The Regiospecific Position of 18:1 *cis* and *trans* Monoenoic Fatty Acids in Milk Fat Triacylglycerols

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ABSTRACT: TAG of butterfat were fractionated according to the type and degree of unsaturation into six fractions by silver-ion HPLC. The fractions containing TAG with either *cis*- or *trans*-monoenoic FA were collected and fractionated further by reversed-phase HPLC to obtain fractions containing *cis* TAG of ACN:DB (acyl carbon number:double bonds) 48:1, 50:1, and 52:1 as well as *trans* 48:1, 50:1, and 52:1. The FA compositions of these fractions were elucidated by GC. The MW distribution of each fraction was determined by ammonia negative-ion CI-MS. Each of the $[M - H]^-$ parent ions was fractionated further by collision-induced dissociation with argon, which gave information on the location of *cis*- and *trans*-FA between the primary and secondary positions of TAG. The results suggest that the *sn*-positions of the monoenoic *cis*- and *trans*-FA depend on the two other FA present in the molecule. With 14:0 FA in the TAG molecule, the 18:1 FA in the *sn*-2 position are mostly present as *cis*-isomers. When there is no 14:0 in the TAG molecule, the *trans*-18:1 isomers seem to be more common in the *sn*-2 position. Also when other long-chain FA are present, the *trans*-isomers are more likely to be located in the secondary (*sn*-2) position.

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KEY WORDS: Bovine, *cis*-monoenoic fatty acids, milk fat, reversed-phase high-performance liquid chromatography, silver ion chromatography, tandem mass spectrometry, *trans*-monoenoic fatty acids, triacylglycerols.

The composition of ruminant milk fat differs greatly from the milk fat of nonruminants. It contains unusually high levels of short-chain, odd-numbered, branched-chain, and *trans*-FA. The *trans*-FA originate in the rumen from the biohydrogenation of unsaturated FA by different anaerobic microbes. The most common *trans*-FA in milk fat are 18:1 C11, 18:1 C15, 18:1 C14, and 18:1 C13. It is not known so far to what extent the geometrical configuration of the double bond affects the location of FA in different *sn*-positions of TAG. Such information is important because the *cis*- and *trans*-FA are likely to exert different biological effects owing to their different chemical and physical properties. *trans*-FA reportedly have negative effects on human and animal health. Numerous studies have shown a connection between factors causing cardiovascular diseases and *trans*-FA (1–3). However, some CLA isomers with *trans* double bonds are a positive exception to this generalization (4,5), and it has been proposed that they be excluded from *trans*-FA nutrition-labeling legislation (6). The *sn*-position affects the metabolic fate of FA in animals and humans. Saturated FA at the secondary (*sn*-2) position are absorbed more efficiently than saturated FA at the primary positions.

Laakso and Kallio (7) observed that butterfat TAG differing in double-bond configuration by one fatty acyl residue were not equally distributed in relation to their molar masses. This indicates that during the biosynthesis of TAG, *cis*- and *trans*-FA are differently processed and are evidently not equally esterified to the three stereospecific positions of TAG molecules. The necessity for liquidity of the milk fat globule requires that most FA exist in TAG in combinations having m.p. at or below 39°C, which is the body temperature of the cow (8). This may be related to the positioning of *cis*- and *trans*-FA in TAG. The molecular structures of *cis*- and *trans*-FA also differ from each other. A *cis*-double bond causes a 30° bend in the fatty acyl chain whereas the chain remains straight with a *trans*-double bond. The bend in *cis*-FA may cause steric hindrance when these FA are esterified to the TAG molecule and in this way affect the positioning of other FA.

