RESULTS AND DISCUSSION
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Contents of 'volatile', 'Total' and 'head space' carbonyls in ghee and changes during storage -

(a) 'Volatile' carbonyls - 'Volatile' carbonyls were isolated (as DNPs) by steam distillation technique from 8 samples each of cow and buffalo ghee stored for 0, 100 and 200 days at 37°C. Their content was estimated spectrophotometrically after extraction of the DNPs with hexane (Table 1). The data were analysed statistically in a factorial experiment conducted in a randomized block design (Table 2). The over-all variations in the contents of 'volatile' carbonyls of cow and buffalo ghee were not significant. Highly significant (P < 0.01) differences were observed in the 'volatile' carbonyl contents due to storage periods. The interaction between the species and storage period was also significant (P < 0.01).

The content of 'volatile' carbonyls in fresh cow ghee (0.33 μM/g fat: 0.31–0.37 μM/g fat) was significantly higher (P < 0.01) than that of fresh buffalo ghee (0.26 μM/g fat: 0.23–0.28 μM/g fat). The PV, TBA values and FFA levels were respectively 0.0, 0.0 and 0.27–0.33 for fresh cow ghee and 0.0, 0.25–0.50 and 0.19–0.24 respectively for fresh buffalo ghee. Both types of ghee, on storage for 100 days, developed mild, off-flavour with
PV, TBA and FFA level varying between 2.5-22.6, 0.30-0.90 and 0.40-0.56 respectively for cow ghee and 3.8-24.00, 0.70-1.48 and 0.25-0.28 respectively for buffalo ghee, followed by a significant (P ≤ 0.01) rise (2-3 fold) in the 'volatile' carbonyl content (from 0.33 μM/g fat to 0.71 μM/g fat in cow ghee and from 0.26 μM/g fat to 0.71 μM/g fat in buffalo ghee). However, the differences in the levels of 'volatile' carbonyls in the two species, after 100 days storage, were non significant.

On storage for 200 days all the ghee samples developed pronounced off-flavour (the PV, TBA values and FFA levels varying between 28.8-39.5, 3.20-4.20 and 0.63-0.96, respectively for cow ghee and 29.5-115.0, 2.90-3.85 and 0.50-0.62, respectively for buffalo ghee) and the 'volatile' carbonyl content showed again a significant rise (P ≤ 0.01). The level rose 4 to 5 folds (from 0.33 μM/g fat to 1.25 μM/g fat in cow ghee and from 0.26 μM/g fat to 1.41 μM/g fat in buffalo ghee). The 'volatile' carbonyls level was also significantly higher (P ≤ 0.05) in buffalo ghee than in cow ghee, after 300 days of storage. Day and Lillard (1960) had also reported an increase in the 'volatile' carbonyl content of milk fat on storage.
(b) 'Total' carbonyls - 'Total' carbonyls were estimated in 8 samples each of cow and buffalo ghee stored for 0, 100 and 200 days at 37°C (Table 3). The data were analysed statistically in a factorial experiment conducted in 'randomized block design' (Table 4). Significant differences (P ≤ 0.01) were observed in the levels of 'total' carbonyls of cow and buffalo ghee. The increase in the level of 'total' carbonyls on storage of ghee was also highly significant (P ≤ 0.01). The interaction between species and storage period was also significant (P ≤ 0.01).

The content of 'total' carbonyls (Table 3) in fresh buffalo ghee (8.84 µM/g fat; 8.43-8.82 µM/g fat) was significantly higher (P ≤ 0.01) than that of fresh cow ghee (7.20 µM/g fat; 6.84-7.61 µM/g fat). In contrast, cow ghee contained significantly higher levels (0.33 µM/g fat) of 'volatile' carbonyls than the buffalo ghee (0.28 µM/g fat), as reported earlier (Table 1). The non-volatile carbonyls thus constituted about 95.15% of the 'total' carbonyls in fresh cow ghee and 97% in fresh buffalo ghee. This was in broad agreement with the earlier reports (Lillard and Day, 1961; Lea and Swoboda, 1962; and Jain et al, 1970).

Both cow and buffalo ghee, on storage for 100 days, developed mild off-flavour with two fold increase (P ≤ 0.01) in the 'total' carbonyl content (from
7.20 μM/g fat to 13.93 μM/g fat in cow ghee and from 8.64 μM/g fat to 14.53 μM/g fat in buffalo ghee.

However, the carbonyl content of cow and buffalo ghee after 100 days of storage were of the same order. At the end of 200 days, all the ghee samples developed marked off-flavours with a further significant increase (P < 0.01) in the 'total' carbonyl content. There was a three fold increase (from 7.20 μM/g fat to 23.05 μM/g fat in cow ghee and from 8.64 μM/g fat to 23.99 μM/g fat in buffalo ghee) in the 'total' carbonyl content of ghee on storage for 200 days, irrespective of the type of ghee (cow or buffalo).

However, the differences in 'total' carbonyl contents of cow and buffalo ghee on 200 days of storage, were not significant. Lillard and Day (1981) had also reported higher carbonyl levels in oxidised milk fat than in fresh milk fat. Increase in the 'volatile' carbonyl content also during storage of ghee has already been observed, but the rate of increase in the case of 'total' carbonyls was somewhat lower than in the case of 'volatile' carbonyls.

(c) "Head space" carbonyls — "Head space" carbonyls were isolated (as DNP's) from 8 samples each of cow and buffalo ghee stored for 0, 100 and 200 days at 37°C, by passing a stream of nitrogen gas through ghee. Variations in their contents have been listed in Table 5,
and the data were analysed statistically (Table 6). Significant differences ($P \leq 0.01$) occurred in the levels of 'head space' carbonyls of cow and buffalo ghee. The increase in the level of 'head space' carbonyls, on storage of ghee, was also highly significant ($P \leq 0.01$). The interaction between species and storage period was also significant ($P \leq 0.01$).

There were significantly higher ($P \leq 0.01$) contents of 'head space' carbonyls in fresh cow ghee ($0.035 \mu M/g fat$ to $0.030-0.044 \mu M/g fat$) than in fresh buffalo ghee ($0.027 \mu M/g fat$ to $0.022-0.029 \mu M/g fat$). In contrast, the 'total' carbonyl content was higher in buffalo ghee than that in cow ghee (Table 3), but the 'volatile' carbonyl content was higher in cow than in buffalo ghee (Table 1). It is noteworthy that 'head space' carbonyl content of fresh ghee was about 1/10th that of the 'volatile' carbonyl content, irrespective of the type of ghee.

On storage for 100 days, there was a significant ($P \leq 0.01$) 4-5 fold rise in the 'head space' carbonyl content (from $0.035 \mu M/g fat$ to $0.141 \mu M/g fat$ in cow ghee and from $0.027 \mu M/g fat$ to $0.126 \mu M/g fat$ in buffalo ghee) and the samples developed mild rancidity. At the end of 100 days, cow ghee contained significantly higher levels ($P \leq 0.01$) of 'head space' carbonyls than
buffalo ghee. On storage for 200 days all the ghee samples developed pronounced off-flavour and the 'head space' carbonyl levels increased 9–10 folds (from 0.035 μM/g fat to 0.319 μM/g fat in cow ghee and from 0.027 to 0.279 μM/g fat in buffalo ghee). The 'head space' carbonyl level was significantly higher (P < 0.01) in cow ghee than in buffalo ghee at the end of 200 days.

Interestingly, the rate of increase of the 'head space' carbonyl content was almost double the rate of increase of 'volatile' and 'total' carbonyl contents, when ghee was stored for 100 and 200 days.

The 'volatile', 'total' and 'head space' carbonyls (Tables 1, 3 and 5) in ghee increased consistently during storage. Earlier, Lillard and Day (1961) had examined the relationship between the off-flavours in milk fat oxidised to different levels and several criteria of chemical change including TBA values, 'total' and 'volatile' carbonyl contents. They reported significant correlation between these chemical tests and oxidised flavour, the best agreement being with carbonyl content. In a separate study from this laboratory also (Gaba and Jain, 1973), the correlation between the off-flavour development and changes in the FFA levels, peroxide values and TBA values, was confirmed. There was a consistent rise in the TBA
value with off-flavour development on storage of ghee. Thus it can be said that carbonyl level coupled with TBA value is a reliable index of the off-flavour development in ghee.

