STUDIES ON LACTONE PROFILE OF GHEE. PART II: STANDARDISATION BY GAS LIQUID CROMATOGRAFY (GLC) FOR FRACTIONATION AND ESTIMATION OF LACTONES

(MRS.) B.K. WADHWA and M.K. JAIN

National Dairy Research Institute, Karnal-132 001

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INTRODUCTION

Paper chromatographic technique was adequate only for identification (Keeney and Patton, 1956a, 1956b; Tharp and Patton, 1960; Patton, 1961) of lactones, but it was not very suitable for the fractionation/estimation of lactones. Forss et al. (1966) used adsorption TLC which resulted in incomplete separation of gamma-lactones from delta-lactones due to large amounts of delta-lactones relative to gamma-lactones in Australian butter oil. They also used partition TLC (Urbach, 1965) for the separation of gamma-and delta-lactones which indicated gamma-C_9-C_14 and delta-C_10-C_15 lactones. However, GLC of the same material led to a complete fractionation of these lactones. GLC has been used extensively for the quantitative estimation of the individual lactones in a lipid sample (Dimick et al., 1966) and has been found to be the best analytical procedure in this laboratory for this purpose (Wadhwa et al., 1978, 1979, 1980a, 1980b).

The present paper reports the GLC fractionation and estimation of lactones isolated from ghee by the developed column chromatographic method of Wadhwa (1983).

MATERIALS AND METHODS

Ghee Samples: Ray milk, drawn from pooled milks of cows maintained at the farm of the Institute, was used as the starting material for the preparation of ghee. Ghee samples were prepared by creamery butter (CB) method essentially according to the procedure outlined by Srinivasan and Anantakrishnan (1968) using the clarification temperature of 120°C/5 min.

Standard lactones: Saturated delta-C_9-C_9, C_18-C_11-C_12 and gamma-C_9-C_10-C_11-C_14, C_15 lactones (C.A. Aromatics Co., U.S.A.)

Saturated delta-C_13-C_14-C_15-C_18 and Gamma-C_15-C_16-C_18 lactones (gift samples from Dr. (Mrs.) G. Urbach, Division of Dairy Research, CSIRO, Melbourne, Australia).

Isolation of lactones from ghee: Lactones were isolated from ghee by the developed column chromatographic method of Wadhwa (1983).

Standardisation by GLC for fractionation of lactones mixture: Pye Unicam Series 304 flame ionization gas chromatograph dual-column) was used. Model mixture of delta-C_6-C_19-C_19 and gamma-C_9-C_12-C_13-C_18-C_18 lactones (each about 1%) in acetonitrile solvent was prepared. Both delta- and gamma-lactones up to C_19 were fractionated at 160°C and those above C_19 were fractionated at 195°C. Model mixture of lactones and lactonic isolates of ghee were fractionated under the conditions as follows:

Column stationary phase: 10%DEGS coated on 100-120 mesh Diatomite-C (AW).
Column dimensions: 1 M x 4 mm
Column temperature programming:
- Initial temperature: 160°C/40 min.
- Rate: 15°C/1 min.
- Upper temperature: 195°C/60 min.
Injector temperature: 210°C
Detector temperature: 225°C
Range: 100
Attenuation: 16
Flow rate of gases:
- Carrier nitrogen = 30 ml/min
- Hydrogen = 33 ml/min
- Air = 330 ml/min

Lactonic isolates of ghee samples were fractionated under the standardised conditions mentioned above.

Characterisation of lactones: The characterisation of lactones of ghee samples was made through retention times.

Estimation of lactones by GLC: Gamma-C_7 lactone was chosen as the internal standard (IS) to be added to the lactonic isolates of different ghee samples. 25 µg of this internal standard was added to the lactonic isolate from 10 gm ghee. The whole material was dissolved in 0.05 ml (50 µl) of acetonitrile. 4 µl of this solution was injected into the column under the standardised conditions of fractionation of lactones described earlier. Individual lactones of ghee samples were quantified according to the equation:

\[
\text{ppm of lactones} = \frac{2 \times \text{peak area of lactone} \times 50}{\text{Peak area of internal standard} \times 4 \times 10} = 2.5 \times \frac{\text{Peak area of lactone}}{\text{Peak area of internal standard}}
\]

Peak areas were calculated by the triangulation method.