Only a few studies have considered the distribution of *trans*-FA between the primary and secondary positions of TAG. Using IR spectroscopy, Woodrow and deMan (9) found only small amounts of *trans*-FA in the secondary position of milk fat. Barbano and Sherbon (10) obtained similar results, with most of the *trans*-18:1 FA at the primary positions in high-melting TAG. Parodi (11) also observed that most of the *trans*-18:1 FA were found at the primary positions in the high-MW fraction of milk TAG, but considerable amounts of *trans*-FA were also esterified to the *sn*-2 position. To our knowledge, there are no published studies comparing the positioning of *cis*- and *trans*-18:1 FA in individual milk fat TAG. The aim of this study was to determine the *sn*-1/3 vs. *sn*-2 positions of monoenoic *cis*- and *trans*-18:1 FA in milk fat TAG in the presence of two saturated FA. This was achieved by using a combination of different chromatographic and mass spectrometric methods.

MATERIALS AND METHODS

Samples and reagents. Fat was separated from 0.25 g of unsalted butter using 9 mL of chloroform/methanol (2:1, vol/vol) and 3 mL of 0.7% sodium chloride. After extraction the butterfat was dried by eluting the sample through a sodium sulfate column.

TAG were separated from other lipid classes by elution through a Florisil™ column with 10 mL of hexane/diethylether

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(4:1, vol/vol). After evaporating the solvent with a stream of nitrogen, the TAG were dissolved in 1,2-dichloroethane. All solvents were of HPLC grade and were supplied by Rathburn (Walkerburn, Scotland), Merck (Darmstadt, Germany), and Labscan (Stillorgan, Ireland).

HPLC. The 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 Sedex 75 light-scattering detector (Alfortville, France), and Shimadzu C-R5A integrator. A stream-splitter was installed between the column and the detector when sample fractions were collected.

The purified TAG were separated into six fractions by silver-ion HPLC (Ag-HPLC) using a cation exchange column (Nucleosil^{TM} 5SA, 25 cm × 4.6 mm i.d., HPLC Technology, Macklesfield, United Kingdom) loaded with silver ions. The column was prepared according to a method described by Christie (12).

The fractionation was carried out at ambient temperature with a binary gradient of (A) dichloroethane/dichloromethane (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 in the next 15 min. The final solvent composition was maintained for an additional 5 min. After this the concentration of B was lowered to 0 % in 5 min, followed by 100% A for 15 min. The flow rate was 1.0 mL/min. Approximately 10–25 μL of purified TAG sample (1 mg in 10 μL 1,2-dichloroethane) was injected into the column. Fractionation was repeated several times to obtain sufficient material for further analyses.

The fractions containing disaturated *trans* monoenoic (fraction 2) and disaturated *cis*-monoenoic TAG (fraction 3) were further fractionated by reversed-phase (RP)-HPLC into several fractions mainly according to the acyl carbon number (ACN). The samples were evaporated with nitrogen and dissolved in isopropanol before the fractionation. The column used was Discovery^{TM} HS C_{18} (25 cm × 4.6 mm, 5 μm; Supelco, Bellefonte, PA). The analysis was performed with a Merck L-6200A solvent delivery system, combined with a Sedex 75 light-scattering detector, Shimadzu C-R3A integrator, and Hitachi LC-Organizer mixer (Kyoto, Japan). A stream-splitter was installed between the column and the detector.

The fractionation was performed at ambient temperature with a binary gradient of (A) isopropanol and (B) methanol. The linear gradient was 80% A–20% B to 20% A–80% B in 30 min, then to 80% A–20% B in 5 min, and this was maintained for an additional 10 min. The flow rate was 0.85 mL/min. Approximately 25 μL of sample was injected into the column, and the fractionation was repeated several times to obtain sufficient material for GC, MS, and tandem MS (MS/MS) analyses.

