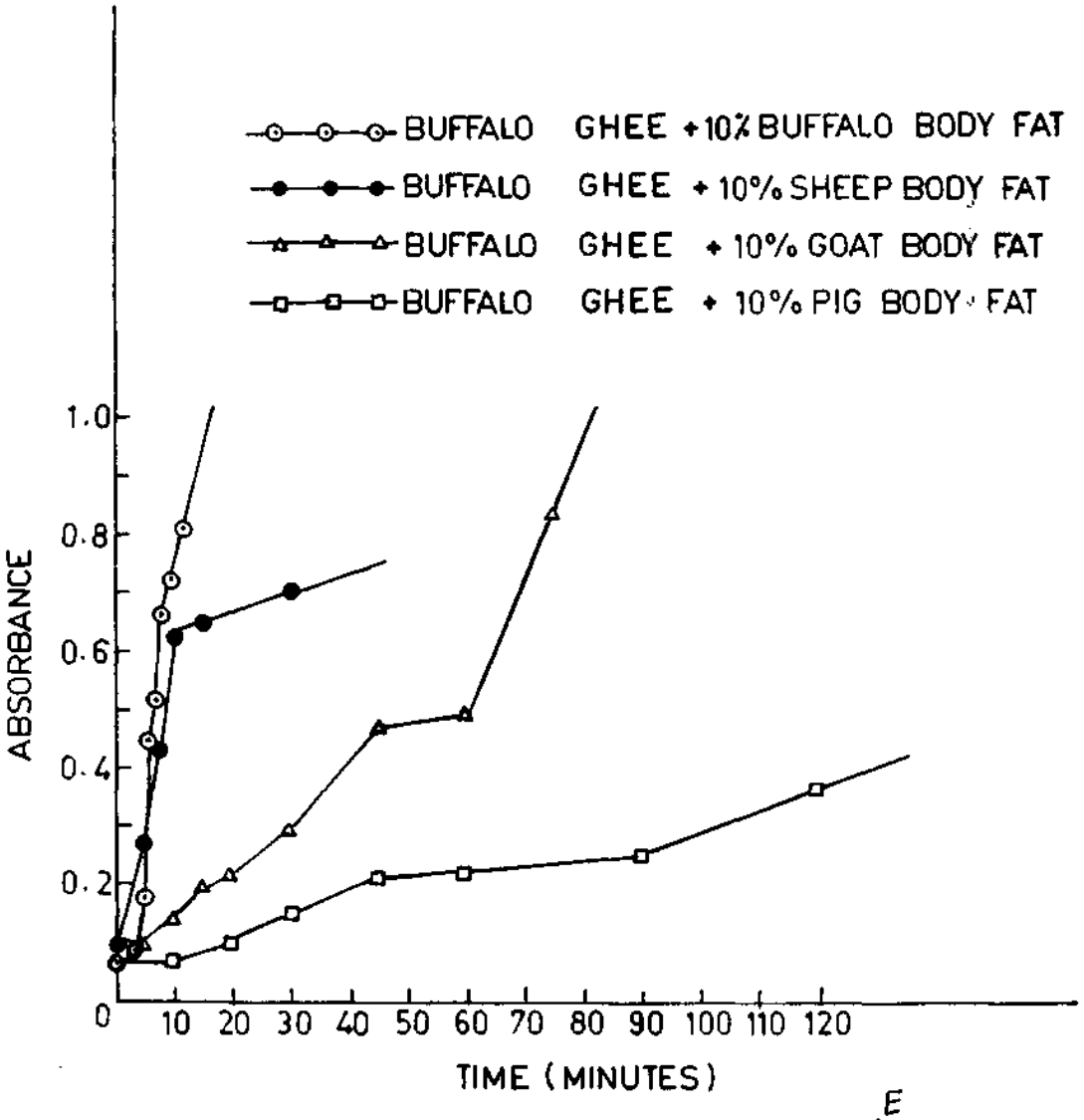


FIG.10 ABSORBANCE (AT 550 nm) OF ADULTERATED BUFFALO GHEE

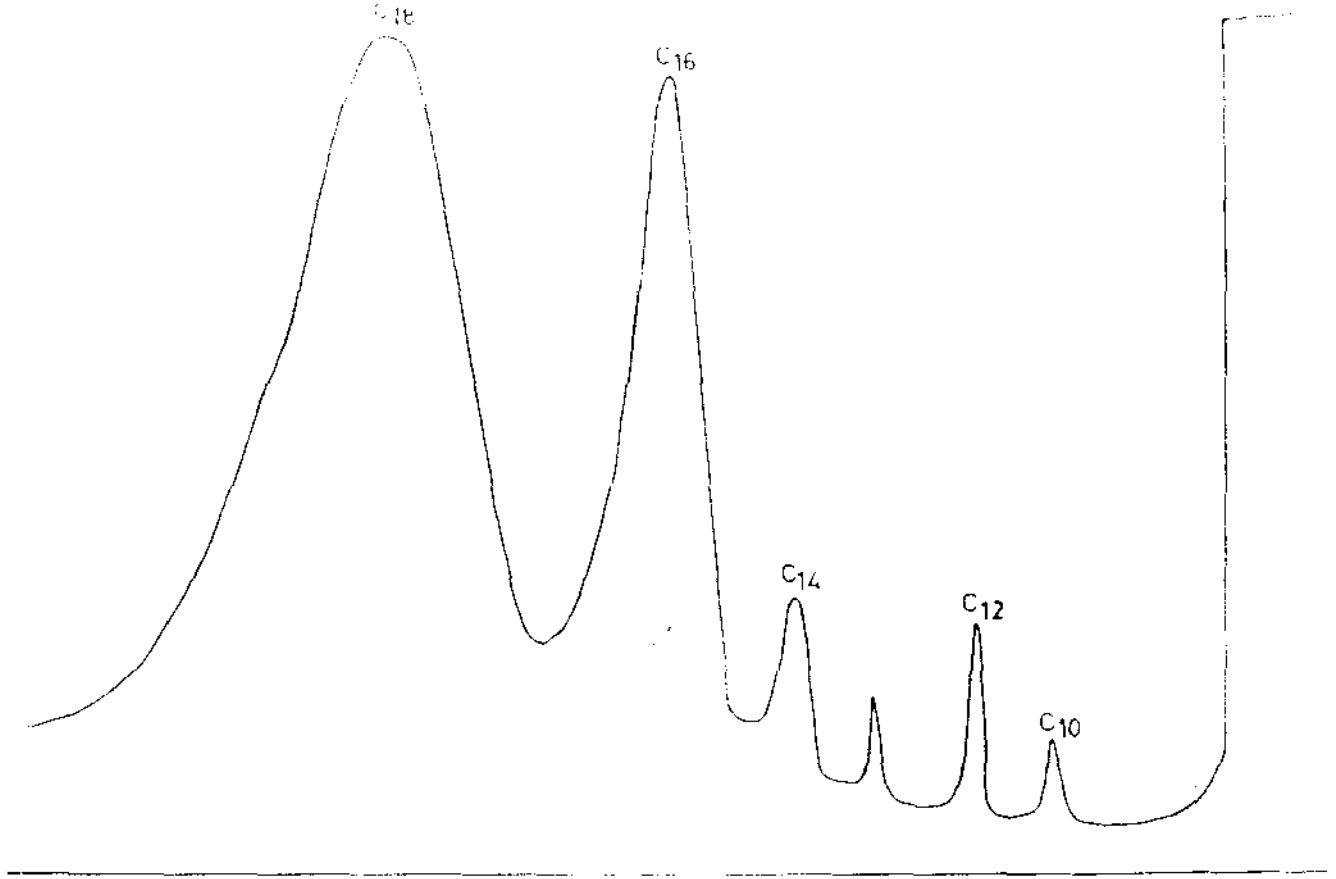


FIG.11 G L C PATTERN OF FATTY ACID METHYL ESTERS OF TURBID MATTER OF ANIMAL BODY FATS

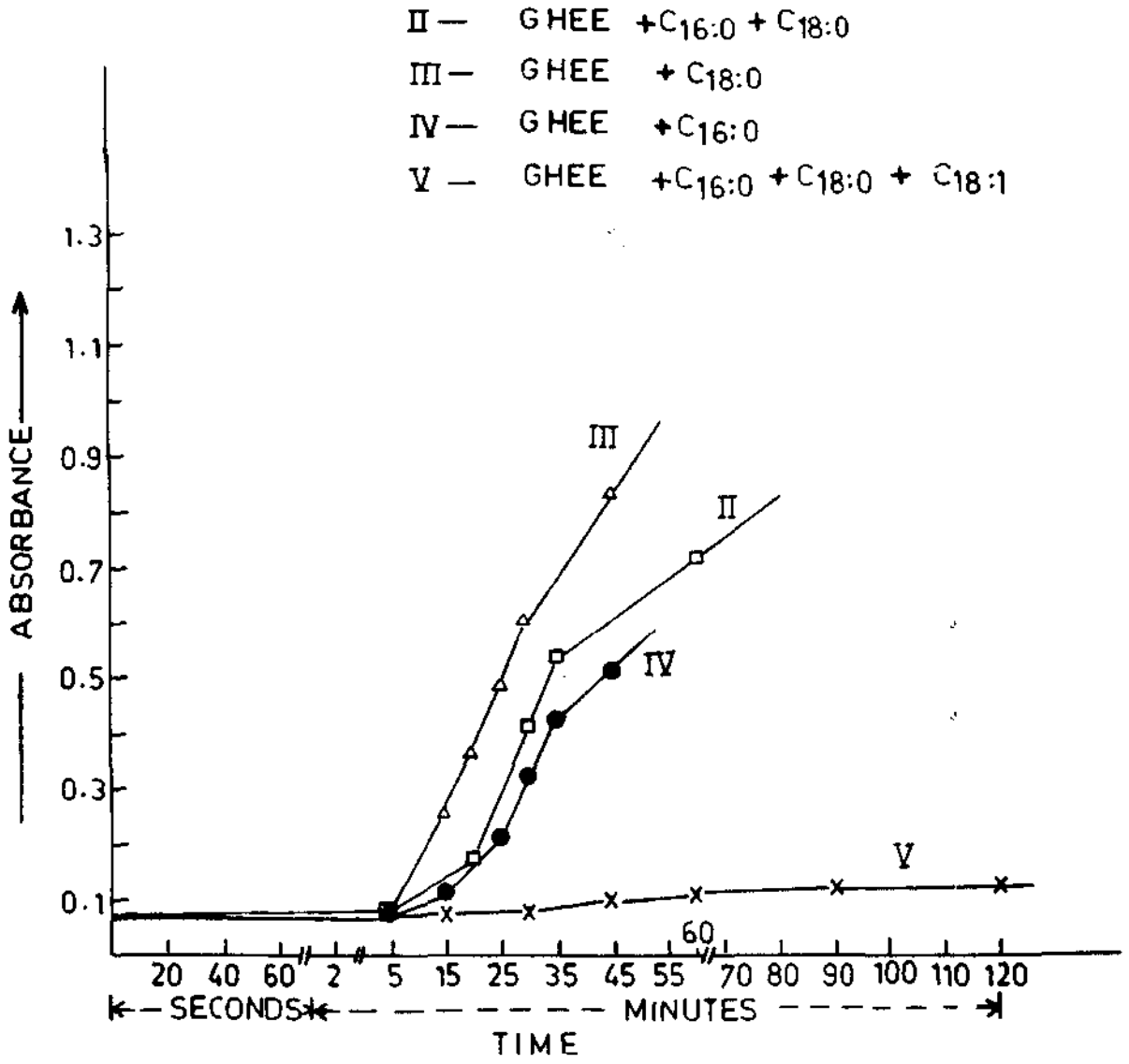


FIG.12 EFFECT OF FATTY ACIDS ADDITION ON TURBIDITY OF BUFFALO GHEE

Fig. 13 TLC of unsaponifiable matter of fat samples

A = Cow ghee

B = Buffalo ghee

C = Sesame oil

D = Vanaspati

Fig. 14 TLC of unsaponifiable matter of adulterated ghee samples

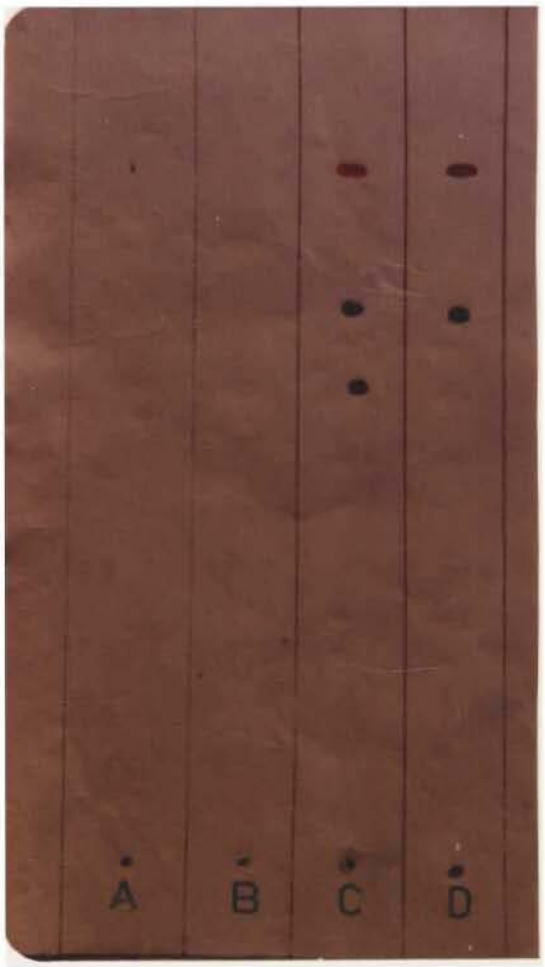
A = Cow ghee + 1% sesame oil

