FLAVOUR POTENTIAL OF GHEE RESIDUE

BY

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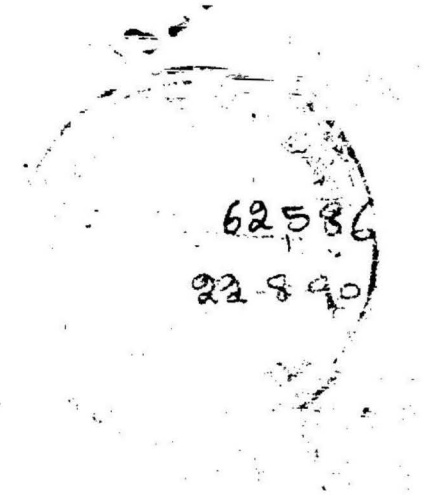
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This is to certify that the thesis entitled "FLAVOUR POTENTIAL OF GHEE-RESIDUE" submitted by Mr.Krishan Kumar Galhotra in partial fulfilment of the requirement for the award of the MASTER OF SCIENCE IN DAIRYING (DAIRY CHEMISTRY) of the National Dairy 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.

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CHAPTER-I

INTRODUCTION

1. INTRODUCTION

Ghee (heat clarified butterfat) holds an important position among edible fats due to its unique pleasing caramelized flavour and granular texture. Nearly 39% of our total milk production is utilised for making ghee due to its heavy demand and long shelf-life. Ghee-residue is the by-product of ghee manufacturing industry. During the manufacture of ghee, the solids not fat (SNF) present in cream or butter appears in the form of small particles known as 'Ghee-residue'. It is obtained as a moist brownish sediment after molten ghee has been strained out. The annual production of ghee-residue in large organised ghee making plants is 6000-8000 tonnes (Dairy India, 1985).

Ghee-residue contains large but varying amounts of entrapped fat, heat denatured milk proteins, caramelized milk sugars, minerals and moisture. The colour of ghee-residue, quality and quantity of its various constituents vary depending upon the method of manufacture and the temperature of clarification of ghee.

The approximate chemical composition (%) of ghee-residue varies as fat (32-70), protein (12-39), moisture (8-30), lactose (2-14) and ash (1-8) (Santha and Narayanan, 1978a; Relwani, 1978; Grewal, 1979). Thus, ghee-residue is a rich source of lipids and proteins.

Further, ghee-residue is a rich source of polar lipids like phospholipids (1-9%) and polar non-lipid constituents

like amino acids, sugars, free sulphydryl compounds etc. which contribute towards its antoxidant property (Santha and Narayanan, 1978a,1979b). During heating of cream/butter, proteins of phospholipid-protein complexes get denatured to give amino acids and sulphydryl compounds. With the removal of moisture, a small portion of phospholipids move into the fat phase whereas a major portion of phospholipids because of their polar nature are retained in the ghee-residue. Also lactose and protein present may undergo browning (Maillard) and caramelization reactions giving rise to browning products. All these constituent together account for the antioxidant property of ghee-residue.

It is reasonable to believe that major portion of the flavour compounds produced from various milk constituents (viz., lipid, protein, carbohydrate, citrate or their interaction products) by the action of heat and/or starter culture during ghee preparation get concentrated in the ghee-residue because of their polar nature. It was, therefore, of interest to determine the flavour potential of ghee-residue with special reference to free fatty acids FFA, carbonyls and lactones level. This basic information would be of applied interest in utilizing ghee-residue (a dairy waste) as flavour concentrate of ghee flavour for flavouring bland products viz. vegetable fats/oils, butter oil etc.

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CHAPTER - II

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

It is proposed to review here the literature of studies to-date under the following heads:

2.1 CHEMISTRY OF GHEE-RESIDUE AND ITS SIGNIFICANCE

The literature on ghee-residue is reviewed up-to-date under the following sub-heads:

2.1.1 Yield

The amount of ghee-residue was found to depend upon the method of preparation of ghee. This was due to the variation of the non-fatty serum constituents of the different raw materials used for the preparation of ghee. The amount of residues varied from 1.5 to 1.7% of the butter handled (Rangappa and Achaya, 1974). The average yield of ghee-residue was maximum (12%) in direct-creamery (DC) method followed by almost the same yield (3.7%) in creamery-butter (CB) and desi-butter (DB) methods. Ripening of cream prior to clarification of cream reduced the yield of ghee-residue (Santha and Narayanan, 1978a).

2.1.2 Particle size and density

Ghee-residue is generally removed by bag filters or muslin cloth, but for the continuous ghee clarification process, centrifugal clarifiers have been designed. It is important to know the particle size distribution and the density of ghee-residue particles for designing such clarifiers. Zaidi et al.

(1980) determined the average particle diameter as 104.79 microns and average density of residue particles as 1.14 g/cm^3.

2.1.3 Composition

There were considerable variations in the chemical composition of ghee-residue depending upon the method of preparation of ghee. The approximate composition (%) of ghee-residue varies as fat (32-70), protein (12-39), moisture (8-30), lactose (2-14) and ash (1-8) (Santha and Narayanan, 1978a; Relwani, 1978; Grewal 1979). Moisture, protein and ash contents were more in CB and DB ghee-residues than in DC ghee-residues. Fat content was higher in DC than in butter ghee-residues. Lactose content was the highest in DB followed by DC and CB ghee-residues (Santha and Narayanan, 1978a). Similar observations were made by Mani (1952) and Prahlad (1954). These studies reveal that ghee-residue is a rich source of lipids and proteins.

2.1.3.1 Lipid fraction of ghee-residue

Lipid fraction of ghee-residue was characterised for the following parameters.

2.1.3.1.1 Physico-chemical constants

Santha and Narayanan (1978b) compared the physico-chemical constants of ghee-residue lipids with those of ghee.Lipids of ghee-residue had low Reichert and Polenske values (24.4, 1.3) and high Iodine value (43.4) in comparison to those of corresponding ghee(30.1, 1.6, 33.9 respectively).

2.1.3.1.2 Fatty acid composition

Santha and Narayanan (1978b) observed slight differences in the fatty acid composition of ghee-residue lipids and ghee. The lipids of ghee-residue contained less of lower chain fatty acids 4:0 to 12:0 (5.3%) especially butyric acid, and total saturated fatty acids (58.7%) and more of unsaturated fatty acids (41.3%) in comparison to those of corresponding ghee (10.1, 66.8 and 33.2% respectively). The neutral lipids of ghee-residue had a similar fatty acid composition to that of ghee. The fatty acid composition of phospholipids showed that it had no fatty acids lower than 12 carbon atoms.

2.1.3.1.3 Polyunsaturated fatty acids (PUFA)

Santha and Narayanan (1978b) reported that irrespective of the method of preparation, PUFA contents of ghee-residue lipids were more than those of corresponding ghee. The average total PUFA contents of lipids of DC ghee-residue, CB ghee-residue and CB ghee were 3.3, 4.4 and 2.8% respectively. Total PUFA contents of ghee-residue phospholipids (11.5%) were more than those of ghee-residue neutral lipids (2.8%).

2.1.3.1.4 Phospholipids

Ghee-residue was observed to be rich in phospholipids (1-9%). Further, the phospholipid content of ghee-residue was dependent upon the method of preparation. In unripened and ripened DC ghee-residues, the phospholipids were about 1.57 and 1.10% respectively of their total fat contents. In CB and DB ghee-residue, the phospholipids were about 17.39 and 4.95%

respectively of their total fat. Thus, phospholipid content was the highest in CB ghee-residue lipids and the least in ripened cream ghee-residue lipids (Rama Murty et al., 1969; Santha and Narayanan, 1978a). These levels are much higher than those in ghee (0.004-0.08) (Rama Murthy and Narayanan, 1966; Pruthi, 1980; Lal et al., 1984). The phospholipid content of ghee-residue decreased as the period of heating increased due to the transfer of phospholipids from ghee-residue to ghee.

While heating cream/butter, only a small fraction of the phospholipids gets transferred to ghee. Most of the phospholipids remain with the residue because of their polar character. The differences observed in the physico-chemical constants, fatty acids and PUFA contents between lipids of ghee-residue and ghee were due to the high phospholipids contents of ghee-residue.

2.1.3.2 Proteins in ghee-residue

2.1.3.2.1 Soluble and non-soluble nitrogen content of ghee-residue

Santha and Narayanan (1979b) reported that soluble nitrogen content of ghee-residue prepared from cream or creamery butter decreased with heating time. It decreased from 0.82 and 0.89% to 0.42 and 0.38% upon heating at 120°C momentarily to 120°C for 30 minutes respectively. The decrease in soluble nitrogen content might be due to the denaturation of the proteins. The average non-protein nitrogen content of CB ghee-residue was 0.81%. By paper chromatographic technique 11 free amino acids, 2 amines and 8 unidentified spots were detected in ghee-residue. These amino acids might have liberated from proteins due to hydrolysis on heat treatment.

2.1.3.2.2 Sulphydryl compounds

The total reducing capacity (expressed as mg of cysteine hydrochloride/g) of CB ghee-residue (26.0) and free sulphydryl content (uM/g) of ghee-residue (2.90) were much higher than those in ghee (0.075 and 0.02 respectively)(Santha and Narayanan, 1979b). These reducing substances or free sulphydryl groups might have liberated from the protein during heat treatment and because of their polar nature are mostly retained in the ghee-residue. Whey proteins, especially β-lactoglobulin is the main source for these sulphydryl compounds.

2.1.3.3 Milk sugars in ghee-residue

Santha and Narayanan (1979c) reported various sugars (weight per cent) in ghee-residue prepared at 120°C/0 min as lactose (76.6), galactose (14.1), glucose (5.3) and two unidentified spots one at the origin (4.0) and second of faster mobility. Further, as the period of heating was increased, the lactose content of ghee-residue decreased with a corresponding increase in galactose and glucose content. The unidentified spots were also found to increase as the period of heating increased. The lower content of glucose in comparison to galactose might be due to the fact that glucose moiety of lactose contains the hemiacetal structure and is involved in sugar-amine reactions. The unidentified spots might be due to some of the breakdown products or sugar fragments formed during browning (Maillard) or caramelization reactions.

2.1.4 Antioxidant properties of ghee-residue

Pagoté and Bhandari (1988) have highlighted the antioxidant properties of ghee-residue. The development of per-

oxides in ghee samples containing 2% ghee-residue at 80°C were slower than in the control showing thereby that ghee-residue contained antioxygenic substances (Santha and Narayanan, 1978c). The overall antioxygenic properties were due to both lipid and non-lipid constituents (Santha and Narayanan, 1979a).

2.1.4.1 Contribution of lipid constituents

In lipid constituents of ghee-residue, phospholipids showed the maximum antioxidant property followed by α-tocopherol and vitamin A. Among the various phospholipid fractions, cephalin showed the greatest antioxidant activity (Santha and Narayanan, 1979a).

Pruthi et al. (1973) described a heat-processing method for the extraction and fortification of ghee with ghee-residue phospholipids. Ghee-residues mixed with equal amounts of fat and heated to 120°C for 5 min gave almost complete recoveries. Santha and Narayanan (1979a) reported that the oxidative stability of ghee was increased by increasing its phospholipid content (0.1%) either through heat-treatment process or through solvent extraction process. Lal et al. (1984) standardised a heat-processing method for the transfer of phospholipids from ghee-residue to ghee. It was observed that heating ghee-residue with ghee in the ratio of 1:4 at 130°C gave maximum transfer of phospholipids from ghee-residue to ghee. These antioxidant concentrates can be added to ghee to give about 0.1% phospholipids so as to increase the keeping quality of ghee.

2.1.4.2 Contribution of non-lipid constituents

Santha and Narayanan (1979a) reported that among the non-lipid constituents, the amino acids proline, lysine, cysteine hydrochloride and tryptophan showed antioxidant property. The contribution of proline as antioxidant was maximum though less than BHA at 0.02% level. Further, the addition of lactose, glucose, galactose and their interaction products with protein and phospholipid to ghee increased the oxidative stability of ghee. Further, Santha and Narayanan (1979b) reported that as ghee-residue contains large amounts of reducing substances including free sulphydryls, such compounds may also contribute to the antioxidant properties of ghee-residues.

2.1.4.3 Antioxidant properties as affected by the temperature of clarification

Santha and Narayanan (1978c) showed that the addition of ghee-residue obtained from ghee manufactured at lower temperature 110°C resulted in lesser development of peroxides than the addition of ghee-residue obtained from ghee manufactured at higher temperature of 150°C. Similar observations were made by El-Sokkary and Ghoneim (1951), El-Sokkary and Zaki (1953) and Rama Murthy et al. (1969). Thus, the antioxidant efficiency of ghee-residue decreases with increase in the temperature of clarification of ghee.

2.1.4.4 Antioxidant properties as affected by the method of preparation

Santha and Narayanan (1978c) observed that CB ghee-residue had the maximum antioxidant property followed by DB and DC ghee-residues.

2.1.4.5 Antioxidant properties as affected by the nature of antioxygenic components

Santha and Narayanan (1978c) observed the antioxidant efficiency of ghee-residues extracted with various solvents as: acetone-extracted ghee-residue > petroleum ether-extracted ghee-residue > solvent ether extracted ghee-residue > lipid free (alcohol-solvent ether-petroleum ether extracted) ghee-residue. Thus the lipid fractions, especially the acetone-insoluble fractions of ghee-residue had the greatest antioxidant effect, although the lipid-free ghee-residue also showed some antioxidant property. Also, the addition of ghee-residue lipids (extracted with chloroform-methanol 2:1) to ghee gave maximum protection to oxidation in ghee.

