Triacylglycerols of Winter Butterfat Containing Configurational Isomers of Monoenoic Fatty Acyl Residues. I. Disaturated Monoenoic Triacylglycerols

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The triacylglycerols of winter butterfat were fractionated according to the type and degree of unsaturation into six fractions by silver ion high-performance liquid chromatography (Ag-HPLC). The acyl carbon number distribution of the triacylglycerols in each fraction was elucidated by reversed-phase HPLC and mass spectrometry (MS). The MS analysis of each fraction gave deprotonated triacylglycerol $[M - H]^-$ ions which were produced by chemical ionization with ammonia. The daughter spectrum of each of the $[M - H]^-$ ions provided information on its fatty acid constituents. Successful fractionation of triacylglycerols differing in the configuration of one fatty acyl residue by Ag-HPLC was important because geometrical isomers could not be distinguished by the MS system used. In addition to the fatty acid compositions, reversed-phase HPLC analysis demonstrated the purity of the collected fractions: molecules having a *cis-trans* difference were separated nearly to the baseline. Triacylglycerols differing in the configuration of one fatty acyl residue were not equally distributed in relation to their acyl carbon numbers. This indicates that during the biosynthesis of triacylglycerols, *cis*- and *trans*-fatty acids are processed differently. Although the fatty acid compositions of the corresponding molecular weight species of disaturated *trans*- and disaturated *cis*-monoenoic triacylglycerols were similar, there may be differences in the amounts of different fatty acid combinations or in the distribution of fatty acids between the primary and secondary glycerol positions. In addition to the main components, it was possible to analyze minor triacylglycerols, such as molecules containing one odd-chain fatty acid, by the MS system used.

KEY WORDS: Bovine, butterfat, *cis*-monoenoic fatty acid, molecular species, reversed-phase high-performance liquid chromatography, silver ion chromatography, tandem mass spectrometry, *trans*-monoenoic fatty acid, triacylglycerols.

The composition of butterfat triacylglycerols is so complex that the separation of all molecules by a single analytical method is impossible. In addition to the major fatty acids, odd and branched-chain as well as *trans*-fatty acids are characteristic constituents of bovine milk fat (1). Generally, combinations of analytical techniques that separate molecules on a different basis are most advantageous to obtain information about molecular species. Silver ion thin-layer chromatography has been used to fractionate milk fat triacylglycerols not only according to the degree of unsaturation but also according to the geometric configuration of the fatty acyl residues (2-8). The utility of silver ion fractionation of triacylglycerols prior to reversed-phase high-performance liquid chromatography (HPLC) (9,10), gas chromatography (GC) (2,3) and gas chromatographic-mass spectrometric (GC-MS) analysis (8) has been demonstrated. Recently, the availability of tandem mass spectrometry (MS/MS) for the analysis of triacylglycerols by collisional activation of negative ions (11,12) or positive ions (13) produced by chemical ionization has been reported. Combined with silver ion chromatography, MS and MS/MS give information about molecular weights of equally unsaturated triacylglycerols and their fatty acid constituents without chromatographic separation.

In the present study, the compositions of winter butterfat triacylglycerols, differing only in the configuration of one fatty acyl residue, were compared by means of reversed-phase HPLC, MS and MS/MS. Prior to these analyses, the triacylglycerols were fractionated according to the type and degree of unsaturation by silver ion HPLC to simplify the interpretation of the results.

MATERIALS AND METHODS

Samples and reagents. Anhydrous butterfat was prepared by rapidly melting fresh winter butter (Sotkamo, Finland) in a microwave oven, washing the oil with deionized water and drying over anhydrous sodium sulfate. The anhydrous oil was flushed with nitrogen before storing at −20°C.

Triacylglycerols were purified by elution from a short column of Florisil™ with 10 mL hexane/diethyl ether (4:1, vol/vol) as the mobile phase. After evaporating the solvent with a stream of nitrogen, the triacylglycerols were dissolved in 1,2-dichloroethane. All solvents were of HPLC grade and were supplied by Merck (Darmstadt, Germany) and Rathburn (Walkerburn, Scotland).

HPLC. HPLC analyses were performed with a Shimadzu LC-9A solvent delivery system (Kyoto, Japan) combined with a Shimadzu FCV-9AL low-pressure gradient elution unit, a Cunow DDL21 light-scattering detector (Cergy-Saint-Christophe, France) and a Shimadzu C-R5A integrator. A stream-splitter was installed between the column and the detector when fractions were collected.

Silver ion chromatography was performed by HPLC as described by Christie (14,15). The triacylglycerols were separated with a cation exchange column (Nucleosil™ 5SA, 25 cm × 4.6 mm i.d.; HPLC Technology, Macclesfield, United Kingdom) loaded with silver ions. The fractionation was carried out at ambient temperature with a binary gradient of (A) dichloromethane/1,2-dichloroethane (4:1, vol/vol) and (B) acetone. The linear gradient was 100% A to 80% A-20% B in 20 min and then to 100% B over a further 15 min. The final solvent composition was maintained for an additional 5 min. The flow rate was 1.0 mL/min. Approximately 1 mg of purified triacylglycerols dissolved in 10 μL 1,2-dichloroethane was injected onto the column. Fractionation was repeated several times, and the corresponding fractions were pooled to obtain sufficient material for further analyses.

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Reversed-phase HPLC analyses of the Ag-HPLC fractions were performed with two columns in series with ODS phase [250 mm × 4.6 mm i.d., spherical 5 μm particles; Zorbax™, Du Pont Inc. (Wilmington, DE); Spheri-5™, Brownlee Labs (Santa Clara, CA]. Triacylglycerols were separated at ambient temperature with a binary solvent gradient of (A) dichloromethane/1,2-dichloroethane (4:1, vol/vol) and (B) acetonitrile. The linear gradient was 30% A–70% B to 55% A–45% B over 65 min, then to 60% A–40% B over 10 min and finally to 65% A–35% B over a further 5 min, and held for 2 min. The flow rate was 0.8 mL/min. Samples were dissolved in 1,2-dichloroethane, and volumes of 10 μL or less were injected onto the column.

Fatty acid analysis. Fatty acid methyl esters were prepared by sodium methoxide-catalyzed transesterification in the presence of internal standard (methyl heneicosanoate; Serva 24576, Heidelberg, Germany) in hexane (16). Methyl esters were separated on a fused-silica capillary column (30 m × 0.32 mm i.d.) coated with 0.2 μm SP-2340™ phase (Supelco, Bellefonte, PA). The analyses were done on a Carlo Erba (Milan, Italy) 5160 gas chromatograph equipped with a Hewlett-Packard (Palo Alto, CA) 7673A injector and a flame-ionization detector. The injector and detector temperatures were 250°C, and the split ratio was about 25:1. The flow rate of the carrier gas (helium, 99.9999%) was raised from 1.5 to 2.1 mL/min after the emergence of methyl butyrate. The temperature was programmed after the elution of methyl butyrate from 70 to 120°C at 30°C/min, to 160°C at 10°C/min, and finally to 190°C at 2°C/min. The response correction factor for each fatty acid methyl ester was determined by analyzing a butter oil of known fatty acid composition (CRM 164; Brussels, Belgium). The fatty acid compositions are expressed as mol%.

MS and MS/MS analysis. Negative ion chemical ionization (NICI) spectra and collision-activated spectra were obtained with a Finnigan MAT TSQ-700 instrument (San Jose, CA). Approximately 1 μg of triacylglycerols dissolved in hexane was injected onto the direct exposure probe (DEP) wire. After the solvent was evaporated, the analysis was started by increasing the DEP temperature in the ion source to 300°C at a rate of 190°C/min. Negative ions were produced at 180°C with ammonia under the ion source pressure of about 8.5 torr. The ionization energy was 70 eV, and emission current was 300 μA. The NICI spectra were averaged and displayed.

Argon was used as the collision gas in the second quadrupole at a pressure of about 0.4 millitorr. The offset for the collision quadrupole was 13 eV. Daughter spectra of $[M - H]^-$ parent ions were obtained over a period of one minute (after 30 s of the sample introduction), while the scan time was 0.5 s, after which the spectra were averaged.