B = Buffalo ghee + 1% sesame oil

C = Cow ghee + 10% vanaspati

D = Buffalo ghee + 10% vanaspati

E = Vanaspati


A
B
C
D


A
B
C
D
E

FIG.15 MODE OF REACTION MECHANISM OF p-HYDROXY BENZALDEHYDE REACTION

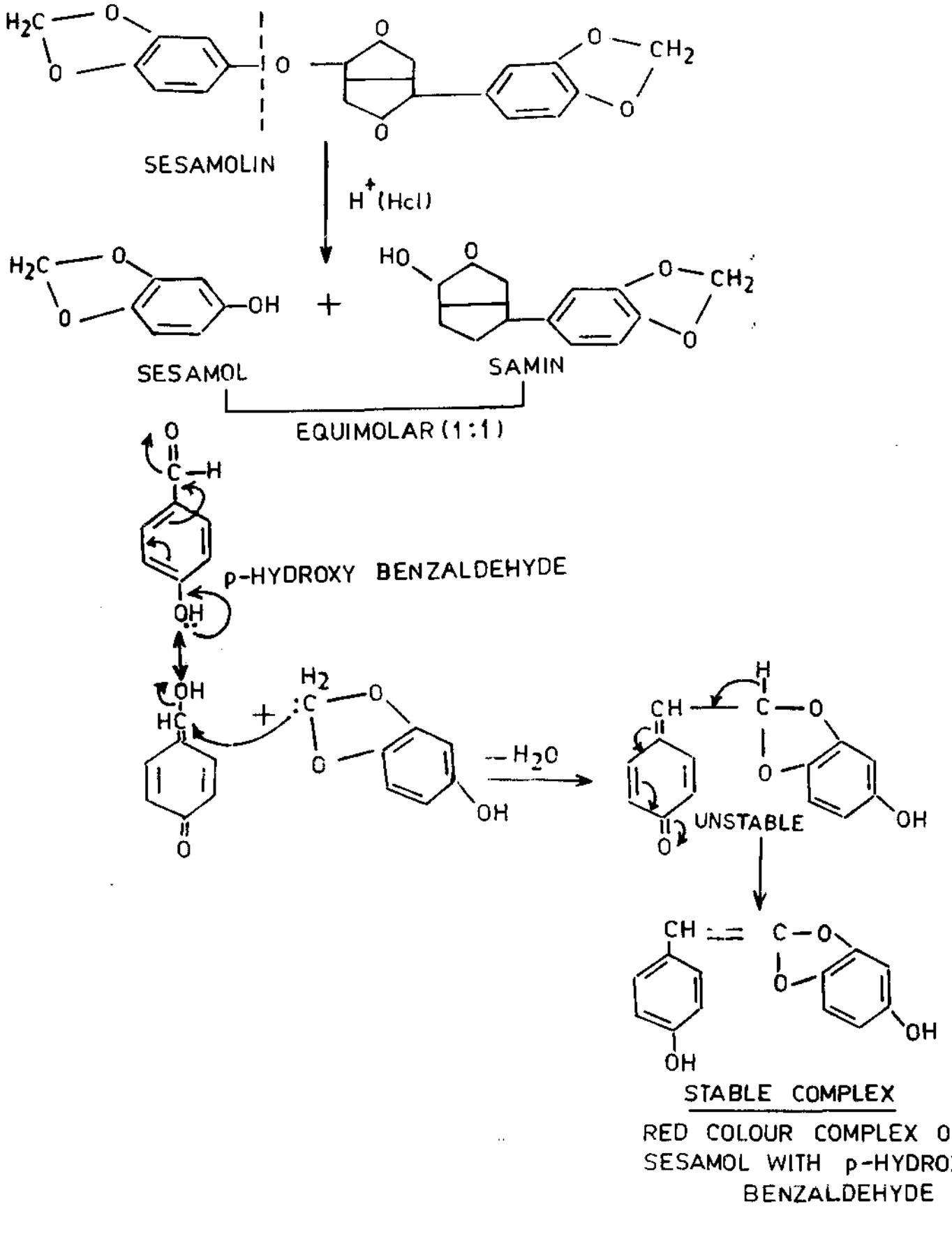


Fig. 16 Paper chromatography of whole fat

A = Buffalo cotton-tract ghee

B = Buffalo ghee

C = Buffalo body fat

D = Pig body fat

E = Cow ghee

F = Goat body fat

Fig. 17 Paper chromatography of pure and adulterated cow ghee samples

A = Cow ghee

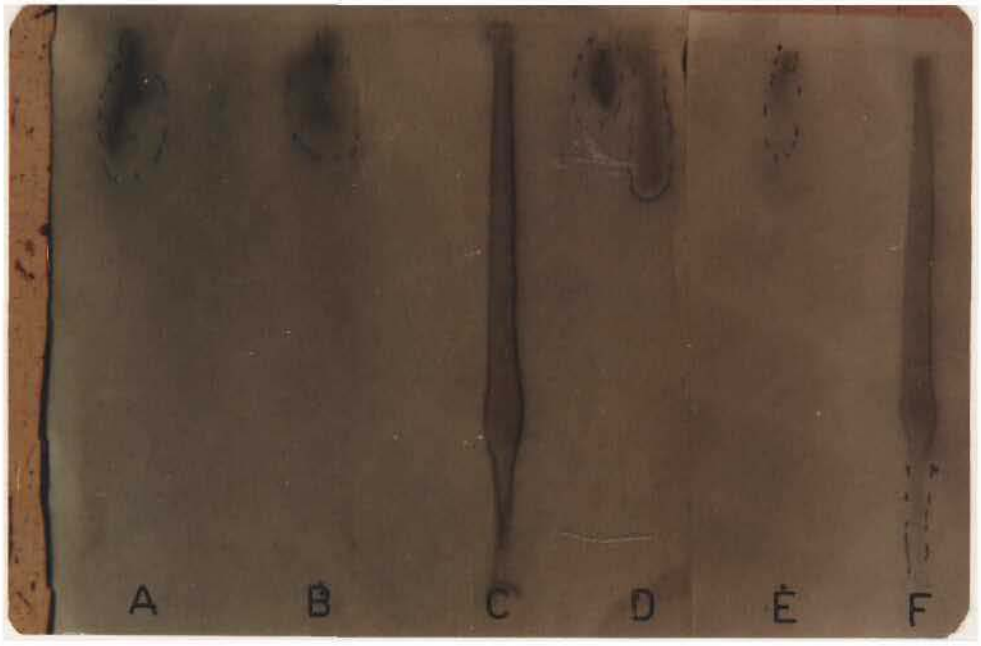
B = Buffalo ghee (cotton-tract)