RESULTS AND DISCUSSION

GLC analysis of lactonic isolates of ghee:
Fig. 1 shows the GLC separation of model mixture of gamma-C_6-C_12, C_15-C_18, and delta-C_8-C_19, C_18 lactones at 160-195°C. Both gamma- and delta-lactones up to C_12 fractionated at 160°C and those above C_13 fractionated at 195°C. Fig. 2 shows the GLC separation of lactonic isolates of ghee under the same conditions as used for model mixture of lactones. The gas

Fig. 1 GLC Separation of model mixture of γ-C_6-12, C_15-C_18, and δ-C_8-C_19, C_18 lactones
chromatograms showed 44 peaks. Of these, 20 peaks were characterised through their retention times as delta-C_6-C_10,C_16 and gamma-C_6-C_12,C_13,C_15,C_18 lactones, 4 peaks were tentatively identified as delta-C_6,C_7 and gamma-C_13-C_16 lactones. Gamma-C_7 lactone was absent in ghee and was, therefore, chosen as the internal standard for the quantification of lactones in ghee. Kinsella et al. (1967) also reported absence of gamma-C_7 lactone in butterfat. However, Dimick et al. (1969) reported traces of this lactone in butterfat.

The homologous series of both delta- and gamma-lactones from C_6-C_10 and C_16 identified in the lactonic isolates of ghee is almost similar to that reported for butterfat in the literature (Kinsella et al., 1967; Kurtz, 1978). Whereas, literature reports indicated the absence of gamma-C_18 lactone in butterfat, we found the C_18 lactones (gamma-as well as delta-) were present in ghee, besides the C_6-C_16 lactones. It is important to note that in our earlier studies (Wadhwa et al., 1979; 1980a, 1980b) only delta-C_10,C_12 and gamma-C_10,C_12 lactones were the major components besides 3-4 unidentified peaks. Of the unidentified peaks at that time, one peak (a major one) could now be identified as delta-C_18 lactone. One of the earlier reports (Wadhwa et al., 1979) had indicated gamma-C_6 lactone also but this lactone was subsequently found to be absent (Wadhwa et al., 1980a, 1980b).

In the present study, out of 44 peaks in the gas chromatograms of lactonic isolates of ghee, we could identify homologous series of both delta- and gamma-lactones from C_6-C_10 and C_16. Gamma-C_5 lactone may or may not be present in 1-7 unidentified peaks (Fig.2).

**Unidentified lactones**: The gas chromatogram (Fig.2) of the lactonic isolate of ghee contained 20 peaks whose retention times differed from those of saturated delta- and gamma-C_6-C_10,C_16 lactones, already identified. These 20 peaks, therefore, remain to be identified and they might include:

(i) Some possible n-saturated lactones like gamma-C_6,C_7,C_17 and delta-C_5,C_17 lactones.

(ii) Substituted saturated lactones.

(iii) Unsaturated lactones.

It has also been reported that some additional lactones having unsaturations and/or alkyl branches are also present in milk fat (Kurtz, 1978). It, therefore, appears that these lactones corresponding to these peaks do not belong to either of the homologous series already reported.
Some of these peaks have been partly characterised and their characterisation considerably narrows down as follows: Peak Nos. 1 to 7: In the gas chromatogram the peaks 1-7 are located before the gamma-C₄ lactone peak. It is, therefore, likely that three of these peaks correspond to lower n-saturated lactones like gamma-C₄, C₅ and delta-C₅. The remaining four peaks may correspond to lower substituted saturated or/and unsaturated lactones.

Peak Nos. 40 to 42: The peak Nos. 40-42 exist between those of delta-C₁₆ and gamma-C₁₈ lactones. GLC separation pattern of model mixture of gamma-and delta-lactones (Fig. 1) has shown that a saturated delta-lactone has a higher retention time than a saturated gamma-lactone of same carbon number. It has also been observed that the sequence of their elution (in order of increasing retention time) is:

gamma-Cₙ, delta-Cₙ, gamma-Cₙ₊₁, delta-Cₙ₊₁, gamma-Cₙ₊₂, delta-Cₙ₊₂, ..............

where n denotes the number of carbon atoms.

