Separation of triglycerides by gas–liquid chromatography

RODNEY WATTS and R. DILS

Department of Medical Biochemistry and Pharmacology, Biology Building, University of Birmingham, Birmingham, England

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.

KEY WORDS triglycerides · gas–liquid chromatography · isothermal · temperature programming · retention indices · elution temperatures · correction factors · separation · milk

In recent years, the GLC of intact glycerides has been developed on stationary phases that are stable at the high temperatures required. Several natural and synthetic triglyceride mixtures have been analyzed according to carbon number (1–15). A comprehensive review of the subject has been published by Kuksis (16), who has very recently commented (17) on the frequent failures to achieve good resolution of triglycerides and suggested improved operating conditions.

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-C_2 to tri-$C_{20:0}$ 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 (ΔH_s) do not vary significantly with temperature, the relationship (20) $\log V_r = a + b/T$ (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-

A preliminary report of some of these data was presented at a symposium organized by the Gas Chromatography Group of the Institute of Petroleum, 22 April 1966.

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; TP, theoretical plates; t_r, retention time; V_r, retention volume; C, number of carbon atoms in a fatty acid or total number of acyl carbon atoms in a triglyceride; C_6–C_{60}, triglycerides with a total number of acyl carbon atoms of 6 to 60.

Terminology: Simple triglycerides (represented as tri-C_x, where x = number of carbon atoms; number of double bonds) possess three identical fatty acid moieties; mixed triglycerides do not.

Downloaded from www.jlr.org by guest, on June 19, 2012

cable, the *isothermal retention index* (I_i) of a triglyceride would be given by the equation:

$$I_i = 100\,(y\text{-}x)\,\frac{\log V_{rc} - \log V_{rx}}{\log V_{ry} - \log V_{rx}} + 100x \qquad (1)$$

where V_{rc} = retention volume of a triglyceride with C acyl carbon atoms; V_{rx} = 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_{pr}) 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_r values in equation 1 to give:

$$I_{pr} = 100(y-x)\,\frac{T_{ec} - T_{ex}}{T_{ey} - T_{ex}} + 100x \qquad (2)$$

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_{pr} values has been questioned (23), partly on the grounds of the difficulty of obtaining I_{pr} 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_r on GLC, but positional isomerization was not determined absolutely. Tri-$C_{5:0}$, tri-$C_{6:0}$, and tri-$C_{7:0}$ 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_{32}) 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 ($C_{4:0}$–$C_{12:0}$) or of the methyl esters on polyethylene glycol adipate ($C_{12:0}$–$C_{20:0}$).

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 × 6.5 mm stainless steel tube, and 10% SE-30 on Gas-Chrom Q in a 53.5 cm × 6.5 mm stainless steel tube. Both were conditioned at 350°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 µl) and 701 NWG (10 µl) syringes were employed. Columns were packed so that, on injection, the tip of the needle came just above the packing.

Triglycerides C_6–C_{21} were applied in about 50% (w/v) solution in benzene, and triglycerides C_{24}–C_{60} in about 10% (w/v) solution in chloroform–methanol 2:1.

In order to determine V_r in the isothermal studies, we measured flow rates through the QF-1 and SE-30 col-

ASBMB JOURNAL OF LIPID RESEARCH

Downloaded from www.jlr.org by guest, on June 19, 2012

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_6–C_{60} at a single temperature, several isothermal analyses were carried out. A direct relationship was obtained between log V_r and C at each temperature on either QF-1 (Fig. 1*a*) or SE-30 (Fig. 1*b*).

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_t) with temperature programs indices (I_{pr}) (22,23). The triglycerides were cochromatographed with *n*-hexadecane (C_{16}), *n*-eicosane (C_{20}), and *n*-dotriacontane (C_{32}) on QF-1 and SE-30. I_t was calculated from equation 1, and the results are shown in Table 1.

Column efficiencies in terms of ΔC, the minimum carbon number difference between two triglycerides which can be completely resolved, and theoretical plates for

TABLE 1 Retention Indices (I_t) of Simple Saturated Triglycerides C_{12}–C_{24} on QF-1 and SE-30

Triglyceride (Carbon Number)	QF-1 at 180°C	SE-30 at			
		140°C	160°C	180°C	220°C
12	2265	1800	1800	1800	—
15	2570	2060	2070	2060	—
18	2890	—	—	2310	2270
21	3200	—	—	—	2570
24	—	—	—	—	2810

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 μg/peak, using the standard mixtures of triglycerides given in Fig. 1 plus suitable quantities of the hydrocarbons C_{16}, C_{20}, and C_{32}.

TABLE 2 Column Efficiencies of QF-1 and SE-30 for the Simple Saturated Triglycerides C_{21} at 180°C and C_{36} at 240°C

Triglyceride (Carbon Number)	Temp.	QF-1		SE-30	
		ΔC	TP	ΔC	TP
	°C				
21	180	1.82 ± 0.01	237 ± 3	1.12 ± 0.01	400 ± 2
36	240	4.59 ± 0.05	144 ± 2	1.46 ± 0.01	325 ± 1

ΔC is the minimum carbon number difference between two triglycerides which can be completely resolved in the C_{18}–C_{21} region (for C_{21}) and in the C_{30}–C_{36} region (for C_{36}). TP is the total theoretical plates of the column. Determinations were in duplicate. Experimental details as in Fig. 1.

glycerides C_{21} and C_{36} as reference compounds on QF-1 and SE-30 are given in Table 2. These triglycerides were chosen since they were also the reference compounds for quantitative work (see below).

