Separation of triglycerides by gas–liquid chromatography

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ABSTRACT The parameters affecting the separation and quantification of triglycerides by gas-liquid chromatography have been investigated with the use of QF-1 and SE-30 as stationary phases and a flame ionization detector.

The isothermal characteristics of a wide variety of triglycerides (carbon number 6 to 60) on both columns show that log retention volume is directly proportional to carbon number and inversely proportional to absolute temperature. Isothermal retention indices of some triglycerides are given, as are column efficiencies (in terms of theoretical plates and ability to separate closely related triglycerides).

When various rates of programmed temperature rise are used, retention indices have been found to be less useful than absolute or relative elution temperatures. The elution temperatures of triglycerides of carbon number 6 to 54 have been determined relative to that of trilaurin. Under optimal separation conditions weight and molar correction factors can be obtained.

Triolein and tristearin have been partially separated, as have certain triglycerides that have the same carbon number but widely different fatty acids. The natural triglycerides of human milk fat have been separated.

Qualitative parameters are largely lacking in the literature of triglyceride GLC. Attention has mainly been devoted to separation of triglycerides on nonpolar stationary phases (1–15), since at the high temperatures required most polar stationary phases are unstable. Only a brief report by Kuksis (16) on the use of QF-1, a fluoroalkyl silicone oil, has appeared, even though this substance has been widely used in the steroid field as a selective stationary phase (18).

We report here qualitative data for good separations of tri-C6 to tri-C54 on 3% QF-1 and data for excellent separations on 10% SE-30, a methyl silicone gum. Under isothermal conditions, the relationship log \( V_r = kC \) is well established (19) for fatty acids and esters; for a homologous series of compounds in which the heats of solution (\( AH_s \)) do not vary significantly with temperature, the relationship (20) \( \log V_r = a/bT \) (\( a \) and \( b \) are constants, \( T \) is in °K) holds. We have now investigated whether these relationships apply to analysis of triglycerides.

In order to standardize measurements of the retention of triglycerides under isothermal conditions we have also applied the retention index system suggested by Wehrli and Kovats (21). This relates the \( V_r \) values of a series of compounds to those of \( n \)-alkanes. In practice this involves choosing \( n \)-alkanes to be added to the sample that would elute before and after any peak investigated. If appli-
cable, the isothermal retention index \( I_i \) of a triglyceride would be given by the equation:

\[
I_i = 100 \left( \frac{\log V_{rc} - \log V_{rr}}{\log V_{ry} - \log V_{rx}} + 100x \right)
\]

where \( V_{rc} \) = retention volume of a triglyceride with \( C \) acyl carbon atoms; \( V_{rr} \) = retention volume of \( n \)-alkane with \( x \) carbon atoms; \( V_{ry} \) = retention volume of \( n \)-alkane with \( y \) carbon atoms.

Since, in practice, temperature programming is needed to separate any wide range of triglycerides, we have standardized retention measurements under these conditions. An extension of isothermal retention indices to temperature programming has been proposed by Guiochon (22). The temperature program retention index \( I_{tp} \) is obtained by substituting the absolute elution temperature \( T_e \) (i.e., the temperature at which the compound and \( n \)-alkanes are eluted during programming) for the \( \log V \) values in equation 1 to give:

\[
I_{tp} = 100 \left( \frac{T_{re} - T_{rr}}{T_{ry} - T_{rx}} + 100x \right)
\]

If this applies to temperature-programmed triglyceride analysis, equation 2 would hold, with the subscripts having the same meaning as in equation 1.

The validity and usefulness of \( I_{tp} \) values has been questioned (23), partly on the grounds of the difficulty of obtaining \( I_{tp} \) from \( I_i \) measurements.

Quantitative aspects of analysis have been carefully studied by others (24) for longer-chain simple triglycerides; we have also studied short-chain simple triglycerides, as well as a modest range of mixed triglycerides.

Since our columns were efficient enough to separate triglycerides of the same carbon number (25) we are able to report considerably better results than those previously given (25). We have also investigated degradation of triglycerides, since this has been reported for saturated and unsaturated triglycerides on 10% SE-30 (26).

**MATERIALS**

SE-30, QF-1, dimethylchlorosilane-treated Chromosorb W (100-120 mesh), and Gas-Chrom Q (100-120 mesh) were purchased from Applied Science Laboratories Inc., State College, Pa. Nitrogen (dried and oxygen-free), air, and hydrogen were obtained from Air Products Ltd., Darlaston, Wednesbury, Staffordshire, or from British Oxygen Ltd., Birmingham, England. No special drying procedures were necessary.