RESULTS AND DISCUSSION

Silver ion chromatography. Silver ion chromatography is a useful method for lipid analysis because it permits the separation of distinct molecular fractions that differ mainly in degree of unsaturation. In addition to the number of double bonds, the isomeric positioning of the double bonds in the fatty acyl residues as well as the position at which the fatty acid is bound to the glycerol backbone are important (17,18). The separation is based on the weak interactions between the π-electrons of the double bonds and the silver ions. The separation of the triacylglycerols of winter butterfat by Ag-HPLC into six fractions is shown in Figure 1. The fatty acid compositions (mol%) of the total butterfat and the six Ag-HPLC fractions as well as their proportions (wt%) are presented in Table 1. The fatty acids representing less than 0.1 mol%, together with unknowns, are reported as "Others." Identification of the fatty acid components was based on the known composition of butterfat (19) and the separation characteristics of the SP-2340™ stationary phase (20). The detector response correction factors determined for the fatty acids were of internationally accepted values (21). The composition of the reconstituted triacylglycerols is similar to that of the original winter butterfat triacylglycerols. Thus no selective losses occurred during silver ion fractionation.

The first Ag-HPLC fraction contained trisaturated molecules and it comprised 39.3% of all the triacylglycerols. The second and the third Ag-HPLC fractions, 4.0 and 37.9% respectively, consisted of molecules with two saturated and one monoenoic fatty acyl residues. Ag-HPLC fractions four (4.5%) and five (9.5%) were comprised mainly of molecules with one saturated and two monoenoic fatty acid constituents. The more unsaturated triacylglycerols were concentrated in the last Ag-HPLC fraction (4.8%).

Compared with monoenoic fatty acids with the *cis*-configuration, *trans*-monoenoic fatty acids form weaker π-complexes and behave more like saturated fatty acids (17,22). Triacylglycerols differing only in the configuration of one fatty acyl moiety have been separated from milk fat by silver ion thin-layer chromatography (2–8). An improved separation was achieved by Ag-HPLC in an analysis of the triacylglycerols of sheep adipose tissue (15). The fatty acid compositions of Ag-HPLC Fractions 2 and 3 were similar, as were the compositions of Ag-HPLC Fractions 4 and 5 (Table 1). The most distinctive difference

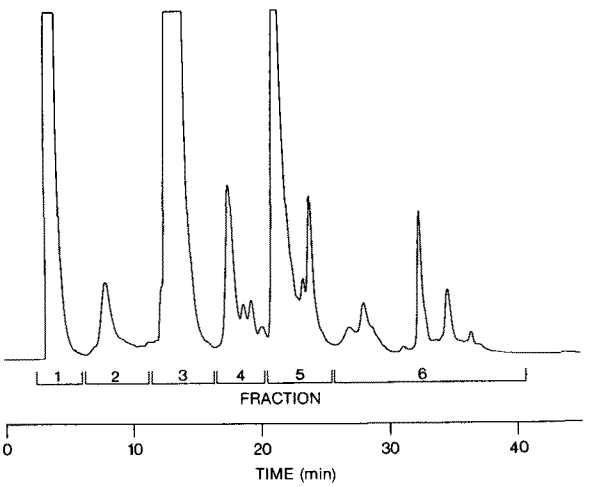

FIG. 1. Separation of the triacylglycerols of winter butterfat into six fractions according to the type and degree of unsaturation, obtained by silver ion high-performance liquid chromatography. The experimental conditions are given in the Materials and Methods section.

TABLE 1

Fatty Acid Composition (mol%) of the Triacylglycerols of Winter Butterfat and of Its Fractions Obtained by Silver Ion High-Performance Liquid Chromatography (HPLC)

Fatty acid	Total	Silver ion HPLC fraction						Reconstructed composition
		1	2	3	4	5	6	
4:0	8.3	10.3	4.8	9.3	3.4	4.3	4.9	8.6
6:0	3.8	4.9	4.8	3.5	1.6	1.9	2.4	3.9
8:0	1.6	2.2	1.9	1.5	0.6	0.8	0.8	1.7
10:0	3.0	4.1	3.2	2.5	1.0	1.3	1.3	3.0
10:1	0.3			0.4	0.3	0.7	0.6	0.3
12:0	2.9	4.2	2.9	2.5	1.0	1.2	1.2	3.0
12:1	0.1			0.1		0.2	0.1	0.1
13:0	0.1	0.1	0.1	0.1				0.1
i[a]-14:0	0.1	0.1	0.1	0.1		0.1		0.1
14:0	10.0	14.7	9.5	8.6	4.0	4.4	3.7	10.4
i-15:0	0.2	0.4	0.3	0.2	0.1	0.1	0.3	0.3
14:1c[b], ai[c]-15:0	1.2	0.5	0.4	1.6	1.0	2.4	1.5	1.1
15:0	0.8	1.2	0.8	0.8	0.4	0.5	0.4	0.9
i-16:0	0.2	0.3	0.2	0.2	0.1	0.1	0.1	0.2
16:0	25.1	36.8	21.6	21.6	12.0	11.8	9.5	26.1
16:1t[d]	0.1		0.8	0.1	0.6		0.1	0.1
i-17:0, 16:1c	1.5	0.4	0.7	2.1	1.9	3.5	2.2	1.5
ai-17:0	0.4	0.5	0.4	0.4	0.2	0.3	0.2	0.4
17:0	0.6	0.8	0.6	0.6	0.4	0.3	0.3	0.6
17:1	0.3			0.4	0.9	0.8	0.5	0.3
18:0	12.2	17.1	12.4	10.7	8.0	6.1	4.7	12.6
18:1t	2.5	0.3	19.7	1.7	18.0	0.1	4.2	2.5
18:1c[e]	20.1	0.1	10.2	28.6	34.9	52.7	36.3	18.9
nmi[f]-18:2	0.6	0.2	3.1	0.4	2.4	0.5	1.2	0.5
19:0, 18:2t,t	0.4	0.1	0.1	0.1	0.3	1.0	1.6	0.2
18:2c,t	0.1			0.1	0.3	0.4	1.0	0.1
19:1t, 18:2t,c	0.1		0.1		0.9	0.2		0.1
18:2c,c	1.3					2.2	15.4	0.9
19:1c	0.1			0.2	0.3	0.3	0.5	0.1
20:0	0.3	0.3	0.2	0.2	0.6	0.1	0.1	0.3
18:3	0.5					0.1	2.5	0.1
20:1	0.2			0.2	0.7	0.5	0.3	0.1
conj[g]-18:2	0.5		0.1	0.9	3.2	0.5	1.0	0.6
Others[h]	0.5	0.3	1.8	0.5	1.4	0.5	1.0	0.4
wt% of the total		39.3	4.0	37.9	4.5	9.5	4.8	

[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* 18:1n-9 Coelutes with some *trans*-isomers, such as 18:1n-3, 18:1n-4 and 18:1n-5 (Ref. 24).
[f]nmi = Nonmethylene interrupted.
[g]conj = Conjugated.
[h]Fatty acids which did not reach 0.1 mol% in any sample, together with the unknowns, are reported as others.

was in the proportion of *cis*- and *trans*-monoenoic fatty acyl moieties, mainly 18:1. The earlier eluting Ag-HPLC Fractions 2 and 4 had higher proportions of *trans*-acids than Ag-HPLC Fractions 3 and 5. Thus, Ag-HPLC Fractions 2, 3, 4 and 5 from butterfat were identified as predominantly disaturated *trans*-monoenoic (SSM^t), disaturated *cis*-monoenoic (SSM^c), saturated *cis,trans*-dimonoenoic (SM^cM^t) and saturated *cis,cis*-dimonoenoic (SM^cM^c) triacylglycerols, respectively. The abbreviation S is a saturated, M^t a *trans*-monoenoic and M^c a *cis*-monoenoic fatty acyl residue. In addition to the main components, Ag-HPLC Fraction 4 contained small amounts of triacylglycerols having one dienoic fatty acyl residue with a conjugated double-bond system. Conjugated dienes were reported to behave as *cis*-monoenoic fatty acyl moieties when methyl esters of fatty acids (23) and triacylglycerols (15) were analyzed.

