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IDENTIFICATION OF THE MORE COMPLEX TRIACYLGLYCEROLS IN BOVINE MILK FAT BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY USING POLAR CAPILLARY COLUMNS

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SUMMARY

The fourth most volatile 2.5% molecular distillate of butteroil obtained by redistillation of the most volatile 10% cut was examined by gas chromatography on a polar capillary column (RSL-300) with electron impact and chemical ionization mass spectrometry. For this purpose the distillate was first freed from the acetyldiacylglycerols by thin-layer chromatography on plain silica gel and the remainder resolved into long and short chain length saturates, *cis*- and *trans*-monoenes, dienes and trienes by thin-layer chromatography on silver nitrate–silica gel. The order of gas chromatographic elution was established for more than 100 major and minor species making up the bulk of the molecular distillate. The results were used to derive the quantitative composition of the triacylglycerol species making up the various peaks obtained by polar capillary column gas chromatography of the total molecular distillate, which closely resembles the lower half of the molecular mass distribution of whole bovine milk fat.

INTRODUCTION

Ruminant milk fat represents one of the most complex mixtures of natural fatty acid esters of glycerol. Because of the high molecular mass and closely similar chemical and physical properties they have yielded even to partial chromatographic separation with great difficulty. The fatty acids range in chain length from C_2 to C_{24}, including odd carbon numbers, and in unsaturation from one to four double bonds, including *cis* and *trans* isomers[1]. In addition, one to four methyl branches may be present on the fatty chains. Commonly about 25 different fatty acids are distinguished, which could theoretically yield 15 625 different triacylglycerols. Since many of the milk fat triacylglycerols are represented only as one of the two possible enantiomers[2,3] the number of species would be reduced by as much as one half. Furthermore, several of the fatty acids are present in small quantities only and are not readily detected in a total

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mixture even after interesterification with other fatty acids in the triacylglycerol molecules. In the past ruminant milk fat triacylglycerols have been resolved on the basis of carbon number using short non-polar packed[4,5] or capillary[6] gas chromatographic (GC) columns. The use of long non-polar capillary columns results in a considerable splitting of the peaks within most carbon numbers[7,8]. However, none of these separations is suitable for triacylglycerol identification because each of the peaks contains many molecular species. GC of butterfat triacylglycerols on polar packed[9] and capillary[10] columns has led to a separation of the triacylglycerol peaks on the basis of both carbon and double bond number, which are readily recognized and quantitated. An extensive separation of peaks within each of the lower carbon numbers is also obtained[9]. As a result many peaks contain two or more major molecular species when total ruminant milk fat is chromatographed prohibiting exact identification and quantitation of any triacylglycerol species.

The present study reports the identification and quantitation of the major molecular species of triacylglycerols in a molecular distillate of butteroil using a combination of thin-layer chromatography (TLC) on silver nitrate–silica gel, polar and non-polar capillary GC and mass spectrometry (MS). A progressive preliminary segregation of the triacylglycerols into small groups of molecular species having well defined chemical differences, and introducing restrictions into the types of possible molecular species that could be present in a specific GC peak, was found to be essential for an unequivocal identification and quantitation of the GC peaks.

EXPERIMENTAL

Milk fats

The fourth most volatile 2.5% distillate, derived by molecular redistillation of the original most volatile 10% cut, had been obtained by distilling 777 pounds of butteroil in 1960 (Distillation Products Industries, Rochester, NY, U.S.A.) and its general properties have been described[11]. This distillate had been stored either at 4°C or −2°C for *ca.* 25 years prior to the present analyses. The sample of total butterfat was from another earlier analysis[9]. Synthetic *rac*-1,2-dipalmitoyl-3-acetyl, 1,3-dipalmitoyl-2-acetyl, *rac*-1,2-dipalmitoyl-3-butyryl and 1,3-dipalmitoyl-2-butyrylglycerols were prepared in the laboratory[12], while the monoacid triacylglycerols ranging in carbon number from C_{24} to C_{54} were prepared by mixing synthetic triacylglycerols of 99%+ purity obtained from NuChek Prep., Elysian, MN, U.S.A. Standards of common fatty acids along with a special Mix 110 containing normal and branched (iso and anteiso) odd and even carbon number saturated fatty acids were obtained from Supelco, Bellefonte, PA, U.S.A.

Solvents and reagents

All solvents were analytical-reagent grade. Hexane, chloroform, acetonitrile and propionitrile were of chromatographic quality. Acidified methanol and *n*-butanol for transesterification of fatty acids were prepared by additon of 5 ml of sulfuric acid to 100 ml of the anhydrous alcohol.

Thin-layer chromatography

TLC was performed on either plain silica gel (G and H, Merck, Darmstadt,

F.R.G.) or on silica gel containing 15% silver nitrate (home made) using 20 × 20 cm glass plates containing a 0.25-mm thick layer prepared in the laboratory. The acetyldiacylglycerols were resolved from the rest of the triacylglycerols in the distillate by chromatography on plain silica gel using hexane–ethyl acetate (88:1) as the developing solvent[13]. The lipid bands representing the acetates and the other triacylglycerols were located by spraying the plates with fluorescein and viewing them under UV light (254 nm). The triacylglycerols were recovered from the silica gel by extraction with chloroform–methanol (2:1). The remainder of the triacylglycerols were resolved into long and short chain lengths saturates, *trans*- and *cis*-monoenes, dienes containing two monoenoic fatty acids, and dienes plus trienes, which were retained at the origin, using chloroform (plus 0.75% ethanol preservative) as the developing solvent[14]. The triacylglycerol bands were located and extracted as above.

Gas chromatography

The GC analyses of triacylglycerols were done on a Hewlett-Packard Model 5880A gas chromatograph equipped with a hydrogen flame ionization detector and an on-column injector (Hewlett-Packard, Palo Alto, CA, U.S.A.). Analyses by carbon number were performed using a flexible quartz column (8 m × 0.32 mm I.D.) coated with a permanently bonded non-polar SE-54 liquid phase (Hewlett-Packard) as previously described for plasma lipid extracts[15]. Analyses by carbon and double bond number were performed using a flexible quartz column (25 m × 0.25 mm I.D.; RSL-300) custom-made by one of us (P.S.). It was conditioned under hydrogen carrier flow by repeatedly programming the oven temperature between 250 and 360°C at 3°C/min in multiprogram-run over a period of 48 h. Samples were injected on-column at 40°C. After 1 min the column was heated ballistically to 290°C. When the microprocessor controller indicated that the column was ready (equilibration time set at 0.5 min) the integration plot was initiated. After an additional 0.5 min the temperature was programmed at 10°C/min to 330°C and then at 2°C/min to 350°C. Single column compensation was used to correct for column bleed. The carrier gas was hydrogen at 0.68 bar. Fatty acid methyl esters were analyzed on a 15 m × 0.32 mm I.D. flexible quartz column (RTx 2330, Supelco) as previously described[16], while the butyl esters were analyzed on the 25 m RSL-300 column using temperature programming (0.5 min after injection at 40°C, 10°C/min to 200°C and then at 5°C/min to 250°C). The carrier gas in all instances was hydrogen at 0.2 bar head pressure.

Gas chromatography–mass spectrometry

The mass spectrometry was performed with a Hewlett-Packard Model 5985B quadrupole instrument interfaced with a capillary gas chromatograph using the above described polar capillary GC column. The samples were injected on-column at 40°C and the temperature allowed to rise to 250°C. The triacylglycerols were resolved by temperature programming from 250 to 340°C at 4°C/min. The transfer line was maintained at 280°C and the ion source at 230°C. Electron impact (EI) spectra were recorded at 70 eV and a hydrogen carrier gas head pressure of 0.34 bar. Chemical ionization (CI) spectra were determined at 210 eV and similar carrier gas head pressure, but the ion source pressure was increased to 0.6 Torr by admitting methane via a separate capillary inlet. Full mass spectra (200–850 mass units) were recorded every 7 s over the entire elution profile. The data were analyzed by means of

a Hewlett-Packard data system (Model HP 1000E) and a graphics terminal (Model HP 2648A) as previously described[17,18].

RESULTS

Table I gives the fatty acid composition of the R-4 distillate and of a representative sample of bovine milk fat as obtained by polar capillary GC. In addition to the major even carbon number saturated and unsaturated long chain and the saturated short chain acids, minor amounts of the odd carbon number saturated and unsaturated acids as well as small amounts of branched-chain acids, are also seen in the distillate. Because of the interesterification of the short and long chain fatty acids in

TABLE I

FATTY ACID COMPOSITION OF R-4 DISTILLATE AND OF A REPRESENTATIVE SAMPLE OF BOVINE MILK FAT TRIACYLGLYCEROLS

GC conditions as in text.

*Fatty acid**	*R-4 distillate (mol%)*	*Milk fat triacylglycerols (mol%)*
4:0	17.32	8.68
6:0	6.58	4.75
8:0	2.25	2.44
10:0	3.12	4.80
10:1	0.44	0.47
11:0	0.01	0.10
12:0	3.03	5.11
13:0	0.10	0.15
14:0 (i)	0.19	0.20
14:0	11.45	12.53
14:1	0.83	0.95
15:0 (i)	0.33	0.36
15:0 (ai)	0.63	0.61
15:0	1.35	1.35
16:0 (i)	0.29	0.23
16:0	29.50	32.71
16:1w9	0.10	0.20
16:1w7	1.17	1.45
17:0 (i)	0.34	0.23
17:0 (ai)	0.45	0.43
Phytanic	0.05	–
17:0	0.67	0.59
17:1	0.31	0.23
18:0 (i)	0.06	0.06
18:0	6.22	7.43
18:1w9 (t)	0.93	1.02
18:1w9 (c)	11.13	11.52
18:2w6	0.82	0.96
18:3w3	0.33	0.29

* i = iso-methyl branched; ai = anteiso-methyl branched; t = *trans*-monounsaturated; c = *cis*-monounsaturated.

butterfat, the selective enrichment of the short chain acids in this fraction is smaller than would have been otherwise anticipated. The distillate also contains small amounts of acetic acid, which is best demonstrated by examining the purified acetate subfraction isolated from the distillate by TLC. The overall composition of the major fatty acids compares closely to previous analyses of the distillate[9,10], where many of the minor acids, however, were not determined. The carbon number distributions of the distillate obtained on non-polar capillary column resembled closely the separations obtained previously on non-polar packed columns[9,10]. On the polar capillary column the total distillate yielded for each carbon number a minimum of two (long chain lengths) to a maximum of eight (short chain lengths) components (see below).