Simple triglycerides (99% pure) were purchased from Sigma Chemical Co., London, England, except for tripropionin (Mann Research Labs Inc., New York). Mixed triglycerides were the generous gift of Dr. C. Barrett, Unilever Research Laboratory, The Frythe, Welwyn, Herts. We also synthesized 2-butyro-1,3-dipalmitin, 1-butyro-2,3-dipalmitin, 2-butyro-1,3-diolein, and 1-butyro-2,3-diolein by the trifluoroacetic acid method of Bourne, Stacey, Tatlow, and Tedder (27) from the corresponding mono- or diglyceride precursors. Isomeric products were identified by their slight differences in \( V_e \) on GLC, but positional isomerization was not determined absolutely. Tri-C<sub>4</sub>:0, tri-C<sub>6</sub>:0, and tri-C<sub>8</sub>:0 were also synthesized by this method. The fatty acids used in these syntheses were obtained from Fluka AG, Buchs S.G., Switzerland (butyric, caproic, and oleic acids) or from British Drug Houses, Poole, England (pentanoic and heptanoic acids). The latter firm supplied \( n \)-hexadecane and \( n \)-eicosane. \( n \)-Dotriacontane (C<sub>32</sub>) was a gift from Koch-Light Laboratories, Ltd., Colnbrook, England.

Before use, triglycerides were purified to the point at which they gave (a) a single spot or band on silica gel TLC in hexane-diethyl ether 60:40; (b) a single peak on GLC (QF-1 or SE-30); and (c) the correct fatty acid composition after hydrolysis and GLC of the free fatty acids on phosphorylated diethylene glycol adipate (C<sub>4</sub>:0-C<sub>12</sub>:0) or of the methyl esters on polyethylene glycol adipate (C<sub>12</sub>:0-C<sub>20</sub>:0).

Rabbit milk was extracted with 20 volumes of chloroform-methanol 2:1. After centrifugation, the lower chloroform-rich phase was removed, dried over anhydrous sodium sulfate, filtered, and taken to dryness at room temperature with a stream of nitrogen. The lipid was dissolved in hexane and the triglycerides were obtained by TLC on silica gel in hexane-diethyl ether 60:40. Human milk and bottled pasteurized cow’s milk were extracted in the same way.

**METHODS**

Two columns were used: 3% QF-1 on acid-washed dimethylchlorosilane-treated Chromosorb W in a 168 cm \( \times \) 6.5 mm stainless steel tube, and 10% SE-30 on Gas-Chrom Q in a 53.5 cm \( \times \) 6.5 mm stainless steel tube. Both were conditioned at 350\(^\circ\)C for 48 hr with a stream of nitrogen at 35 ml/min. Nitrogen was used as carrier gas throughout. Unless otherwise stated, analyses were carried out on the QF-1 and SE-30 columns described above.

A Pye 204 dual column chromatograph with dual flame ionization detectors was used. Hamilton 7001 NCH (1 \( \mu l \)) and 701 NWG (10 \( \mu l \)) syringes were employed. Columns were packed so that, on injection, the tip of the needle came just above the packing.

Triglycerides C<sub>6</sub>-C<sub>9</sub> were applied in about 50% (w/v) solution in benzene, and triglycerides C<sub>24</sub>-C<sub>40</sub> in about 10% (w/v) solution in chloroform-methanol 2:1.

In order to determine \( V_e \) in the isothermal studies, we measured flow rates through the QF-1 and SE-30 col-
umns at temperature intervals over the range used. The flow rate changed very little through the SE-30 column over the temperature range 120°C-330°C, but the flow rate through QF-1 column decreased greatly with increasing temperature. \( V_r \) values have been calculated from the flow rates and \( t_r \) measurements.

For the nominal setting of the programmer for a range of 110°C to 330°C at 2°C, 4°C, and 6°C/min, the true program (as given in the Results) was found to be 118°C-322°C at 1.85°C, 3.71°C, and 5.56°C/min.

Peak areas were measured by triangulation to about ±4% accuracy.