The fatty acid compositions presented in Table 1 were obtained by analysis with the SP-2340™ stationary phase. With this phase, the proportions of *trans*-acids were underestimated because of incomplete separation of geometrical isomers, especially 18:1 (24). The main *trans*-iosmer present in milk fat, 18:1n-7, was well separated, but *trans* 18:1n-3, *trans* 18:1n-4 and *trans* 18:1n-5 isomers coeluted with the major *cis*-isomer 18:1n-9. An improved separation of geometrical isomers with the SP-2560™ phase has been reported (25), but *trans* 18:1n-4 and *trans* 18:1n-5 still coelute with *cis* 18:1n-9. A preliminary

analysis with this phase confirmed that none of the major components in Ag-HPLC Fraction 2 had a retention time equal to that of *cis* 18:1n-9.

Reversed-phase HPLC. Each of the fractions obtained by Ag-HPLC was analyzed by reversed-phase HPLC, in which the order of elution is related mainly to the combined chainlengths of the fatty acyl residues. Generally, reversed-phase HPLC separates molecules also according to the degree of unsaturation, the double bonds reducing the retention of the molecule by the stationary phase.

The reversed-phase HPLC resolution of the triacylglycerol fractions of butterfat was not as complete as expected. The chainlength asymmetry of the triacylglycerols arising from their short-chain fatty acid moieties interfered with the separation. To facilitate the identification of the molecules, retention indices were calculated for each chromatographic peak according to the saturated, monoacid triacylglycerols 3 × 8:0, 3 × 10:0, 3 × 12:0, 3 × 14:0, 3 × 16:0 and 3 × 18:0 (26). These indices gave information on the approximate acyl carbon numbers of the corresponding saturated triacylglycerols. The odd-chain fatty acid constituents of butterfat made the identification of its components even more difficult.

The reversed-phase HPLC profiles of Ag-HPLC Fractions 2 and 3 were much alike (Fig. 2A and B). When the samples were mixed and reanalyzed, each of the original peaks formed a double peak (Fig. 2C). This separation has to be due to the difference in the configuration of one fatty acyl residue, because these two fractions differed substantially only in the proportion of *cis*- and *trans*-monoenoic fatty acids. Because the chemical properties of *trans*-monoenoic fatty acids resemble those of saturated ones, it is evident that triacylglycerols containing *trans*-acids elute after the corresponding molecules having *cis*-acids. Baseline separations of triolein-trielaidin (27) and triolein-tripetroselin (28) mixtures have been reported, as well as partial separations of molecules differing in the configuration of one fatty acyl residue (28,29). In the present study, a near-baseline separation of SSM^t and SSM^c molecules of butterfat was achieved. Although the SSM^t fraction was a minor one, it was possible to isolate it by Ag-HPLC without contamination by the larger SSM^c fraction: the reversed-phase HPLC chromatogram of SSM^t showed the absence of any component eluting just before each major peak (Fig. 2A). On the other hand, the SSM^c fraction may have been contaminated with SSM^t, because the main peaks carried small, tailing shoulders (Fig. 2B).

The reversed-phase HPLC analyses of Ag-HPLC Fractions 4 and 5 confirmed the conclusions, based on their fatty acid analyses, that these two fractions differ essentially only in the configuration of one monoenoic fatty acyl residue (Fig. 3A and B). The analysis of the mixture of SM^cM^t and SM^cM^c gave a peak dualism (Fig. 3C) similar to that of the disaturated monoenoic triacylglycerols. Compared with Ag-HPLC Fraction 5, the reversed-phase chromatogram of Ag-HPLC Fraction 4 contained a few extra peaks, possibly representing molecules that contained a conjugated diene.

Reversed-phase HPLC proved to be useful in checking the purity of the Ag-HPLC fractions. When disaturated *cis*- and disaturated *trans*-monoenoic triacylglycerols were collected as one fraction in real proportions, the presence of molecules having *trans*-acids could still be detected. The same phenomenon was observed with saturated *cis,cis*- and saturated *cis,trans*-dimonoenoic triacylglycerols. With the reversed-phase HPLC system used, the effect of one double bond on the retention of the molecule was equivalent to approximately two to three methylene groups: 18:0-18:0-18:1 eluted midway between triacylglycerols of acyl carbon numbers 50 and 52, and 18:0-18:1-18:1 coeluted with molecules of 48 acyl carbons. The difference in the configuration of one fatty acyl residue resulted in a difference of 0.3 to 0.4 in the retention indices of the molecules, which is in good agreement with the results reported by Podhala and Töregård (29). These data were used to classify the triacylglycerols according to their acyl carbon numbers.

MS and MS/MS. NICI-MS is a fast and sensitive method for the analysis of mixtures of triacylglycerols (11,12,30,31). The triacylglycerols of winter butterfat were analyzed with this method after fractionation of the sample by Ag-HPLC. In the first stage of the analysis, the triacylglycerols were chemically ionized with ammonia to produce pseudomolecular $[M - H]^-$ ions. The total number of acyl carbon atoms and the number of double bonds in the fatty acyl residues in each $[M - H]^-$ ion could be measured.

The major ions of the NICI spectra of the trisaturated, disaturated *cis*-monoenoic and disaturated *trans*-monoenoic triacylglycerols (Fig. 4A-C), 28 mass units apart from each other, represented the deprotonated triacylglycerols. Ions with *m/z* values one or two mass units higher than that of the corresponding $[M - H]^-$ represented molecules that contained one or two ^{13}C isotopes, respectively. The minor ions, such as *m/z* 596, 624, 652 and 680 (Fig. 4A) represented triacylglycerols with an odd number of acyl carbons. Trisaturated molecules contained the highest proportion of odd carbon number molecules (16%), but similar compounds were detected in lower abundances from each NICI spectra (Fig. 4B and C).

The upper part of the NICI spectrum was clear and readily interpreted. The area of low-molecular weights, $[M - H]^-$ smaller than 578-582, was more complex because of the formation of pyrolysis products of the higher-molecular weight compounds during distillation of the triacylglycerols from the heated direct exposure probe. For quantitative purposes, the selected $[M - H]^-$ ions were monitored over the whole period of analysis. As an example, the displayed scans of SSM^t are shown (Fig. 5). The peaks of the mass chromatogram of the low mass ions had two maxima: the first originated from the deprotonated low-molecular weight triacylglycerol, and the latter represented the pyrolysis products. In this way, the proportion of the pyrolysis products can be deducted from the proportion of $[M - H]^-$ ions. The $[M - H]^-$ ions were produced typically between 40 and 80 s, depending on the molecular size (Fig. 5). When the analysis was repeated, the positions and duration of the maxima showed slight variability. Thus, it is important to average the NICI spectra over the whole period of evaporation. In this study, the ions *m/z* 552, 580 and 608 of the fractions SSM^t and SSM^c only were corrected for pyrolysis products.

In the second stage of the MS analysis, each $[M - H]^-$ parent ion was collision-activated with argon to produce fragment ions, mainly RCO_2^-, $[M - H - RCO_2H - 100]^-$, $[M - H - RCO_2H - 74]^-$ and $[M - H -$