Non-enzymatic browning occurs in most of the heat processed foods. Ghee prepared at higher temperatures 120°C/10 min developed brown colour and also showed greater oxidative stability than ghee prepared at lower temperatures 110°C/10 min. During the manufacture of ghee dicarbonyls and amino acids might interact and produce browning compounds (Rama Murthy, 1968). Nath and Rama Murthy (1988) observed that browning compounds produced due to interaction of dicarbonyls and amino acids have a considerable antioxidant activity in ghee. It is quite reasonable to believe that browning compounds in ghee-residue formed during browning and caramelization reactions may also contribute towards the antioxidant properties of ghee-residue.

Hence, ghee-residue is a rich source of natural antioxidants and its antioxidant properties are due to the mixture of its constituents affected by various technological parameters.

2.1.5 Nutritive value of ghee-residue

The by-product ghee-residue thrown as a waste matter is nutritionally a rich source of fat, proteins and minerals. As ghee-residue is rich in milk proteins and nitrogenous compounds, the product has been assessed for its nutritive value as human dietary supplement.

Relwani (1979) showed by depletion technique studies that skim milk power (SMP), SMP:ghee-residue (2:1), SMP:Ghee-residue (1:2) and ghee-residue diets had protein efficiency ratio (PER) of 3.44, 3.07, 2.46 and 0.66 respectively. Further supplementation of ghee-residue with 0.5% lysine-HCl resulted in little improvement in the nutritional response of the animals.

Grewal (1979) observed that lack of lysine in ghee-residue was the most dominant factor in depressing the PER of ghee-residue and even 4% lysine supplement was effective in improving its nutritional quality.

Malhotra (1980) observed that the nutritive value of ghee-residue was definitely low as compared to SMP, but supplementing ghee-residue with a combination of lysine (8%), methionine (2.5%) and tryptophane (1.4%) increased its nutritional value even slightly higher than that of SMP.

Kapoor and Pal (1979) determined the PER of ghee-residue and casein as 2.55 and 2.62 respectively and found that the quality of protein in ghee-residue was good and that it can serve as a good source of animal proteins to the vegetarian population of our country. Singhal and Mudgal (1983) studied detailed amino acid composition of ghee-residue and reported 17 amino acids. Though essential amino acids contents

of ghee-residue were low, yet it can be served as a feed to the animals in combination with other feeds. Deodhar (1986) fed the albino rats with 1/3 proteins from ghee-residue and 2/3 from skim milk powder and got PER as 3.07 and 2.46 respectively. The low protein nutritional value of ghee-residue might be due to the damage of some essential amino acids. This shows that nutritive value of ghee-residue can be made parallel to SMP by supplementing ghee-residue with SMP or lysine or a combination of various amino acids.

2.1.6 Utilization of ghee-residue

2.1.6.1 Recovery of ghee from ghee-residue

Viswanathan et al. (1973) suggested two methods for the recovery of ghee from ghee-residue. The centrifugal method gave yield of ghee as 25% (efficiency 46%) whereas pressure methods (hand screw press and hydraulic press) gave yield of ghee 44.2 and 46.7% (efficiency 61 and 74%) respectively. Hand screw-press method was recommended as it was simple, efficient, practical and economical.

2.1.6.2 Preparation of sweets, etc.

Ghee-residue was utilized for preparing chosidu-burfi by mixing it with skim milk powder (SMP), khoa, chocolate and sugar (Verma and De, 1978). It can also be utilized for preparing coconut-burfi,candies, toffees, pinni, etc. after mixing with other ingredients. The nutritious by-product should be utilised as a food supplement in variety of foods, soups, etc.

2.1.6.3 Source of natural antioxidants

Wadhwa et al. (1990b-communicated) reported that shelf-life of flavoured butter oil was enhanced by incorporating ghee-residue (15-20% level) at 120°C/3 min and was comparable to that of ghee. Further, it was observed that oxidative stability imparted by ghee-residue was parallel to that imparted by BHA and BHT (Wadhwa et al., 1990a-communicated). Thus, ghee-residue can be used as a source of natural antioxidants for improving the shelf-life of food products including dairy products where use of synthetic antioxidants is generally not preferred because of their toxic effects. Apart from this, ghee-residue treatment further enhanced the flavour of flavoured butter oil probably due to the transfer of some flavour compounds, concentrated in the ghee-residue. Hence, the present study on the flavour potential of ghee-residue was undertaken.

2.2 FLAVOUR POTENTIAL OF GHEE

The unique feature of ghee (heat clarified butterfat) is its pleasant caramelized flavour and granular texture. It is proposed to review here briefly the flavour potential of ghee with special reference to fatty acids, carbonyls and lactones levels. The levels reported in the present review generally cover the overall range as reported for different types of ghee samples from the recent review on the 'Chemistry of ghee flavour' by Wadhwa and Jain (1990c-communicated).

2.2.1 Fatty acids

Ghee owes its pleasing flavour to several free fatty acids (FFA). These compounds are produced from fatty acid

glycerides by the mechanism involving hydrolysis/lipolysis through Lactic streptococci (Kinsella, 1969a; Wadhwa and Jain, 1989a and Law, 1981) during fermentation of milk or cream and/or processing treatments while preparing ghee.

Several workers (Gaba and Jain, 1973,1975a; Bector and Narayanan, 1977; Singh et al., 1979; Wadhwa et al., 1979a; Yadav and Srinivasan, 1984; Chauhan and Wadhwa, 1987; Arora and Singh, 1987) used titrimetric method as described in IS:3508 (ISI 1966) for the estimation of FFA level in ghee and reported the levels as 0.14-0.60% Oleic acid. Chander and Bindal (1987) modified the titrimetric method which involved alkali titration of ghee (less than 0.2 gm) taken in 12 ml solvent mixture of benzene and neutral alcohol (5:7) to phenolphthalein end point and reported FFA level in ghee as 31-166 uM Oleic acid/g fat. Also, Chander (1982) estimated FFA level in ghee by colorimetric method of Lowry and Tinsley (1976) as 21-121 uM Oleic acid/g fat. The interference of yellow colour of cow ghee during FFA estimation was observed to be minimum at fat level of 0.2 gm.

Singhal and Jain (1973) isolated FFA from ghee as their methyl esters by ion-exchange resin technique and analysed subsequently over gas liquid chromatograph (GLC). The short chain fatty acids (6:0-12:0) accounted for only 5-10% and long chain fatty acids (14:0-18:2) accounted for 90-95% of total free fatty acids in ghee.

Sharma and Bindal (1987) developed a simple method for the preparation of methyl esters of FFA of ghee without their prior isolation for subsequent analysis by GLC. Ghee (0.1 ml) and an internal standard (19:0 acid) was incubated with

BF_3-methanol reagent in presence of methyl urea at 100°C for 5 min followed by extraction of the resultant methyl esters with petroleum ether. The methyl esters of FFA were analysed by GLC over 10% diethylene glycol succinate (DEGS) coated on diatomite C-AW (100-120 mesh) at 68°C for 4:0 acid and then isothermally at 170°C. The FFA make up of ghee revealed the presence of 16 fatty acids from 4:0 to 18:2, 4:0 being absent in fresh ghee and present in rancid ghee. The FFA level of fresh ghee was 6-12 mg/g. The lower fatty acids 6:0-12:0 though present in low concentration (0.1-1.0 mg/g) accounting only 5-10% of total free fatty acids contribute significantly to ghee flavour.

2.2.2 Carbonyls

Carbonyls play important role in flavours and off-flavours of fat-rich dairy products like ghee. This class of compounds includes broadly monocarbonyls further constitute alkan-2-ones, alkanals, alk-2-enal;s and alka-2,4-dienals. Alkan-2-ones or methyl ketones are reported in several dairy products especially heat processed. They are now regarded as integral components of good quality butter and ghee.

Alkan-2-ones are produced by the hydrolysis of ketonoggenic glycerides (B-ketoglycerides) followed by decarboxylation of B-keto carboxylic acids (Kinsella, 1969a; Wadhwa and Jain, 1939a) during various processing treatments involved in the preparation of ghee. Also lipolysis of triglycerides through penicillin moulds (Law, 1981) during fermentation process may be the source of alkan-2-ones. Penicillium lipases liberate free saturated fatty acids and further oxidise them at B-position to form B-keto acids which on decarboxylation yield methyl ketones. Aldehydes namely n-alkanals, alk-2-enals and alka-2, 4-dienals

are formed by the autoxidation of unsaturated fatty acids of milk fat.

Polar carbonyls which include (i) dicarbonyls, viz., diacetyl (ii) alpha-keto acids, (iii) glyoxals and furfurals are produced as fermentation products of (i) lactose and citrate (ii) proteins (amino acids) (Hillier and Jago, 1978) and (iii) browning products of lactose caramelization (Keeney et al., 1960), respectively and seem to play an important role in the flavour of ghee.

Total carbonyls in ghee were determined by the flask method of Rama Murthy and Jain (1973). Total carbonyls were converted to their dinitriphenyl hydrozones DNPs by keeping the fat in contact with celite-dinitrophenyl hydrazine-phosphoric acid sluffy foddered by spectrophotometric estimation at 340 nm and using molar extinction coefficient ϵ = 22,500. Total carbonyls in ghee were found to be in the range of 3.3-9.7 uM/g fat (Gaba and Jain, 1974a, 1975a, 1976b; Singh and Ram, 1978; Sharma, 1981; Yadav and Srinivasan, 1984; Arora and Singh, 1987). Head space carbonyls of ghee were trapped as DNPs by allowing the stream of nitrogen gas along with volatiles to bubble through acidified 2:4 dinitrophenyl hydrazine solution followed by their spectrophotometric estimation. Head space carbonyls in ghee were estimated to be 0.031 uM/g (Gaba and Jain, 1976a). Volatile carbonyls were collected as DNPs by steam distilling the ghee followed by their spectrophotometric estimation. Volatile carbonyls in ghee were 0.32 uM/g (Gaba and Jain, 1974b).

Total head space and volatile carbonyls were separated into monocarbonyl-DNPs and dicarbonyl-DNPs followed by fractiona-

tion/estimation of monocarbonyl-DNPs into classes and then into individual components by the chromatographic procedures of TLC and GLC over 25% Carbowax 20M coated on diatoport W (60-80 mesh) at a column temperature of 125°C. Total volatile monocarbonyls of ghee (cow/buffalo) consisted of 90% alkan-2-ones and remaining 10% as alkanals (6%), alk-2-enals (2%) and alka-2,4-dienals (2%). The corresponding values for head space monocarbonyls of cow ghee were 85, 11, 2, 2% and that of buffalo ghee were 79, 19, 1, 1%.

The clarification of butter into ghee showed a sharp increase in the level of alkan-2-ones from 56 to 87% (Gaba and Jain, 1977). The level of alkan-2-ones in ghee (87%) was much higher than that of butter oil (62%) (Gaba and Jain, 1976b). It appears that among the monocarbonyls, alkan-2-ones play an important role in the flavour of fresh ghee.

Out of 49 carbonyls detected, 39 were monocarbonyls and 10 were polar carbonyls. So far 34 monocarbonyls have been identified in ghee. These were alkan-2-ones from C_3-C_{10}, C_{12}(9) alkanals from C_2-C_9(8), alk-2-enals from C_4-C_{12}(9) and alka-2,4-dienals from C_5-C_7, C_9-C_{12} and C_{14} (Gaba and Jain, 1974a, 1974b, 1975a, 1976a).

Rao and Rama Murthy (1984) trapped polar carbonyls as their 2,4-DNP's from the aqueous washings of ghee followed by their spectrophotometric estimation at 340 nm and using extinction co-efficient $E^{1\%}_{1cm}$ = 156.4. Six components were detected over TLC in ghee prepared at 100°C and three of them were identified as diacetyl, methyl glyoxal and alpha-ketoglutaric acid. Ten components were detected in ghee prepared at 120°C. Among them, in addition to the above ones, furfural and hydroxy methyl

furfural were also identified. Gaba and Jain (1975b) also identified diacetyl and methyl glyoxal among the six components of dicarbonyl profile of ghee. Ghee prepared at clarification temperature of 100°C and 120°C for 10 min contained (per 100 gm) about 1.9 mg and 31.5 mg of polar carbonyls respectively in DC ghee prepared from fresh cream, 6.1 mg and 75.8 mg in DC ghee prepared from ripened cream and 1.4 mg and 3.2 mg in CB ghee.

Thus, the qualitative and quantitative nature of polar carbonyls is significantly influenced by factors such as method of preparation, temperature of clarification and acidity of cream or butter. As these factors influence the flavour and keeping quality of ghee, these polar carbonyls seem to play an important role in the flavour and keeping quality of ghee.

2.2.3 Lactones

Lactones contribute significantly to the flavour of fat-based dairy products especially the heat processed ones like ghee. These compounds are produced by the hydrolysis of lactonogenic glycerides followed by dehydration (lactonization) of hydroxy acids (Kinsella, 1969a; Wadhwa and Jain, 1989a). Addition of microbial lipase accelerates the lactone production and thus flavour is enhanced (Jolly and Kosikowski, 1975). The lipolysis of delta-hydroxy acid glycerides yields free delta-hydroxy acids which ungergo ring closure to form lactones. Alternatively, delta-keto acid glycerides undergo lipolysis to yield delta-keto acids which undergo reduction to form hydroxy acids and then converted to lactones (Boldingh and Taylor, 1962; Law, 1981). Ferretti et al. (1970) identified lactones among the

products of Maillard type reaction in non-enzymatic browning in a lactose-casein model system.

Among the dairy products, ghee owes its unique flavour to variety of lactones. Wadhwa et al. (1979b,1980a,1980b) used as such the method of Ellis and Wong (1975) for the isolation of lactones from ghee. They expressed the relative proportion of various lactones as affected by the method of preparation, temperature of clarification and storage period, etc. Wadhwa and Jain (1984a, 1989b) developed celite-digitonin-alumina column chromatographic method for the quantitative isolation of lactones from ghee. The acetonitrile eluant from the column was concentrated and purified over silica gel-G TLC plates.