Fig. 1 illustrates the separations obtained for the distillate by TLC on plain silica gel and on silica gel treated with silver nitrate. On the plain gel the triacylglycerols were resolved on the basis of overall polarity, with the acetates retarded more strongly than the butyrates, hexanoates, octanoates and decanoates, which overlapped, while any long chain triacylglycerols migrated ahead. On silver nitrate TLC the triacylglycerols were resolved according to chain length, the number of double bonds and their geometric configuration. The saturates are separated into butyrates (band 2) and all others (band 1), which are clearly resolved from the monoenes and dienes. The monoenes and dienes are divided into the *cis* and *trans* isomers, which overlap with the chain length isomers. Not all of these separations were fully realized. It would be anticipated, however, that the *trans*-monoenes of the non-butyrates would migrate ahead of the *trans*-monoenes of butyrates, which would overlap with the *cis*-monoenes of non-butyrates. The *cis*-monoenes of butyrates would be expected to overlap with the *trans*-dienes of the non-butyrates (band 5). The *cis*-dienes of the butyrates would overlap with any *trans*-trienes of the non-butyrates (band 6), while the *cis*-trienes of the butyrates would remain at the origin (band 7). The anticipated separations were partly confirmed by analysis of the TLC fractions. It is possible that the actual separations were more complex. In any event Table II gives the relative amounts of material recovered from the different TLC bands.

Fig. 2 shows the polar capillary GC resolution of the acetate fraction of the distillate, which was recovered as a minor slow moving component by TLC on plain silica gel (TLC band 3, Fig. 1A). The chromatographic identification of the associations of the major fatty acids in each of the acetate peaks obtained on polar

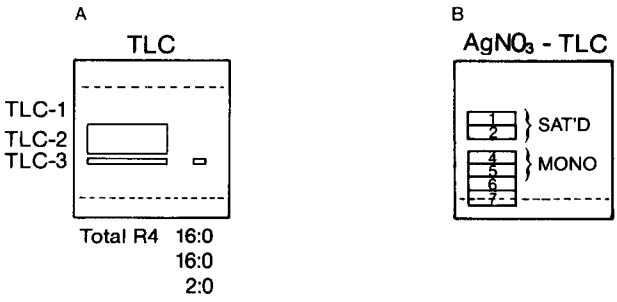

Fig. 1. Fractionation of R-4 butteroil distillate triacylglycerols by TLC on plain (A) and silver nitrate-treated (B) silica gel G. Band identification as shown in figure. TLC conditions: (A) hexane–ethyl acetate (88:1)[13]; (B) chloroform plus 0.75% ethanol preservative (15% silver nitrate).

TABLE II

RELATIVE AMOUNTS OF TRIACYGLYCEROLS RECOVERED FROM SILVER NITRATE TLC RESOLUTION OF R-4 DISTILLATE

TLC conditions as in text.

Silver nitrate TLC fraction	*R-4 (%)*	*Characterization*
1	23.7	Saturates; C_6 and longer chain fatty acids
2	31.3	Saturates; C_4 and longer chain fatty acids
3	0.0	
4	4.0	Monoenes: 72% *trans*; 28% *cis*
5	32.0	Monoenes: 100% *cis*
6	5.2	Dienes containing two monoenoic fatty acids
7	3.8	Other dienes and trienes

capillary GC was confirmed by GC–MS. Table III gives the composition of the major molecular species in the acetyldiacylglycerol fraction.

Fig. 3 shows the polar capillary GC profile of band 1 from the silver nitrate TLC. It is made up exclusively of the triacylglycerol species containing two long chain-length saturated fatty acids in combination with one residue of caproic, caprylic or capric

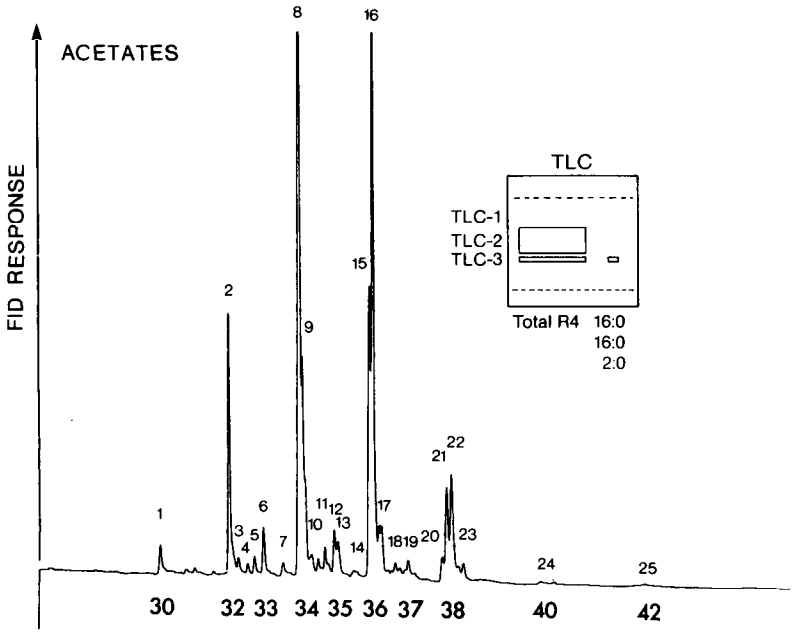

Fig. 2. Polar capillary GC profile of the acetyldiacylglycerol fraction isolated from R-4 butteroil distillate by TLC on plain silica gel. Peak numbers are identified in Table III. GC conditions: instrument, Hewlett-Packard Model 5880; column, 25 m × 0.25 mm I.D. RSL-300; carrier gas, hydrogen at 0.68 bar head pressure; on-column injection at 40°C, then ballistically heated to 290°C. Integration plot initiated at 290°C, then after 0.5 min oven programmed at 10°C/min to 330°C and then at 2°C to 350°C. Abscissa indicates the carbon number and is linear with time. Peak 22 is eluted in 5.56 min after the start of the integration plot.

TABLE III

COMPOSITION OF THE MAJOR MOLECULAR SPECIES OF ACETYLDIACYLGLYCEROL FRACTION

Peak No.	*Species type**	*RRT***	*Mol%*	*Major molecular species*
1	30 (0, 0, 0)	0.530	0.96	
1a		0.546	0.10	
1b	ai 31 (0, 0, 0)	0.628	0.26	
1c		0.662	0.26	
1d	i 32 (0, 0, 0)	0.737	0.11	
2	32 (0, 0, 0)	0.798	8.08	14, 16, 2 + 12, 18, 2
3		0.831	0.75	
4	i 33 (0, 0, 0)	0.869	0.56	i 15, 16, 2 + i 17, 14, 2
5	ai 33 (0, 0, 0)	0.899	0.80	ai 15, 16, 2 + ai 17, 14, 2
6	33 (0, 0, 0)	0.933	1.80	15, 16, 2 + 17, 14, 2
7	i 34 (0, 0, 0)	1.008	0.78	
7a		1.031	0.23	
8	34 (0, 0, 0)	1.072	21.21	16, 16, 2 + 14, 18, 2
9	34 (0, 0, 1)	1.072	6.85	14, 18:1, 2 + 16:1, 16, 2
9a	34 (shoulder)	1.095		
10		1.110	0.57	
10a		1.121	0.93	
10b	i 35 (0, 0, 0)	1.144	0.87	i 15, 18, 2 + i 17, 16, 2
10c	i 35 (0, 0, 1)	1.159	0.41	i 15, 18:1, 2 + i 17, 16:1, 2
11	ai 35 (0, 0, 0)	1.170	1.25	ai 15, 18, 2 + ai 17, 16, 2
11a	ai 35 (0, 0, 1)	1.185	0.82	ai 15, 18:1, 2 + ai 17, 16:1, 2
12	35 (0, 0, 0)	1.204	1.82	15, 18, 2 + 17, 16, 2
13	35 (0, 0, 1)	1.219	2.22	15, 18:1, 2 + 17, 16:1, 2 + 17:1, 16, 2
14	i 36 (0, 0, 0)	1.279	0.99	
14a	i 36 (0, 0, 1)	1.293	0.78	
15	36 (0, 0, 0)	1.347	8.69	16, 18, 2
16	36 (0, 0, 1)	1.362	17.36	16, 18:1, 2 + 16:1, 18, 2
17	36 (0, 1, 1)	1.385	1.59	16:1, 18:1, 2
17a	36 (0, 0, 2)	1.395	1.68	
17b	i 37 (0, 0, 0)	1.415	0.66	
17c	37 (0, 0, 1)	1.433	0.48	
18	ai 37 (0, 0, 0)	1.444	0.85	ai 17, 18, 2
18a	ai 37 (0, 0, 1)	1.464	0.99	
18b	37 (0, 0, 0)	1.482	0.75	17, 18, 2
19	37 (0, 0, 1)	1.494	1.58	17, 18:1, 2
20	38 (0, 0, 0)	1.629	1.06	18, 18, 2
21	38 (0, 0, 1)	1.648	3.16	18, 18:1, 2
22	38 (0, 1, 1)	1.666	4.14	18:1, 18:1, 2
22a	38 (0, 0, 2)	1.693	0.71	18, 18:2, 2
23	38 (0, 1, 2)	1.712	1.00	18:1, 18:2, 2
23a		1.757	0.31	
24	40		0.84	
25	42		0.58	

* Species type indicated by the total acyl carbon number and, in brackets, the degree of unsaturation of each of the three acyl chains.