Analysis by Temperature Programming. From the isothermal result on QF-1 and SE-30, a temperature range of 118°C to 322°C was chosen to elute the wide range of triglycerides C_6–C_{54}. Temperature program indices (I_{pr}) (22,23) were determined at different rates of programming for the triglycerides C_{12}–C_{24} by means of equation 2. The hydrocarbon standards were those used in the isothermal studies (i.e. C_{16}, C_{20}, and C_{32}). I_{pr} values are shown in Table 3.

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

Relative column efficiencies of QF-1 and SE-30 for triglycerides C_{36}–C_{42} have been determined. A standard mixture of simple saturated triglycerides C_{24}–C_{34} containing 2.5–10 μg/μl 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). ΔC for QF-1 was 2.46 ± 0.09 and for SE-30 was 1.36 ± 0.01. The range C_{36}–C_{42} 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_{21}) 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.

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB
JOURNAL OF LIPID RESEARCH
JLR

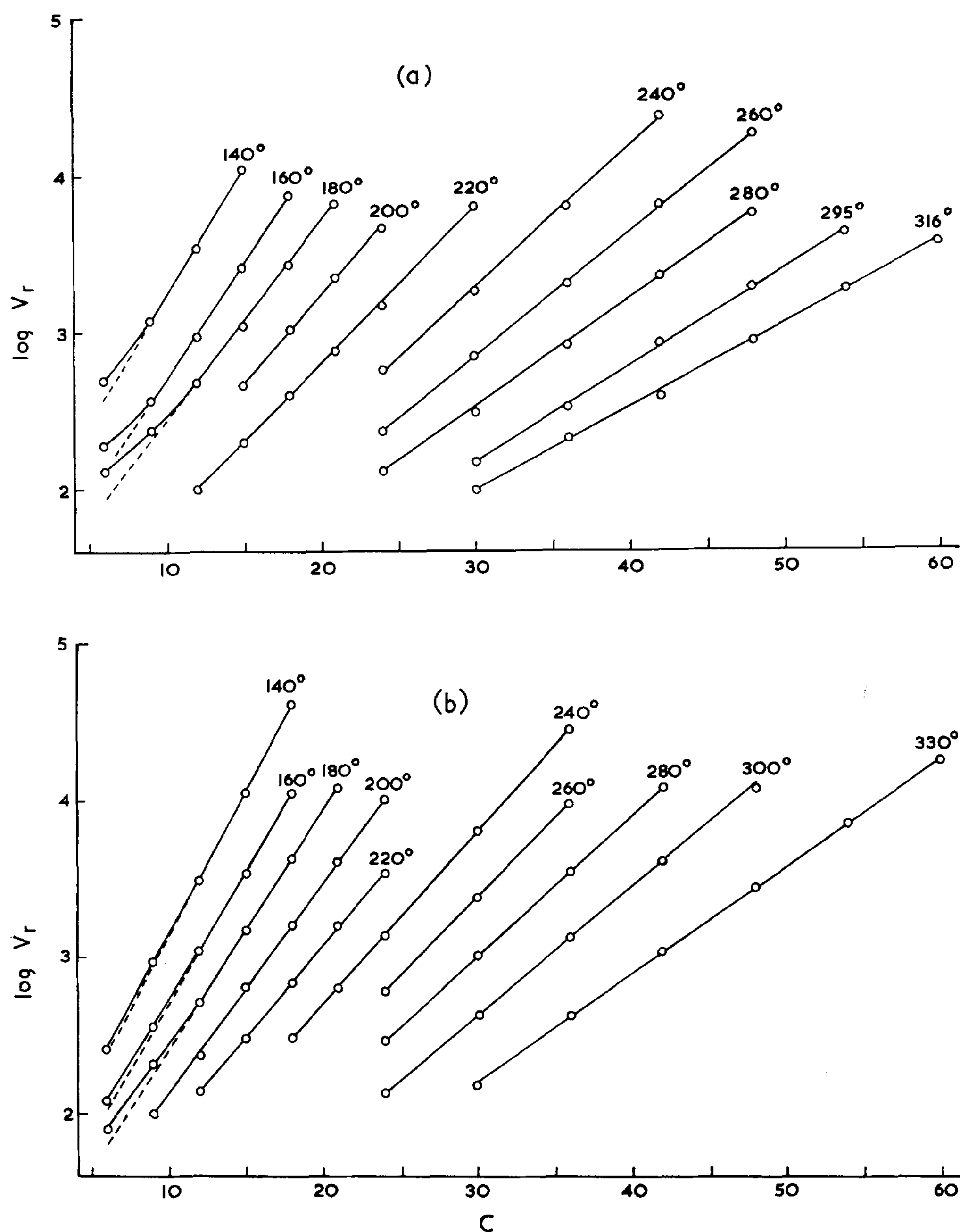

FIG. 1. Relationship of log V_r and C at various temperatures for simple saturated triglycerides. *a*, QF-1; *b*, SE-30. Duplicate or triplicate samples were chromatographed at each temperature. Reproducibility was within ± 1%. Sample load was 5–25 μg/peak in standard mixtures (by weight) of triglycerides C_6–C_{21} and C_{24}–C_{60}.

Fig. 3*a* shows a linear detector response to increasing loads of triglycerides C_{24}, C_{30}, C_{36}, C_{42}, and C_{46}. Intermediate even-numbered triglycerides in the sample showed similar linear plots which passed through or near the origin (except C_{46}). A similar result was obtained for the short-chain simple saturated triglycerides C_6–C_{21} (Fig. 3*b*).

Since adsorption on the column became readily apparent for milk triglycerides higher than C_{96}, 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 = weight %/area % and f_m = mole %/area %.

