Composition of the Triacylglycerols of Butterfat and Its Fractions Obtained by an Industrial Melt Crystallization Process

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The compositions of two solid and two liquid fractions of butterfat obtained by the Tirtiaux melt fractionation process were studied. The triacylglycerols of each of the four fractions and butterfat were separated by silver ion high-performance liquid chromatography (Ag-HPLC) into six subfractions. Each was analyzed for fatty acid composition by gas chromatography and was separated by reversed-phase HPLC. The liquid Tirtiaux fractions contained higher proportions of saturated short-chain and monoenoic fatty acids, especially *cis*-18:1, than the solid ones. Every Tirtiaux fraction comprised at least 64% saturated fatty acids. The trisaturated (35.0–58.9%) and disaturated *cis*-monoenoic (27.9–44.1%) triacylglycerols were the most abundant Ag-HPLC fractions, together representing at least 79%. The other unsaturated fractions were composed of disaturated *trans*-monoenoic (2.5–3.4%), saturated *cis,trans*-dimonoenoic (1.8–4.4%), saturated *cis,cis*-dimonoenoic (5.2–9.1%), and the more unsaturated molecules (2.8–5.0%). The greatest differences between the Tirtiaux fractions were found in the trisaturated triacylglycerols. There were differences too, albeit less pronounced, in the disaturated *cis*- and disaturated *trans*-monoenoic molecules. Although the di- and polyunsaturated molecules were not evenly distributed between the solid and liquid Tirtiaux fractions, no clear selectivity according to their triacylglycerol structure was observed.

INTRODUCTION

Butter and, to a smaller extent, butter oil are the traditional fat products of the dairy industry. Declining consumption and surpluses have created a need to develop a wider selection of products, but the functional properties of butterfat are not ideal for several potential applications. Modifying butterfat by chemical methods such as hydrogenation or interesterification, processes which are permitted by food regulations in many countries, has not been popular because such processing damages the "image" of natural butterfat. However, butterfat has a broad melting range (Taylor et al., 1978) and is therefore a suitable material for physical fractionation.

There are several methods from which to choose: short-path distillation (Arul et al., 1988), supercritical fluid extraction (Kaufmann et al., 1982), melt crystallization (Makhlouf et al., 1987) and solvent crystallization (Timms, 1980), or emulsion fractionation (Banks et al., 1985) processes. The absence of organic solvents or process aids, the reasonable cost of processing, a relatively simple instrumentation, and less fractionation or loss of aroma components compared with the other processes have made the melt crystallization method the only significant industrial process so far (commercialized under the name of the Tirtiaux process).

In the Tirtiaux process pure melted fat is gradually cooled according to a specified temperature program with agitation until a suspension of large fat crystals in clear oil has been formed at the final temperature. Solid and liquid phases are then separated at this same temperature by vacuum filtration using a continually rotating, perforated stainless steel belt (Tirtiaux, 1991). Typically with butterfat, the first solid fraction (S1), "stearin", and the first liquid fraction (L1), "olein", are separated at a temperature between 22 and 28 °C, depending on the composition of the original fat and the final uses of the products. The liquid fraction can be crystallized again at a lower temperature, 14–18 °C (S2 and L2). Usually the amount of solid fraction in suspension is between 20 and 40%.

Because of the commercial significance of the Tirtiaux process, its users are interested also in the chemical selectivity of the method. Usually the products are studied by means of fatty acid analyses, but, at least with butterfat, the remarkable differences in melting profiles between the fractions and the original fat are not reflected much in their fatty acid compositions (Amer et al., 1985). This is to be expected, since the process is based on the different melting points of the triacylglycerols in the mixture and only indirectly on the melting points of the fatty acids they are made of. The melting point of a triacylglycerol is a function of the chain lengths of its three fatty acyl residues, their type of unsaturation (number and configuration of double bonds), and their position on the glycerol backbone. Therefore, analytical methods that separate molecules according to these parameters should be more informative.

Theoretically, assuming random distribution of fatty acids, there would be more than 83 million (437^3) different triacylglycerols in butterfat (Patton and Jensen, 1975), and the number detectable by modern techniques and instrumentation too is enormous. There is no method or combination of methods capable of quantitating them all in a reasonable time; neither is the human brain capable of comprehending such information, even if it were available. We have to be satisfied with a rougher grouping. Both reversed-phase high-performance liquid chromatography (HPLC) and high-temperature capillary gas chromatography (GC) have been used for the characterization of the Tirtiaux fractions of butterfat (Deffense, 1987). Both methods separate triacylglycerols simulta-

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neously according to chain length and type of unsaturation, which complicates the interpretation of the results. Silver ion chromatography separates molecules solely according to type of unsaturation and can be used in the preparative mode and followed by other analytical procedures (Christie, 1987). The purpose of this study was to investigate the chemical selectivity of the butterfat fractionation of the Tirtiaux process by using silver ion HPLC, reversed-phase HPLC, and GC analysis. Such a combination has been used for the analysis of other fats (Nikolova-Damyanova et al., 1990; Laakso and Christie, 1991) but not, as far as we know, for the analysis of butterfat fractions.

MATERIALS AND METHODS

Samples and Reagents. Industrial-scale crystallization of butterfat, prepared from lactic butter, into a solid (S1) and a liquid (L1) fraction was performed according to the Tirtiaux melt fractionation process (Normilk Oy, Äänekoski, Finland). The liquid fraction (L1) was further crystallized, yielding a solid (S2) and a liquid (L2) fraction. The S2 and L2 samples used in this research were not from the same batch as the other products. According to the manufacturer, the melting points (Mettler dropping points) of the samples were 42 (S1), 21 (L1), 27 (S2), 16 (L2), and 34 °C (butterfat). All samples were stored at −20 °C until analyzed.

Triacylglycerols (about 10 mg) of each of the five samples were purified, prior to HPLC and GC analyses, on short Florisil columns by elution with 10 mL of hexane–diethyl ether (4:1 v/v). All solvents were of HPLC grade and were supplied by Merck and Rathburn. Pure triacylglycerols were purchased from Sigma.

High-Performance Liquid Chromatography (HPLC). The HPLC analyses were carried out with a Shimadzu (Kyoto, Japan) LC-9A pump together with a Shimadzu FCV-9AL low-pressure gradient elution unit and a Cunow (Cergy-Saint-Christophe, France) DDL21 light scattering detector. Chromatograms were recorded with a Shimadzu C-R5A Chromatopac integrator. For preparative purposes a stream-splitter was inserted between column and detector.

Silver ion high-performance liquid chromatography (Ag-HPLC) was performed according to the procedure described by Christie (1987, 1988). The separation was achieved with a cation-exchange column (4.6 mm i.d. × 250 mm) of Nucleosil 5SA (HPLC Technology, Macclesfield, U.K.) loaded with silver ions. Triacylglycerols (1–2 mg) dissolved in 10 μL of 1,2-dichloroethane were applied to the column and then eluted at ambient temperature with a binary gradient of (A) dichloromethane–1,2-dichloroethane (4:1 v/v) and (B) acetone at a flow rate of 1.0 mL/min. The linear gradient was 100% A to 80% A − 20% B in 20 min and then to 100% B in 15 min (held at 100% B for 5 min). The preparative collection was repeated several times to get sufficient material for further analyses.

Total triacylglycerols of the five samples as well as their fractions obtained by Ag-HPLC were separated by reversed-phase HPLC. The samples were dissolved in 1,2-dichloroethane, and volumes of 10 μL or less were injected on to the column. Two columns (4.6 mm i.d. × 250 mm) with ODS phase (5-μm particles, Zorbax, Du Pont, Willmington, DE; Spheri-5, Brownlee Labs, Santa Clara, CA) in series were utilized at ambient temperature with a binary gradient of (A) dichloromethane–1,2-dichloroethane (4:1 v/v) and (B) acetonitrile as the mobile phase at a flow rate of 0.8 mL/min. The linear gradient was 30% A–70% B to 55% A–45% B in 65 min, to 60% A–40% B in 10 min, and then to 65% A–35% B in 5 min. The final solvent composition was held for 2 min.

Gas Chromatography (GC). Whole triacylglycerol fractions or aliquots, weight 100–400 μg, plus 75 μg of internal standard (methyl heneicosanoate, Serva 24576), in 100 μL of hexane and 3 μL of methyl acetate were methylated by adding 1 μL of 1 M methanolic sodium methoxide (freshly prepared from Fluka 71748 reagent) and vortexing for a few minutes (Christie, 1982). The turbid preparation was acidified with oxalic acid and transferred to a 200-μL insert in a 2-mL vial for immediate gas chromatographic analysis with a fused silica column (0.32 mm i.d. × 30 m) coated with 0.2 μm SP-2340 phase (Supelco, Bellefonte, PA). A Carlo Erba (Milan, Italy) 5160 gas chromatograph fitted with a Hewlett-Packard (Palo Alto, CA) 7673A injector was used. The injector and detector temperatures were 250 °C, and the split ratio was about 25:1. Helium (99.9999%) was the carrier gas, at 50 kPa (flow rate 1.5 mL/min) until the emergence of methyl butyrate and thereafter at 80 kPa (2.1 mL/min). The temperature was held at 70 °C until the emergence of methyl butyrate, followed by programming at 30 °C/min to 120 °C, 10 °C/min to 160 °C, and 2 °C/min to 190 °C. The response correction factor for each fatty acid methyl ester, used to convert peak area percent to weight percent, was determined by analyzing a butter oil of known fatty acid composition (CRM 164). The identification of the fatty acid components was based on the known composition of butterfat (Strocchi and Holman, 1971) and the separation characteristics of the SP-2340 stationary phase (Slover and Lanza, 1979). The detector response correction factors determined for the fatty acids were of internationally accepted values (Badings and de Jong, 1988). The final fatty acid compositions are expressed as mole percent.

RESULTS AND DISCUSSION

The physical properties of butterfat fractions are defined by their triacylglycerol compositions. Fatty acid analyses alone do not provide enough information about the molecular species. Because of the complexity of butterfat triacylglycerols, combinations of chromatographic techniques which separate molecules in different ways are advantageous. In the present study, the compositions of differently unsaturated triacylglycerols isolated by Ag-HPLC from butterfat and its Tirtiaux fractions were elucidated by means of reversed-phase HPLC and GC analysis.

Fatty Acids of the Tirtiaux Fractions. The fatty acid compositions of the butterfat and the Tirtiaux fractions obtained from it are shown in Table I. Tirtiaux fractionation is based on the crystallization properties of triacylglycerols. Thus, it is not surprising that the liquid fractions contained larger proportions of low-melting short-chain-length saturated and monoenoic fatty acids than the solid fractions. The fraction L1 contained 19.7 mol % and S1 15.4% saturated C_4–C_{10} fatty acids, for example. Monoenoic fatty acids represented 24.7–26.0% of L1 and 18.8–19.9% of S1. Fraction L2, with the lowest melting range, contained approximately 30% monoenoic fatty acids. Generally, in all samples the saturated fatty acids were the most abundant, varying from 64.1–66.0% in L2 to 77.6–78.9% in S1, the solid fractions having greater proportions of total saturates, especially 14:0, 16:0, and 18:0, than the liquid ones. The characteristic patterns of the fatty acid compositions of solid and liquid butterfat fractions analyzed in this study were in good agreement with those reported elsewhere (Norris et al., 1971; Badings et al., 1983; Amer et al., 1985; Deffense, 1987).

Because of the differences in physical properties of the geometrical isomers of the fatty acids, *cis*- and *trans*-18:1 have been determined in some studies. Norris et al. (1971) noted that *trans*-18:1 was concentrated in the solid fraction, whereas Banks et al. (1985) and Amer et al. (1985) did not. However, the proportion of *trans*-18:1 in the total 18:1 was greater in the solid than in the liquid fractions, as reported by Amer et al. (1985). These results can be explained by the melting properties of the monoenoic isomers. In comparison to the main *cis* isomer 18:1(n−9), mp 16 °C, present in bovine milk fat (Hay and Morrison, 1970), the most abundant trans isomer 18:1(n−7), mp 43.5–44.1 °C, has a melting point closer to that of the saturated C_{18} fatty acid, mp 69.7 °C. We found no enrichment of *trans*-18:1 in the solid fractions, in terms of mole percent of fatty acid, but the proportion of *trans*-18:1 in the total 18:1 was higher in S1 (9.4%) than in butterfat (8.1%) and L1 (7.2%).

Table I. Fatty Acid Compositions (Mole Percent) of the Triacylglycerols of Butterfat and Its Solid (S1, S2) and Liquid (L1, L2) Tirtiaux Fractions

fatty acid	S1	butter	L1	S2	L2
4:0	7.4	8.5	9.8	8.5	9.7
6:0	3.4	4.0	4.5	4.0	4.5
8:0	1.6	1.8	2.0	1.7	2.0
10:0	3.0	3.2	3.4	2.9	3.6
10:1	0.3	0.3	0.4	0.3	0.4
12:0	3.3	3.2	3.2	2.7	3.3
12:1	0.1	0.1	0.1	0.1	0.2
13:0	0.1	0.1	0.1	0.1	0.1
i[a]-14:0	0.1	0.1	0.1	0.1	0.2
14:0	11.3	10.3	10.0	10.9	9.6
i-15:0	0.2	0.2	0.2	0.2	0.3
14:1c,[b] ai[c]-15:0	1.1	1.3	1.3	1.4	1.6
15:0	0.8	0.8	0.7	0.9	0.8
i-16:0	0.2	0.2	0.2	0.2	0.2
16:0	30.1	26.2	24.8	25.9	20.0
16:1t[d]		0.1	0.1		0.1
i-17:0	0.4	0.4	0.4	0.5	0.5
16:1c	0.9	1.1	1.2	1.0	1.4
ai-17:0	0.4	0.4	0.3	0.4	0.4
17:0	0.6	0.5	0.6	0.6	0.5
17:1	0.3	0.2	0.4	0.3	0.4
18:0	13.8	11.3	10.0	12.5	8.2
18:1t	1.6	1.7	1.6	1.8	2.5
18:1c[e]	15.5	19.4	20.5	18.1	24.7
nmi[f]-18:2	0.6	0.9	0.5	0.8	0.5
19:0, 18:2tt	0.2	0.3	0.2	0.4	0.3
18:2ct	0.1	0.1	0.1	0.4	0.1
19:1t, 18:2tc		0.1		0.2	
18:2cc	0.8	1.4	1.3	1.3	1.4
19:1c		0.2	0.3	0.1	0.2
20:0	0.9	0.3	0.2	0.2	0.2
18:3	0.3	0.4	0.4	0.4	0.5
20:1	0.1	0.3	0.1	0.1	0.3
conj[g] 18:2	0.3	0.3	0.4	0.6	0.8
others[h]	0.4	0.5	0.5	0.5	0.5

[a] i, iso, methyl branched at the $n-1$ position. [b] c, cis. [c] ai, anteiso, methyl branched at the $n-2$ position. [d] t, trans. [e] *cis*-18:1(n−9) coelutes with some trans isomers, such as 18:1(n−3), 18:1(n−4), and 18:1(n−5) (Ratnayake and Beare-Rogers, 1990). [f] nmi, non-methylene-interrupted. [g] conj, conjugated. [h] Fatty acids that did not reach 0.1 mol % in any sample, together with the unknowns, are reported as "others".

Silver Ion HPLC Fractionation. To obtain more information about the molecular species, the triacylglycerols of butterfat and its solid and liquid fractions were fractionated according to degree of unsaturation by Ag-HPLC. As an example, the separation of the original butterfat is shown in Figure 1. The triacylglycerols were divided into six subfractions, each of which was manually collected via the stream-splitter for GC and reversed-phase HPLC analyses. Silver ion chromatography separates molecules according to the complexing of the π-electrons in the double bonds by silver ions (Morris, 1966; Litchfield, 1972). These interactions are weak and can be broken by chromatographic procedures. The Ag-HPLC fractions were analyzed and quantified by the determination of fatty acid compositions by GC in the presence of internal standard; the fatty acid results are shown in Tables II–VII. To check the recoveries, the composition of the reconstituted triacylglycerols (data not shown here) was calculated from the proportions of fatty acids in each silver ion fraction. No selective losses occurred during silver ion fractionation, because the expected fatty acid composition of each sample, found by a calculated reconstitution, agreed well with the composition found by direct analysis.

The order of elution was trisaturated molecules (Ag-HPLC fraction 1) followed by disaturated monoenoic (Ag-HPLC fractions 2 and 3), saturated dimonoenoic (Ag-HPLC fractions 4 and 5), and the higher unsaturated molecules (Ag-HPLC fraction 6). Although Ag-HPLC fractions 2 and 3 were cleanly separated, their fatty acid compositions were very similar (Tables III and IV). The only distinct difference was that the proportion of *trans*-monoenoic fatty acids, mainly 18:1, in Ag-HPLC fraction 2 was higher, whereas Ag-HPLC fraction 3 consisted mainly of *cis*-monoenoic fatty acids. It is known that trans fatty acids form weaker complexes with silver ions than cis acids (Morris, 1966; Christie and Breckenridge, 1989), and separations of disaturated *cis*- and disaturated *trans*-monoenoic (Taylor and Hawke, 1975; Parodi, 1980, 1981) as well as saturated *cis,trans*- and saturated *cis,cis*-dimonoenoic triacylglycerols (Parodi, 1980, 1981) from milk fat have been achieved by silver ion thin-layer chromatography. Later, configurational isomers of disaturated monoenoic, saturated dimonoenoic, and tri-

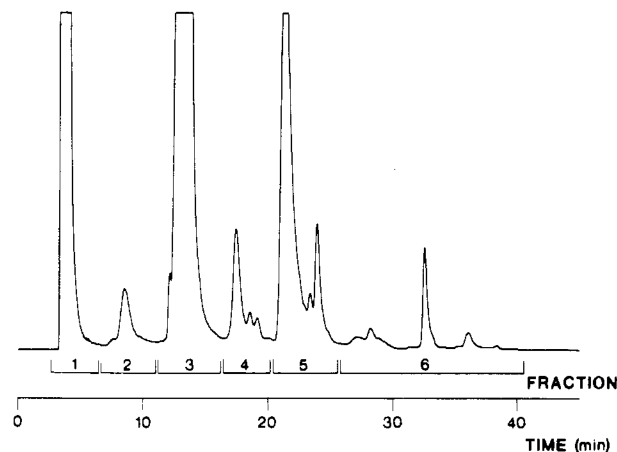

Figure 1. Separation of butterfat triacylglycerols into six fractions by means of preparative silver ion HPLC. The experimental conditions are given under Materials and Methods. Fractions: 1, trisaturated; 2, disaturated *trans*-monoenoic; 3, disaturated *cis*-monoenoic; 4, saturated *cis,trans*-dimonoenoic; 5, saturated *cis,cis*-dimonoenoic; 6, the more highly-unsaturated triacylglycerols.

Table II. Fatty Acid Compositions (Mole Percent) of the Saturated Triacylglycerols (Ag-HPLC Fraction 1) of Butterfat and Its Solid (S1, S2) and Liquid (L1, L2) Tirtiaux Fractions

fatty acid	S1	butter	L1	S2	L2
4:0	8.1	11.0	14.4	12.5	14.4
6:0	3.9	5.3	6.4	5.8	7.2
8:0	1.9	2.4	2.8	2.4	3.3
10:0	3.9	4.5	4.8	4.1	6.0
11:0			0.1	0.1	0.1
12:0	4.4	4.5	4.6	3.7	5.7
13:0	0.1	0.1	0.1	0.1	0.1
i[a]-14:0	0.1	0.2	0.1	0.2	0.2
14:0	15.2	14.6	14.3	12.8	15.8
i-15:0	0.3	0.3	0.3	0.3	0.4
ai[c]-15:0	0.4	0.5	0.5	0.5	0.9
15:0	1.1	1.0	1.0	1.2	1.3
i-16:0	0.3	0.3	0.2	0.3	0.3
16:0	39.7	36.7	35.0	34.9	30.1
i-17:0	0.4	0.4	0.3	0.5	0.5
ai-17:0	0.4	0.4	0.4	0.4	0.6
17:0	0.7	0.8	0.7	0.8	0.8
i-18:0	0.1	0.1		0.1	
18:0	18.4	15.6	12.8	18.2	10.7
18:1t[d]		0.3	0.1	0.4	0.5
18:1c[e]	0.1	0.2	0.2	0.2	0.2
nmi[f]-18:2		0.2	0.1		0.2
19:0, 18:2tt	0.1	0.1	0.1	0.1	0.1
20:0	0.3	0.2	0.3	0.3	0.3
others[h]		0.5	0.4	0.3	0.5

[a,c–f,h] Footnotes: see Table I.

Table III. Fatty Acid Compositions (Mole Percent) of the Disaturated *trans*-Monoenoic Triacylglycerols (Ag-HPLC Fraction 2) of Butterfat and Its Solid (S1, S2) and Liquid (L1, L2) Tirtiaux Fractions

fatty acid	S1	butter	L1	S2	L2
4:0	1.7	3.8	6.9	4.8	8.0
6:0	2.4	3.9	5.5	4.4	6.6
8:0	1.2	1.7	2.4	1.9	2.7
10:0	2.5	3.2	4.2	3.4	4.6
12:0	2.5	3.0	3.4	3.0	3.7
13:0		0.1		0.1	0.1
i[a]-14:0	0.1	0.1	0.1	0.1	0.2
14:0	9.9	9.9	9.7	9.7	9.5
i-15:0	0.3	0.3	0.3	0.3	0.4
ai[c]-15:0	0.3	0.4	0.5	0.5	0.6
15:0	0.8	0.8	0.7	0.9	0.8
i-16	0.2	0.2	0.2	0.2	0.2
16:0	27.6	24.3	20.5	21.8	17.2
16:1t[d]	0.6	0.7	0.8	0.9	1.0
i-17:0, 16:1c[b]	0.8	0.8	0.8	0.8	0.8
ai-17:0	0.3	0.3	0.3	0.4	0.4
17:0	0.6	0.6	0.5	0.6	0.6
18:0	14.1	12.1	9.2	12.1	8.3
18:1t	15.5	16.4	17.3	19.3	20.9
18:1c[e]	12.9	11.5	10.9	9.6	9.1
nmi[f]-18:2	3.7	3.7	3.5	3.7	2.7
19:0, 18:2tt	0.1	0.1	0.1	0.1	0.1
19:1t, 18:2tc	0.1	0.2	0.4	0.1	0.1
20:0	0.2	0.2	0.2	0.2	0.1
20:1	0.1	0.1			
conj[g] 18:2	0.1	0.4	0.1	0.1	0.1
others[h]	1.1	1.0	1.4	1.1	1.3

[a–h] Footnotes: see Table I.

Table IV. Fatty Acid Compositions (Mole Percent) of the Disaturated *cis*-Monoenoic Triacylglycerols (Ag-HPLC Fraction 3) of Butterfat and Its Solid (S1, S2) and Liquid (L1, L2) Tirtiaux Fractions

fatty acid	S1	butter	L1	S2	L2
4:0	6.8	7.9	8.0	6.9	8.8
6:0	3.0	3.6	3.6	2.8	3.8
8:0	1.4	1.6	1.6	1.2	1.7
10:0	2.4	2.8	2.7	2.2	3.0
10:1	0.5	0.5	0.5	0.5	0.4
12:0	2.4	2.6	2.7	2.3	2.9
12:1	0.1	0.1	0.1	0.1	0.1
13:0	0.1	0.1	0.1		0.1
i[a]-14:0	0.1	0.2	0.1	0.2	0.1
14:0	8.8	8.9	8.9	8.6	9.3
i-15:0	0.2	0.2	0.2	0.3	0.3
14:1c,[b] ai[c]-15:0	1.7	1.8	1.8	1.8	1.8
15:0	0.7	0.7	0.7	0.9	0.8
i-16:0	0.2	0.2	0.2	0.1	0.3
16:0	24.7	23.3	22.8	23.5	20.1
16:1t[d]		0.1		0.1	0.1
16:1c	2.3	2.3	2.1	2.3	2.3
ai-17:0	0.4	0.4	0.4	0.4	0.4
17:0	0.6	0.5	0.5	0.6	0.6
17:1	0.4	0.4	0.4	0.4	0.5
18:0	11.2	10.1	9.8	12.2	8.9
18:1t	1.0	1.0	0.3	1.5	1.3
18:1c[e]	28.6	28.9	30.2	28.6	29.6
nmi[f]-18:2	0.5	0.4	0.4	0.3	0.3
19:0, 18:2tt	0.1	0.1	0.1	0.1	0.1
18:2ct	0.1	0.1	0.1	0.1	
19:1c	0.2	0.1	0.1	0.3	0.2
20:0	0.2	0.2	0.2	0.2	0.1
20:1	0.2	0.2	0.2	0.2	0.2
conj[g] 18:2	0.6	0.5	0.7	1.1	1.0
others[h]	0.6	0.5	0.6	0.6	0.6

[a–h] Footnotes: see Table I.

monoenoic triacylglycerols of sheep adipose tissue were resolved more efficiently by Ag-HPLC (Christie, 1988). Ag-HPLC fractions 2 and 3 of the present study were identified as SSM^t and SSM^c, respectively, consisting of two saturated (S) and one *trans*-monoenoic (M^t) or one

Table V. Fatty Acid Compositions (Mole Percent) of the Saturated *cis,trans*-Dimonoenoic Triacylglycerols (Ag-HPLC Fraction 4) of Butterfat and Its Solid (S1, S2) and Liquid (L1, L2) Tirtiaux Fractions

fatty acid	S1	butter	L1	S2	L2
4:0	3.4	3.9	3.9	3.6	4.1
6:0	1.6	1.9	2.0	1.7	2.0
8:0	0.7	0.8	0.8	0.7	0.8
10:0	1.9	1.3	1.5	1.2	1.3
10:1	0.4	0.4	0.4	0.4	0.3
12:0	1.2	1.3	1.3	1.2	1.4
12:1		0.6	0.1		0.1
13:0				1.2	
i[a]-14:0		0.2	0.1	0.3	0.2
14:0	5.9	5.5	4.6	4.4	4.4
i-15:0		0.3	0.2	0.2	0.2
14:1c,[b] ai[c]-15:0	1.9	1.5	1.4	1.2	1.4
15:0	0.6	0.4	0.4	0.5	0.5
i-16:0	0.2	0.1	0.1	0.2	0.2
16:0	11.9	12.1	12.2	12.2	10.6
16:1t[d]	0.6	0.7	0.8	0.2	0.8
i-17:0	0.5	0.6	0.7	0.5	0.7
16:1c	2.2	1.5	1.7	1.2	1.4
ai-17:0	0.3	0.2	0.4	0.5	0.2
17:0	0.4	0.7	1.0	0.5	0.4
17:1	0.2	0.4	0.4	0.3	0.6
18:0	7.0	5.8	6.4	7.0	6.4
18:1t	14.3	14.0	14.3	14.5	15.2
18:1c[e]	36.3	35.0	35.3	37.1	36.0
nmi[f]-18:2	4.0	2.8	4.0	2.8	2.2
18:2ct	0.5	0.8	0.4	0.3	0.3
19:1t, 18:2tc	1.1	1.7	0.8	0.3	0.9
19:1c	0.6	0.5	0.2		0.3
20:0	0.2	1.0	0.1	0.5	0.1
18:3		0.6	0.4		0.3
20:1	0.2	0.3	0.3	0.2	0.3
conj[g] 18:2	1.3	2.1	1.7	3.0	4.1
others[h]	1.0	1.0	2.4	2.4	2.3

[a–h] Footnotes: see Table I.

cis-monoenoic (M^c) fatty acyl residues. Fractions 4 and 5 were well separated by Ag-HPLC, although their only essential difference was in the proportions of *trans*- and *cis*-monoenoic acids (Tables V and VI). Ag-HPLC fraction 4 was identified as saturated *cis,trans*-dimonoenoic (SM^cM^t) and Ag-HPLC fraction 5 as saturated *cis,cis*-dimonoenoic (SM^cM^c) triacylglycerols.

The fatty acid compositions presented in the tables were obtained by analysis with SP-2340 stationary phase. It is probable that the proportion of trans acids was underestimated, especially in the samples which contained high proportions of trans isomers. According to Ratnayake and Beare-Rogers (1990), *trans*-18:1(n–7), which is the main trans isomer present in milk fat, is fairly well resolved from the major cis isomer 18:1(n–9). However, the trans isomers 18:1(n–3), 18:1(n–4), and 18:1(n–5), representing approximately 20% (Lund and Jensen, 1983) or even more (Hay and Morrison, 1970) of the total *trans*-18:1 isomers in butter, coelute with *cis*-18:1(n–9). An improved separation of geometrical isomers with another cyanopropylsiloxane stationary phase, SP-2560, is possible (Ratnayake et al., 1990). Still, *trans*-18:1(n–4) and *trans*-18:1(n–5) isomers coelute with *cis*-18:1(n–9). A preliminary analysis with this phase showed that none of the major components of Ag-HPLC fraction 2 of butterfat had a retention time equal to that of methyl oleate [*cis*-18:1(n–9)], for when the sample was spiked with methyl oleate, the shape of the overlapped peak changed.

The results of the reversed-phase HPLC analyses of disaturated monoenoic and saturated dimonoenoic molecules supported the conclusions made above: a molecule containing a trans fatty acid remained in a nonpolar stationary phase longer than the corresponding molecule

Table VI. Fatty Acid Compositions (Mole Percent) of the Saturated *cis,cis*-Dimonoenoic Triacylglycerols (Ag-HPLC Fraction 5) of Butterfat and Its Solid (S1, S2) and Liquid (L1, L2) Tirtiaux Fractions

fatty acid	S1	butter	L1	S2	L2
4:0	4.3	4.3	4.2	4.3	4.4
6:0	1.8	1.8	1.8	1.8	1.9
8:0	0.8	0.8	0.8	0.8	0.8
10:0	1.4	1.3	1.3	1.4	1.9
10:1	0.8	0.8	0.7	0.8	0.8
12:0	1.2	1.2	1.2	1.3	1.3
12:1	0.2	0.2	0.2	0.2	0.2
i[a]-14:0				0.1	0.1
14:0	4.3	4.2	4.3	4.4	4.6
i-15:0	0.3	0.1	0.1	0.1	0.1
14:1c,[b] ai[c]-15:0	2.6	2.5	2.6	2.6	2.9
15:0	0.4	0.4	0.4	0.5	0.5
i-16:0	0.1	0.2	0.1	0.2	0.1
16:0	12.0	12.1	11.7	11.0	10.8
i-17:0	0.5	0.8	0.5	0.5	0.6
16:1c	3.2	3.6	3.3	2.8	3.0
ai-17:0	0.3	0.6	0.3	0.4	0.2
17:0	0.3	0.8	0.3	0.4	0.3
17:1	0.8	0.7	0.8	0.8	0.9
18:0	5.8	5.0	5.2	5.6	5.2
18:1t[d]	0.5	0.2	0.2		
18:1c[e]	53.2	53.4	55.6	54.5	54.7
nmi[f]-18:2	0.6	0.6	0.4	1.0	0.5
19:0, 18:2tt	0.8	0.8	0.8	1.2	0.9
18:2ct	0.4	0.6	0.4	0.5	0.4
19:1t, 18:2tc	0.8	0.1	0.4	0.5	0.6
19:1c	0.4	0.7	0.3	0.3	0.4
20:0	0.1	0.1	0.1	0.1	0.1
18:3	0.2	0.2	0.1	0.1	
20:1	0.5	0.4	0.4	0.2	0.4
conj[g] 18:2	0.9	0.4	0.7	0.8	0.8
others[h]	0.5	1.0	0.7	0.8	0.8

[a–h] Footnotes: see Table I.

with a cis acid (Laakso and Kallio, 1992). Thus, the SSM^t and SSM^c triacylglycerols were composed of different chromatographic peaks, as were the SM^cM^t and SM^cM^c molecules. In addition to the main components, Ag-HPLC fractions 3 and 4 apparently contained small amounts of triacylglycerols with one conjugated diene as their fatty acyl constituents. In silver ion chromatography, conjugated dienes were reported to behave as *cis*-monoenoic fatty acids when methyl esters of fatty acids (Christie, 1973) or triacylglycerols (Christie, 1988) were analyzed. The proportion of 18:2 with non-methylene-interrupted double bonds (nmi-18:2) in Ag-HPLC fractions 2 and 4 was elevated. Presumably also these dienoic fatty acids behave as monoenoic fatty acids.

The relative proportions of the triacylglycerols in relation to the degree of unsaturation in each Tirtiaux fraction are given in Table VIII. The high-melting solid fractions contained more than 50% saturated triacylglycerols, while the proportion was only 35–40% in the liquid fractions. The proportions of Ag-HPLC fractions containing unsaturated fatty acids, except for the disaturated *trans*-monoenoic triacylglycerols, were all increased in the liquid fractions. The most important triacylglycerols affecting the physical properties of butterfat are evidently the saturated and disaturated *cis*-monoenoic molecules, representing together 79 (L2) to 87% (S1, S2) of the total triacylglycerols. Comparable results were obtained by gas chromatographic analyses of the solid and liquid fractions of butterfat produced by the Tirtiaux process (Kankare and Antila, 1988).

Reversed-Phase HPLC Analyses. After Ag-HPLC fractionation, the triacylglyercols were separated by reversed-phase HPLC. In this case, because of the uniform unsaturation of the sample molecules, separation is based

Table VII. Fatty Acid Compositions (Mole Percent) of the Polyunsaturated Triacylglycerols (Ag-HPLC Fraction 6) of Butterfat and Its Solid (S1, S2) and Liquid (L1, L2) Tirtiaux Fractions

fatty acid	S1	butter	L1	S2	L2
4:0	3.9	4.1	4.1	3.9	4.3
6:0	1.9	2.0	2.6	1.8	2.3
8:0	0.9	0.9	1.1	0.8	0.8
10:0	1.9	1.6	1.5	1.2	1.7
10:1	0.7	0.7	0.7	0.7	0.9
12:0	1.4	1.5	1.4	1.2	1.2
12:1	0.1	0.2	0.1	0.1	0.4
i[a]-14:0	0.3	0.1	0.1		0.1
14:0	4.6	4.6	4.6	4.0	3.7
i-15:0	0.1	0.1	0.3	0.1	0.1
14:1c,[b] ai[c]-15:0	1.4	1.4	1.5	1.4	1.7
15:0	0.4	0.4	0.4	0.4	0.4
i-16:0	0.1	0.2	0.1	0.1	0.1
16:0	12.4	12.0	11.6	10.8	9.2
16:1t[d]		0.1	0.1	0.1	0.4
i-17:0	0.3	0.3	0.4	0.2	0.6
16:1c	1.7	1.8	1.8	1.7	2.0
ai-17:0	0.2	0.2	0.2	0.2	0.3
17:0	0.3	0.6	0.4	0.3	0.4
17:1	0.5	0.8	0.5	0.4	0.6
18:0	6.6	5.5	5.5	6.4	4.3
18:1t	3.4	2.3	2.2	3.3	3.3
18:1c[e]	32.8	32.5	32.9	35.5	37.7
nmi[f]-18:2	0.9	1.0	0.9	1.5	1.5
19:0, 18:2tt	1.4	2.0	1.5	2.2	2.4
18:2ct	0.7	1.0	0.9	1.0	1.0
18:2cc	17.2	16.6	17.0	16.1	14.2
19:1c		0.7	0.2		
20:0	0.2	0.2	0.2		0.2
18:3	2.2	2.6	2.6	2.1	1.6
20:1	0.2	0.3	0.3	0.3	0.2
conj[g] 18:2	0.6	0.5	0.7	1.4	1.1
others[h]	0.5	1.4	1.6	1.0	1.5

[a–h] Footnotes: see Table I.

Table VIII. Proportions (Weight Percent) of Triacylglycerol Fractions Obtained by Silver Ion HPLC from Butterfat and Its Solid (S1, S2) and Liquid (L1, L2) Tirtiaux Fractions

silver ion HPLC fraction[a]	S1	butter	L1	S2	L2
1 (SSS)	58.9	44.9	39.7	53.8	35.0
2 (SSM^t)	2.8	3.4	2.7	2.5	2.5
3 (SSM^c)	27.9	38.2	41.4	33.3	44.1
4 (SM^cM^t)	2.1	2.5	2.7	1.8	4.4
5 (SM^cM^c)	5.4	6.1	8.7	5.2	9.1
6 (others)	2.8	4.9	4.8	3.4	5.0

[a] Abbreviations: S, saturated; M^c, *cis*-monoenoic, M^t, *trans*-monoenoic fatty acyl residue.

mainly on the combined chain lengths of the fatty acyl residues. The reversed-phase HPLC analyses of the most abundant Ag-HPLC fractions, SSS and SSM^c, of each Tirtiaux fraction and butterfat were repeated three times. The other Ag-HPLC fractions were analyzed only once due to the small sample size. In addition, each SSS and SSM^c sample was analyzed after spiking with a mixture of monoacid-saturated triacylglycerols (3 × C8:0, 3 × C10:0, 3 × C12:0, 3 × C14:0, 3 × C16:0, 3 × C18:0) to calculate the retention index (I_R) of each chromatographic peak

$$I_R(x) = \mathrm{ACN}(z) + B\frac{\log t_r(x) - \log t_r(z)}{\log t_r(z+1) - \log t_r(z)}$$

where x is the particular triacylglycerol, z is the reference triacylglycerol eluting before x, $z + 1$ is the reference triacylglycerol eluting after x, ACN(z) is the acyl carbon number of z, B is ACN(z + 1)–ACN(z), and t_r is the retention time in minutes.

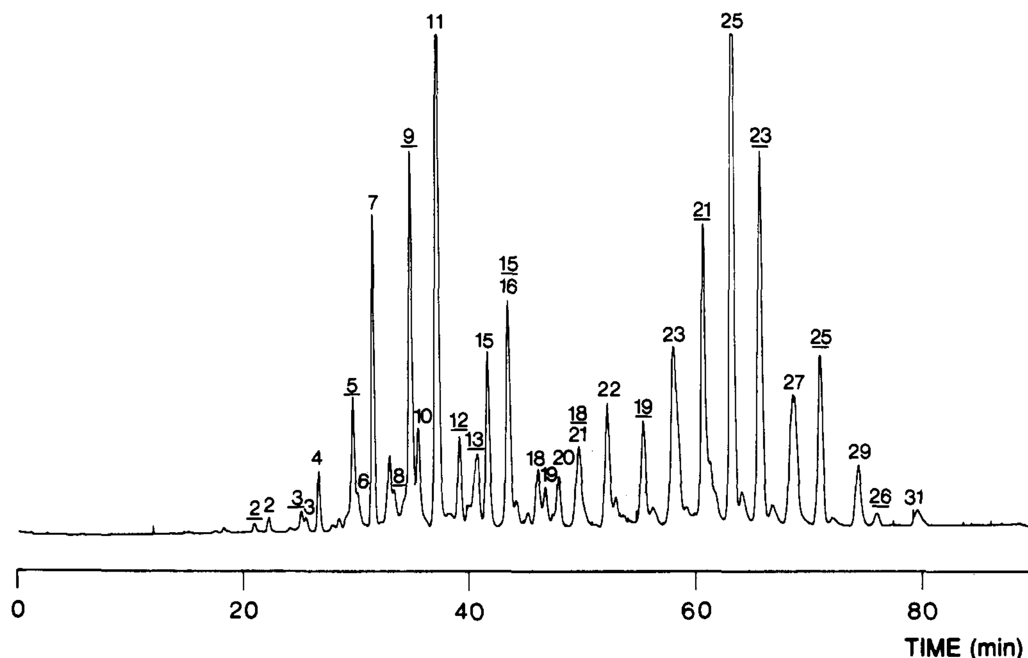

Figure 2. Separation of total butterfat triacylglycerols by reversed-phase HPLC and light-scattering detection. The experimental conditions are given under Materials and Methods. The places where the main trisaturated (nonunderlined numbers) and disaturated *cis*-monoenoic (underlined numbers) triacylglycerols elute are shown with numbers which refer to Figures 3 and 4, respectively.

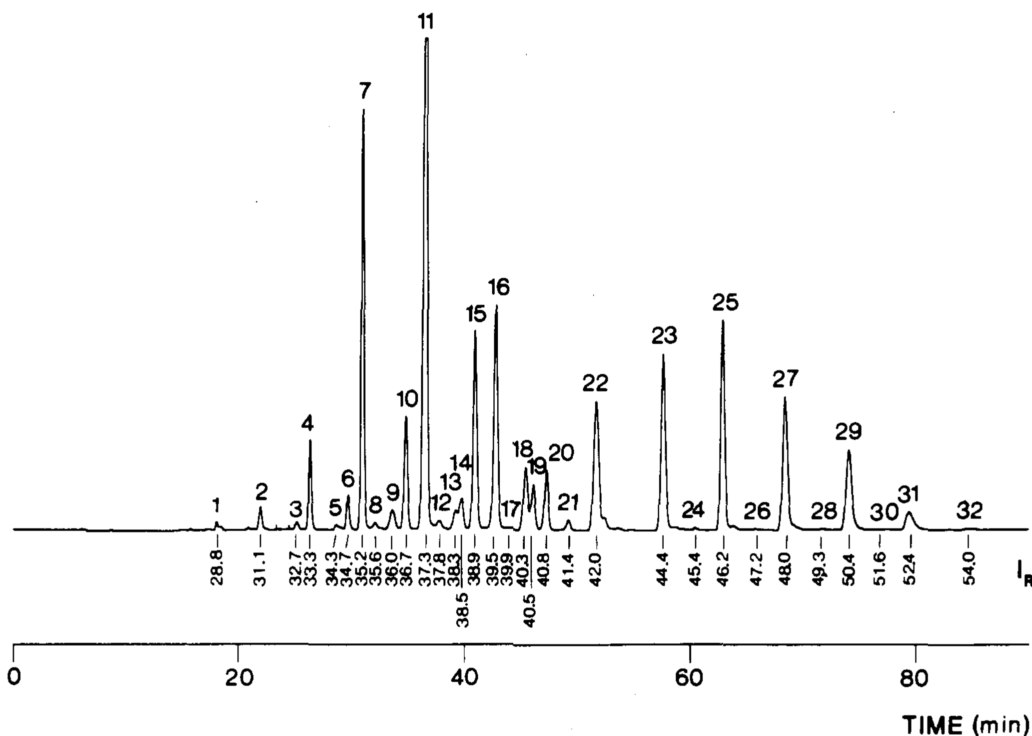

Figure 3. Reversed-phase HPLC separation and retention indices (I_R) of the trisaturated triacylglycerols (Ag-HPLC fraction 1) of butterfat obtained by reversed-phase HPLC. The experimental conditions are given under Materials and Methods.

The retention indices approximated to the acyl carbon numbers of the saturated triacylglycerols. The retention indices of the unsaturated molecules were not true estimates of the combined chain lengths of their fatty acyl residues, because each double bond reduced the retention of the triacylglycerol by the stationary phase in reversed-phase analyses. We found that the effect of one double bond was equivalent to that of two to three methylene groups. The separation of total butterfat triacylglycerols by reversed-phase HPLC is shown in Figure 2. Characterization of the components is difficult because of the separation according to both degree of unsaturation and combined chain lengths of the fatty acyl moieties. Simplified reversed-phase HPLC separations of the SSS and SSM[c] fractions of butterfat obtained by Ag-HPLC, together with the retention indices, are shown in Figures 3 and 4, respectively. Although the separation was based merely on the total number of acyl carbons, the asymmetry of the molecules containing short-chain-length fatty acids had an interfering effect. Also, the presence of odd-chain fatty acids rendered the interpretation problematic. The proportion of each chromatographic peak was calculated from its peak area with no correction for detector response. This procedure was satisfactory for the intercomparison of butterfat and its solid and liquid fractions.

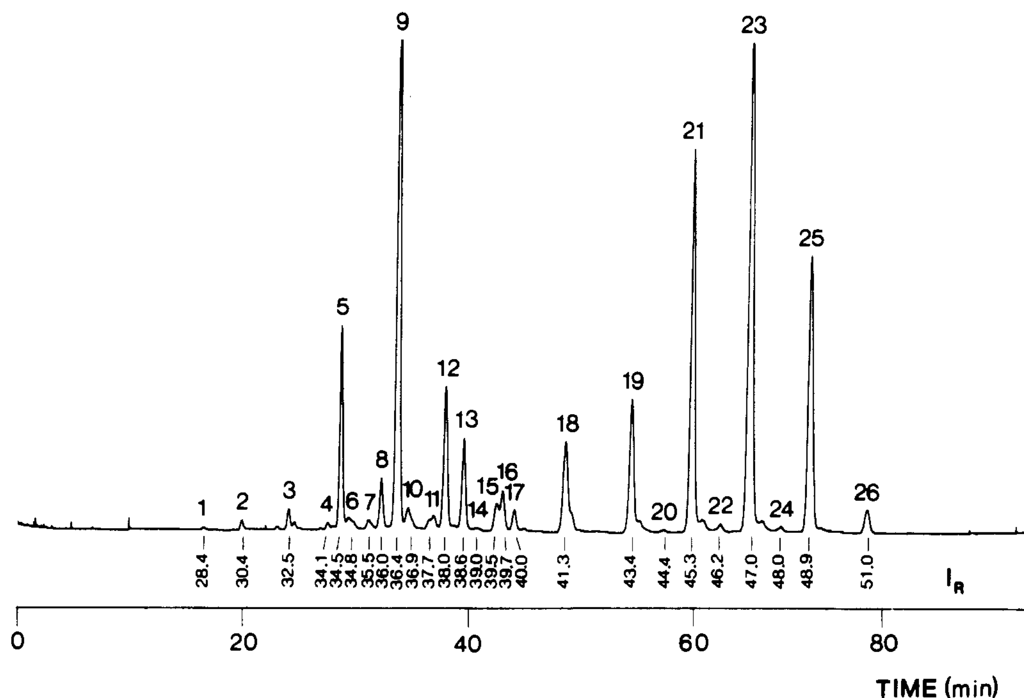

Figure 4. Reversed-phase HPLC separation and retention indices (I_R) of the disaturated *cis*-monoenoic triacylglycerols (Ag-HPLC fraction 3) of butterfat obtained by reversed-phase HPLC. The experimental conditions are given under Materials and Methods.

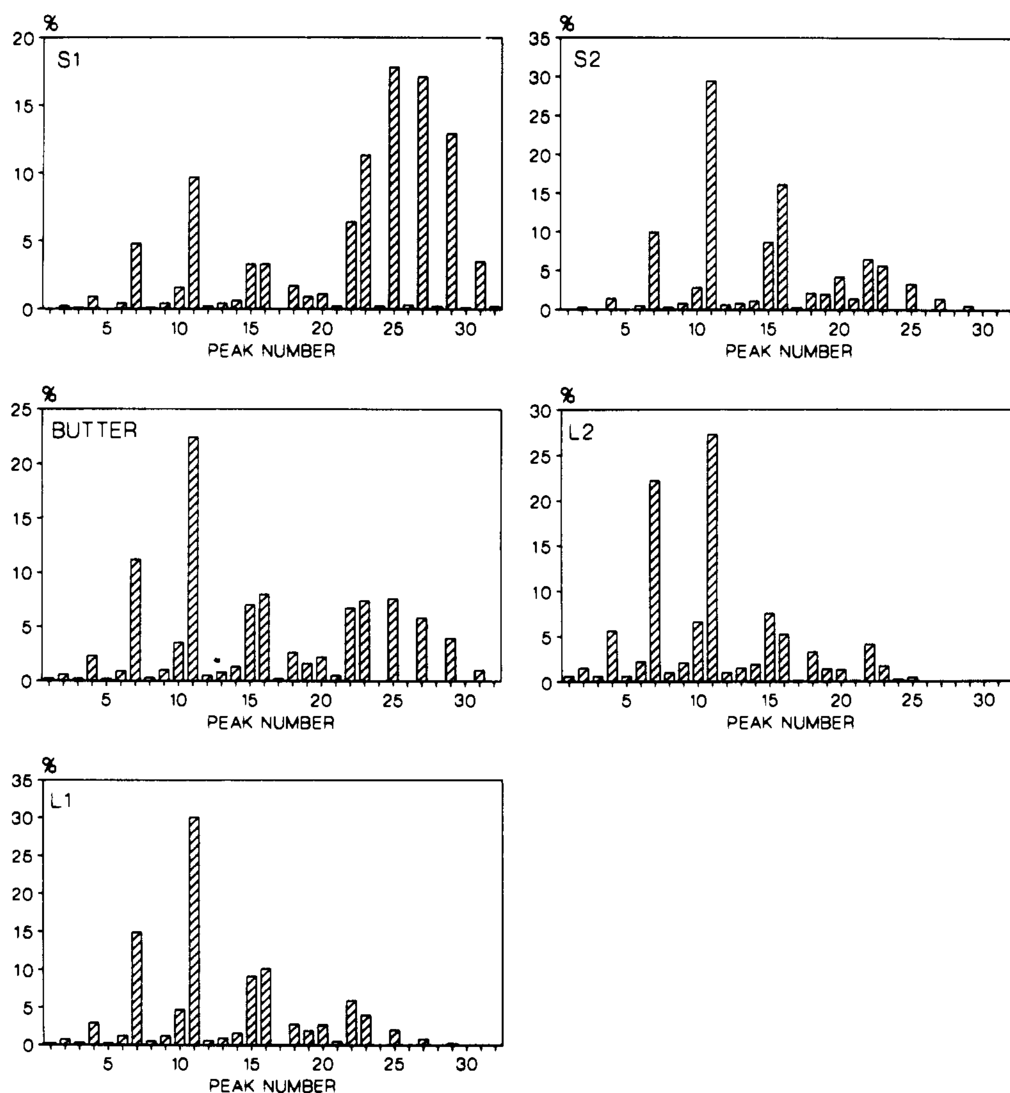

Figure 5. Distribution of the saturated triacylglycerols of butterfat and its solid (S1, S2) and liquid (L1, L2) fractions in relation to their separation by reversed-phase HPLC. Peak numbers refer to those shown in Figure 3.

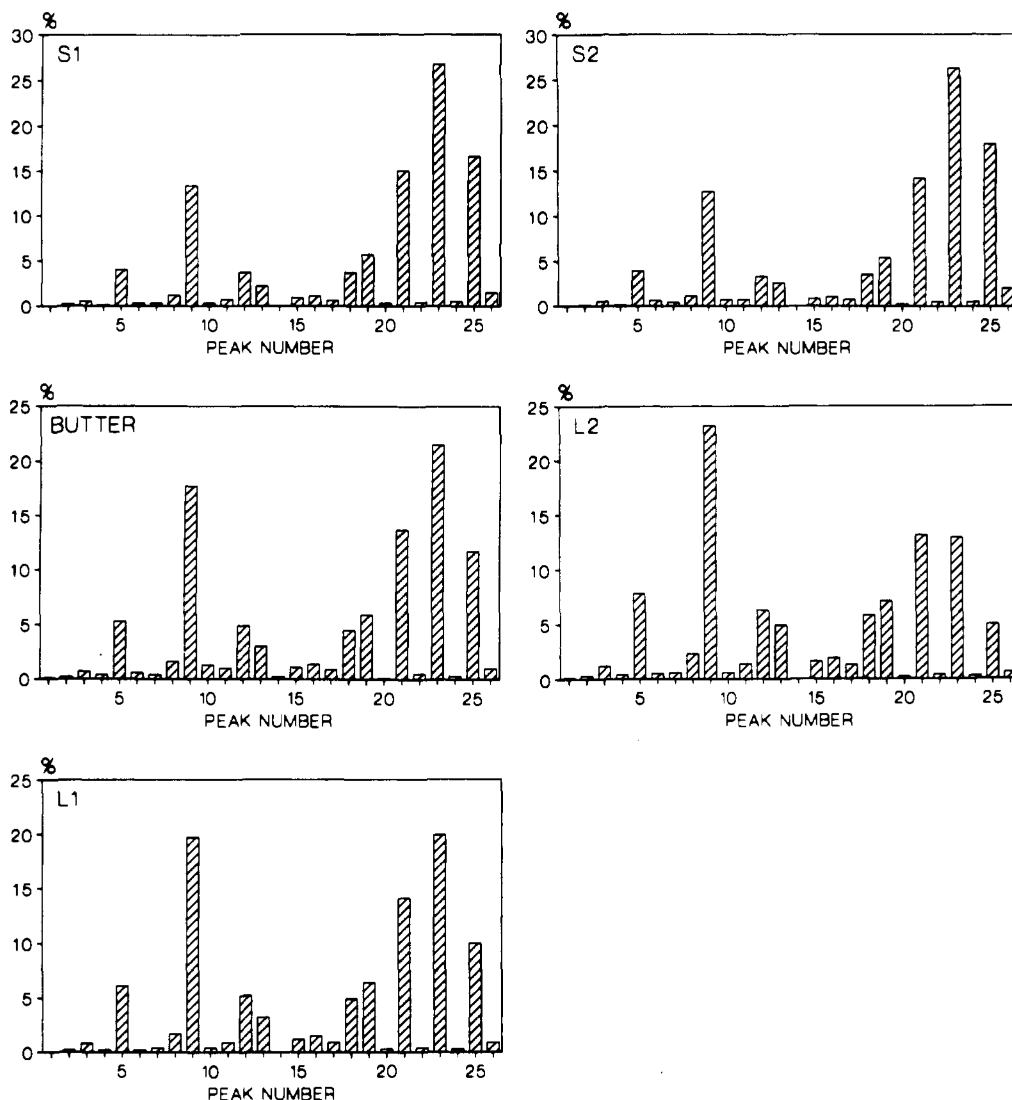

Figure 6. Distribution of the disaturated *cis*-monoenoic triacylglycerols of butterfat and its solid (S1, S2) and liquid (L1, L2) fractions in relation to their separation by reversed-phase HPLC. Peak numbers refer to those shown in Figure 4.

The distributions of the trisaturated and disaturated *cis*-monoenoic triacylglycerols in relation to the reversed-phase HPLC separations are given in Figures 5 and 6. The peak numbers in Figures 3 and 4 correspond to the numbers of the columns in Figures 5 and 6, respectively. The trisaturated molecules of butterfat and its solid and liquid fractions were composed of identical reversed-phase HPLC peaks in varying proportions. The same phenomenon was observed with all of the other silver ion HPLC fractions. Supporting evidence was the high reproducibility of the retention indices of the corresponding chromatographic peaks of a certain Ag-HPLC subfraction isolated from each of the Tirtiaux fractions. The reversed-phase HPLC method used was highly reproducible: the relative standard deviations of peaks representing 1.0 mol % or more averaged 2.3%. As expected, the deviations of small components (less than 1.0 mol %) were greater, in this case 11.7% on average.

Saturated Triacylglycerols. Because the majority of the triacylglycerols of butterfat and its Tirtiaux fractions are saturated and disaturated *cis*-monoenoic molecules, their composition will be discussed in more detail. The saturated triacylglycerols of butterfat had I_R values of 28.8–52.4 (Figure 3). The most abundant components were peaks 11 (I_R = 37.3) and 7 (I_R = 35.2), comprising 22.4 and 11.2% of the total, respectively (Figure 5). The composition of the S1 fraction was different, nearly 64% of it having I_R values of 44.4 or higher (peaks 23–32), compared with 26% in butterfat. Molecules with a maximum of 54 acyl carbon atoms including tristearin and some high molecular weight compounds containing, possibly, odd-chain fatty acyl residues, were found only in S1. Also, Banks et al. (1987) reported the absence of components with 54 acyl carbons from the saturated molecules of total milk fat. Among the low molecular weight components of S1, the most abundant (peaks 7 and 11) were the same as those which dominated in butterfat.

The distribution profile of the saturated triacylglycerols in the L1 fraction was very similar to that of butterfat, up to those molecules containing approximately 42 acyl carbons (peak 22). The two components present in the largest amounts in butterfat (peaks 7 and 11) dominated in L1 also, together representing 45% of the total. Similarly to S1, the saturated molecules of S2 consisted of more late-eluting and fewer early-eluting triacylglycerols than the starting material L1. The proportion of low molecular weight components in the L2 fraction was essentially greater than in L1: 70% of the components had I_R values of 37.3 (peak 11) or less. Compared with L1, the most marked differences in S2 and L2 were the proportions of peaks 7 and 16. Peak 7 (I_R value 35.2) comprised 10% of S2, 15% of L1, and 22% of L2. Peak 16 (I_R value 39.5) varied from 16% in S2 to 5% in L2. The proportion of the main component (peak 11) was almost

the same in all three fractions. Although the S2 and L2 fractions are compared here with L1, it should be noted that the L1 studied here was not their starting material.

All fractions except S1 showed a unimodal distribution of triacylglycerols in relation to the I_R values (Figure 5). The solid fractions manufactured by the Tirtiaux process always contain some liquid (20–30%) but not vice versa, which gives rise to the bimodal characteristics of the S1 fraction. The most abundant components of L1 (peaks 7 and 11) were present in relatively high proportions in S1 also. The same unimodal distribution was reported also by Banks et al. (1987), who studied the trisaturated molecules of whole milk fat obtained by preparing mercuric acetate adducts of the unsaturated components.

The average retention index, defining the number of acyl carbons, of the average saturated triacylglycerol in butter was 40.2, which falls between the 44.2 of S1 and the 38.2 of L1. The corresponding values for S2 and L2 were 39.1 and 37.2. The total proportion of molecules possibly containing butanoic acid (peaks 1–19) varied from 29% in S1 to over 90% in L2. According to the fatty acid analysis, 24% of S1 and 43% of L2 consisted of esters of butanoic acid. The results presented above agree well with the fatty acid compositions shown in Table II. The proportions of short-chain-length C_4–C_{10} fatty acids in the liquid fractions (28% in L1, 31% in L2) were higher than in the solid ones (18% in S1, 25% in S2). In contrast, the proportions of 14:0, 16:0, and 18:0 were higher in the solid fractions, with the exception that the largest 14:0 figure was found for L2. As expected, the composition of the butterfat lies between S1 and L1.

Disaturated *cis*-Monoenoic Triacylglycerols. The disaturated *cis*-monoenoic (SSM^c) triacylglycerols were resolved into 26 peaks by reversed-phase HPLC (Figure 4). The retention indices of the components varied from 28.4 to 51.0. The last component must have been composed of molecules with 54 acyl carbon atoms, because it coeluted with the reference triacylglycerol, 18:0–18:1–18:0. In this study the retention indices underestimate the real acyl carbon numbers by two to three carbon atoms. The profiles of the disaturated *cis*-monoenoic triacylglycerols (Figure 6) were substantially different from those of the saturated molecules. For instance, the distributions of the molecules were bimodal, and all samples contained the full range of SSM^c molecules, including high molecular weight components. Only the SSS and SSM^c profiles of the S1 fraction were intercomparable. The distributions of the SSM^c molecules in relation to the retention indices were much the same in all fractions except L2. The late-eluting peaks 21, 23, and 25 were among the most abundant components in all samples: 47% in butterfat, nearly 60% in solid fractions, 44% in L1, and 31% in L2. The differences were due mainly to the increased proportions of peaks 23 (I_R = 47.0) and 25 (I_R = 48.9) in the solid fractions, whereas the proportion of peak 21 (I_R = 45.3) was nearly the same in all samples. The major early-eluting component of the disaturated *cis*-monoenoic molecules eluted with a retention index of 36.4 (peak 9). The amount of this component in the liquid fractions was greater than in the solid fractions. It was also the most abundant component (23%) in the L2 fraction.

