Analysis of *sn*-1(3)- and *sn*-2-Short-Chain Acyl Isomers of Triacylglycerols in Butteroil by Gas–Liquid Chromatography

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ABSTRACT: The aim of the study was to determine major triacylglycerols (TG), and *sn*-1(3) and *sn*-2 isomers of butyryl and caproyl TG in butteroil (BO) and interesterified butteroil (IBO) by gas–liquid chromatography (GLC) and silver ion column chromatography. Altogether, 112 molecular species of TG were synthesized by interesterification and their retention indices were determined. Molar empirical correction factors for TG were determined using linear calibration. Retention indices showed that *sn*-1(3) and *sn*-2 isomers of the TG containing one short-chain acyl (butyrate, caproate) and two long-chain acyls (lauroate, myristate, palmitate, stearate, and oleate) were separated on a phenyl (65%) methylsilicone column. The difference between retention indices of 1(3)- and 2-short-chain acyl isomers ranged from 14 to 19, and from 9 to 16 for butyrates and caproates, respectively. The proportion of *sn*-2 isomers of butyrates averaged 1.4%, but only traces of *sn*-2 isomers of caproates were detected in butteroil. The ratio of *sn*-1(3)- to *sn*-2-butyrates and caproates in interesterified butteroil averaged 2.0:1. The most abundant molecular species of mono-short-chain TG in butteroil were BPP + BMS (5.6 mol%), BPO + BSPo (4.8 mol%), BMP + BLaS (3.4 mol%), BMO + BPPo (2.7 mol%), BPS (2.5 mol%), and CoPP + CoMS (2.3 mol%).
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KEY WORDS: Butteroil, gas–liquid chromatography, interesterification, linear calibration, quantitation, retention index, silver ion column chromatography, *sn*-1(3) and *sn*-2 isomers, triacylglycerols.

Triacylglycerols (TG) containing short-chain acyl groups (butyryl, caproyl), together with phospholipids and proteins of milk fat, influence the whipping and organoleptic properties of dairy products and the wetting properties of dried dairy products (1). The composition of short-chain TG in milk fat has been extensively studied since the 1960s; the first stereospecific analyses were carried out by Pitas *et al.* (2) and Breckenridge and Kuksis (3) at the end of the 1960s, when they showed that butyryl and caproyl groups were located predominantly at the *sn*-3 position. Several analytical methods have been used to determine the asymmetrical distribution of short-chain acyls: pancreatic lipase deacylation (2,3), proton nuclear magnetic resonance (NMR) with (4) and without chiral chemical shift reagents (5), ^{13}C NMR (6), Grignard degradation and chiral-phase high-performance liquid chromatography (HPLC) (7), and mass spectrometry (MS) (8). Kuksis and Breckenridge (9) reported the separation of positional isomers of palmitoyldibutyrylglycerol on an apolar packed column by gas–liquid chromatography (GLC) and, later, Myher *et al.* (10) discussed the separation of the positional isomers of acetate and butyrate TG on a polar capillary column in relation to their equivalent carbon numbers. Recently, two studies have been published in which unspecified short-chain TG isomers (11) and 1(3)-butyrate and 2-butyrate isomers (5) have been shown to be separated on a polarizable phenylmethylsilicone column. In milk fat, 94–100% of butyryl groups and 74–100% of caproyl groups have been reported to be in the *sn*-3 position (2–4,6,12–14). In the present study, our aim was: (i) to investigate to what extent the molecular species of TG can be identified by GLC and by silver ion column chromatography, and (ii) to determine the major TG, and *sn*-1(3)- and *sn*-2-butyryl and caproyl TG in butteroil (BO) and interesterified (randomized) butteroil (IBO).

EXPERIMENTAL PROCEDURES

Materials. BO samples were of commercial origin (Valio Ltd., Helsinki, Finland) and they were kept at −18°C under argon atmosphere before analysis. Monoacid TG standards were purchased in 99% purity from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland). All solvents used in chromatographic analysis were purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland, United Kingdom) and Merck and were of HPLC or pro-analysis grade.

Interesterification. Altogether, 23 equimolar mixtures of three monoacid TG (Table 1) were interesterified as follows: TG mixtures were dried under reduced pressure (25 torr) at 95°C for 100 min and bleached at 95°C for 30 min with 2% bleaching earth prior to interesterification in order to remove the impurities of monoacid TG, which may retard the interesterification. Mixtures were interesterified with 1% sodium

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TABLE 1
Interesterified Equimolar Mixtures of Three Monoacid Triacylglycerols (TG) for the Determination of Retention Indices[a]

Saturated TG mixtures		Unsaturated TG mixtures	
Name	Interesterified mixture of	Name	Interesterified mixture of
BMP	BBB + MMM + PPP	BMO	BBB + MMM + OOO
BLaS	BBB + LaLaLa + SSS	BPO	BBB + PPP + OOO
BMS	BBB + MMM + SSS	BSO	BBB + SSS + OOO
BPS	BBB + PPP + SSS	CoMO	CoCoCo + MMM + OOO
CoMP	CoCoCo + MMM + PPP	CoPO	CoCoCo + PPP + OOO
CoMS	CoCoCo + MMM + SSS	CoSO	CoCoCo + SSS + OOO
CoPS	CoCoCo + PPP + SSS	CyLaO	CyCyCy + LaLaLa + OOO
CyLaM	CyCyCy + LaLaLa + MMM	CyMO	CyCyCy + MMM + OOO
CyLaS	CyCyCy + LaLaLa + SSS	CiLaO	CiCiCi + LaLaLa + OOO
CyMP	CyCyCy + MMM + PPP		
CyMS	CyCyCy + MMM + SSS		
CiLaP	CiCiCi + LaLaLa + PPP		
CiMP	CiCiCi + MMM + PPP		
CiLaS	CiCiCi + LaLaLa + SSS		

[a]Abbreviations for fatty acids: B = 4:0, Co = 6:0, Cy = 8:0, Ci = 10:0, La = 12:0, M = 14:0, P = 16:0, S = 18:0, and O = 18:1.

methoxide as catalyst at 85–90°C for 1 h under argon atmosphere. Most interesterification reactions yielded random TG mixture. Interesterification of 200 mg BO samples were carried out according to the principles described above.