These factors were obtained by chromatography of

Downloaded from www.jlr.org by guest, on June 19, 2012

TABLE 3 Programmed Retention Indices (I_{pr}) of Simple Saturated Triglycerides C_{12}–C_{24} on QF-1 and SE-30

Triglyceride (Carbon Number)	Corrected Program Rate (°/min) 1.85		3.71		5.56	
	QF-1	SE-30	QF-1	SE-30	QF-1	SE-30
12	2255 ± 20 (3)	1770 ± 25 (2)	2275 ± 5 (2)	1795 ± 5 (2)	2315 ± 20 (3)	1805 ± 10 (2)
15	2575 ± 35 (3)	2050 ± 10 (2)	2600 ± 0 (2)	2070 ± 5 (2)	2615 ± 25 (3)	2095 ± 25 (2)
18	2860 ± 30 (3)	2340 ± 20 (2)	2865 ± 5 (2)	2400 ± 10 (2)	2910 ± 20 (3)	2380 ± 10 (2)
21	3100 ± 25 (3)	2590 ± 30 (2)	3110 ± 5 (2)	2680 ± 15 (2)	3145 ± 5 (3)	2675 ± 10 (2)
24	—	2830 ± 50 (2)	—	2935 ± 5 (2)	—	2940 ± 10 (2)

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_{RE}) of Simple Saturated Triglycerides C_6–C_{54} on QF-1 and SE-30

Triglyceride (Carbon Number)	Corrected Program Rate (°/min) 1.85		3.71		5.56	
	QF-1	SE-30	QF-1	SE-30	QF-1	SE-30
6	0.493 ± 0.004	0.493 ± 0.001	0.485 ± 0.002	0.425 ± 0.009	0.485 ± 0.004	0.418 ± 0.004
9	0.523 ± 0.003	0.463 ± 0.002	0.533 ± 0.004	0.469 ± 0.011	0.535 ± 0.003	0.482 ± 0.004
12	0.576 ± 0.004	0.522 ± 0.006	0.593 ± 0.001	0.534 ± 0.010	0.596 ± 0.003	0.549 ± 0.007
15	0.648 ± 0.002	0.599 ± 0.002	0.664 ± 0.001	0.610 ± 0.008	0.668 ± 0.002	0.628 ± 0.004
18	0.710 ± 0.004	0.671 ± 0.001	0.724 ± 0.001	0.679 ± 0.006	0.729 ± 0.001	0.691 ± 0.001
21	0.763 ± 0.005	0.735 ± 0.001	0.778 ± 0.002	0.744 ± 0.007	0.781 ± 0.004	0.754 ± 0.000
24	0.813 ± 0.005	0.796 ± 0.004	0.826 ± 0.002	0.802 ± 0.004	0.825 ± 0.001	0.811 ± 0.004
30	0.932 ± 0.004	0.908 ± 0.003	0.932 ± 0.001	0.906 ± 0.005	0.929 ± 0.005	0.914 ± 0.002
36	1.00	1.00	1.00	1.00	1.00	1.00
42	1.08 ± 0.01	1.08 ± 0.01	1.08 ± 0.01	1.09 ± 0.01	1.07 ± 0.01	—
48	1.14 ± 0.01	—	1.14 ± 0.01	—	—	—
54	1.20 ± 0.01	—	—	—	—	—

All results are triplicates with standard deviations given. Triglyceride C_{36} (trilaurin) was chosen as the internal standard and given arbitrarily the value of unity. Sample load and composition as in Fig. 1.

standard weighed mixtures, and are expressed graphically in Fig. 4*a* for simple and some mixed triglycerides, C_{24}–C_{54}, on SE-30. For comparison, Fig. 4*b* expresses f_w and f_m for these triglycerides on QF-1. The variation of f_w with short-chain simple saturated triglycerides, C_6–C_{21}, on SE-30 is shown graphically in Fig. 5. The large values of f_m for small carbon number make it impossible to show these on the same scale as the f_w values given in Fig. 5. They can be readily calculated from data given. Values of f_w on QF-1 are shown for comparison with SE-30 in Fig. 5.

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-$C_{12:0}$ 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 C_{40} 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 C_{54} isomers was that of triolein and tristearin.

We were also able to separate C_{36} isomers (tri-$C_{12:0}$ and 2-butyro-1,3-dipalmitin) to an extent similar to that shown in Fig. 6*a* on QF-1, but attempts to reproduce the partial separation of triolein and tristearin (Fig. 6*b*) 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 C_{40}, cited above, cochromatography with the expected peak occurred with no distinct resolution. This was even so in the case of the C_{36} isomers, where only partial resolution was found. Overlap with C_{37} and C_{41} 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.

Downloaded from www.jlr.org by guest, on June 19, 2012

TABLE 5 Attempted Separation of Triglycerides of the Same Carbon Number but Different Fatty Acid Composition

Sample No.	Sample	Retention Time		
		285°C	305°C	330°C
			mm	
1	Tri-$C_{12:0}$	71 ± 0.5 (3)	—	—
2	2-Butyro-1,3-dipalmitin	82 ± 0.5 (5)	—	—
3	1-Butyro-2,3-dipalmitin	77 ± 0.5 (5)	—	—
4	2-Butyro-1,3-diolein	—	74 ± 1 (3)	—
5	1-Butyro-2,3-diolein	—	71 ± 1 (2)	—
6	2-Butyro-1,3-distearin	—	81 ± 0 (2)	—
7	Triolein	—	—	201 ± 0.5 (2)
8	Tristearin	—	—	213 ± 0.5 (2)
9	Trilinolein	—	—	201 ± 1 (2)
10	Trilinolenin	—	—	201 ± 1 (2)

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. 6*b* was obtained.