Isolation, fractionation, characterization and estimation of the 'volatile', 'total' and 'head space' carbonyls in ghee and changes during storage

Isolation - The carbonyls were isolated (as DNPs) from cow and buffalo ghee samples which had been stored for 0, 100 and 200 days at 37°C. Six samples each of cow and buffalo ghee were analysed for each type of carbonyls. 'Volatile' carbonyls, including both monocarbonyls and dicarbonyls, were obtained from ghee by steam distillation technique. Compared with fresh ghee, steam distillation for a much longer period was necessary for the isolation of 'volatile' carbonyls from stored (rancid) ghee samples. The residual ghee after steam distillation still was not completely devoid of off-flavours. Possibly, non-volatile carbonyls present in the oxidised ghee samples were responsible for this off-flavour. A similar observation was made by Berry and McKerrigan (1958). Forss (1969a) has also reported that non-volatile components augmented or modified the flavour due to 'volatile' components.
The 'total' carbonyls included both monocarbonyls and dicarbonyls. The 'total' monocarbonyls were isolated from ghee essentially according to the method of Schwartz et al. (1963). However, Schwartz et al. (1963) did not isolate dicarbonyl-DNPs adsorbed on the top of the magnesia-celite column. In the present study, the isolation of dicarbonyls from magnesia-celite column was attempted by elution with solvents such as nitromethane-chloroform (1:2, 1:1, 2:1, 3:1), methanol-chloroform (1:1), nitromethane, chloroform, ethanol and methanol. However, all the dicarbonyl-DNPs adsorbed on the column could not be eluted out quantitatively with any of these solvents. Better recoveries of dicarbonyl-DNPs were obtained when the adsorbent was extracted with methanol (5 x 50 ml per 10 g). The dicarbonyl DNPs were thus obtained in the form of a dark-brown sticky material which was subsequently purified as reported later.

The 'head space' carbonyls, including both mono- and di-carbonyls, were isolated by bubbling N₂ gas through ghee and trapping the effluent in the 2,4-dinitrophenylhydrazine reagent as described earlier.

Fractionation and characterization - The carbonyls, 'volatile' and 'head space', contained
monocarbonyls as well as dicarbonyls. Several TLC methods were available in literature for the separation into classes of monocarbonyl-DNPs (Schwartz and Parks, 1963$^3$; Badings and Wassink, 1963) and dicarbonyl-DNPs (Cobb, 1964$^4$; and Schwartz et al., 1964). However, separation of monocarbonyl-DNPs from dicarbonyl-DNPs had not been reported except that Schwartz et al. (1963) had only indicated the possible separation of dicarbonyl-DNPs from monocarbonyl-DNPs. As indicated above, the Schwartz column technique when applied to ghee DNPs, was not adequate in recovering the dicarbonyl-DNPs completely and in a pure form. The separation of monocarbonyl-DNPs from dicarbonyl-bis-DNPs was, therefore, attempted and the method had to be standardized.

Standardization of the TLC method for the separation of monocarbonyl-DNPs from dicarbonyl-bis-DNPs - A series of trials using adsorbents like silica gel G, kieselguhr G and magnesia-celite mixture (1:1, 1:2, and 2:1) were made to achieve this separation. Solvent systems such as hexane containing varying proportions of methanol, hexane containing varying proportions of chloroform, methanol containing varying proportions of chloroform and chloroform containing varying proportions of nitromethane were tried (Tables 7a, 7b, 7c and 7d).
Effect of heating the plates for varying lengths of time on the separation was also examined. Carbowax impregnation of the plates was also attempted. Authentic carbonyls used in these experiments included DNP's of representative alkan-2-ones, alkanals, alk-2-enals alka-2,4-dienals, \( \alpha \)-diketones and \( \alpha \)-ketoaldehydes. Best separation was obtained on magnesia-celite (2:1) plates heated for 2 hours at 110\(^\circ\)C, and using the solvent system, nitromethane-chloroform (1:3) (Fig.2). Heating the plates at 110\(^\circ\)C for 2-3 minutes immediately before spotting the DNP's on the plates also improved the separation. Heating the developed plates at 110\(^\circ\)C for 2-3 minutes increased the colour intensity of the spots thereby facilitating the identification of spots. An equally satisfactory separation was also possible on Kieselguhr-G plates, using the solvent system, chloroform-hexane (7:5: 92.5). The magnesia-celite method was, however, preferable because the separation could be followed visually on magnesia-celite plates. Monocarbonyl-DNPs and dicarbonyl-bis-DNPs had similar yellow colour on kieselguhr G plates but they had different colours on magnesia-celite plates, yellow for the monocarbonyl-DNPs and bluish-violet for the dicarbonyl-bis-DNPs. The bis-DNPs of diacetyl and methylglyoxal after elution from magnesia-celite plates gave spectral data identical
to that of authentic bis-DNPs of diacetyl and methylglyoxal, viz., 390 nm and 410 nm in neutral medium (methanol) and 520 nm and 528 nm in alkaline medium (Fig. 1). The bis-DNPs of diacetyl and methylglyoxal eluted from kieselguhr G plates, it may be noted, showed absorption maxima at lower wavelengths, viz., 383 nm and 400 nm in neutral medium and 513 nm and 520 nm in alkaline medium.

Schwartz et al. (1983a) had reported that during the isolation of monocarboxyls (as DNP's) from oils/fats over a column of magnesia-celite (1:1), certain DNP-components remained adsorbed on the column. These were possibly dicarboxyl-DNPs as suggested by them, but they did not examine these DNP-component, further.

The 'volatile' and 'head space' carboxyls (as DNPs) isolated from six samples each of cow and buffalo ghee stored for 0, 100 and 200 days were separated into monocarboxyl-DNPs and dicarboxyl-DNPs by preparative TLC on magnesia-celite (2:1) plates using nitromethane-chloroform (1:3) (Fig. 2). The crude 'total' dicarboxyls (as DNPs), isolated from these ghee samples over magnesia-celite (1:1) column as described earlier, were further purified by the above TLC procedure (Fig. 2). The identification of the monocarboxyl-DNPs and dicarboxyl-DNPs was made by
comparative TLC of authentic carbonyl-DNPs, their characteristic colours on magnesia-celite plates and also by comparative ultraviolet and visible spectra of the unknown and authentic carbonyl-DNPs.

The monocarbonyl-DNPs and dicarbonyl-DNPs from ghee (cow/buffalo) gave respective absorption maxima at 363-365 nm and 390-393 nm in neutral medium (methanol) and 430 nm and 530-535 nm in alkaline medium. Fig. 3 depicts the spectra of monocarbonyl-DNPs and dicarbonyl-DNPs of a typical ghee sample. The absorption maximum of the dicarbonyl-bis-DNPs, so isolated, was almost identical to that of an equimolar mixture of diacetyl-bis-DNP and methylglyoxal-bis-DNP, viz., 395 nm in neutral medium and 535 nm in alkaline medium (Fig. 1). The absorption maxima of the monocarbonyl-DNPs isolated from ghee corresponded to those of alkan-2-one-DNPs (Fig. 5).

Class separation of monocarbonyl-DNPs – A survey of literature revealed that separation of aliphatic monocarbonyl-2,4-DNPs into classes has been accomplished by paper chromatography (Gaddis and Ellis, 1959), by adsorption chromatography on columns of zinc carbonate (Duin, 1958) and activated magnesia (Schwartz et al., 1962) and by TLC on aluminium oxide-G (Urbach, 1963), Kieselguhr G plates impregnated with
zinc carbonate or silver nitrate (Badings and Wassink, 1963) and magnesia-celite (Schwartz and Parks, 1963b and Schwartz et al., 1968).

The adsorption column chromatographic method of Dian (1958) was a two stage process involving (a) separation based on chain length by partition chromatography and (b) separation of the single fractions by adsorption chromatography on zinc carbonate. In the paper chromatographic method of Gaddis and Ellis (1958) and the adsorption chromatographic method of Schwartz et al. (1962), short chain members behaved anomalously, moving into the class immediately following. The technique of TLC offers an advantage over both paper and closed column techniques in being faster and adaptable to the detection of smaller amounts of compounds. Urbach (1963) separated the DNP mixture of alkan-2-ones, alkanals and alk-1-en-3-ones on aluminium oxide G plates, using 4% diethyl ether in light petroleum as the solvent. For the separation of DNP mixture of alkanals, alk-2-enals, alka-2,4-dienals and nona-2,6-dienals, aluminium oxide G containing 20% silver nitrate with solvent system 16% diethyl ether in light petroleum was used. Two sets of experiments were thus used for the separation of the DNP of alkan-2-ones, alkanals, alk-3-enals and alka-2,4-dienals. This method also
suffered from the limitation that the lower members of different classes merged with the class immediately following. Two dimensional TLC reported by Urbach (1963) for the separation of carbonyl classes also had similar limitations.