C = Cow ghee +10% buffalo body fat

D = Buffalo ghee

E = Cow ghee + 10% goat body fat

F = Cow ghee + 10% pig body fat


A
B
C
D
E
F

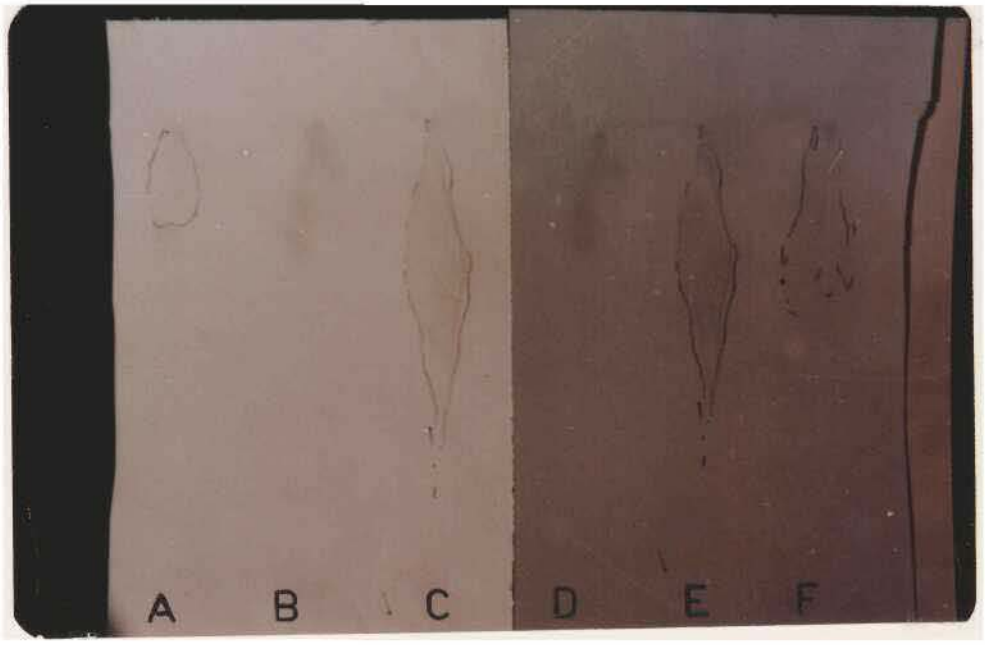

A
B
C
D
E
F

Fig. 18 Paper chromatography of adulterated buffalo ghee samples

A = Buffalo ghee

B = Buffalo ghee + 10% buffalo body fat

C = Buffalo ghee + 10% goat body fat

D = Buffalo ghee + 10% pig body fat

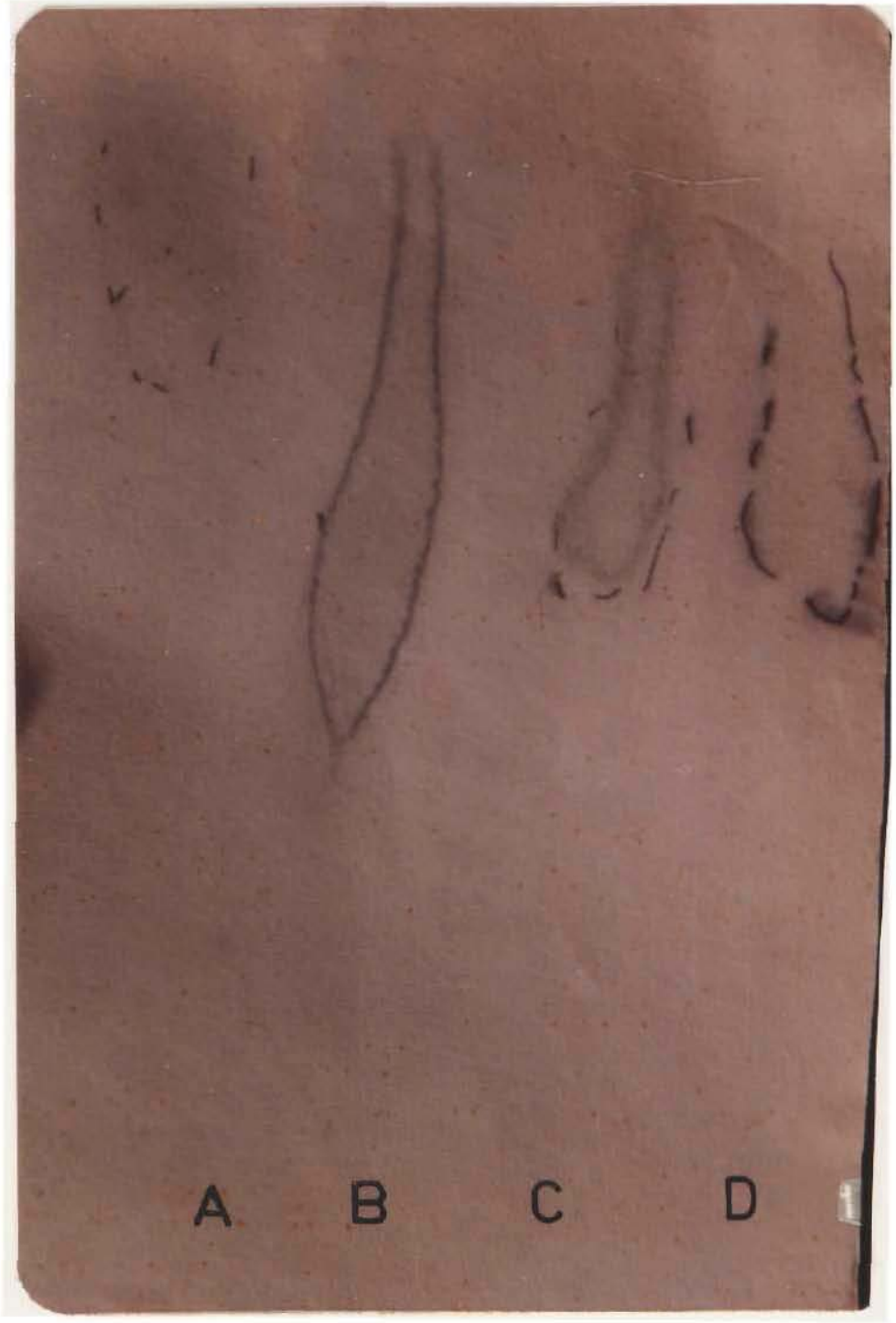

A
B
C
D

Fig. 19 Paper chromatography of unsaponifiable matter of ether soluble fractions of fat samples

A = Cow ghee

B = Buffalo ghee

C = Buffalo body fat

D = Pig body fat

Fig. 20 Paper chromatography of unsaponifiable matter of ether insoluble fractions of fat samples

A = Cow ghee

B = Buffalo ghee

C = Buffalo body fat

D = Pig body fat


A
B
C
D

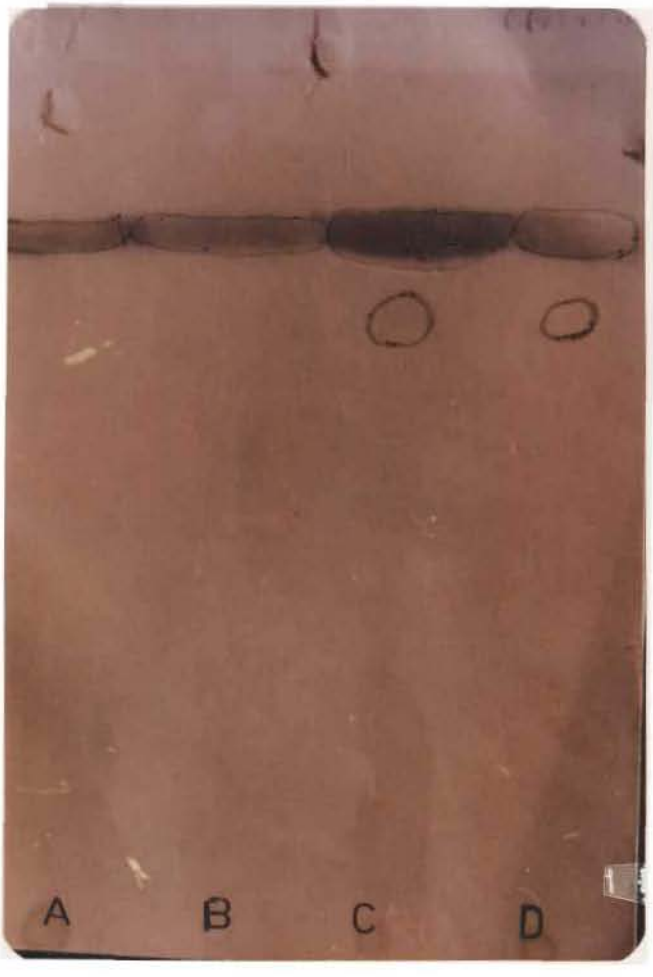

A
B
C
D

Fig. 21 TLC of fat samples

B = Groundnut oil

C = Vanaspati

D = Cow ghee

E = Buffalo ghee

F = Coconut oil

Fig. 22 TLC of coconut oil and ghee/ vanaspati adulterated with coconut oil

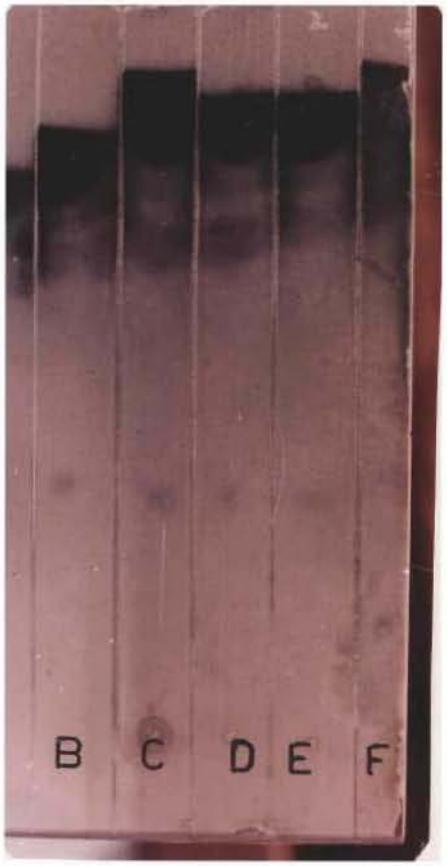
A = Cow ghee + 10% coconut oil

B = Buffalo ghee + 10% coconut oil

C = Vanaspati + 10% coconut oil

D = Coconut oil

E = Standard stigmasterol (Sigma Grade)