**RESULTS**

**Qualitative Characteristics of GLC of Triglycerides**

**Isothermal Analysis.** Since it was not possible to chromatograph the series of triglycerides \( C_n-C_m \) at a single temperature, several isothermal analyses were carried out. A direct relationship was obtained between the logarithm of the retention volume and the reciprocal of the absolute temperature at which they were obtained (Fig. 2).

As a test of the relationship \( \log V_r = a + b/T \), the isothermal values for \( \log V_r / C = k \) were related to the reciprocal of the absolute temperature at which they were obtained (Fig. 2).

At all temperatures used, smaller retention volumes were observed on 3% QF-1 than on 10% SE-30.

Kovats’ indices (21) have been determined for the simple saturated triglycerides \( C_{12}-C_{24} \) in order to compare isothermal retention indices \( (I_r) \) with temperature programs indices \( I_p \). The triglycerides were cochromatographed with o-hexadecane \( (C_{16}) \), \( \sigma \)-eicosane \( (C_{20}) \), and \( \sigma \)-dotriacontane \( (C_{32}) \) on QF-1 and SE-30. \( I_r \) was calculated from equation 1, and the results are shown in Table 1.

Column efficiencies in terms of \( \Delta C \), the minimum carbon number difference between two triglycerides which can be completely resolved, and theoretical plates for the hydrocarbon standards were those used in the isothermal studies (i.e. \( C_{16}, C_{20}, \) and \( C_{32} \)). \( I_p \) values are shown in Table 3.

The elution temperatures for triglycerides, relative to that of trilaurin \( (T_{RR}) \) are shown in Table 4.

Relative column efficiencies of QF-1 and SE-30 for the simple saturated triglycerides \( C_{12}-C_{18} \) have been determined. A standard mixture of simple saturated triglycerides \( C_{12}-C_{18} \) containing 2.5-10 \( \mu g/\mu l \) of each component was used. The columns were run at 250°C isothermally for 10 min and then programmed at 1.85°C/min to 322°C (final equilibrium temperature 330°C). \( \Delta C \) for QF-1 was 2.46 ± 0.09 and for SE-30 was 1.36 ± 0.01. The range \( C_{12}-C_{18} \) was chosen as being representative of most milk triglycerides [see accompanying paper (28)].

**Quantitative Characteristics of GLC of Triglycerides**

Detector response to variation in loading for triglycerides of different carbon numbers has been investigated. We considered it more realistic to use a biological sample than a mixture of synthetic triglycerides. A natural mixture of rabbit milk triglycerides was employed because it was readily available in this laboratory. For completeness, detector response to short-chain simple triglycerides \( (C_6-C_{12}) \) has also been determined.

Since the flow rate through the SE-30 column did not vary substantially with temperature, and excellent separations can be obtained (see Qualitative Data), this column was used rather than the QF-1 for this part of the work.

<table>
<thead>
<tr>
<th>Table 1: Retention Indices ((I_r)) of Simple Saturated Triglycerides (C_{12}-C_{24}) on QF-1 and SE-30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride (Carbon Number)</td>
</tr>
<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>15</td>
</tr>
<tr>
<td>18</td>
</tr>
<tr>
<td>21</td>
</tr>
<tr>
<td>24</td>
</tr>
</tbody>
</table>

In no case did the error exceed ± 5 indices in triplicate determinations on QF-1 and duplicate determinations on SE-30. Sample size was 5-25 \( \mu g/\)peak, using the standard mixtures of triglycerides given in Fig. 1 plus suitable quantities of the hydrocarbons \( C_{16}, C_{20}, \) and \( C_{32} \).
Fig. 1. Relationship of log $V_t$ and $C$ at various temperatures for simple saturated triglycerides. $a$, QF-1; $b$, SE-30. Duplicate or triplicate samples were chromatographed at each temperature. Reproducibility was within ± 1%. Sample load was 5–25 µg/peak in standard mixtures (by weight) of triglycerides $C_{24}$–$C_{60}$.

Fig. 3a shows a linear detector response to increasing loads of triglycerides $C_{24}$, $C_{30}$, $C_{48}$, $C_{54}$, and $C_{60}$. Intermediate even-numbered triglycerides in the sample showed similar linear plots which passed through or near the origin (except $C_{48}$). A similar result was obtained for the short-chain simple saturated triglycerides $C_6$–$C_{21}$ (Fig. 3b).