Schwartz and Parks (1963b) separated monocarbonyl-DNPs into four classes namely, alkan-2-ones, alkanals, alk-2-enals and alka-2,4-dienals on magnesia-celite plates using chloroform-hexane (85:15) as the solvent system. This method was time consuming as it required heating of the plates for 16-20 hrs at 110°C and saturation of the chromatographic chamber for at least 16 hrs. It also required development of the plates in the direction of application of the slurry. Further, the lowest members of each class interfered with the classes immediately following.

Badings and Wassink (1963) described TLC techniques for the separation of the aliphatic monocarbonyls into different classes using basic zinc carbonate or silver nitrate impregnated kieselguhr G plates. They observed that zinc carbonate plates did not give clear separation between the classes, and silver nitrate impregnated kieselguhr G plates did not at all separate the ketone-DNPs from other classes.

In view of above, it was necessary to develop a more convenient and simpler method if possible, for
the class separation of monocarbonyl-DNPs. Several trials were carried out on magnesia-celite (2:1) plates using a variety of solvent systems like chloroform, dichloromethane, methanol, acetone, nitromethane, acetic acid, solvent ether, ethyl acetate and hexane (individually and in combination). The effect of heating the plate was also evaluated. Table 8 records some promising solvent systems for class separation of monocarbonyl-DNPs. The best separations were obtained with methanol-hexane (1:25:100) on magnesia-celite (2:1) plates heated for 2 hrs at 110 °C. It was also found that hexane in these solvents could be successfully replaced by petroleum ether (60/80).

Individual DNPs of all the four monocarbonyl classes, viz., alkan-2-ones (C₃–C₁₇), alkanals (C₁–C₁₄), alk-2-enals (C₄–C₁₀) and alka-2,4-dienals (C₅–C₁₄), were subjected to TLC using newly developed solvent system (Methanol: hexane: 1:25:100). The order in which the various classes of monocarbonyl-DNPs appeared on chromatoplates was alkan-2-ones (farthest from the base line), alkanals, alk-2-enals and alka-2,4-dienals (nearest the base line). It was observed that acetone (C₃-ketone), formaldehyde (C₁-alkanal), acetaldehyde (C₂-alkanal) and but-2-enal (C₄-alk-2-enal) DNPs had Rf values lower than those of other members of their respective classes, so that
there was a possibility of these members contaminating the members belonging to the next lower class on the chromatoplate. In order to confirm the scope of this method for the class separation of monocarbonyl-DNPs, different combinations of mixtures of DNPs having possible interfering components were chromatographed by the TLC method described above on preparative scale. The adsorbent zone corresponding to each separated band was carefully scraped off, the DNPs eluted out with methanol AnalR and their spectra examined (Table 9). Methanal and ethanal-DNPs interfered with the DNPs of alk-2-enal class, propanone-DNP formed a separate band, though very close to that of the other alkan-2-one-DNPs, and but-2-enal-DNP gave a separate band except when it occurred with methanal-DNP. While analysing the monocarbonyl-DNPs isolated from ghee, it was observed that (i) propanone-DNP band sometimes merged with the other alkan-2-one-DNP band, depending on the relative concentrations of both, (ii) ethanal-DNP came along with the other alkanal-DNPs. Thus it was only the methanal-DNP which really interfered with the DNPs of alk-2-enal class. The results were better when the plates were irrigated upto about 15 cm length in an air tight chamber saturated with the solvent system for about 10 minutes prior to insertion of plates.
The method standardised above was more convenient and less time consuming than those mentioned earlier. It may be mentioned here that the TLC method of Schwartz et al. (1968), which came to our notice as this work was in progress, for the separation of monocarbonyl-DNPs into the four classes on magnesia-celite (3:7) plates and with hexane-chloroform (95:25) as the solvent system was more time consuming. Further, in their method the colours of the DNP-classes did not contrast well on the developed chromatograms. When their method was used in this laboratory, butan-2-one-DNP and propanone-DNP moved separately from the other members of their class. Ethanal-DNP moved separately from the other members of its class. Methanal-DNP almost moved with the alk-2-enal-DNPs. Propenal (acrolein)-DNP and but-2-enal-DNP moved separately from other members of their class. Moreover, their method was suitable only when low and equimolar concentrations of the class members were used. The method standardized in this laboratory was equally good when used on preparative scale. The identification of various classes by this method improved considerably as they gave widely different colours on magnesia-celite (2:1) plates. DNPs of alkan-2-ones gave yellowish grey, alkanals light brown,
alk-2-enals, peach and alka-2,4-dienals, red colour and these were similar to those reported by Schwartz and Parks (1963b). In this case also, heating the plates at 110°C for 2-3 minutes immediately before spotting the DNP-mixture improved the separation. Heating of the developed chromatogram further intensified the colours of the various classes.

Fig. 4 depicts a typical TLC pattern of the 'volatile' monocarbonyl-DNPs obtained from ghee (cow/buffalo) stored for varying periods (0, 100 and 200 days). The patterns of both cow and buffalo ghee were similar. The patterns of the 'total' and 'head space' monocarbonyl-DNPs were also similar to those of 'volatile' monocarbonyl-DNPs and have not been shown here. All the four classes of monocarbonyls, viz., alkan-2-ones, alkanals, alk-2-enals and alka-2,4-dienals were detected in each type of ghee examined in this study and these were confirmed by (a) comparative TLC of authentic class-DNPs, (b) the characteristic colours (Schwartz and Parks, 1963b) of the DNPs class on magnesia-celite plates and (c) comparative ultraviolet and visible spectra of the monocarbonyl-DNP classes isolated from ghee and the relevant authentic monocarbonyl-class DNPs. Repeated preparative TLC followed by the elution of the respective extruded zones with methanol.
was necessary to get adequate amounts of various monocarbonyl-DNP classes for characterization and identification.

The spectra in methanol of a representative member of each of the four monocarbonyl-DNP-classes are shown in figures 5, 6, 7 and 8. It may be noted that the spectra of two to three more members of each class was scanned and found identical. Each class of monocarbons-DNPs had characteristic $\lambda_{\text{max}}$ and $\varepsilon_{\text{max}}$, as shown below:

- **Alkan-2-one-DNPs**: $\lambda_{\text{max}}$ 363 nm ($\varepsilon 2.18 \times 10^4$).
- **Alkanal-DNPs**: $\lambda_{\text{max}}$ 360 nm ($\varepsilon 2.06 \times 10^4$).
- **Alk-2-enal-DNPs**: $\lambda_{\text{max}}$ 378 nm ($\varepsilon 2.84 \times 10^4$).
- **Alka-2,4-dienal-DNPs**: $\lambda_{\text{max}}$ 392 nm ($\varepsilon 2.90 \times 10^4$).

**Estimation of monocarbonyl classes in ghee** - The proportions of various monocarbonyl classes in ghee were calculated from the above values of $\lambda_{\text{max}}$ and $\varepsilon_{\text{max}}$ of authentic monocarbonyl-DNP classes.

(a) 'Volatile' monocarbonyl classes Six samples each of cow and buffalo ghee were analysed after 0, 100 and 200 days of storage (Tables 10 and 11). Alkan-2-ones constituted about 90% of the total 'volatile' monocarbons in fresh (storage 0 days) cow and buffalo
ghee. Fresh cow ghee contained 8.8% alkanals, 2.0% alk-2-enals and 1.8% of alka-2,4-dienals. The proportions of these three classes of monocarbonyls in fresh buffalo ghee were of the same order (5.9, 2.1 and 1.7% respectively).

On storage, there were marked changes in the relative proportions of the four classes of monocarbonyls in the two types of ghee. Alkan-2-one level decreased from 89.8 to 90.0% in case of cow ghee and from 90.2 to 54.0% in buffalo ghee after storage for 100 days. There was a further decline to 23.3% and 24.4% in cow and buffalo ghee respectively after storage for 200 days. In contrast, the other three classes showed a marked increase in their relative proportions irrespective of the type of ghee. Alkanal level increased from 6.6 to 33.5% (about 5 fold) in cow ghee and from 5.9 to 30.0% (about 5 fold) in buffalo ghee after 100 days of storage. Further storage for 200 days led to a seven fold increase in the alkanal level (45.3% in cow ghee and 44.5% in buffalo ghee).