B C D E F

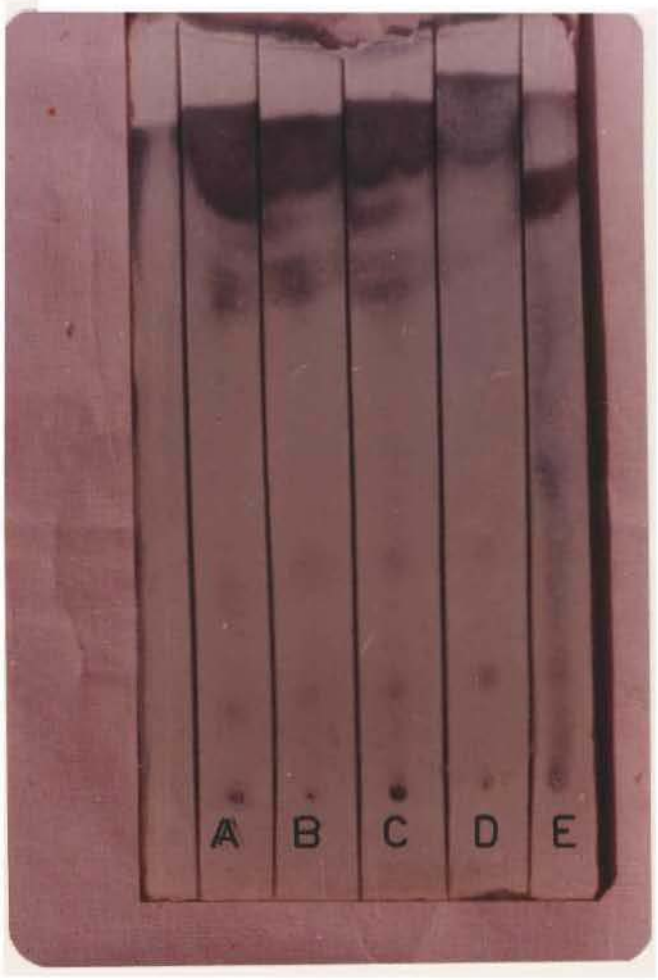

A B C D E

Fig. 23 TLC of unsaponifiable matter of fat samples

A = Cow ghee

B = Buffalo ghee

C = Buffalo body fat

D = Goat body fat

E = Sheep body fat

F = Pig body fat

Fig. 24 TLC of unsaponifiable matter of pure and adulterated ghee samples

A = Cow ghee

B = Cow ghee + 10% buffalo body fat

C = Cow ghee + 10% goat body fat

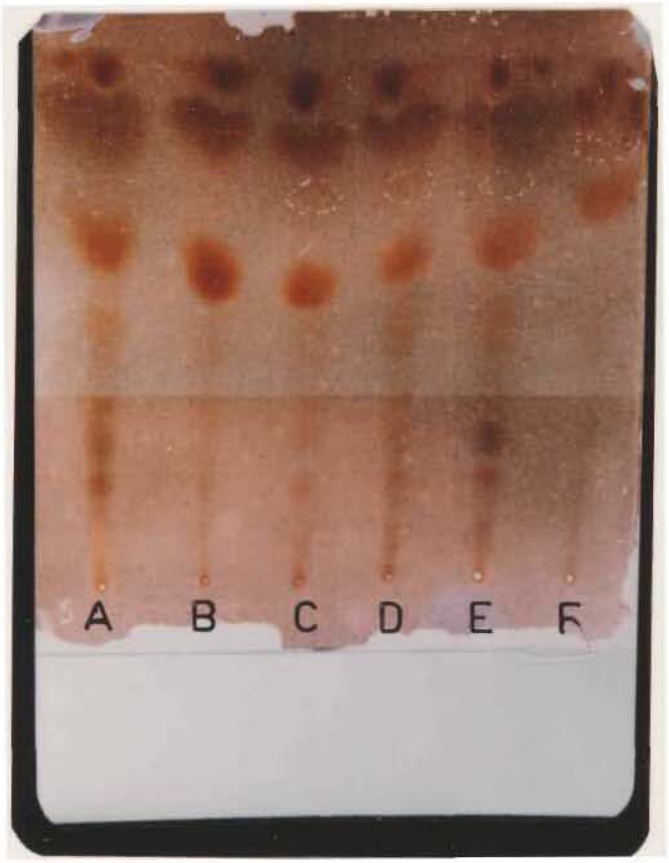
D = Cow ghee + 10% sheep body fat

E = Buffalo ghee

F = Buffalo ghee + 10% buffalo body fat

G = Buffalo ghee + 10% goat body fat

H = Buffalo ghee + 10% sheep body fat


A B C D E F

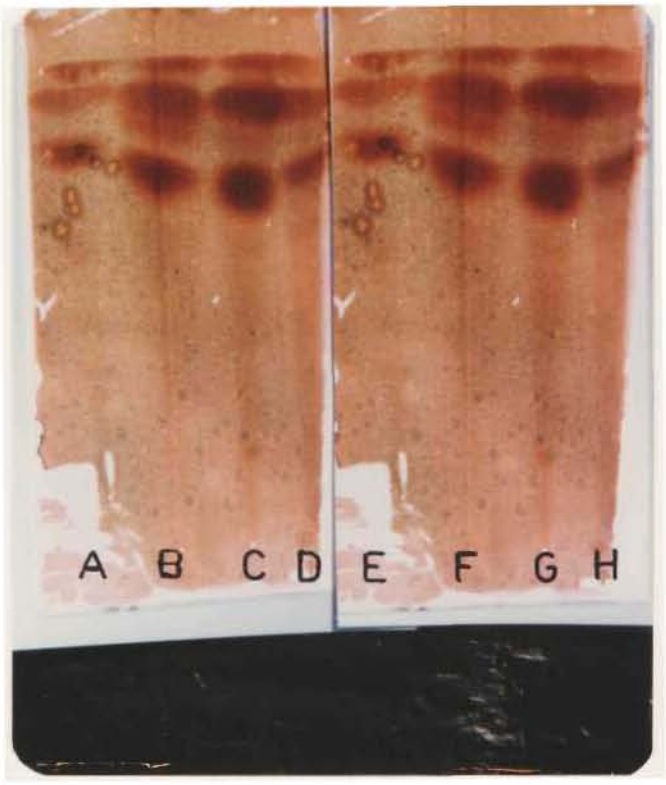

A B C D E F G H

Fig. 25 TLC of unsaponifiable matter of adulterated ghee samples

A = Cow ghee + 10% pig body fat

B = Buffalo ghee + 10% pig body fat

C = Cow ghee + 10% buffalo body fat

D = Buffalo ghee + 10% buffalo body fat

Fig. 26 TLC of standard sterol and unsapnifiable matter of fat samples

A = Cholesterol

B = Dihydrocholesterol

C = 7-dehydrocholesterol

D = Lanosterol

E = Buffalo body fat

F = Pig body fat


A
B
C
D

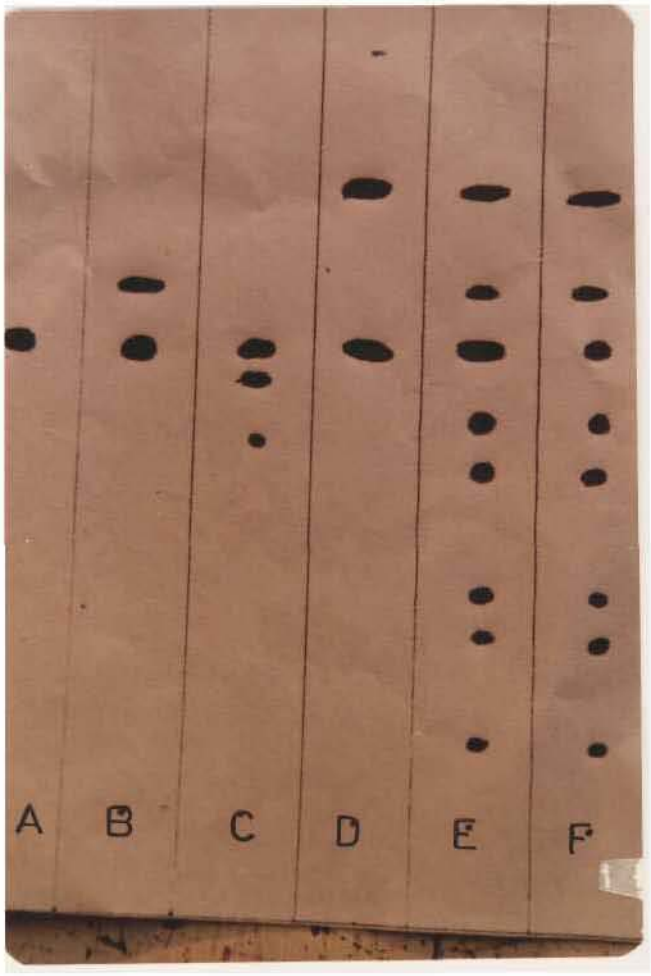

A
B
C
D
E
F

Fig. 27 TLC of sterol digitonides of fat samples

A = Cow ghee

B = Buffalo ghee

C = Pig body fat

D = Sheep body fat

E = Goat body fat

F = Buffalo body fat

Fig. 28 TLC of steryl acetate of fat samples

A = Cow ghee