** Relative to 14:0 16:0 4:0 on the polar capillary column (RSL-300).

TABLE IV

MAJOR TRIACYLGLYCEROLS FROM SILVER NITRATE TLC BAND 1

Peak No.	*Species type**	*RRT***	*Mol%*	*Major species*
1	32	0.681	0.19	
1a	32	0.699	0.11	
2	34 (8, *X*, *X*)	0.933	0.73	8, 10, 16 + 8, 12, 14
3	34 (8, *X*, *X*)	0.948	0.68	
4	34 (6, *X*, *X*)	0.960	1.72	6, 14, 14 + 6, 18, 10 + 6, 16, 12
4a		1.034	0.10	
4b		1.073	0.30	
5	35 (6, *X*, *X*)	1.094	0.42	
6		1.165	0.23	
7	36 (8–10, *X*, *X*)	1.206	4.24	8, 14, 14 + 8, 12, 16 + 10, 14, 12
8	36 (6, *X*, *X*)	1.230	10.31	6, 14, 16 + 6, 12, 18
9		1.260	0.46	
10	i 37 (6, *X*, *X*)	1.298	0.55	
11	ai 37 (6, *X*, *X*)	1.325	1.244	
12	37 (6, *X*, *X*)	1.360	1.770	
13		1.396	0.151	
13a		1.411	0.156	
13b		1.434	0.43	
14	38 (10, *X*, *X*)	1.461	3.17	10, 14, 14 + 10, 12, 16
15	38 (8, *X*, *X*)	1.473	6.31	8, 14, 16 + 8, 12, 18
16	38 (6, *X*, *X*)	1.503	18.95	6, 16, 16 + 6, 14, 18
17	38 (4, *X*, *X*)	1.535	1.28	
18		1.577	0.89	
19		1.606	1.86	
20	39 (6, *X*, *X*)	1.645	1.16	
20a		1.683	0.29	
20b		1.698	0.17	
21	40 (10–12, *X*, *X*)	1.758	7.71	10, 14, 16 + 12, 14, 14
22	40 (8, *X*, *X*)	1.775	5.86	8, 16, 16 + 8, 14, 18
23	40 (6, *X*, *X*)	1.808	6.12	6, 16, 18
24		1.849	0.65	
25		1.879	0.41	
25a		1.897	0.22	
26		1.921	0.94	
27		1.941	0.29	
27a		1.977	0.16	
28		2.018	0.28	
29	42 (10, *X*, *X*)	2.104	8.24	10, 16, 16 + 10, 14, 18
30	42 (8, *X*, *X*)	2.128	1.45	8, 16, 18
31	42 (6, *X*, *X*)	2.167	0.42	
31a		2.190	0.19	
32		2.238	0.18	
33		2.285	0.35	
34	i 44	2.395	0.14	
35	44 (10–14, *X*, *X*)	2.499	4.58	10, 16, 18 + 12, 16, 16 + 14, 14, 16
36	i 45	2.614	0.17	
37	i 45	2.665	0.21	
38	45	2.723	0.31	
39	i 46	2.848	0.10	
40	46	2.966	1.98	14, 16, 16
41	i 47	3.088	0.10	
42	ai 47	3.160	0.13	
43	47	3.224	0.12	
44	48	3.503	0.65	16, 16, 16
45	50	4.290	0.17	16, 16, 18

* Species type indicated by the total acyl carbon number and, in brackets, the chain of each of the three alkyl chains. Unspecified chain lengths are indicated by *X*.

** Retention time relative to 16:0 14:0 4:0.

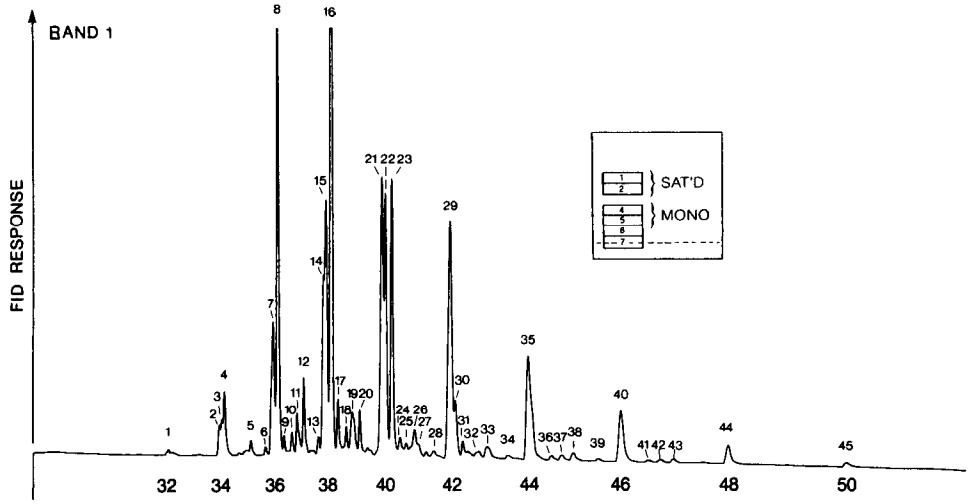

Fig. 3. Polar capillary GC profile of the saturated long chain-length triacylglycerol fraction isolated from R-4 butteroil distillate by silver nitrate TLC (band 1). Peak numbers are identified in Table IV. GC conditions as in Fig. 2. TLC conditions as in Fig. 1.

acid, which have been partially resolved within the corresponding carbon numbers on the polar capillary column. Between the major even carbon number peaks are seen minor peaks, which are due to odd carbon number triacylglycerols arising from the substitution of one of the long chain acids by a C_{15} or C_{17} fatty acid of normal, iso or anteiso structure. The identity of the peaks was confirmed by MS (see below), which also provided estimates for the relative proportions of the molecular species within

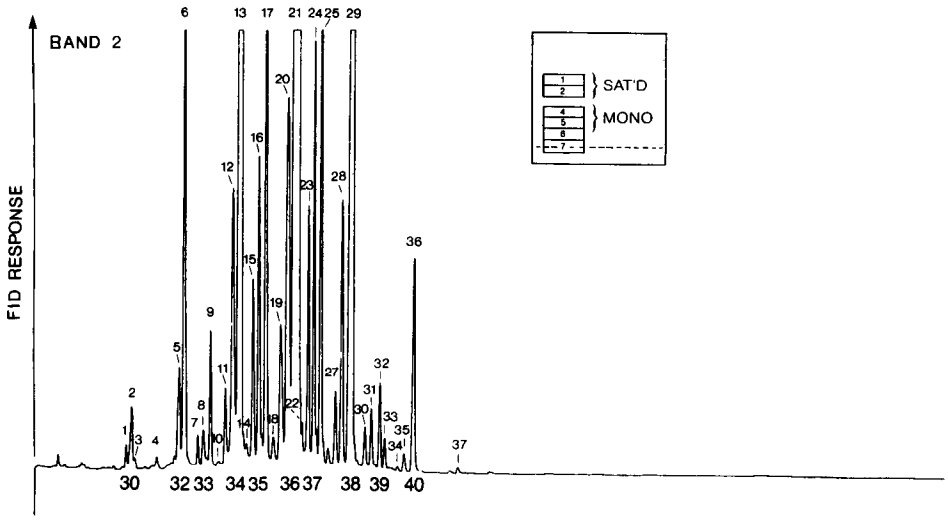

Fig. 4. Polar capillary GC profile of the saturated short chain-length triacylglycerols isolated from R-4 butteroil distillate by silver nitrate TLC (band 2). Peak numbers are identified in Table V. GC conditions as in Fig. 2. TLC conditions as in Fig. 1.

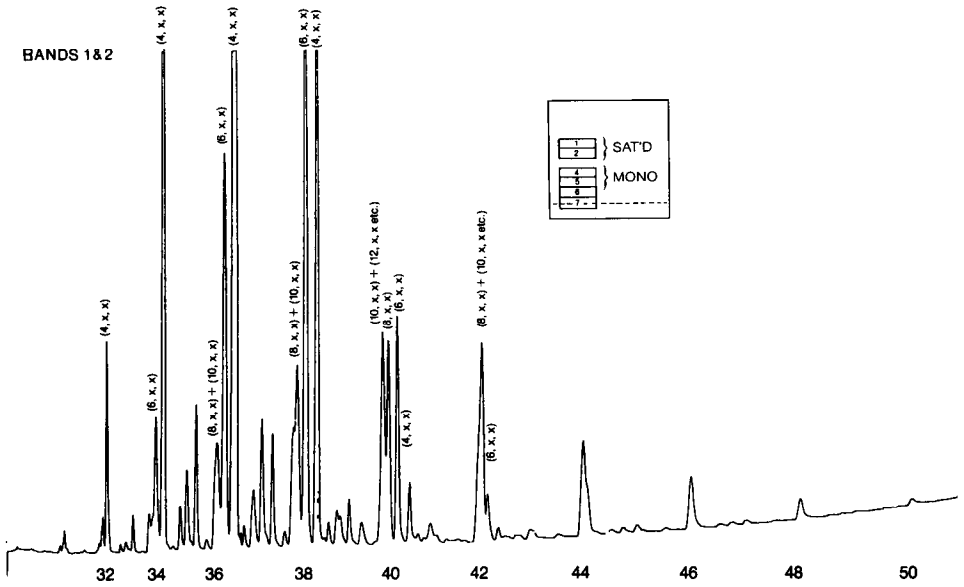

Fig. 5. Polar capillary GC profile of a 1:1 mixture of triacylglycerols from band 1 (Fig. 1) and band 2 (Fig. 1). Peak identification as shown on figure. GC conditions as in Fig. 2. TLC conditions as in Fig. 1.

each carbon number. Table IV gives the identity and quantity of the major molecular species as estimated by GC and GC–MS using the polar capillary column.