TABLE 6 Triglyceride Composition (Moles %) of Human Milk, 7 Days' Lactation, as Determined by Chromatography on QF-1 and SE-30 Columns

Triglyceride (Carbon Number)	QF-1	SE-30
34	0.1	0.2
36	0.3	0.5
38	0.9	1.4
40	2.1	2.9
42	4.6	5.4
44	8.2	8.6
46	12.8	13.6
48	16.0	16.0
50	20.3	18.0
52	25.4	23.6
54	9.0	8.1
56	0.9	1.8
Average fatty acid carbon number*	16.3	16.2

* Obtained from the formula: $C_a = 1/100 \times \Sigma_n (C/3 \times \text{moles } \%)$ where C_a = average fatty acid carbon number; C = triglyceride carbon number; n = 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. 8*c* 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_{12}–C_{60} on QF-1 (Fig. 1*a*) and SE-30 (Fig. 1*b*) at the temperatures shown. The lower molecular weight triglycerides (C_6 and C_9) 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-

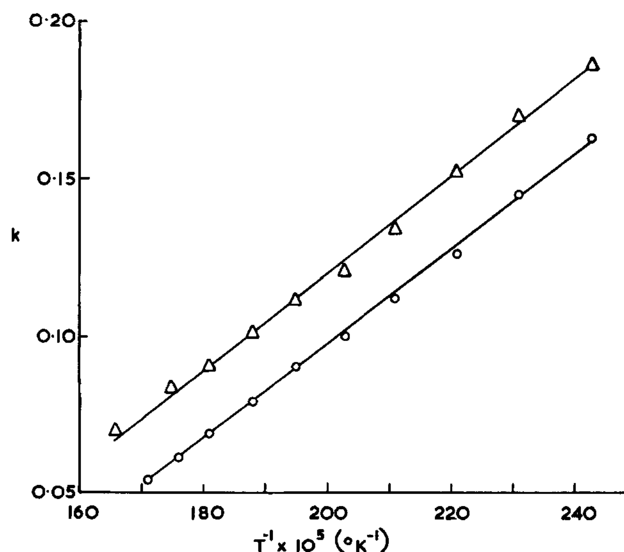

Fig. 2. Relationship between k and T^{-1} for simple saturated triglycerides. ○, QF-1; Δ, SE-30. k is the constant of the relationship $\log V_r = kC$, obtained from Fig. 1.

Downloaded from www.jlr.org by guest, on June 19, 2012
ASBMB JOURNAL OF LIPID RESEARCH

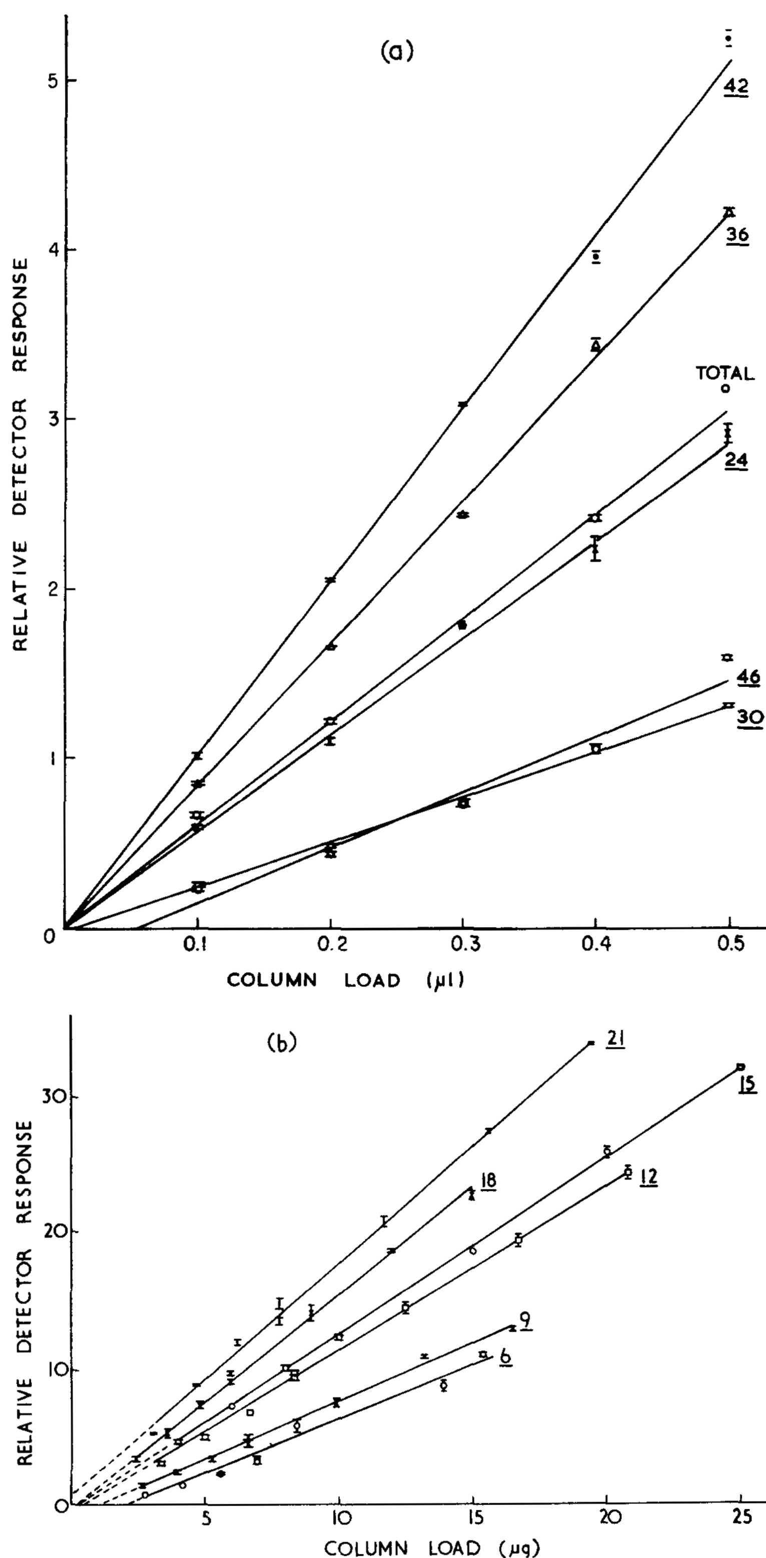

FIG. 3. Relationship between detector response and column load for various triglycerides. *a*, Natural mixture of triglycerides of rabbit milk. SE-30 column, run at 250°C isothermally for 10 min and then temperature programmed at 1.85°C/min to 322°C (330°C final equilibrium temperature). Duplicate samples 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_{21}. 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_9, 18.24% of C_{12}, 21.92% of C_{15}, 13.10% of C_{18}, and 17.05% of C_{21}.

