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Reversed-phase liquid chromatography–mass spectrometry of the uncommon triacylglycerol structures generated by randomization of butteroil

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Abstract

The test triacylglycerols were prepared by randomization of natural butterfat followed by silver ion TLC segregation of the saturates, monoenes and dienes. The molecular species were identified by on-line positive chemical ionization mass spectrometry. Within a series of isologous triacylglycerols, those containing the shortest fatty acid chain were eluted last from a reversed-phase column (XX10 > XX8 > XX6 > XX4, where X = long-chain acid), although common experience would have predicted that the shorter and more polar species would be eluted first. A comparable order of elution was obtained for isologous triacylglycerols containing two short- and one long-chain (X88 > X86 > X66 > X84 > X64 > X44) or three short-chain fatty acids per molecule, when compared to triacylglycerols containing medium or long-chain fatty acids in combination with short-chain acids. Since this resolution is similar to that achieved on adsorption chromatography, it is suggested that the C_{18} reversed-phase column possesses residual adsorptive activity. No discernible separation was seen among the corresponding positional or reverse isomers of short-chain triacylglycerols with the present reversed-phase system.

1. Introduction

The presence of high proportions of the short-chain (C_4–C_8) fatty acids in butterfat has been long known [1] as has been their overall positional distribution and molecular association. Early studies by high-temperature gas chromatography (GC) [2] and silver ion thin-layer chromatography ($AgNO_3$-TLC) [3] showed that the bulk of these acids occurred with the frequency of one short-chain fatty acid per triacylglycerol molecule. Furthermore, evidence was obtained that the short-chain fatty acids were confined largely to the *sn*-3 position [4,5]. More recently butterfat composition has been analyzed by capillary GC on polarizable liquid phases [6,7], reversed-phase high-performace liquid chromatography (HPLC) [8,9] and GC and HPLC with mass spectrometry (MS) [10], as well as by MS–MS [11]. These studies have led to an extensive resolution of butterfat triacylglycerols, but the possible presence of small amounts of positional and reverse isomers as well as of species containing two or three short-chain acids per molecule has not been addressed.

As an aid in the identification of such triacylglycerols in butterfat, we have investigated the reversed-phase LC–MS behavior of randomized butterfat, which contains the various isomeric triacylglycerols in known and sufficient amounts

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for analysis. The results show that conventional C_{18} reversed-phase columns would not resolve the positional and reverse isomers of mixed acid triacylglycerols, but that the resolution of isologous triacylglycerols is retained also when two or three short-chain fatty acids occur per molecule.

2. Materials and methods

2.1. Butterfat samples

The rearranged butteroil sample had been prepared by Distillation Products Industries, Rochester, NY, USA, and had been stored in solid form in a sealed container at 7°C and −20°C. The natural butterfat sample was from a local Canadian creamery and also had been stored at −20°C. Synthetic triacylglycerols of short, medium and long chain length were available in the laboratory from previous studies [7,12].

2.2. Reagents and solvents

All reagents and solvents were of analytical or chromatographic grade and were obtained from reputable laboratory supply houses. Acetonitrile was from Caledon (Canada), while propionitrile was from Romil (UK) or Fluka (Switzerland).

2.3. Gas chromatography

GC analyses of fatty acid butyl esters and intact triacylglycerols by carbon number were performed on non-polar capillary columns [7] while the separations based on both carbon and double bond number of the fatty acids were done on polar capillary columns as previously described in detail [7].

2.4. High-performance liquid chromatography

The triacylglycerols were resolved by reversed-phase HPLC with a Supelcosil LC-18 reversed-phase column (5 μm, 25 cm × 0.46 cm I.D.; Supelco, Mississauga, Canada) using a linear gradient of 0–90% propionitrile in acetonitrile or 10–90% propionitrile in acetonitrile (2.2 ml/min). The column was installed in a Hewlett-Packard Model 1084B liquid chromatograph connected via a direct liquid inlet interface to a mass spectrometer as previously described [10]. Alternatively, these analyses were performed with a Hewlett-Packard Model 1050 liquid chromatograph equipped with the reversed-phase HPLC column coupled to a Varex ELSD II light-scattering detector (Varex, Burtonsville, MD, USA). This column was operated at a flow-rate of 1 ml/min (90 min) using a linear gradient of 10–90% isopropanol in acetonitrile.

2.5. $AgNO_3$-TLC

The rearranged butteroil was segregated on basis of degree of unsaturation by TLC on silica gel G impregnated with 15% $AgNO_3$ (20 × 20 cm, 250 μm thick layer) using chloroform containing 0.75% ethanol as the developing solvent. The resolved triacylglycerols were located by spraying the plate with a 0.05% solution of 2,7-dichlorofluorescein in methanol–water (50:50) and viewing the plates under UV light. A total of five fractions were collected [3,5] by eluting with chloroform–methanol (2:1) the silica gel scraped from areas corresponding to the following R_F values: 0.70–0.85 (band 1); 0.55–0.70 (band 2); 0.35–0.55 (band 3); 0.25–0.37 (band 4); and 0.06–0.25 (Band 5).

2.6. Mass spectrometry

On-line LC–MS was performed with a Hewlett-Packard Model 5985B quadrupole mass spectrometer coupled to a Hewlett-Packard 1084B liquid chromatograph via a direct liquid inlet interface [10] as previously described. For LC–MS analysis about 1% of the column effluent was admitted to the mass spectrometer. The LC–MS analyses were made in both positive and negative chemical ionization mode as previously described [7,10], with the HPLC solvent providing the reagent gas. Specifically, full mass spectra were taken every 7 s in the mass range 200–900 over the entire elution profile and were stored in the computer. Subsequently single-ion

profiles were recalled from the data stored in the computer for both molecular and fragment ions.