Fig. 4 shows the polar capillary GC profile of the triacylglycerols in band 2 from the silver nitrate TLC separation. On the basis of the fatty acid composition these triacylglycerols are largely butyrates with small amounts of caproates overlapping from silver nitrate TLC band 1. Fig. 5 shows the polar capillary GC profile of a mixture of bands 1 and 2. It is seen clearly that the butyrates are eluted later than the longer chain-length species of triacylglycerols within each carbon number. On the basis of the mass chromatograms recorded for the peaks obtained on the polar column for band 2, it was possible to confirm the identity of all major molecular species in each major resolved peak. Table V gives the identities and quantities of the major molecular species in band 2 as determined by GC on the polar capillary columns and confirmed by GC–MS.

Fig. 6 illustrates the use of GC–MS for the identification of the milk fat triacylglycerols. It shows the mass chromatograms recorded for the $(M-RCOOH)^+$ ions for the major species of the triacylglycerols in the silver nitrate TLC band 2. It can be seen that C_{32} yields two major ions with m/z 327 and 495, which correspond to C_{16} and C_{28} diacylglycerols, respectively, representing a 16:0 12:0 4:0 triacylglycerol as a sole major component. C_{34} yields ions with m/z 327, 355, 383 and 523 as major products and 411, 439 and 467 as minor products, representing C_{16} to C_{30} diacylglycerols, respectively. Within the C_{34}, it is therefore possible to construct the triacylglycerols 18:0 12:0 4:0, 16:0 14:0 4:0 as major components preceded by 16:0 12:0 6:0 as a minor component. Likewise, it is seen that C_{36} contains 16:0 16:0 4:0 as a major component, which is preceded by 16:0 14:0 6:0 and 14:0 14:0 8:0 as minor components. Similarly, C_{38} is seen to be made up of 18:0 16:0 4:0 as a major component, preceded by

TABLE V

MAJOR TRIACYLGLYCEROLS FROM SILVER NITRATE TLC BAND 2

Peak No.	*Species type**	*RRT***	*Mol%*	*Major molecular species*
1		0.441	0.03	
2	30 (4, *X*, *X*)	0.467	0.06	
3		0.481	0.01	
4		0.586	0.11	
4a		0.661	0.05	
4b		0.673	0.08	
5	32 (6, *X*, *X*)	0.693	0.69	
6	33 (4, *X*, *X*)	0.717	3.56	16, 12, 4 + 14, 14, 4
7	i 33 (4, *X*, *X*)	0.789	0.19	i 15, 14, 4 + i 17, 12, 4
8	ai 33 (4, *X*, *X*) + 33 (6, *X*, *X*)	0.815	0.28	
9	33 (4, *X*, *X*)	0.848	0.74	15, 14, 4 + 17, 12, 4
10		0.890	0.10	
11		0.923	0.52	
12		0.958	1.99	
13	34 (4, *X*, *X*)	1.000	19.06	14, 16, 4 + 18, 12, 4
14		1.027	0.20	
15	i 35 (4, *X*, *X*)	1.060	0.98	i 15, 16, 4 + i 17, 14, 4
16	ai 35 (4, *X*, *X*)	1.086	1.68	ai 15, 16, 4 + ai 17, 14, 4
17	35 (4, *X*, *X*)	1.125	3.10	15, 16, 4 + 17, 14, 4
18		1.161	0.28	
19	36 (8, *X*, *X*)	1.193	1.02	
20	36 (6, *X*, *X*)	1.229	3.07	
21	36 (4, *X*, *X*)	1.283	38.30	16, 16, 4 + 14, 18, 4
22		1.304	0.22	
23	i 37 (4, *X*, *X*)	1.336	1.17	i 15, 18, 4 + i 17, 16, 4
24	ai 37 (4, *X*, *X*)	1.363	1.66	ai 15, 18, 4 + i 17, 16, 4
24a		1.375	0.16	
25	37 (4, *X*, *X*)	1.396	2.44	15, 18, 4 + 17, 16, 4
26		1.438	0.18	
27		1.473	0.45	
28	38 (6, *X*, *X*)	1.503	1.41	
29	38 (4, *X*, *X*)	1.554	13.86	16, 18, 4
29a		1.580	0.07	
30	i 39 (4, *X*, *X*)	1.619	0.26	i 17, 18, 4
31	ai 39 (4, *X*, *X*)	1.649	0.31	ai 17, 18, 4
32	39 (4, *X*, *X*)	1.690	0.39	17, 18, 4
33		1.714	0.16	
33a		1.732	0.03	
33b		1.747	0.03	
34	i 40 (4, *X*, *X*)	1.777	0.04	
35	40 (6, *X*, *X*)	1.809	0.10	
36	40 (4, *X*, *X*)	1.854	1.01	18, 18, 4
37		2.071	0.03	

* As in Table IV.

** Retention time relative to 16:0 14:0 4:0.

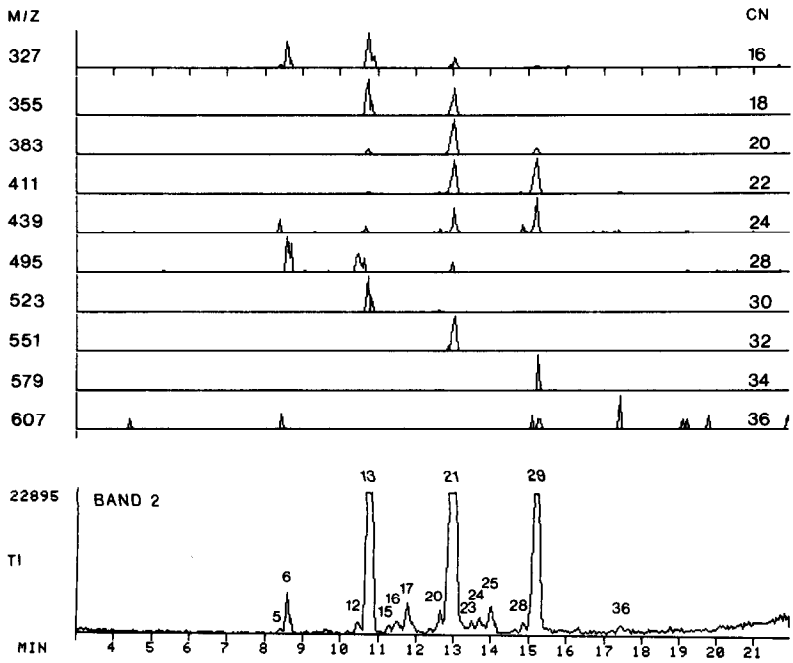

Fig. 6. Mass chromatograms of the mixed saturated triacylglycerols (Fig. 4). Peak identification by carbon number and by $(M-RCOOH)^+$ fragment ions. GC–MS conditions: instrument, Hewlett-Packard quadrupole mass spectrometer interfaced with a gas chromatograph equipped with the same capillary column as the GC instrument in Fig. 2; carrier gas, hydrogen; methane chemical ionization; other conditions as given in text. Full spectra were recorded every 7 s and the appropriate ions were recalled from storage in a computer.

16:0 16:0 6:0 as another major component, which is further preceded by 14:0 14:0 10:0 and 16:0 12:0 10:0 as minor components. The three major peaks within C_{40} (Fig. 5) gave GC–CI-MS fragments which could have come only from a sequential elution of the capric, caprylic and caproic acid containing triacylglycerol species. Within each peak the short chain acid was combined, however, with two or more different long chain acids to give a total carbon number of 40 (*e.g.* 8:0 14:0 18:0; 8:0 16:0 16:0). In all instances the GC–MS findings were consistent with the results of silver nitrate TLC separation and analyses of the fatty acids of the individual TLC bands. The minor even carbon number peaks were identified on the basis of the GC retention times and the rules of resolution just established. The minor odd carbon number peaks were due to the presence of significant amounts of C_{15} and C_{17} acids, which were combined with the short and long chain length even carbon number acids in the triacylglycerol molecules. The separation within the carbon number was due to a resolution of the iso, anteiso and normal chains, in order of increasing retention time. This was consistent with the order of resolution of the simple fatty acid esters and the finding of "diacylglycerol" ions with identical masses in all three peaks for carbon numbers 35 and 37. Fig. 7 illustrates the GC–CI-MS identification of the saturated odd carbon number butyrates in silver nitrate TLC band 2 (Fig. 1B). These are due to the combination of one residue of butyric acid with one residue of the odd carbon number

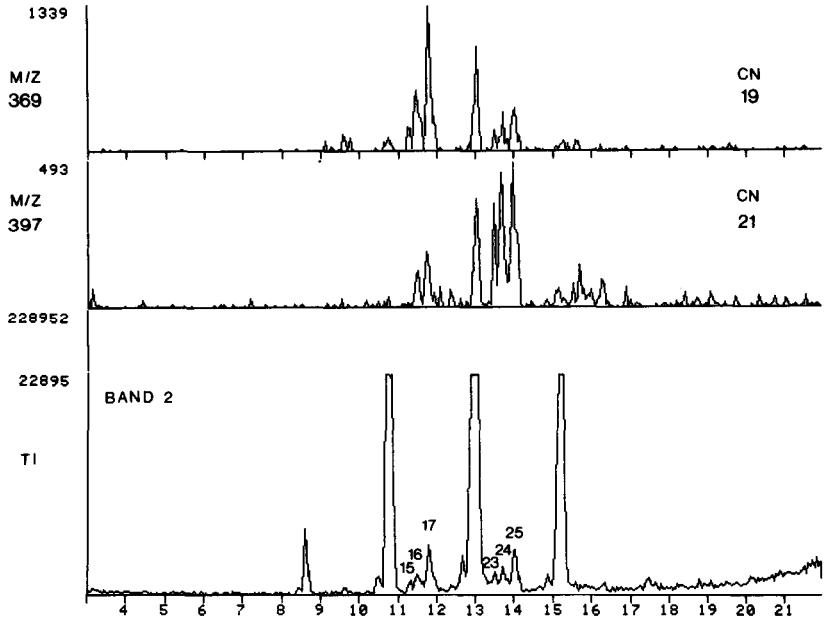

Fig. 7. Mass chromatograms of the odd carbon number triacylglycerols resolved from the saturated short chain-length fraction (Fig. 4). Peak identification by carbon number and by $(M - RCOOH)^+$ fragment ions. GC–MS conditions as in Fig. 6.