GC. The FA compositions of TAG fractions *cis* ACN:DB (ACN:double bonds) 48:1, 50:1, 52:1, and *trans* 48:1, 50:1, 52:1 obtained by RP-HPLC were determined by GC. FAME were prepared by the sodium methoxide method. The column was a fused-silica capillary column SP-2340^{TM} (30 m × 0.32 mm i.d., coated with 0.2 μm phase; Supelco). The analysis was performed with PerkinElmer gas chromatograph (Norwalk, CT) equipped with a FID and split/splitless system. The linear flow rate of the carrier gas (helium) was 30 cm/s. The detector temperature was 240°C. The initial injector temperature was 170°C, which was raised at the beginning of the analysis at the rate of 200°C/min to 250°C and maintained there during the whole analysis. The oven temperature program started at 120°C, was held there for 3 min, and was then raised to 240°C at 4°C/min and held there for 20 min. One microliter samples were injected into the column. A mixture of reference compounds, CRM 164 (Commission of the European Community Bureau of References, Brussels, Belgium), was used.

MS and MS/MS. Negative ion CI spectra and collision-induced dissociation spectra were obtained with a Finnigan MAT TSQ-700 mass spectrometer (San Jose, CA). In the MS analysis, approximately 0.5 μL of sample was injected onto the direct exposure probe wire. The conditions were as follows: pressure of the ammonia 8500 mtorr, temperature of the ion source 200°C, ionization energy 70 mV, and emission current 400 μA. In the MS analyses, the scanning area was 700–950 *m/z* (7).

The MS/MS analyses were performed by dissociating the chosen $[M - H]^-$ ions with argon collision in the second quadrupole of the instrument at a pressure of 1.97 mtorr. The offset for the collision quadrupole was 18 eV, and the scanning area was 200–900 *m/z*. Appropriate correction factors for determination of proportions of FA by the intensities of $[RCOO]^-$ ions were applied. The results were calculated by TAGS-100® program (Nutrifen Ltd, Turku, Finland) (13).

Statistical analysis. The MS/MS results were analyzed statistically with SPSS for Windows 10.0 (SPSS Inc., Chicago, IL). The samples were compared with each other in pairs, using paired samples *t*-test and nonparametric tests. Differences of $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Ag-HPLC. Silver-ion chromatography is a useful method to separate TAG according to the degree of unsaturation and the type of double bonds in the FA. The π-electrons of *trans*-FA form weaker bonds with silver ions than those of *cis*-FA and behave more nearly like saturated FA (14,15). The separation of the six fractions obtained by Ag-HPLC is shown in Figure 1. Fraction 1 contained trisaturated TAG. Fractions 2 and 3 contained molecules with two saturated and one monoenoic fatty acyl residues. In fraction 2 the monoenoic fatty acyl residue had a *trans* configuration and in fraction 3 a *cis* configuration. Fractions 4 and 5 consisted of molecules with one saturated and two monoenoic fatty acyl residues, and fraction 6 contained more unsaturated TAG than fractions 2 or 3. The resolution of fractions 2 and 3 was >99%, and these fractions could thus be separated satisfactorily from each other.

RP-HPLC. Ag-HPLC fractions 2 and 3 were further separated successfully by RP-HPLC (Figs. 2, 3). Identification of the MW of the fractions was based on MS analysis. The TAG fractions of ACN:DB 48:1, 50:1, and 52:1 were collected in both samples. In the *cis*-monoenoic sample, fractions 48:1, 50:1, and 52:1 constituted 12.1, 18.3, and 4.7% of the total

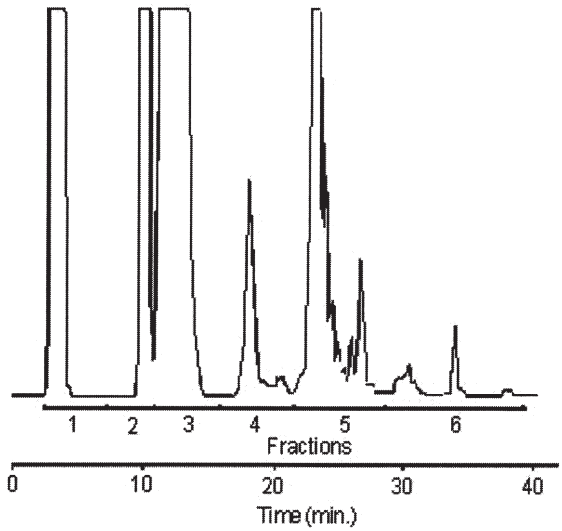

FIG. 1. The separation of milk fat TAG by Ag-HPLC.