Since adsorption on the column became readily apparent for milk triglycerides higher than $C_{44}$, the observed detector response in these cases is a combination of the amount of material reaching the detector and the weight % of carbon in the molecule (29, 30). To achieve overall quantification for as wide a range of triglyceride chain-lengths as possible, we applied the method of internal normalization (31) to give (24) weight correction factors ($f_w$) and molar correction factors ($f_m$), where $f_w = \text{weight} %/\text{area} \%$ and $f_m = \text{mole} %/\text{area} \%$.

These factors were obtained by chromatography of

Watts and Dils  Separation of Triglycerides by GLC  43
Triglyceride absolute deviation is given. Sample load and composition as in Table 1.

They can be readily calculated from data given. Values of $fw$ with short-chain simple saturated triglycerides, CB-Cz1, on SE-30 is shown graphically in Fig. 5. The large values of $fw$ for small carbon number make it impossible to show these on the same scale as the $fw$ values given in Fig. 5. They can be readily calculated from data given. Values of $fw$ on QF-1 are shown for comparison with SE-30 in Fig. 6.

Separation of Triglycerides of the Same Carbon Number

Triglycerides of the same carbon number but widely different fatty acid compositions have been examined. The partial separation of tri-C12:0 and 2-butyro-1,3-dipalmitin and of triolein and tristearin have been achieved on SE-30 with temperature programming (Fig. 6). Under the same conditions, tripalmitolein and tripalmitin were not separated.

When cow milk triglycerides were chromatographed together with 2-butyro-1,3-distearin on SE-30 with temperature programming, the C40 peak was partially split (Fig. 7). Results of the isothermal separation of isomeric triglycerides on SE-30 are given in Table 5. Because of the broad peaks of trilinolein and trilinolenin, the only distinguishable separation of C54 isomers was that of triolein and tristearin.

We were also able to separate C56 isomers (tri-C15:0 and 2-butyro-1,3-dipalmitin) to an extent similar to that shown in Fig. 6a on QF-1, but attempts to reproduce the partial separation of triolein and tristearin (Fig. 6b) on QF-1 were unsuccessful.

Since these partial separations indicated that natural fats might not, in fact, separate only according to carbon number on our columns, mixed and simple triglycerides were cochromatographed with samples of cow and rabbit milk fat. Except in the case of C40, cited above, cochromatography with the expected peak occurred with no distinct resolution. This was even so in the case of the C54 isomers, where only partial resolution was found. Overlap with C47 and C44 was slight. Hence, even if the peaks observed with natural triglycerides are a spectrum of triglycerides of the same carbon number, they overlap peaks of different carbon number to a negligible extent.

### Table 3: Programmed Retention Indices ($f_p$) of Simple Saturated Triglycerides C12–C44 on QF-1 and SE-30

<table>
<thead>
<tr>
<th>Triglyceride (Carbon Number)</th>
<th>QF-1</th>
<th>SE-30</th>
<th>QF-1</th>
<th>SE-30</th>
<th>QF-1</th>
<th>SE-30</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>2255 ± 20 (3)</td>
<td>1770 ± 25 (2)</td>
<td>2275 ± 4 (2)</td>
<td>1795 ± 5 (2)</td>
<td>2315 ± 20 (3)</td>
<td>1805 ± 10 (2)</td>
</tr>
<tr>
<td>15</td>
<td>2575 ± 35 (3)</td>
<td>2050 ± 10 (2)</td>
<td>2600 ± 2 (2)</td>
<td>2070 ± 5 (2)</td>
<td>2615 ± 25 (3)</td>
<td>2095 ± 25 (2)</td>
</tr>
<tr>
<td>18</td>
<td>2860 ± 30 (3)</td>
<td>2340 ± 20 (2)</td>
<td>2865 ± 5 (2)</td>
<td>2400 ± 10 (2)</td>
<td>2910 ± 20 (3)</td>
<td>2380 ± 10 (2)</td>
</tr>
<tr>
<td>21</td>
<td>3100 ± 25 (3)</td>
<td>2590 ± 30 (2)</td>
<td>3110 ± 5 (2)</td>
<td>2680 ± 15 (2)</td>
<td>3145 ± 5 (3)</td>
<td>2675 ± 10 (2)</td>
</tr>
<tr>
<td>24</td>
<td>—</td>
<td>2830 ± 50 (2)</td>
<td>—</td>
<td>2935 ± 5 (2)</td>
<td>—</td>
<td>2940 ± 10 (2)</td>
</tr>
</tbody>
</table>

The figures in parentheses represent the number of determinations. For triplicates, standard deviation is quoted; for duplicates, the absolute deviation is given. Sample load and composition as in Table 1.