(C_{15} or C_{17}) and one residue of the corresponding even carbon number long chain length fatty acid. The mass spectra indicate that C_{35} is made up of peaks containing 4:0 15:0 16:0 and 4:0 17:0 14:0 triacylglycerol species, which have been resolved according to the nature of the odd carbon number fatty acid (*e.g.* normal chains preceded by iso and anteiso chains). Similar identifications were made of the components in C_{37}. The odd carbon number triacylglycerols were identified and quantitated similarly in band 1 where they were shown to consist of the 6, 8 and 10 carbon short chain along with the corresponding even carbon number long chain acids. Fig. 8 gives the full mass spectra recorded for the major components of C_{35} from the silver nitrate TLC band 2. The major triacylglycerols in each of the peaks yield the same "diacylglycerol" fragments, although some contamination from the preceding and succeeding major peaks is also seen. The major ions represent the 14:0 17:0 and 16:0 15:0 (*m/z* 537); 17:0 4:0 (*m/z* 397), 14:0 4:0 (*m/z* 365), 16:0 4:0 (*m/z* 383) and 15:0 4:0 (*m/z* 369) "diacylglycerols". The parent ions of these species were not seen under the present working conditions.

Fig. 9 gives the polar capillary GC profile of silver nitrate TLC band 5 (Fig. 1B), which represents the major monoene fraction. On the basis of the fatty acid analyses the monoenes were the *cis* isomers of 18:1 and lower carbon number monounsaturates. Unlike the saturates, the monoenes were not resolved into the butyrate and longer chain length species as effectively as the saturates, presumably because of the overriding influence of the π-bonding to the silver ions. As a result each carbon number on GC is resolved into the butyrate, caproate, caprylate and caprate subfractions. GC–MS examination of the major peaks showed combinations of the mono-unsaturated fatty acid with a short chain and a long chain length acid corresponding to

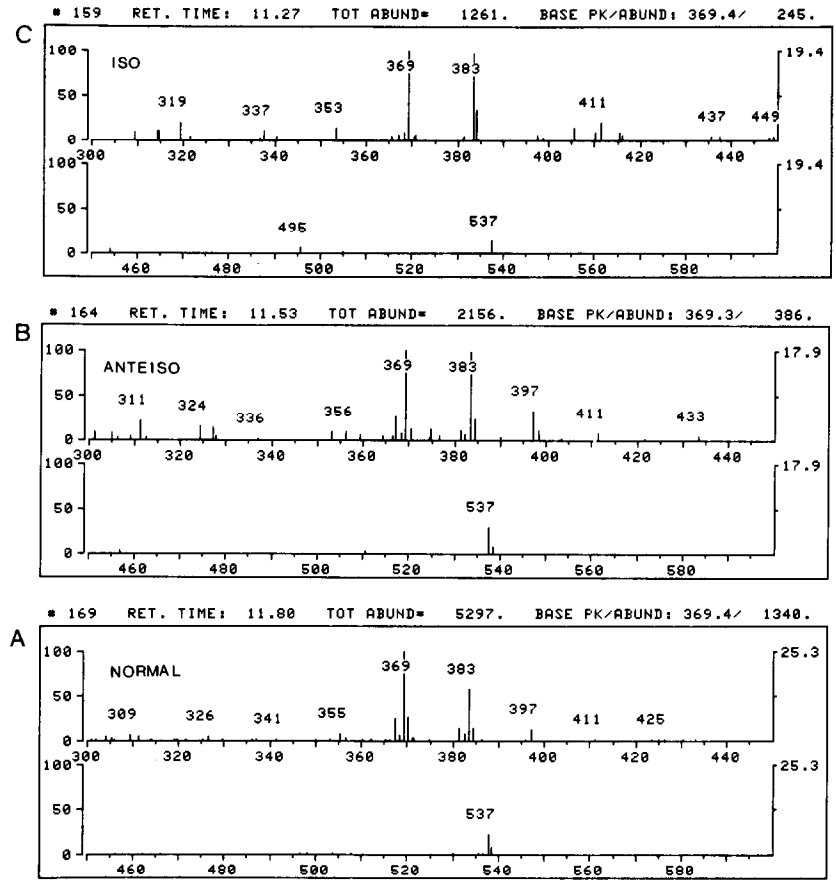

Fig. 8. Full GC–MS spectra of odd carbon number triacylglycerols resolved under C_{35} from saturated short chain length fraction (Fig. 4). (A) Normal chain C_{15} and C_{17} triacylglycerols; (B) anteiso chain C_{15} and C_{17} triacylglycerols; (C) iso chain C_{15} and C_{17} triacylglycerols. GC–MS conditions as in Fig. 6.

the appropriate carbon number (*e.g.* C_{38} = 18:1 16:0 4:0, preceded by 18:1 14:0 6:0 and 18:1 12:0 8:0). The odd carbon number triacylglycerols again were resolved according to the configuration of the odd carbon number fatty chain into the normal, iso and anteiso species. However, among these peaks were interdigitated the peaks arising from the separation of the *cis* and *trans* monoenes of the butyrates, caproates and caprylates in accordance with the appropriate carbon number. This complexity was not seen for the odd carbon number triacylglycerols of silver nitrate TLC band 2, which contained butyric as the only short chain-length acid. The odd carbon number resolution obtained with band 5 also contrasted with that recorded for band 1, which showed in addition to the normal, iso and anteiso separation also a segregation of the caproates and caprylates present in this saturated triacylglycerol fraction. Since it was not possible to confirm the identity of each minor peak by GC–MS, the identities of the minor odd carbon number peaks were assigned on the basis of their relative retention times and consistency with other chromatographic data and chemical composition. An

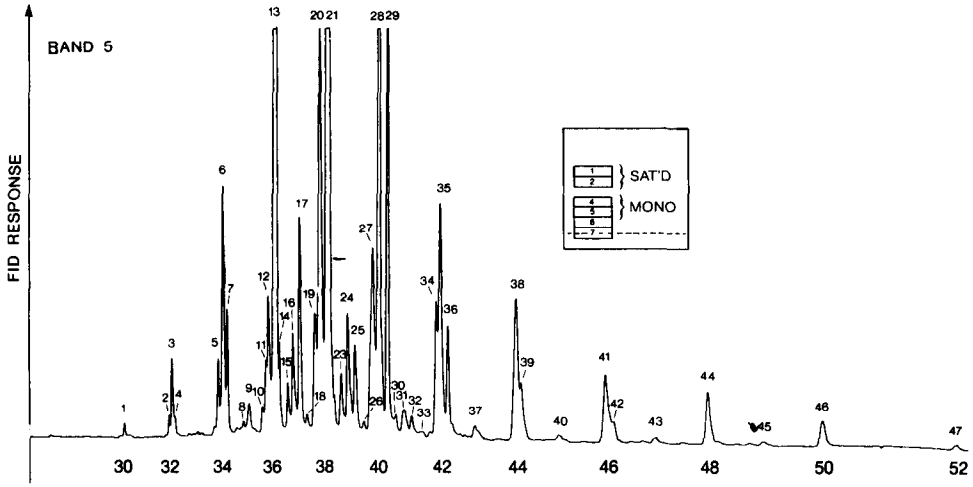

Fig. 9. Polar capillary GC profile of the *cis*-monoene fraction isolated from R-4 butteroil distillate by silver nitrate TLC (band 5). Peak numbers identified in Table VIII. GC conditions as in Fig. 2.

essentially identical elution pattern was obtained for band 4, which was identified as a monoene fraction containing *trans*-18:1 as the major monounsaturated fatty acid. This band made up *ca.* 11% of the total monoenoic triacylglycerols and is consistent with the ratio of elaidic to oleic acid in the distillate. Greater mobility of elaidic compared to oleic acid-containing butterfat triacylglycerols on silver nitrate TLC plates has been previously reported by Breckenridge and Kuksis[14]. The identity of the major peaks in bands 4 and 5 were confirmed by GC–MS as described above. In addition, the GC–MS revealed the presence of small amounts of monoenoic triacylglycerols containing medium chain length monounsaturated fatty acids. The identity and quantity of the major triacylglycerol species in bands 4 and 5 are listed in Table VI.

The polar capillary GC profile of the triacylglycerols from silver nitrate TLC band 6, which made up 8.1% of the total, indicated (chromatogram not shown) a C_{34}–C_{52} range, with only one or two peaks each: butyrates and caproates at the lower end and caprylates, caprates and laurates at the upper end as the major components. On the basis of the fatty acid composition, the fraction represents a diene band containing two monounsaturated fatty acids per triacylglycerol molecule. The requirement for two monounsaturated fatty acids per molecule also explains the relative simplicity of the elution pattern, which is due to the predominance of the 18:1 over the other monoenoic fatty acids in the mixture. The chromatographic pattern also shows evidence for small quantities of odd carbon number triacylglycerols, which reflect the trace amount of 17:1 expected to occur in this fraction. Table VII gives the identity and estimated triacylglycerol composition of band 6.