The lactonic isolates of ghee showed a complex GLC profile (44 peaks) and included homologous series of n-saturated delta- and gamma-lactones (24 peaks) from C_6-C_{16} and C_{18}. The remaining 20 peaks were partly characterised as mostly unsaturated lactones. Delta-lactones were the major components in ghee, the chief components being delta-C_{10}, C_{12}, C_{14}, C_{16} and C_{18} at 1.20, 3.07, 2.25, 1.09 and 8.14 ppm levels respectively. Gamma-lactones were the minor components, the ratio of delta- to gamma-lactones being 10:1. The gross lactone level of ghee was 30 ppm. Total delta (17.90 ppm), total gamma (1.76 ppm), saturated delta + gamma (19.66 ppm) and unidentified (10.60 ppm) lactones constituted 59, 6 and 35% of the gross lactone level in ghee respectively (Wadhwa and Jain, 1984b, 1989b).

The lactone profile of ghee was affected rather strongly, but only quantitatively by various technological parameters, viz., method of ghee preparation, temperature of

clarification and storage. The changes in the flavour quality of ghee due to various technological parameters are closely related with corresponding changes in the lactone level of ghee (Wadhwa and Jain, 1984c; 1985a, 1986, 1989c). The contribution of synthetic lactones to the flavour of ghee was enhanced when they were used in combination with synthetic fatty acids and carbonyls (Wadhwa and Jain 1985b, 1989d).

Review of literature under Sections 2.1 and 2.2 has revealed that FFA, carbonyls and lactones are important components of ghee flavour and so far no work has been done on the flavour potential of ghee-residue. Hence, the present study on the evaluation of flavour potential of ghee-residue with special reference to FFA, carbonyls and lactones level was undertaken.

2.3 ESTIMATION METHODS OF LACTONES

It is proposed to review here briefly the estimation methods of lactones from the recent review on 'Isolation, characterisation and estimation methods of lactones' by Wadhwa and Jain (1989b). The various techniques reported for the estimation of lactones are:

2.3.1 Isotope dilution

Jurriens and Oele (1965) and Beers and Zijdan (1966) quantified the absolute amounts of lactones in butterfat by isotope dilution method according to the equation:

$$X = P(S_1/S_2-1)$$

Where, X = amount of lactone in butterfat in ppm

P = amount of labelled lactone added in ppm

S_1 = Specific activity of the labelled lactone added

S_2 = Specific activity of the lactone isolated

The specific activities were determined on the radio gas chromatograph by dividing the measured radioactivity by the areas of the mass peaks.

2.3.2 Gas liquid chromatography(GLC)

Smits (1965) developed a GLC procedure for simultaneous quantitative analysis of lactone-oxo acid mixtures. Oxo acids were converted into their methyl esters by diazomethane treatment. Lactone-methyl ester mixtures were chromatographed on 10% Apiezon coated on celite (150-178 u) column at 175°C. Oxo acids and lactones in the sample were quantified by the internal standard method. Siek and Lindsay (1970) analysed the neutral volatile fraction of fresh sweet-cream butter semi-quantitativelyby capillary GLC. Stark **et al.** (1970a,1970b,1973) used calibration curves established with synthetic mixtures for the estimation of lactones of butterfat by GLC.

Wong **et al.** (1973,1975) and Ellis and Wong (1975) estimated lactones in cheddar cheese, butter and butter oil by GLC using 7.5% ethylene glycol adipate and 2% phosphoric acid on 80-90 mesh Anakram ABS at column temperature of 130-180°C. Area counts of the lactones were plotted against ug of the lactones and straight line relationship was obtained.

Stark **et al.** (1976) used a formula:

$$C = 10 \frac{mU}{S} \times \frac{100}{r}$$

for calculating the concentration of lactones in butter oil, where

C = Concentration of lactones in ppm,

m = Weight of lactone in mg in 2 ml of standard solution.

u = height of lactone GC peak from butter oil distillate

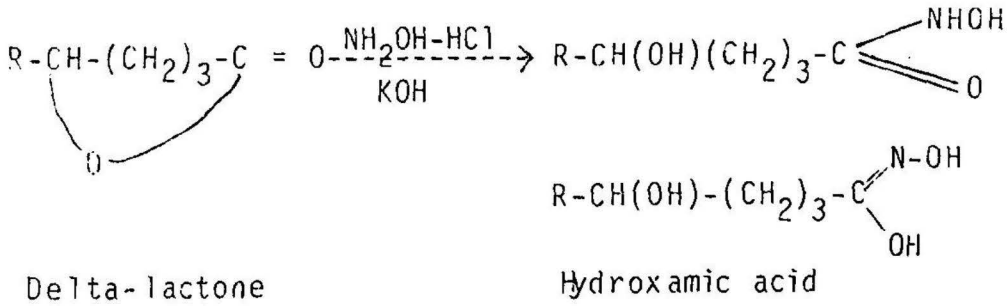
S = height of lactone GC peak from standard solution

r = % recovery of lactone from butter oil

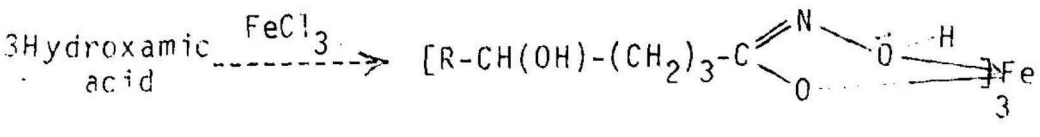
Wadhwa and Jain (1984b) quantified the individual lactones in ghee by GLC over 10% diethylene glycol succinate DEGS coated on 100-120 mesh Diatomite-C(AW) at column temperature of 160-195°C. Gamma-C_7 lactone was used as the internal standartd for the estimation of lactones in ghee.

2.3.3 Spectrophotometry

Linstead and Weedon in 'Qualitative Organic Chemical Analysis' (1956) have described a hydroxamic acid test as a qualitative test for the detection of esters (carboxylic ester or lactone). Boldingh and Taylor (1962) and Urbach (1965) characterised the lactones as their ferrichydroxamate derivaties by paper and thin layer chromatography.



Delta-lactone Hydroxamic acid



Ferric hydroxamate of delta-lactone

Smits (1965) discussed the possibility of spectrophotometric estimation of lactones as their ferric hydroxamate derivatives as reported by Francke (1963). Francke (1963) in his paper on the 'Enzymatic reduction of delta-keto acids to the corresponding optically active hydroxy acids' has just mentioned about the estimation of optically active delta-hydroxy acids as their ferric hydroxamic acid complex at 515 nm. Neither details of the method nor any cross reference for it is given in the literature.

Review of literature has revealed that generally GLC has been used for the estimation of lactones. It was, therefore, of interest to standardise the spectrophotometric method for the estimation of lactones. The method would be more convenient and appropriate to use when one is interested in the total lactone level isolated from a sample rather than individual lactone levels.

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CHAPTER - III

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 GHEE-RESIDUE/GHEE SAMPLES

Ghee-residue samples (four) and their respective ghee samples were collected directly from the Experimental Dairy of the National Dairy Research Institute (Karnal), prepared in practice as follows:

Cream was churned into butter and heated in steam jacketted double walled stainless steel vessel at a temperature of 120^{o}C flash. Ghee was kept undisturbed for about half an hour in the same vessel. Ghee (upper fat layer) was decanted off carefully and passed through bag filters. It was further passed through 55 disc clarifier (Alfa-Laval, Max.speed = 1500 rpm). Finally, ghee-residue left in stainlees steel vessel and bag filters was pooled and kept over four-folds of muslin cloth over a bucket overnight at room temperature (RT) for an effective recovery of ghee. Next day, ghee-residue samples and their respective ghee samples (from the main lot) were collected.

3.2 SOLVENTS

3.2.1 Carbonyl free n-hexane

Hexane (LR,BDH) was made carbonyl free (CF) essentially by the procedure of Schwartz and Parks (1961) modi-

fied as follows:

Hexane was refluxed with 2,4 dinitrophenyl hydrazine (5g/lit) and trichloroacetic acid (1 ml/lit) for 3 hr and then distilled at 68°C.

3.2.2 Carbonyl free benzene

Benzene (LR,BDH) was made CF by the same procedure as described above.

3.2.3 Neutral alcohol

Alcohol (95%) was kept over sodium hydroxide (AnalaR,BDH) overnight and distilled at 76°C. It was made further neutral (if necessary) at a time of use by titrating against N/10 sodium hydroxide to phenolphthalein end-point.

3.2.4 Distilled acetonitrile

Acetonitrile (LR,S.Merck) was kept over phosphorus pentoxide (E.Merck) overnight and distilled at 82°C.

3.2.5 Petroleum ether (40°-60°C)(LR,BDH)

3.2.6 Diethyl ether (LR,BDH)

3.2.7 Methanol (AnalaR,BDH)

3.2.8 Benzene(LR,HPC)

3.2.9 Ethyl acetate(AnalaR,BDH)

3.2.10 Chloroform (AnalaR,BDH)

3.2.11 Dichloromethane (LR,S.Merck)

3.3 REAGENTS AND ADSORBENTS

3.3.1 Cupric acetate (LR,BDH)

3.3.2 Pyridine (AR,Polypharma)

3.3.3 2,4-dinitrophenylhydrazine (LR,BDH)

3.3.4 Hydroxylamine hydrochloride(AnalaR,BDH)

3.3.5 Potassium hydroxide (AnalaR,BDH)

3.3.6 Ferric chloride (LR, S.Merck)

3.3.7 Hydrochloric acid (LR,BDH)

3.3.8 Orthophosphoric acid (AnalaR,BDH)

3.3.9 Iodine (S.Merck)

3.3.10 Trichloroacetic acid (E.Merck)

3.3.11 Silica gel G (Centron)

3.3.12 Celite-545(Loba-Chemie)

3.4 STANDARDS

3.4.1 Lactones

Saturated delta - C_8, C_9, C_{10}, C_{11}, C_{12} lactones (C.A.Aromatics Co., USA).

Saturated delta - C_{13}, C_{14}, C_{15}, C_{16}, C_{18} and gamma - C_{15}, C_{16}, C_{18} lactones (gift samples from Dr(Mrs) G.Urbech, Division of Dairy Research, CSIRO, Melbourne, Australia).

3.4.2 Oleic acid (E.Merck)

3.4.3 Cholesterol (E.Merck)

3.4.4 Tripalmitin (Sigma Chemical Co.)

3.4.5 1,3 Dipalmitin (Sigma Chemical Co.)

1,2 Dipalmitin (Sigma Chemical Co.)

3.4.6 Monopalmitin (Sigma Chemical Co.)

3.5 INSTRUMENTS

3.5.1 pH LI-10 (Elico)

3.5.2 Electronic balance (Anamed)

3.5.3 Clinical centrifuge (Remi T-8)

3.5.4 Rotary flash evaporator (Tempo Industrial Corporation)

3.5.5 Balanced-cell colorimeter 102(Systronics)

3.5.6 Spectronic-20

3.5.7 Spectrophotometer (JASCO)

3.5.8 Thin layer chromatograph TLC applicator(Desaga)

3.5.9 Gas Liquid Chromatograph GLC (Aimil Nucon Series 5700)

3.6 EXTRACTION OF GHEE-RESIDUE LIPIDS

Lipids were extracted from ghee-residue samples by the following two methods:

3.6.1 Mojonnier Method

Lipids were extracted essentially according to the conventional procedure of Mojonnier (ISI, 1961) with a few modifications. Accurately weighed ghee-residue sample (about 1.0g) was taken in 100 ml conical flask. Added to it 10 ml of neutral alcohol and warmed the contents over a waterbath. Cooled to RT and added 25 ml of diethyl ether and 25 ml of pet. ether

and slightly warmed the contents to about 30-40^{o}C for few minutes with occasional shakings. The solvent layer was filtered while hot through ordinary filter paper taking care that most of the ghee-residue remained in the conical flask. The filterate was collected in a pre-weighed standard joint long-neck round bottom flask. Repeated the extraction 3-4 times with 15, 15 ml of diethyl ether and pet. ether in the similar manner till complete extraction of fat was ensured. Appearance of clear solvent layer and separated fine particles of ghee-residue indicated complete extraction of fat. The pooled filterate (solvent) was evaporated under rotary flash evaporator. Weighed the extracted lipids till constant weight. Dissolved the lipids in dichloromethane and made up the volume to 15 ml for further analysis. Lipids extracted from ghee-residue samples were labelled as GR_1, GR_2, GR_3 and GR_4. A sample blank GR_0 was also prepared under similar conditions.

3.6.2 Single solvent (Methanol) Method

Accurately weighed ghee-residue sample (about 1.0g) was taken in a 100 ml conical flask. Extracted the lipids with 50, 30, 30, 20, 20, 20, 15, 15 ml of methanol while warming the contents every time to 65-70^{o}C for few minutes with occasional shakings. The disappearance of fogginess in the solvent during extraction and appearance of separated fine particles of ghee-residue indicated complete extraction of fat. In every extraction, the solvent layer filtered while hot through oridinary filter paper taking care that most of the ghee-residue remained in the conical flask. The filterate was collected in a pre-weighed standard joint long-neck round bottom flask. The pooled filterate (solvent) was evaporated under rotary flash evaporator. Weighed

the extracted lipids till constant weight. Dissolved the lipids in dichloromethane and made the volume to 15 ml for further analysis. Lipids extracted from ghee-residue samples were labelled as GR_1', GR_2', GR_3' and GR_4'. A sample blank GR_0' was also prepared under the similar conditions.

3.7 GHEE SAMPLE SOLUTIONS

Accurately weighed ghee samples (about 5.0g) were dissolved in benzene and volume made to 25 ml and labelled as G_1, G_2, G_3 and G_4. A sample blank G_0 was also prepared.