The level of alk-2-enals increased six fold (from 2.0 to 11.9% in cow ghee and from 2.1 to 12.1% in buffalo ghee) after storage for 100 days. Storage for 200 days resulted in a 12-13 fold increase in the alk-2-enal level (23.9% in cow ghee and 23.9% in buffalo ghee).
Alka-2,4-dienals increased from 1.8 to 4.5% (about 2.5 fold) after 100 days of storage and then to 7.5% (about 4 fold) after 200 days storage in the case of cow ghee. There was a similar increase in the alka-2,4-dienal content in case of buffalo ghee. It rose from 1.7 to 4.0% (about 2.5 fold) after 100 days of storage and then to 7.1% (about 5 fold) after 200 days of storage.

There was thus a marked decrease in the level of alkan-2-ones and a marked increase in the levels of alkanals, alk-2-enals and alka-2,4-dienals during storage of ghee. Lillard and Day (1981) reported an increase in the levels of 'volatile' saturated aldehydes and unsaturated aldehydes, on oxidation of milkfat.

(b) 'Total' monocarbonyl classes - Tables 12 and 13 record the proportions of 'total' monocarbonyls estimated from six samples each of cow and buffalo ghee after 0, 100 and 200 days of storage. Alkan-2-ones constituted about 90% of the total monocarbonyls in fresh (storage for 0 day) cow and buffalo ghee. Fresh ghee contained 6.4% alkanals, 2.2% alk-2-enals and 1.8% alka-2,4-dienals; whereas fresh buffalo ghee, contained 6.4, 2.2 and 1.9% respectively of the latter three classes.
On storage, there was marked changes in the relative proportions of the four classes of monocarbonyls in the two types of ghee. After storage for 100 days, alkan-2-one level declined from about 90 to 45.8% in cow ghee and to 53.4% in buffalo ghee. There was a further decline to 25.1% in cow ghee and to 23.8% in buffalo ghee on storage for 200 days. The contents of the other three classes increased, irrespective of the type of ghee. Alkanals increased almost 5-6 fold (from 6.4 to 35.4% in cow ghee and from 6.4 to 30.0% in buffalo ghee) after 100 days of storage. It further increased to 44.6 and 44.8% respectively in cow and buffalo ghee after 200 days of storage. The level of alk-2-enals increased almost 6 fold (from 2.2 to 13.2% in cow ghee and from 2.2 to 12.2% in buffalo ghee) on 100 days of storage. Storage for 200 days led to a further 10-12 fold increase in the alk-2-enal level (to 22.8% in cow ghee and to 24.4% in buffalo ghee). The level of alka-2,4-dienals increased about three fold (from 1.8 to 5.5% in cow ghee and from 1.9 to 4.3% in buffalo ghee) after storage for 100 days. Storage for 200 days resulted in a 4-5 fold increase in the alka-2,4-dienal levels (to 7.7% in cow ghee and to 6.9% in buffalo ghee).

The relative distribution of the four classes of monocarbonyls was thus similar in the case of
'volatile' and 'total' (hence non-volatile also) monocarboxyls of fresh as well as stored ghee (cow as well as buffalo). Ahmed et al. (1971) reported that the relative proportions of methyl ketones, saturated aldehydes, 2-enals and 2,4-dienals were 55, 25, 12 and 7% respectively in oxidised butter oil and 40, 40, 11 and 9% respectively in oxidised samosa. These results are in close agreement with the findings reported in this study for ghee stored for 100 days.

(c) 'Head space' monocarboxyl classes - Six samples each of cow and buffalo ghee were analysed after 0, 100 and 200 days of storage for relative proportions of monocarboxyl classes among the 'head space' carbonyls (Tables 14 and 15). Alkan-2-ones constituted 85.3 and 79.3% of the 'head space' monocarboxyls isolated from cow and buffalo ghee respectively. Fresh cow ghee contained 11.0% alkanals, 1.9% alk-2-enals and 1.8% alka-2,4-dienals, whereas fresh buffalo ghee contained 19.3%, 0.7% and 0.7% respectively of the latter three classes of monocarboxyls. The proportion of alkanals was comparatively higher in fresh buffalo ghee than in fresh cow ghee, but the proportions of alkan-2-ones, alk-2-enals and alka-2,4-dienals were comparatively lower in buffalo ghee than in cow ghee. Some of the fresh buffalo ghee samples
did not at all show the presence of alk-2-enal and alka-2,4-dienal classes. The relative distribution of the four classes of ‘head space’ monocarbonyls was very different from that of ‘volatile’ and ‘total’ monocarbonyl classes in ghee. The level of alkan-2-one was comparatively lower and that of alkanals higher (2 times for cow ghee and 3 times for buffalo ghee) in the ‘head space’ monocarbonyls. The proportions of alk-2-enals and alka-2,4-dienals were very much lower in ‘head space’ monocarbonyls than those in ‘volatile’ and ‘total’ monocarbonyls, the differences being more marked in case of buffalo ghee.

On storage changes in the relative proportions of the four classes of ‘head space’ monocarbonyls were similar to those observed in the case of ‘volatile’ and ‘total’ monocarbonyls. After 100 days of storage, alkan-2-one level declined from 85.30 to 55.8% in cow ghee and from 79.3 to 50.5% in buffalo ghee. The alkan-2-one level declined further to 29.7% in cow ghee and to 26.3% in buffalo ghee after 200 days of storage. Alkanals increased from 11.0 to 30.1% in cow ghee and from 19.3 to 42.8% in buffalo ghee after 100 days of storage. The level increased still further to 43.5% and 54.7% respectively in cow and buffalo ghee after 200 days of storage. The level of alk-2-enals increased from 1.9 to 10.2% in cow ghee and from
After 200 days of storage, the level increased to 19.5% in cow ghee and 12.3% in buffalo ghee. The level of alka-2,4-dienals increased from 1.8 to 3.9% in cow ghee and from 0.7 to 2.8% in buffalo ghee after storage for 100 days. Storage for 200 days led to a further increase in the alka-2,4-dienal level to 7.3% and 6.7% respectively in cow and buffalo ghee.

**TLC separation of the monocarbonyl-DNP-classes into individual components** - The 'volatile', 'total' and 'head space' monocarbonyl-DNP classes isolated from six samples each of cow and buffalo ghee stored for 0, 100 and 200 days were analysed for their individual components.

The separation was achieved by partition TLC using carbowax impregnated kieselguhr G plates and hexane-pentane (1:1) as the solvent system. Figures 9, 10, 11 and 12 depict the TLC patterns of the four monocarbonyl DNP classes obtained from cow/buffalo ghee (fresh and stored). The TLC patterns of 'volatile', 'total' and 'head space' monocarbonyl-DNP-classes were similar for both cow and buffalo ghee (fresh and stored). However, the concentrations of the various components differed in these three types of carbonyls obtained from cow and buffalo ghee (fresh and stored).
Fraction I (Fig. 4), corresponding to alkan-2-one-DNP mixture from fresh ghee, gave seven spots (Fig. 9) which were tentatively identified as propanone, butan-2-one, pentan-2-one, hexan-2-one, heptan-2-one, octan-2-one and nonan-2-one (or higher homologues). The TLC patterns of alkan-2-one-DNP mixtures obtained from 100 day and 200 day old ghee samples were also qualitatively similar, except that they gave an additional faint spot in between the identified spots of DNPs of propanone and butan-2-one. The additional spot was possibly an artefact or a polymorphic form of propanone-DNP.

Fraction 2 (Fig. 4) corresponding to alkanal-DNP mixture from both fresh and stored ghee separated into eight spots (Fig. 10) which were tentatively identified as DNPs of ethanal, propanal, butanal, pentanal, hexanal, heptanal, octanal and nonanal (or higher homologues).

Fraction 3 (Fig. 4) corresponding to alk-2-enal-DNP mixture from both fresh and stored ghee gave nine spots (Fig. 11), of which eight were identified tentatively as DNPs of but-2-enal, pent-2-enal, hex-2-enal, hept-2-enal, oct-2-enal, non-2-enal, dec-2-enal and dodec-2-enal. The unidentified spot 'a' could be assigned the structure of propenal-DNP on the basis of its relative position on thin-layer
chromatogram. However, the possibility of this spot being methanal DNP could not be ruled out.