### Table 4: Relative Elution Temperatures ($T_R$) of Simple Saturated Triglycerides C12–C44 on QF-1 and SE-30

<table>
<thead>
<tr>
<th>Triglyceride (Carbon Number)</th>
<th>QF-1</th>
<th>SE-30</th>
<th>QF-1</th>
<th>SE-30</th>
<th>QF-1</th>
<th>SE-30</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.493 ± 0.004</td>
<td>0.493 ± 0.001</td>
<td>0.485 ± 0.002</td>
<td>0.425 ± 0.009</td>
<td>0.485 ± 0.004</td>
<td>0.418 ± 0.004</td>
</tr>
<tr>
<td>9</td>
<td>0.523 ± 0.003</td>
<td>0.463 ± 0.002</td>
<td>0.533 ± 0.004</td>
<td>0.469 ± 0.011</td>
<td>0.535 ± 0.003</td>
<td>0.482 ± 0.004</td>
</tr>
<tr>
<td>12</td>
<td>0.576 ± 0.004</td>
<td>0.522 ± 0.006</td>
<td>0.593 ± 0.001</td>
<td>0.534 ± 0.010</td>
<td>0.596 ± 0.003</td>
<td>0.549 ± 0.007</td>
</tr>
<tr>
<td>15</td>
<td>0.684 ± 0.002</td>
<td>0.559 ± 0.002</td>
<td>0.664 ± 0.001</td>
<td>0.610 ± 0.008</td>
<td>0.682 ± 0.001</td>
<td>0.628 ± 0.004</td>
</tr>
<tr>
<td>18</td>
<td>0.710 ± 0.004</td>
<td>0.671 ± 0.001</td>
<td>0.724 ± 0.001</td>
<td>0.679 ± 0.006</td>
<td>0.722 ± 0.004</td>
<td>0.691 ± 0.001</td>
</tr>
<tr>
<td>21</td>
<td>0.763 ± 0.005</td>
<td>0.735 ± 0.001</td>
<td>0.778 ± 0.002</td>
<td>0.744 ± 0.007</td>
<td>0.781 ± 0.004</td>
<td>0.754 ± 0.000</td>
</tr>
<tr>
<td>24</td>
<td>0.813 ± 0.005</td>
<td>0.796 ± 0.004</td>
<td>0.826 ± 0.002</td>
<td>0.802 ± 0.004</td>
<td>0.825 ± 0.001</td>
<td>0.811 ± 0.004</td>
</tr>
<tr>
<td>30</td>
<td>0.932 ± 0.004</td>
<td>0.908 ± 0.003</td>
<td>0.932 ± 0.001</td>
<td>0.906 ± 0.005</td>
<td>0.929 ± 0.005</td>
<td>0.914 ± 0.002</td>
</tr>
<tr>
<td>36</td>
<td>1.00 ± 0.01</td>
<td>1.00 ± 0.01</td>
<td>1.00 ± 0.01</td>
<td>1.00 ± 0.01</td>
<td>1.00 ± 0.01</td>
<td>1.00 ± 0.01</td>
</tr>
<tr>
<td>42</td>
<td>1.08 ± 0.01</td>
<td>1.08 ± 0.01</td>
<td>1.08 ± 0.01</td>
<td>1.09 ± 0.01</td>
<td>1.07 ± 0.01</td>
<td>—</td>
</tr>
<tr>
<td>48</td>
<td>1.14 ± 0.01</td>
<td>—</td>
<td>1.14 ± 0.01</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>54</td>
<td>1.20 ± 0.01</td>
<td>—</td>
<td>1.20 ± 0.01</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

All results are triplicates with standard deviations given. Triglyceride C40 (trilaurin) was chosen as the internal standard and given arbitrarily the value of unity. Sample load and composition as in Fig. 1.
TABLE 5 ATTEMPTED SEPARATION OF TRIGLYCERIDES OF THE SAME CARBON NUMBER BUT DIFFERENT FATTY ACID COMPOSITION