Downloaded from www.jlr.org by guest, on June 19, 2012
JOURNAL OF LIPID RESEARCH
ASBMB

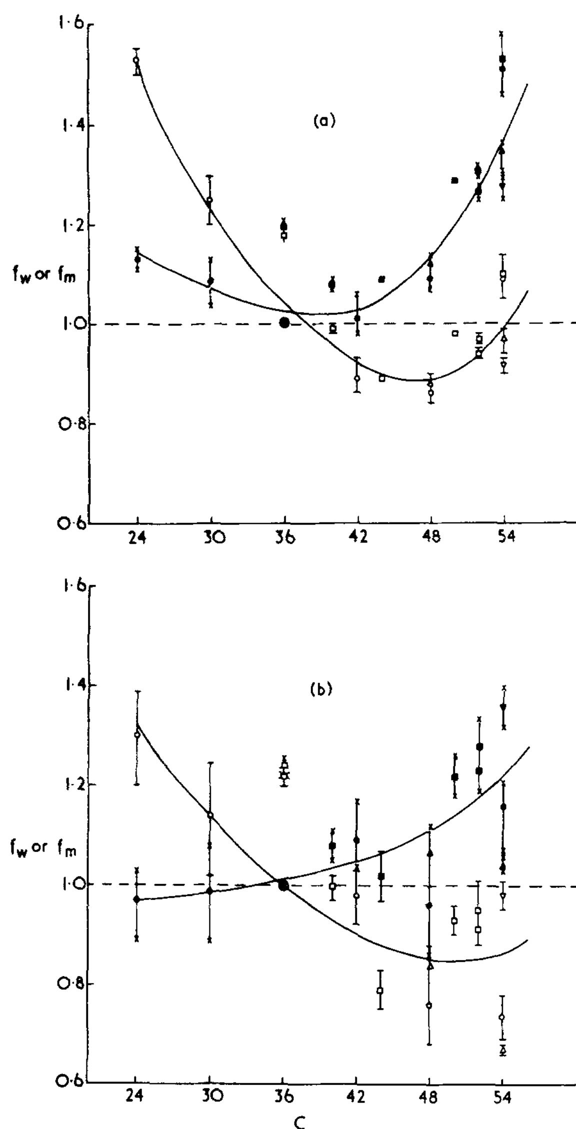

FIG. 4. Weight (f_w) and molar (f_m) correction factors for a range of triglycerides C_{24}–C_{54}. *a*, SE-30; *b*, QF-1. The columns were run at 250°C isothermally for 10 min and then temperature programmed at 1.85°C/min to 322°C (final equilibrium temperature 330°C). Triplicate samples of standard mixtures containing 2.5–10 μg/μl of each component were used. Each mixture contained only triglycerides of different molecular weight. Solid symbols with absolute limits represent f_w and open symbols with absolute limits represent f_m. ●, ○, simple saturated triglycerides; ▲, △, tri-$C_{16:1}$ and tri-*cis*-$C_{18:1}$; ▼, ▽, tri-*trans*-$C_{18:1}$; ■, □, mixed triglycerides (2-butyro-1,3-dipalmitin, *36;* 2-butyro-1,3-distearin, *40;* 2-caprylo-1,3-distearin, *44;* 1-oleo-2,3-dipalmitin, *50;* 1-palmito-2-oleo-3-stearin and 1-palmito-2,3-diolein, *52;* 2-oleo-1,3-distearin, *54*).

tionary phase present was about the same for the two columns.

The relationship $\log V_r = a + b/T$ is well obeyed by simple saturated triglycerides C_{12}–C_{60} on QF-1 and SE-30 (Fig. 2). Thus, by using standard triglycerides, one can predict the V_r of these triglycerides at different temperatures and, by extrapolation, the V_r of triglycerides not in the mixture, but in the homologous series.

If one takes ±5 units as an acceptable standard of reproducibility and agreement for I_i and I_{pr}, then both QF-1 and SE-30 gave satisfactory I_i 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_{pr} 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_i (Table 1) and I_{pr} (Table 3) shows this in the case of QF-1. For SE-30, good agreement was obtained only for C_{12} and C_{15}. 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