TLC. TG of BO, IBO, and interesterified standard mixtures were isolated by TLC on 20 × 20 cm silica gel plates (Kieselgel 60; Merck) with 0.25 mm layer thickness, which were developed with hexane/diethyl ether/formic acid (80:20:2, vol/vol/vol). After spraying with 0.2% 2,7-dichlorofluorescein in ethanol, both short- and long-chain TG bands were separated off together from the plates, and TG were eluted from silica gel matrix by chloroform/methanol (98:2, vol/vol).

Silver ion chromatography. TG of BO and IBO were fractionated into saturated (S), monoene (M), and polyene (P) fractions by silver ion column chromatography. Fractionation was carried out on solid-phase extraction columns loaded with 2 g of Bulk Isolute™ SCX sorbent (International Sorbent Technology Ltd., Hengoed, United Kingdom). Impregnation of the columns with silver ions and washing with solvents prior to sample application were performed according to the principles described by Christie (15). A total amount of 2.4 (3 × 0.8) mg of BO and IBO was fractionated according to the method described by Kemppinen and Kalo (16), except that the fractionation procedure was carried out only once for each fraction. The stepwise elution scheme is shown in Table 2.

TABLE 2
Stepwise Elution Scheme for the Fractionation of TG of Butteroil (BO) and Interesterified Butteroil (IBO) by Silver Ion Column Chromatography[a]

Solvent mixture in elution order	Volume		TG fraction
	Volume ratio	mL	
Pentane/dichloromethane	25:75	35	Saturated
Acetone/dichloromethane	1:99	45	Monoenes
Acetone	100	40	Polyenes

[a]For abbreviation see Table 1.

GLC. TG were analyzed with a Carlo Erba 5300 gas chromatograph (Milano, Italy) equipped with a flame-ionization detector and constant pressure constant flow cp-cf 516 control module using a 25 m × 0.25 mm i.d. polarizable phenyl(65%)methylsilicone column with 0.1-µm film thickness (Quadrex, New Haven, CT). Cold on-column injection was made with constant hydrogen pressure at high-oven temperature (170°C), using carbon dioxide with a pressure of 5 bar for 100 s for secondary cooling and a 10 cm long aluminum-foil sleeve fixed to the cooling tube to intensify the cooling (16). Immediately after injection, the cp-cf-module was changed to cf-mode and linear velocity of carrier gas was set at 61 cm/s. The temperature program was: 1 min at 170°C, 10°/min to 310°C (hold 1 min), 0.5°/min to 315°C (hold 1 min), 8°/min to 360°C (hold 12 min). All samples were dissolved in isooctane solution containing trinonanoylglycerol (TG 27:0, 83.6 ng/µL) as internal standard. Because a multistep temperature program was used in the analyses, retention indices were calculated for TG species with the Micman program (Sunicom Ltd., Helsinki, Finland) using the cubic spline curve-fitting method. For determination of the index values, monoacid tritetranoylglycerol (TG 12:0), trihexanoylglycerol (TG 18:0), trioctanoylglycerol (TG 24:0), tridecanoylglycerol (TG 30:0), tridodecanoylglycerol (TG 36:0), tritetradecanoylglycerol (TG 42:0), trihexadecanoylglycerol (TG 48:0), and trioctadecanoylglycerol (TG 54:0) were added to the analytical samples, which did not contain them. Calculation of unknown indices were based on the index values for monoacid TG which were 100 times the number of acyl carbons.

Calculations. Data acquisition and integration of chromatograms in GLC analysis were performed by SC Chromatographic Workstation 1.2B (Sunicom Ltd., Helsinki, Finland). If the peaks were not baseline-separated, they were in-

tegrated by detecting the valleys between the peaks and dropping a perpendicular down to the baseline. However, very small peaks on the tail of larger peaks were integrated by tangential skimming. All statistical calculations were carried out by Microsoft Excel 5.0 (Microsoft® Corp., Redmond, WA) and Microcal Origin 4.0 (Microcal Software Inc., Northampton, MA).

RESULTS

Identification of TG by retention indices. In order to identify major molecular species and *sn*-1(3)- and *sn*-2-short-chain acyl isomers of TG in BO and modified BO, 112 different molecular species of TG were synthesized by interesterification, and the retention index for each synthesized TG was determined by GLC (Table 3).

In general, the increase in difference between the length of acyl chains resulted in higher retention indices for TG with the same number of acyl carbons and the same degree of unsaturation. Retention indices indicated partial overlapping of some monoene TG and the most polar saturated TG, e.g., CoMO and MCoS, BSO and SBS, CoSO and SCoS (for abbreviations, see Table 1). The same trend was also observed with some diene TG and the most polar monoene TG, e.g., BOO and SBO.