Fraction 4 (Fig. 4) corresponding to alka-2,4-dienal-DNP mixture from fresh and stored ghee separated into ten spots (Fig. 12) of which seven were identified tentatively as DNP's of penta-2,4-dienal, hexa-2,4-dienal, nona-2,4-dienal, deca-2,4-dienal, undeca-2,4-dienal, dodeca-2,4-dienal and tetradeca-2,4-dienal. Out of the remaining three spots (a, d, and e in Fig. 12), two spots (d and e) could be assigned the structures of hepta-2,4-dienal-DNP and octa-2,4-dienal-DNP on the basis of their relative positions on the thin-layer chromatograms. The spot (a), however, could not be identified and it could be an artefact.

GLC separation of monocarbonyl-DNP-classes into individual components - Schwartz et al (1968) reported that TLC technique was not adequate to separate DNP's of higher homologues of the four classes of monocarbonyls. This was confirmed in this laboratory also (Jain and Rama Murthy, 1971). GLC separation of alkan-2-one-DNP's and other classes of monocarbonyl-DNP's obtained from ghee was attempted to see if some additional information could be obtained in regard to the carbonyl make up of ghee. GLC patterns of the 'volatile', 'total' and 'head space'
monocarbonyl-DNPs from both cow and buffalo ghee (fresh and stored) were similar.

Fraction 1 (Fig. 4), corresponding to alkan-2-one-DNPs, gave nine peaks (Fig. 13). Of these, seven peaks ('a' to 'g' in Fig. 13) corresponded to the alkan-2-ones already identified by TLC. The remaining two peaks ('h' and 'i') were those of decan-2-one and dodecan-2-one, as found by comparative retention times.

Fraction 2 (Fig. 4), corresponding to alkanal-DNPs, separated into ten peaks (Fig. 14). Of these, eight peaks ('a' to 'h' in Fig. 14) corresponded to alkanal-DNPs identified earlier by TLC. The peak 'i' was assigned the structure of decanal on the basis of comparative retention time. Peak 'j', however, could not be identified.

Fraction 3 (Fig. 4), corresponding to alk-2-enal-DNPs, gave seven peaks (Fig. 15), six ('b' to 'g' in Fig. 15) of which corresponded to alk-2-enals ('b' to 'g' in Fig. 11) already detected by TLC. Peak 'a' (Fig. 15), however, could not be identified.

Fraction 4 (Fig. 4), corresponding to alka-2,4-dienal-DNPs, gave six peaks (Fig. 16). Of these, two peaks ('b' and 'c' in Fig. 16) corresponded to penta-2,4-dienal and hexa-2,4-dienal, already identified.
by TLC. The peak 'd' was assigned the structure hepta-2, 4-dienal, on the basis of comparative GLC. The remaining three peaks 'a', 'e' and 'f' could not, however, be identified.

Thus out of 38 monocarbonyls detected in case of fresh ghee and 39 monocarbonyls detected in case of stored ghee (cow and buffalo), 34 were identified by comparative TLC/GLC.

Winter et al. (1963) isolated 'volatile' constituents of fresh butter by steam-distillation under reduced pressure. They converted the carbonyl compounds in the aqueous distillate into DNPs, which were separated by column and paper chromatography. The identified compounds included 2-mananone, nonanal, hexanal, formaldehyde, acetaldehyde, iso-butylaldehyde, phenylacetaldehyde, acetone, 2-heptanone and isovaleraldehyde. Forss et al. (1967) isolated 'volatile' compounds from Australian butter oil by high vacuum degassing and reported the presence of C3, C5, C7, C9 and C11-alkan-2-ones, diacetyl and various alkanolic acids and lactones, etc., using gas-chromatography and mass spectrometry. Schwartz and Virtanen (1968) isolated 37 carbonyl compounds (as DNPs) in the 'volatiles' from milkfat: (i) eight methyl-ketones (C3 to C7, C9, C11 and C13) (ii) eleven saturated aldehydes C1 to C4 and C6 to C12.
(iii) ten 2-enals C₃ to C₁₂, and (iv) an unidentified class containing eight members.

Lillard and Day (1961) reported that carbonyl compounds were most effective in producing the oxidized flavours and that most of these carbonyls were monocarbonyls belonging to four classes, alkan-2-ones, alkanals, alk-2-enals and alka-2,4-dienals. Forns et al. (1960c) had reported the presence of C₅ to C₁₀ alkanals, C₅ to C₁₀ alk-2-enals and hepta-2,4-dienal, in the butterfat with tallowy flavour. Day and Lillard (1960) conclusively identified C₁ to C₁₀ alkanals, C₅ to C₁₁ alk-2-enals and propanone among the 'volatile' material isolated from oxidised milkfat. In addition, but-2-enal and odd numbered C₅ to C₁₅ alkan-2-ones were also tentatively identified. Parks et al. (1963) also studied the carbonyl compounds in the butter oil made from oxidised whole milk, and C₅ to C₁₈ saturated aldehydes, C₉ to C₁₁ alk-2-enals and C₈ to C₁₂ alka-2,4-dienals were identified in such butter oil. The earlier reports from this laboratory (Jain and Bindal, 1968; Jain and Singhal, 1969; and Jain et al., 1971) also gave a gross picture of the 'volatile' carbonyls present in ghee, but no attempt was made in those studies to fractionate total 'volatile' carbonyl-NPs into different classes.
Variations in the contents of individual monocarbonyls in ghee during storage - Repeated preparative TLC of the four classes of monocarbonyl-DNPs from ghee was performed on kieselguhr G plates impregnated with carbowax, using hexane-pentane (1:1) as the solvent system. The relative proportions of individual monocarbonyls were estimated colorimetrically as described earlier.

(a) 'Volatile' monocarbonyls - The relevant data have been summarised in Tables 18, 17, 18 and 19.

The relative proportions of individual alkan-2-ones (except butan-2-one) were similar in both cow and buffalo ghee (fresh). The butan-2-one level in fresh cow ghee (2.7%) was markedly lower than that in buffalo ghee (5.1%). The contents of other alkan-2-ones were: propanone (about 19, 20%)*, pentan-2-one (about 22, 21%), hexan-2-one (about 2,2%), heptan-2-one (about 34, 34%) and Octan-2-one, including higher homologues (about 20,18%). On storage for 100 and 200 days, there was a significant increase in the

* The two values given in this parenthesis and in parentheses on pages 103 - 111, 121 and 122, represent the data for cow and buffalo ghee respectively.
level of butan-2-one. While there was an appreciable increase in the levels of heptan-2-one and octan-2-one including higher homologues, the levels of propanone and pentan-2-one showed a marked decline during storage, (Table 16), irrespective of the types of ghee.

The relative proportions of individual alkanals were similar in both cow and buffalo ghee (fresh and stored). The contents of ethanal, propanal, butanal, pentanal, hexanal, heptanal and octanal (including higher homologues) were about 2, 9, 8, 29, 21, 13 and 19% respectively in fresh ghee (cow and buffalo). The levels of ethanal and propanal increased markedly and those of hexanal and octanal (including higher homologues) appreciably during storage of ghee. The proportions of butanal, pentanal and heptanal, however, decreased during storage, the decrease being more pronounced in pentanal and heptanal (Table 17).

The trend in the relative proportions of individual alk-2-enals was similar in both cow and buffalo ghee (fresh and stored). However, the level of the unidentified member (propanal or methanal) in fresh cow ghee (about 25%) was higher than that in fresh buffalo ghee (about 18%) and that of oct-2-enal less in cow ghee (about 11%) as compared to buffalo ghee (about 16%). The proportions of other alk-2-enals
were but-2-enal (about 3, 3%), pent-2-enal (about 12, 10%), hex-2-enal (about 10, 9%), hept-2-enal (12, 14%), non-2-enal (about 14, 16%) and dec-2-enal, including higher homologues (about 12, 14%) in fresh ghee. The levels of but-2-enal and hex-2-enal increased considerably, but those of pent-2-enal, oct-2-enal and dec-2-enal (including higher homologues) increased to a much less extent during storage of ghee. Significantly, propenal (or methanal) decreased to about one half level on storage for 100 days and one third to one fourth level on storage for 200 days. There was an appreciable decrease, in hept-2-enal content through the decrease in non-2-enal content was much less (Table 18).