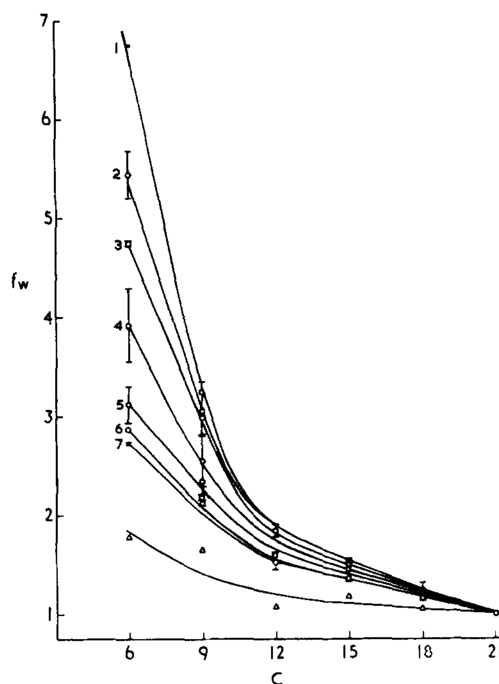

FIG. 5. Weight (f_w) correction factors for simple saturated triglycerides C_6–C_{21} on SE-30. Column conditions and samples are given in Fig. 3*b*. Triglyceride C_{21} has been assigned an f_w of unity. Curves were determined using 0.4 μl (*1*), 0.6 μl (*2*), 0.8 μl (*3*), and 1.0 μl (*4*) of benzene containing 45.62 μg/μl triglyceride mixture, and 0.4 μl (*4*), 0.6 μl (*5*), 0.8 μl (*6*), and 1.0 μl (*7*) of benzene containing 114.05 μg/μl triglyceride mixture. △: for comparison, a 1 μl sample of concentration 45.62 μg/μl was applied to QF-1 under conditions identical to those for SE-30. For the sake of clarity, limits are not shown when they lie inside the mean symbol.

JOURNAL OF LIPID RESEARCH ASBMB

Downloaded from www.jlr.org by guest, on June 19, 2012

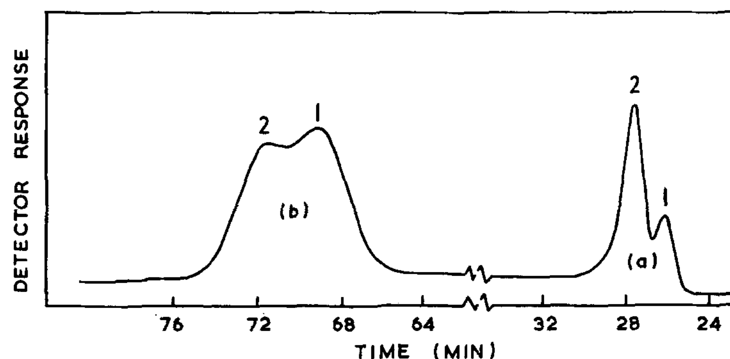

FIG. 6. Attempted separation of triglycerides of the same carbon number but different fatty acid composition (SE-30, temperature programmed). *a*, About 1 μg of tri-$C_{12:0}$ (peak *1*) and 4 μg of 2-butyro-1,3-dipalmitin (peak *2*) in 0:6 μl of benzene. *b*, About 7.5 μg each of triolein (peak *1*) 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.

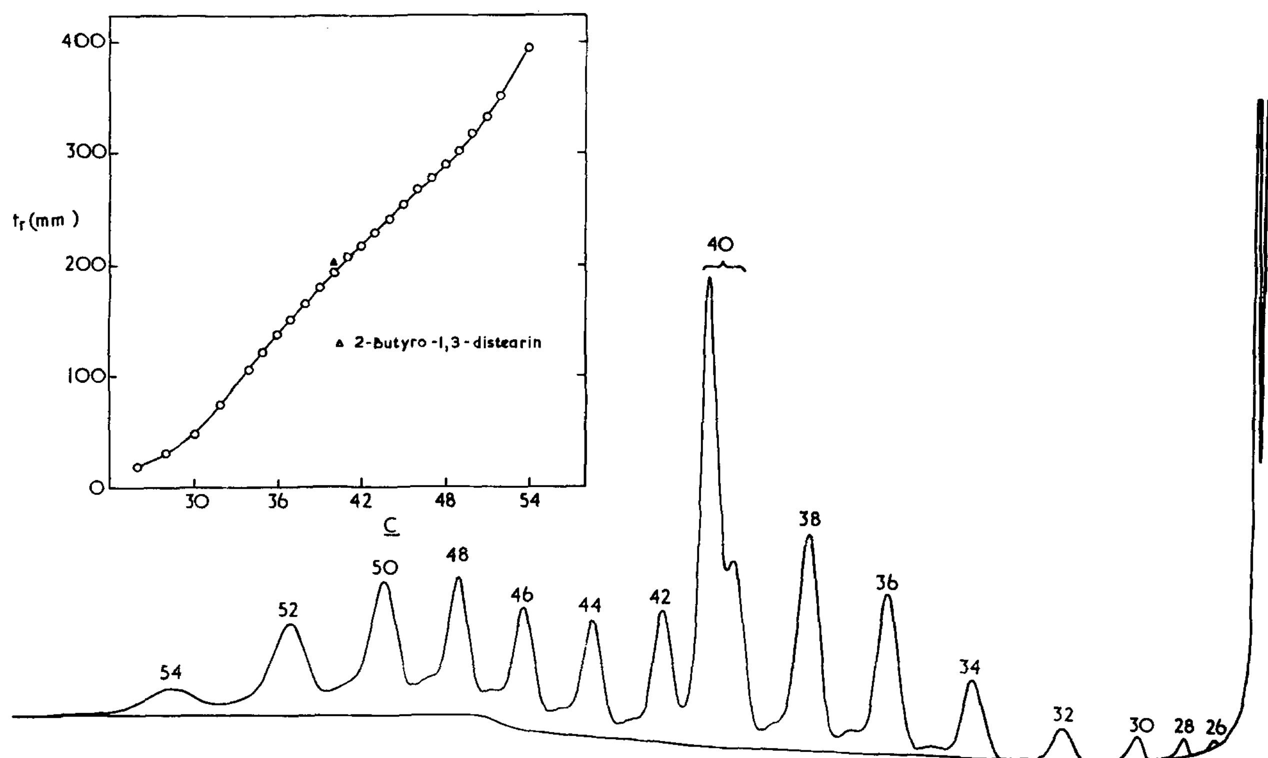

FIG. 7. Cochromatography of a sample of cow's milk triglycerides (100 μg) and 2-butyro-1,3-distearin (10 μg) in 1 μl of hexane. 10% SE-30 column, programmed from 250 to 322°C at 1.85°C/min (final equilibrium temperature 330°C). Retention data are given in the inset as t_r to give ready combination of programmed and isothermal characteristics.