Retention index data showed that positional isomers of molecular species of the TG that were composed of two long-chain acyl groups (lauroyl, myristoyl, palmitoyl, stearoyl, and oleoyl) and one short-chain acyl group (butyryl and caproyl) eluted in two separate peaks on a phenyl(65%)methylsilicone column. In general, TG isomers did not separate from each other, as the number of carbon atoms of the shortest acyl was eight or higher. However, some positional isomers of dibutyryl TG and those of capryoyldioleoylglycerol (CyOO) were shown to separate from each other, shown as shouldering in chromatogram.

The difference between retention indices of *sn*-1(3)-butyryl and *sn*-2-butyryl TG ranged from 14 to 18 and from 16 to 19 in saturated and unsaturated TG, respectively. The difference ranged from 9 to 10 and from 9 to 16 between *sn*-1(3)-caproyl and *sn*-2-caproyl acyl isomers of saturated and unsaturated TG, respectively. Hence, the carbon chainlength of two long-chain acyl groups esterified to the glycerol moiety seemed to have less influence on the difference between retention indices of *sn*-1(3) and *sn*-2 positional isomers than that of a short-chain acyl group.

Quantitation of the molecular species of TG. For determination of experimental molar correction factors for the quantitation of TG in BO and IBO, five different concentrations of the calibration mixtures of nine monoacid TG (12:0, 18:0, 24:0, 30:0, 36:0, 42:0, 48:0, 54:0, and 54:3) were analyzed using the linear calibration method. The amounts of TG in 5 mL of the calibration mixtures were 125, 250, 500, 1250, and 2500 µg for TG 12:0, 54:0 and 54:3, and 62.5, 125, 250, 625, and 1250 µg for all other TG, respectively. The amount of internal standard in all calibration mixtures was 167 µg/5 mL. The molar ratio (y) of each TG to internal standard (TG 27:0) was plotted against the area ratio (x) of each TG to TG 27:0 (Fig. 1), and the slope of linear regression curve ($y = ax$) was calculated on the basis of triplicate measurements. The values for the slope of the linear regression models of other TG were calculated by nonlinear curve fitting (Fig. 2). Slope values showed high parabolic ($y = 0.00152x^2 - 0.127x + 3.35$, $r^2 = 0.966$) dependence on the acyl carbon number.

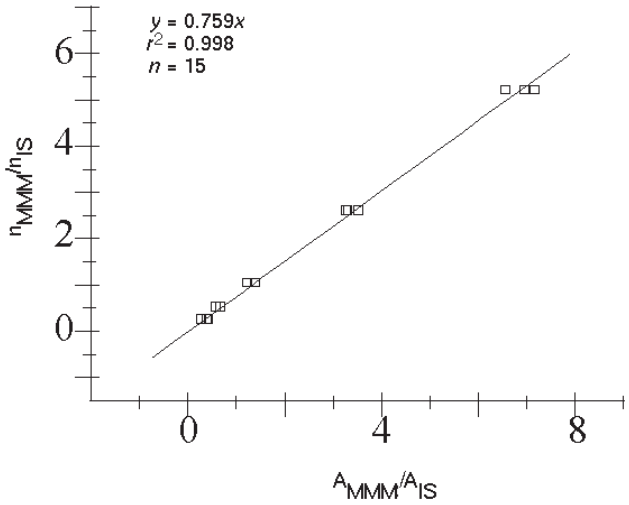

FIG. 1. Determination of empirical correction factor for trimyristoylglycerol (MMM) as an example of linear calibration method. A_{MMM}/A_{IS} = the ratio of the area of MMM to the area of internal standard (trinonanoylglycerol); n_{MMM}/n_{IS} = molar ratio of MMM to internal standard; n = number of determination; r^2 = coefficient of determination.

The coefficient of determination of the linear regression models for all TG showed excellent reproducibility for the

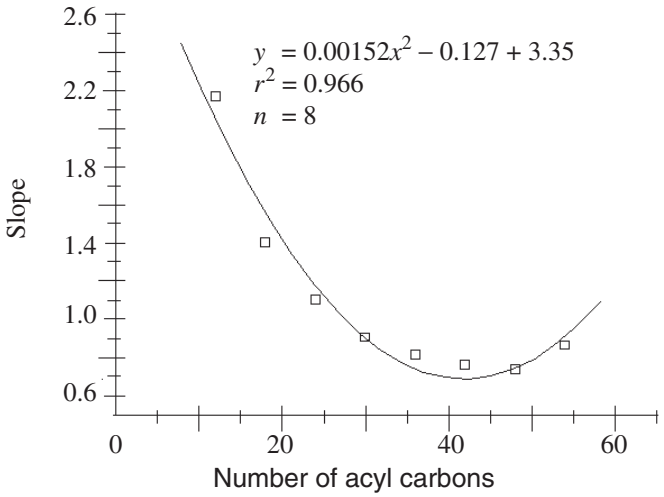

FIG. 2. Dependence of slope values on the number of acyl carbons of saturated triacylglycerols determined by nonlinear curve fitting; n = number of determination; r^2 = coefficient of determination.