TAG, respectively, and 17.7, 24.9, and 8.5% in the *trans*-monoenoic sample.

GC. The FA compositions of TAG fractions were determined by GC using a known mixture of reference compounds. Only the major C_{14}–C_{18} FA were determined, and without identification of the detailed structures of the various 18:1 *trans*-isomers. Only the FA MW could be directly determined from the MS analyses used. The major difference between the *cis* and *trans* samples was the presence of four small peaks in the *trans* samples instead of the one oleic acid peak in the *cis* samples. One of the four peaks in the *trans* samples contained *cis*-18:1n-9 FA. This had to be taken into account when analyzing the results. The FA composition of different samples is shown in Table 1.

MS and MS/MS analysis. Negative-ion CI-MS is a sensitive and rapid method for the analysis of the MW distribution according to ACN and the number of DB. The MS results showed that the fractionations by Ag-HPLC and RP-HPLC had been successful. As an example, the spectra of the samples *cis* 48:1 and *trans* 48:1 are shown in Figure 4. The MW distribution of each sample showed one major peak and a few minor ones that were disregarded in the further analysis. The results of MS analysis are shown in Table 2.

Each fraction analyzed contained a TAG species with a MW that was 14 units higher compared with the major TAG. This indicates the existence of one odd-carbon FA in these minor TAG species, the structures of which were, however, not determined. The contents of these minor TAG species varied between 1.6 and 7.3% of the total TAG, indicating the proportion of odd-carbon FA varied from 0.5 to 2.4% of the total FA within the fractions.

The MS/MS method is useful in determining the MW of a FA species and the regiospecific positions of FA in TAG molecules without enzymatic fragmentation. There are some limitations in the method, though. The method cannot be used to differentiate between TAG containing *cis*- and *trans*-FA, which is why HPLC resolution, off-line or on-line, is necessary prior to the MS/MS analysis. The variation in MS/MS analyses can also be substantial, but the effect of this was minimized by quadruplicate analyses.

The MS/MS results were obtained by collision-induced dissociation of selected $[M - H]^-$ ions in the second quadrupole and by scanning the resulting ions with the third quadrupole. As an example, the daughter ion spectra of the samples *cis* 48:1 and *trans* 48:1 are shown in Figure 5. In addition to the parent ions, the spectra consisted mainly of $[RCOO]^-$ and $[M - H - RCOOH - 100]^-$ ions. The corrected intensities for $[RCOO]^-$ ions were used for calculations. No quantitative correction factors were needed for the $[M - H - RCOOH - 100]^-$ ions (13). The distribution of FA of different MW between *sn*-2 and *sn*-1/3 positions was obtained with our program (TAGS-100®) that calculates the FA combinations and their proportions, and regiospecific positions of different FA in the TAG species of the selected MW.