On the basis of above pattern of elution of lactones, it can be suggested that gamma-C₁₇ and delta-C₁₇ lactones are possibly present among peak Nos. 40-42. Nevertheless, it is difficult to characterise the third peak among this bunch of three peaks.

Peak No. 10: The minor peak No. 10 exists between those of delta-C₄ (peak No. 9) and gamma-C₇ (peak No. 11) lactones. Honkanen et al. (1968) have reported the presence in butterfat of substituted saturated lactone, trans-4-methyl-5-hydroxy-hexanoic acid lactone and the position of this lactone was between delta-C₄ and delta-C₇ lactones in gas chromatograms. This peak No. 10, therefore, appears to be trans-4-methyl-5-hydroxy-hexanoic acid lactone on the basis of the evidence cited above.

Peak No. 28: In the gas chromatogram, peak No. 28 exists between that of delta-C₁₃ (peak No. 27) and gamma-C₁₃ (peak No. 29) lactones. Boldingh and Taylor (1962) and Zijden et al. (1966) have reported the occurrence of the unsaturated delta-lactone of 9-dodecenoic acid, and the gamma-lactone of 6-dodecenoic acid.

Possibly, peak No. 28 is an unsaturated C₁₂-lactone. Peak No. 29 cannot be characterised, gamma-dodecenoic-lactone as this compound has lower retention time than the saturated delta-C₁₂ lactone (Zijden et al., 1966). Therefore, delta-dodecenoic-9-lactone appears to be the likely structure of the lactone corresponding to peak 28.

Peak Nos. 31 and 34: Urbach and Stark (1978b) have identified four unsaturated C₁₄-lactones in goat butterfat by the combination of GLC, IR and MS data, and these are:

1. cis delta-tetradec-8-enolactone
2. trans delta-tetradec-8-enolactone
3. non-conjugated cis, trans-gamma-tetradecadienolactone
4. conjugated cis, trans-delta-tetradecadienolactone

Gas chromatography on Apienzon 'L gave the order of elution of these lactones with respect to saturated gamma and delta-C₁₄ lactones (in order of increasing retention times) as:

1. cis delta-tetradec-8-enolactone gamma-C₁₄ saturated lactone
2. trans delta-tetradec-8-enolactone delta-C₁₄ saturated lactone
3. non-conjugated cis, trans-gamma-tetradecadienolactone
4. conjugated cis, trans-delta-tetradecadienolactone
Lactone Profile of Ghee

It shows that:

- trans-monoenolactone has higher retention time than the cis-isomer [cf. (2) and (1)]

- conjugated dienolactone has higher retention time than the monounsaturated (cis or trans) lactone or same carbon number [cf. (4), (1) and (2)]

- trans-monounsaturated lactone has lower retention time than the corresponding saturated lactone [cf. (2) and delta-C14 saturated lactone]

- conjugated dienolactone has higher retention time than the corresponding saturated lactone [cf. delta-C14 saturated lactone and (4)].

An examination of Fig. 2 shows that there are two peaks, Nos. 31 and 34, in the vicinity of gamma-C14 and delta-C14 lactones. The order of elution (in order of increasing retention times) is:

- peak no. 31
- gamma-C14
- delta-C14
- peak No. 34

A critical comparison of A and B suggests that peak No. 31 possibly corresponds to either the non-conjugated cis, trans-gamma-tetradecadienolactone (3), or cis-delta-tetradec-8-enolactone (1). Similarly, peak No. 34 appears to correspond to the conjugated cis, trans-delta-tetradecadienolactone.

The presence of delta-tetradec-9-enolactone in butterfat has also been indicated by Zijden et al. (1966). Monounsaturated lactone has lower retention time than the corresponding saturated lactone [cf. (2) and delta-C14 saturated lactone]. Peak No. 34 has higher retention time than the saturated delta-C14, saturated lactone. Hence, the structure of peak 34 as delta-tetradec-9-enolactone is ruled down. Thus, peak No. 34 appears to correspond to the conjugated cis, trans-delta-tetradecadienolactone.