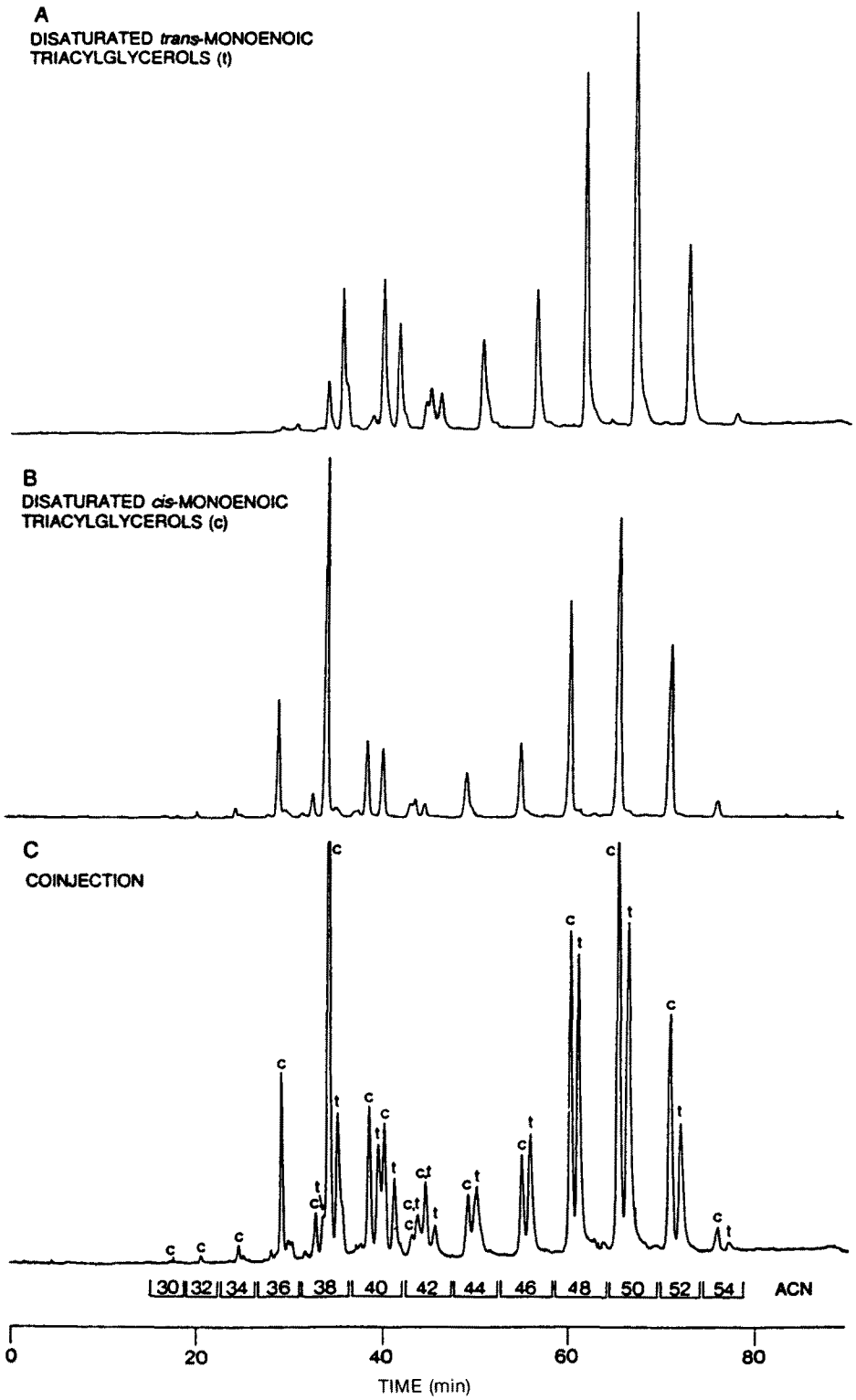

FIG. 2. Reversed-phase high-performance liquid chromatography separations of (A) the disaturated *trans (t)*-monoenoic, (B) disaturated *cis (c)*-monoenoic triacylglycerols and (C) a mixture of samples A and B. ACN = acyl carbon number. The experimental conditions are given in the Materials and Methods section.

$RCO_2H]^-$, providing information on the fatty acyl moieties. The molecular weights of the fatty acids and their combinations in the triacylglycerols were determined according to the RCO_2^- ions. The positions of the double bonds and the configurations of the fatty acids could not be determined by the MS/MS method used. Detailed information about the structure of fatty acids is available by GC and GC–MS analysis.

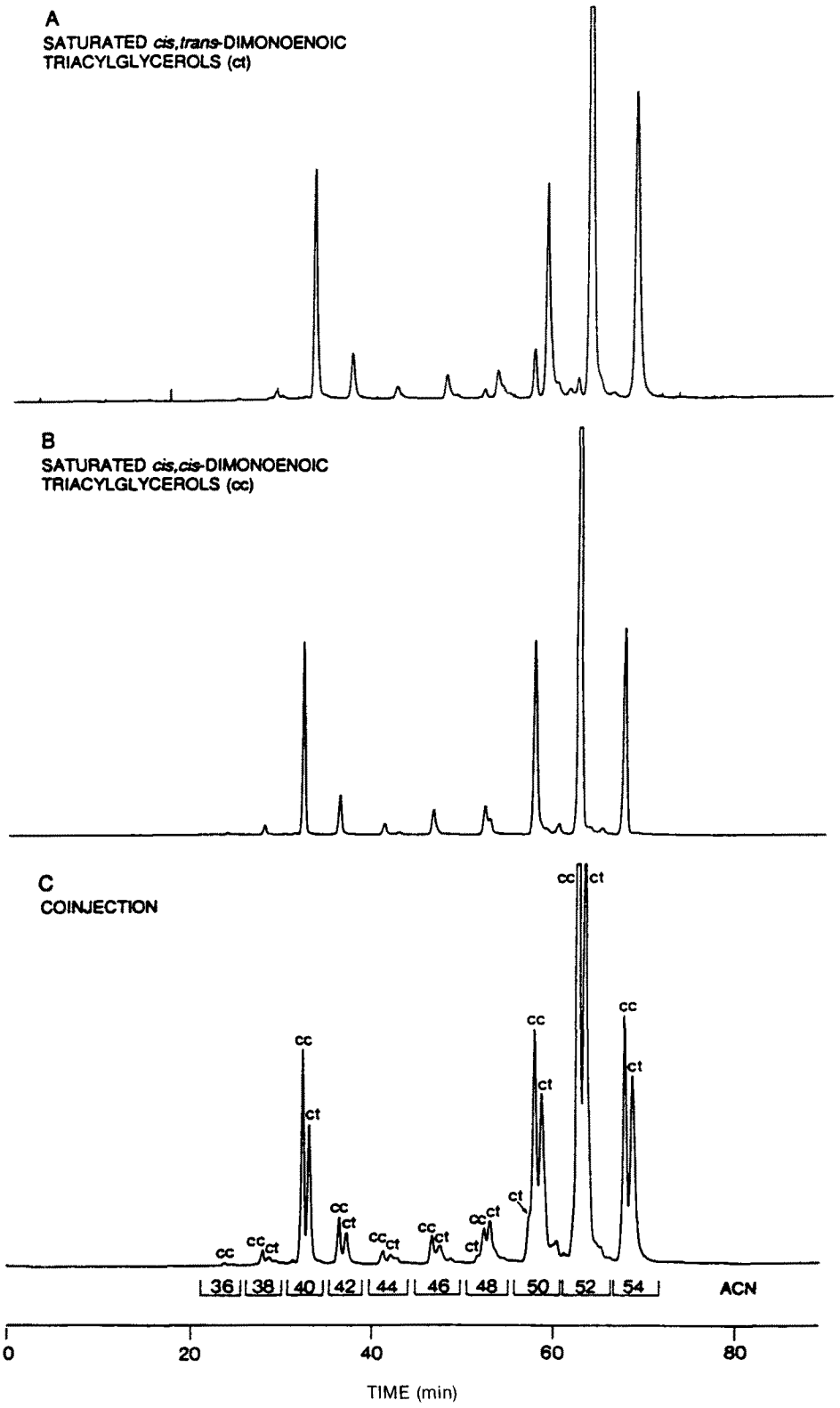

FIG. 3. Reversed-phase high-performance liquid chromatography separations of (A) the saturated *cis,trans (c,t)*-dimonoenoic, (B) saturated *cis,cis (c,c)*-dimonoenoic triacylglycerols and (C) a mixture of samples A and B. ACN = acyl carbon number. The experimental conditions are given in the Materials and Methods section.