The relative proportions of individual alka-2,4-dienals were also similar in both cow and buffalo ghee (fresh and stored). The levels were: unidentified component (about 15, 14%), penta-2,4-dienal (about 7, 8%), hexa-2,4-dienal (about 6, 9%), hepta-2,4-dienal (about 19, 18%), octa-2,4-dienal (about 7, 7%), nona-2,4-dienal (about 8, 7%), deca-2,4-dienal (about 15, 13%), undeca-2,4-dienal (about 4, 6%), dodeca-2,4-dienal (about 9, 8%) and tetradeca-2,4-dienal, including higher homologues (about 11, 12%) in fresh ghee. The levels of hepta-2,4-dienal and deca-2,4-dienal increased significantly, but octa-2,4-dienal increased to a lesser
extent during storage. The contents of unidentified alka-2,4-dienal, nona-2,4-dienal, dodeca-2,4-dienal and tetradeca-2,4-dienal (including higher homologues) showed a marked decline during storage. However, the decrease in the penta-2,4-dienal, hexa-2,4-dienal and undeca-2,4-dienal levels, during storage, was less marked (Table 19).

(b) 'Total' monocarboxyls - Tables, 20, 21 and 22 give the relative proportions of individual monocarboxyls (except alka-2,4-dienals) among the 'total' monocarboxyl in ghee. Individual alka-2,4-dienals could not be estimated as it was too laborious to obtain adequate quantities of these dienal-DNPs by preparative TLC.

Propanoate (about 74, 74%) was the most abundant alkan-2-one in fresh ghee (cow as well as buffalo) whereas pentan-2-one (about 1.5, 1.5%) was minimum (Table 20). The contents of other alkan-2-ones were: octan-2-one and higher homologues (about 17, 17%), hexan-2-one (about 3, 3%), heptan-2-one (about 2, 2%) and butan-2-one (about 2, 2%). 'Volatile' alkan-2-ones in fresh ghee (Table 16) consisted mainly of heptan-2-one (about 34, 34%), followed by pentan-2-one (about 21, 21%) and then propanone (about 19, 20%).

The increased levels of pentan-2-one and heptan-2-one
in 'volatile' carbonyls in ghee appeared to suggest that appreciable amounts of pentan-2-one and above are possibly formed during steam distillation of ghee. The reports of Lawrence (1963), Langrer and Day (1964) and Parks et al. (1964a) that these methyl ketones are formed during the heat treatment of milkfat, especially in the presence of moisture, also support this suggestion.

On storage for 100 and 200 days, there was a marked decrease in propanone content, but an appreciable increase in the levels of butan-2-one through heptan-2-one. The increase in the content of octan-2-one (and for higher homologues) was, however, marginal. The changes were comparatively more in buffalo ghee (Table 20). In contrast, the level of pentan-2-one in the 'volatile' monocarbonyls of ghee had shown a marked decrease on storage (Table 16).

The levels of individual alkanals were similar in both cow and buffalo ghee (fresh), except that ethanal level was somewhat higher in cow ghee (about 20%) than in buffalo ghee (about 15%), and the content of octanal (including higher alkanals) was lower in cow ghee (about 30%) than in buffalo ghee (about 38%) (Table 21). The levels of other alkanals were: propanal (about 9, 8%), butanal (about 9, 9%), pentanal (about 10, 9%), hexanal (about 5, 5%) and heptanal (about 17, 18%). Marked differences were
observed in the levels of alkanals among the 'total' and 'volatile' monocarbonyls of fresh ghee. The levels of pentanal (9%) and hexanal (5%) in 'total' monocarbonyls (Table 21) were much lower than those in 'volatile' monocarbonyls (29%). On the other hand, the contents of ethanal and octanal including higher homologues in 'total' carbonyls were much higher than those in 'volatile' carbonyls (Table 17).

On storage, the proportions of propanal, hexanal and heptanal increased, irrespective of the type of ghee, although the increase in the hexanal content was relatively more marked. On the other hand, the levels of ethanal and octanal (and/or higher homologues) declined. There was, however, no marked change in the contents of butanal and pentanal (Table 21). It is interesting that the levels of ethanal, propanal, hexanal and octanal (and/or higher homologues) among the 'volatile' monocarbonyls of ghee had shown an increase on storage (Table 17).

The proportions of individual alk-2-enals were similar in both cow and buffalo ghee (fresh), though the levels of non-2-enal and/or higher alk-2-enals were comparatively lower in cow ghee (about 30%) than in buffalo ghee (about 37%) (Table 22). The levels of other alk-2-enals were: unidentified component
(about 21, 18%), but-2-enal (about 13, 11%), pent-2-enal (about 7, 7%), hex-2-enal (about 9, 10%) and oct-2-enal (about 10, 10%) and oct-2-enal (about 9, 8%).

There were differences in the proportions of individual alk-2-enals among 'total' and 'volatile' monocarbonyls of fresh ghee. The proportions of but-2-enal (13, 11%) and of non-2-enal and/or higher homologues in 'total' monocarbonyls (Table 22) were higher than those in 'volatile' monocarbonyls (Table 18). The proportions of pent-2-enal and oct-2-enal in 'total' monocarbonyls were, on the other hand, lower than those in 'volatile' monocarbonyls.

On storage, the proportions of pent-2-enal, hex-2-enal, oct-2-enal and non-2-enal (and/or higher homologues) increased in both cow and buffalo ghee, whereas those of unidentified component (propenal or methanal) and hept-2-enal decreased. There were, however, no marked changes in the proportions of but-2-enal during storage especially in case of buffalo ghee. Similar changes in the proportions of these alk-2-enals (except but-2-enal) among 'volatile' monocarbonyls have been observed during storage of ghee (Table 18).
(c) 'Head space' monocarbonyls - Tables 23 and 24 give the relative proportions of individual 'head space' monocarbonyls (alkan-2-one and alkanals) in ghee. Individual alk-2-enals and alka-2,4-dienals could not be estimated among the 'head space' monocarbonyls as it was too laborious to obtain adequate quantities of these carbonyl-ONPs, due to rather low concentrations of these in ghee.

The levels of individual alkan-2-ones were similar in both cow and buffalo ghee (Table 23). The contents in decreasing order were: propanone (about 43, 42%), octan-2-one and/or higher homologues (about 21, 23%), heptan-2-one (about 21, 21%), pentan-2-one (about 7, 7%), butan-2-one (about 6, 6%) and hexan-2-one (about 2, 2%). Marked differences were observed in the levels of alkan-2-ones among the 'head space', 'total' and 'volatile' monocarbonyls of fresh ghee. The propanone level in 'head space' monocarbonyls (Table 23) was markedly higher than that in 'volatile' monocarbonyls (Table 16) but lower than that in 'total' monocarbonyls (Table 2). The level of butan-2-one was also higher in 'head space' monocarbonyls than in 'volatile' and 'total' monocarbonyls of fresh ghee.

On storage for 100 and 200 days, a marked decrease in the propanone content with a concomitant increase in the contents of butan-2-one through
octan-2-one (and/or higher homologues) was observed (Table 23). These changes were similar to those observed in the case of 'total' monocarbonyls.

The trend in the relative proportions of individual alkanals was similar in both cow and buffalo (fresh as well as stored) (Table 24). The levels of alkanals, in decreasing order were: pentanal (about 22, 23%), ethanal (about 21, 19%), octanal including higher alkanals (about 19, 19%), propanal (about 14, 14%), hexanal (about 11, 11%), heptanal (about 8, 8%) and butanal (about 5, 6%). There were differences in the proportions of individual alkanals among 'head space', 'total' and 'volatile' monocarbonyls in ghee. The levels of ethanal and propanal in 'head space' monocarbonyls were higher than in 'total' as well as 'volatile' monocarbonyls (Tables 21 and 17), whereas the reverse was the case with butanal and heptanal contents. The levels of pentanal and hexanal were, however, lower than those in 'volatile' monocarbonyls and higher than those in 'total' monocarbonyls. The level of octanal (and/or higher homologues) was almost the same as that in 'volatile' monocarbonyls, though it was much less in 'total' monocarbonyls.

On storage for 100 and 200 days, the levels of propanal, butanal and hexanal increased and those of ethanal, pentanal, heptanal and octanal (and/or higher
homologues) decreased. Increase in the levels of propenal and hexanal was also observed in 'volatile' and 'total' monocarbonyls (Tables 17 and 21) of ghee.