TABLE 3
Retention Indices (RI) for TG Determined by Gas–Liquid Chromatography[a]

CN[a]	TG	RI Average	RI Min.	RI Max.	n^b
	Saturated TG				
12	BBB	1200	1200	1200	14
18	CoCoCo	1800	1800	1800	12
20	BBLa	2037	2036	2037	2
22	BBM	2252	2251	2253	6
24	CyCyCy	2400	2400	2400	12
24	BBP	2466	2465	2467	4
26	CoCoM	2633	2627	2638	5
26	BBS	2680	2678	2683	7
28	CyCyLa	2813	2807	2816	6
28	BLaLa[c]	2838	2838	2838	2
28	CoCoP	2842	2839	2845	5
28	LaBLa[c]	2852	2852	2852	2
30	CiCiCi	3000	3000	3000	8
30	CyCyM	3014	3012	3017	8
30	CoCoS	3048	3045	3050	4
32	CiCiLa	3189	3187	3190	6
32	CyLaLa	3194	3190	3200	6
32	CyCyP	3207	3206	3208	2
32	BMM	3237	3234	3239	6
32	MBM	3251	3249	3253	6
34	CiLaLa	3389	3387	3391	6
34	CiCiM	3394	3392	3395	2
34	CyLaM	3400	3399	3400	2
34	CoMM	3412	3408	3415	5
34	CyCyS	3416	3413	3419	4
34	MCoM	3421	3420	3422	3
34	BMP	3444	3444	3444	2
34	BLaS	3448	3447	3449	2
34	MBP	3460	3460	3460	2
34	LaBS	3465	3464	3466	2
36	LaLaLa	3600	3600	3600	14
36	CiCiP	3613	3612	3614	4
36	CyMM	3616	3613	3619	8
36	CoMP	3632	3631	3632	2
36	MCoP	3641	3640	3641	2
36	BPP	3658	3656	3660	6
36	BMS	3664	3662	3666	2
36	PBP	3675	3673	3677	6
36	MBS	3682	3679	3685	2
38	CiMM	3810	3803	3816	2
38	LaLaM	3814	3813	3814	2
38	CiLaP	3818	3817	3818	2
38	CiCiS	3824	3822	3825	2
38	CyMP	3831	3828	3833	2
38	CyLaS	3832	3832	3832	2
38	CoPP	3845	3841	3848	6
38	CoMS	3847	3845	3848	2
38	PCoP	3855	3852	3858	6
38	MCoS	3856	3856	3856	2
38	BPS	3869	3868	3870	2
38	PBS	3885	3884	3885	2
40	LaLaP	3997	3996	3997	2
40	CiMP	4002	3992	4012	2
40	LaMM	4005	4004	4006	2
40	CiLaS	4010	4009	4011	2
40	CyPP	4018	4016	4019	2
40	CyMS	4025	4024	4025	2
40	CoPS	4038	4033	4043	2
40	PCoS	4047	4042	4051	2
40	BSS	4068	4057	4078	6
40	SBS	4085	4074	4094	6
42	MMM	4200	4200	4200	22
42	LaLaS	4200	4200	4200	6
42	CiPP	4215	4214	4216	2
42	CoSS	4251	4243	4260	6
42	SCoS	4262	4254	4271	6
44	LaPP	4449	4448	4450	2
44	MMP	4454	4449	4458	3
44	CySS	4475	4468	4482	4
46	MMS	4642	4627	4651	6
46	MPP	4646	4641	4651	4
46	CiSS	4648	4648	4648	2
48	PPP	4800	4800	4800	18
48	LaSS	4800	4800	4800	6
50	PPS	4966	4964	4966	4
50	MSS	4971	4966	4978	6
52	PSS	5159	5158	5160	4
54	SSS	5400	5400	5400	20
	Monoene TG				
26:1	BBO	2683	2681	2686	6
30:1	CoCoO	3056	3054	3059	4
34:1	CyCyO	3429	3428	3429	4
36:1	BMO	3678	3676	3680	2
36:1	MBO	3697	3694	3700	2
38:1	CiCiO	3836	3831	3840	2
38:1	CyLaO	3848	3847	3849	2
38:1	CoMO	3853	3848	3858	2
38:1	MCoO	3862	3857	3867	2
38:1	BPO	3889	3887	3891	2
38:1	PBO	3907	3906	3908	2
40:1	CiLaO	4023	4010	4035	2
40:1	CyMO	4049	4046	4051	2
40:1	CoPO	4060	4058	4061	2
40:1	PCoO	4069	4067	4070	2
40:1	BSO	4091	4087	4094	2
40:1	SBO	4107	4103	4110	2
42:1	LaLaO	4225	4220	4231	4
42:1	CoSO	4273	4270	4276	2
42:1	SCoO	4287	4284	4290	2
46:1	MMO	4648	4640	4658	6
50:1	PPO	4984	4975	4995	4
54:1	SSO	5432	5422	5436	4
	Polyene TG				
40:2	BOO	4101	4100	4104	4
40:2	OBO	4120	4117	4123	6
42:2	CoOO	4282	4266	4307	6
42:2	OCoO	4298	4275	4321	6
44:2	CyOO	4489	4474	4499	4
46:2	CiOO	4681	4678	4683	2
48:2	LaOO	4836	4834	4840	4
50:2	MOO	5009	4998	5021	6
52:2	POO	5208	5200	5217	4
54:2	SOO	5474	5469	5482	4
54:3	OOO	5498	5487	5514	18

cont.

[a]Number of acyl carbons.
[b]Number of determinations.
[c]BLaLa = 3-butyryl-1,2-dilauroyl-*rac*-glycerol; LaBLa = 2-butyryl-1,3-dilauroylglycerol. For chromatographic conditions see text. See Table 1 for abbreviations.