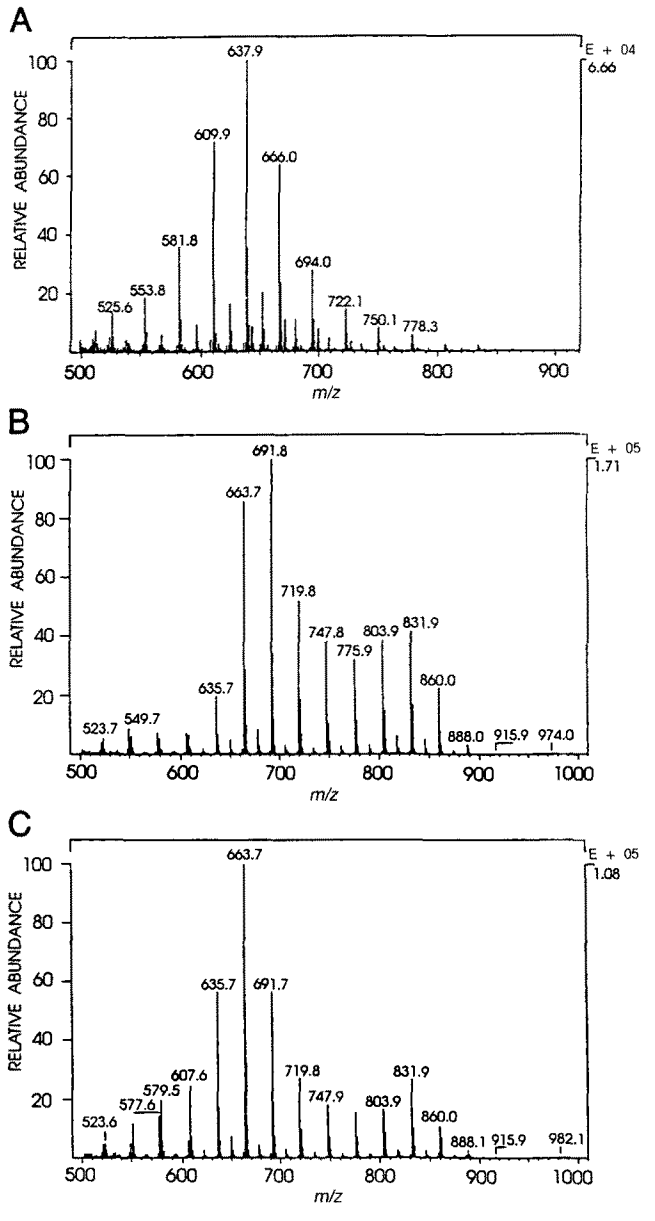

FIG. 4. Mass spectra of the negative ions produced by chemical ionization with ammonia of the triacylglycerol fractions of winter butterfat obtained by silver ion high-performance liquid chromatography (Ag-HPLC): (A) trisaturated Ag-HPLC fraction 1), (B) disaturated *trans*-monoenoic (Ag-HPLC Fraction 2) and (C) disaturated *cis*-monoenoic (Ag-HPLC Fraction 3) triacylglycerols. The ions of the displayed areas represent the deprotonated triacylglycerols, $[M - H]^-$ ions. The experimental conditions are given in the Materials and Methods section.

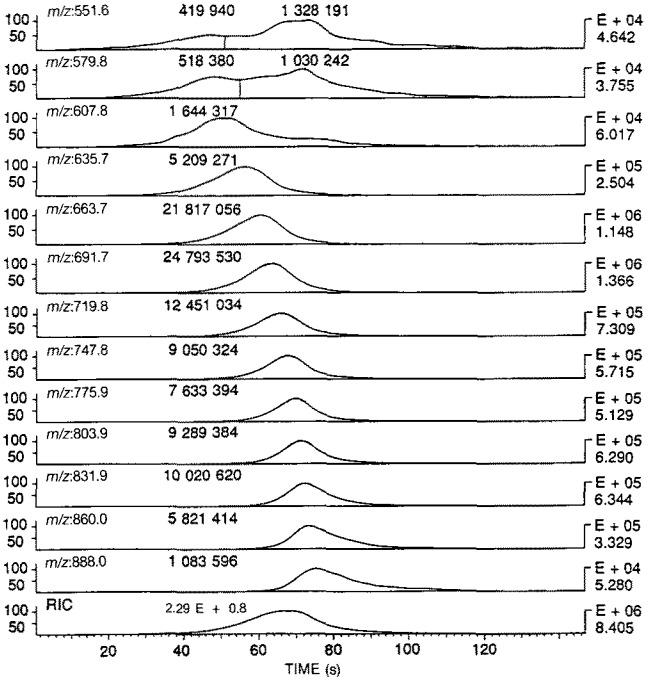

FIG. 5. Selected ion chromatograms of the major $[M - H]^-$ ions and the reconstructed ion chromatogram (RIC) of the disaturated *trans*-monoenoic triacylglycerols of winter butterfat.

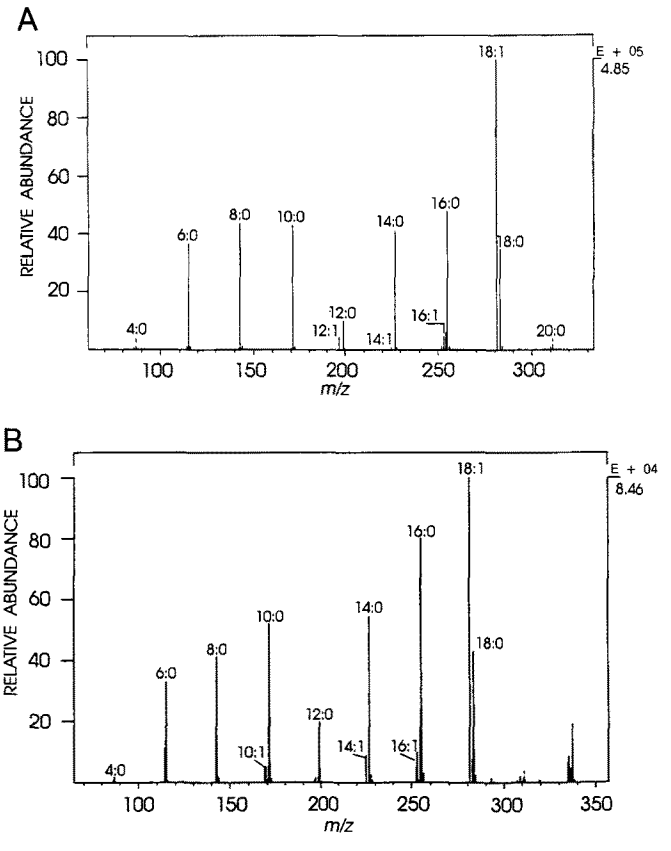

FIG. 6. Collision-activated daughter spectra of the parent ion 719.8 from disaturated *trans*-monoenoic (A) and disaturated *cis*-monoenoic triacylglycerols (B). The ions of the displayed areas represent the deprotonated fatty acids, RCO_2^- ions. The experimental conditions are given in the Materials and Methods section.

As an example, areas of the collision-activated mass spectra representing deprotonated fatty acids originating from SSM^t and SSM^c triacylglycerols of molecular weight 720.6 are shown in Figure 6A and B. The spectra were extremely logical, and they expressed the formation of deprotonated fatty acid ions only on the displayed areas. Some of the signals were split, such as 6:0, 8:0 and 10:0 (Fig. 6B). This was due to the tuning of the instrument but it did not affect the results. Even when certain fatty acids dominated the spectra, minor fatty acids could

still be positively identified because of the extremely low background noise.

Combining the HPLC and MS results: saturated triacylglycerols. In the present study, NICI-MS was used to study the molecular species of differently unsaturated triacylglycerols of winter butterfat. The distribution of saturated molecules according to their even and odd acyl carbon numbers, based on the uncorrected intensities of the $[M - H]^-$ ions, is presented in Figure 7A. A corresponding distribution based on the uncorrected areas of the reversed-phase HPLC peaks is shown in Figure 7B. The profiles of Figure 7A and B are different. With both methods, the proportions of the small molecules were overestimated and those of the large ones underestimated. The discrimination in the MS analysis was greater than in the reversed-phase HPLC. The response of the $[M - H]^-$ ions of the ammonia NICI spectra is known to decrease strongly with increasing molecular weights of the triacylglycerols (12). The reproducibility of the HPLC analysis was better, providing smaller standard deviations, than that of the MS analysis.