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Sample</th>
<th>Retention Time (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tri-C12:0</td>
<td>71 ± 0.5 (3)</td>
</tr>
<tr>
<td>2</td>
<td>2-Butyro-1,3-dipalmitin</td>
<td>82 ± 0.5 (5)</td>
</tr>
<tr>
<td>3</td>
<td>1-Butyro-2,3-dipalmitin</td>
<td>77 ± 0.5 (5)</td>
</tr>
<tr>
<td>4</td>
<td>2-Butyro-1,3-diolein</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>1-Butyro-2,3-diolein</td>
<td>71 ± 1 (2)</td>
</tr>
<tr>
<td>6</td>
<td>2-Butyro-1,3-distearin</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>Triolein</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>Tristearin</td>
<td>—</td>
</tr>
<tr>
<td>9</td>
<td>Trilinolein</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>Trilinolenin</td>
<td>—</td>
</tr>
</tbody>
</table>

SE-30 column run isothermally at temperature shown. Chart speed was 380 mm/hr. Samples were 4–6 μg/μl. The figures in parentheses are the number of samples run. Though a partial resolution of 1 and 2 was achieved, there was no resolution of 2 + 3 (duplicate experiment). No resolution of any combination of 4 + 5 + 6 was achieved (duplicate experiments). A partial separation of 7 and 8 similar to that shown in Fig. 6a was obtained.

TABLE 6 TRIGLYCERIDE COMPOSITION (MOLES %) OF HUMAN MILK, 7 DAYS' LACTATION, AS DETERMINED BY CHROMATOGRAPHY ON QF-1 AND SE-30 COLUMNS

<table>
<thead>
<tr>
<th>Triglyceride (Carbon Number)</th>
<th>QF-1</th>
<th>SE-30</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>36</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>38</td>
<td>0.9</td>
<td>1.4</td>
</tr>
<tr>
<td>40</td>
<td>2.1</td>
<td>2.9</td>
</tr>
<tr>
<td>42</td>
<td>4.6</td>
<td>5.4</td>
</tr>
<tr>
<td>44</td>
<td>8.2</td>
<td>8.6</td>
</tr>
<tr>
<td>46</td>
<td>12.8</td>
<td>13.6</td>
</tr>
<tr>
<td>48</td>
<td>16.0</td>
<td>16.0</td>
</tr>
<tr>
<td>50</td>
<td>20.3</td>
<td>18.0</td>
</tr>
<tr>
<td>52</td>
<td>25.4</td>
<td>23.6</td>
</tr>
<tr>
<td>54</td>
<td>9.0</td>
<td>8.1</td>
</tr>
<tr>
<td>56</td>
<td>0.9</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Average fatty acid carbon number* 16.3 16.2

* Obtained from the formula: \( C_a = \frac{1}{100} \times \sum C_i \times x_i \) where \( C_a \) = average fatty acid carbon number; \( C_i \) = triglyceride carbon number; \( x_i \) = number of triglycerides present.

Application to Biological Materials

Using human milk fat and a combination of isothermal and temperature programmed conditions, we compared the separating abilities of QF-1 and SE-30 columns (Fig. 8). Retention data are given as insets. Quantitative results from the two chromatograms, obtained with the aid of correction factors, are given in Table 6. Fig. 8c illustrates a separation of the triglycerides on another QF-1 column which had been balanced against a second QF-1 column, and is included to show what can be expected in routine practice.

DISCUSSION

Qualitative Data

The relationship \( \log V_r = kC \) is seen to hold for simple saturated triglycerides \( C_{16}-C_{40} \) on QF-1 (Fig. 1a) and SE-30 (Fig. 1b) at the temperatures shown. The lower molecular weight triglycerides \( (C_6 \text{ and } C_8) \) deviate from the straight line relationship in having slightly greater \( V_r \) than predicted. This phenomenon has been observed for the lower members of other homologous series of compounds, especially polar ones (32), and may be due to interactions between adjoining groups. Such interactions would be more evident the more polar the stationary phase. However, other factors, such as adsorption on the column support and (or) walls may be operative. At all temperatures used, smaller \( V_r \) values were observed on the QF-1 column than on the SE-30. This would reflect the greater polarity of the QF-1, since the amount of sta-
Fig. 3. Relationship between detector response and column load for various triglycerides. 

(a) Natural mixture of triglycerides of rabbit milk. SE-30 column, run at 250°C isothermally for 10 min and then temperature programmed at 1.85°C/min to 322°C (330°C final equilibrium temperature). Duplicate samples were applied without solvent. Detector response per peak was measured for the triglycerides shown.