The polar capillary GC elution patterns recorded for the triacylglycerols recovered in silver nitrate TLC band 7, which contributed 3.3% of the total distillate and represented the origin of the plate, indicated a very complex pattern (chromatogram not shown). On the basis of the fatty acid composition and the relative retention times of the triacylglycerols, it contained mainly tri-unsaturated species plus

TABLE VI

MAJOR MONOENOIC TRIACYLGLYCEROLS FROM SILVER NITRATE TLC BANDS 4 AND 5

Peak No.	*Species type**	*RRT***	*Band 4 (mol%)*	*Band 5 (mol%)*	*Major molecular species*
1	30	0.468		0.10	
2		0.716		0.18	
3	32 (4, *X*, *X*)	0.735	0.25	0.78	8, 18:1, 4 + 10, 16:1, 4
4		0.755	0.06		
5	34 (6, *X*, *X*)	0.986	0.96	0.73	10, 18:1, 6
6	34 (4, *X*, *X*)	1.011	1.52	2.51	12, 18:1, 4 + 14, 16:1, 4
7	34 (4, *X*, *X*)	1.036	0.28	1.01	16, 14:1, 4
8	35	1.130	0.99	0.26	
9	35	1.152	0.24	0.59	
10	36	1.223		0.28	
11		1.241	3.34	0.65	
12	36 (6, *X*, *X*)	1.256		1.54	12, 18:1, 6 + 14, 16:1, 6 + 16, 14:1, 6
13	36 (4, *X*, *X*)	1.292	11.56	13.20	14, 18:1, 4 + 16, 16:1, 4
14	36 (4, *X*, *X*)	1.312	1.58	0.76	18, 14:1, 4
15	ai 37 (6, *X*, *X*) + i 37 (4, *X*, *X*)	1.355	1.15	0.56	ai 15, 16:1, 6 + i 15, 18:1, 4
16	ai 37 (4, *X*, *X*) + 37 (6, *X*, *X*)	1.380	1.00	1.23	ai 15, 18:1, 4 + 15, 16:1, 6
16a		1.393	0.72		
17	37 (4, *X*, *X*)	1.408	1.79	2.28	15, 18:1, 4 + 17, 16:1, 4
18		1.455		0.31	
19	38 (8–10, *X*, *X*)	1.495	2.11	2.08	16:1, 14, 8 + 12, 16:1, 10
20	38 (6, *X*, *X*)	1.520	6.82	6.87	18:1, 14, 6 + 16:1, 16, 6
21	38 (4, *X*, *X*)	1.569	26.57	31.569	18:1, 16, 4 + 16:1, 18, 4
22		1.595	3.14	0.44	
23	ai 39 (6, *X*, *X*) + i 39 (4, *X*, *X*)	1.634	0.88	0.82	ai 15, 18:1, 6 + i 17, 18:1, 4
24	ai 39 (4, *X*, *X*) + 39 (6, *X*, *X*)	1.666	1.55	1.55	ai 17, 18:1, 4 + 15, 18:1, 6
25	39 (4, *X*, *X*)	1.700	0.51	1.13	17, 18:1, 4
25a		1.711	0.87		
26		1.749	0.18	0.24	
27	40 (8–10, *X*, *X*)	1.796	3.19	2.94	18:1, 14, 8 + 16:1, 14, 10
28	40 (6, *X*, *X*)	1.832	8.53	7.52	18:1, 16, 6
29	40 (4, *X*, *X*)	1.876	1.07	4.57	18:1, 18, 4
30		1.919		0.34	
31	ai 41 (6, *X*, *X*) + (8–10, *X*, *X*)	1.959	0.84	0.57	
32	41 (6, *X*, *X*)	1.998	0.32	0.34	
33		2.052	0.30	0.10	
34	42 (10, *X*, *X* + 12, *X*, *X*)	2.128	2.70	1.37	10, 18:1, 14 + 12, 16:1, 14
35	42 (8, *X*, *X*)	2.152	1.80	2.72	18:1, 16, 8
36	42 (6, *X*, *X*)	2.190	1.46	1.03	18:1, 18, 6
37	43	2.322	0.35	0.31	
38	44	2.543	1.97	2.01	10, 18:1, 16
39	44	2.567	0.28	0.67	18:0, 18:1, 8
40	45	2.770		0.10	
41	46	3.002	1.05	1.11	16, 18:1, 12 + 14, 14, 18:1
42	46	3.043		0.22	16, 18:1, 10
43	47	3.267		0.10	
44	48	3.540	0.60	0.74	14, 16, 18:1
45	49	3.829		0.07	15, 16, 18:1
46	50	4.135	0.23	0.35	16, 16, 18:1
47	52	4.824	0.07	0.12	

* As in Table IV.

** Retention time relative to 16:0 14:0 4:0.

TABLE VII

MAJOR TRIACYLGLYCEROLS FROM SILVER NITRATE TLC BAND 6

Species type	*RRT**	*Mol%*	*Major molecular species*
30	0.495	0.12	
32	0.761	0.19	
	0.775	0.26	
34	0.958	0.06	
	1.011	0.49	
	1.035	1.12	
36	1.227	0.13	
	1.268	1.31	
	1.306	6.10	16:1, 16:1, 4
	1.377	0.19	
37	1.410	0.83	
	1.439	0.96	
	1.483	0.21	
	1.516	0.93	
38	1.551	3.1	14:1, 16:1, 8
	1.578	7.1	14:1, 18:1, 6
38	1.589	10.4	16:1, 18:1, 4
39	1.663	0.77	
	1.698	0.85	
	1.725	2.40	
40	1.899	32.67	18:1, 18:1, 4
	1.920	4.54	18:2, 18, 4
41	2.029	1.50	
	2.062	0.45	
42	2.186	2.25	18:1, 16:1, 8
	2.213	8.97	18:1, 18:1, 6
43	2.385	0.29	
44	2.602	4.38	18:1, 18:1, 8 + 16:1, 18:1, 10
46	3.072	2.59	18:1, 18:1, 10
47		0.22	
48	3.602	0.79	18:1, 18:1, 12
	3.644	0.77	14:1, 18:1, 16
49	3.894	0.14	
50	4.200	1.31	18:1, 18:1, 14
51	4.522	0.16	18:1, 18:1, 15
52	4.902	0.97	18:1, 18:1, 16
54	5.792	0.07	18:1 18:1, 18

* Retention time relative to 16:0 14:0 4:0.

dienes, which were not completely resolved from band 6. However, triacylglycerols containing one butyric, caproic or caprylic acid residue in combination with one monounsaturated and one diunsaturated fatty acid could be clearly identified, as could be some dienoic odd carbon number triacylglycerols containing only one di-unsaturated long chain length fatty acid per molecule. The chromatographic identifications of the major components were again confirmed by GC–MS. Table VIII gives the identities and quantities of the major triacylglycerols in silver nitrate TLC band 7.

Fig. 10 gives the polar capillary GLC profile of the total R-4 distillate of butteroil

TABLE VIII

MAJOR TRIACYLGLYCEROLS FROM SILVER NITRATE TLC BAND 7

*Species type**	*RRT***	*Mol%*	*Molecular species*
32	0.764	0.62	
	0.793	0.10	
	0.819	0.17	
	0.921	0.01	
34	0.982	0.06	
	1.003	0.53	
	1.035	0.54	Band 6 overlap
	1.052	0.74	
	1.084	0.77	
	1.111	0.10	
	1.157	0.10	
	1.178	0.12	
	1.219	0.12	
36	1.254	0.61	
	1.268	0.27	
	1.306	4.55	Band 6 overlap: 16:1, 16:1, 4
	1.323	0.94	14:1, 18:2, 4
	1.353	4.76	
	1.394	0.57	
	1.434	0.85	
	1.455	0.50	
	1.490	0.70	
38	1.507	0.76	
	1.551	2.18	14:1, 16:1, 8
	1.575	7.44	14:1, 18:1, 6 + 16:1, 18:1, 4
	1.589		
	1.607	4.10	
	1.652	9.82	16:1, 18:2, 4
	1.676	1.54	
	1.723	1.48	
	1.765	1.27	
	1.812	1.21	
40 (4, Δ1, Δ1)	1.895	8.82	18:1, 18:1, 4
40 (4, Δ1, Δ2)	1.954	17.76	18:1, 18:2, 4
40 (4, Δ0, Δ3)	1.996	3.50	
	2.020	5.07	
41	2.067	1.48	
42	2.168	1.38	
	2.213	1.01	18:1, 18:1, 6
	2.278	5.97	18:1, 18:2, 6
	2.338	1.23	
	2.362	2.09	
44	2.596	1.04	
	2.674	1.50	(includes front and back shoulder)
	2.775	0.46	
46	3.705	0.35	
	4.323	0.33	
	5.048	0.47	

* Species type indicated by total acyl carbon number and, in brackets, carbon number of the short chain acid and degree of unsaturation of the long chain acids.

** Retention time relative to 16:0 14:0 4:0.

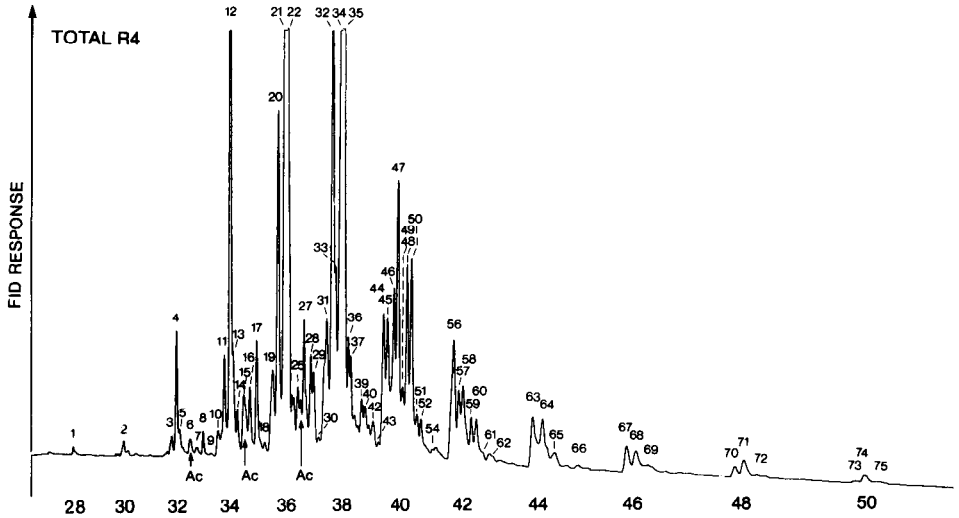

Fig. 10. Polar capillary GC profile of total R-4 distillate triacylglycerols. Peak numbers are identified in Table IX. Acetates (Ac) are identified by arrows. GC conditions as in Fig. 2.