MS is an ideal tool for the analysis of components present in small amounts, such as saturated triacylglycerols containing odd-chain fatty acids (16% of SSS) (Fig. 7A). Typically, butterfat contains approximately 3% fatty acids with odd carbon numbers. A similar certainty in identifying triacylglycerols containing odd-chain fatty acids cannot be achieved by reversed-phase HPLC without MS

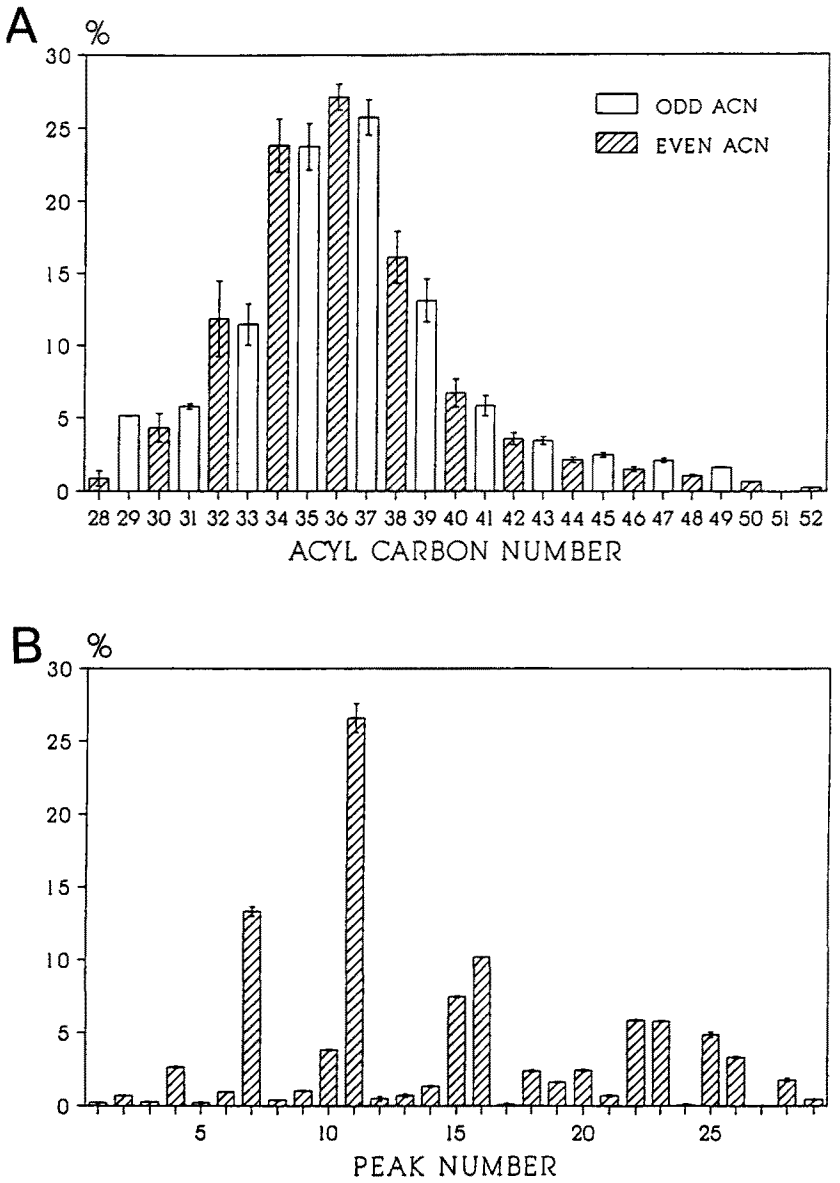

FIG. 7. Distribution of the trisaturated triacylglycerols of winter butterfat according to (A) the intensities of the uncorrected $[M - H]^-$ ions in relation to their even and odd acyl carbon numbers (ACN), analyzed by mass spectrometry and (B) the uncorrected areas of the chromatographic peaks obtained by reversed-phase high-performance liquid chromatography.

detection. Thus in Figure 8B, based on reversed-phase HPLC analyses, the molecules containing, probably, odd-chainlength fatty acids are included with those having an even carbon number.

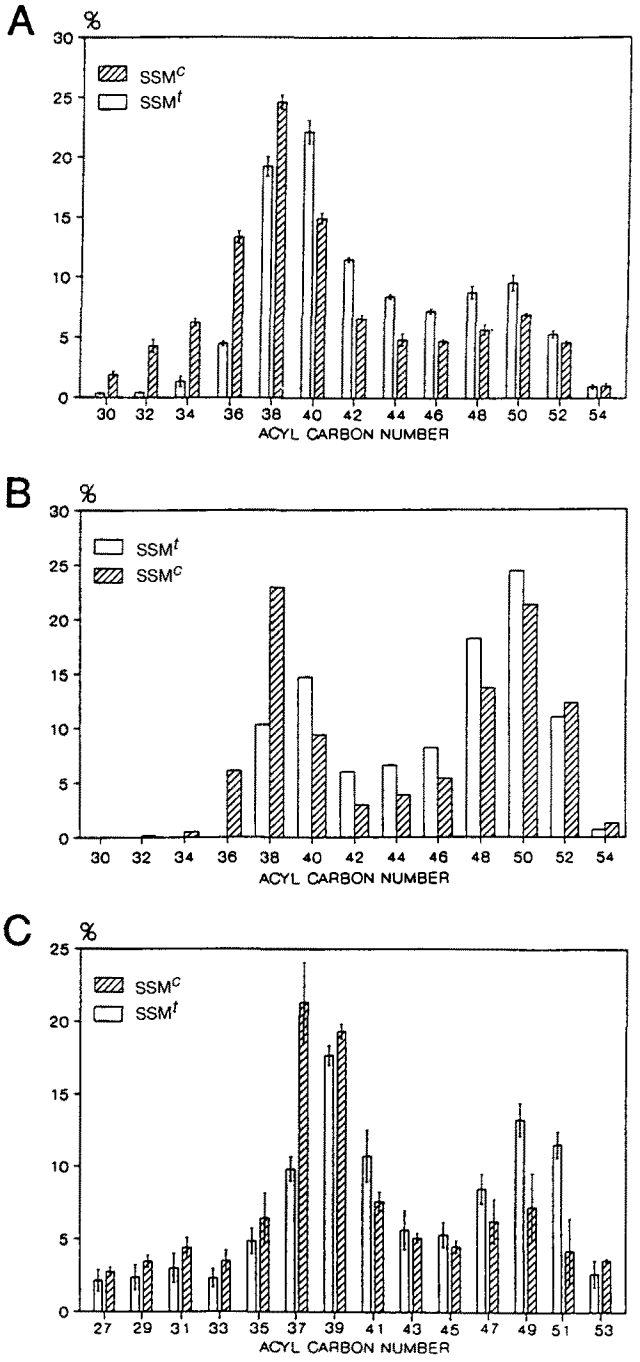

FIG. 8. Distribution of the disaturated *trans*-monoenoic (SSM^t) and disaturated *cis*-monoenoic (SSM^c) triacylglycerols according to (A) the intensities of the uncorrected $[M - H]^-$ ions in relation to their even acyl carbon numbers, analyzed by mass spectrometry (MS), (B) the uncorrected areas of the chromatographic peaks obtained by reversed-phase high-performance liquid chromatography, in relation to their acyl carbon numbers and (C) the intensities of the uncorrected $[M - H]^-$ ions in relation to their odd acyl carbon numbers, analyzed by MS.

Disaturated monoenoic triacylglycerols. The winter butterfat studied contained about 40% triacylglycerols with two saturated plus one monoenoic fatty acyl residues (Table 1). A small portion of these molecules contained a *trans*-acid instead of a *cis*-monoenoic fatty acid. Fractionation of the triacylglycerols according to the *cis* and *trans* difference in one fatty acyl residue by Ag-HPLC prior to MS analysis was important because configurational isomers could not be distinguished with the MS/MS system used. The distribution of disaturated *cis*- and disaturated *trans*-monoenoic triacylglycerols in relation to their acyl carbon numbers analyzed both by MS and reversed-phase HPLC are shown in Figure 8A and B. The profiles differ considerably, because of differences in the discrimination of the methods. For example, molecules with an acyl carbon number of 40 or less comprised 48% of the SSM^t fraction and 65% of the SSM^c fraction when analyzed by MS, whereas the corresponding proportions were, respectively, 25 and 39% by reversed-phase HPLC analysis. It is also worth remembering that classification of the molecules according to their acyl carbon numbers determined by HPLC analyses was not unambiguous.