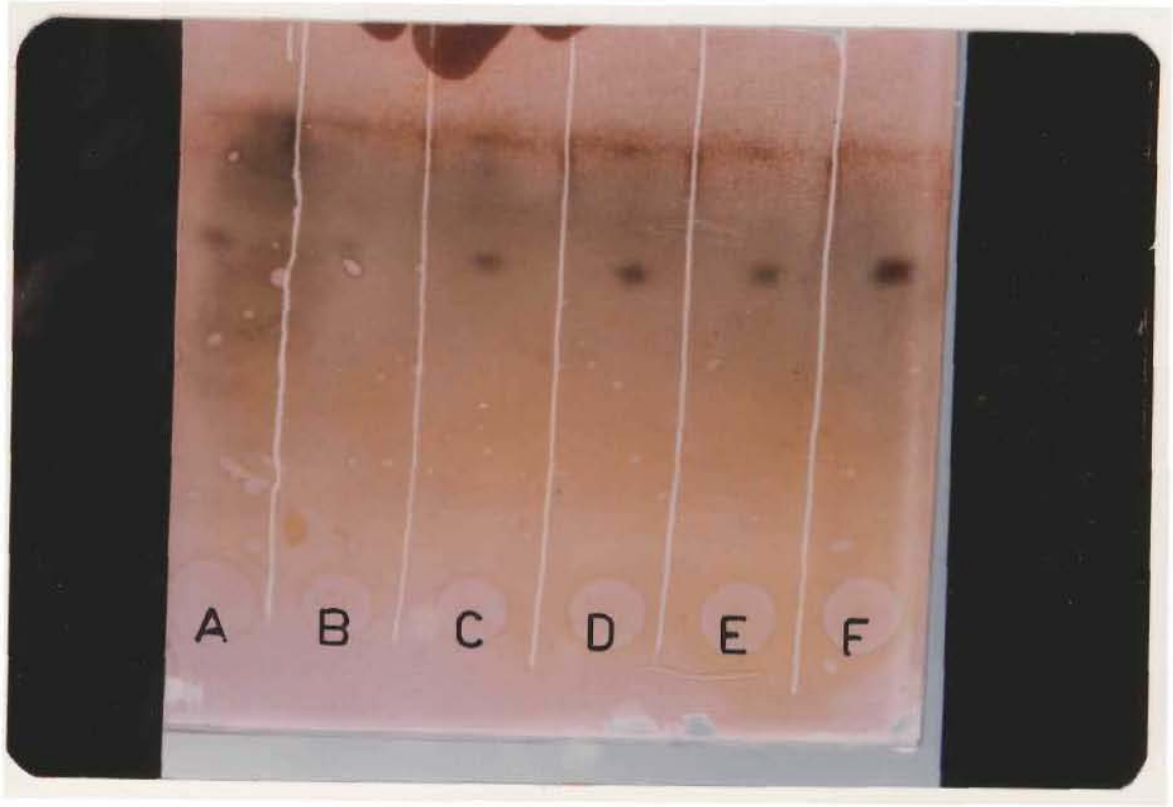
B = Buffalo ghee

C = Pig body fat

D = Sheep body fat

E = Goat body fat

F = Buffalo body fat


A
B
C
D
E
F

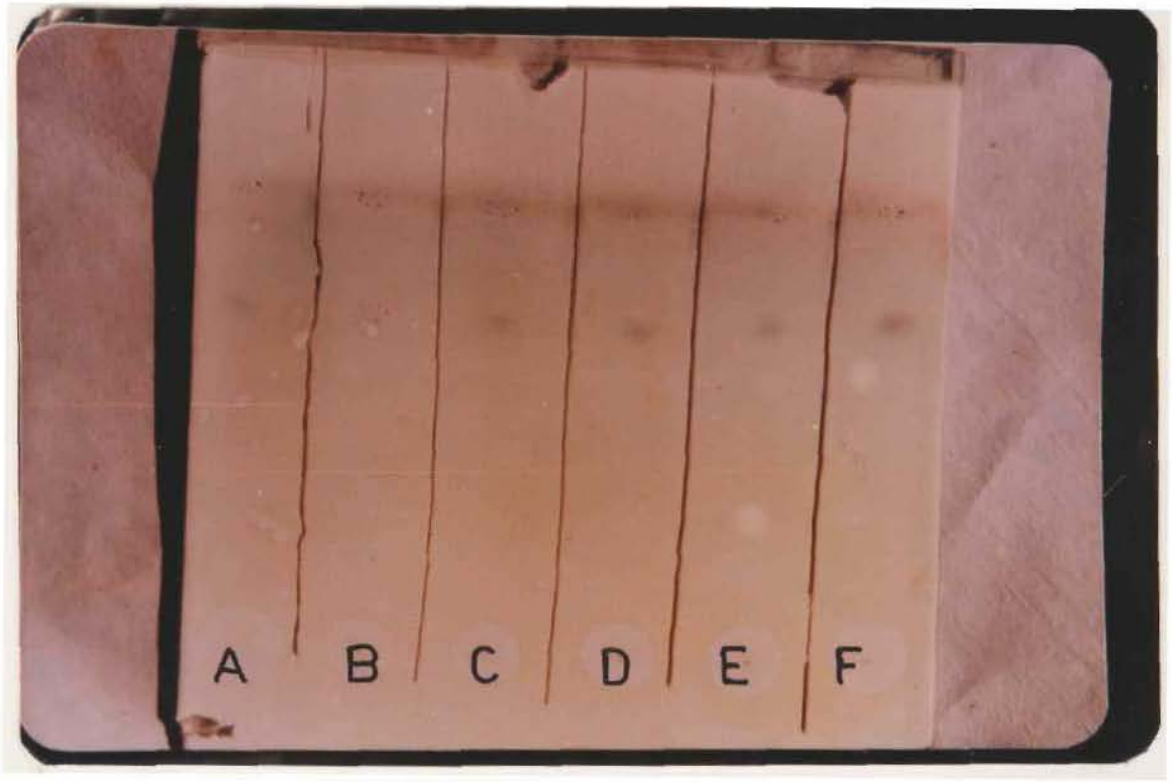

A
B
C
D
E
F

Fig. 29 TLC of free sterol digitonides of fat samples

A = Dihydrocholesterol

B = Cholesterol

C = Sheep body fat

D = Goat body fat

E = Buffalo body fat

F = Pig body fat

G = Buffalo ghee

H = Cow ghee

Fig. 30 TLC of free sterol digitonides of adulterated ghee samples

A = Cow ghee + 10% goat body fat

B = Cow ghee + 10% sheep body fat

C = Cow ghee + 10% pig body fat

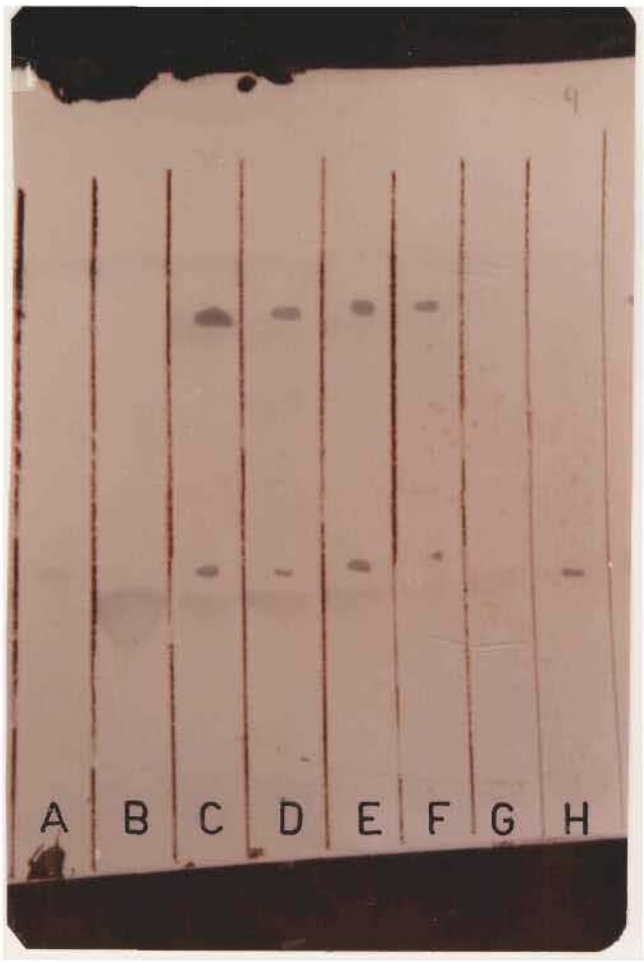
D = Cow ghee + 10% buffalo body fat

E = Buffalo ghee + 10% buff.body fat

F = Buffalo ghee + 10% goat body fat

G = Buffalo ghee + 10% sheep body fat

H = Buffalo ghee + 10% pig body fat


A B C D E F G H

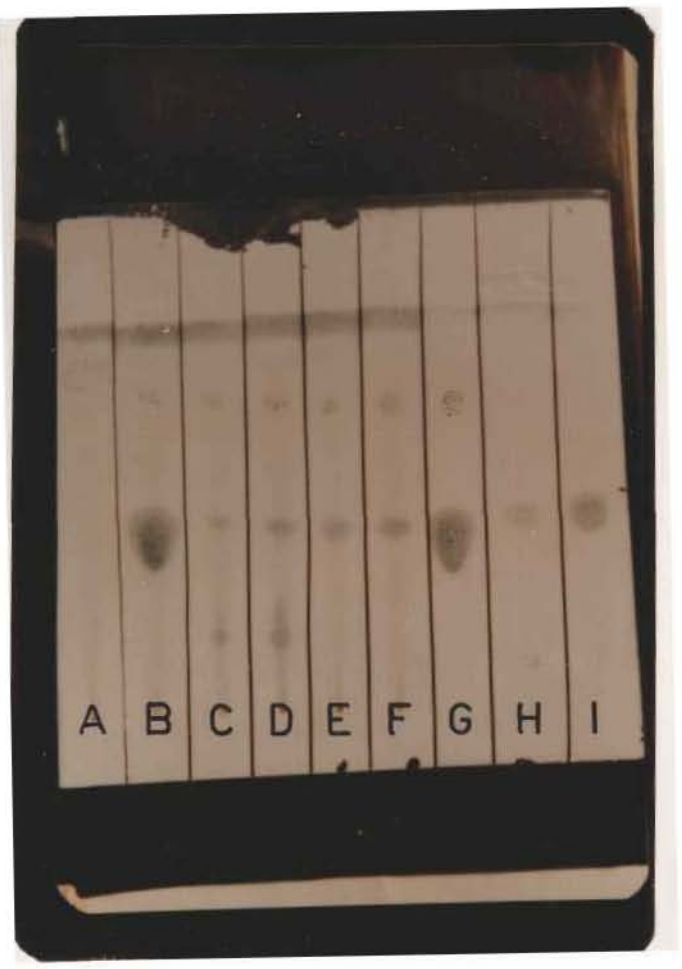

A B C D E F G

Fig. 31 TL of esterified sterol digitonides of fat samples

A = 7-dehydrocholesterol digitonides
B = Pig body fat
C = Sheep body fat
D = Goat body fat
E = Buffalo body fat
F = Buffalo body fat
G = Pig body fat
H = Buffalo ghee
I = Cow ghee

Fig. 32 TLC of esterified sterol digitonides of adulterated cow ghee samples

A = Cholesterol digitonide
B = 7-dehydrocholesterol digitonide
C = Cow ghee + 10% buffalo body fat
D = Cow ghee + 10% goat body fat
E = Cow ghee + 10% sheep body fat
F = Cow ghee + 10% pig body fat