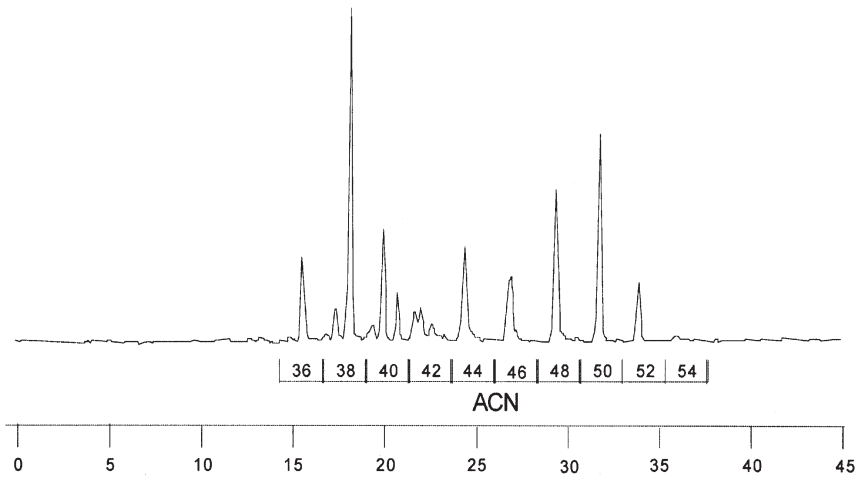

FIG. 2. The separation of *cis*-monoenoic TAG by reversed phase (RP)-HPLC. ACN, acyl carbon number.

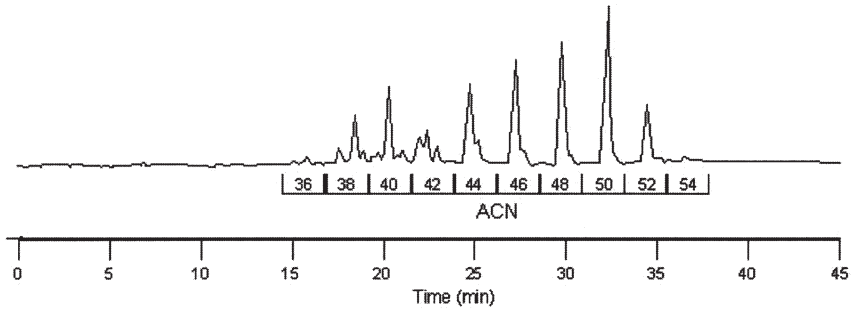

FIG. 3. The separation of *trans*-monoenoic TAG by RP-HPLC. For abbreviations see Figure 2.

Combining the GC and MS/MS results. The GC chromatograms showed that the *trans*-TAG samples contained small amounts of oleic acid, *cis*-18:1n-9 FA. This had to be taken into account when interpreting the MS/MS results. The proportion of oleic acid in all the 18:1 FA in the *trans*-sample was determined. The results suggest that the proportion of *cis*-18:1n-9 FA was 32.9, 31.6, and 30.9% in *trans* 48:1, *trans* 50:1, and *trans* 52:1, respectively, i.e., approximately 30% in all *trans* samples. The corrected values for the concentrations of different regioisomers of *trans* samples were obtained by Equation 1,

$$0.7X \times 0.3A = B \quad [1]$$

in which A is the average value of a specific TAG regioisomer of four parallel *cis*-samples and B is the value of one parallel *trans*-sample. The X for which Equation 1 may be solved equals the corresponding *trans*-regioisomer.

The equation was directly applicable to the *trans* 52:1 sample because all of its isomers contained 18:1 FA. For *trans* 48:1 and 50:1 samples, we had to correct the A and B values before using the equation. This was achieved by first disregarding TAG isomers that did not contain 18:1 FA, and then calculating proportions of regioisomers of the TAG containing 18:1 FA (Table 3).

The results show that the most common TAG consisted of FA combinations 16:0/14:0/18:1 in TAG fraction 48:1, 16:0/16:0/18:1 in TAG fraction 50:1, and 18:1/16:0/18:0 in TAG fraction 52:1. The 18:1 FA were located mainly in the primary positions as is typical in milk fat, and 14:0 and 16:0 were located mostly in the secondary position. In TAG fraction 52:1, 18:0 and 18:1 were located in the secondary position more frequently than in other fractions.

The only statistical difference between the *cis*- and *trans*-18:1 samples ($P = 0.002$) was found in pair 6 (TAG 50:1). In this TAG regioisomer, *trans*-18:1 FA are located more frequently in the primary positions than *cis*-18:1 FA. This might suggest that when a TAG molecule contains myristic and stearic acids, *trans*-18:1 FA are esterified more frequently to the primary positions compared with oleic acid.