Fractionation of dicarboxyl-DNPs isolated from ghee - The 'volatile', 'total' and 'head space' dicarboxyl-bis-DNPs isolated, as described earlier, from 6 samples each of cow and buffalo ghee after 0, 100 and 200 days of storage at 37°C were subjected to thin-layer chromatography on magnesia-celite (2:1) plates. Solvent systems such as methanol-hexane, chloroform-methanol, chloroform-hexane and hexane-methanol-chloroform in varying proportions were tried. Effect of heating the plates at 110°C for 0, 1 and 2 hours was also studied in relation to the separation of these DNPs. The best separation (Fig. 17) was obtained on magnesia-celite (2:1) layers (250 µ thick) coated on plates heated for 2 hours at 110°C and using the solvent system, hexane-methanol-chloroform (5:5:90). Separation was better if the plates were run to about 8-12 cm in 15-25 minutes. Heating the plate at 110°C for 2-3 minutes prior to spotting the DNP-mixtures and also after development of the plate also appeared to improve the separation. Six components were detected in each type of ghee. Out of
these, two components were tentatively identified as diacetyl-bis-DNP and methylglyoxal-bis-DNP on the basis of the comparative TLC of the authentic DNPs (Fig. 17).

Winter et al (1983) and Steinsholt et al (1971) reported the presence of diacetyl and acetoin in fresh butter. Sick and Lindsay (1970) also reported the presence of diacetyl in the neutral 'volatile' fraction of fresh sweet cream butter. The presence of diacetyl and acetoin has also been reported in stored butter by Steinsholt et al (1971).

Variations due to storage, in the 'volatile' and 'total' carbonyl contents of ghee prepared from fresh and 'ripened' butter

Eight samples each of cow and buffalo ghee prepared from fresh and 'ripened' butter were analysed for 'volatile' and 'total' carbonyl contents before and after storage for 100 days (Tables 25 and 27). The data were analysed statistically in 2³ factorial experiment (Tables 26 and 28). Fresh ghee (cow/buffalo) prepared from 'ripened' butter had slightly better flavour. Fresh cow ghee (from fresh and 'ripened' butter) had nil PV and TBA value. However, the FFA
levels varied between 0.35-0.38 and 0.30-0.38 respectively in ghee prepared from fresh and 'ripened' butter. After 100 days of storage, ghee (from fresh butter) developed mild off-flavour with PV, TBA value and FFA level varying between 3.6-11.1, 0.70-2.15 and 0.52-0.69 respectively and ghee (from 'ripened' butter) developed strong off-flavour with PV, TBA value and FFA level varying between 12.8-32.0, 0.70-3.00 and 1.15-1.34 respectively. Fresh buffalo ghee (from fresh and 'ripened' butter) had PV, zero; and TBA value, 0.05-0.25. However, the FFA level varied between 0.21-0.26 and 0.32-0.35 respectively in ghee prepared from fresh and 'ripened' butter. The buffalo ghee (from fresh butter), on 100 days of storage, developed off-flavour (PV, TBA value and FFA level varying between 9.8-17.5, 2.50-3.00 and 0.33-0.36 respectively) whereas the ghee (from 'ripened' butter) developed highly strong off-flavour (PV, TBA value and FFA level varying between 13.8-40.5, 3.30-3.60 and 0.50-0.55 respectively).

The variations in the 'volatile' and 'total' carbonyl contents due to species were significant (P ≤ 0.05 in case of 'volatile' and P ≤ 0.01 in case of 'total' carbonyls).

The 'volatile' and 'total' carbonyl contents of ghee prepared from 'ripened' butter were also
significantly different \((P \leq 0.01)\) than those of ghee prepared from fresh butter. The interaction between species and type was also found to be significant \((P \leq 0.05)\) in case of 'volatile' and \(P \leq 0.01\) in case of 'total' carbonyls).

For comparing the two species with regard to carbonyl level of fresh ghee, unpaired 't' test was applied separately on ghee prepared from fresh and ripened butter. Further, the comparison of the levels of carbonyls of fresh ghee prepared from fresh and ripened butters was made separately for both the species, with the help of paired 't' test.

The 'volatile' carbonyl content (Table 25) of fresh ghee (cow as well as buffalo) prepared from ripened butter was significantly higher \((P \leq 0.01)\) than that of ghee prepared from fresh butter, though the differences were more marked in cow ghee prepared from ripened and fresh butters. The content of 'volatile' carbonyls in fresh cow ghee prepared from fresh butter (Average 0.33 \(\mu M/g\) fat) and ripened butter (Average 0.42 \(\mu M/g\) fat) were significantly higher \((P \leq 0.01)\) than the corresponding levels (Average 0.26 \(\mu M/g\) fat and 0.28 \(\mu M/g\) fat in ghee prepared from fresh and ripened butter, respectively) of buffalo ghee.

* type: ghee prepared from fresh or 'ripened' butter.
The 'total' carbonyl content (Table 27) of fresh cow ghee prepared from 'ripened' butter (Average 9.66 µM/g fat) was significantly higher (P < 0.01) than that prepared from fresh butter (Average 7.26 µM/g fat). However, no significant differences were observed in the 'total' carbonyl content of buffalo ghee prepared from fresh (Average 8.60 µM/g fat) and 'ripened' (Average 8.62 µM/g fat) butter. The 'total' carbonyl content of buffalo ghee prepared from fresh butter was significantly higher (P < 0.01) than that of corresponding cow ghee. On the other hand, the 'total' carbonyl level was significantly lower (P < 0.01) in buffalo ghee prepared from 'ripened' butter than that of corresponding cow ghee.

The carbonyl ('volatile' and 'total') level increased significantly (P < 0.01) on storage of ghee. The effect of storage was significantly different (P < 0.01) for species (cow and buffalo ghee) and type (ghee prepared from fresh and 'ripened' butter) (Tables 26 and 28).

'Volatile' carbonyl content showed a rise of about 2 fold (Average 0.71 µM/g fat) and 3 fold (Average 0.78 µM/g fat) respectively in cow and buffalo ghee prepared from fresh butters, after 100 days.
of storage. The rise was of about 4 fold (Average 1.66 µM/g fat) and 5-6 fold (Average 1.67 µM/g fat) respectively in cow and buffalo ghee prepared from 'ripened' butters (Table 25).

On storage for 100 days, 'total' carbonyl content increased (Table 27) by about 2 fold (Average 13.93 µM/g fat) and 2.5 fold (Average 24.85 µM/g fat) in cow ghee prepared from fresh and 'ripened' butters, respectively. The increase was about 2 fold (Average 18.12 µM/g fat) and 4 fold (Average 34.68 µM/g fat) in buffalo ghee prepared from fresh and 'ripened' butters, respectively, on 100 days of storage.

The ripening of butter (cow/buffalo) thus increased the contents of 'volatile' and 'total' carbonyls of fresh ghee (exception, 'total' carbonyls of buffalo ghee). Further, the rise in the contents of 'volatile' and 'total' carbonyls of such ghee, on storage, was comparatively more than those of ghee prepared from fresh butter.

Isolation, fractionation, characterization and estimation of 'volatile' and 'total' carbonyls of ghee prepared from fresh and 'ripened' butter, and changes in the make-up on storage

(a) Isolation of monocarbonyls and dicarbonyls (as DNP's) - The carbonyls were isolated
in the usual manner from cow and buffalo ghee prepared from fresh and 'ripened' butters after 0 and 100 days of storage.

The 'volatile' carbonyl DNP's were separated into monocarbonyl- and dicarbonyl-DNP's by the preparative TLC procedure, on magnesia-celite plates, as already described.

The 'total' monocarbonyl-DNP's were isolated essentially according to the method of Schwartz et al. (1983a), the dicarbonyl-bis-DNP's being isolated from the magnesia-celite(1:1) column as already described. The dicarbonyl-DNP's, so obtained, were further purified by preparative TLC, on magnesia-celite plates as already described.

(b) Fractionation into classes and their estimation -

The 'volatile' and 'total' monocarbonyl-DNP's were separated into different classes namely, alkan-2-ones, alkanals, alk-2-enals and alka-2,4-dienals and their presence confirmed by procedures already described. The relative proportions of the four classes of 'volatile' and 'total' monocarbonyls (Tables 29, 30, 31 and 32), were estimated spectrophotometrically. The data showed that there were no major differences in the relative proportions of the four classes of 'volatile' and 'total' monocarbonyls of fresh ghee (cow and buffalo) prepared from fresh and 'ripened' butters, except that the alkanal levels were
lower in cow ghee prepared from fresh butter. The decreasing order in which various monocarbonyls occurred in fresh ghee (all types) was: alkan-2-ones (about 86-95%), alkanals (about 2-7%), alk-2-enals (about 1-3%) and alka-2,4-dienals (about 1-3%).