2.7. Calculations

The 1,2,3-random calculation of the molecular association of fatty acids in the rearranged butteroil was performed as described elsewhere [13]. The triacylglycerol peaks were summed by carbon and by partition number and compared to the distributions determined experimentally by polar capillary GC and reversed-phase HPLC.

3. Results

3.1. General characteristics of rearranged butteroil

Table 1 gives the fatty acid composition of the rearranged in relation to natural butterfat triacylglycerols as obtained by polar capillary GC. There are close similarities between these two samples in the quantitative composition of the fatty acids, except for a reversal in the proportions of the stearic and oleic acids. The overall composition of the natural fat is not unlike that reported previously for blended bovine milk fats [14].

Table 2 compares the carbon number distribution in the two butterfat samples as obtained by GC on a non-polar capillary column. This column gives an essentially quantitative recovery for all triacylglycerols in the C_{20}–C_{56} range and the peak areas provide a true account of the carbon number proportions, as evidenced from a comparison of the experimentally determined and calculated proportions of the rearranged triacylglycerols. The triacylglycerol peak proportions are similar to those reported previously from analyses on packed columns containing a non-polar liquid phase [14], except for the estimates of the odd-carbon-number triacylglycerols, which were not very well resolved in the earlier work. It is seen that the rearranged fat is increased in the proportion of both short- and long-chain triacylglycerols and decreased in the proportion of the medium-chain triacylglycerols.

Table 1
Fatty acid composition of randomized and reference butterfat

Fatty acid	Butterfat (mol%)[a]	
	Reference	Randomized
4:0	8.76	6.21
6:0	4.79	3.34
8:0	2.44	1.84
10:0	4.80	3.37
10:1	0.47	0.39
11:0	0.10	0.10
12:0	5.11	3.90
12:1		
13:0	0.16	0.13
14:0 *iso*	0.21	0.02
14:0 *n*	13.39	13.89
14:1	1.02	0.39
15:0 *iso*	0.36	0.41
15:0 *anteiso*	0.60	0.65
15:0 *n*	1.34	1.46
16:0 *iso*	0.23	0.42
16:0	32.36	34.51
16:1	1.58	0.54
17:0 *iso*	0.23	0.53
17:0 *anteiso*	0.43	0.62
17:0 *n*	0.58	0.97
18:0 *iso*	0.06	0.30
18:0 *n*	7.35	5.23
18:1 *trans*	1.01	1.35
18:1 *cis*-9	11.40	8.86
18:2	0.95	0.07
18:3	0.29	0.00

[a] Determined by polar capillary GC as the *n*-butyl esters following acid transesterification. The relative standard deviations for repeat analyses were $<1\%$ for components making up more than 1% of total, and $<5\%$ for components making up less than 1% of total.

Fig. 1 shows the separation of the natural and rearranged butterfat triacylglycerols by reversed-phase HPLC using a linear gradient of 10–90% isopropanol in acetonitrile and light-scattering detection. Comparable elution profiles were obtained with a linear gradient of 10–90% [10] or 30–90% [15] propionitrile in acetonitrile. The natural triacylglycerol distribution differs from the rearranged one mainly in the molecular

Table 2
Carbon number distribution of butterfat triacylglycerols

Carbon No.	Butterfat (mol%)[a]		
	Reference	Randomized	Calculated[b]
18	0.00	0.13	0.07
20	0.00	0.19	0.10
22	0.04	0.49	0.25
24	0.13	1.21	0.66
25	0.00	0.12	0.06
26	0.39	1.75	0.97
27	0.03	0.09	0.06
28	0.89	1.53	1.00
29	0.07	0.00	0.08
30	1.50	1.60	1.26
31	0.12	0.01	0.12
32	2.83	2.29	1.83
33	0.39	0.26	0.25
34	6.13	3.75	3.17
35	1.11	0.70	0.60
36	11.13	6.46	5.57
37	1.64	0.97	0.89
38	12.84	8.11	6.88
39	1.17	0.72	0.78
40	9.96	6.84	6.04
41	0.71	0.42	0.71
42	6.61	5.12	5.61
43	0.76	0.69	0.93
44	5.72	5.93	6.93
45	0.87	0.60	1.47
46	5.82	8.35	9.83
47	1.09	1.83	2.40
48	6.62	11.58	13.32
49	1.42	2.34	2.77
50	7.77	12.51	13.14
51	1.41	1.80	1.77
52	6.72	8.39	7.62
53	0.62	0.29	0.48
54	2.99	2.64	2.03
55	0.15	0.01	0.02
56	0.28	0.23	0.10

[a] Determined by non-polar capillary GC. The relative standard deviations for repeat determinations were $< 1\%$ for components making up more than 2% of total and $< 2\%$ for components making up less than 2% of total.
[b] 1,2,3-Random calculation based on the fatty acid composition of the randomized butteroil.

association of the fatty acids, although discrepancies are also present in the proportions of the 18:0 and 18:1 fatty acids (Table 1).

3.2. Peak identification in total oil

The individual triacylglycerols in the various HPLC peaks were identified by on-line LC–MS using a variety of strategies [10,16]. Single-ion profiles for the diacylglycerol type of fragments were systematically matched against those recorded for the total ion current and the partition number and elution order established for all triacylglycerols by reference to the fatty acid moiety lost during fragmentation. The identification of individual molecular species was confirmed by examining the full mass spectra taken from the ascending and descending limbs or the heart section of each peak for the presence of characteristic diacylglycerol fragment ions and the molecular ions, if any.

Fig. 2A gives the LC–MS profile for the saturated diacylglycerol moieties of the triacylglycerol peaks in the randomized butteroil. The major saturated even-carbon-number diacylglycerols determined as the $[MH - RCOOH]^+$ ions range from 8:0 to 36:0 and are combined randomly with all the fatty acids present in the rearranged oil. The relative proportions of the various diacylglycerols are indicated by the ion counts given in the left hand corner of each ion plot, while the nature of the fatty acid lost during fragmentation is indicated by its carbon and double bond number attached to each diacylglycerol peak. The splittings in the partition numbers of the triacylglycerols correspond to the splittings in the peaks for the diacylglycerol fragment ions and are due to differences in elution times of the species containing fatty acids of different chain length within isologous triacylglycerols. There is a clear indication of the separation of various critical pairs of species, *e.g.* the palmitates from oleates and the myristates from palmitoleates. Among the triacylglycerols containing short-chain fatty acids, *e.g.* butyrates, caproates and caprylates, there is a clear-cut resolution of a triplet, with the butyrates being preceded by caproates, which are preceded by caprylates and higher chain lengths within each isologous series of triacylglycerols. Thus, the triacylglycerols yielding a diacylglycerol (DG) fragment DG22:0 were made up of a triplet of peaks within the

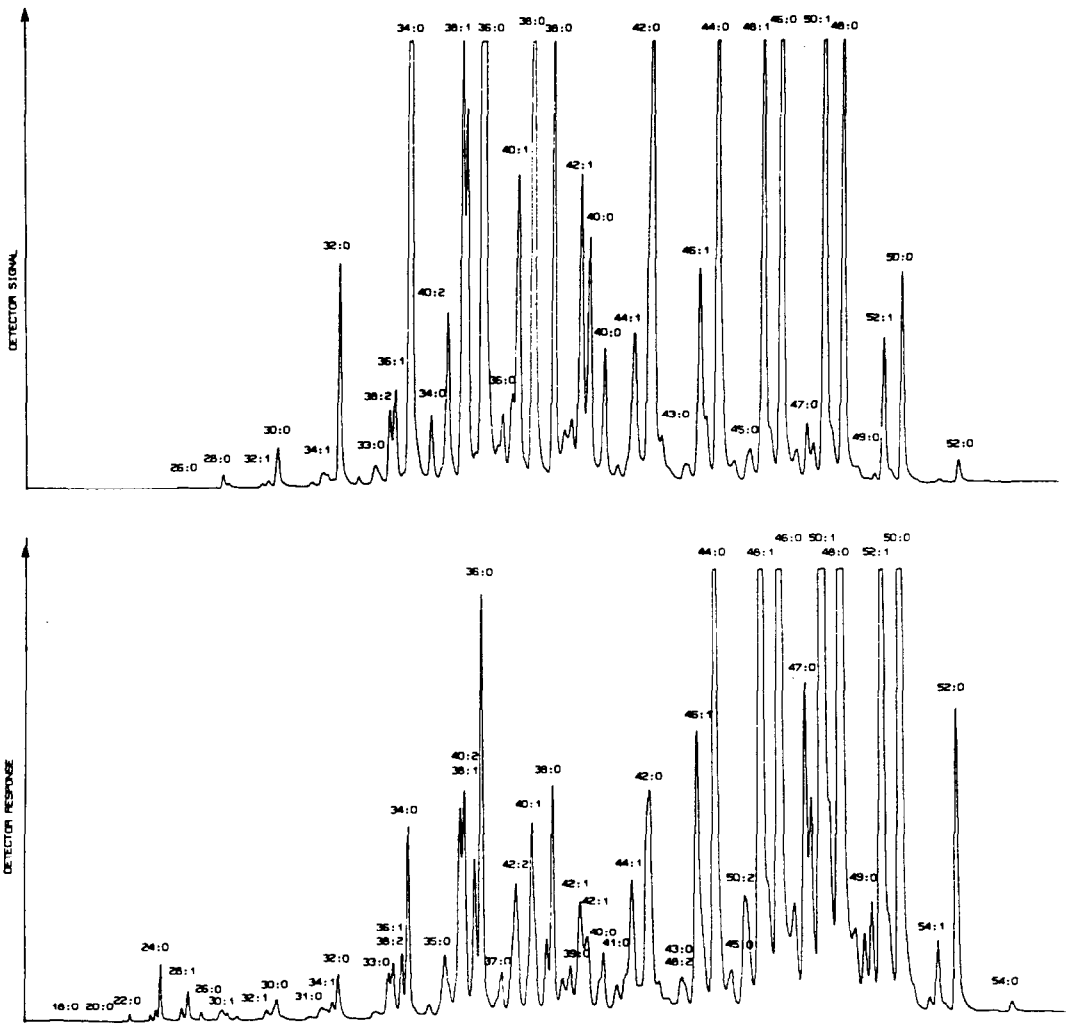

Fig. 1. Reversed-phase HPLC profiles of natural (top) and rearranged (bottom) butterfat triacylglycerols as obtained with light-scattering detector. HPLC conditions: Hewlett-Packard Model 1050 liquid chromatograph equipped with a Supelcosil LC-18 column (25 cm × 0.46 cm I.D.) coupled to a Varex ELSD II light-scattering detector. Solvent: linear gradient of 10–90% isopropanol in acetonitrile at 25°C over a period of 90 min (1 ml/min); recording stopped at 70 min. Peak identification by carbon and double bond number of triacylglycerols.

partition numbers 26–40. This was due to the presence of diacylglycerols containing such combinations of saturated fatty acids as 18:0–4:0, 16:0–6:0, 14:0–8:0 and 12:0–10:0, which are well resolved when combined with 4:0 to 18:0 saturated fatty acids and the corresponding unsaturated fatty acids in the original triacylglycerols. Likewise, there is resolution among isologous triacylglycerols containing two short-chain fatty acids. Thus, DG8:0, mainly 4:0–4:0, when combined with 18:0, is retained longer than DG10:0, mainly 6:0–4:0, when combined with 16:0, which is retained slightly longer than DG12:0, mainly 6:0–6:0 and 8:0–4:0, when combined with 14:0. The shouldering is due to the earlier elution of 14:0–6:0–6:0 when com-

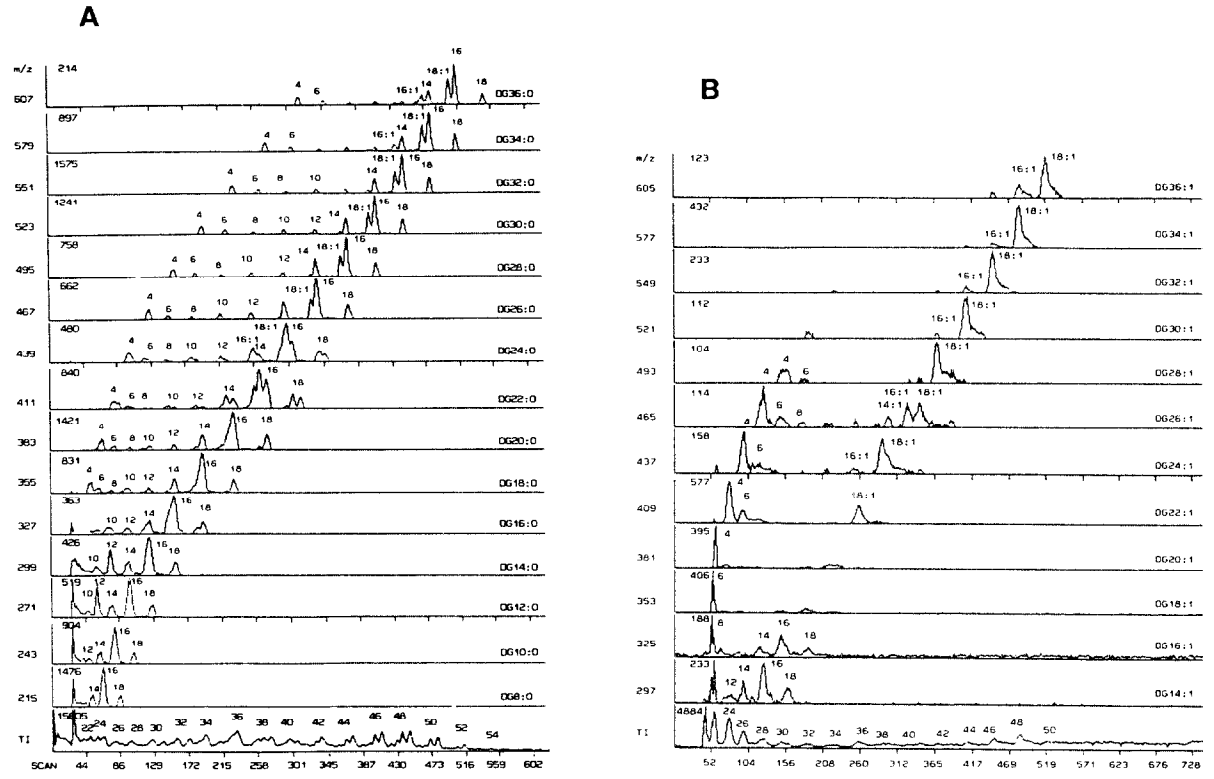

Fig. 2. (A) Mass chromatograms of the saturated diacylglycerol fragment ions, $[MH - RCOOH]^+$, as obtained for rearranged butterfat triacylglycerols by LC–MS. DG36:0–DG8:0 = Diacylglycerols corresponding to the fragment ions (m/z 607-215); FA4–FA18 = fatty acids lost from triacylglycerols during formation of diacylglycerol fragment ions; TI = total positive chemical ionization current. Numbers in upper left hand corner of individual ion plots indicate the ion counts for the most intense peak. LC–MS conditions: Hewlett-Packard Model 1048B liquid chromatograph equipped with a Supelcosil LC-18 column (25 cm × 0.46 cm I.D., Supelco). Solvent: linear gradient of 10–90% propionitrile in acetonitrile (2.2 ml/min) at 30°C over a period of 60 min. Sample: 25 μl of a 0.01% solution in tetrahydrofuran. (B) Mass chromatograms of the monounsaturated and diunsaturated diacylglycerol fragment ions, $[MH - RCOOH]^+$, as obtained for rearranged butterfat triacylglycerols by LC–MS using a linear gradient of 0–90% propionitrile in acetonitrile. Other details and peak identification as in (A).

pared to that of the isologous 14:0–8:0–4:0. Similar orders of elution can be recognized among other short-chain triacylglycerols.

The split in the partition number 50 is due to the presence of two major triacylglycerols, 18:0–18:1–16:0 and 18:0–16:0–16:0, with the 18:1-containing species being eluted ahead of the 16:0-containing species. In addition, the front part of this triacylglycerol peak contained also 18:0–18:0–16:1 and the rear part 18:0–14:0–18:0 as major species. The composition of the diacylglycerol moieties derived from the rear peak of partition number 48 revealed the presence of 18:0–16:0, 14:0–16:0 and 14:0–18:0 diacylglycerol species in nearly equal proportions, corresponding to 14:0–16:0–18:0. The front of partition peak 48 yielded diacylglycerol ions for 16:1–18:1, 18:1–18:1, 16:0–18:1, 18:0–18:1 and 18:0–16:1, corresponding to triacylglycerols 16:1–18:0–18:1 and 16:0–18:1–18:1. The triacylglycerols eluted between the even partition number peaks were due to odd-carbon number species.

Fig. 2B shows the single-ion plots for the fragment ions corresponding to the even-carbon-number monounsaturated diacylglycerols. Again the diacylglycerol moieties can be readily matched to the triacylglycerol parent molecules from which the various saturated fatty acids have been

lost in proportion to their content in the total randomized oil. The plots confirm further the effective resolution of the critical pairs, and of the isologous triacylglycerols containing one or two short-chain fatty acids. Due to low 18:2 content, it was not possible to obtain reliable plots for the 22:2 due to 18:2–4:0, and 24:2 due to 18:2–6:0. However, the combinations of 18:1–18:1 and 18:1–16:1 with 4:0 and 6:0 were clearly recognized and could be traced to the front limb of the corresponding triacylglycerol triplet within the appropriate partition number of the parent triacylglycerols. The above resolution of the critical pairs and isologous triplets in the even series was faithfully reproduced in the odd-carbon-number series. Furthermore, separate series were apparent for the normal- and branched-chain odd-carbon-number species, the *iso* and *anteiso* isomers not being resolved (results not shown).

3.3. Peak identification in subfractions

Fig. 3 shows the LC–MS profiles of the five major triacylglycerol bands resulting from randomization of butteroil. TLC band 1 contained the long-chain saturates, TLC band 2 the short-chain saturates and long-chain *trans*-monoenes, TLC band 3 the short-chain *trans*-monoenes and long-chain *cis*-monoenes, TLC band 4 the short-chain *cis*-monoenes and the long-chain *trans*-dienes, while TLC band 5 contained long-chain *cis*-dienes, short-chain *cis*-dienes and both *cis*- and *trans*-trienes of both long and short chain length. Such an overlap of natural butterfat triacylglycerols had been recognized previously during $AgNO_3$-TLC [3] as had been the separation of *cis*- and *trans*-monounsaturated triacylglycerols.

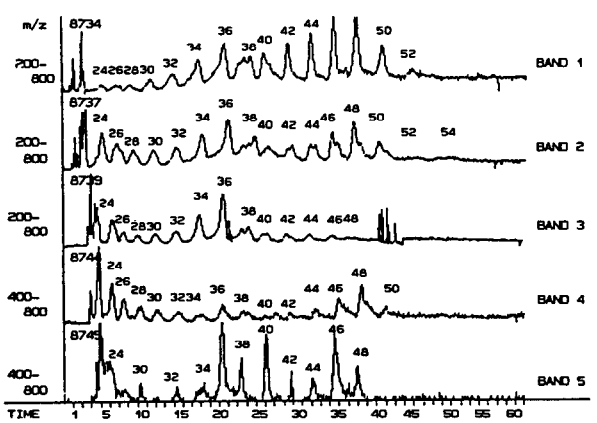

Fig. 3. Total ion current profiles of selected mass ranges as obtained by reversed-phase LC–MS for rearranged butterfat triacylglycerols recovered in $AgNO_3$-TLC bands 1–5. Peaks are identified by triacylglycerol partition numbers. LC–MS conditions as in Fig. 2A. Time in min.

The single-ion plots for the diacylglycerol moieties of the saturated long-chain triacylglycerols in TLC band 1 (results not shown) indicated the presence of diacylglycerols ranging from DG10:0 to DG36:0. The single-ion plots obtained for the saturated diacylglycerol moieties of the short-chain saturated and the long-chain *trans*-monounsaturated triacylglycerols recovered from TLC band 2 (results not shown) indicated that the short-chain triacylglycerols were largely butyrates, caproates and caprylates. TLC band 2 contained a high proportion of the saturated medium-chain triacylglycerols that possessed the same composition of molecular species in both TLC band 1 and TLC band 2. The small amounts of long-chain monoenoic triacylglycerols found in TLC band 2 were characterized largely by the presence of 16:1 and *trans*-18:1t and to a lesser extent 14:1. Characteristically, these triacylglycerols were eluted from the HPLC column between the fully saturated and the *cis*-monounsaturated triacylglycerols of the same partition number. The *trans*-monoenoic and the *cis*-monoenoic triacylglycerols were resolved into long- and short-chain species, which facilitated their identification and determination of elution order. These conclusions were confirmed by the single-ion plots for the monounsaturated diacylglycerol moieties derived from the *trans*-monounsaturated long-chain triacylglycerols in relation to the parent molecules in the total ion current profile of TLC band 2 (results not shown). Interestingly, the single-ion plots for the monounsaturated diacylglycerols showed a symmetrical repetitive pattern representing the combination of *trans*-18:1- with two saturated fatty acids, which were seen in the

proportions in which they occur in the total mixture (see also Fig. 2B). The combinations of the *trans*-monoenoic acids with the shorter-chain saturated acids were found in TLC band 3.

Fig. 4A shows the single-ion plots for the saturated diacylglycerol moieties derived from the short-chain *trans*-monoenoic and the long-chain *cis*-monoenoic triacylglycerols recovered from TLC band 3. The short-chain triacylglycerols are characterized by the exclusive association of the *trans*-18:1 (elaidic) acid with the short-chain saturated diacylglycerols, which is consistent with a high proportion of butterfat *trans*-monoenes contributed by elaidic acid [17]. In contrast, the *cis*-monounsaturated triacylglycerols were made up of the saturated diacylglycerols in combination with a variety of monounsaturated fatty acids ranging from 10:1 to 18:1. The monounsaturated fatty acids were combined with each diacylglycerol moiety in proportion to the content of the fatty acids and the proportion of the specific diacylglycerols in the randomized butteroil. Fig. 4B shows the single-ion plots for the monounsaturated diacylglycerol moieties of the short-chain *trans*-monoenoic and the long-chain *cis*-monoenoic triacylglycerols from TLC band 3. Perpendicular lines dropped from the various monoenoic-diacylglycerol-like fragment ion peaks to the corresponding triacylglycerol peaks reveals them to be of two types. One being retained somewhat longer (*trans*) than the other (*cis*), when compared within the same partition number, both being eluted ahead of the fully saturated triacylglycerols of same partition number.

Single-ion plots for the saturated diacylglycer-

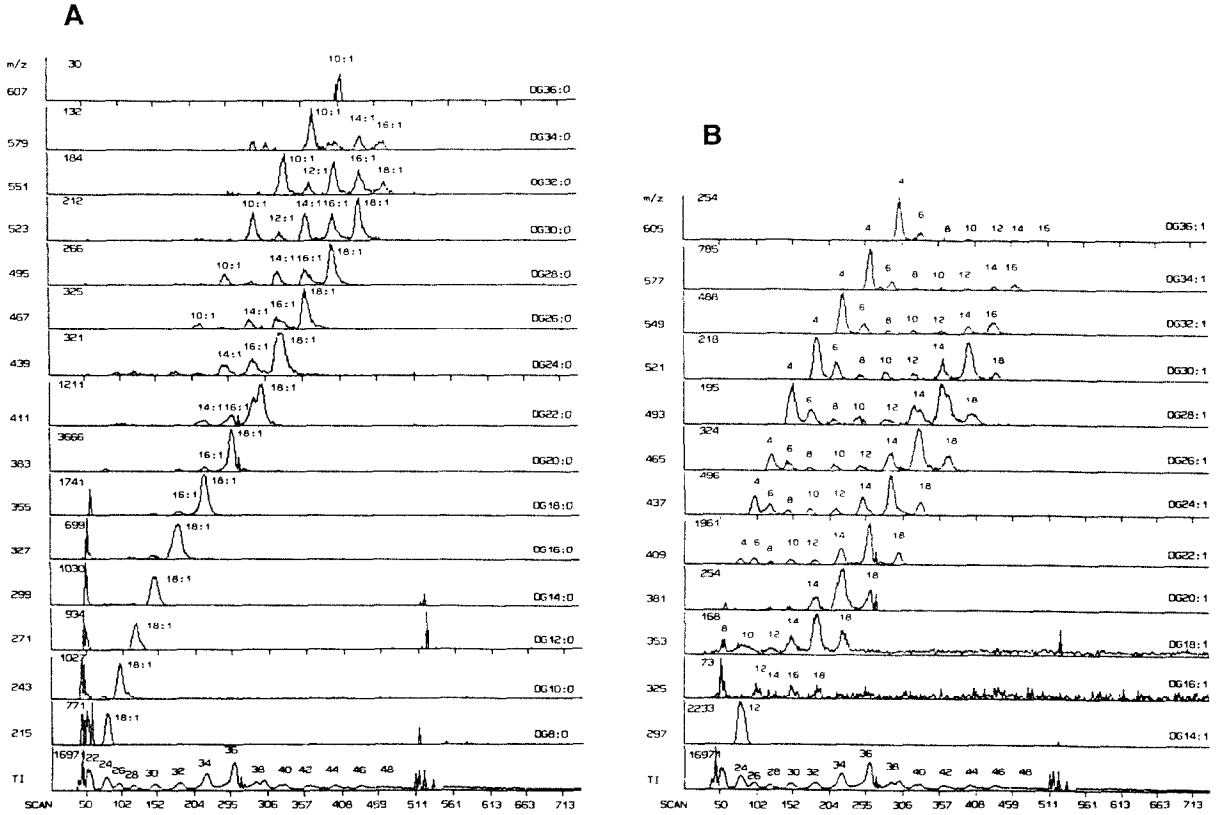

Fig. 4. (A) Mass chromatograms of saturated diacylglycerol fragment ions, $[MH - RCOOH]^+$, as obtained for TLC band 3 by LC–MS. LC–MS conditions and peak identification as in Fig. 2. (B) Mass chromatograms of monounsaturated diacylglycerol fragment ions, $[MH-RCOOH]^+$, as obtained for TLC band 3 by LC–MS. LC–MS conditions and peak identification as in Fig. 2.

ol moieties of the short-chain *cis*-monoenoic and the long-chain *trans*-dienoic triacylglycerols from TLC band 4 (results not shown) indicated that the long-chain *trans*-dienes were minor components. The short-chain *cis*-monoenoic triacylglycerols corresponded to the *trans*-monoenoic triacylglycerols in an apparent near-exclusive combination of the short-chain saturated diacylglycerols with *cis*-18:1 acid. In addition, TLC band 4 contained long-chain dienoic triacylglycerols. Single-ion plots for the dienoic diacylglycerol moieties showed that they are combined with the major long-chain saturated fatty acids. The dienoic diacylglycerols were made up largely of two monounsaturated fatty acids, *e.g.* 18:1–18:1, 16:1–18:1, 16:1–16:1, 14:1–16:1 and 14:1–18:1, presumably mainly *trans* isomers. The single-ion plots for the monoenoic diacylglycerols derived from the short-chain *cis*-monoenoic and the long-chain *trans*-dienoic triacylglycerols in TLC band 4 (results not shown) led to the finding that the 26:1 monoenoic diacylglycerol occurred in two peaks due to a resolution of the species containing the 6:0–18:1 and the 8:0–16:1 diacylglycerol moieties in the triacylglycerols of the same partition number. A similar doubling of several other triacylglycerol peaks was seen because of a resolution of other isologous monoenoic triacylglycerols.

Fig. 5 shows the single-ion plots for the saturated, monounsaturated and some diunsaturated diacylglycerol moieties derived from the long-chain *trans*-dienoic and the short- and long-chain *cis*-dienoic triacylglycerols and traces of trienoic triacylglycerols in TLC band 5. All of these separations are also highly regular based on the principles recognized earlier in the resolution of the saturated and monoenoic triacylglycerols. It should be noted, however, that in several instances the monounsaturated and diunsaturated diacylglycerol peaks coincide fully with the corresponding peak for the parent triacylglycerol, when previous discussion would suggest doubling or tripling of the triacylglycerol peak as well as differences in the elution times for the diacylglycerol moieties. This is not an exception, but a result of the origin of all these diacylglycerol moieties from a single triacylglycerol species.

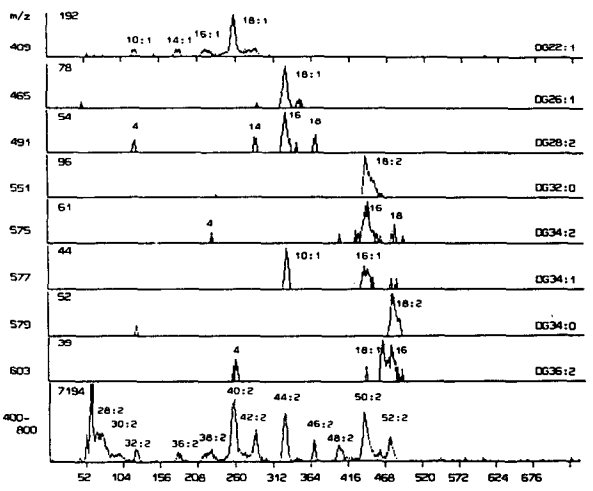

Fig. 5. Mass chromatograms of selected saturated, monounsaturated and diunsaturated diacylglycerol fragment ions, $[MH - RCOOH]^+$, as obtained for TLC band 5 by LC–MS. LC–MS conditions and peak identification as in Fig. 2.

Thus, the 18:1–DG26:1 combination overlapped with the 16:0–DG28:2 combination and the combination of 10:1–DG34:1, all of which corresponded to the triacylglycerol (TG) TG44:2. The simple explanation for this observation was the presence of a single triacylglycerol, 10:1–18:1–16:0, in this chromatographic peak.

No evidence was obtained for the resolution of enantiomers or reverse isomers of unsaturated triacylglycerols of the type 16:0–18:1–18:1 and 18:1–16:0–18:1, which had been observed on $AgNO_3$-TLC [18]. Also, the 15% $AgNO_3$-TLC system employed here did not permit this delicate resolution of the molecular species.

3.4. Quantitation

The quantitative estimates of the various triacylglycerol species are given in Table 3 as obtained by HPLC–light-scattering detection, LC–MS and by calculation. The uncorrected estimates show considerable divergence from the calculated values, which, however, do not indicate non-randomness of fatty acid association. This is due to a variable response of the different molecular species in the two different detector types, none of which shows a true mass or mole composition. A more accurate quantitation

Table 3
Composition of randomized butteroil as estimated by reversed-phase HPLC–light-scattering detection and reversed-phase LC–MS

Molecular species	Determined (%, w/w)		Calculated (%, w/w)[b]
	HPLC–LSD[a]	LC–MS	
10:0 (4–4–2)		0.08	
12:0 (4–4–4)		0.14	0.02
14:0 (4–4–6)		0.32	0.04
16:0 (4–4–8; 4–6–6)		0.12	0.04
18:0 (4–4–10; 4–6–8)		0.30	0.07
20:0 (4–4–12; 4–6–10)		0.36	0.10
22:0 (4–4–14; 4–6–12)	0.16	0.73	0.24
26:1 (4–4–18:1)	0.17	0.62	0.13
24:0 (4–4–16; 4–6–14)	0.50	1.17	0.64
28:1 (4–6–18:1)	0.26	0.75	0.16
26:0 (4–6–16; 4–4–18)	0.42	1.63	0.83
30:1 (4–8–18:1)	0.32	0.61	0.18
28:0 (4–8–16; 4–6–18)	0.20	1.80	0.80
32:1 (4–10–18:1)	0.30	0.40	0.25
30:0 (4–10–16; 4–12–14)	0.50	2.20	1.07
31:0 (4–15–12)	0.31	0.25	0.12
34:1 (4–12–18:1)	0.32	0.75	0.33
32:0 (4–12–16; 4–14–14)	0.64	3.90	1.55
33:0 (4–15–14)	0.59	0.61	0.24
36:1 (4–14–18:1)	0.68	1.39	0.73
38:2 (4–16–18:2)	0.70		0.03
34:0 (4–14–16; 6–12–16)	1.48	5.13	2.80
35:0 (4–15–16)	1.10	0.53	0.58
40:2 (4–18:1–18:1)	1.68	0.19	0.23
38:1 (4–16–18:1; 6–14–18:1)	1.76	1.09	1.82
36:0 (4–16–16; 6–14–16)	2.63	7.84	4.72
37:0 (4–15–18; 4–17–16)	0.60	0.33	0.77
42:2 (6–18:1–18:1)	1.73	0.04	0.16
40:1 (4–18–18:1; 6–16–18:1)	2.05	2.90	1.73
38:0 (4–16–18; 6–16–16)	1.78	6.75	4.92
39:0 (4–17–18)	0.67	0.50	0.62
42:1 (6–18–18:1; 8–16–18:1)	1.33	0.76	1.33
40:0 (6–16–18; 10–14–16)	1.07	6.29	4.01
41:0 (6–17–18)	0.90	0.87	0.59
44:1 (10–16–18:1)	1.76	1.54	1.51
42:0 (10–16–16; 12–14–16)	2.68	3.25	4.04
43:0 (16–12–15)	0.68	0.41	0.78
48:2 (12–18:1–18:1)	0.66	0.21	0.23
46:1 (12–16–18:1	2.79	2.03	1.95
44:0 (12–16–16; 10–16–18)	4.56	3.74	5.15
45:0 (16–14–15)	0.66	0.38	1.27
50:2 (14–18:1–18:1)	1.51	0.88	0.49
48:1 (14–16–18:1)	5.90	3.85	3.43
46:0 (14–16–16; 12–16–18)	7.90	5.03	7.47
47:0 (16–16–15)	2.47	0.98	2.02
52:2 (16–18:1–18:1)	n.d.	1.57	1.17
50:1 (16–16–18:1)	9.04	5.42	5.26
48:0 (14–16–18; 16–16–16)	10.14	5.66	9.32