The results from pairs 3, 5, 9, and 10 indicate that in pair 5, as in pair 6, *trans*-FA are located more frequently in the primary positions than *cis*-isomers; the other FA in the molecule are myristic and stearic acids. In pair 3, with both myristic and palmitic acids, *cis*-18:1 FA is more common in the secondary position than *trans*-18:1 FA. In pair 9 the case is opposite, i.e. *trans*-18:1 seem to be more common in the *sn*-2 position. In

TABLE 1
FA Compositions of TAG Fractions Obtained by GC

TAG	FA in the TAG	Relative proportion (%) of FA
cis 48:1	14:0	20.0
	14:1	1.1
	16:0	34.8
	16:1	2.4
	18:0	6.5
	18:1n-9	28.5
	Others	6.8
cis 50:1	14:0	7.0
	16:0	49.0
	18:0	11.1
	18:1n-9	31.3
	Others	1.7
cis 52:1	16:0	35.8
	18:0	28.7
	18:1n-9 *cis*	27.0
	Others	8.5
trans 48:1	14:0	23.1
	16:0	33.4
	18:0	4.9
	18:1 *trans*	14.1
	18:1n-9 *cis*- + *trans*-isomers	10.6
	18:1 *trans*	6.2
	18:1 *trans*	1.3
	Others	6.5
trans 50:1	14:0	8.7
	16:0	48.3
	18:0	10.0
	18:1 *trans*	16.5
	18:1n-9 *cis*- + *trans*-isomers	9.0
	18:1 *trans*	2.2
	18:1 *trans*	0.7
	Others	5.3
trans 52:1	16:0	34.4
	18:0	25.1
	18:1 *trans*	13.1
	18:1n-9 *cis*- + *trans*-isomers	7.1
	18:1 *trans*	2.3
	18:1 *trans*	0.4
	Others	18.0

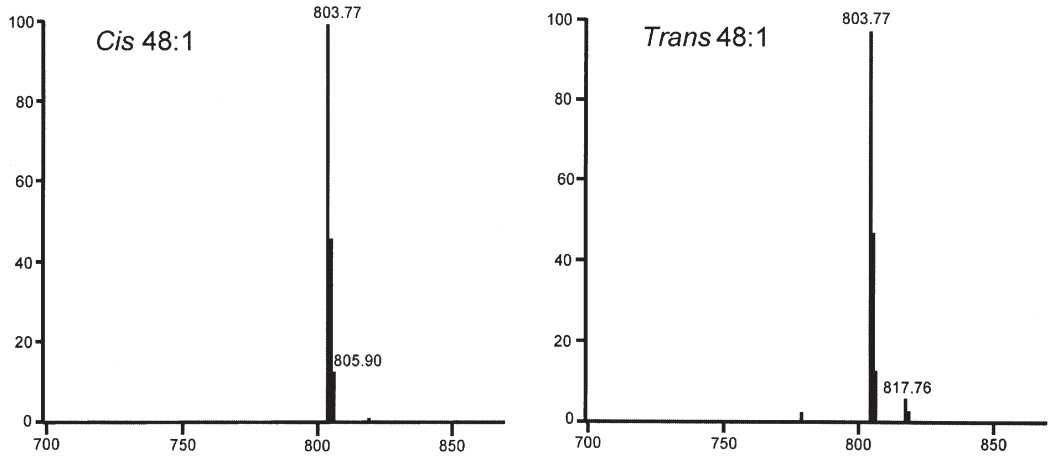

FIG. 4. The MS spectra of the *cis* and *trans* 48:1 samples.

pair 10, in which the molecule contains 16:0 and 18:0 FA, the results indicate that oleic acid is located more frequently in the primary positions than *trans*-18:1 FA.