On storage for 100 days alkan-2-ones level fell to about one half to one third in ghee (cow/buffalo) prepared from fresh butter and to about one-fourth, to one-fifth in ghee (cow/buffalo) prepared from 'ripened' butter. The concentration of the other three classes increased, irrespective of the type of ghee. The proportions of alkanals, alk-2-enals and alka-2,4-dienals were respectively 36, 20 and 8% cow ghee (from fresh butter) and 45, 22 and 8% respectively in cow ghee (from 'ripened' butter). The corresponding values were 41, 28 and 9% in buffalo ghee (from fresh butter) and 47, 28 and 9% in buffalo ghee (from 'ripened' butter). The increase due to storage, in the level of alkanals was thus more significant in ghee (cow/buffalo) prepared from 'ripened' butter.

(c) Separation of the monocarbonyl-DNP-classes into individual DNP's and their identification

The monocarbonyl-DNP-classes, obtained above, were separated into individual components by TLC on kieselguhr G plates impregnated with carbowax-400 and GLC on carbowax(20M) column. Identification of the
individual components of different classes was achieved by comparative TLC and comparative GLC of authentic monocarboxyl-DNPs. Similar components of the four classes of 'volatile' and 'total' monocarboxyls were detected and identified in all types of ghee (ghee prepared from fresh and 'ripened' butters), except that an additional component was observed in the alkan-2-one-DNP fraction from stored ghee by TLC. The TLC and GLC patterns were similar to those obtained earlier in case of 'volatile' monocarboxyl classes (Figs. 9, 10, 11, 12, 13, 14, 15 and 16).

The individual monocarbonals of various classes identified by TLC and GLC are given below:

<table>
<thead>
<tr>
<th>Alkan-2-ones</th>
<th>Alkanals</th>
<th>'Alk-2-enals</th>
<th>'Alka-2,4-dienals</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLC GLC</td>
<td>TLC GLC</td>
<td>TLC GLC</td>
<td>TLC GLC</td>
</tr>
<tr>
<td>C2</td>
<td>C3</td>
<td>C3</td>
<td>Unidentified</td>
</tr>
<tr>
<td>C4</td>
<td>C4</td>
<td>C4</td>
<td>C4</td>
</tr>
<tr>
<td>C5</td>
<td>C5</td>
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<td>C8</td>
<td>C8</td>
<td>C8</td>
<td>C8</td>
</tr>
<tr>
<td>C9 &amp; C10</td>
<td>C9 &amp; C10</td>
<td>C9 &amp; C10</td>
<td>C9</td>
</tr>
<tr>
<td>C11</td>
<td>-</td>
<td>-</td>
<td>C11</td>
</tr>
<tr>
<td>C12</td>
<td>-</td>
<td>-</td>
<td>C12, C12</td>
</tr>
</tbody>
</table>

(7)* (9)* (3)* (10)* (9)* (7)* (10)* (6)*

* Figures in parentheses are the number of components detected.
Day et al. (1962) identified the presence of ethanal, propanal, butanal, pentanal, acetone, butanone, diacetyl in the flavour volatiles from ripened cream butters. Lindsay (1966) proposed that the flavour of cultured butter was due to a combination of compounds contributing to the flavour of sweet cream or non-cultured butter and the compounds produced by the starter bacteria used to prepare the cultured butter; Kawanish and Saito (1965b) also identified propanal, hexanal, octanal, hexanone-2- and heptanone-2 from the flavour concentrate prepared from ripened butter.

(d) Contents of individual monocarbonyls in ghee and changes on storage — The relative proportions of individual monocarbonyls (volatile' and 'total') of different classes (except alka-2,4-dienals) are given in Tables 33 to 44. Individual alka-2,4-dienals of 'total' monocarbonyls could not be estimated as it was too laborious to obtain adequate quantities of these dienal-DNPs in this aspect of the study.

Individual 'volatile' alkan-2-ones of fresh ghee (cow and buffalo) prepared from fresh and 'ripened' butters, showed similar distribution patterns. The 'volatile' alkan-2-ones of fresh ghee (Tables 33 and 34) consisted mainly of heptan-2-one (about 36.34%), followed by pantan-2-one (about 21-23%), propanone (about 17.21%), octan-2-one
including higher homologues (about 21.18%), butan-2-one (about 2.5%) and hexan-2-one (about 2.2%). Distribution of 'total' alkan-2-ones was also similar for ghee (cow/buffalo) prepared from fresh and 'ripened' butter. The 'total' alkan-2-ones of fresh ghee (Tables 35 and 36) consisted mainly of propanone (about 74.76%), followed by octan-2-one and higher homologues (about 17.16%), butan-2-one (about 2.2%), hexan-2-one (about 2.2%), heptan-2-one (about 2.2%) and pentan-2-one (about 2.2%).

On storage, ghee (cow/buffalo) prepared from fresh and 'ripened' butter again gave identical distribution patterns for 'volatile' (Tables 33 and 34) and 'total' alkan-2-ones (Table 35 and 36). In case of 'volatile' alkan-2-ones of stored ghee (from fresh and 'ripened' butter), the levels of propanone and pentan-2-one decreased markedly and those of butan-2-one, hexan-2-one, heptan-2-one and octan-2-one (including higher homologues) increased. In case of 'total' alkan-2-ones also, the level of propanone decreased markedly, but those of butan-2-one, pentan-2-one, hexan-2-one, heptan-2-one and octan-2-one (including higher homologues) increased appreciably.

The proportions of individual alkanals ('volatile' as well as 'total') in general, were similar in cow and buffalo ghee prepared from fresh and 'ripened' butters.
(Tables 37, 38, 39 and 40). However, the contents of ethanal and octanal (and/or higher homologues) were higher and those of pentanal and hexanal lower in 'total' monocarbonyls as compared to those of 'volatile' monocarbonyls of ghee prepared from fresh and 'ripened' butters. On storage, an increase in the proportions of ethanal, propanal, hexanal and octanal (and/or higher homologues) and decrease in the proportions of pentanal and heptanal in 'volatile' monocarbonyls was observed, irrespective of the type of ghee. In contrast, the proportions of heptanal increased and those of ethanal and octanal decreased in 'total' monocarbonyls, irrespective of the type of ghee.

The trend in the relative proportions of individual alk-2-enals was also similar in both cow and buffalo ghee prepared from fresh and 'ripened' butters (Tables 41, 42, 43 and 44). The proportions of but-2-enal and non-2-enal (and/or higher homologues) were higher and those of pent-2-enal and oct-2-enal lower in 'total' monocarbonyls than those in 'volatile' monocarbonyls of ghee prepared from fresh as well as 'ripened' butters. On storage the proportions of oct-2-enal and non-2-enal (and/or higher homologues) increased and those of the unidentified component and hept-2-enal decreased in the 'volatile' and 'total' monocarbonyls, irrespective of the type of ghee. No marked changes were observed in the but-2-enal levels in 'total'
monocarbonyls on storage of ghee. In contrast, but-2-enal level in 'volatile' carbonyls increased during storage.

The ripening of butter (cow/buffalo) thus did not influence the carbonyl ('volatile' and 'total') make-up of ghee qualitatively. However, it caused significant changes in the quantitative patterns of the carbonyls. The contents of 'volatile' and 'total' carbonyls of fresh ghee prepared from 'ripened' butter were significantly higher than those of ghee prepared from fresh butter (exception: 'total' carbonyls of buffalo ghee). The increase in the contents of 'volatile' and 'total' carbonyls due to storage was also comparatively more in such ghee samples. Further, on storage, the decrease in the contents of alkan-2-ones and a concomitant increase in the contents of alkanals, alk-2-enals and alka-2,4-dienals was more marked in case of ghee (cow/buffalo) prepared from 'ripened' butter. Thus, the highly pronounced off-flavour developed, on storage for 100 days, in ghee prepared from 'ripened' butter was possibly due to the higher contents of carbonyls and marked changes in the distribution patterns of the four monocarbonyl classes.