3.8 ANALYSIS

Ghee-residue lipid sample solutions GR_1, GR_2, GR_3, GR_4; GR_1', GR_2', GR_3', GR_4' and respective ghee sample solutions G_1, G_2, G_3, G_4 alongwith their corresponding sample blanks GR_0; GR_0'; G_0 prepared above were analysed for FFA and total carbonyl levels as described below:

3.8.1 Estimation of FFA

FFA were estimated essentially according to the procedure of Lowry and Tinsley (1976) as follows:

3.8.1.1 Preparation of standard curve

Cupric acetate pyridine(CAP) reagent: CAP reagent was an aq.solution of cupric acetate (5% w/v) with pH adjusted to 6.0-6.2 with pyridine.

Oleic acid: Its stock solution was prepared by dissolving 6.34 ml of Oleic acid in benzene to make up the volume to 100 ml so as to provide 200 mM oleic acid per 100 ml. Its working solution was prepared by further diluting 1 ml of stock solution to 100 ml with benzene so as to provide 20 uM oleic acid per ml.

Pipetted out 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 ml of working solution of Oleic acid (equivalent to 0, 10, 20, 30, 40, 50, 60, 70 and 80 uM) in separate long glass stoppered tubes. Total volume was made to 10 ml with benzene in each tube. Added 2 ml CAP reagent to each tube and shook the contents vigorously for 2 min. Centrifuged the biphasic system for 5 min at 1450 rpm. Klett reading of upper benzene layer containing blue colour complex was taken cautiously in Balanced-Cell Colorimeter-102 using red filter and standard curve was plotted (Fig.1).

3.8.1.2 Samples analysis

Took 1 ml of each ghee-residue lipid solutions and their corresponding sample blanks in separate long glass stoppered tubes and evaporated the solvent carefully. To each tube, added 10 ml benzene along the sides of the tube to dissolve the fat. Developed the blue colour complex in each tube by adding 2 ml CAP reagent as mentioned above. Noted the Klett reading of samples against their corresponding sample blanks and estimated FFA from the standard curve (Fig.1).

FFA in ghee samples were estimated by taking 1 ml of ghee solutions in benzene, making final volume to 10 ml with

benzene and following the rest of the procedure as mentioned above.

FFA level (uM/g ghee-residue) = $\frac{15 \times C}{W}$

FFA level(uM/g ghee-residue lipid) = $\frac{15 \times C}{W} \times \frac{100}{F}$

FFA level (uM/g ghee) = $C \times \frac{25}{W'}$

Where,

C = uM of Oleic acid corresponding to observed KR (Fig.1)

W = Weight of ghee-residue sample taken for extraction

W' = Weight of ghee sample taken

F = Fat (%) of ghee-residue sample

3.8.2 Estimation of total carbonyls

Total carbonyls were estimated by the method of Rama Murthy and Jain (1973) known as flask method described below:

3.8.2.1 Preparation of celite-dinitrophenylhydrazine phosphoric acid mixture(reaction mixture)

The reaction mixture was prepared and washed according to the procedure of Schwartz and Parks (1961) as follows:

2,4-dinitrophenylhydrazine (0.5g) was dissolved in 6 ml of 85% (v/v) orthophosphoric acid (6 ml = 5.8 ml of 88% acid + 0.2 ml distilled water) by grinding in a pestle mortar. Added 4 ml glass distilled water and the precipitates formed were redissolved by further grinding. Celite-545(10g) was added

into the solution and mixed the contents thoroughly by grinding until a homogeneously damp preparation was obtained. The material thus obtained was packed into the column (2 x 26 cm) containing a plug of glass wool at the bottom and CF hexane (25 ml). The column was packed by gentle pressure and it was then flushed immediately with CF benzene (50 ml) followed by washing with CF hexane till the effluent appeared colourless. The reaction mixture thus packed in the column was taken out and kept in the desiccator for further use.

3.8.2.2 Samples analysis

Took 0.5 ml of each ghee-residue lipid solutions, ghee solutions and their corresponding sample blanks in 25 ml glass stoppered conical flasks and evaporated the solvent carefully. Added 0.5 ml of CF hexane and 0.5g of the reaction mixture. Shook the contents and allowed to stand under periodic shakings for 24 hr at RT. Added 9.5 ml of CF hexane to make up the total volume of the solution to 10 ml. The optical density (OD) of the resulting yellow solution of 2,4-dinitrophenylhydrazones was determined at 340 nm against their corresponding sample blanks. The readings so obtained were converted into uM/g using ϵ = 22,500 by the general formulae:

$$\text{Total Carbonyl level (uM/g ghee-residue)} = \frac{D}{22500} \times \frac{10^6}{1000} \times 10 \times \frac{15}{0.5} \times \frac{1}{W}$$

$$\text{Total carbonyl level(uM/g ghee-residue lipid)} = \frac{D}{22500} \times \frac{10^6}{1000} \times 10 \times \frac{15}{0.5} \times \frac{1}{W} \times \frac{100}{F}$$

$$\text{Total carbonyl level(uM/g)ghee)} = \frac{D}{22500} \times \frac{10^6}{1000} \times 10 \times \frac{25}{0.5} \times \frac{1}{W}$$

Where,

D = Observed optical density

W = Weight of ghee-residue sample taken for extraction

W' = Weight of ghee sample taken

F = Fat(%) of ghee-residue sample

3.8.3 Standardisation of spectrophotometric method for the estimation of lactones

A method was standardized for the estimation of lactones as their ferric hydroxamates as follows:

3.8.3.1 Determination of absorption maxima(λmax)

Took 1 ml of delta-C_{12} lactone solution (100 mg/100 ml in chloroform) in a glass test tube and evaporated the solvent carefully on a water bath. Added 0.1 ml methanolic hydroxylamine hydrochloride (5.5% w/v) and 0.1 ml methanolic potassium hydroxide (10% w/v) to lactone and heated the contents on the boiling water bath for 1-2 min cooled to RT. Added 9 ml glass distilled water along the sides of the tube so as to dissolve all the precipitated material. Acidified the solution by adding 0.1 ml of 2N hydrochloric acid. Added 0.3 ml aqueous ferric chloride (5% w/v). Red-violet colour complex of ferric hydroxamate was obtained. Determined λmax by observing optical density (OD) against the reagent blank at various wave lengths (400-550 nm) on Spectronic-20. The λmax was found to be 515 nm (Fig.2).

3.8.3.2 Preparation of standard curve

Took 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml delta-C_{12} lactone solution (100 mg/100 ml in chloroform) equivalent to 0, 200, 400, 600, 800 and 1000 ug of lactone in separate test tubes. Evaporated the solvent carefully on water bath. Developed the red-violet colour by the procedure mentioned above and noted OD of different concentrations at 515 nm on spectronic-20. Addition of aq.ferric chloride was done simultaneously in various reaction tubes and recorded OD immediately after the colour development. A standard curve was obtained by plotting concentration (ug) against OD (Fig.3).

3.8.4 Isolation of lactones from ghee-residue lipids by TLC

Since spectrophotometric estimation of lactones as their ferric hydroxamate derivatives is not possible in presence of fat, lactones were isolated from ghee-residue lipids sample solutions GR_1', GR_2', GR_3', GR_4' by TLC procedure of Wadhwa and Jain (1984a).

0.2 ml of ghee-residue lipid solution (obtained by methanol extraction method) was spo-tted quantitatively over about two 20 cm x 20 cm silica gel G plates (0.25 mm thickness, silica gel G:water::1:2) activated at 110^{0}C for 1 hr. The plates were developed in the solvent system benzene:ethyl acetate (9:1) saturated with acidified water (10 ml water + 1 drop of HCl). After development, the plates were completely air dried and then sprayed with methanolic iodine (2% w/v). The lactonic region (Fig.4) parallel to standard lactone was scratched from all the TLC plates. The pooled scratched material was extracted with

25, 15, 10, 10 ml of dichloromethane, filtered through Whatman filter paper No.40 to remove silica gel particles followed by evaporation of solvent under rotary flash evaporator to get the lactonic isolate of ghee-residue. Similarly, the corresponding sample blank GR'_0 (0.2 ml) was developed quantitatively over about two TLC plates and exactly the same area of silica gel parallel to standard lactone was scratched and extracted with dichloromethane followed by evaporation of the solvent as done for ghee-residue lipid sample solutions.

3.8.5 Estimation of lactones in ghee-residue samples by spectrophotometric method

Lactonic isolates obtained from 0.2 ml ghee-residue lipid sample solutions GR'_1, GR'_2, GR'_3. GR'_4 and corresponding sample blank GR'_0 were taken in test tubes. Red-violet colour of ferric hydroxamate complex was developed simultaneously in all the tubes by the standardised procedure mentioned in Section 3.8.3. The OD of various samples was taken at 515 nm against the corresponding sample blank processed under exactly similar conditions. Lactones were estimated from the standard curve (Fig.3) using the general formulae:

$$\text{Lactones level(ug/g ghee-residue)} = \frac{C \times 5 \times 15}{W}$$

$$\text{Lactones level(ug/g ghee-residue lipid)} = \frac{C \times 5 \times 15}{W} \times \frac{100}{F}$$

Where,

C = ug of lactones corresponding to observed OD from the graph (Fig.3)

W = Weight of ghee-residue sample taken for extraction,

F = Fat (%) of ghee-residue sample

3.8.6 Estimation of lactones in ghee-residue samples by GLC method

3.8.6.1 Standardisation by GLC for fractionation of standard lactones mixture

Most mixture of delta-C_8-C_{16}, C_{18} and gamma-C_6-C_{12}, C_{15}, C_{16}, C_{18} lactones in acetonitrile solvent was prepared and fractionated (Fig.5) under the conditions as follows:

Type of detector : Flame ionization detector(FID)

Column stationary phase : 15% DEGS(diethylene glycol succinate) coated on 100-120 mesh Chromosorb W(AW-DMCS)

Column dimensions : 6' x ¼", Glass column

Column temperature : Initial temperature 160°C for 21 min.

Rate 20°C/min.

Final temperature 195°C for 179 min.

Injector temperature : 210°C

Detector temperature : 220°C

Chart speed : 0.5 cm/min.

Attenuation : 1

Sensitivity : 1000

Volts full scale : 0.01 mV

Flow rate of gasses: : Carrier Nitrogen = 30 ml/min

Nitrogen = 33 ml/min.

Air = 330 ml/min.

3.8.6.2 Samples analysis

Lactonic isolates obtaioned from 1.0 ml ghee-residue lipid sample solutions GR_1', GR_2', GR_3', GR_4' by the procedure mentioned under Section 3.8.4 were subjected to GLC analysis under the standardised conditions mentioned above.

Gamma-C_7 lactone was chosen as the internal standard (IS) to be added to the lactonic isolates of different ghee-residue lipid samples. 5 ul (equivalent to 50 ug) of this IS (1% solution in acetonitrile) was added to the lactonic isolate from 1 ml ghee-residue lipid sample solution. The volume was made to 0.1 ml (100 ul) with acetonitrile. 10 ul of this solution was injected into the column under the standardized conditions of fractionation oflactones described earlier. The characterisation of lactones in ghee-residue was made through retention times (Fig.6). Individual lactones were quantities according to the general formulae:

$$\text{Lactone level (ug/g ghee-residue)} = \frac{P_2}{P_1} \times 5 \times \frac{100}{10} \times \frac{15}{W}$$

$$\text{Lactone level (ug/g ghee-residue lipid)} = \frac{P_2}{P_1} \times 5 \times \frac{100}{10} \times \frac{15}{W} \times \frac{100}{F}$$

Where,

P_1 = Peak area of I.S.

P_2 = Peak area of lactone in sample

W = Weight of ghee-residue sample taken for extraction

F = Fat (%) of ghee-residue

Peak areas were calculated by the triangulation method.

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CHAPTER - IV

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 EXTRACTION OF GHEE-RESIDUE LIPIDS

Ghee-residue lipids were extracted by:

4.1.1 Mojonnier Method

The original method of Mojonnier (ISI, 1961) for fat extraction was subjected to a few modifications for extracting ghee-residue lipids:

i) The use of liquid ammonia was eliminated to avoid neutralisation of FFA present. More so, in the absence of fat globule membrane, use of ammonia was not necessary for extracting ghee-residue lipids.

ii) Ordinary alcohol (95%) was replaced by neutral alcohol as the former develops acidity (acetic acid) on standing.

iii) The extraction of lipids was done under slightly warm conditiions (30-40^{o}C) which eliminated vigorous shakings and required only ocassional shakings so as to facilitate lipid extraction.

iv) The extractions were done in an ordinary conical flask rather than the conventional Mojonnier tube.

4.1.2 Single solvent (Methanol) method

Single solvent extraction method was standardised for extracting ghee-residue lipids with a view to replace mixture

of solvents as used in Mojonnier method described above. Preliminary trials were done with polar solvents like dichloromethane, acetone, acetonitrile of polarity indices (PI)* 3.4, 5.1, 5.9 respectively for extracting ghee-residue lipids. As methanol is the most polar organic solvent of PI 6.6, its use was preferred over all solvents. More so, it is commonly available and can be easily evaporated (B.P. = 65°C). Further, Methanol being more polar than diethyl ether of PI 2.8 could be expected to extract flavour compounds more effectively alongwith ghee-residue lipids.

Lipids were extracted from accurately weighed ghee-residue (about 1.0g) using 200 ml methanol solvent in suitable instalments. Ghee-residue lipid extractions were standardised with 50,30, 30, 20, 20, 20, 15, 15 ml of methanol under warm conditions were very much necessary because as such the solutibility of fat in Methanol is less at RT. The complete disappearance of fogginess in the solvent during extraction and appearance of separated fine particles of ghee-residue indicated complete extraction of lipids. Ghee-residue left over various extractions when tested for flavour compounds gave negative results.