A small portion of the *cis*-monoenoic TAG fraction 3 contaminated the *trans*-monoenoic fraction 2 because of deficiencies in the Ag-HPLC collection procedure. According to Ratnayake and Beare-Rogers (16), *trans*-18:1n-3, *trans*-18:1n-4, and *trans*-18:1n-5 co-elute with oleic acid (*cis*-18:1n-9) when using the SP-2340^{TM} column for GC analyses. This means that the calculations contain a small error that affects the results to some extent. The differences between *cis*- and *trans*-samples might have been clearer if the *trans*-samples had been pure.

The results indicate, however, that the entire FA combination affects the positioning of *trans*- and *cis*-18:1 FA in the TAG molecule. If the largest outliers of the replicated analyses within each regioisomer are disregarded, the differences between the *cis*- and *trans*-samples become even clearer. Particularly in TAG fraction 52:1, the difference between the *cis*- and *trans*-isomers is obvious. *Trans*-isomers are located more frequently in the secondary position than *cis*-isomers. This implies that when other long-chain FA are present in the TAG, the *trans*-18:1 FA are more likely located at *sn*-2 position.

The results suggest that when myristic acid is present in TAG (TAG fractions 48:1 and 50:1), the 18:1 FA in the *sn*-2 position is mostly the *cis*-isomer and the *trans*-isomers are more common in the primary positions. The opposite seems to be true when no myristic acid is present in the TAG molecule. However, these observations are speculative and more research is needed with TAG molecules having different structures before making final conclusions.

The synthesis of milk TAG by ruminants occurs mainly through the glycerol-3-phosphate pathway, and the enzymes catalyzing the esterification process (17) are at least partly specific to different FA. Other factors such as the concentration of FA in the mammary gland also have been reported to affect the FA positioning (11,18). Enjalbert *et al.* (19) reported that mammary gland desaturation of 18:0 was lowered by uptake of *trans*-18:1, suggesting there is less *cis*-18:1n-9 formed and available for TAG synthesis when *trans*-18:1 is taken up into the mammary gland. This implies that the appearance of 18:0

TABLE 2
The MW Distribution of the Samples by MS

TAG sample	MW	TAG	Relative proportion (%)	SD
cis 48:1	803.8	48:1	96.9	0.1
	817.6	49:1	3.1	0.1
cis 50:1	831.8	50:1	95.6	0.1
	845.8	51:1	4.4	0.1
cis 52:1	859.8	52:1	94.6	0.6
	873.8	53:1	2.8	0.2
trans 48:1	777.7	46:0	2.3	0.1
	803.8	48:1	91.6	1.4
	817.6	49:1	6.2	1.4
trans 50:1	805.8	48:0	1.6	0.6
	831.8	50:1	91.1	2.1
	845.8	51:1	7.3	1.7
trans 52:1	859.8	52:1	95.9	0.1
	873.8	53:1	4.1	0.1

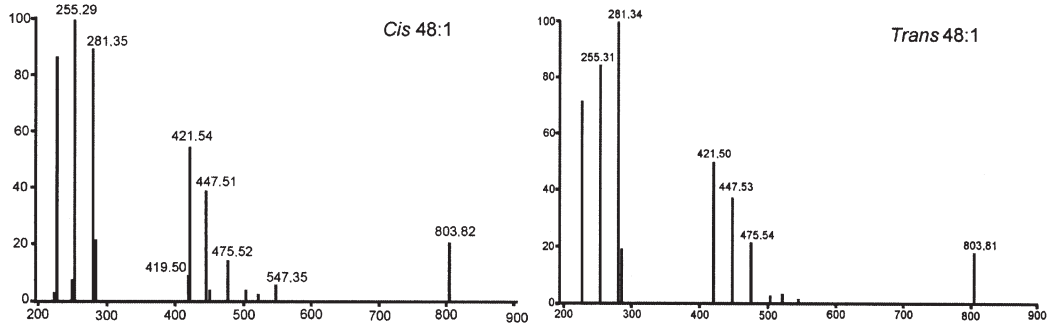

FIG. 5. The MS/MS spectra of the TAG samples *cis* and *trans* 48:1.