reproducibility for triplicate determinations of the same triglyceride. Hence measurement of relative elution temperatures (T_{RE}), 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_{18}–C_{21} and C_{30}–C_{36} (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_{24}–C_{46} (Fig. 3*a*) although the plot for C_{46} 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. 3*b*). However, the

Downloaded from www.jlr.org by guest, on June 19, 2012

JOURNAL OF LIPID RESEARCH ASBMB

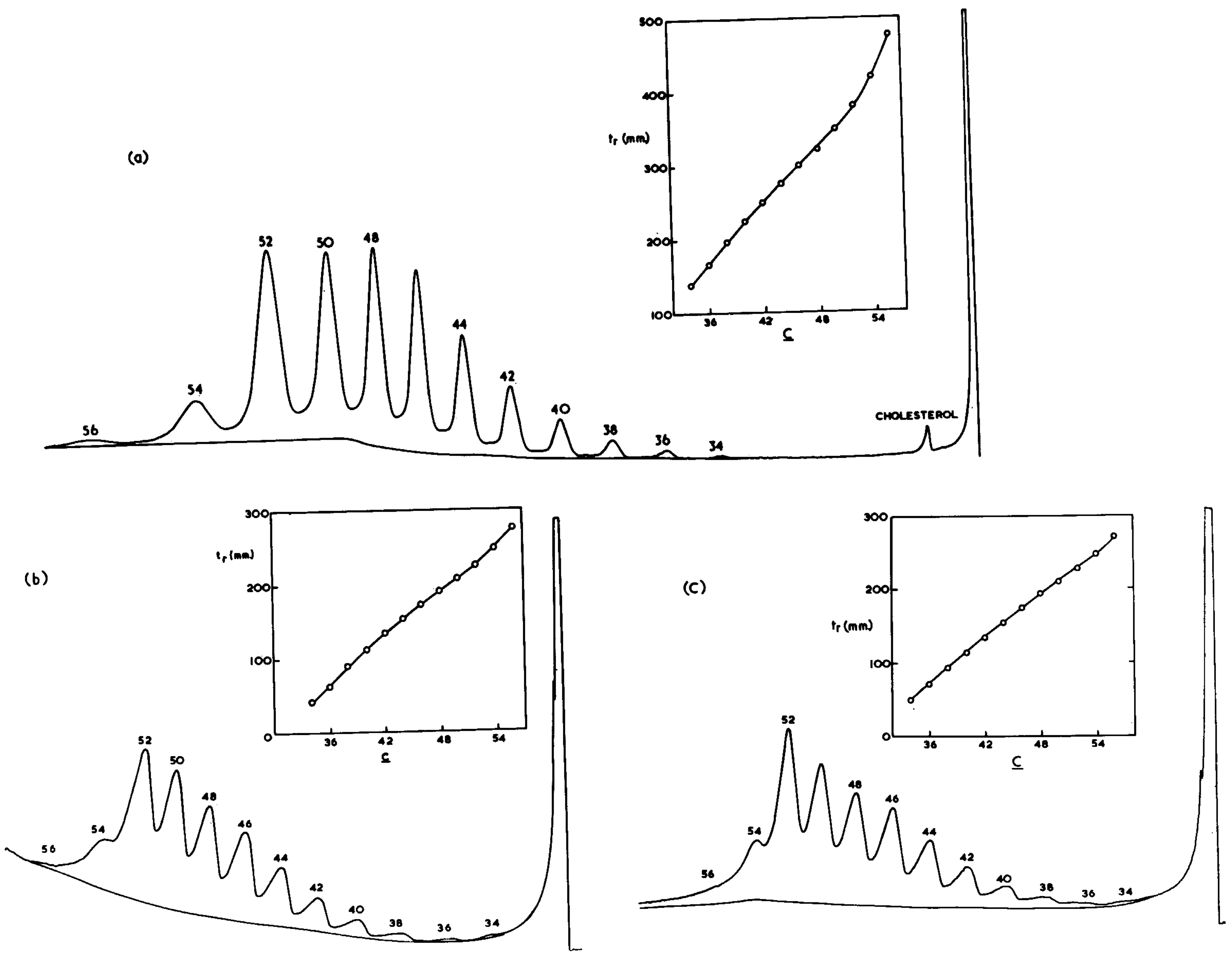

Fig. 8. A sample of human milk triglycerides, obtained on the 7th day of lactation, chromatographed on *a*, SE-30 and *b*, QF-1. Column and sample conditions as in Fig. 4. Retention data are given in the inset as t_r to give ready combination of programmed and isothermal characteristics. *c*, Chromatogram obtained on QF-1, column balanced against another QF-1 column, with temperature program upper limit of 305°C.

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB
JOURNAL OF LIPID RESEARCH

plots for C_6 and C_9 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. 4*a*) for the simple triglycerides C_{24}–C_{54} showed greatest deviation from linearity above C_{48}. 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. 4*a*) indicate that although agreement may be as good as $\pm 0.5\%$ or as bad as $\pm 7\%$ (obtained for 2-butyro-1,3 dipalmitin, Fig. 4*a*, six of the seven triglycerides had f_m values which were within $\pm 4\%$ of the expected values.