4.1.3 Comparison between the two methods

Table 1 depicts the lipid (%) in ghee-residue extracted by Mojonnier method and Single solvent (Methanol) method. Lipid (%) in ghee-residue extracted by Mojonnier method

*Data collected from 'Handbook Chemistry and Physics' 61st edition CRC 1980-81

was 53.51 (48.25-57.14). Lipid (%) in ghee-residue extracted by Single solvent (Methanol) method was 58.39 (56.55-62.66). Santha and Narayanan (1978a) reported 36.2% fat in creamery butter ghee-residue extracted by conventional Mojonnier method.

Statistical analysis (Table 1) revealed that the average lipid (%) obtained by Single solvent (Methanol) method was significantly higher ($P \leq 0.05$) than that obtained by Mojonnier method. Repeated extractions of ghee-residue with small amounts of methanol under warm conditions resulted in higher recovery of lipids. This might be due to he high polar character of methanol which would have resulted in effective extraction of flavour compounds alongwith ghee-residue lipids.

4.2 ANALYSIS

Ghee-residue lipid sample solutions GR_1, GR_2, GR_3, GR_4 (obtained by Mojonnier Method); GR'_1, GR'_2, GR'_3, GR'_4 (obtained by Single solvent method) and respective ghee sample solutions G_1, G_2, G_3, G_4 alongwith their corresponding sample blanks GR_0; GR'_0; G_0 prepared under section 3.6.1, 3.6.2, 3.7 were analysed for FFA and total carbonyl levels. Results were expressed as uM/g ghee-residue as well as uM/g ghee-residue lipid and compared with uM/g ghee.

4.2.1 Estimation of FFA

FFA were estimated as blue coloured copper complexes from the standard curve of Oleic acid (Fig.1) obtained by the colorimetric method of Lowry and Tinsley (1976). The results have been tabulated in Table 3. FFA levels expressed as uM/g ghee-residue in lipid samples extracted by Mojonnier method and Single solvent (Methanol) method were 229.59 (163.52-349.31) and

627.48 (543.75-682.38) respectively. The corresponding FFA levels expressed as uM/g ghee-residue lipid were 405.52 (311.61-724.04) and 1077.37 (945.65-1206.52) respectively. This revealed that methanol extraction method was more effective in extracting ghee-ressidue lipids alongwith free fatty acids as flavour compounds than the Mojonnier method. Methanol being more polar solvent could extract the flavour compounds more quantitatively than diethyl ether-petroleum ether mixture and hence confirmed other hypothesis based upon which methanol was used for extracting ghee-residue lipids.

FFA levels (uM/g) in respective ghee samples were 53.55 (49.59-61.68). Thus, FFA levels expressed as uM/g ghee-residue and uM/g ghee-residue lipid in lipid samples (extracted by methanol method) were 10.75 times and 18.45 times respectively that in ghee. The study indicated that a major portion of FFA as flavour compounds, generated from fatty acid glycerides by hydrolytic/lipolytic mechanism (Kinsella, 1969a; Law, 1981; Wadhwa and Jain, 1989a,1990c) during processing treatments while preparing ghee are retained in the ghee-residue because of their polar nature whereas only a small portion of FFA passes into ghee. Thus, it was concluded that level of free fatty acids was higher in ghee-residue than in ghee.

4.2.1 Estimation of total carbonyls

Total carbonyls were estimated as their 2,4-dinitrophenylhydrazine derivates by flask method of Rama Murthy and Jain (1973). The results are depicted in Table 4. Total carbonyl levels expressed as uM/g ghee-residue in lipid samples extracted by Mojonnier method and Single solvent

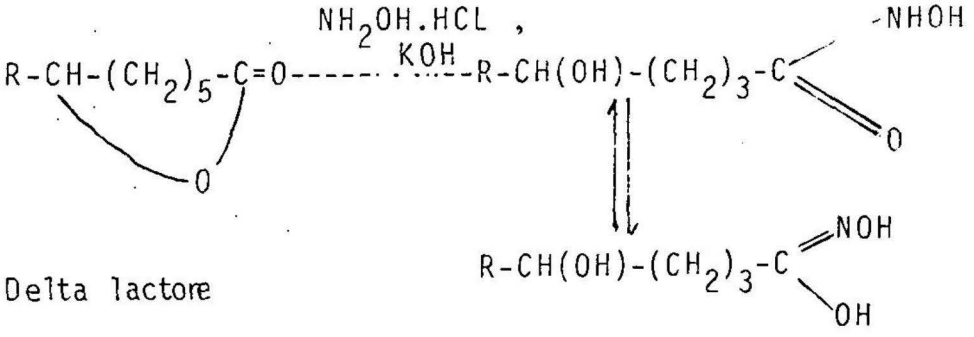
(Methanol) method were 15.28(12.27-20.46) and 43.65(27.90-55.45) respectively. The corresponding total carbonyl levels expressed as uM/g ghee-residue lipid were 28.38(23.38-36.41) and 75.30 (48.52-97.85) respectively. Thus, it further confirmed that methanol method was more effective for extracting ghee-residue lipids along with carbonyls as flavour compounds than the Mojonnier method because of high polar character of Methanol solvent.

Total carbonyl levels (uM//g) in respective ghee samples were 4.30(3.50-4.97). Thus, total carbonyl levels expressed as uM/g ghee-residue and uM/g ghee-residue lipid in lipid samples (extracted by methanol method) were 13.15 and 17.51 times respectively that in ghee. The study revealed that a major portion of carbonyls generated during the manufacture of ghee are retained in the ghee-residue whereas only a small portion of carbonyls passes into ghee. These carbonyls are generated from unsaturated fatty acid glycerides through oxidative mechanism, from β-ketoglycerides through hydrolytic/lipolytic and decarboxylative mechanisms (Kinsella, 1969a; Law, 1981; Wadhwa and Jain, 1989a,1990c) and from non-lipid precursors viz. proteins, lactose etc. as fermentation and browning products (Hillier and Jago, 1978; Keeney et al., 1950).

Thus it was inferred that level of carbonyls was higher in ghee-residue than in ghee.

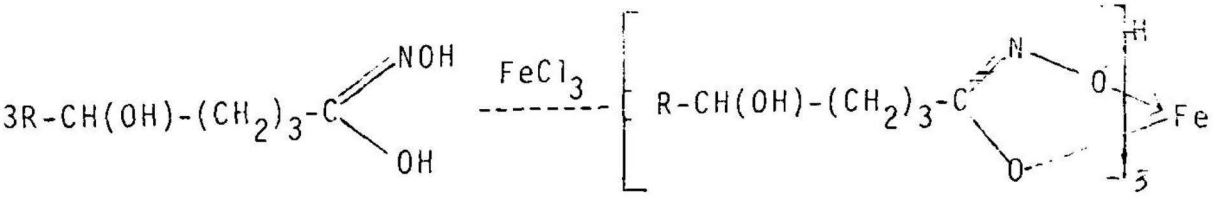
4.2.3 Standardization of spectrophometric method for the estimation of lactones

The method was based upon the hydroxamic acid test as a qualitative test for the detection of esters (carboxylic ester or lactone) described by Linstead and Weedon in 'Qualitative Organic Chemical Analysis' (1956). Boldingh and Tayler (1962) and Urbach (1965) characterized the lactones as their ferric hydroxamate derivatives through paper and TLC by spraying with i) equal volumes of 5% methanolic hydroxalamine hydrochloride and 12.5% methanolic sodium hydroxide, ii) 1% methanolic ferric chloride, and iii) glacial acetic acid.



Delta lactore

Hydroxamic acid



Hydroxamic acid

Ferric hydroxamate of delta-lactone

First, a qualitative reaction was performed by the method of Linstead and Weedson (1956) by heating a drop of delta-C_{12}lactone with six drops of saturated methanolic hydroxylamine

hydrochloride and six drops of saturated methanolic potassium hydroxide follo.-wed by acidification (9 ml water + 1 ml 2NHCl) and developing the red-violet colour with few drops (five) of 5% aq.ferric chloride reagent. The absorption maxima was observed at 515 nm against the reagent blank which was in accordance with that reported by Francke (1963).

In the present study, the standardisation of spectrophotometric method for the estimation of lactones was done using reagents.

(a) 0.05%-1 ml of 5.5% methanolic hydroxylamine hydrochloride (5.5% concentration was nearer to saturation point).

(b) 0.05-1 ml of 10% methanolic KOH (10% concentration was nearer to saturation point).

(c) 0.05-1 ml of 2NHCl

(d) 0.1-1 ml of 5% aq.ferric chloride.

under various combinations in different trials. 200, 600 and 1000 ug concentration tubes of lactones were used after evaporating the solvent carefully and performing he reaction as mentioned under 3.8.3.1, unless stated otherwise. OD was taken immediately at 515 nm against the reagent blank and results are summarised in Table 5.

Trial 1: With 0.05, 0.05, 0.05 and 0.1 ml of reagents (a), (b), (c) and (d) respectively.

Trial 2: With 0.05, 0.05, 0.05 and 0.2 ml of reagents (a), (b), (c) and (d) respectively.

Trials 1 and 2 gave low absorbance values not proportionate to concentrations indicating that the amount of reagents added were probably not sufficient.

Trial 3: With 0.1, 0.1, 0.1 and 0.3 ml of reagents (a), (c) and (d), respectively.

The absorbance values appeared to be in proportion with concentrations.

Trial 4: With 0.5, 0.5, 0.5 and 0.5 ml of reagents (a),(b), (c) and (d), respectively.

Trial 5: With 1.0, 1.0, 1.0 and 1.0 ml of reagents (a), (b), (c) and (d), respectively.

Trials 4 and 5 were performed with a view to see if there was any improvement in absorbance values of various concentrations of lactones. It was observed that addition of 0.5-1 ml of 5% aq.ferric chloride was not desirable as it imparted much yellow colour in the blank. The absorbance values observed were very much erratic probably there was shift in the absorption maxima due to higher concentration of ferric chloride. The study revealed that amount of 5% aq.ferric chloride reagent addition was very much critical and that Trial 2 was the best possible trial. The absorption maxima under Trial 2 was again confirmed as 515 nm (Fig 2). Accordingly, the standard curve was prepared at 515 nm (Fig 3) against the reagent blank using 200, 400, 600, 800 and 1000 ug concentrations of delta-C_{12} lactone.

Some important observations were made during the standardisation process:

i) All glasswares used in the experiment should be thoroughly washed with dichromate solution or nitric acid followed by final rinsing with glass distilled water and drying in oven, as ferric hydroxamate test is given by more than one class of

compounds (carboxylic esters, lactones, anhydrides, acid chlorides, oxalates, glycerides). Any such type of contamination from detergents etc. may interfere in the reaction.

ii) Solvent should be evaporated carefully from the reacting lactone material before treating with various reagents.

iii) OD of different tubes containing 0.2, 0.4, 0.6, 0.8 and 1 ml of delta-C_{12} lactone solution (100 mg/100 ml chloroform) against a common reagent blank (containing no chloroform) was same as that observed with different blanks containing 0.2, 0.4, 0.6, 0.8 and 1 ml of chloroform (reaction performed after evaporation of solvent).

iv) OD should be taken immediately after the reaction as it decreased with time (Table 6).

v) Klett readings obtained on colorimeter-102 using green filter indicated that one can also use colorimeter for the estimation of lactones as their ferric hydroxamate derivatives (Table 7). However, absorbance values obtained with different concentrations of lactones on colorimeter-102 and spectronic-20 were not exactly following the relation Klett reading $K = \frac{OD}{500}$

4.2.4 Isolation of lactones from ghee-residue lipids by TLC

Wadhwa and Jain (1984a) developed a Celite-digitonin-alumina column chromatographic method for the quantitative isolation of lactones from 10g ghee. The acetonitrile eluant from the column was concentrated and purified over silica gel TLC plates. They confirmed the purity of the lactonic isolates over infra-red spectroscopy followed by their GLC analysis.

As ghee-residue lipids showed higher levels of flavour compounds viz. FFA and total carbonyls in comparison to ghee, lactones in ghee-residue lipids were also expected to be in higher levels than those in ghee. Hence, lactones were isolated from ghee-residue lipids (Fig.4) directly by TLC procedure of Wadhwa and Jain (1984a). Isolation of lactones from ghee-residue lipids was necessary because spectrophotometric estimation of lactones as their ferric hydroxamate derivatives is not possible in presence of fat.

4.2.5 Estimation of lactones in ghee-residue samples by spectrophotometric method

Linstead and Weedon (1956) have described the hydroxamic acids of long-chain glycerides are insoluble in water and give negative ferric hydroxamate test under aqueous conditions i.e., when 9 ml of water is used. However, when 9 ml of alcohol is used instead of water, this test is positive.

In the present study, ferric hydroxamate tests were performed with standard tripatmitin; 1,3,dipalmitin; 1,2 dipalmitin and monopalmitin separately and negative tests were obtained under aqueous conditions. Also, this test was performed with fat which gave positive result. This was due to short-chain glycerides and other esters in fat.

In the lactonic isolates of ghee-residue (Fig.4) obtained through TLC (of Section 3.8.4, 4.2.4), the possible contaminants were 1,3 and 1,2 diglycerides as Rf values of these glycerides were almost same as that of lactones in the solvent system used in this study. To clear this point, 0.2 ml of ghee solution (5g/25 ml benzene) was spotted over TLC plate and

developed in the solvent system. The region parallel to standard lactone was tested for ferric hydroxamate test which gave negative result. This showed that there were no lactones in this region (as concentration of lactones in ghee is too low and needs concentration from 10g ghee by column chromatography as described by Wadhwa and Jain, 1984a,b). Also that in this region 1,3 and 1,2 diglycerides of long-chain fatty acids apopeared which gave negative test under aq.conditions. Hence, the lactonic isolates of ghee-residue could be possibly contaminated with 1,3 and 1,2 diglycerides of long-chain fatty acids which do not interfere in the ferric hydroxamate reaction under aqueous condition.