Peak No. 18: The minor peak No. 18 occurs between gamma-C9 (Peak No. 17) and delta-C9 (peak No. 19) lactones. By reasoning similar to that used above in the case of peak Nos. 31 and 34, it can be shown that peak No. 18 appears to be transmonounsaturated delta-C9 lactone [cf. (2) and delta-C14 saturated lactone above]. There is yet another possibility of peak 18 being a conjugated dien-gamma-C9 lactone [cf. delta-C14 saturated lactone and (4) above]. Boldingh and Taylor (1962) and Lardelli et al. (1966) reported a compound characteristic of butter flavour as, 2,3-dimethyl-2, 4-nonadien-4-olide (bovolide). This compound with a strong odour reminiscent of celery has been associated with butters of widely different origins. Interestingly, this compound is a disubstituted conjugated gamma-nonadienolactone. As there is, sufficient information about the occurrence of this compound in butterfat, it is reasonable to suggest peak No. 18 corresponding to ‘bovolide’ of ghee.

It is significant to point out at this stage that Jurrians and Oele (1965) have also reported the occurrence of small peaks whose retention times differed from those of saturated gamma- and delta-lactones. They also concluded tentatively that these small peaks were of unsaturated lactones. Unsaturated lactones have also been reported in natural products like peach essence (Sevensants and Jennings, 1971) and amongst the volatile components of the lactose-casein browning system (Ferretti and Flanagan, 1971).

It is not unreasonable to assume that unsaturated lactones other than those discussed above might also contribute to the flavour of ghee. A similar observation based on mass
Spectral data was made by Urbach et al. (1972) in respect to the natural flavour of butter. Possibly peak Nos. 13, 15, 20, 24 and 38 belong to the category of unsaturated lactones, but it needs a more extensive study to clinch this issue.

Lactone profile of ghee: Lactone levels (ppm) of cow ghee prepared by CB method are given in Table 1. Data on the four replicate samples and their average values are also tabulated. Lactone contents in ppm are given in parentheses.

(a) Individual lactones: ‘Even’ lactone contents in ghee can be arranged in the increasing order.

delta-lactones: \( C_8 \) (0.26), \( C_6 \) (0.73), \( C_{16} \)

**TABLE 1**

<table>
<thead>
<tr>
<th>C-No.</th>
<th>Delta-lactones</th>
<th>Gamma-lactones</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample No. 1 2 3 4 Average</td>
<td>Sample No. 1 2 3 4 Average</td>
</tr>
<tr>
<td>6</td>
<td>1.12 0.92 0.32 0.55 0.73 t t t t t</td>
<td>t t t t t</td>
</tr>
<tr>
<td>7</td>
<td>0.26 0.30 0.17 0.30 0.26 t t t t t</td>
<td>t t t t t</td>
</tr>
<tr>
<td>8</td>
<td>0.05 0.10 0.10 0.06 0.08 0.02 0.02 0.04 0.02 0.02</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.03 1.70 1.03 1.05 1.20 0.14 0.30 0.32 0.28 0.26</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.26 0.30 0.34 0.13 0.26 0.17 0.20 0.17 0.15 0.25</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>3.07 3.42 3.07 2.74 3.07 0.47 0.16 0.22 0.34 0.30</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.04 0.02 0.08 0.05 0.05 t t t t t</td>
<td>t t t t t</td>
</tr>
<tr>
<td>13</td>
<td>2.08 2.88 1.95 2.11 2.25 t t t t t</td>
<td>t t t t t</td>
</tr>
<tr>
<td>14</td>
<td>0.85 0.94 0.53 0.78 0.77 0.50 0.60 0.54 0.20 0.36</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.80 1.24 1.21 1.19 1.09 0.41 0.38 0.43 0.38 0.40</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>7.36 8.34 8.62 8.23 8.14 0.25 0.12 0.10 0.19 0.17</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>16.92 20.16 17.42 17.10 17.90 2.26 1.78 1.42 1.56 1.76</td>
<td></td>
</tr>
</tbody>
</table>