TABLE IX

MAJOR TRIACYLGLYCEROL TYPES AND SPECIES IDENTIFIED AND QUANTITATED IN R-4 DISTILLATE

Peak No.	*Species type**	*RRT***	*Mol%*	*Major molecular species*
1	28	0.72	0.07	
2	30	1.57	0.33	
3	32 (6, *X*, *X*)	0.694	0.26	
4	32 (4, *X*, *X*)	0.717	1.24	
5		0.735	0.37	
6	i 33 (4, *X*, *X*)	0.791	0.09	
7	n 33 (6, *X*, *X*) + ai 33 (4, *X*, *X*)	0.891	0.15	
8	n 33 (4, *X*, *X*)	0.855	0.28	
9	ai 33 (2, *X*, *X*)	0.895	0.07	
10	n 33 (2, *X*, *X*) +	0.935	0.32	
11	34 (6, *X*, *X*)	0.966	1.27	
12	34 (4, *X*, *X*)	1.000	7.07	}
13	(shoulder)			}
14		1.037	0.49	
15	i 35 (4, *X*, *X*) + 34 (2, *X*, *X*)	1.073	0.43	
16	n 35 (6, *X*, *X*) + ai 35 (4, *X*, *X*) + 34 (2, *X*, *X*) Δ1	1.100	0.76	
17	n 35 (4, *X*, *X*)	1.134	1.25	
18	n 35 (2, *X*, *X*) +	1.176	0.20	
19	36 (8, *X*, *X*) + 35 (2, *X*, *X*) Δ1	1.216	1.32	
20	36 (6, *X*, *X*)	1.243	3.92	
21	36 (4, *X*, *X*) + 36 (6, *X*, *X*) Δ1	1.283	12.90	
22	36 (4, *X*, *X*) Δ1	1.294	4.30	
23	i 37 (6, *X*, *X*) + 36 (4, *X*, *X*) Δ1	1.314	0.44	

(continued on p. 112)

TABLE IX (*continued*)

Peak	*Species type**	*RRT***	*Mol%*	*Major molecular species*
24		1.325	0.49	
25	ai 37 (6, *X*, *X*) +	1.341	0.70	
26	36 (2, *X*, *X*) +	1.355	0.16	
27	36 (2, *X*, *X*) Δ1 + 37 (6, *X*, *X*) + ai 37 (4, *X*, *X*)	1.369	1.11	
27a	36 (2, *X*, *X*) Δ1,1 +	1.383	0.43	
28	n 37 (4, *X*, *X*) +	1.403	0.92	
29		1.413	0.98	
30		1.439	0.25	
31	38 (8, *X*, *X*) + 38 (10, *X*, *X*)	1.483	2.53	8, 14, 16 + 10, 14, 14 + 10, 12, 16
32	38 (6, *X*, *X*) + 38 (8, *X*, *X*) Δ1	1.503	5.37	6, 14, 18 + 6, 16, 16 + 8, 16:1, 14
33	38 (6, *X*, *X*) Δ1	1.513	1.48	6, 18:1, 14 + 6, 16:1, 16
34	38 (4, *X*, *X*)	1.542	5.40	4, 16, 18
35	38 (4, *X*, *X*) Δ1	1.557	9.65	4, 16, 18:1 + 4, 16:1 18
36	38 (4, *X*, *X*) Δ1,1	1.581	0.96	4, 16:1 18:1
37	39 +	1.592	0.83	
38			0.70	
39	38 (4, *X*, *X*) Δ1,2 + (2, *X*, *X*) Δ1 + 39	1.648	0.63	
40	38 (2, *X*, *X*) Δ1,1	1.669	0.61	
41		1.695	0.34	
42		1.704	0.57	
43		1.731	0.18	
44	40 (10, *X*, *X*)	1.757	1.74	10, 14, 16
45	40 (8, *X*, *X*)	1.775	1.61	8, 16, 16 + 8, 14, 16
46	40 (6, *X*, *X*) + (8, *X*, *X*) Δ1 + (10, *X*, *X*) Δ1	1.810	2.18	6, 16, 18 + 8, 18:1, 14 + 10, 18:1, 12
47	40 (6, *X*, *X*) Δ1	1.828	2.90	6, 18:1 16
48	40 (4, *X*, *X*) +	1.851	0.83	
49	40 (4, *X*, *X*) Δ1	1.875	1.08	4, 18:1, 18
50	40 (4, *X*, *X*) Δ1,1	1.895	2.14	4, 18:1, 18:1
51	40 (4, *X*, *X*) Δ2 + 41 (10, *X*, *X*)	1.922	0.59	
52	40 (4, *X*, *X*) Δ1,2	1.945	0.79	
53				
54	41	2.001	0.18	
55	41	2.018	0.37	
56	42 (10, *X*, *X*)	2.104	2.04	10.14, 18 + 10, 16, 16
57	42 (8, *X*, *X*) + (10, *X*, *X*) Δ1	2.130	0.92	8, 16, 18 + 10, 18:1, 14
58	42 (8, *X*, *X*) Δ1	2.151	1.34	8, 18:1, 16
59	42 (6, *X*, *X*) Δ1 + (8, *X*, *X*) Δ1,1	2.192	0.62	6, 18:1, 18 + 8, 16:1, 18:1
60	42 (6, *X*, *X*)	2.220	0.74	6, 18:1, 18:1
61	43			
62	42 (6, *X*, *X*) Δ1,2	2.283	0.29	6, 18:1, 18:2
63	44 (10–14, *X*, *X*)	2.498	1.23	10, 1, 18 + 12, 16, 16 + 14, 14, 16
64	44 (10, *X*, *X*) Δ1	2.547	1.32	10, 18:1, 16
65	44 (8, *X*, *X*) Δ1,1	2.594	0.45	8, 18:1, 18:1
65a		2.647	0.55	
66	45	2.727	0.66	
67	46 (14, *X*, *X*)	2.970	0.55	14, 16, 16
68	46 (12, *X*, *X*) Δ1 + (14, *X*, *X*) Δ1	3.012	0.66	12, 16, 18:1 + 14, 14, 18:1
69	46 (10, *X*, *X*) Δ1,1	3.07	0.18	10, 18:1, 18:1
70	48	3.505	0.26	16, 16, 16
71	48 (14, *X*, *X*) Δ1	3.549	0.54	14, 16, 18:1
72	48 (12, *X*, *X*) Δ1,1	3.609	0.06	12, 18:1, 18:1
73	50 (16, *X*, *X*)	4.111	0.09	16, 16, 18
74	50 (16, *X*, *X*) Δ1	4.157	0.33	16, 16, 18:1
75	50 (14, *X*, *X*) Δ1,1	4.224	0.16	14, 18:1, 18:1

* Species type indicated as in Table IV with fatty acid (*X*, *X*) degree of unsaturation specified by Δ1, Δ1,1, Δ2, and Δ1,2.

** Retention time relative to 16:0 14:0 4:0.

including the identity of the major peaks. The peak pattern resembles very closely that of the lower-molecular mass triacylglycerols in total bovine milk fat published previously by Geeraert and Sandra[10], where these peaks, however, were not identified. Table IX gives the composition of the total triacylglycerol mixture of the R-4 distillate. The mole percentages of 75 different peaks are estimated from the knowledge of the carbon number distribution and the flame ionization response in the gas chromatograph. The triacylglycerol species were identified on the basis of GC and GC–MS analysis of the intact triacylglycerols recovered from the various silver nitrate TLC bands and the knowledge of the fatty acid composition of the distillate. Over 100 individual triacylglycerol species were recognized, which accounted for a large percentage of the total distillate. Only the major triacylglycerol species have been specifically listed.

Table X illustrates the method used in the reconstitution of the mass of the triacylglycerols recovered from the various TLC bands. In the example the composition of the C_{40} triacylglycerol grouping in the R-4 distillate is compared to that reconstituted from the analysis of the various silver nitrate TLC bands. A total of eleven peaks are recognized, each of which contains more than one triacylglycerol species. Only the major components have been identified in Table X. Although the grouping appears very complex, only the third peak of the first seven major peaks had major contributions from more than one silver nitate TLC band. For the reconstitution the contribution of each C_{40} peak in the TLC band was multiplied by the mass proportion of the corresponding TLC band and the products summed. The resulting composition was compared to the composition of the C_{40} triacylglycerol grouping in the total R-4 distillate. An excellent agreement was obtained. Similar reconstitution of the composition of the other triacylglycerol carbon numbers gave equally good agreement with the values measured directly in the total R-4 distillate.