Lactonic isolates obtained from different samples of ghee-residue were estimated as their ferric hydroxamates at 515 nm using standard curve (Fig.3). Results are depicted in Table 8. Lactone levels expressed as ug/g ghee-residue and ghee-residue lipid were 3850.56 (33688.52-4263.70) and 6612.28 (5904.88-7499.91) respectively. Gross lactone level (ug/g) in ghee as reported by Wadhwa and Jain (1984b) was 30.26. Thus, lactone levels expressed as ug/g ghee-residue and ug/g ghee-residue lipid were 127.55 times and 218.52 times, respectively than that in ghee. The study showed that a major portion of lactones generated during various processing treatments in the manufacture of ghee are retained in the ghee-residue because of their highly polar character, whereas only a small portion of lactones passes into ghee. These lactones are produced from lactonogenic glycerides through hydrolytic/lipolytic and dehydrative mechanisms (Kinsella, 1969a; Law, 1981; Wadhwa and Jain, 1989a;1990c).

Thus, it was concluded that level of lactones was higher in ghee-residue than in ghee.

The validity of the spectrophotometer method for the estimation of lactones in ghee-residue was further confirmed by GLC analysis.

4.2.6 Estimation of lactones in ghee-residue samples by GLC method

Wadhwa and Jain (1984b) analysed lactonic isolates of ghee by GLC over 10% DEGS coated on 100-120 mesh Diatomite C (AW) at column temperatures of 160°C for 40 min and 195°C for 60 min. Both gama- and delta-lactones from C_6 to C_{12} fractionated at 160°C and those above C_{12} fractionated at 195°C.

In the present study, Fig.5 shows the GLC separation of model mixture of gamma-C_6 to C_{12}, C_{15}, C_{16}, C_{18} and delta-C_8 to C_{16}, C_{18} lactones over 15% DEGS Coated on 100-120 mesh Chromosorb W(AW-DMCS) at 160-195°C. Gamma-C_6, C_7, C_8 lactones fractionated at 160°C for 21 min and delta-C_8 to C_{16}, C_{18} and gamma-C_9 to C_{12}, C_{15}, C_{16}, C_{18} fractionated at 195°C for 79 min. Fig.6 shows the GLC separation of lactonic isolates of ghee-residue under the same conditions as used for model mixture of lactones. The gas chromatograms showed 47 peaks. Of these 20 peaks were characterized through their retention times as delta-C_8 to C_{16}, C_{18} and gamma-C_6 to C_{12}, C_{15}, C_{16}, C_{18} lactones. 4 peaks were tentatively identified as delta C_6, C_7 and gamma-C_{13}, C_{14} lactones. Gamma-C_7 lactone was absent in lactonic isolates of ghee-residue and was, therefore, chosen as the internal standard for the quantification of lactones in ghee-residue. Wadhwa andJain (1984b) also reported the absence of gamma-C_7 lactone in ghee.

Work from the same laboratory (Wadhwa and Jain, 1984b) showed a complex GLC profile containing 44 peaks in the lactonic isolates of ghee. These included homologous series of n-saturated delta- and gamma-lactones (24 peaks) from C_6 to C_{16} and C_{18}. The remaining 20 peaks (unidentified lactones) were partly characterized and were mostly unsaturated lactones. Though GLC profile of lactonic isolates of ghee-residue showed 47 peaks, the labelling was done as 1-12, 14-16, 16a, 16b, 17, 18, 18a, 18b, 19-44 (Fig.6) for comparing it with the GLC profile of lactonic isolates of ghee (Wadhwa and Jain, 1984b) conveniently. The homologous series of both n-saturated delta- and gamma-lactones from C_6-C_{16} and C_{18} (24 peaks) identified in the lactonic isolates of ghee-residue were qualitatively similar to that reported for ghee. Out of remaining 23 peaks (unidentified lactones), position of 19 peaks was simiar to that reported for ghee. Lactonic isolates of ghee-residue showed the presence of four additional peaks 16a, 16b (between delta-C_8 and gamma-C_9), 18a, 18b between (unidentified peak 18 and delta-C_9). Also, an unidentified peak 13 as reported by Wadhwa and Jain (1984b) in the lactonic isolates of ghee was found to be absent in the lactonic isolates of ghee-residue.

Lactone levels expressed as ug/g ghee-residue and ug/g ghee-residue lipid are given in Table 9. Even lactones (delta- as well as gamma-) were predominant lactones in ghee-residue. Further, delta-lactones (even as well as odd) were present in higher amounts than their corresponding gamma-lactones. However, delta-C_{12} (237.32), C_{14}-(2859.42) and C_{18}-(533.62) expressed as ug/g ghee-residue were the major lactones in ghee-residue. The corresponding values expressed as ug/g ghee-residue lipid were 4-7.49, 4904.50 and 920.43 respectively. However, Wadhwa and

Jain (1984b) have reported delta-C_{10} (1.20), C_{12} (3.07), C_{14} (2.25), C_{16} (1.09) and C_{18} (8.14) as major lactones (ug/g) in ghee. The highest lactone levels of delta-C_{14} expressed as ug/g ghee-residue and ug/g ghee-residue lipid were 1270.85 times and 2179.78 times that in ghee respectively.

Total delta-lactone levels expressed as ug/g ghee-residue and ug/g ghee-residue lipid were 3696.96 (3524.64-4012.72) and 6346.13 (5754.73-7058.50) respectively. The corresponding levels for total gamma-lactones were 77.96 (57.57-103.01) and 132.96 (101.25-179.16) respectively. Total delta-lactone levels (ug/g) and total gamma-lactone levels (ug/g) in ghee were 17.90 and 1.76 respectively (Wadhwa and Jain, 1984b).

Saturated delta + gamma-lactone levels as ug/g ghee-residue and ug/g ghee-residue lipid were 3774.91 (3627.65-4070.29) and 6479.10 (5902.36-7159.75) respectively. The corresponding levels for unidentified lactones were 217.94 (158.50-355.45) and 375.78 (265.73-642.83) respectively. Saturated delta + gamma-lactone levels (ug/g) and unidentified lactone level (ug/g) in ghee as reported by Wadhwa and Jain (1984b) was 30.26. Thus, lactone levels expressed as ug/g ghee-residue and ug/g ghee-residue lipid were 131.95 times and 226.53 times that in ghee respectively.

4.2.7 Comparison of spectrophotometric method and GLC method for the estimation of lactones in ghee-residue

Comparison of lactone levels in ghee-residue estimated by the two methods (Cf Table 8,9) revealed that lactone levels

obtained by spectrophotometric method were 3.55% (1.55%-4.48%) lower than that obtained by GLC method. However, statistical analysis showed that differences in the lactone levels estimated by the two methods were non-significant (Table 10).

Hence, the validity of the spectrophotometric method for the estimation of lactones in ghee-residue was confirmed.

4.2.8 Conclusions and future research

The present study has opened a new chapter in our basic knowledge on the flavour potential of ghee-residue. The study has revealed beyond doubt that ghee-residue is a rich source of flavour compounds. Levels of flavour compounds viz. FFA, carbonyls and lactones are higher in ghee-residue than those in ghee. Ghee-residue lipids alongwith flavour compounds extracted by a highly polar solvent like methanol can be exploited as flavour concentrates of ghee-flavour for flavouring bland products viz. vegetable fats/oils, butter oil etc. Alternatively, enrichment of flavour concentrates through heat treatment process can also be used for inducing ghee-flavour in bland products. The shelf-life of flavoured products thus obtained would obviously be higher as ghee-residue is a rich source of natural antioxidants also.

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CHAPTER - V

SUMMARY

5. SUMMARY

5.1 EXTRACTION OF GHEE-RESIDUE LIPIDS

Ghee-residue lipids were extracted by suitably modified Mojonnier method and a newly developed Single solvent (Methanol) method. Lipid (%) in ghee-residue extracted by Methanol method (58.39) was significantly higher ($P \leq 0.05$) than that obtained by Mojonnier method (53.51). Hence, Methanol extraction method was more effective in extracting ghee-residue lipids than the Mojonnier method.

5.2 ESTIMATION OF FFA

FFA levels expressed as uM/g ghee-residue and uM/g ghee-residue lipid in lipid samples extracted by Methanol method (627.48, 1077.37 respectively) were higher than those by Mojonnier method (229.59, 405.52 respectively). Thus, Methanol extraction method was more effective in extracting ghee-residue lipids alongwith free fatty acids as flavour compounds than the Mojonnier method.

FFA levels expressed as uM/g ghee-residue and uM/g ghee-residue lipid in lipid samples (extracted by Methanol method) were 10.75 times and 18.45 times respectively that in ghee (53.55 uM/g). Thus the level of free fatty acids was higher in ghee-residue than in ghee.

5.3 ESTIMATION OF TOTAL CARBONYLS

Total carbonyl levels expressed as uM/g ghee-residue and uM/g ghee-residue lipid in lipid samples extracted by Methanol method (43.65, 75.30 respectively) were higher than those by Mojonnier method (15.28, 28.38 respectively). Thus, Methanol extraction was more effective in extracting ghee-residue lipids alongwith carbonyls as flavour compounds than the Mojonnier method.

Total carbonyl levels expressed as uM/g ghee-residue and uM/g ghee-residue lipid in lipid samples (extracted by Methanol method) were 10.15 and 17.51 times respectively that in ghee (4.30 uM/g). Thus, level of carbonyls was higher in ghee-residue than in ghee.

5.4 STANDARDIZATION OF SPECTROPHOTOMETRIC METHOD FOR THE ESTIMATION OF LACTONES

A spectrophometric method was standardized for the estimation of lactones at 515 nm as their red-violet ferric hydroxamate derivatives.

5.5 ISOLATION OF LACTONES FROM GHEE-RESIDUE LIPIDS BY TLC

Lactones were isolated from ghee-residue lipids (extracted Methanol method) directly through TLC.

5.6 ESTIMATION OF LACTONES IN GHEE-RESIDUE SAMPLES BY SPECTROPHOTOMETRIC METHOD

Lactone levels expressed as ug/g ghee-residue and ug/g ghee-residue lipid were 3850.56 and 6612.28 respectively by spectrophotometric method. These levels were 127.25 times and

218.52 times that in ghee (30.26 ug/g cf. literature report Wadhwa and Jain, 1984b). Thus, level of lactones was higher in ghee-residue than in ghee.

5.7 ESTIMATION OF LACTONES IN GHEE-RESIDUE SAMPLES BY GLC METHOD

Lactone levels expressed as ug/g ghee-residue, ghee-residue lipid were 3992.85 and 6854.88 respectively by GLC method.

5.8 VALIDITY OF SPECTROPHOTOMETRIC METHOD FOR THE ESTIMATION LACTONES IN GHEE-RESIDUE

Statistical analysis revealed that differences in the lactone levels in ghee-residue estimated by spectrophotometric method and GLC method were non-significant. Hence, the validity of the spectrophotometric method for the estimation of lactones in ghee-residue was confirmed.

5.9 CONCLUSIONS AND FUTURE RESEARCH

- Ghee-residue is a rich source of flavour compounds.
- Levels of flavour compounds viz. FFA, carbonyls and lactones are many times in ghee-residue than in ghee.
- Ghee-residue lipids alongwith flavour compounds extracted a highly polar solvent like methanol can be exploited as flavour concentrate of ghee-flavour for flavouring bland products viz. vegetable fats/oils, butter oil etc.
- Alternatively, enrichment of flavour concentrate through heat treatment process can also be used for flavouring bland products.