Similar results were obtained with QF-1 (Fig. 4*b*), and the slightly positive slope (24) for f_w would indicate the expected greater adsorption of triglycerides on the longer column. By analogy with f_w 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, C_{12}–C_{21} (Fig. 5), gave straight-line plots of f_w versus carbon number, with a negative slope. As expected, the points for C_6–C_9 did not lie on the straight line (30). This deviation, which decreases with load, suggests adsorption on the column, as do the results for C_6 and C_9 in Fig. 3*b*. 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 $\pm 5\%$. The use of digital integrators would possibly help to eliminate the inaccuracy in peak area measurement [$\pm 4\%$ (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 C_{34} and C_{36} 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 C_{40} and C_{36} 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 C_{40} and C_{36} would overlap only slightly with peaks from C_{41} and C_{37}, 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 shouldering observed by Kuksis and Breckenridge (25) might therefore be due to a partial separation of the C_{34} and C_{36} 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 C_{36} 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.

Dr. C. B. Barrett, Unilever Research Laboratory, Welwyn, Herts. generously provided a number of samples of mixed triglycerides. Professor A. C. Frazer gave support and encouragement throughout this work.

We thank the Medical Research Council of Great Britain and

Downloaded from www.jlr.org by guest, on June 19, 2012

the Faculty of Medicine of the University of Birmingham for financial support.

Manuscript received 17 May 1967; accepted 29 August 1967.

References

1. Fryer, F. H., W. L. Ormand, and G. B. Crump. 1960. *J. Am. Oil Chemists' Soc.* **37:** 589.
2. Kuksis, A., and M. J. McCarthy. 1962. *Can. J. Biochem. Physiol.* **40:** 679.
3. McCarthy, M. J., A. Kuksis, and J. M. R. Beveridge. 1963. *Can. J. Biochem. Physiol.* **40:** 1693.
4. McCarthy, M. J., and A. Kuksis. 1963. *Proc. Can. Fed. Biol. Soc.* **6:** 42.
5. Kuksis, A., M. J. McCarthy, and J. M. R. Beveridge. 1963. *J. Am. Oil Chemists' Soc.* **40:** 530.
6. Kuksis, A. 1964. *Can. J. Biochem.* **42:** 419.
7. Kuksis, A., and M. J. McCarthy. 1964. *J. Am. Oil Chemists' Soc.* **41:** 17.
8. Youngs, C. G., and M. R. Subbaram. 1964. *J. Am. Oil Chemists' Soc.* **41:** 218.
9. Subbaram, M. R., and C. G. Youngs. 1964. *J. Am. Oil Chemists' Soc.* **41:** 445, 595.
10. Subbaram, M. R., M. M. Chakrabarty, C. G. Youngs, and B. M. Craig. 1964. *J. Am. Oil Chemists' Soc.* **41:** 691.
11. Litchfield, C., M. Farquhar, and R. Reiser. 1964. *J. Am. Oil Chemists' Soc.* **41:** 588.
12. Harlow, R. D., C. Litchfield, H. Fu., and R. Reiser. 1965. *J. Am. Oil Chemists' Soc.* **42:** 747.
13. Culp, T. W., R. D. Harlow, C. Litchfield, and R. Reiser. 1965. *J. Am. Oil Chemists' Soc.* **42:** 974.
14. Tallent, W. H., R. Kleiman, and D. G. Cope. 1966. *J. Lipid Res.* **7:** 531.
15. Lefort, D., R. Perron, A. Pourchez, C. Madelmont, and J. Petit. 1966. *J. Chromatog.* **22:** 266.
16. Kuksis, A. 1965. *J. Am. Oil Chemists' Soc.* **42:** 269.
17. Kuksis, A., and W. C. Breckenridge. 1966. *J. Lipid Res.* **7:** 576.
18. Horning, E. C., and W. J. A. Vandenheuvel. 1964. *J. Am. Oil Chemists' Soc.* **41:** 707.
19. James, A. T., and A. J. P. Martin. 1952. *Biochem. J.* **50:** 679.
20. Littlewood, A. B., C. S. G. Phillips, and D. T. Price. 1955. *J. Chem. Soc.* 1480.
21. Wehrli, A., and E. Kovats. 1959. *Helv. Chim. Acta.* **42:** 2709.
22. Guiochon, G. 1964. *Anal. Chem.* **36:** 661.
23. Habgood, H. W., and W. E. Harris. 1964. *Anal. Chem.* **36:** 663.
24. Litchfield, C., R. D. Harlow, and R. Reiser. 1965. *J. Am. Oil Chemists' Soc.* **42:** 849.
25. Kuksis, A., and W. C. Breckenridge. 1965. *J. Am. Oil Chemists' Soc.* **42:** 978.
26. Jurriens, G., and A. C. J. Kroesen. 1965. *J. Am. Oil Chemists' Soc.* **42:** 9.
27. Bourne, E. J., M. Stacey, J. C. Tatlow, and J. M. Tedder. 1949. *J. Chem. Soc.*, 2976.
28. Smith, S., R. Watts, and R. Dils. 1968 *J. Lipid Res.* **9:** 52.
29. Ackman, R. G., and J. C. Sipos. 1964. *J. Am. Oil Chemists' Soc.* **41:** 377.
30. Ackman, R. G., and J. C. Sipos. 1964. *J. Chromatog.* **16:** 298.
31. Burchfield, H. P., and E. E. Storrs. 1962. Biochemical Applications of Gas Chromatography. Academic Press, London. 120.
32. Kaiser, R. 1963. Gas Phase Chromatography. Butterworths, **I:** 18.
33. Schmit, J. A. and R. B. Wynne. 1966. *J. Gas Chromatog.* **4:** 325.
34. Gill, J. M., F. Baumann, and C. H. Hartmann. 1966. *Varian Aerograph Research Notes*, Autumn.
35. Boudreau, A., and J. M. de Man. 1965. *Can. J. Biochem.* **43:** 1799.
36. Kumar, S., T. I. Pynadath, and K. Lalka. 1960. *Biochim. Biophys. Acta.* **42:** 373.

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH