Fractionation of the Triacylglycerols of Lipase-Modified Butter Oil

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Triacylglycerols (TGs) of lipase-modified butter oil were separated into saturated, monoene, diene and triene fractions on a *p*-propylbenzene sulfonic acid solid-phase extraction column loaded with silver ions. Fatty acid analysis of the fractions showed that the amounts of saturated TGs (98.4 mol%) and monoene TGs (26.0 mol%) in the saturated and monoene fractions, respectively, were close to the theoretical amounts of TGs in pure fractions. The column method provides a useful alternative to $AgNO_3$-thin-layer chromatography as a means of separating the TGs of butterfat and producing relatively pure TG fractions for further analysis by gas chromatography (GC) or GC–mass spectrometry.

KEY WORDS: Argentation chromatography, benzenesulfonate columns, interesterified butter oil, triacylglycerols.

The resolving power of polarizable phenylmethylsilicone capillary columns in gas–liquid chromatography (GLC) in analysis of the triacylglycerol (TG) composition of butterfat (1) and modified butterfats (2,3) has been demonstrated. However, a complete resolution of even major TG species is difficult to achieve without prefractionation of TG samples (2). Argentation chromatography has been used since the early 1960s by a number of lipid researchers to fractionate various classes of lipids according to the degree of unsaturation. The technique has been comprehensively reviewed by Morris (4). Some of the first papers on argentation chromatography of lipids described the separation of TGs by silver ion column chromatography (5) and by silver nitrate–thin-layer chromatography ($AgNO_3$-TLC) (6). TLC is generally regarded as superior to column chromatography because of the higher resolving power for separating highly unsaturated TGs (4,7).

There are, however, certain problems in using $AgNO_3$-TLC to fractionate the TGs of milk fats according to the degree of unsaturation. Because of the wide molecular weight range of the TGs, their prior separation on silicic acid TLC plates is essential for effective fractionation on $AgNO_3$-TLC plates (8). Separation of milk fats into high- and low-molecular weight fractions (9) or high-, medium- and low-molecular weight fractions (8,10) is the most commonly used technique. Although prefractionation is a rather tedious and lengthy analytical procedure, without it, argentation TLC of TGs of milk fats results in highly impure fractions (8).

Christie (11) has recently demonstrated that stable ion-exchange columns loaded with silver ions in high-performance liquid chromatography (HPLC) separate even highly unsaturated natural TGs with good resolution. Adaptation of stable ion-exchange materials in silver-ion form to low-pressure column chromatography has allowed clean fractionation of relatively saturated natural TGs (12) and fatty acid methyl esters (FAMES) (13,14).

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The objective of the present study was to develop a simple method for the fractionation of TGs of lipase-modified butter oil (LMBO) according to degree of unsaturation, to be used before the analysis of TG composition by GLC and GLC–mass spectrometry (MS). The method—fractionation on a *p*-propylbenzene sulfonic acid solid-phase extraction column (BondElut™; Analytichem International, Harbor City, CA) loaded with silver ions—provided a useful, rapid alternative to the $AgNO_3$-TLC method.

EXPERIMENTAL PROCEDURES

Materials. Dried and bleached butter oil was interesterified with *Pseudomonas fluorescence* lipase as catalyst according to the procedure described by Kalo *et al.* (15).

Argentation chromatography. TGs of the lipase-modified butter oil were fractionated into saturated, monoene, diene and triene fractions on *p*-propylbenzene sulfonic acid solid-phase extraction columns (BondElut™ SCX; Analytichem International). The columns were impregnated with silver ions and washed with solvents as described by Christie (13) before sample application; 200 μg of LMBO in 200 μL dichloromethane (DCM) was applied to the columns, and fractionations were carried out according to the stepwise elution scheme shown in Table 1. All solvents were HPLC- or pro-analysis-grade and all except *n*-pentane (Merck, Darmstadt, Germany) were purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland, United Kingdom).

A plastic syringe (10 mL) was fitted to the top of a BondElut™ column for applying eluents to the column. The eluents were allowed to flow through the column freely without extra pressure. Elution rate was about 0.5 mL/min. The TG fractions were collected in 10-mL glass bottles. Four successive fractionations were carried out with each of the two columns used for the fractionation. A total amount of 1600 (8 × 200) μg LMBO was fractionated. The columns were flushed with 10 mL of DCM between fractionations.

The respective fractions were pooled, solvents were evaporated under a nitrogen stream and the fractions were dissolved in a small amount of DCM. Each of the four fractions was then purified by the fractionation procedure described above. The purified fractions were dissolved in a known volume of standard solution of trinonanoylglycerol (TG27:0, 80 ng/μL) in *iso*-octane for further gas chromatographic analysis.

TABLE 1

Stepwise Elution Scheme for the Fractionation of Triacylglycerols of Lipase-Modified Butter Oil with *p*-Propylbenzene Sulfonic Acid Solid-Phase Extraction Column (BondElut™ SCX)

Solvent mixture in elution order	Volume ratio	Volume mL	Fraction
Pentane/dichloromethane	50:50	6	Saturated
Acetone/dichloromethane	1:99	5	Monoenes
Acetone/dichloromethane	5:95	7.5	Dienes
Acetone	100	5	Trienes

GLC. Small portions of the TG fractions were converted to FAMEs (16). The fatty acid composition of TG samples was determined with a MicroMat Model HRGC 412 gas chromatograph (HNU^R-Nordion Ltd., Helsinki, Finland), equipped with a flame-ionization detector (FID, 225°C) and a split injector (split 1:20, 250°C). The FAMEs were separated on an NB-351 (HNU^R-Nordion) capillary column (25 m × 0.32 mm i.d., 0.50 μm) with helium as carrier gas (inlet pressure 0.7 bar) and the following temperature program: After 1 min at 60°C, the temperature was increased at 10°C/min to 250°C (hold 7 min).

Cis-trans isomers of FAMEs were separated with a Carlo Erba 5300 gas chromatograph (Milano, Italy), equipped with split injector (split 1:25, 225°C), FID (220°C) and SP-2560 (Supelco, Inc., Bellefonte, PA) capillary column (100 m × 0.25 mm i.d., 0.2 μm). Hydrogen was used as carrier gas (linear velocity 21 cm/s).

The TG composition was analyzed with a Carlo Erba 5300 gas chromatograph in a 25 m × 0.25 mm i.d. immobilized phenyl (65%)methyl-silicone capillary column (Quadrex, New Haven, CT) with 0.1 μm film thickness, an FID for detection and a constant-pressure/constant-flow cp-cf 516 control module. A high-oven temperature cold on-column injection was made at 200°C with constant carrier gas (hydrogen) pressure. Carbon dioxide for secondary cooling was applied with a pressure of 5 bar for 100 s. An aluminum-foil sleeve (*ca.* 10 cm long and 0.5 cm i.d.) was fixed on the secondary cooling tube of the on-column injector. Immediately after injection, the cp-cf module was changed to constant-flow mode (linear velocity 62 cm/s). Temperature program was: 1 min at 200°C, 15°C/min to 320°C (hold 1 min), 7°C/min to 360°C (hold 14 min). For calculation of retention indices, symmetric trioctanoylglycerol (TG24:0), tridecanoylglycerol (TG30:0) and tridodecanoylglycerol (TG36:0) were added to the saturated fraction and to LMBO. All these, plus symmetric tritetradecanoylglycerol (TG42:0), trihexadecanoylglycerol (TG48:0) and trioctadecanoylglycerol (T54:0), were added to the unsaturated fractions. The TG standards were purchased from Nu-Chek-Prep (Elysian, MN).

RESULTS

Christie's successful experiment (13) on fractionation of FAME on benzenesulfonate extraction columns loaded with silver ions encouraged us to adapt the technique for the separation of TGs. The step-wise elution scheme shown in Table 1 was developed from the elution scheme described by Christie (13). Initially, we tried to separate the LMBO into five fractions, with eluents consisting of DCM and acetone in the following proportions: 100:0, 97:3, 94:6, 90:10 and 0:100. In further studies, the number of fractions was reduced to four. Because of an unsatisfactory resolution of the TG fractions, a change was sought in the initial scheme that would lower the polarity of the eluent mixtures. Eluent mixtures of DCM and *n*-pentane, ranging from 100% DCM to 100% *n*-pentane and combined in different elution volumes, were tested in the hope of obtaining a pure saturated fraction. The purest saturated fraction was achieved with equal volumes of DCM and *n*-pentane. The proportion of acetone in DCM ranged from 1 to 3% in the eluent mixture for the monoene fraction and from 3 to 6% in the eluent mixture for the diene fraction.

TABLE 2

Empirical Correction Factors for Calculation of Wt% of Fatty Acid[a]

Fatty acid	ECF[b]	SD[c]	CV (%)[d]
4:0	1.64	0.037	2.27
6:0	1.26	0.025	2.02
8:0	1.12	0.022	1.93
10:0	1.05	0.020	1.88
12:0	1.02	0.019	1.82
14:0	1.02	0.017	1.62
16:0	0.99	0.010	0.98
18:0	1.00	0.000	0.00
18:1	0.99	0.002	0.24
18:2	1.05	0.003	0.28
18:3	1.17	0.003	0.25
20:0	1.02	0.011	1.08
22:0	1.05	0.012	1.10

[a]Values are given in relation to methyl stearate (18:0) and are means of six separate determinations.
[b]Empirical correction factor.
[c]SD of ECF.
[d]Coefficient of variation.

The purity of the fractions was monitored by GC analysis of TG and fatty acid (FA) compositions of the fractions. Correction factors for quantitation of FAs were determined by repeating the analysis of a FAME standard (GLC-74; Nu-Chek-Prep, Inc.) six times. Results of the analysis are summarized in Table 2.

The empirical correction factors (ECFs) agreed with the theoretical response factors (7), although the ECFs of methyl butyrate and polyunsaturated methyl esters were slightly higher than the theoretical values.

For determination of correction factors for the quantitation of TG composition, a calibration mixture that contained symmetric TG 24:0, 30:0, 36:0, 42:0, 48:0, 54:0 and 54:3 was analyzed. The column showed excellent thermal stability, allowing a temperature program up to 360°C without significant bleeding of the stationary phase. The column stability was also reflected in the only moderate increase of the correction factors with increasing molecular weight (Table 3).

The fatty acid compositions of the separated fractions and LMBO expressed in mol%, are presented in Table 4. Saturated and branched or odd-carbon number FAs were tentatively identified in the group "Other." The relative proportions of each fraction were calculated from the FA composition by the internal standard method. The relative

TABLE 3

Empirical Correction Factors for Calculation of Mol% of Triacylglycerols[a]

Triacylglycerol	ECF[b]	SD[c]	CV (%)[d]
24:0	1.294	0.021	2.2
30:0	1.104	0.018	1.8
36:0	1.000	0.000	0.0
42:0	0.939	0.015	1.4
48:0	1.021	0.034	2.6
54:0	1.569	0.129	5.9
54:3	1.721	0.342	14.3

[a]Values are given in relation to tridodecanoylglycerol (TG36:0) and are means of six separate determinations. Footnotes as in Table 2.

TABLE 4

The Fatty Acid (FA) Composition (means ± SD of three fractionations) of the Saturated (S), Monoene (M), Diene (D) and Triene (T) Fractions and the Original Lipase-Modified Butter Oil (LMBO)

	FA composition (mol%)					
Fatty acid	S	M	D	T	LMBO	Calculated[a]
4:0	15.1 ± 0.58	15.0 ± 1.05	5.1 ± 0.54	5.1 ± 0.06	12.8 ± 0.34	13.2
6:0	6.1 ± 0.22	5.6 ± 0.27	2.3 ± 0.25	1.9 ± 0.03	4.8 ± 0.08	5.2
8:0	2.7 ± 0.08	2.3 ± 0.07	1.1 ± 0.06	0.8 ± 0.02	2.1 ± 0.04	2.3
10:0	4.9 ± 0.19	3.6 ± 0.05	1.9 ± 0.09	1.3 ± 0.02	3.6 ± 0.07	3.8
12:0	4.6 ± 0.12	3.3 ± 0.04	2.0 ± 0.06	1.3 ± 0.07	3.4 ± 0.06	3.6
14:0	14.6 ± 0.35	10.0 ± 0.12	6.7 ± 0.14	4.5 ± 0.22	11.0 ± 0.14	11.4
14:1		1.2 ± 0.08	2.0 ± 0.12	1.8 ± 0.05	0.9 ± 0.22	0.8
15:0	1.2 ± 0.04	0.9 ± 0.03	0.7 ± 0.04	0.5 ± 0.10	0.9 ± 0.22	1.0
16:0	32.2 ± 0.33	21.4 ± 0.81	15.4 ± 0.44	11.6 ± 1.79	24.5 ± 0.15	25.0
16:1	0.1 ± 0.03	1.9 ± 0.10	4.3 ± 0.39	4.7 ± 0.35	1.3 ± 0.34	1.6
17:0	0.5 ± 0.02	0.4 ± 0.02	0.3 ± 0.01	0.2 ± 0.02	0.4 ± 0.10	0.4
17:1		0.4 ± 0.01	0.8 ± 0.06	0.8 ± 0.10	0.3 ± 0.07	0.3
18:0	13.0 ± 0.53	8.2 ± 0.51	5.8 ± 0.24	3.6 ± 0.40	10.2 ± 0.02	9.8
18:1	1.2 ± 0.25	22.5 ± 0.75	43.8 ± 1.19	38.8 ± 2.26	18.8 ± 0.08	16.2
18:2	0.1 ± 0.02	0.2 ± 0.09	2.7 ± 0.23	11.5 ± 0.18	1.0 ± 0.04	1.2
18:3	0.2 ± 0.10			4.8 ± 0.66	0.5 ± 0.01	0.4
20:0	0.2 ± 0.01	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.05	0.2 ± 0.00	0.2
Other	3.3 ± 0.82	3.0 ± 0.01	4.9 ± 0.49	6.7 ± 0.89	3.3 ± 0.06	3.6
Saturated	95.1	70.8	41.4	31.0	73.9	
Monoene	1.3	26.0	50.9	46.1	21.3	
Diene	0.1	0.2	2.7	11.5	1.0	
Triene	0.2			4.8	0.5	
Other	3.3	3.0	4.9	6.7	3.3	

[a]The FA composition was calculated on the basis of FA compositions and relative proportions of each fractions.

proportions of saturated, monoene, diene and triene fractions (means ± SD of three fractionations) were 45.8 ± 3.52, 36.2 ± 3.34, 11.0 ± 0.33 and 7.0 ± 0.04%, respectively. The FA composition of LMBO, which was calculated from the FA composition of each fraction, was close to that of the analyzed FA composition (Table 4), indicating that recovery of TGs from the columns was adequate.

Detection of unsaturated FAs in the saturated fraction of TGs revealed a premature migration of unsaturated TGs within the first eluent mixture. Excess of saturated FAs in the monoene fraction compared to the theoretical value of 67% likewise was clear evidence of overlapping of the first two fractions. On the basis of the FA composition, the degree of overlapping of the saturated fraction with monoene TGs, diene TGs and triene TGs can be estimated to be approximately 3.9, 0.3, and 0.6 mol%, respectively. The monoene fraction contained 73.8 mol% saturated, 26.0 mol% monoene and 0.2 mol% diene FA; the diene fraction contained 46.3 mol% saturated, 50.9 mol% monoene and 2.7 mol% diene FA; and the triene fraction contained 37.7 mol% saturated, 46.1 mol% monoene, 11.5 mol% diene and 4.8 mol% triene FA. These values suggest the presence of more saturated TGs than predicted by theory in all unsaturated fractions.

The tentatively analyzed composition of the *cis-trans* isomers of FA showed that the *cis/trans* ratio for C18:1 was *ca.* 10:1 in both LMBO and the monoene fraction. A relative abundance of *trans* isomers was evident in the saturated fraction, where the *cis/trans* ratio for C18:1 was *ca.* 1:1.

The gas chromatograms of the TG fractions clearly show that each fraction contains, in addition to major peaks, small peaks with the same retention time (index) as a major peak in another argentation fraction (Fig. 1). Influence of fractionation on the TG composition was most evident in the low-resolution area of the TG chromatograms containing TGs with acyl carbon number 44 or higher. The effect of fractionation was less clearly seen in the high-resolution area of chromatograms up to acyl carbon number 42 because of the more substantial number of different isomers with the same acyl carbon number and degree of saturation and because of partial overlapping of the peaks of different degree of saturation. The amounts of the major saturated TGs with acyl carbon numbers of 46, 48 and 50 in the saturated fraction were 7.9, 8.8 and 6.2 mol%, respectively. In the monoene fraction the amounts of the major monoene TGs with acyl carbon numbers of 48, 50 and 52 were 9.7, 13.0 and 7.9 mol%, respectively. The major saturated and monoene TGs of LMBO were the same as those of the fractions. Identification of the peaks was based on the GC and GC-MS analysis as described elsewhere (17).

The overlapping of the fractions was also clearly evident in the TG composition of the fractions. The degree of overlapping was 18 and 21 mol% in the saturated and monoene fractions, respectively, with only the identified peaks considered. These values were slightly higher than those of the FA analysis. Overlapping was seen in both fractions throughout the whole range of molecular species of TGs.

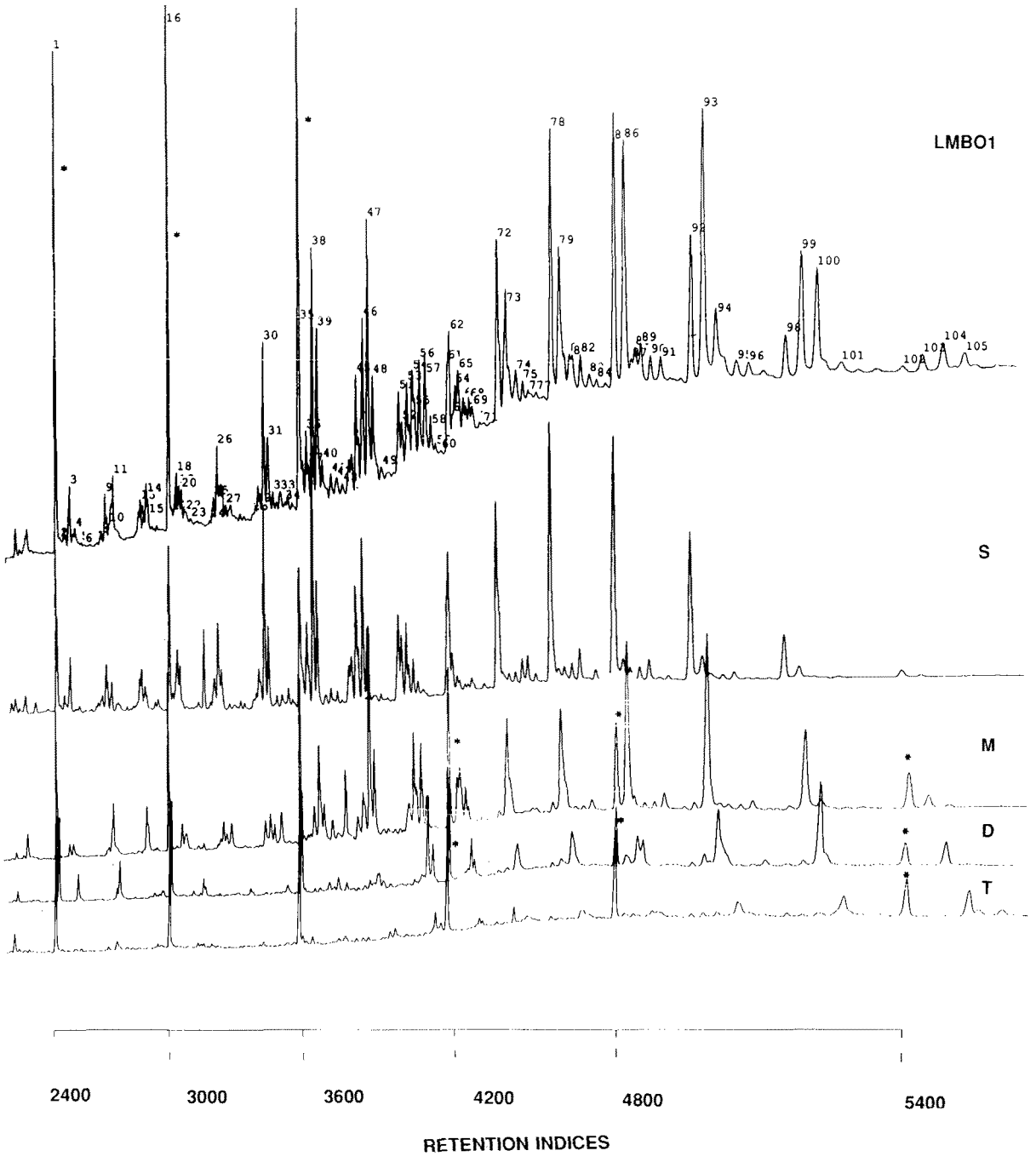

FIG. 1. Gas chromatograms of lipase-modified butter oil (LMBO1), and of the triene (T), diene (D), monoene (M) and saturated (S) fractions.