Paralleling the results for the saturated molecules, the average retention indices of the average SSM^c triacylglycerol of the solid fractions were greater (S1 = 43.7 and S2 = 43.8) than those of the liquid ones (L1 = 42.1 and L2 = 40.7). From a comparison of the SSM^c distributions, it is not surprising that the average I_R value of L1 was close to that of butterfat (42.4). The similarity of the

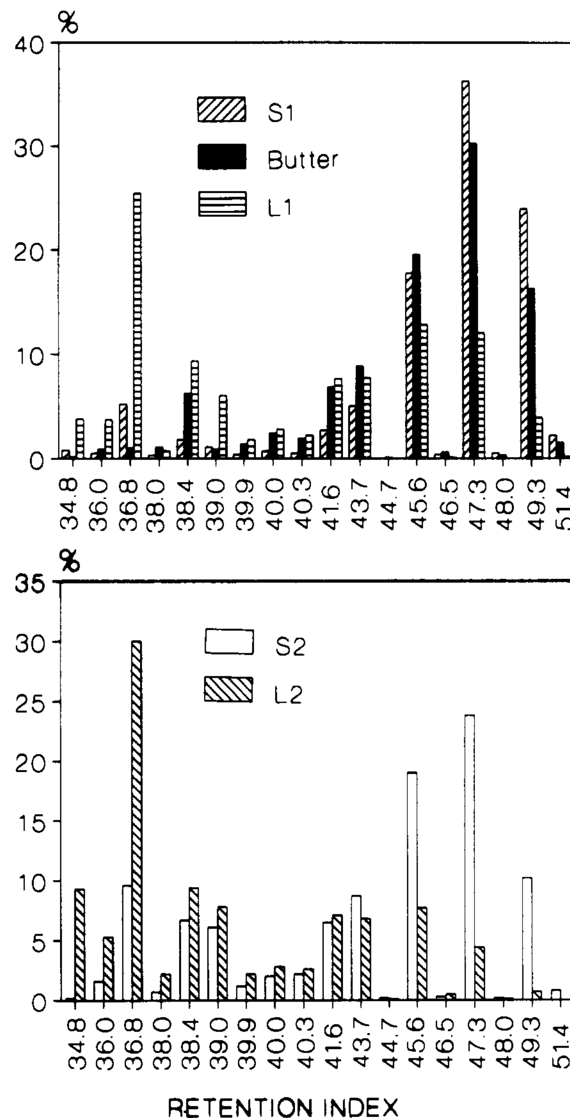

Figure 7. Distribution of the disaturated *trans*-monoenoic triacylglycerols of butterfat and its solid (S1, S2) and liquid (L1, L2) fractions in relation to their retention indices.

fatty acid compositions of butterfat and L1 (Table IV) supported the results obtained by reversed-phase HPLC. In addition, the S1 and S2 fractions had nearly identical fatty acid compositions, which was predictable from Figure 6. In general, the proportions of the short-chain-length fatty acids C_4–C_{12} and *cis*-18:1 in the liquid fractions were elevated, whereas the amounts of 16:0 and 18:0 were reduced.

Other Triacylglycerols. In addition to trisaturated and disaturated *cis*-monoenoic triacylglycerols, about 13% of the solid fractions and 20% of the liquid fractions were composed of SSM^t, SM^cM^t, SM^cM^c, and the more unsaturated molecules (Table VIII). The differences in the reversed-phase distributions of the disaturated *trans*-monoenoic triacylglycerols of butterfat and its fractions (Figure 7) were more pronounced than those of the disaturated *cis*-monoenoic triacylglycerols (Figure 6). The SSM^t molecules of the S1 fraction and butterfat contained elevated proportions of high molecular weight compounds, while low molecular weight compounds were enriched in the liquid fractions. In addition, the average acyl carbon numbers for the average SSM^t molecules were approximately two units smaller for the liquid fractions and two units greater for butterfat and S1 than the values for the corresponding SSM^c fractions. The fatty acid analyses

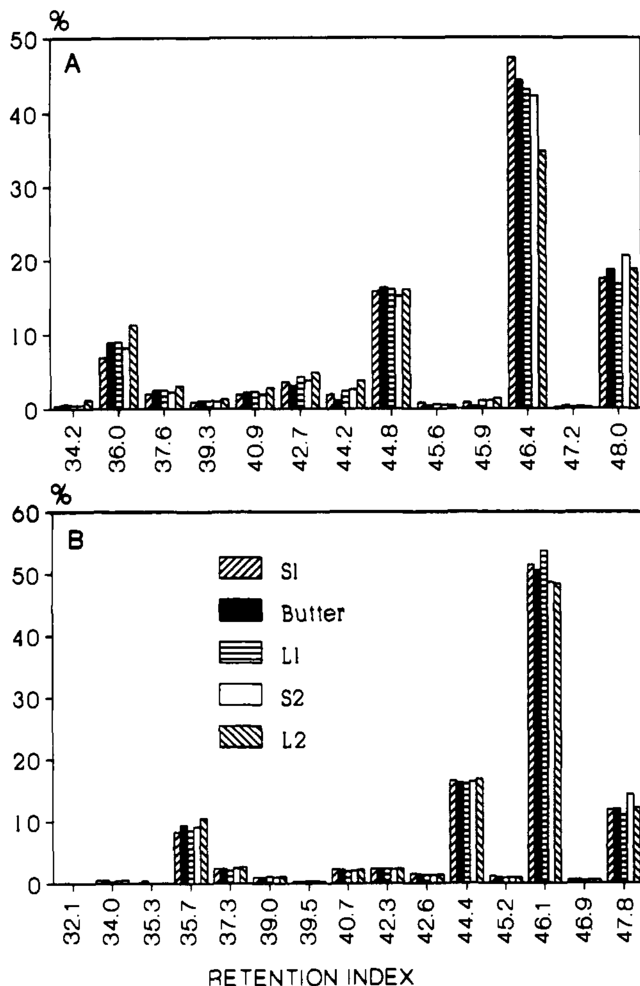

Figure 8. Distribution of (A) the saturated *cis,trans*-dimonoenoic and (B) the saturated *cis,cis*-dimonoenoic triacylglycerols of butterfat and its solid (S1, S2) and liquid (L1, L2) fractions in relation to their retention indices.

(Table III) were in good agreement with these results. Compared with disaturated *cis*-monoenoic molecules, the proportion of short-chain fatty acids (C_4–C_{12}) in the disaturated *trans*-monoenoic triacylglycerols of the liquid fractions was high, whereas in those of S1 and butterfat it was low. The differences in the proportion of the configurational isomers in the disaturated monoenoic triacylglycerols of S2 were not as distinct.

The three most abundant components, representing at least 70% of the saturated dimonoenoic triacylglycerols in each sample, consisted of molecules with 50, 52, and 54 acyl carbon atoms. All profiles of the reversed-phase HPLC separations of butterfat and its fractions were similar (Figure 8), as were the average retention indices (44.2–45.1). The differences in fatty acid composition between the SM^cM^t fractions (Table V) were small, and those between the SM^cM^c fractions (Table VI) even smaller. Also, the fatty acid compositions of the polyunsaturated triacylglycerols eluting in the last Ag-HPLC fraction (Table VII) were alike.

At the temperatures used for the Tirtiaux fractionation, saturated dimonoenoic and polyunsaturated molecules remain liquid because of their low melting points. After the crystals are separated from the liquid by filtration, the solid fraction always contains liquid, whereas the liquid fraction should be pure. This explains why the compositions of di- and polyunsaturated triacylglycerols of each Tirtiaux fraction are so similar.

CONCLUSIONS

The sequential combination of the chromatographic methods used in this study provides a better understanding of the chemical selectivity of the Tirtiaux process than all of these methods used in parallel. However, the incomplete separation of the liquid phases from the solid ones, the complexity of butterfat, and the large amount of data produced make the interpretation of results rather difficult. The most pronounced differences in the composition of the Tirtiaux fractions were found in the trisaturated triacylglycerols, which are the highest-melting components. There were differences too, albeit less pronounced, in the disaturated *cis*- and disaturated *trans*-monoenoic triacylglycerols. Although the di- and polyunsaturated molecules were not evenly distributed between the solid and liquid fractions, no clear selectivity according to their triacylglycerol structure was observed.

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