TABLE 4
Empirical (molar) Correction Factors (ECF) for Monoacid TG with 12, 18, 24, 30, 36, 42, 48, and 54 CN Determined by Linear Calibration (number of determinations $n = 15$)[a]

TG	ECF		Coefficient of determination
	Slope value	Error	
BBB	2.165	0.039	0.995
CoCoCo	1.405	0.022	0.997
CyCyCy	1.102	0.011	0.999
CiCiCi	0.904	0.008	0.999
LaLaLa	0.811	0.006	0.999
MMM	0.759	0.009	0.998
PPP	0.736	0.016	0.993
SSS	0.864	0.018	0.994
OOO	0.905	0.017	0.995

[a]For abbreviations, see Tables 1 and 3.

GLC analysis (Table 4), even though the triplicate measurements were carried out by two different analytical GLC columns. Neither molecular weight nor the degree of unsaturation had marked influence on the reproducibility of TG analysis by GLC on the basis of coefficient of determination. Slight increases in the slope values with increasing molecular weight and increasing degree of unsaturation indicated only moderate thermal degradation or polymerization during GLC analysis. Because there was only a slight difference between saturated and unsaturated TG in the values of the parameters of the linear regression models, the same values for slope were used for saturated and unsaturated TG with the same number of acyl carbons in the quantitative analysis of TG.

Determination of sn-*1(3)- and* sn-*2-short-chain acyl isomers of TG in BO and IBO*. Randomization of the specific distribution of acyl groups of TG in BO by chemical interesterification decreased the intensity of most peaks in the range of 28–42 acyl carbons and increased the number of peaks in the same range (Figs. 3,4). The effects were due to the marked increase in the amount of *sn*-2-butyryl and caproyl isomers and the decrease in the amount of *sn*-1(3)-butyryl and caproyl isomers of TG with two long-chain acyl, and also to the increased number of detectable molecular species of TG resulting from interesterification.

Analysis of the saturated fraction of IBO (Fig. 3) and also the retention index data (Table 3) showed that *sn*-1(3)- and *sn*-2-butyryl isomers separated from those of caproyl isomers with the same number of acyl carbons. BPS and BSS eluted as individual molecular species. (For these and subsequent abbreviations see Table 1.) However, several butyrate TG (BMP and BLaS, BMS and BPP, and most probably BMM and BLaP) can elute in the same peak. The same trend was observed with caproate TG: CoPS and CoSS eluted in separate peaks, but CoMS and CoPP, CoMM and CoLaP, CoMP and CoLaS eluted in a peak composed of two molecular species. Other short-chain TG (BLaLa, and most probably BLaM, CoLaLa, CoLaM) overlapped with other molecular species of TG with the same acyl carbon number. The results showed that most abundant monobutyryl and monocaproyl TG could be separated from each other and from the other molecular species of TG and quantitated as separated molecular species or as a mixture of TG species.

In Table 5, identification and quantitation of molecular species of saturated TG with 32–42 acyl carbons are presented as average values of duplicate measurements. Identifi-

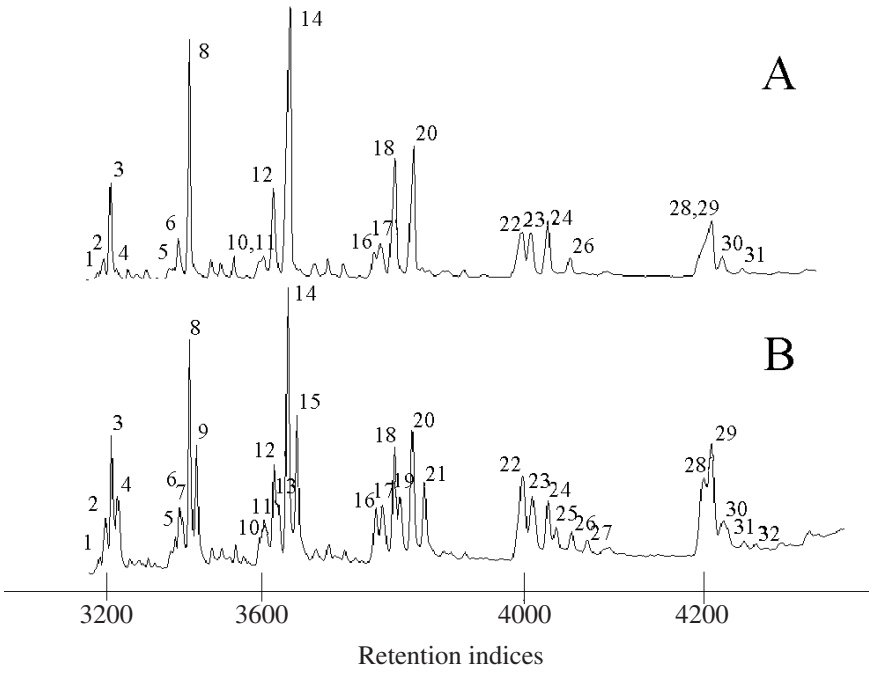

FIG. 3. The gas chromatograms of the saturated fraction of butteroil (A) and interesterified butteroil (B). See text for chromatographic conditions.

TABLE 5
Proportions (mol%) of Saturated TG with 32–42 CN in BO and IBO[a]

TG	Peak number	RI	Mol% BO	Mol% IBO	Ratio of *sn*-1(3) and *sn*-2 acyl isomers in IBO
TG 32					
CyCyP, CiCiLa	1	3193	0.11	0.06	
CyCyP	2	3209	0.36	0.32	
BMM	3	3226	1.57	0.79	1.8:1
MBM	4	3241	n.d.	0.45	
TG 34					
CiCiLa, CiLaLa, CiCiM, CyLaM	5	3389	0.31	0.24	
CoMM/MCoM, CyCyS	6	3397	0.73	0.28	1.2:1
CoMM/MCoM, CyCyS	7	3411	n.d.	0.24	
BMP, BLaS	8	3437	3.41	1.19	1.8:1
MBP, LaBS	9	3454	trace	0.66	
TG 36					
LaLaLa	10	3600	0.72	0.19	
CyMM, CiCiP	11	3610	(0.72)	0.38	
CoMP	12	3629	1.78	0.62	1.8:1
MCoP	13	3637	n.d.	0.34	
BPP, BMS	14	3659	5.59	1.72	1.7:1
PBP, MBS	15	3674	0.05	1.03	
TG 38					
CiMM, LaLaM, CiLaP	16	3808	0.50	0.41	
CyMP, CyLaS, CiCiS	17	3829	0.74	0.33	
CoPP, CoMS	18	3838	2.32	0.66	2.2:1
PCoP, MCoS	19	3847	n.d.	0.30	
BPS	20	3865	2.41	0.75	1.9:1
PBS	21	3880	0.07	0.40	
TG 40					
LaLaP, CiMP, LaMM, CiLaS, CyPP	22	4005	1.31	1.31	
CyMS, CyPP	23	4017	0.92	0.41	
CoPS	24	4033	0.99	0.44	2.2:1
PCoS	25	4047	trace	0.20	
BSS	26	4055	0.34	0.20	1.7:1
SBS	27	4073	0.04	0.12	
TG 42					
MMM, LaLaS	28	4200	2.53	0.83	
CiPP, LaLaS	29	4214	(2.53)	1.14	
—	30	4225	0.45	0.46	
CoSS	31	4243	0.15	0.08	1.3:1
SCoS	32	4256	0.03	0.06	
Saturated TG 32–42			27.43	16.61	

[a]See Figure 3 for peak numbers. Abbreviation: n.d. = not detected. See Tables 1–3 for other abbreviations.

cation is based on the retention indices of synthesized TG, but often other TG species which are not present in the synthesized mixtures will elute in the same peak. The analysis showed that in BO in the range of TG with 32–42 acyl carbons the amounts of monobutyryl TG and monocaproyl TG were 13.3 mol% and 6.0 mol%, respectively, which were much higher than respective values in randomized BO (7.3 mol% for monobutyryl TG, 3.2 mol% for monocaproyl TG). The most abundant (quantities greater than 2 mol%) saturated monobutyryl and monocaproyl TG in BO were BPP + BMS 5.64 mol%, BMP + BLaS 3.41 mol%, BPS 2.48 mol%, and CoPP + CoMS 2.32 mol%. In IBO, only the proportion of BPP + BMS was higher than 2 mol% (2.75 mol%).

The GLC analysis of *sn*-1(3) and *sn*-2 isomers showed clearly that the butyryl and caproyl acyl groups were almost entirely esterified to the *sn*-1(3) position in BO. The proportion of butyryl groups in the *sn*-2 position ranged mostly from 0 to 3, but only traces of *sn*-2 isomers of monocaproates were detected (Table 5). The high proportions of *sn*-2 isomers of butyryldistearoylglycerol and caproyldistearoylglycerol were most probably due to the partial overlapping of saturated and monoene TG fractions and, hence, overlapping with saturated *sn*-2 and monoene *sn*-1(3) isomers. In interesterified butteroil, the ratio of saturated *sn*-1(3)- to *sn*-2-monobutyryl and caproyl isomers aver-

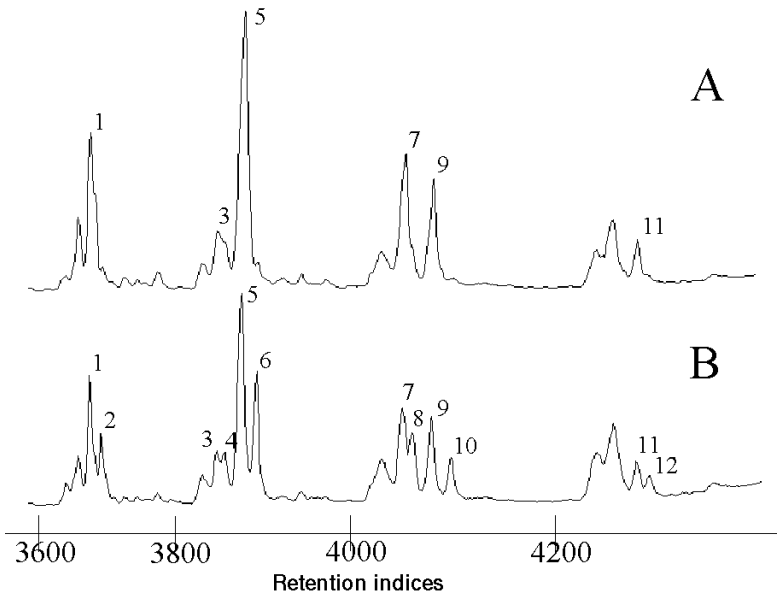

FIG. 4. The gas chromatograms of the monoene fraction of butteroil (A) and interesterified butteroil (B). See text for chromatographic conditions.

aged 1.8:1, which is close to the expected random ratio 2:1.