Table 3. (*Continued*)

Molecular species	Determined (%, w/w)		Calculated (%, w/w)[b]
	HPLC–LSD[a]	LC–MS	
49:0 (16–16–17)	0.82	1.02	1.95
54:2 (18–18:1–18:1)	n.d.	1.07	0.51
52:1 (16–18–18:1)	5.26	3.07	3.48
50:0 (16–16–18)	7.03	3.54	7.05
54:1 (18–18–18:1)	0.88	0.64	0.86
52:0 (16–18–18)	2.25	1.28	2.83
54:0 (18–18–18)	0.27	0.29	0.54
55:0 (18–17–20)			0.01
56:0 (18–18–20)		Trace	0.01
Total	92.05	97.21	93.18

[a] Reversed-phase HPLC with light-scattering detection. The relative standard deviations for repeat analyses were >2% for components making up more than 5% of total and <10% for components making up less than 5% of total.
[b] 1,2,3-Random calculation followed by appropriate summation of corresponding molecular species.

would require much more extensive calibration of the detector response which was not possible in the absence of pure reference compounds.

4. Discussion

The purpose of this investigation was to determine the relative order of elution of the molecular species of rearranged butterfat triacylglycerols for which the exact quantitative composition can be calculated by statistical considerations. The initial part of the study was concerned with the verification that a random distribution of the fatty acids had indeed been obtained, which was established by detailed GC and HPLC resolution and MS identification of the species.

Previous work had shown that the short- and long-chain triacylglycerols of butterfat undergo extensive resolution within the isologous series and within a given calculated partition number due to a longer retention of the molecular species containing short-chain fatty acids [7,12]. In order to simplify the LC–MS resolution and identification, we used $AgNO_3$-TLC for a preliminary segregation and isolation of triacylglycerol molecules differing in the number and geometric configuration of double bonds [3,5]. $AgNO_3$-TLC also permitted a separation of the short- and long-chain triacylglycerols within each degree of unsaturation and type of geometric configuration of the double bond.

The present study confirms the separation of isologous short-chain triacylglycerols based on the presence of butyric, caproic or caprylic and longer-chain fatty acids. The order of peak elution within an isologous series of saturated triacylglycerols can be summarized as follows: XX10 < XX8 < XX6 < XX4, where X stands for any other saturated fatty acid. A similar order of resolution was observed for the monoenoic and dienoic species within isologous series. A similar order of elution was obtained for isologous triacylglycerols containing two short-chain fatty acids: X88 < X86 < X66 < X84 < X64 < X44. The acetates were eluted last in each isologous series of triacylglycerols. Since this resolution is similar to that achieved on adsorption chromatography, it is possible that adsorptive sites remaining on the support of the conventional reversed-phase columns are responsible for the longer retention of the short-chain species.

Reverse isomers of triacylglycerols may be defined as isologous series of triacylglycerols of opposite fatty acid distribution in the primary and secondary positions of the glycerol molecule. Normally reverse isomers of triacylglycerols are not resolved by chromatographic methods, ex-

cept for certain unsaturated reverse isomers of triacylglycerols by $AgNO_3$-TLC [18]. Despite much attention to detail, no evidence was obtained for a resolution of the reverse isomers of short-chain triacylglycerols under the present experimental conditions. Specifically, the anticipated double peaks were not observed for such triacylglycerol pairs as 1,3-dibutyryl-2-palmitoylglycerol and its reverse isomer, 1,2-dibutyryl-3-palmitoylglycerol, and 1,3-dibutyryl-2-oleoylglycerol and its reverse isomer, 1,2-dibutyryl-3-oleoylglycerol.

There was also no evidence for an effect of the placement of the *cis*- and *trans*-monoenoic fatty acids in the primary and secondary positions of the glycerol molecules, although the *cis*- and *trans*-monoenoic species were partially resolved. Butterfat contains up to 20% of the 18:1 as elaidic (*trans*-9) and over 80% as oleic (*cis*-9) acids, while the other monoenoic fatty acids are believed to contain a somewhat smaller proportion of the *trans*-isomer, except 10:1, which contains none because of the terminal double bond [17]. Specifically, separation was obtained for such medium-chain triacylglycerols as 16:0–18:1t–4:0 (t = *trans*) and 16:0–18:1c–4:0, (c = *cis*) as well as the long-chain 16:0–18:1t–16:0 and 16:0–18:1c–16:0 isomers. Since conventional chromatographic liquid phases do not resolve enantiomers, there was no need to distinguish between the fatty acid compositions of the *sn*-1 and *sn*-3 positions.

The utilization of the rearranged butteroil for the chromatographic separations allowed the identification and characterization of the elution times of several unusual triacylglycerol species. Thus, the lowest-molecular-mass triacylglycerol that we identified in the rearranged oil was 4:0–4:0–2:0, followed by 4:0–4:0–4:0, 4:0–4:0–6:0, 4:0–4:0–8:0, etc. These triacylglycerols were found to conform to the order of elution of mixed saturated fatty acid isologous triacylglycerols of their higher-molecular-mass homologues. Acetic acid had been previously found to be present in butterfat triacylglycerols, where it had been reported to occur in combination with long-chain fatty acids [7,19].

This investigation establishes the empirical chromatographic elution order of all major mixed acid triacylglycerols found in butterfats. In parallel studies Myher *et al.* [20] have derived retention factors for each of the fatty acids, which can be used to determine the order of elution of one triacylglycerol species relative to any other. A comparison of the observed and predicted order of elution for a variety of molecular species showed good agreement.

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