The distributions of both disaturated *cis*- and disaturated *trans*-monoenoic molecules consisting of one odd-chain fatty acyl residue, in relation to their acyl carbon numbers (Fig. 8C), were similar to those of the corresponding molecules with even carbon numbers (Fig. 8A). With the MS method used, the standard deviations for minor fragments were higher than those for major ones.

The distribution profiles of SSM^t and SSM^c triacylglycerols (Fig. 8A and C) obtained by MS were slightly bimodal, whereas those of the trisaturated molecules (Fig. 7A) were clearly unimodal. The most abundant even-number triacylglycerols of SSM^c had 38 acyl carbons and those of SSM^t had 40. Triacylglycerols with 36 acyl carbons formed the main fraction of SSS. The SSM triacylglycerols containing odd-chainlength fatty acids had maxima at 37–39 acyl carbons (Fig. 8C). In addition, the SSM^t fraction showed a distinct second maximum at 49–51 acyl carbons. The distribution profiles of SSM molecules (Fig. 8B) obtained by HPLC were clearly bimodal. Molecules with acyl carbon numbers of 38 and 50 were the most abundant in SSM^c and those with 40, 48 and 50 acyl carbons in SSM^t. According to our results, the distribution of molecules containing *trans*-fatty acids was dissimilar to that of the trisaturated molecules. Actually, the distribution of SSM^c, rather than that of SSM^t, was closer to the distribution of SSS.

Although correction factors were not used in the analysis of the triacylglycerols, it was informative to calculate the ratio of SSM^t to SSM^c within each acyl carbon number. Regardless of the method of analysis, the ratios were analogous for all triacylglycerols having an even number of carbon atoms (Fig. 9A). The smallest molecules, containing butanoic acid and other short-chain fatty acids, preferred *cis*- to *trans*-monoenoic acid. When the chainlengths of the fatty acyl residues increased, the proportion of *trans*-acids increased up to an acyl carbon number of 42. When the acyl carbon number increased further, the ratio of SSM^t to SSM^c decreased slowly, passing a value of 1.0.

The ratio of SSM^t to SSM^c for those triacylglycerols having an odd-carbon number within each acyl carbon number is shown in Figure 9B. Again, *cis*- and *trans*-acid-

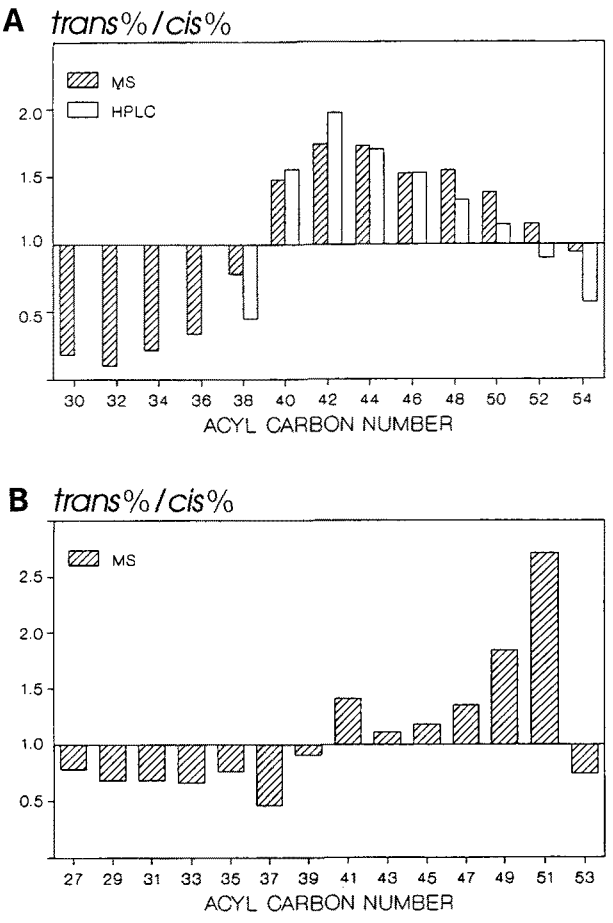

FIG. 9. The ratios of the proportion of the disaturated *trans*- to disaturated *cis*-monoenoic triacylglycerols within each (A) even acyl carbon number and (B) odd acyl carbon number. Based on mass spectrometric (MS) analysis, the ratio was calculated with the proportions of the corresponding $[M - H]^-$ ions. Based on reversed-phase high-performance liquid chromatography (HPLC) analysis, the ratio was calculated with the proportions of the corresponding chromatographic peaks.

containing molecules were not evenly distributed. The relative proportion of SSM^t in the molecules containing long-chainlength fatty acyl moieties was higher than in those containing short-chainlength fatty acyl moieties.

Further information about the composition of SSM^t and SSM^c triacylglycerols was obtained by collisional activation of each of the $[M - H]^-$ parent ions in the second stage of the MS analysis. The uncorrected proportions of RCO_2^- ions, representing fatty acids of the disaturated monoenoic molecules with even acyl carbon numbers, are shown in Table 2. The fatty acids were identified by their total number of carbon atoms and double bonds only. Configurational or positional isomers could not be distinguished by the MS/MS method used.

The 13 investigated molecular weights of the disaturated monoenoic triacylglycerols varied from 552.4 (measured *m/z* of $[M - H]^-$ 551.5) to 888.8 (measured *m/z* of $[M - H]^-$ 888.1) representing acyl carbon numbers from 30 to 54. Although the collision-activated spectra of the four lowest molecular weights were obtained only from SSM^c, corresponding molecules were also recorded from SSM^t (Fig. 8A). Within each molecular weight, 18:1 was

TABLE 2

Fatty Acid Composition of the Disaturated *trans*-Monoenoic (SSM^t) and Disaturated *cis*-Monoenoic (SSM^c) Triacylglycerols of Winter Butterfat Obtained by Collisional Activation of Each of the Major $[M - H]^-$ Ions by Mass Spectrometry

	$[M - H]^-$																					
Fatty acid[a]	551.5		579.7		607.7		635.7	663.7		691.7		719.8		747.8		775.8		803.9		831.9		859.7		888.1
	SSM^c		SSM^c		SSM^c		SSM^c	SSM^t	SSM^c	SSM^t	SSM^c	SSM^t	SSM^c	SSM^t	SSM^c	SSM^t	SSM^c	SSM^t	SSM^c	SSM^t	SSM^c	SSM^t	SSM^c	SSM^c
4:0	15.0		13.0		11.6		11.6	9.4	13.6	6.5	7.5	trace	trace											
6:0	9.6		5.6		4.9		2.4	8.4	4.3	14.5	14.4	10.7	8.1	0.6	trace		trace							
8:0	20.0		5.6		1.8		0.9	1.1	0.5	3.9	3.6	11.0	11.2	4.6	4.3		trace							
10:1	17.5[b]		2.0		0.7		trace		trace				1.4		0.4		trace			trace				
10:0	3.8		21.6		6.3		1.3	2.2	1.0	2.9	2.7	11.2	10.6	23.6	19.4	9.9	6.8	0.2	trace					
12:1	2.4[b]		13.7[b]		0.8		trace					1.0	trace	1.8	trace	0.5	trace							
12:0	2.3		3.5		18.0		2.8	0.9	0.8	2.5	1.6	2.9	3.5	8.2	8.0	12.8	14.1	5.2	4.9	trace	trace			
14:1	2.6[b]		4.4[b]		15.9[b]		2.2		0.4		0.7		2.1	0.4	3.2	0.4	4.2	trace	1.4		0.2			
14:0	1.9		4.0		6.9		29.5	11.4	7.2	4.9	4.4	11.0	11.0	7.8	11.1	19.6	19.9	28.1	29.1	11.0	13.3	1.4	1.0	0.5
16:1	3.0[b]		3.1[b]		6.0[b]		16.6[b]	2.4	2.1	1.0	1.4	1.4	2.1	1.4	1.9	2.4	2.6	1.2	2.4	1.0	1.2	trace	0.7	
16:0	2.1		2.4		7.3		7.1	25.0	32.1	19.5	20.1	12.0	17.6	21.6	23.9	19.8	24.1	29.7	31.4	43.8	47.0	37.1	38.3	5.3
18:1	19.8[b]		19.9[b]		18.6[b]		23.8[b]	37.3[b]	37.0[b]	28.9	28.9	27.5	22.9	24.8	21.6	25.1	20.3	30.1	23.6	33.9	26.8	29.7	28.1	32.0
18:0			1.2		1.2		1.9	2.0	1.0	15.5	14.8	10.5	9.5	5.0	6.3	9.5	8.0	5.5	7.2	10.3	11.2	30.6	31.0	57.9
20:1														0.2	trace		trace				0.2	0.7	0.4	0.8
20:0												0.7	trace		trace		trace				trace	0.7	0.6	3.5