DISCUSSION

Cis/trans isomerism (11), double bond positions in the carbon chain of FA (18), the symmetry of TG molecules (19) and the degree of unsaturation and acyl carbon number of TG affect the separation of TG by HPLC in the silver-ion mode. The same factors influence the separation of TGs on silver nitrate TLC (2,4). The basis for the resolution of TGs in BondElut™-type solid-phase columns loaded with silver ions is the same as in $AgNO_3$-TLC on silica gel plates, but some additional interactions occur as well. The separation of TGs in solid-phase column loaded with silver ions is mainly due to the complex formation between silver ions and double bonds of unsaturated TGs and, partly to the nonpolar interactions of the *p*-propylbenzene ring. In addition, some of the polar properties of the silica gel matrix affect the resolution of the most polar TGs. Most of the properties are masked by the bonded *p*-propylbenzene sulfonic acid ion-exchange sorbent.

Christie (12) demonstrated the suitability of BondElut™ solid-phase extraction columns for separation of the TGs of relatively saturated fats such as palm oil and cocoa butter. His elution scheme differed considerably

from ours. In our work, an eluent mixture of DCM and *n*-pentane (50:50, vol/vol) proved superior to DCM alone for the separation of saturated TGs. The suitability of DCM for the separation of saturated TGs appears to depend strongly on the composition of the experimental fats. The greater overlap of the saturated and monoene TG fractions in our study could be due to the more complex TG composition of LMBO and, especially, to the larger amount of short-chain FA residues in LMBO TGs than in the TGs of Christie's (12) relatively saturated fats.

Because of the near random distribution of the FAs among the TGs of the LMBO (15), the proportional mole ratio of individual saturated FAs to the total amount of other saturates should theoretically be constant in every fraction. However, the amount of butyrate relative to the total amount of other identified saturated acids was 15.8 and 21.1% in the saturated and monoene fractions, respectively. The respective percentages of other saturated FAs of the two fractions differed at most by 1.5%, except those of palmitate and stearate, which differed by 3.5 and 2.0%, respectively. The relative abundance of butyrate in the monoene fraction could be due to the retarded migration of the saturated TG molecules consisting of polar butyrate component. The retarding influence of the most polar FAs on migration of saturated TGs on $AgNO_3$-TLC plates was demonstrated by Myher *et al.* (2).

Although the TG fractions in the present study were not totally pure, the separation was adequate when compared with the results of studies carried out with milk fats on $AgNO_3$-TLC plates. In Kuksis and Breckenridge's (8) separation of the TGs of the most volatile 2.5% distillate of butteroil by $AgNO_3$-TLC, the monoene fraction contained a total of only 18.8 mol% monounsaturated FAs, compared to the theoretical 33.3 mol% or 26.0 mol% of our study. The overlapping of the fractions was due to polar saturated short-chain TGs in their study (8). Our finding of a proportional excess of butyrate in the monoene TG fraction is in agreement with this.

The overlapping of the monoene fraction with dienes was only moderate (0.3 mol%) in the present study. Parodi (10) reported for milk fat a contamination of *trans* monoene and *cis* monoene TG fractions with diene TGs, ranging from 1.2% in the high-molecular weight fraction to 11.4% in the medium fraction for *trans* monoenes and from 1.8% in the low-molecular weight fraction to 3.6% in the high-molecular weight fraction for *cis* monoenes. The high proportion of *trans* isomers of monoene FAs in the saturated TG fraction of the present study indicated that *cis* isomers were more strongly retarded than *trans* isomers in solid-phase extraction columns. The effect of the configuration of double bonds on the migration of TG molecules is, however, more clearly demonstrated on $AgNO_3$-TLC plates (2,10).

BondElut™ solid-phase extraction columns loaded with silver ions provide a useful alternative to $AgNO_3$-TLC for the separation of TGs of butterfats according to the degree of unsaturation of TGs. Relatively pure fractions up to triunsaturated TGs are achieved, without the need for prefractionation on silicic-acid TLC plates. Column chromatography of TGs on BondElut™ extraction columns currently lacks the resolving power of $AgNO_3$-TLC, but it offers a rapid and sound technique for the prefractionation of TGs into saturated, monoene, diene and triene fractions suitable for further qualitative analysis by GC and GC-MS.

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