A B C D E F G H I

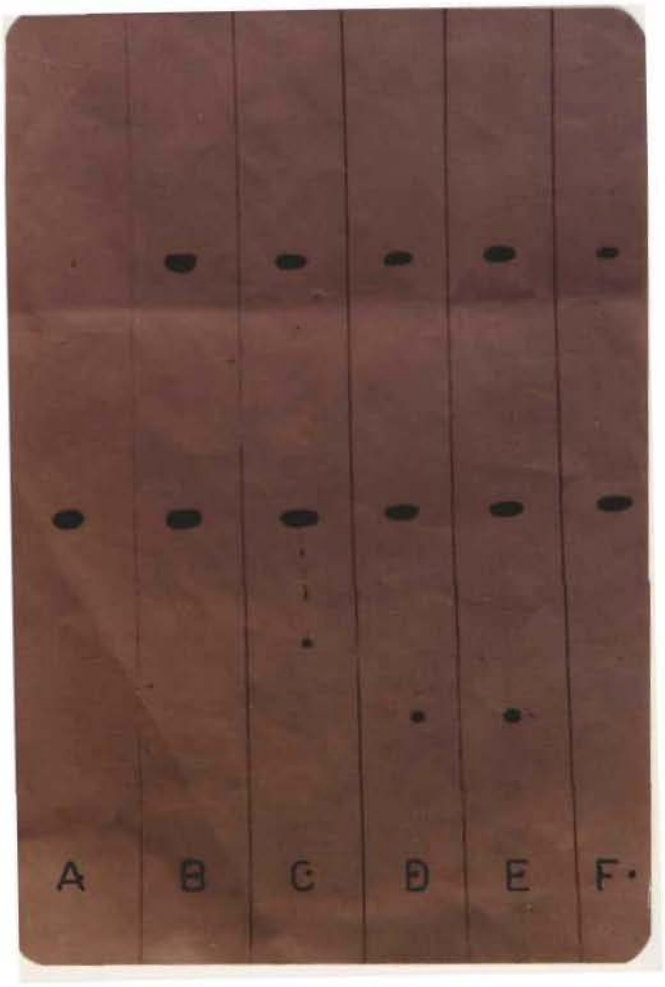

A B C D E F

Fig. 33 TLC of esterified sterol digitonide of adulterated buffalo ghee samples

A = Buffalo ghee + 10% pig body fat

B = Buffalo ghee + 10% buffalo body fat

C = Buffalo ghee + 10% goat body fat

D = Buffalo ghee + 10% sheep body fat

Fig. 34 TLC of trimethyl silyl ethers of unsaponifiable matter of fat samples

A,B = Goat body fat

C,D = Sheep body fat

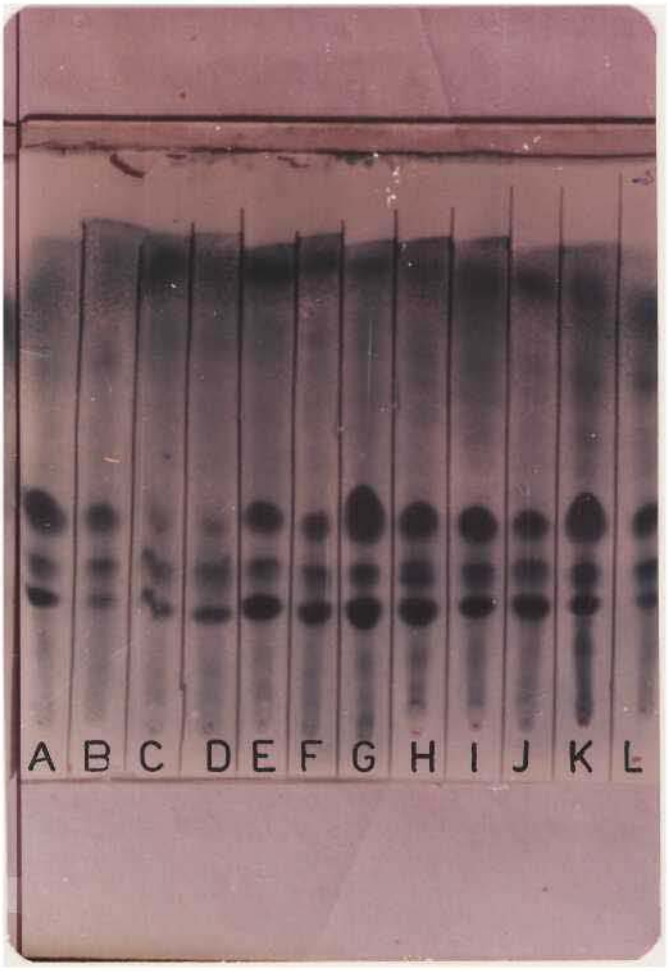
E,F = Cow ghee

G,H = Buffalo ghee

H,I = Buffalo body fat