[a]Fatty acids were identified only by the number of carbon atoms and double bonds. Identification of the fatty acids and calculation of their proportions were based on the intensities of the uncorrected RCO_2^- ions. Trace = less than 0.2%.
[b]The RCO_2^- in question was interfered with some $[M - H - RCO_2H - 100]^-$ ions.

the main monoenoic fatty acyl residue. Considerable amounts of 10:1 and 8:0, 12:1 and 10:0, 14:1 and 12:0, and 16:1 and 14:0 were recorded among the deprotonated molecular weight species of 551.5, 579.7, 607.7 and 635.7, respectively. The chainlengths of the saturated fatty acids increased systematically toward the high-molecular weight compounds containing predominantly 18:1 as their monoenoic acid. In addition to the fatty acids presented in Table 2, small amounts of acetic acid were detected from the triacylglycerols with the lowest molecular weights.

Within each molecular weight, the proportions of the fatty acids of the SSM^c and SSM^t molecules were similar. The same fatty acyl residues, with the exception of some minor components, were identified in the corresponding SSM^c and SSM^t fractions. There was no difference in average chainlength of fatty acids between the disaturated *cis*- and the disaturated *trans*-monoenoic triacylglycerols of a certain molecular weight. The 10:1 and 14:1 were located almost entirely in the SSM^c fractions, whereas 16:1 was more evenly distributed. Some SSM^c fractions contained lower proportions of 18:1 than the corresponding SSM^t fractions. According to these results, there is no substantial difference in fatty acyl residues between SSM^c and SSM^t molecules. The variation in the amounts of different fatty acid combinations and the distribution of the fatty acyl residues between the primary and secondary glycerol positions remain to be elucidated.

The major pathways for triacylglycerol synthesis in animal tissues are the *sn*-glycerol-3-phosphate, the dihydroxyacetone phosphate and the monoacylglycerol pathways. The *sn*-glycerol-3-phosphate pathway is the principal route for triacylglycerol synthesis in ruminant mammary tissues (32). Acyltransferases play a key role when fatty acids are esterified to the glycerol backbone. In all pathways, the *sn*-3 position is the last one to be esterified. Our results indicate that during the biosynthesis of bovine milk triacylglycerols the *cis*- and *trans*-monoenoic fatty acids are not esterified equally. Thus it appears that the acyltransferases metabolize configurational isomers differently. The *cis*-acids predominate in small molecules. The less hindered sterical structure may permit the esterification of a longer fatty acid to a molecule already containing a *trans*-acid, or *vice versa*. When the chainlengths of the fatty acyl moieties increase further, the relative proportion of *cis*-acids increases again. These effects may be the result of the requirement that the bovine produces triacylglycerols with favorable physical properties; compared with a *trans*-acid, a *cis*-acid lowers the melting point of the triacylglycerol and thus may improve its physiological usefulness.

Variable results on the distribution of *trans*-18:1 between the primary and secondary glycerol positions have been reported. Woodrow and deMan (33), using infrared spectroscopy, found no measurable amount of *trans*-unsaturation in the *sn*-2 position of milk fat. Barbano and Sherbon (34) found small amounts of *trans*-18:1 in the *sn*-2 position when they analyzed high-melting triacylglycerols of bovine milk fat. Parodi (7) reported that considerable amounts of *trans*-18:1 were esterified at the *sn*-2 position of the disaturated *cis*- and disaturated *trans*-monoenoic triacylglycerols. He suggested that there is no acyltransferace positional specificity for geometrical isomers of octadecenoic acid during the biosynthesis of bovine triacylglycerols. By means of the MS and MS/MS method described in the present study, it is possible to elucidate the distribution of the configurational isomers of 18:1 within each acyl carbon number of triacylglycerols by recording $[M - H - RCO_2H - 100]^-$ ions (12), after fractionation of the sample by Ag-HPLC. Determination of the distribution of fatty acids in triacylglycerols with this method is in progress in our laboratory.

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REFERENCES

1. Patton, S., and R.G. Jensen, *Progr. Chem. Fats Lipids 14*:163 (1975).
2. Breckenridge, W.C., and A. Kuksis, *Lipids 3*:291 (1968).
3. Breckenridge, W.C., and A. Kuksis, *Ibid. 4*:197 (1969).
4. Taylor, M.W., and J.C. Hawke, *N. Z. J. Dairy Sci. Technol. 10*:40 (1975).
5. Parodi, P.W., *Austr. J. Dairy Technol. 35*:17 (1980).
6. Parodi, P.W., *J. Dairy Res. 48*:131 (1981).
7. Parodi, P.W., *Ibid. 49*:73 (1982).
8. Myher, J.J., A. Kuksis, L. Marai, and P. Sandra, *J. Chromatogr. 452*:93 (1988).
9. Nikolova-Damyanova, B., W.W. Christie, and B. Herslöf, *J. Am. Oil Chem. Soc. 67*:503 (1990).
10. Laakso, P., and W.W. Christie, *Ibid. 68*:213 (1991).
11. Kallio, H., and G. Currie, in *Short Course on HPLC of Lipids*, American Oil Chemists' Society, Bloomingdale, May 9–11, 1991.
12. Kallio, H., and G. Currie, *Lipids 28*:207 (1993).
13. Duffin, K.L., J.D. Henion, and J.J. Shieh, *Anal. Chem. 63*:1781 (1991).
14. Christie, W.W., *J. High Resolut. Chromatogr., Chromatogr. Commun. 10*:148 (1987).
15. Christie, W.W., *J. Chromatogr. 454*:273 (1988).
16. Christie, W.W., *J. Lipid Res. 23*:1072 (1982).
17. Morris, L.J., *Ibid. 7*:717 (1966).
18. Litchfield, C., *Analysis of Triglycerides*, Academic Press, New York, 1972.
19. Strocchi, A., and R.T. Holman, *Riv. Ital. Sostanze Grasse 48*:617 (1971).
20. Slover, H.T., and E. Lanza, *J. Am. Oil Chem. Soc. 56*:933 (1979).
21. Badings, H.T., and C. de Jong, *Ibid. 65*:659 (1988).
22. Christie, W.W., and G.H. Breckenridge, *J. Chromatogr. 469*:261 (1989).
23. Christie, W.W., *Biochim. Biophys. Acta 316*:204 (1973).
24. Ratnayake, W.M.N., and J.L. Beare-Rogers, *J. Chromatogr. Sci. 28*:633 (1990).
25. Ratnayake, W.M.N., R. Hollywood, E. O'Grady, and J.L. Beare-Rogers, *J. Am. Oil Chem. Soc. 67*:804 (1990).
26. Laakso, P.H., K.V.V. Nurmela, and D.R. Homer, *J. Agric. Food Chem. 40*:2472 (1992).
27. El-Hamdy, A.H., and E.G. Perkins, *J. Am. Oil Chem. Soc. 58*:867 (1981).
28. Phillipis, F.C., W.L. Erdahl, J.D. Nadenicek, L.J. Nutter, J.A. Schmit and O.S. Privett, *Lipids 19*:142 (1984).
29. Podhala, O., and B. Töregård, *J. Chromatogr. 482*:215 (1989).
30. Currie, G., and H. Kallio, *Lipids 28*:217 (1993).
31. Kallio, H., in *Contemporary Lipid Analysis*, Stockholm, Sweden, January 23, 1992.
32. Moore, J.H., and W.W. Christie, *Progr. Lipid Res. 17*:347 (1979).
33. Woodrow, I.L., and J.M. deMan, *Biochim. Biophys. Acta 152*:472 (1968).
34. Barbano, D.M., and J.W. Sherbon, *J. Dairy Sci. 58*:1 (1975).

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