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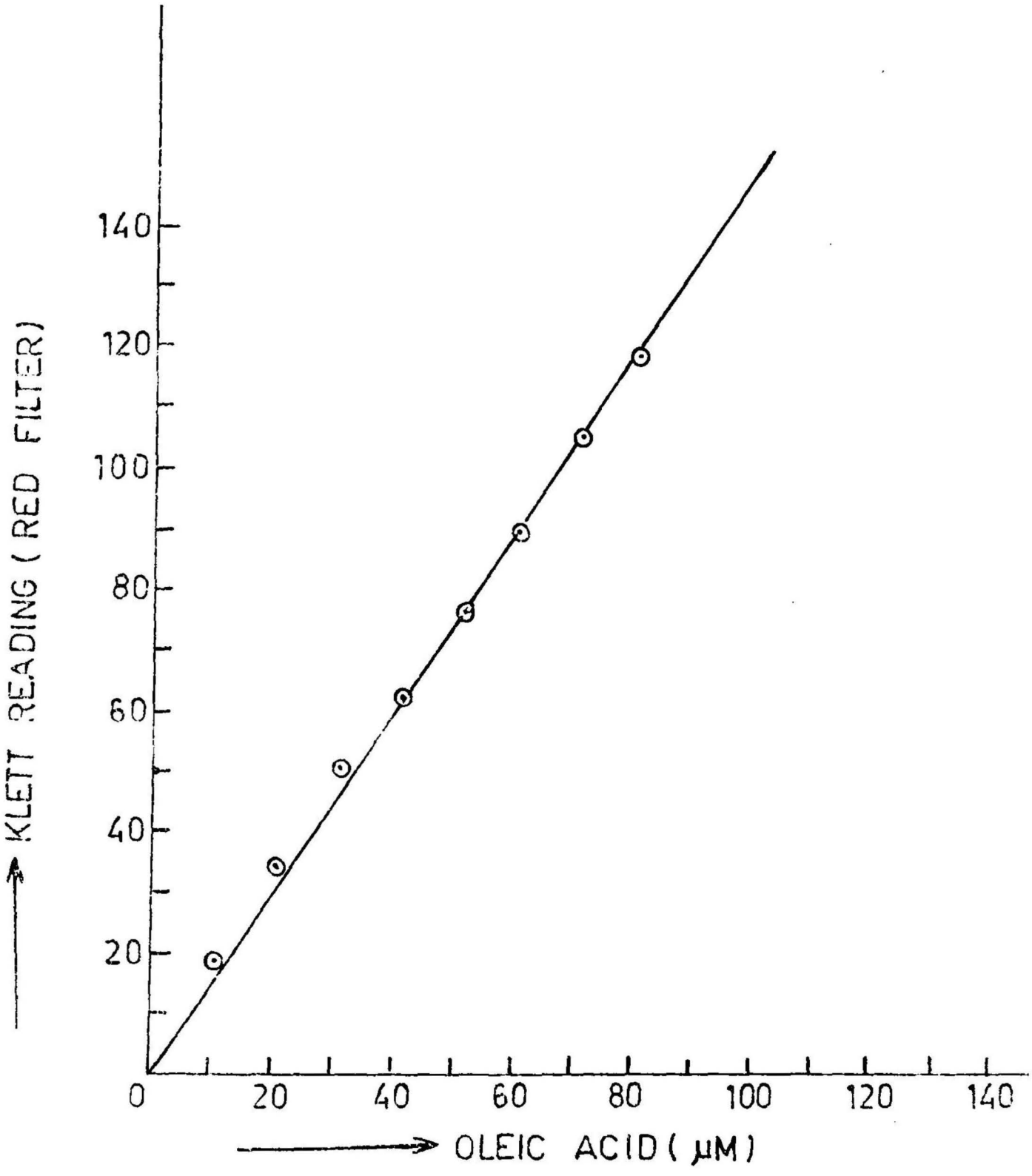
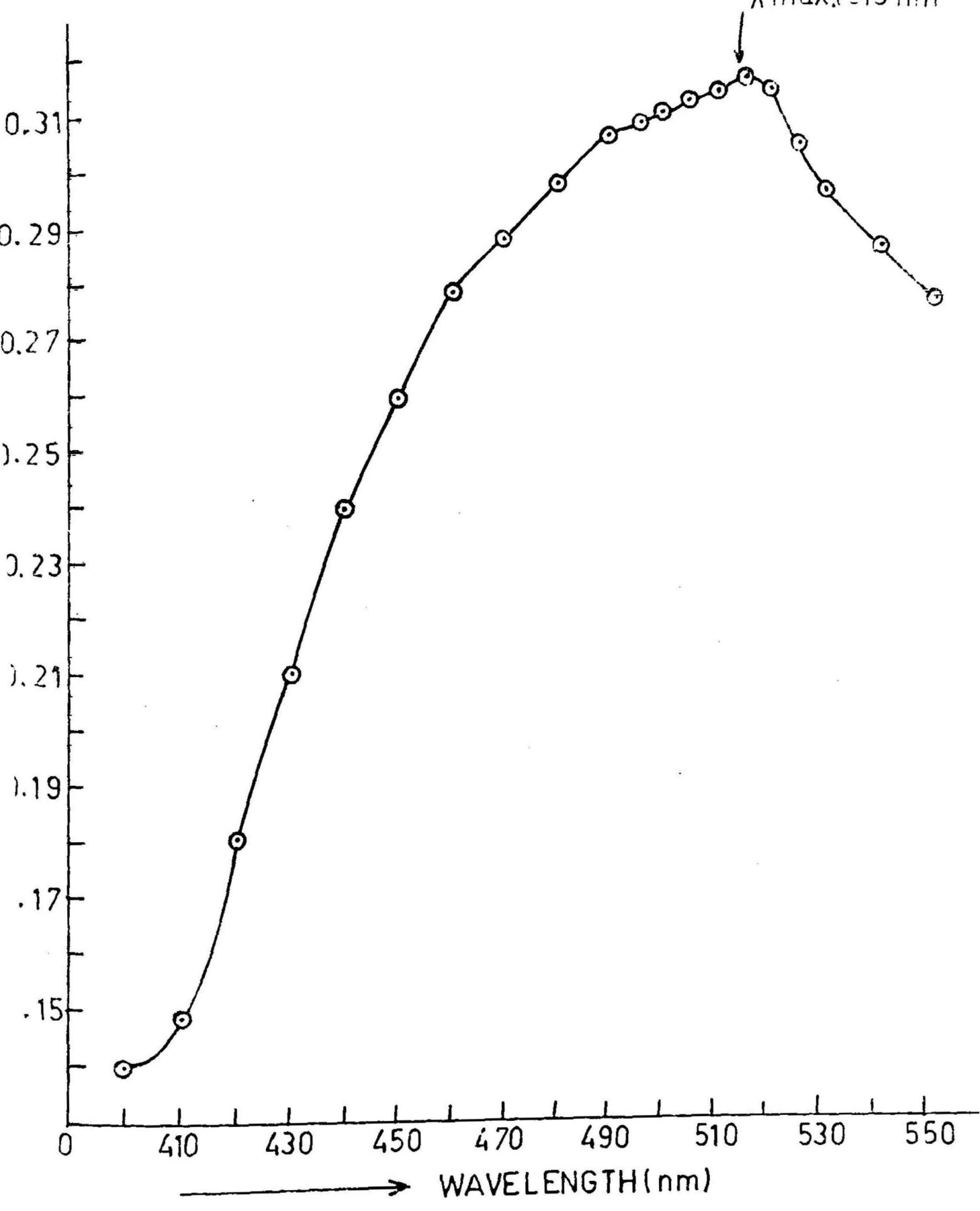
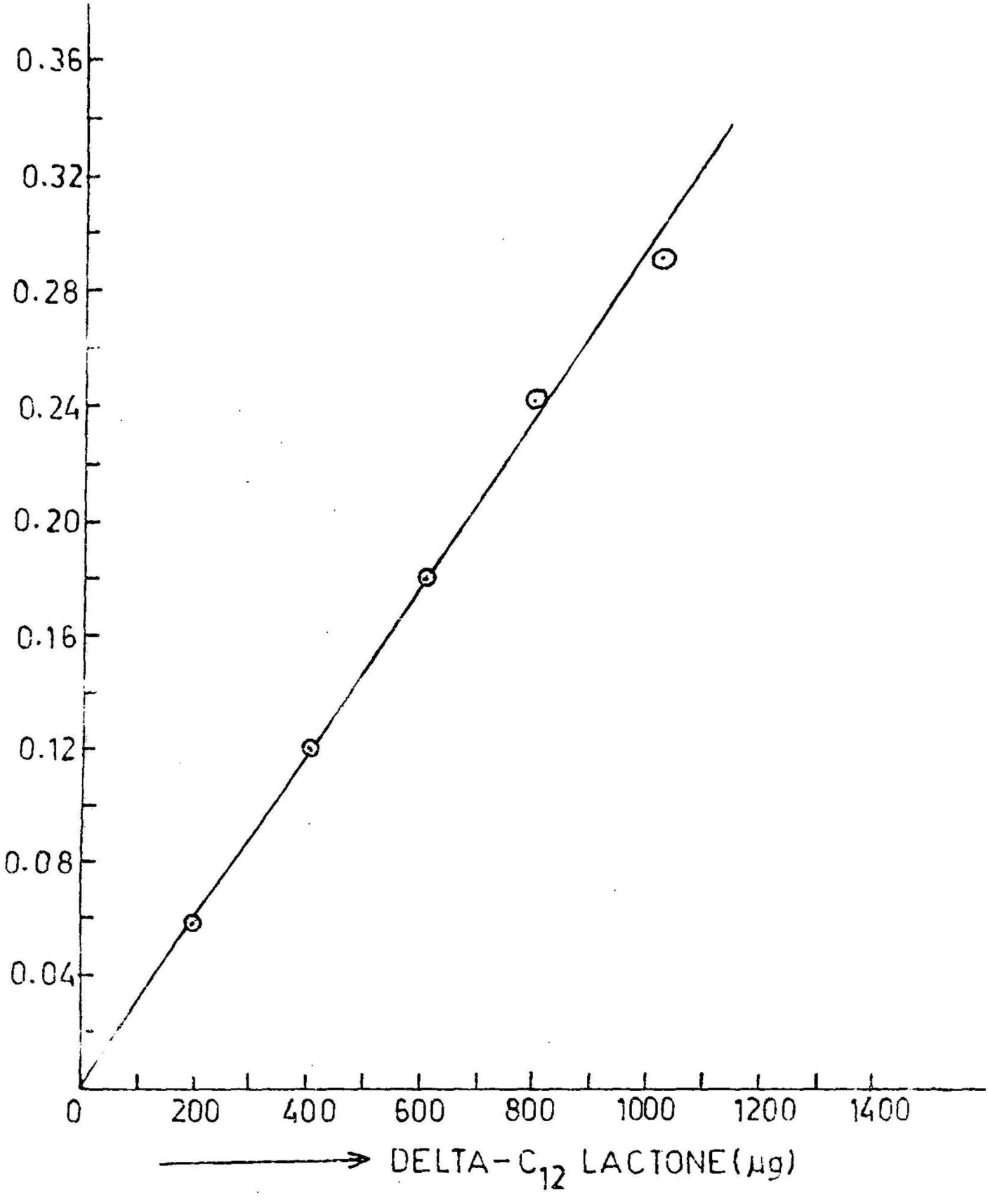


FIG 1 STANDARD CURVE FOR FFA ESTIMATION



2 ABSORPTION MAXIMA (λ max.) OF FERRIC HYDROXAMATE COMPLEX OF DELTA-C_{12} LACTONE



.3 STANDARD CURVE FOR LACTONES ESTIMATION

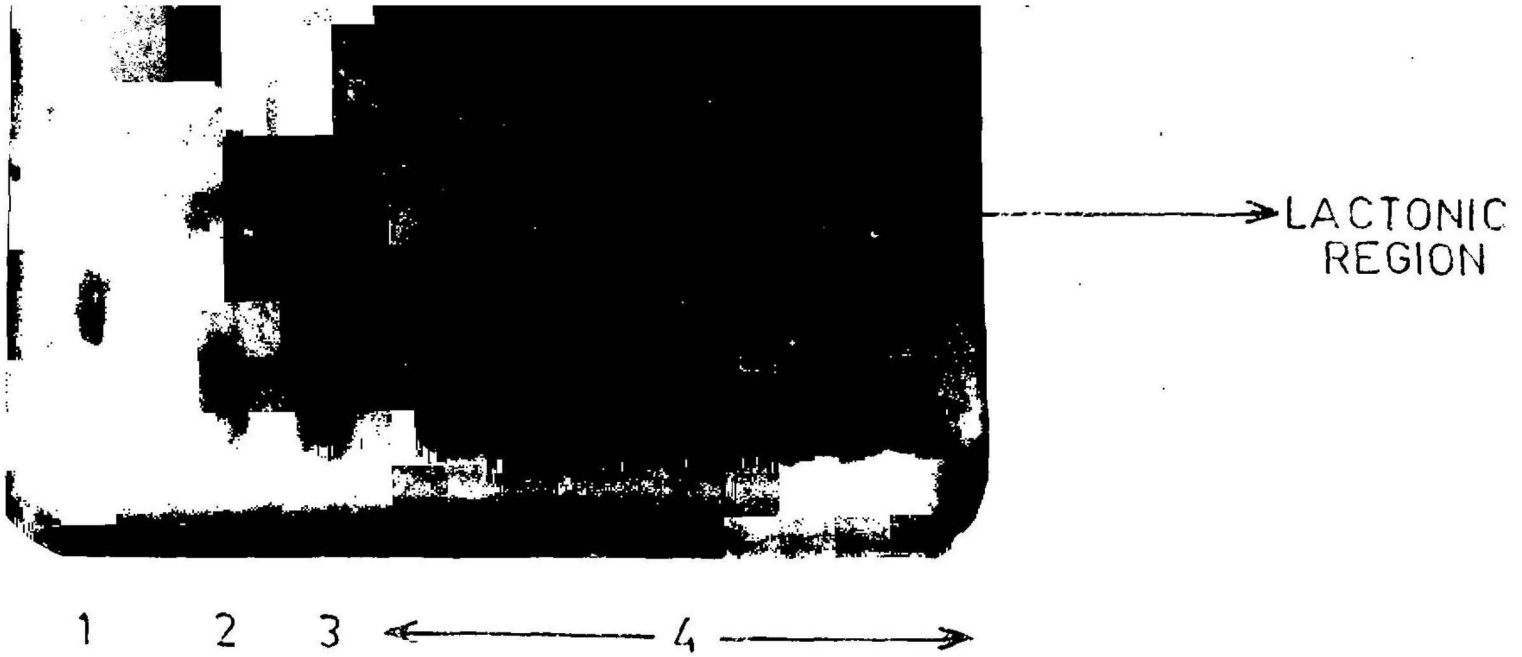


FIG.4 ISOLATION OF LACTONES FROM GHEE-RESIDUE LIPIDS BY T.L.C

1-STANDARD CHOLESTEROL
2-STANDARD LACTONE
3-MIXTURE OF 1 AND 2
4-GHEE-RESIDUE LIPIDS

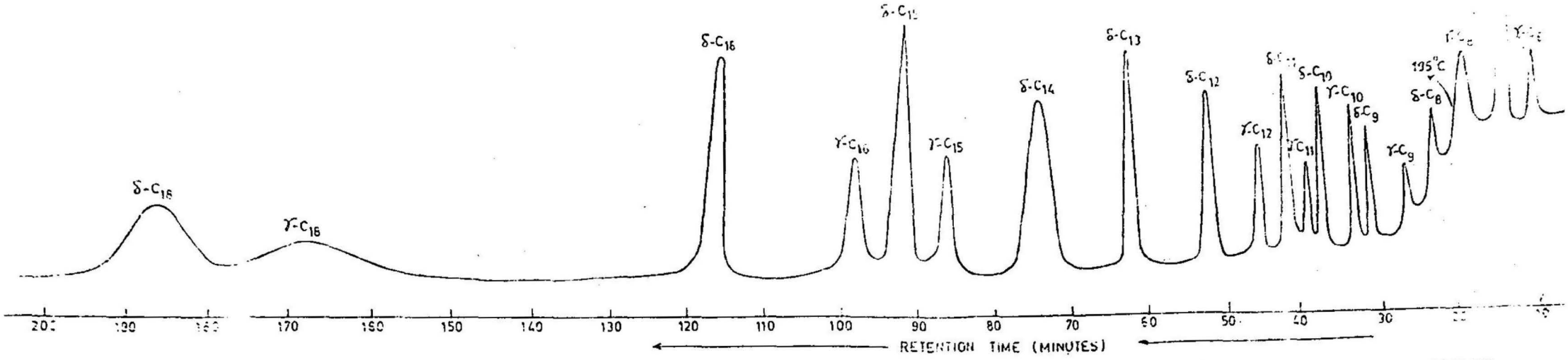


FIG. 5 GLC SEPARATION OF MODEL MIXTURE OF γ-$C_{6,7,8,9,10,11,12,15,16,18}$ AND δ-$C_{8,9,10,11,12,13,14,15,16,18}$ LACTONES