J,K = Pig body fat


A
B
C
D


A B C D E F G H I J K L

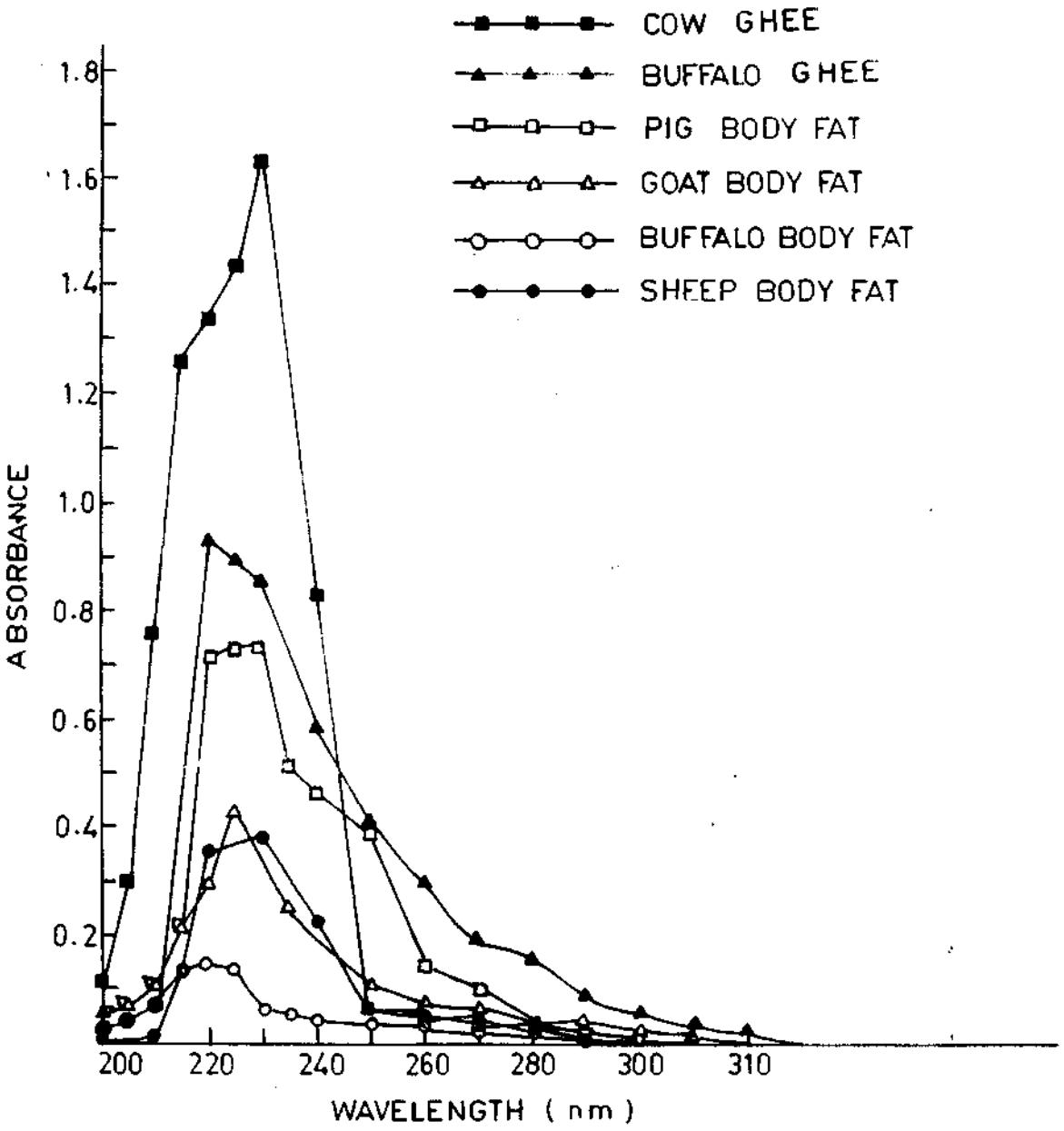


FIG. 35 UV ABSORPTION SPECTRA OF GHEE AND ANIMAL BODY FAT

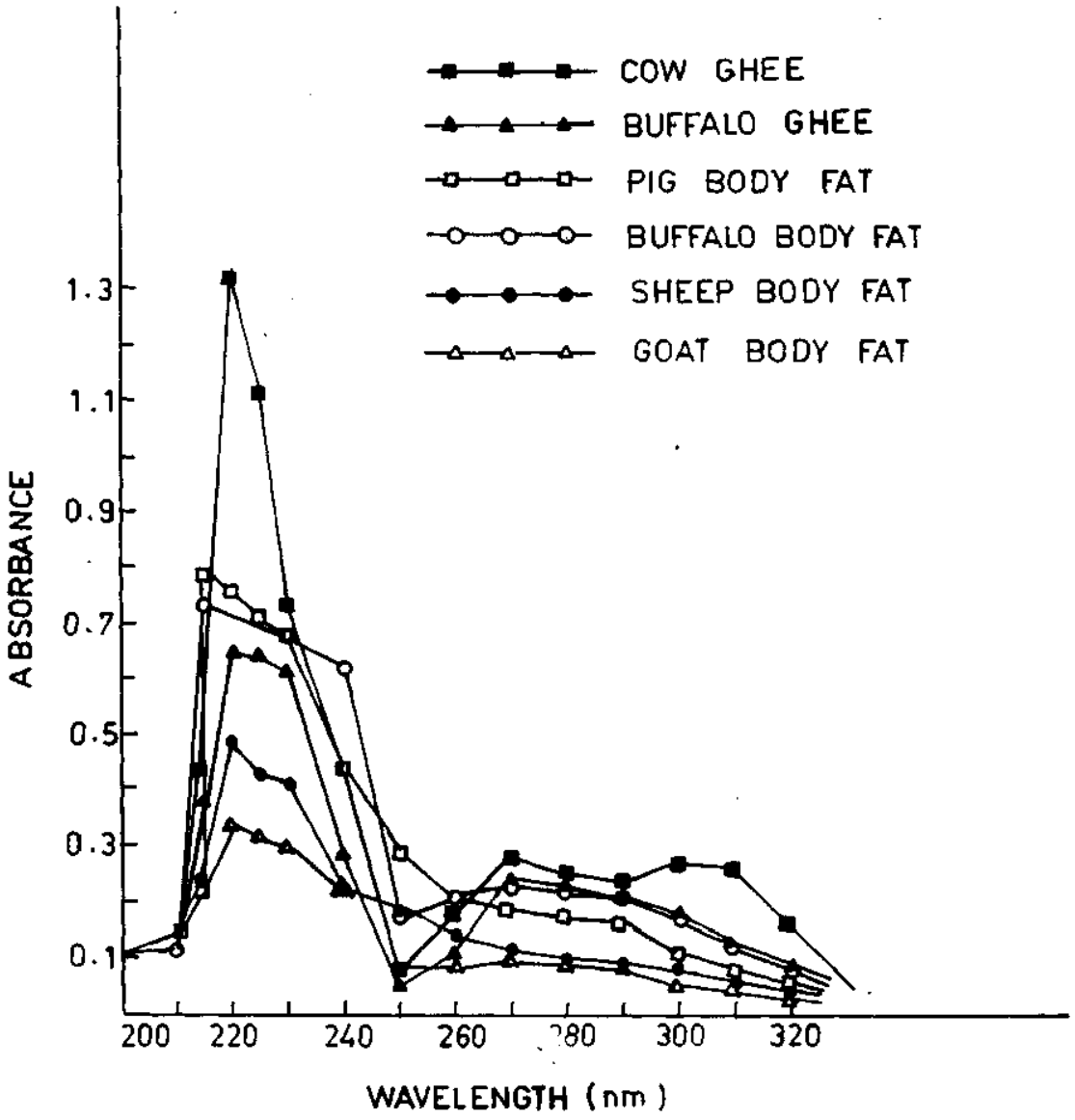


FIG.36 UV ABSORPTION SPECTRA OF UNSAPONIFIABLE MATTER OF GHEE AND ANIMAL BODY FAT

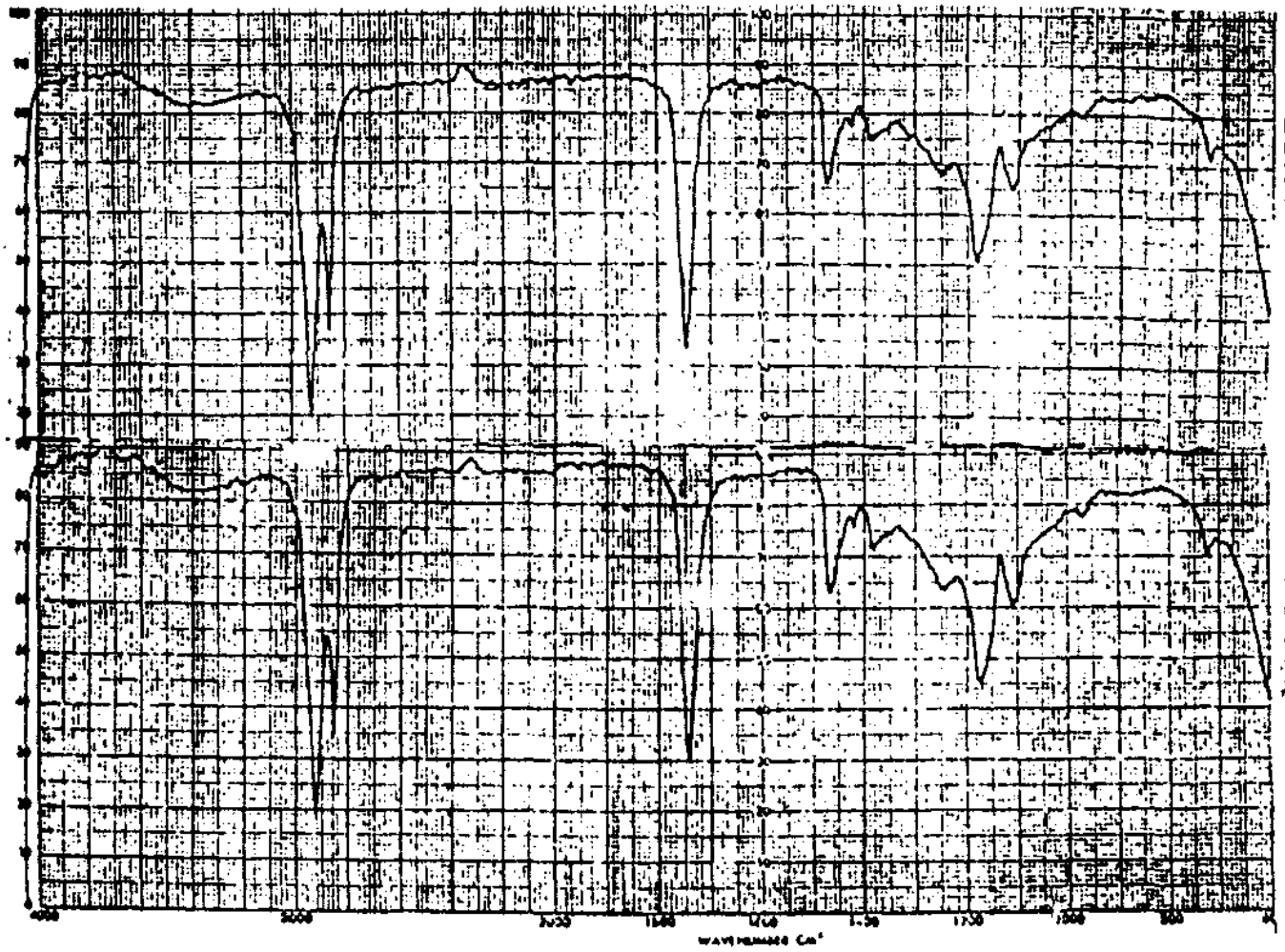


FIG.37 I R SPECTRA OF GHEE

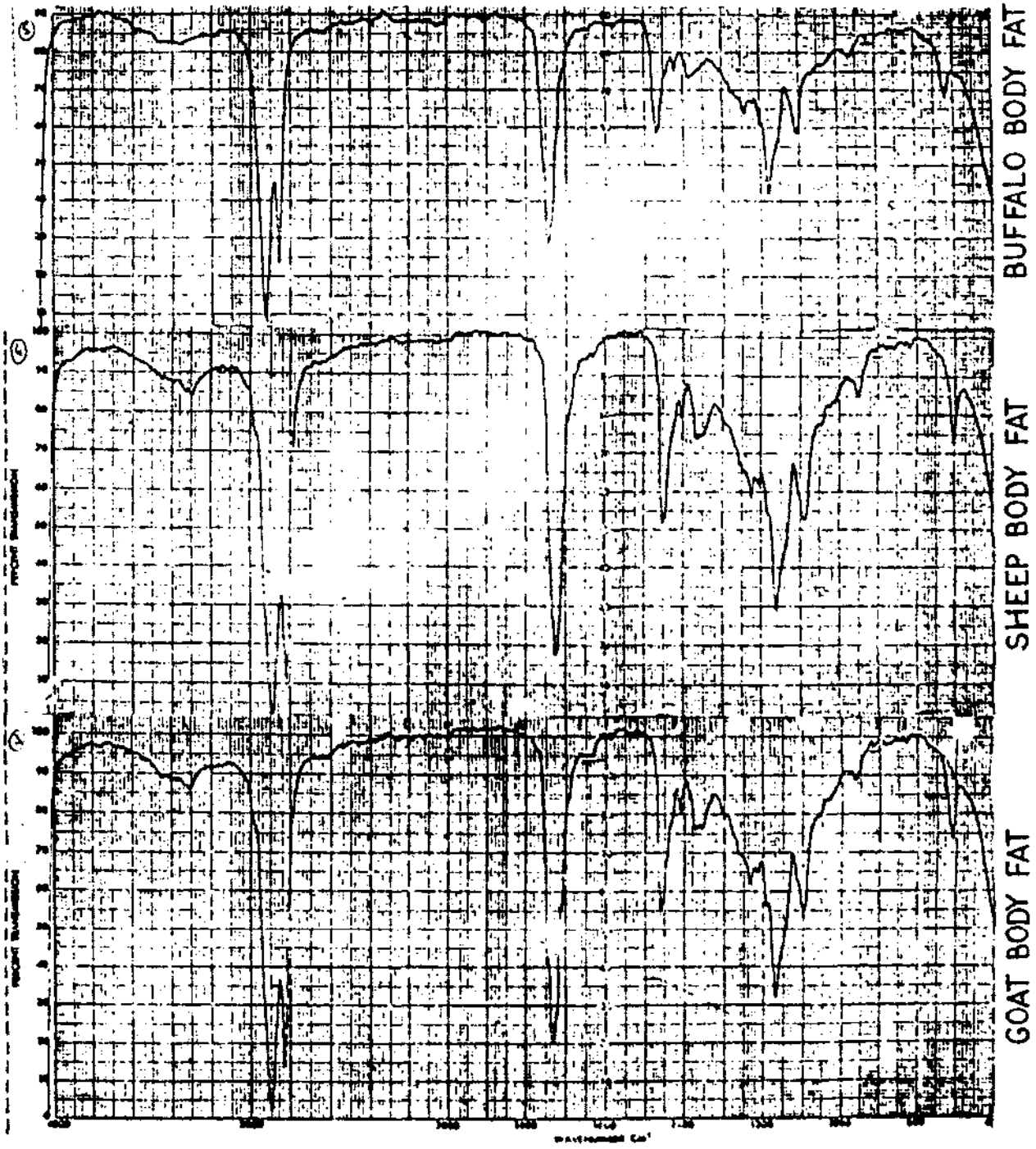


FIG.38 I R SPECTRA OF ANIMAL BODY FAT

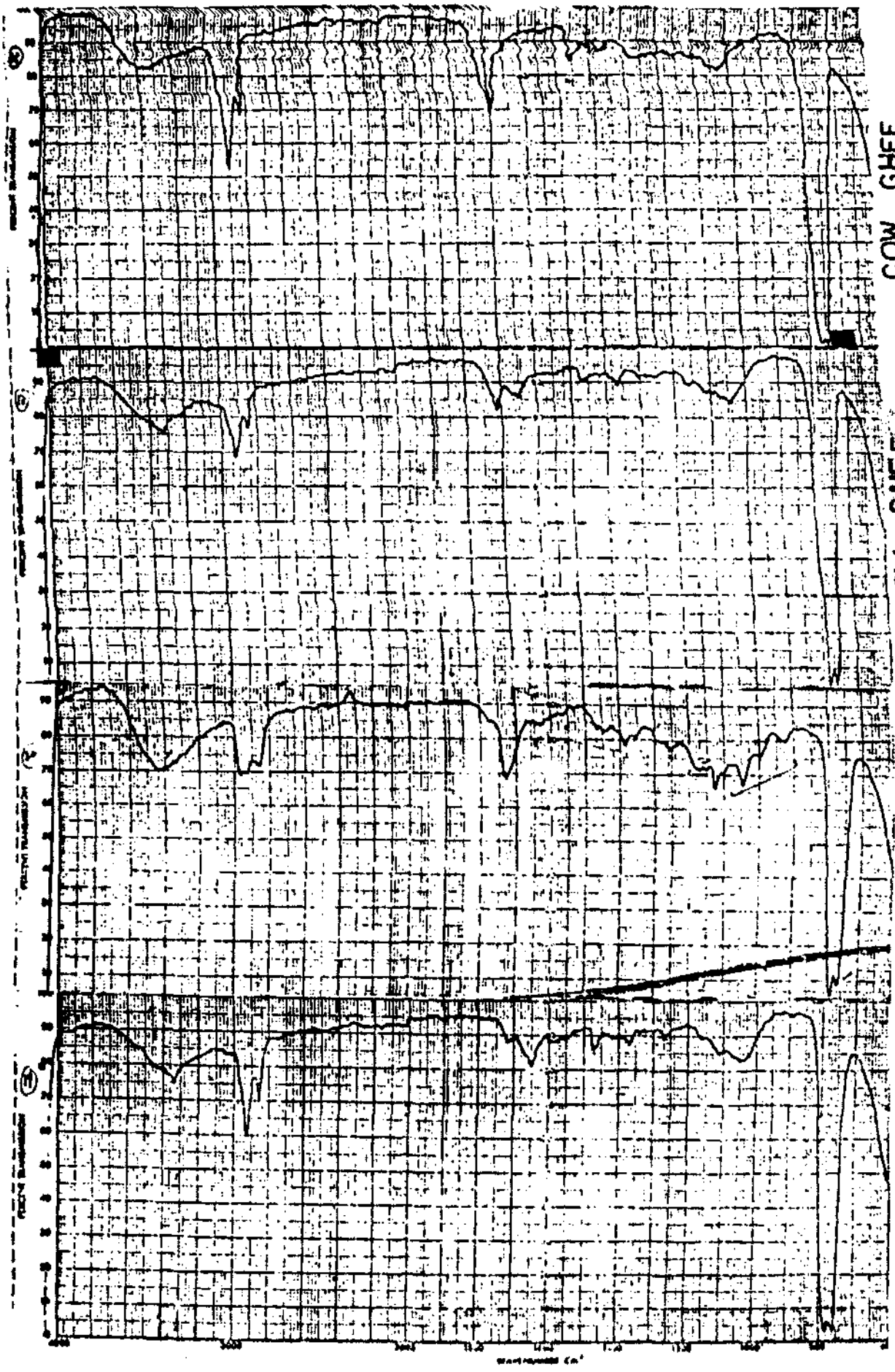


FIG.42 I R SPECTRA OF UNSAPONIFIABLE MATTER OF ADULTERATED COW AND BUFFALO GHEE