The retention indices (Table 3) and the chromatograms of monoene fraction of BO and IBO (Fig. 4) show that *sn*-1(3)- and *sn*-2-monobutyryl and caproyl isomers of BSO and CoSO eluted as individual peaks. On the basis of retention indices (Table 3, Fig. 4), the other abundant monoene short-chain TG (BMO, BPO, CoMO, CoPO) eluted in well-separated peaks, but may coelute with other butyrates or caproates. The most abundant diene short-chain TG (BOO, CoOO) separated well from each other and from the other TG species. Among the most abundant molecular species of TG in BO were BPO (+BPPo) (4.80 mol%) and BMO (+BSPo) (2.66 mol%) (Table 6).

The ratio of monoene *sn*-1(3)- and *sn*-2-butyryl and caproyl isomers in randomized BO (Table 6) was on average 2.3:1, which was in accordance with the random ratio and with the observations in saturated TG. In BO, the proportion of unsaturated *sn*-2 monobutyrates ranged from 0 to 3%, and only traces of *sn*-2 monocaproates were detected.

The total proportions of saturated, monoene, and diene monobutyrate and monocaproate TG in BO were 19.5, 13.4, and 1.8 mol%, respectively. The total proportions of monobutyryl (24.2 mol%) and monocaproyl TG (10.5 mol%) in BO were much higher than those in randomized BO (14.9 and 6.6 mol%, respectively) indicating clearly the nonrandom distribution of acyl groups in TG of BO. Altogether, the amount of 15 TG (or TG isomers eluting in the same peak) in BO was higher than 2 mol%, and eight of them were other than monobutyryl or monocaproyl isomers (PPO + others, 4.88 mol%; MPO + others, 3.60 mol%; POO + others, 3.43 mol%; PSO + others, 3.08 mol%; CiPP + LaLaS + others, 2.53 mol%; MPP + MSS + others, 2.43 mol%; MOO + others, 2.18 mol%; MMO + LaPO + others, 2.03 mol%).

DISCUSSION

Retention indices. The principles of the elution order of TG species on polarizable GC columns was well established by GC–MS studies (10,17,18). Chainlength, positional placement, and unsaturation of the fatty acids are considered to cause the variation in retention times of TG within the same number of acyl carbons (10). Short-chain TG were shown to elute in order of *xx*Cy > *xx*Co > *xx*B (where *x* = long-chain acyl) both on a polarizable GC column (10) and on a reversed-phase HPLC column (18,19). Myher *et al*. (10) discussed the variations in relation to the equivalent carbon numbers (ECN): the effect of changing esterified caproyl for butyryl group resulted in 0.29 units increase in ECN, and changing the short-chain group from *sn*-1(3) to *sn*-2 position in monobutyrates resulted in 0.16 units increase in ECN. The changes in the retention indices determined in the present study agree well with the changes in ECN: the difference between the retention indices of butyrates and caproates averaged 29 units, and the difference between the retention indices of *sn*-1(3) and *sn*-2 butyrate TG averaged 17 units.

The applicability of retention indices for the identification of TG species was proven in our previous studies (17). Comparison of the results showed that the retention indices within the range of 36–54 acyl carbons agreed reasonably well, but some variation was observed with smaller TG. The use of a different analytical column and temperature program and different index compounds (TG 12:0, 18:0, 24:0, 30:0, ... vs. TG 24:0, 30:0, ...) for the calculations was the most probable reason for the variation. When the difference between the reten-

TABLE 6
Proportions (mol%) of Unsaturated TG with One Short-Chain Acyl (B, Co) and Two Long-Chain Acyls (M, P, S, O) in BO and IBO

TG	Peak number	RI	Mol%		Ratio of *sn*-1(3) and *sn*-2 acyl isomers in IBO
			BO	IBO	
TG 36					
BMO	1	3671	2.58	1.22	1.9:1
MBO	2	3688	0.08	0.65	
TG 38					
CoMO	3	3857	0.81	0.45	1.4:1
MCoO	4	3866	n.d.	0.33	
BPO	5	3882	4.73	1.99	2.5:1
PBO	6	3898	0.07	0.79	
TG 40					
CoPO	7	4050	1.75	1.00	2.1:1
PCoO	8	4058	trace	0.48	
BSO	9	4074	1.50	0.88	2.8:1
SBO	10	4092	0.04	0.31	
TG 42					
CoSO	11	4266	1.18	0.33	2.2:1
SCoO	12	4278	n.d.	0.15	
Diene TG					
TG 40					
BOO		4089	1.07	0.91	2.3:1
OBO		4108	n.d.	0.39	
TG 42					
CoOO		4286	0.68	0.45	2.0:1
OCoO		4298	n.d.	0.22	
Unsaturated short-chain acyl isomers of TG 36–42			14.49	10.55	

[a]See Figure 4 for peak numbers. See Tables 1–3 for abbreviations.

tion indices of two peaks (TG species) was smaller than 5 units, the peaks separated at most as shoulders in gas chromatograms. When the difference between the retention indices was 10 or higher, the peaks could be easily integrated. However, the analyses of BO and IBO showed that TG species with differences in retention indices even higher than 10 units tended to merge as a single peak when several molecular species eluted at the same range of retention indices. A high concentration of eluted component also tended to cause some variation in the retention indices.