TABLE 3
The Proportions[a] and the Regioisomeric Structures of Selected Monoenoic *cis/trans* TAG in Milk Fat

TAG	Regioisomer of TAG	Sample	Avg	SD	Pair	*P*
48:1	*sn*-14:0/16:0/18:1 +	*cis* 48:1	16.0	8.3		
	sn-18:1/16:0/14:0	*trans* 48:1	23.1	6.0	1	0.250
	sn-16:0/14:0/18:1 +	*cis* 48:1	82.4	9.3		
	sn-18:1/14:0/16:0	*trans* 48:1	76.9	6.0	2	0.250
	sn-14:0/18:1/16:0 +	*cis* 48:1	1.7	1.6		
	sn-16:0/18:1/14:0	*trans* 48:1	0	0	3	0.136
50:1	*sn*-14:0/18:1/18:0 +	*cis* 50:1	1.6	1.1		
	sn-18:0/18:1/14:0	*trans* 50:1	0.7	1.3	4	0.500
	sn-18:1/14:0/18:0 +	*cis* 50:1	27.2	1.0		
	sn-18:0/14:0/18:1	*trans* 50:1	31.8	2.8	5	0.059
	sn-14:0/18:0/18:1 +	*cis* 50:1	1.4	1.2		
	sn-18:1/18:0/14:0	*trans* 50:1	5.0	1.6	6	0.002
	sn-16:0/16:0/18:1 +	*cis* 50:1	66.0	7.0		
	sn-18:1/16:0/16:0	*trans* 50:1	57.5	4.7	7	0.250
		cis 50:1	4.0	6.8		
	sn-16:0/18:1/16:0	*trans* 50:1	5.0	4.7	8	0.705
52:1	*sn*-16:0/18:1/18:0 +	*cis* 52:1	8.7	7.6		
	sn-18:0/18:1/16:0	*trans* 52:1	23.0	10.7	9	0.180
	sn-18:1/16:0/18:0 +	*cis* 52:1	75.5	8.0		
	sn-18:0/16:0/18:1	*trans* 52:1	56.8	6.7	10	0.069
	sn-16:0/18:0/18:1 +	*cis* 52:1	15.9	5.9		
	sn-18:1/18:0/16:0	*trans* 52:1	20.2	5.6	11	0.461

[a]The proportion of each regioisomer is expressed as mole-percentage. The total sum of the average values of each *cis*- or *trans*-regioisomer in every TAG fraction is 100. $n = 4$.

and *trans*-18:1 might be connected to some extent and explain why *trans*-18:1 FA have been observed more frequently in high MW TAG (10,11).

Barbano and Sherbon (10) reported that *trans*-18:1 FA were located mainly in the *sn*-1 position in low-melting TAG and in *sn*-3 positions in high-melting TAG. However, we could not prove this suggestion because we were not able to discriminate the positioning of FA between the *sn*-1 and *sn*-3 positions with our methods. Parodi (11) also reported that most of the 18:1 FA were esterified in the *sn*-1 position in low MW TAG and in the *sn*-3 position in high MW TAG, but substantial amounts of *trans*-18:1 FA were also found in the *sn*-2 position when the MW increased. There were no observed differences in the positioning of *cis*- and *trans*-FA, and Parodi (11) suggested that there is no acyltransferase positional specificity for geometrical isomers of 18:1. Laakso and Kallio (7) observed that there were differences between the MW distributions of *cis*- and *trans*-FA, which is contrary to the suggestion by Parodi (11). Our study also suggests that *cis*- and *trans*-FA are processed differently in milk fat biosynthesis, with other FA affecting the regiospecific position of the monoenoic C_{18} FA.

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