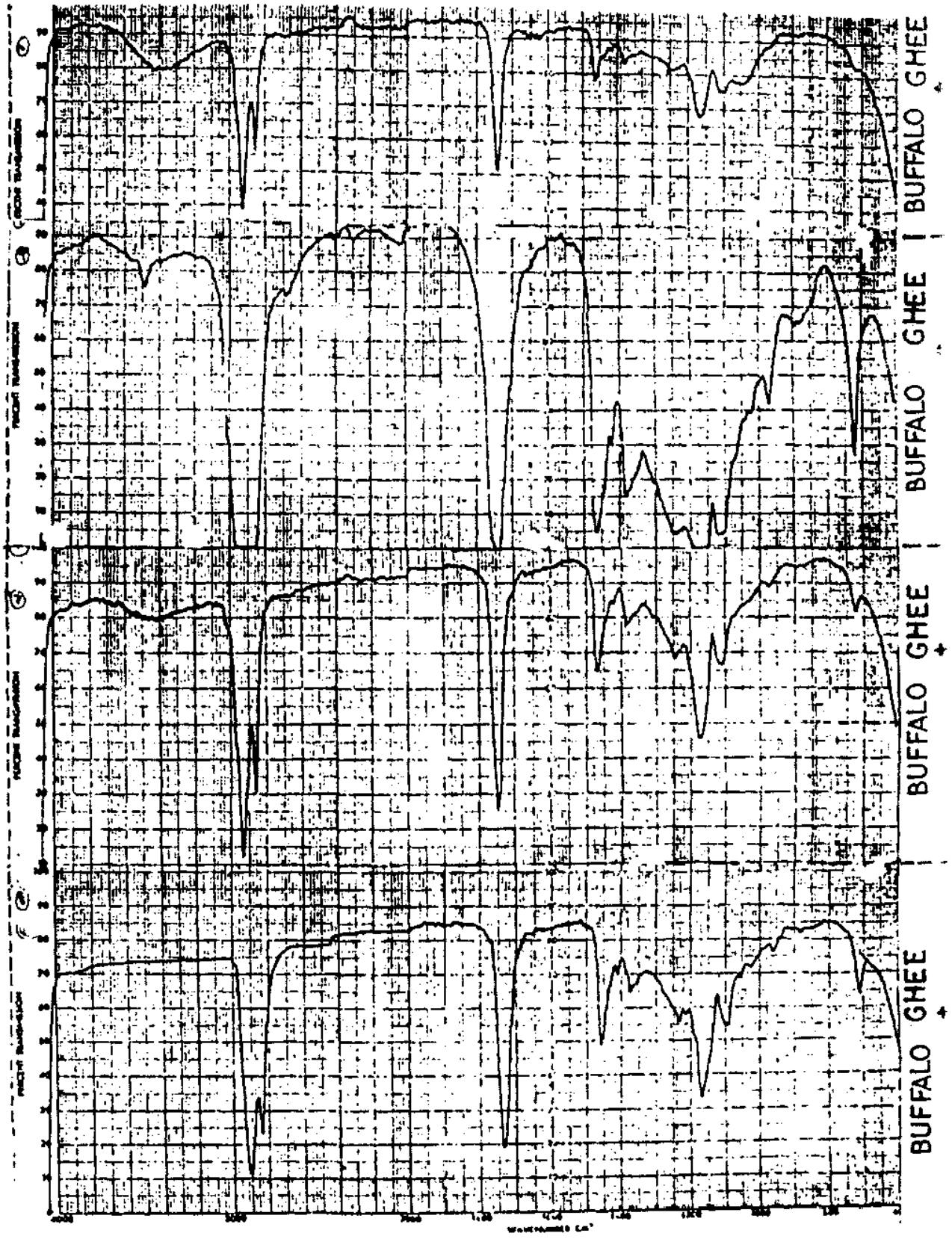


FIG.40 IR SPECTRA OF ADULTRATED BUFFALO GHEE

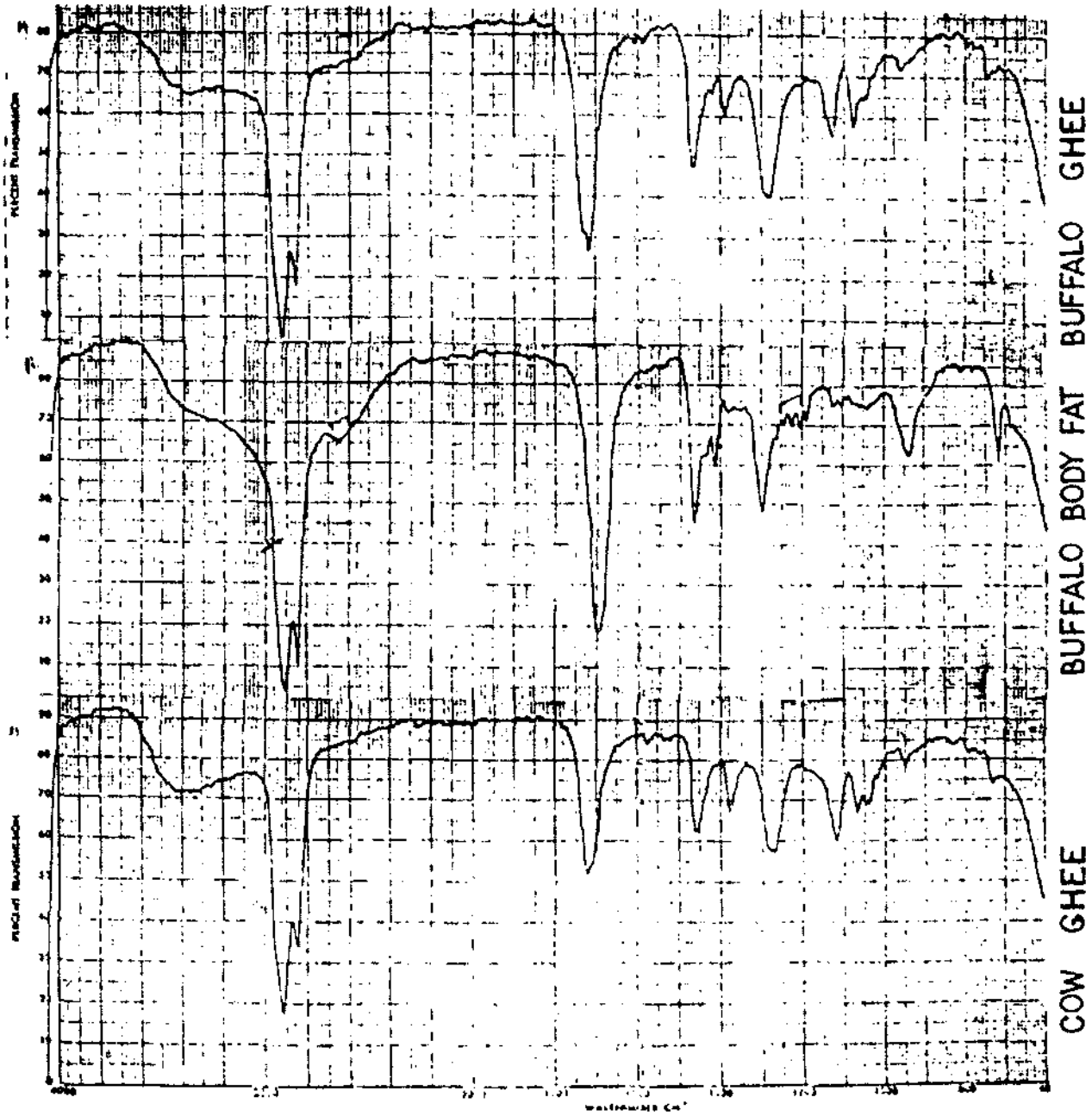


FIG.41 I R SPECTRA OF UNSAPONIFIABLE MATTER OI
FAT SAMPLE

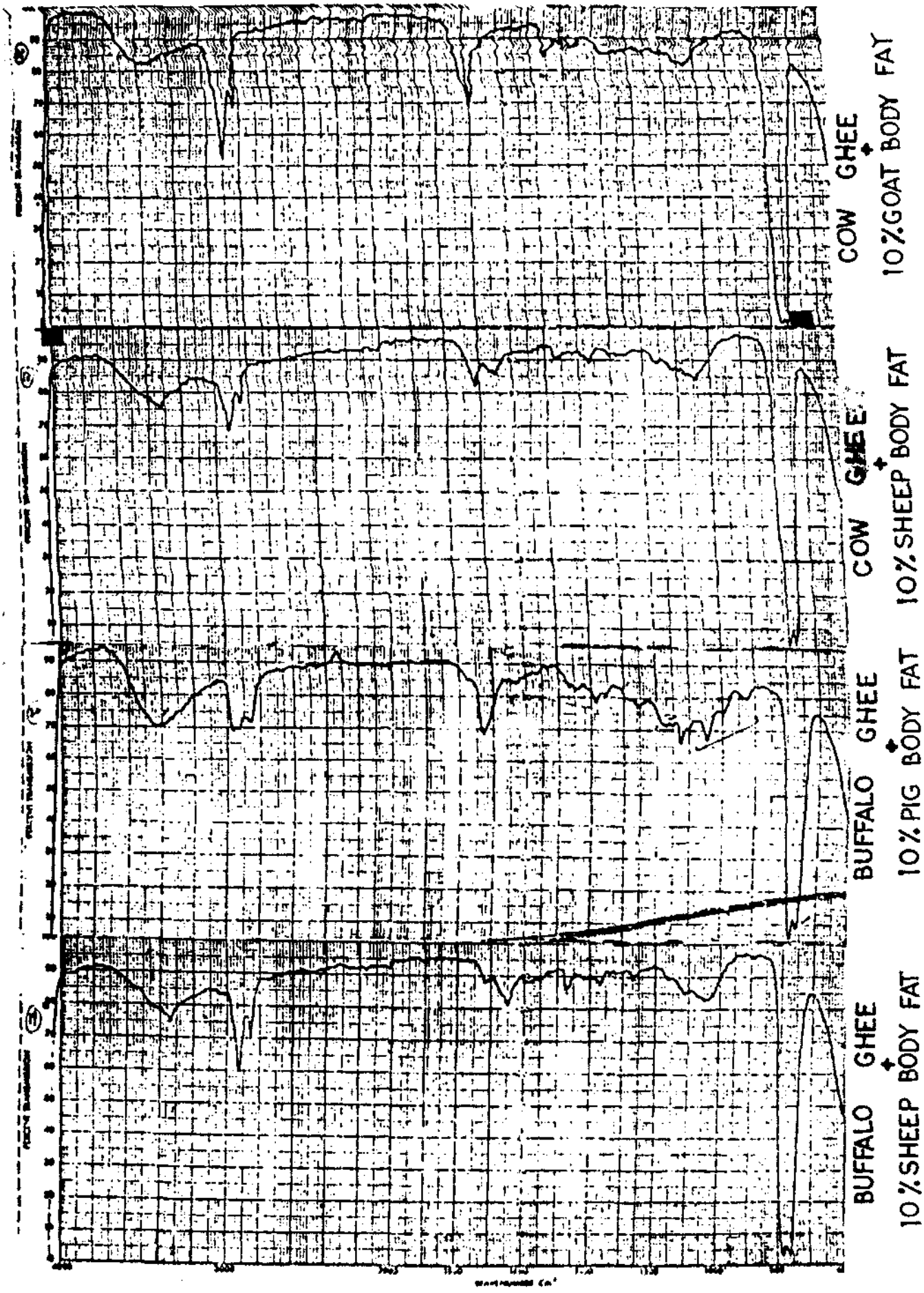


FIG.42 I R SPECTRA OF UNSAPONIFIABLE MATTER OF ADULTERATED COW AND BUFFALO GHEE

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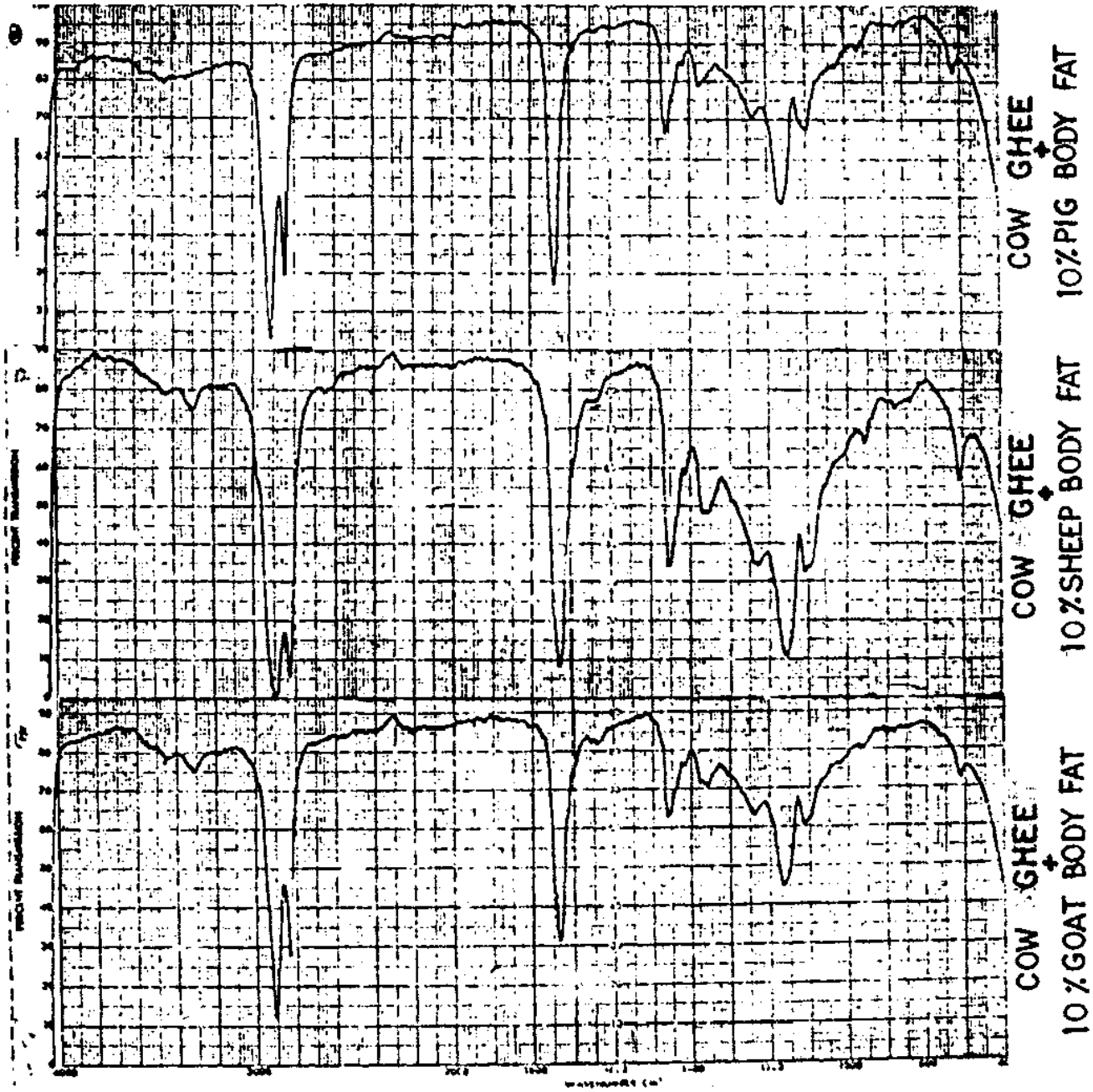

FIG.39 I R SPECTRA OF ADULTRATED COW GHEE

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