In our previous studies, the positional isomers of butyrates have been isolated by HPLC and identified with ^{1}H NMR, and the monobutyrates having a short-chain acyl in the secondary position have been demonstrated to elute on a polarizable column later than the monobutyrates having a short-chain acid in the primary position (5). In the present study, the same trend was confirmed for caproates and, in one case (CyOO/OCyO), for caprylates. We observed that the TG which consist of two butyrate groups and one long-chain acyl group separated partly as shoulders on a phenyl(65%)methylsilicone column. Total proportion of TG consisting of two short-chain acyl moieties has been shown to be very low at 0.32 mol% (20). Thus, the optimization of analytical conditions for the determination of their positional isomers was considered to be unnecessary.

Linear calibration. In his review article (21), Mares emphasized the importance of the use of empirical correction factors (ECF) in capillary GC in order to achieve precise quantitative analysis of TG. Mares and Husek (22) have shown the ECF for the TG with 30–54 acyl carbons to be practically independent of the amount of analyzed TG and of the carrier gas flow rate when good-quality capillary columns (OV-1 stationary phase) are used in combination with cold on-column injection. This is in accordance with the observations of the excellent linearity of ECF throughout the whole range of even-numbered TG (TG 12:0–54:3), and the wide range of concentrations (62.5–2500 μg/5 mL/component) with a phenyl(65%)methylsilicone column used in the present study. The use of constant carrier gas flow rate probably improved further the linearity of ECF for high molecular weight TG.

Quantitative analysis of the molecular species of TG in BO and IBO. The quantitative analysis of most major short-chain TG and their *sn*-1(3) and *sn*-2 isomers by combining GLC and silver ion column chromatography was demonstrated in the present study. Theoretically, there exist 40 different molecular species (including positional isomers) of saturated TG consisting of one short-chain acyl group (butyryl or caproyl) and two long-chain acyl groups (lauroyl, myristoyl, palmitoyl, or stearoyl) in BO. Accordingly, there exist 16 different molecular species (including positional isomers) of unsaturated TG consisting of one short-chain, one saturated long-chain, and one oleoyl acyl group, and further, four more TG species (including positional isomers) composed of one short-chain and two oleoyl groups. In this study, retention indices were determined for 44 of those 60 TG and 42 of them were quantitated.

Retention index data (Table 3) show that in all cases butyryl and caproyl TG separate from each other and, also, *sn*-1(3) and *sn*-2 isomers separate from each other. However, partial overlapping will occur between some saturated and monoene TG, and some monoene and diene TG. Thus, prefractionation of BO according to the degree of unsaturation must be carried out prior to the GLC analysis.

A recent study by Gresti *et al.* (20) showed clearly the abundance of the TG composed of one short-chain fatty acid and two long-chain fatty acids. Their proportion was 36 mol% in total, including three major TG in milk fat (BPO, BPP, and BMP). The proportion of short-chain TG in the present study (34.7 mol% in total) agrees quite well with the study by Gresti *et al*. (20), even though the combination of GLC and silver ion chromatography could not separate the molecular species of TG in BO as far as the combination of HPLC and GLC (20). Our study showed that 8 of 30 (BPS, BSS, CoPS, CoSS, BSO, CoSO, BOO, CoOO) of the mono-short-chain TG, and their *sn*-1(3) and *sn*-2 isomers (see above), eluted as individual species, and most of the others as a coeluting pair of TG species on a phenyl(65%)methylsilicone column. The proportion (mol%) of the most common mono-short-chain molecular species of TG in BO according to the two studies (the present study and the study of Gresti *et al.*) were 5.6/4.6, 4.8/4.4, 3.4/3.4, 2.3/2.2, 2.5/2.5, and 2.7/2.2 for BPP + BMS, BPO (+PSPo), BMP + BLaS, CoPP + CoMS, BPS, and BMO (+BPPo), respectively.

The proportion of *sn*-2-butyrates in BO TG was reported to range from 0 to 6% in the first stereospecific analysis by enzymatical methods (2,3). Later investigations by enzymatical analyses (12–14), Grignard degradation, and chiral-phase HPLC (7) and by direct measurements from untreated fats by NMR measurements (4–6) have shown butyryl groups locate almost entirely at the *sn*-3 position. In the present study, the proportion of *sn*-2 isomers of butyrates ranged from 0 to 3% (averaging 1.4%), depending somewhat on the molecular species of TG. No marked differences were observed between saturated and unsaturated TG species. The detected small amount of *sn*-2-butyrates was most probably due to the observed partial overlap of saturated and monoene fractions, and monoene and polyene fractions.

The proportion of *sn*-2-caproates in BO TG was reported to range from 0 to 26% (2,3,12–14). All the studies have been based on the use of enzymatic methods, and no direct measurements by NMR have been reported. The problems of enzymatical determination due to the acyl migration and higher rate of hydrolysis of the TG consisting of both short-chain and long-chain acyls than that of the TG with only long-chain acyls have been discussed thoroughly (2,3) and suggest a possible overestimation of the proportion of *sn*-2 caproyl isomers. The use of GLC in combination with silver ion chromatography provides a more direct method than enzymatic deacylation in analyzing the fatty acid distribution between *sn*-1(3) and *sn*-2 positions. Our observation that only traces of *sn*-2 caproates were present in BO supports the results of Breckenridge and Kuksis (3), Parodi (13), and Itabashi *et al.* (7).

In conclusion, the combination of GLC using a phenyl(65%)-methyl silicone column and silver ion column chromatography enabled direct quantitation of most of the major monobutyrate and monocaproate TG in BO as individual molecular species or as a group of molecular species. Further, the method provided a useful analytical technique for determining *sn*-1(3) and *sn*-2-short-chain acyl isomers of TG without chemical or enzymatic treatment of BO.

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