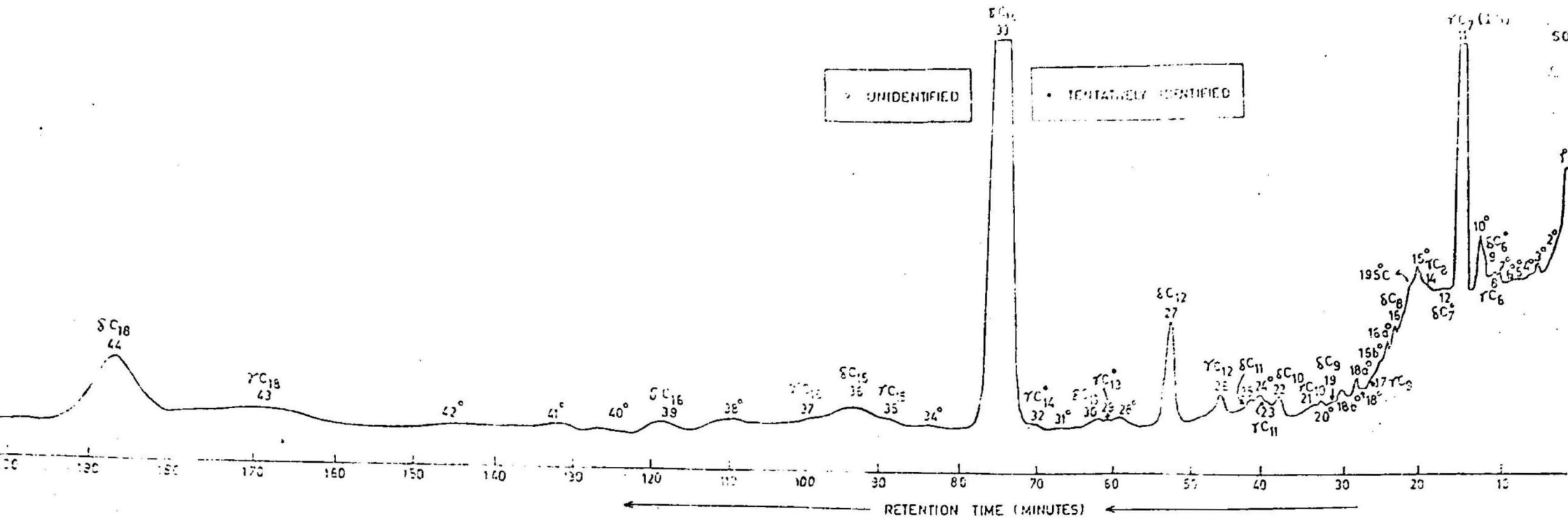


FIG.6 GLC PROFILE OF LACTONES IN GHEE - RESIDUE

Table 1. Lipids (%) in ghee-residue extracted by Mojonnier method and Single solvent (Methanol) method

Method	Sample No				
	1	2	3	4	Average
Mojonnier	52.47	48.25	57.14	56.19	53.51
Single solvent(Methanol)	56.85	57.50	62.66	56.55	58.39

Table 2. Analysis of variance for lipid(%) in ghee-residue extracted by Mojonnier method and Single solvent (Methanol) method

Source of variations	d.f.	Sum of squares	Mean sum of squares	F-value (M_1/M_2)
Between methods	1	72.14	72.14 (M_1)	7.13*
Within methods	6	60.73	10.12 (M_2)	

*Significant ($P/0.05$)

Table 3. FFA levels expressed as uM/g ghee-residue, ghee-residue lipid and ghee

Method	Ghee-residue					Ghee-residue lipid					Ghee				
	Sample[a] No.					Sample[a] No.					Sample No.				
	1	2	3	4	Average	1	2	3	4	Average	1	2	3	4	Average
											50.82	52.09	61.68	49.59	53.55
Mojonnier	163.52	349.31	204.85	254.32	229.59	311.61	724.04	358.49	452.54	405.52					
Single solvent	665.64	543.75	618.16	682.38	627.48	1170.89	945.65	986.42	1206.52	1077.37					

a - Analysis done in duplicate

Table 4. Total carbonyl levels expressed as uM/g ghee-residue, ghee-residue lipid and ghee

Method	Ghee-residue					Ghee-residue lipid					Ghee				
	Sample[a] No.					Sample[a] No.					Sample No.				
	1	2	3	4	Average	1	2	3	4	Average	1	2	3	4	Average
											3.50	4.31	4.97	4.42	4.30
Mojonnier	12.27	12.53	15.86	20.46	15.28	23.38	25.96	27.75	36.41	28.38					
Single solvent	55.45	27.90	35.90	55.34	43.65	97.54	48.52	57.28	97.85	75.30					

a - Analysis done in duplicate

Table 5. Absorbance values (OD) of various trials used for standardising spectrophotometric method for the estimation of lactones

Lactone concentration (ug)	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
200	0.03	0.04	0.058	0.16	0.31
600	0.08	0.10	0.18	0.35	0.79
1000	0.10	0.15	0.30	1.18	1.17

Table 6. Change in OD of ferric hydroxamate of lactone with time

Time (min)	OD
0	0.18
10	0.18
20	0.17
30	0.16
60	0.15

Table 7. Klett readings (green filter) of ferric hydroxamates of lactones over colorimeter-102

Lactone concentration(ug)	Klett reading
100	11
200	20
300	33
400	38.5
500	52
600	62
700	78
800	90
900	100
1000	114

Table 8. Lactone levels expressed as ug/g ghee-residue and ghee-residue lipid estimated by spectrophotometric method

Ghee-residue					Ghee-residue lipid				
Sample No.					Sample No.				
1	2	3	4	Average	1	2	3	4	Average
4263.70	3750.00	3700.00	3688.52	3850.56	7499.91	6521.74	5904.88	6522.58	6612.28

Table 9. Lactone levels expressed as ug/g ghee-residue lipid and ghee-residue estimated by GLC

C-No.	Ghee-residue									
	Delta-Lactone Sample No.					Gamma-lactones Sample No.				
	1	2	3	4	Average	1	2	3	4	Average
6	5.69	1.75	7.58	1.13	4.04	t	t	t	t	t
7	1.95	1.46	1.70	2.04	1.79	-	-	-	-	-
8	9.10	5.49	5.46	2.55	5.43	6.82	1.17	3.33	1.36	3.17
9	t	t	t	t	t	t	t	t	t	t
10	19.90	10.94	20.02	28.57	19.86	5.12	6.13	14.56	2.55	7.09
11	5.69	4.38	7.89	3.82	5.45	0.57	0.59	0.69	0.63	0.62
12	272.91	246.58	225.67	204.12	237.32	9.95	32.68	7.89	16.67	16.80
13	6.25	14.01	10.01	4.59	8.72	2.56	9.92	6.42	4.08	5.75
14	3002.02	2737.06	2891.90	2806.71	2859.42	5.69	26.26	5.03	2.72	9.93
15	9.49	8.25	10.21	8.52	9.12	t	t	t	t	t
16	6.25	8.75	18.20	15.64	12.21	t	t	t	t	t
18	673.47	485.87	407.67	566.45	533.62	26.86	26.26	54.60	30.71	34.61
Total	4012.72	3524.64	3606.31	3644.14	3696.96	57.57	103.01	92.52	58.72	77.35

C-No.	Ghee-residue									
	Delta-Lactone Sample No.					Gamma-lactones Sample No.				
	1	2	3	4	Average	1	2	3	4	Average
6	5.69	1.75	7.58	1.13	4.04	t	t	t	t	t
7	1.95	1.46	1.70	2.04	1.79	-	-	-	-	-
8	9.10	5.49	5.46	2.55	5.43	6.82	1.17	3.33	1.36	3.17
9	t	t	t	t	t	t	t	t	t	t
10	19.90	10.94	20.02	28.57	19.86	5.12	6.13	14.56	2.55	7.09
11	5.69	4.38	7.89	3.82	5.45	0.57	0.59	0.69	0.63	0.62
12	272.91	246.58	225.67	204.12	237.32	9.95	32.68	7.89	16.67	16.80
13	6.25	14.01	10.01	4.59	8.72	2.56	9.92	6.42	4.08	5.75
14	3002.02	2737.06	2891.90	2806.71	2859.42	5.69	26.26	5.03	2.72	9.93
15	9.49	8.25	10.21	8.52	9.12	t	t	t	t	t
16	6.25	8.75	18.20	15.64	12.21	t	t	t	t	t
18	673.47	485.87	407.67	566.45	533.62	26.86	26.26	54.60	30.71	34.61
Total	4012.72	3524.64	3606.31	3644.14	3696.96	57.57	103.01	92.52	58.72	77.96

Lactone levels	Sample No.				
	1	2	3	4	Average
Saturated delta- + gamma-	4070.29	3627.65	3698.83	3702.86	3774.91
Unidentified	365.45	181.29	166.52	158.50	217.94
Gross	4435.74	3808.94	3865.35	3861.36	3992.85

.....contd. Table 9

C-No.	Ghee-residue lipid									
	Delta-lactone Sample No.					Gamma-lactone Sample No.				
	1	2	3	4	Average	1	2	3	4	Average
6	10.00	3.05	12.10	2.00	6.79	t	t	t	t	t
7	3.43	2.54	2.71	3.61	3.07	-	-	-	-	-
8	16.00	8.00	8.71	4.51	9.31	12.00	2.03	5.32	2.41	5.44
9	t	t	t	t	t	t	t	t	t	t
10	35.00	19.03	31.95	50.52	34.13	9.00	10.66	23.23	4.51	11.85
11	10.00	7.61	12.58	6.77	9.24	1.00	1.02	1.10	1.08	1.05
12	480.06	428.84	360.12	360.92	407.49	17.50	56.84	12.58	29.47	29.10
13	11.00	24.36	15.97	8.12	14.86	4.50	17.26	10.25	7.22	9.80
14	5280.67	4760.00	4614.73	4962.59	4904.50	10.00	45.67	8.02	4.81	17.13
15	16.69	14.35	16.29	15.06	15.60	t	t	t	t	t
16	11.00	15.23	29.04	27.67	20.74	t	t	t	t	t
18	1184.65	844.98	650.53	1001.54	920.43	47.25	45.68	87.13	54.31	58.59
									103.81	132.96

.....contd. Table 9

C-No.	Ghee-residue lipid									
	Delta-lactone Sample No.					Gamma-lactone Sample No.				
	1	2	3	4	Average	1	2	3	4	Average
6	10.00	3.05	12.10	2.00	6.79	t	t	t	t	t
7	3.43	2.54	2.71	3.61	3.07	-	-	-	-	-
8	16.00	8.00	8.71	4.51	9.31	12.00	2.03	5.32	2.41	5.44
9	t	t	t	t	t	t	t	t	t	t
10	35.00	19.03	31.95	50.52	34.13	9.00	10.66	23.23	4.51	11.85
11	10.00	7.61	12.58	6.77	9.24	1.00	1.02	1.10	1.08	1.05
12	480.06	428.84	360.12	360.92	407.49	17.50	56.84	12.58	29.47	29.10
13	11.00	24.36	15.97	8.12	14.86	4.50	17.26	10.25	7.22	9.80
14	5280.67	4760.00	4614.73	4962.59	4904.50	10.00	45.67	8.02	4.81	17.13
15	16.69	14.35	16.29	15.06	15.60	t	t	t	t	t
16	11.00	15.23	29.04	27.67	20.74	t	t	t	t	t
18	1184.65	844.98	650.53	1001.54	920.43	47.25	45.68	87.13	54.31	58.59
Total	7058.50	6127.99	5754.73	6443.31	6346.13	101.25	174.16	147.63	103.81	132.96

Lactone levels	Sample No. 1	2	3	4	Average
Saturated delta- + gamma	7159.75	6307.15	5902.36	6547.12	6479.10
Unidentified	642.83	314.25	265.73	280.30	375.78
Gross	7802.58	6621.40	6168.09	6827.42	6854.88

Table 10. Analysis of variance for lactone levels estimated by spectrophotometric and GLC methods

Source of variation	d.f.	Sum of squares	Mean sum of squares	F-value(M_1/M_2)
Between methods	1	117709.52	117709.52(M_1)	0.26^{NS}
Within methods	6	2729084.58	454847.43(M_2)	

NS = Non-significant