DISCUSSION

Chromatographic characteristics of short chain length triacylglycerols

The present study reveals that the complex chromatographic elution patterns of the short chain triacylglycerols of butteroil arise from the resolution of its triacylglycerols into several groups of molecular species within most carbon numbers. This is due to both the characteristic composition and molecular association of the fatty acids. Essentially all of the triacylglycerols are mixed acid species, in which the occurrence of the short chains is limited to one per molecule. From stereospecific analyses[2,3] we know that the short chain acids are confined specifically to the *sn*-3-position of the triacylglycerol molecule, which renders them enantiomeric. The association of the butyric acid residue with the *sn*-3-position has been confirmed by NMR using chiral shift reagents[19,20]. Further complications occur from the polarity differences between the short and long chain length fatty acid substituents in the triacylglycerol molecules, which become manifested under certain chromatographic conditions, resulting in a segregation of the triacylglycerols on the basis of chain length on plain silica gel, or on polar capillary columns. The short and long chain lengths of saturated triacylglycerols are also resolved on silver nitrate TLC, but the unsaturated triacylglycerol bands were too complex to see clearly the anticipated resolution. Such chain length separations on plain silica gel have been observed previously by Nutter and Privett[21] and Brecken-

TABLE X

RECONSTITUTION OF TRIACYLGLYCEROL MASS RECOVERED BY POLAR CAPILLARY GC FROM THE VARIOUS SILVER NITRATE TLC BANDS

Representative triacylglycerols	*RRT**	*ECN***	*Band No.****						*Sum*	*R4*
			1	*2*	*4*	*5*	*6*	*7*		
16, 14, 10	1.757	40.000	1.83	0.01				0.05	1.89	1.73
16, 16, 8 + 18, 14, 8	1.778	40.13	1.39	0.01					1.40	1.59
18, 16, 6 + 14, 18:1, 8 + 12, 18:1, 10	1.811	40.33	1.45	0.03	0.13	0.94		0.05	2.60	2.16
16, 18:1, 6	1.828	40.43			0.34	2.41			2.75	2.87
18, 18, 4	1.852	40.58	0.16	0.32					0.48	0.82
18, 18:1, 4	1.876	40.72	0.10		0.04	1.46			1.60	2.06
18:1, 18:1, 4	1.897	40.84	0.05		0.03		1.70	0.34	2.12	2.12
16, 15, 10 + 18, 18:2, 4	1.924	41.00	0.22		0.11		0.24		0.57	0.58
18:1, 18:2, 4	1.947	41.13	0.07		0.03		0.18	0.67	0.95	0.78
Unknown	2.000	41.40			0.01	0.11		0.13	0.25	0.18
Unknown	2.018	41.51	0.07				0.08	0.19	0.34	0.35

* Retention times relative to 16:0 14:0 4:0.

** Equivalent carbon number based on the set of long chain saturated triacylglycerols, such as 10:0 16:0 14:0 (ECN = 40.00) and 10:0 16:0 16:0 (ECN = 42.00).

*** Values are in mol%.

ridge and Kuksis[14]. The separations on silver nitrate TLC were accompanied by a resolution of triacylglycerols containing *cis*-18:1 (oleic) and *trans*-18:1 (elaidic) acids, as already reported by Breckenridge and Kuksis[14].

Chain length separations beyond the carbon number on polar packed columns were first observed by Kuksis *et al.*[9], who demonstrated that within a carbon number the butyrates were preceded by caproates, caprylates, caprates and mixtures of triacylglycerols containing exclusively long chain length fatty acids. The present separations on polar capillary columns, supported by MS, have permitted a further characterization of this resolution. Thus, the shifts in equivalent carbon number (ECN) that result from differences in chain length, positional placement and unsaturation of the fatty acids have been assessed. It was found that for species with the same total carbon and double bond number, the ECN decreased as the minimum chain length increased (*e.g.* ΔECN are observed as follows: 2,*X*,*X* − 4,*X*,*X* = 0.47; 4,*X*,*X* − 6,*X*,*X* = 0.29; 6,*X*,*X* − 8,*X*,*X* = 0.20). The acetates and butyrates having the short chain acid in the secondary position were eluted later than the equivalent species having the short chain acid in the primary position (*e.g.* *X*,2,*X* − 2,*X*,*X* = 0.29; *X*,4,*X* − 4,*X*,*X* = 0.16). The effects of unsaturation on the separation of the short chain triacylglycerols on the polar capillary columns paralleled those described by Geeraert and Sandra[7,10]. Thus, the triacylglycerol 4, 18:0, 18:1 was retained longer than 4, 18:0, 18:0 (ΔECN = 0.13) and 4, 18:1, 18:1 longer than 4, 18:0, 18:1 (ΔECN = 0.12), while 4, 18:0, 18:2 was retained much longer than 4, 18:1, 18:1 (ΔECN = 0.17), and 4, 18:1, 18:2 very much longer than 4, 18:1, 18:1 (ΔECN = 0.29). Similar resolution factors were observed for the caproates and caprylates of the C_{18} fatty acids and the mixtures of C_{16} and C_{18} acids. Furthermore, the polar capillary columns permitted an effective resolution of the odd carbon number triacylglycerols into normal, anteiso and iso isomer-containing odd carbon number fatty acids, which occurred with a stoichiometry of only one per molecule. In all instances the species containing the normal chain isomers were preceded by species containing the anteiso isomers, which were preceded by species containing the iso isomers of the odd carbon fatty acids, These separations are similar to those seen for the simple fatty acid methyl and butyl esters on the polar capillary column. The finding of these odd carbon number and branched chain fatty acids in butterfat is in agreement with the original report of Ryhage[22]. Small amounts of phytanic (C_{20}) acid were also noted among the fatty acids of the distillate, and presumably accounted for small peaks in the total GC pattern of the triacylglycerols. The chromatographic behaviour of the tetramethyl-substituted C_{15} (pristanic) and C_{16} (phytanic) saturated fatty acids in bovine milk fat have been previously characterized by Avigan[23] and Hansen and Ackman[24], respectively.

The substitution of a *trans*- for a *cis*-18:1 acid in the butterfat triacylglycerols did not result in a significant change in the retention times for the corresponding species. However, the GC run of silver nitrate TLC band 4, which contained both *cis* and *trans* monounsaturates, showed peaks that were slightly broadened in comparison to runs obtained for silver nitrate TLC band 5 (pure *cis*-species). Other monounsaturated fatty acids in bovine milk fat have been identified as positional isomers of oleic acid, as well as of palmitoleic acid. In addition, trace amounts of even and odd carbon number medium chain length fatty acids have also been reported in bovine milk fat[1,22]. No unidentified fatty acids appeared to accumulate in significant amounts in any of the major triacylglycerol fractions isolated and identified in the present study. There was

little loss of linoleic and linolenic acids in the distillate in comparison to the total bovine milk fat.

GC–MS characteristics of short chain length triacylglycerols

The MS behaviour of short and long chain triacylglycerols has been extensively studied in the past and the principles of fragmentation under EI[25,26] and CI[27] conditions have been well established. The use of the polar capillary GC for the admission of the triacylglycerols to the ion source of the mass spectrometer was anticipated to provide a superior method of insuring the purity of the analyte, any remaining overlaps being confined to clearly defined chromatographic and molecular weight analogues. In general this was true. However, due to the close spacing and uneven proportions of the peaks, and the need for overloading the low-capacity capillary column to ensure sufficient ions for the minor components, there was considerable overlap between adjacent peaks, and uncertainty arose regarding the exact identity and order of elution of closely spaced species. It was therefore necessary to effect a fractionation of the samples prior to the GC–MS analysis. Isolation of the acetates by TLC on plain silica gel and of the long and short chain saturates, monoenes, dienes and trienes by TLC on silver nitrate-treated silica gel helped to simplify the interpretation of the mass spectra for all species including the odd carbon number triacylglycerols. In most instances the GC–MS spectra did not give a molecular ion in either EI or CI mode and the triacylglycerol species present had to be identified from the "diacylglycerol" type of fragment ions. Within the restrictions of carbon and double bond numbers and the chromatographic retention times of the peaks, however, the identification of the parent triacylglycerols did not present problems. In many instances the diacylglycerol fragments were unique to the triacylglycerols present, while in other instances the full complement of triacylglycerol species could be recognized by appropriate allowance for minor diacylglycerol species.

We had previously shown[28] that the ion yields vary with the triacylglycerol type and the diacylglycerol fragment selected, and that exact quantitation of the molecular species of triacylglycerols in a mixture requires calibration of the system with appropriate standards. Since this was not practical, we relied upon the hydrogen flame ionization response in the gas chromatograph for the quantitation of the total amount of material in each GC peak and used the ion current response only for obtaining the relative proportions of the unresolved components in a GC peak. The overall validity of each estimate was established by reference to the mass proportions of the species in the corresponding silver nitrate TLC fraction.

Previously a GC–MS analysis of bovine milk fat had been reported[6] using a non-polar capillary column for admission of triacylglycerols to the ion source, but the limited resolution had not given any extensive identifications. Despite the great resolving power of the polar capillary column and its exceptional suitability for analysis of natural triacylglycerols[10], few meaningful identifications of bovine milk fat triacylglycerols could be made without a preliminary fractionation of the sample.

Previous analysis of R-4 distillate

The fatty acid composition and carbon number distribution of the R-4 distillate was reported some 25 years ago[11]. At various later times this butteroil fraction had been employed to demonstrate the specific location of the short chain fatty acids in the

sn-3-position[3], the TLC separation of short and long chain length triacylglycerols of bovine milk fat[14], the separation of short and long chain length-containing triacylglycerols within a carbon number on packed polar GC columns[9], and more recently the liquid chromatography–MS identification of short and medium chain length triacylglycerols in ruminant milk fat[29]. These studies had shown that the composition and chemical structure of the triacylglycerols in the R-4 distillate closely resembles that of the lower-molecular-mass triacylglycerols of bovine milk fat, as far as the analytical methods of the time had permitted to determine.

Significance of present analyses

The present study establishes the chromatographic behaviour of the short chain length triacylglycerols on GC and GC–MS using polar capillary columns. The molecular distillate of butteroil provides a secondary reference standard for the identification of the mixed short and long chain triacylglycerols in bovine milk fat. We had noted in the past that the R-4 distillate closely resembles the early part of the carbon number profile of whole butterfat and of ruminant milk fats in general[9,11,14]. The elution profiles obtained on the polar capillary columns for the R-4 distillate in the present study and for whole butterfat in the present study and by Geeraert and Sandra[10] are almost superimposable over the C_{24} to C_{46} range of carbon numbers, including the fine details of the pattern within many of the carbon numbers. The analyses of the distillate account for the species in the most complex part of the butterfat profile. Polar capillary GC appears superior to the reversed-phase HPLC resolution of the milk fat triacylglycerols[29] because of the ease of quantitation provided by the flame ionization detector and the much larger number of theoretical plates, which can be profitably exploited for the resolution of closely related species. However, a complete resolution of even all major species is not obtained without prefractionation by silver nitrate TLC. It would be of interest to submit to polar capillary GC the milk fat triacylglycerol fractions obtained by reversed-phase HPLC.

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