CHAPTER IV

EXPERIMENTAL
Experimental animals - In this study, all the lactating cows and buffaloes of National Dairy Research Institute Karnal, were included. The herd comprised of 320 cows (Sahiwal 30%, Tharparkar 30%, Red Sindhi 15% and cross bred of Sahiwal and Brown swiss 25%) and 60 Murrah buffaloes. All the animals were managed and housed under identical conditions in the open type barn.

All the animals were fed balanced ration consisting of roughages and concentrate mixture. The requirements for maintenance and for the first four kg milk production were met from the roughages, and further one kg concentrate was allotted for every additional 2.5 kg of milk produced in case of cows and 2 kg of milk produced in case of buffaloes. Both leguminous and non-leguminous roughages were provided to animals, depending upon availability. Non-leguminous fodders consisted of maize, oats and Jowar (green or in silage forms) and leguminous fodders were berseem, lucerne and cowpea (green or silage forms). The concentrate mixture contained groundnut cake (35%), Wheatbran (25%), barley (38%), common salt (1%) and mineral mixture (1%). The concentrate mixture was offered to the animals at
the time of milking and roughages were given twice daily. All the animals had free access to drinking water.

**Milkfat samples** — Raw milk, drawn from separately pooled milks of cows and buffaloes maintained at the farm of the Institute, was used as the starting material for the preparation of ghee. Ghee was prepared by desi method essentially according to the method of Srinivasan and Anantakrishnan (1964). DRC-I (*Streptococcus diacetilactis*), at the level of 1%, was used as the starter culture for the preparation of dahi (curd) and the dahi obtained was churned into butter. Butter was clarified into ghee at 110°C, the temperature of clarification being maintained for 5-7 minutes. Molten ghee, after filtration, was transferred into lacquered tin containers, stoppered loosely and then stored at 37°C in an incubator for 200 days.

For comparative studies on the carbonyls of ghee (fresh and stored) prepared from fresh and ripened desi-butter (cow and buffalo), butter was divided into two parts. The first part was immediately clarified into ghee at 110°C as before. The second part was allowed to 'ripen' at room temperature for 3 days. The acidity levels (as percentage oleic acid) of fresh butter from cow and buffalo milk were respectively,
0.056-0.067 and 0.044-0.063 and those of 'ripened' butter were respectively, 0.118-0.147 and 0.045-0.085. It may be noted that no appreciable changes occurred in the acidity levels of buffalo butter under conditions comparable to those used for cow butter. The butter was then clarified into ghee at 110°C as before. The molten ghee was transferred into open tin containers lacquered from inside and stored for 100 days at 37°C.

Solvents - All the solvents used were of AnalaR grade, unless stated otherwise. n-Hexane and Petroleum ether were made carbonyl-free, where necessary, by the procedure of Schwartz and Parks (1961). Solvent ether and ethanol were made carbonyl-free essentially according to the procedure of Gordon et al (1951).

Adsorbents - Kieselguhr G (E.M.), celite 545 (Koch-Light), magnesium oxide light for TLC (M&B) and magnesium oxide for chromatographic adsorption analysis (BDH) were used. Magnesium oxide (Seaworb-43) for chromatographic adsorption analysis was also obtained through the courtesy of Dr. O.W. Parks (U.S.A.).

Aluminium oxide - Aluminium oxide, standard for chromatographic adsorption analysis according to Brockmann (E.M.) was activated by heating for 24 hours.
at 150°C, then partially deactivated by addition of 6% (W/W)
distilled water. The wet alumina was shaken until all
lumps were broken and allowed to equilibrate overnight.

2,4-Dinitrophenylhydrazine (DNPH) reagent - This was
prepared by dissolving DNPH (1.2 g) in 2 N HCl (300 ml).

2,4-Dinitrophenylhydrazones (DNPs) of authentic carbonyls -
DNPs of alkan-2-ones (C₃-C₉), alkanals (C₁, C₂, C₄, C₆),
alk-2-enals (C₄), diacetyl, methylglyoxal and acetaldehydes
were prepared from pure samples, obtained from commercial
sources, by the general procedure outlined by Vogel (1971).

Alkanals (C₇-C₁₀), alk-2-enals (C₉-C₁₀), alka-2,4-dienals
(C₅-C₆) were the generous gifts of Dr. D. A. Forss of
International Flavour and Fragrances, U.S.A., and were used
to prepare the corresponding DNPs by the method of Vogel
(1971). The carbonyl-DNPs prepared in this laboratory were
checked by comparative chromatographic, spectroscopic and
mixed melting point data of the authentic carbonyl-DNPs
kindly supplied by Dr. O. W. Parks of U.S.D.A., Department of
Agriculture, U.S.A.

DNPs of alkan-2-ones (C₁₂-C₁₃, C₁₅, C₁₇ and C₁₉),
alkanals (C₃, C₁₀, C₁₂, C₁₄, and C₁₉), alk-2-enals (C₅, C₁₀-C₁₂)
and alka-2,4-dienals (C₉-C₁₂ and C₁₄) were also the generous
gifts by Dr. O. W. Parks (U.S.A.).
The diacetyl-DNP (m.p 316-317°C) and methyl glyoxal-DNP (m.p 308°C), prepared in this laboratory, corresponded to diacetyl-bis-DNP (m.p 315°C, Lit.) and methylglyoxal-bis-DNP (m.p 310°C, Lit.), respectively.

Thin layer chromatography (TLC) - Desaga Applicator (adjustable) was used for the preparation of TLC plates.

Spectra - Ultraviolet and visible spectrophotometer (VSU-2, Zeiss) was used to record the spectra reported in this study.

Gas liquid chromatography (GLC) - The analyses involving GLC were performed on F & M, Model 609, flame ionization, gas chromatograph. The stationary liquid phase was carbowax-20M (Sigma) supported on diatoport W (60-80 mesh) (F & M corp.).

Sensory evaluation of ghee samples - Ghee samples were subjected to sensory evaluation by a Flavour Panel consisting of seven judges. For this purpose, ghee samples were kept in 100 ml beakers at a temperature of 40°C and then examined independently by the members of the Panel in an environment free from extraneous odours. Not more than four samples were analysed at a time for sensory evaluation. The samples were rated: Very good (readily acceptable); or good (acceptable); or poor (doubtful acceptability) or mildly rancid (doubtful acceptability and off-flavour
becomes perceptible) or highly rancid (not acceptable).

**Determination of free fatty acid (FFA) levels, peroxide value (PV) and thiobarbituric acid (TBA) values** - FFA level (as percentage oleic acid) and PV were determined according to ISI method (Anon, 1960). TBA value was determined by the method of Gaba and Jain (1973).

**Estimation of 'volatile' carbonyls** - Ghee (10g) was steam distilled in an all-glass apparatus and the distillate (150 ml) was collected in a receiver containing DNP reagent (50 ml). The distillate was allowed to stand overnight and the DNP reagent extracted with 4 x 25 ml n-hexane (carbonyl-free). The extract, washed with distilled water (3 x 25 ml), was dried over anhydrous sodium sulphate. Carbonyl level was estimated in the resulting yellow extract from its optical density measurements at 340 nm (λ22,500). A blank experiment was run simultaneously.

**Estimation of the 'total' carbonyls** - The total carbonyl content of ghee was estimated essentially according to the procedure of Schwartz et al (1963) as modified by Ramamurthy and Jain (1973).

**Estimation of the 'head space' carbonyls** - Dry nitrogen gas was passed for 8-10 hours through ghee (50g) maintained at 40°C. The 'head space' carbonyls were trapped as DNPs by allowing the effluent nitrogen gas to bubble through DNP...
The solution was allowed to stand overnight in refrigerator and extracted with carbonyl-free n-hexane (4 x 25 ml). The extract was washed with distilled water (3 x 25 ml) until the washings were free of yellow colour. The extract was dried over anhydrous sodium sulphate. The optical density of the resulting solution was measured at 340 nm and the carbonyl level expressed as micromoles (\mu\text{moles}) using the value of \( \varepsilon = 22,500. \)

**Isolation of the 'volatile' carbonyls** - Ghee (1 kg, in the case of fresh ghee or 500 g in the case of oxidized ghee) was steam distilled and the distillate (1.5 L in case of fresh ghee and 4 L in the case of oxidized ghee) was trapped in the DNPH reagent (300 ml for fresh ghee and 4 x 300 ml for oxidized ghee). The distillate was allowed to stand overnight and the precipitated DNPs were collected by filtration under suction.

**Isolation of the 'head space' carbonyls** - The 'head space' carbonyls were isolated (as DNPs) by trapping the volatiles, removed from ghee (500 g) by a stream of nitrogen gas, in the DNPH reagent (100 ml) as described earlier. The solution was allowed to stand overnight and extracted with n-hexane (carbonyl-free) and the hexane removed to obtain DNPs.
TLC separation of the ‘volatile’ and ‘head space’ carbonyls into monocarbonyl- and dicarbonyl - DNP's - A mixture of sieved magnesia (150 mesh) and celite 545(2:1) was slurred into distilled water (5 parts) and the slurry was immediately coated on to glass plates (10 x 20 cms) to get a thickness of about 250 μm (if the plates are to be prepared by hand, the ratio of the adsorbent mixture and water should be 1:6). The plates were air dried, heated at 110°C for 2 hours, and then stored in a desiccator over anhydrous calcium chloride.

The mixture of DNP's (in chloroform) was spotted on the above plates and separated into monocarbonyl-DNP’s and dicarbonyl-bis-DNP’s, using the solvent system nitromethane (Eastman Kodak, USA)-Chloroform(1:3). The development of the plates was carried out in a chromatographic chamber equilibrated with the above solvent system for about ten minutes prior to insertion of plates. The development was stopped after about 20 minutes. The plates were removed from the chamber, air dried and heated for 2-3 minutes at 110°C. The spots were detected visually.

Isolation of the ‘total’ carbonyls from ghee - ‘Total’ monocarbonyls were isolated from ghee (as DNP’s) essentially according to the method of Schwartz et al(1963). The method involved formation of DNP’s of the ‘total’ carbonyls in ghee by direct reaction on celite-hydrazine-phosphoric acid column of Schwartz and Parks(1961); adsorption of the
resulting DNPs on magnesia-celite(1:1) column while eliminating the bulk of fat followed by desorption of the DNPs (other than dicarbonyl DNPs) and separation of the monocarbonyl-DNPs by column chromatography on alumina (Schwartz and Parks, 1961). The 'total' dicarbonyl DNPs were isolated as follows: The adsorbent mixture (magnesia-celite, 1:1), after the desorption of monocarbonyl DNPs (above) with solvent mixture nitromethane-chloroform (1:3), was removed from the column and extracted with methanol (5 x 5 ml). Evaporation of the solvent left a dark-brown sticky material which was then subjected to TLC on magnesia-celite (2:1) plates, using the solvent system nitromethane-chloroform (1:3). Dicarbonyl-bis-DNPs moved as a violet band on the plate.

TLC separation of monocarbonyl-DNPs into different classes, their characterization and estimation - The above monocarbonyl-DNPs were subjected to preparative TLC, on magnesia-celite (2:1) plates, prepared as usual and heated for 2 hours at 110°C prior to spotting, using methanol-hexane (1:25:100) as the solvent system. The plates were developed in a chamber which had been saturated for about 10 minutes with the solvent system prior to insertion of plates. The development was stopped when the solvent front had travelled about 15 cm (in about 1/2 hour). The plates were removed from the chamber, air dried and heated at 110°C for 2-3 minutes.
For estimation of the relative proportions of the monocarbonyl classes, the above mentioned preparative TLC on magnesia-celite(2:1) layers was repeated several times.

The appropriate bands were scrapped and the respective monocarbonyl-DNPs eluted out with  A. The proportion of each class was estimated spectrophotometrically as usual.

**TLC separation of the monocarbonyl-DNP classes into individual components and their estimation**

Glass plates (10 x 20 cm), coated with Kieselguhr G layers (250 µ thick), were air dried and impregnated with carbowax-400 (10% solution in acetone). The DNP-mixture in chloroformic solution was spotted on the plate. The chromatogram was developed in a pre-equilibrated chamber, with hexane-pentane(1:1) as the solvent system. The development was stopped when the solvent had travelled about 10-15 cm; plates removed and air dried. Authentic DNPs were also run simultaneously.

For estimation of the relative proportions of individual components of each class, repeated preparative TLC on Kieselguhr G layers was performed. The appropriate bands were scrapped and the extruded zones shaken thoroughly with n-hexane to elute out the respective carbonyl-DNPs.

The proportion of each DNP was estimated spectrophotometrically as usual.
Separation by GLC of the monocarbonyl-DNP classes into individual components - The monocarbonyl-DNP mixture (about 10 mg) of each class was dissolved in laevulinic acid (0.2 ml) by heating at 60-70°C for 2-4 minutes. The resulting mixture (10 μl) was injected directly into the gas chromatograph using stainless steel column (150 x 15/ cm) packed with carbowax 20 M (35%) on Diatoport W (60-80 mesh) at a column temperature of 125°C. The rate of flow of carrier gas, nitrogen, was 60 ml/min. The temperature of both the injection port and detector was 250°C. The various peaks were identified by comparative GLC of the authentic carbonyl-DNPs carried out under identical conditions.

TLC separation of the dicarbonyl-DNPs - The dicarbonyl-bis-DNPs, isolated from ghee, were chromatographed over magnesia-celite (2:1) plates prepared as before and heated for 2 hours at 110°C, using the solvent system hexane-methanol-chloroform (5:5:90). Authentic dicarbonyl-DNPs were also run simultaneously.