**Sample No.**

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated delta- + gamma-(24 Peaks)</td>
<td>19.18</td>
<td>21.94</td>
<td>18.84</td>
<td>18.66</td>
<td>19.66</td>
</tr>
<tr>
<td>Unidentified (20 peaks)</td>
<td>10.81</td>
<td>12.11</td>
<td>8.97</td>
<td>10.48</td>
<td>10.60</td>
</tr>
<tr>
<td>Gross (44 peaks)</td>
<td>29.99</td>
<td>31.08</td>
<td>27.81</td>
<td>29.14</td>
<td>30.26</td>
</tr>
</tbody>
</table>

\( t = \text{traces (< 0.01 ppm)} \)
(1.09), C₁₉ (1.20), C₁₄ (2.25), C₁₂ (3.07), C₁₈ (8.14)

**Gamma-lactones**  
C₇ (0.17), C₁₉ (0.26), C₁₂ (0.30), C₁₆ (0.40)

This showed that there was no definite correlation between the carbon numbers and the contents of 'even' lactones (delta as well as gamma-).

'Odd' lactone contents in cow ghee arranged in the increasing order, were:

**Delta-lactones**  
C₇ (0.05), C₉ (0.08), C₁₁ (0.26), C₁₅ (0.77)

**Gamma-lactones**  
C₁₃ (0.02), C₁₉ (0.25), C₁₅ (0.36)

The levels of 'odd' delta-lactones appeared to increase with the increase in their carbon numbers except for delta-C₁₃ lactone. Similarly, the levels of 'odd' gamma-lactones appeared to increase with the increase in their carbon numbers except for gamma-C₁₂ lactone.

It is apparent that 'even' lactones (delta- as well as gamma-) were the predominant constituents in ghee. However, delta-C₁₀ (1.20), C₁₃ (3.07), C₁₁ (2.25), C₁₈ (1.09), C₁₆ (8.14) lactones were the major components in ghee. These lactones have also been regarded as major constituents in butterfat (Kinsella et al., 1967; Kurtz, 1973). Ellis and Wong (1973) have reported in fresh butter, delta-C₁₀, C₁₂, C₁₃ lactones at 1.0, 2.1 and 3.9 ppm levels respectively.

**Total delta-lactone levels**: Total delta-lactone levels (17.90:16.92-20.16) were about 59% of the gross lactone levels (30.26:27.81-34.08) in ghee.

**Total gamma-lactone levels**: Total gamma-lactone levels (1.76:1.42-2.26) were about 6% of the gross lactone levels in ghee.

**Saturated delta- + gamma-lactone levels**: The combined delta-and gamma-lactone levels (19.66:18.66-21.94) were about 65% of the gross lactone levels in ghee.

**Unidentified lactone levels**: Unidentified lactone levels (10.60:8.97-12.14) were about 35% of the gross lactone levels in ghee.

Thus, it can be concluded that saturated delta- + gamma-and gross lactone levels in ghee were 20 and 30 ppm respectively.

It again infers from Table 2 that delta-lactones, compared to gamma-lactones, were predominant constituents in ghee. Ratio of delta-to gamma-lactones was 10:1. Forss et al. (1966) have reported the ratio of delta-to gamma-lactones as 4:1 in Australian butter oil. Kinsella (1969) reported the ratio of delta-to gamma-lactones as 19:1 in butterfat.

**SUMMARY**

The gas chromatograms of lactic isolates of ghee showed a complex profile (44 peaks). The homologous series of n-saturated delta-and gamma-lactones (24 peaks from C₆-C₁₈ and C₁₃ have been characterised in ghee. The 20 peaks have been partly characterised and these may include n-saturated, substituted saturated and unsaturated lactones.

Delta-lactones, compared to gamma-lactones, were the major components in ghee. Predominant delta-lactones in ghee were delta-C₁₀,C₁₂,C₁₄,C₁₆,C₁₈ at 1.20, 3.07, 2.25, 1.09 and 8.14 ppm levels, respectively. Ratio of delta-to gamma-lactones was 10:1. Saturated delta- + gamma-and gross lactone levels in ghee were 20 and 30 ppm respectively. Gross lactone levels constituted about 65% of saturated delta- + gamma-and about 35% of unidentified (partly characterised as mostly unsaturated) lactones in ghee.
ACKNOWLEDGEMENTS

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REFERENCES