(b) A standard mixture of simple saturated triglycerides \( C_6-C_{31} \). SE-30 column, temperature programmed at 3.71°C/min from 118°C. (The ordinate is not comparable to that of a). Duplicate samples were applied in 0.4, 0.6, 0.8, and 1.0 μl of benzene at concentration of 45.62 and 114.05 μg/μl. The weight % of the mixture was 15.21% of \( C_6 \), 14.47% of \( C_7 \), 18.24% of \( C_8 \), 21.92% of \( C_{19} \), 13.10% of \( C_{18} \), and 17.05% of \( C_{31} \).
If one takes ± 5 units as an acceptable standard of reproducibility and agreement for $I_1$ and $I_{pr}$, then both QF-1 and SE-30 gave satisfactory $I_1$ values (Table 1) but $I_{pr}$ values for different rates of temperature program that are outside the acceptable limits (Table 3). The disagreement is less pronounced for the two higher rates of program with SE-30. Reproducibility for triplicate or duplicate determinations of $I_1$ is poor, though less so with SE-30 (Table 3). This is not due to experimental technique but primarily to the erratic $T_e$ of the hydrocarbon standards used (possibly because of partial adsorption of hydrocarbon on steel walls and hydrocarbon or triglyceride acting as a stationary phase), and the nonlinearity of $T_e$ with carbon number (Figs. 7 and 8). A comparison of $I_1$ (Table 1) and $I_{pr}$ (Table 3) shows this in the case of QF-1. For SE-30, good agreement was obtained only for C12 and C16. These results support the view (23) that $I_{pr}$ values are of limited use.

Since a more reliable method of determining peak retentions during temperature programming was needed, the relative elution temperature ($T_{RE}$) measurement of Schmit and Wynne (33) was applied. The results (Table 4) show good agreement between different rates of temperature program for the same triglyceride and excellent...
FIG. 6. Attempted separation of triglycerides of the same carbon number but different fatty acid composition (SE-30, temperature programmed). a, About 1 µg of tri-C48:1 (peak 7) and 4 µg of 2-butyro-1,3-distearin (peak 2) in 0.6 µl of benzene. b, About 7.5 µg each of triolein (peak 7) and tristearin (peak 2) in 0.6 µl of chloroform–methanol 2:1. Column conditions for SE-30 as in Fig. 4. Peaks were identified by chromatography of the samples separately.

Fig. 7. Cochromatography of a sample of cow’s milk triglycerides (100 µg) and 2-butyro-1,3-distearin (10 µg) in 1 µl of hexane. 10% SE-30 column, programmed from 250 to 322°C at 1.85°C/min (final equilibrium temperature 330°C). Retention data are given in the inset as t, to give ready combination of programmed and isothermal characteristics.

reproducibility for triplicate determinations of the same triglyceride. Hence measurement of relative elution temperatures (T_{rel}), or graphical plots of absolute elution temperatures (T_e) against carbon number, C, are the methods of choice when temperature programming is used.

Relative column efficiencies of QF-1 and SE-30 for the ranges of triglycerides C_{16–C_{30}} (Table 2) indicate our general experience that the 10% SE-30 column gives somewhat better resolution than the 3% QF-1 under similar operating conditions. Despite the apparently low number of theoretical plates of both columns (Table 2), each gave adequate resolution of the simple saturated triglycerides used.

Quantitative Results
Quantification of detector response to load showed linearity for the range of triglycerides C_{16–C_{30}} (Fig. 3a) although the plot for C_{36} did not pass through the origin. Load is plotted as µl of milk triglyceride sample; this represents linearity up to about 30 µg for each triglyceride component of the sample. Shorter-chain triglycerides show similar linearity (Fig. 3b). However, the
Fig. 8. A sample of human milk triglycerides, obtained on the 7th day of lactation, chromatographed on a, SE-30 and b, QF-1. Column and sample conditions as in Fig. 4. Retention data are given in the inset as $t_r$ to give ready combination of programmed and isothermal characteristics. c, Chromatogram obtained on QF-1, column balanced against another QF-1 column, with temperature program upper limit of 305°C.
plots for C₄ and C₅ do not pass through the origin, and this cannot be explained simply by experimental error.

Weight (f_w) and molar (f_m) correction factor determinations on SE-30 (Fig. 4a) for the simple triglycerides C₄₋₅C₉ showed greatest deviation from linearity above C₆. However, there are no significant differences between our results and those of Litchfield, Harlow, and Reiser (24). The latter workers have also assumed that all saturated triglycerides of the same carbon number have the same f_m, and that for triglycerides with a combination of saturated and unsaturated acids an average f_m may be estimated. Our results with a limited range of seven mixed triglycerides (Fig. 4a) indicate that although agreement may be as good as ±0.5% or as bad as ±7% (obtained for 2-butyro-1,3 dipalmitin, Fig. 4a, six of the seven triglycerides had f_m values which were within ±4% of the expected values.

Similar results were obtained with QF-1 (Fig. 4b), and the slightly positive slope (24) for f_w would indicate the expected greater adsorption of triglycerides on the longer chain. By analogy with f_m values of methyl esters of fatty acids (29), a slightly negative slope (of f_w versus carbon number, C) with increasing carbon number was expected. The spread of the correction factors was greater than in the case of the SE-30 column, and reflects the greater error inherent in measuring areas of peaks with considerable tailing.

The short-chain triglycerides on SE-30, C₁₅₋₁₆C₉ (Fig. 5), gave straight-line plots of f_w versus carbon number, with a negative slope. As expected, the points for C₆-C₉ did not lie on the straight line (30). This deviation, which decreases with load, suggests adsorption on the column, as do the results for C₈ and C₉ in Fig. 3b. The unexpected adsorption here may explain the anomalous results of Ackman and Sipos (30) who used the corresponding short-chain fatty acids and methyl esters.

It would thus appear that at the present time, even if correction factors are used, quantitative accuracy cannot be claimed to be better than ±5%. The use of digital integrators would possibly help to eliminate the inaccuracy in peak area measurement [±4% (34)]. A good check on accuracy is made by comparing the average fatty acid chain length in the triglyceride sample, obtained from both triglyceride and fatty acid ester data. This is illustrated in the following paper (28).

Separation of Triglycerides of the Same Carbon Number
For the resolution of triglycerides of the same carbon number (25) conditions approaching those of gas-solid chromatography were used. Shoulders were obtained for peaks from C₄ and C₅ of butter oil distillate and were attributed to partial resolution of triglycerides containing both 1- and 2-butyrate residues. This supported the finding (35), by enzymic hydrolysis of milk fat, that butyrate could be located on any of the three positions of glycerol. [Previous studies (36) had indicated that they are exclusively situated on the 1 or 3 position.]

Figs. 6 and 7 and Table 5 indicate that it is quite feasible under suitable conditions to obtain partial separation of triglyceride isomers. The absence of a noticeable resolution of C₄₀ and C₉₈ in the natural triglyceride milk sample indicates that two likely fatty acid combinations (monobutyro-diolein and monobutyro-dipalmitin) are not present to any appreciable extent. Since the butyrate-containing isomers of C₄₀ and C₉₈ would overlap only slightly with peaks from C₄₈ and C₇₈, respectively (Fig. 7, inset), the small peaks between the major ones are not due to even-numbered triglycerides (Fig. 7).

The attribution of partial resolution (25) to the difference in position of butyrate would also appear in question. Though some small differences in retention characteristics were observed according to unsaturation or position of the butyrate group (Table 5), these were not sufficient to give resolution of 1- and 2-butyro isomers when they were chromatographed together. The shoulder observed by Kukis and Breckenridge (25) might therefore be due to a partial separation of the C₄₆ and C₆₆ isomers on the basis of presence or absence of a butyrate residue.

The basis of separation of triglycerides of the same carbon number is not clear. For although triolein and tristearin could be separated partially (Table 5 and Fig. 6), no separation of tripalmitin and tripalmitolein was observed under the same conditions. Furthermore, the relative retention times of the C₆₆ isomers on SE-30 and QF-1 were not reversed. Thus triglyceride polarity or constellation differences would not appear to be sufficient explanations for separation of triglycerides of the same carbon number.

Application to Biological Materials
The technique has been applied with success to the partial analysis of a number of milk fats and the results are reported in the following paper (28). An example is given here of human milk fat (Fig. 8 and Table 6). The agreement of results between QF-1 and SE-30 are seen to be quite good. 10% SE-30 would be the column of choice for routine triglyceride separation, but laboratories already using the QF-1 column for selective steroid analysis (18) or for rapid triglyceride analysis could use the latter column. Stainless steel should be substituted for glass columns, which tend to fracture in use.

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