Determination of Positional Distribution of Short-Chain Fatty Acids in Bovine Milk Fat on Chiral Columns

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The positional distribution of acetic and butyric acids in bovine milk fat triacylglycerols was determined by chiral-phase high-performance liquid chromatography (HPLC) of the derived diacylglycerols. Enriched fractions of acetic and butyric acid-containing triacylglycerols were isolated by normal-phase thin-layer chromatography (TLC) from a molecular distillate of butter oil, and they were fully hydrogenated. Mixed *sn*-1,2(2,3)- and *X*-1,3-diacylglycerols of short- and long-chainlength, which were generated by partial Grignard degradation of the hydrogenated triacylglycerols, were isolated by borate-TLC. The enantiomeric *sn*-1,2- and *sn*-2,3-diacylglycerols and the *X*-1,3-diacylglycerols as their 3,5-dinitrophenylurethanes were resolved by HPLC on chiral columns. Both acetic and butyric acids were exclusively associated with the *sn*- 2,3- and *X*-1,3-diacylglycerols of short and long chainlength. These results establish the presence of acetic and butyric acids in the *sn*-3-position of bovine milk fat triacylglycerols. Other short- and medium-chainlength acids were found in progressively increasing proportions also in the *sn*-1- and *sn*-2-positions.

KEY WORDS: Butterfat, chiral-phase HPLC, dinitrophenylurethanes, enantiomeric diacylglycerols, fatty acids, Grignard degradation, positional analysis.

Stereospecific analyses have indicated that butyric and other short- and medium-chain fatty acids are located largely or exclusively in the *sn*-3-position of the triacylglycerols of total bovine milk fat (1) or butteroil distillate (2), while certain subfractions of butterfat obtained by crystallization (3) have shown the presence of short-chain acids in the *sn*-1- and *sn*-2-positions as well. Proton nuclear magnetic resonance (NMR) with a chiral shift reagent (4) and C-13 NMR (5) have also demonstrated the presence of butyric acid in the *sn*-3-position and in the primary positions, respectively. Because the stereospecific analyses depend on enzymatic fatty acid positional selectivity, which is partly dependent on the fatty acid structure and chainlength of the triacylglycerols (6), and because the NMR technique is rather insensitive (7), we have used chiral-phase high-performance liquid chromatography (HPLC) with a sensitive ultraviolet (UV) detector to redetermine the stereospecific location of butyric acid in the previously analyzed molecular distillate of butteroil (2). In addition, we have determined the positional location of acetic acid, which had not been established previously.

MATERIALS AND METHODS

Materials. Synthetic *rac*-1-acetyl-2-palmitoyl-, *sn*-2-palmitoyl-3-acetyl- and *sn*-1-acetyl-3-palmitoylglycerols, as well as *rac*-1-butyryl-2-palmitoyl-, *sn*-2-palmitoyl-3-butyryl- and *sn*-1-butyryl-3-palmitoylglycerols were prepared in the laboratory by partial Grignard degradation of the corresponding acetyl- and butyryldipalmitoylglycerols (8). Commercially available synthetic *rac*-1,2- and 1,3-dipalmitoylglycerols (Sigma Chemical Co., St. Louis, MO) were also used. The butteroil distillate (R-4) was as previously described (8). It had been obtained as the fourth most volatile 2.5% redistillate from an initial most volatile 10% distillate.

Methods. The acetyl- and butyryl-enriched triacylglycerol fractions were isolated by normal-phase thin-layer chromatography (TLC) with hexane/ethyl acetate (88:12) as developing solvent (9). After the initial TLC separation, the fractions were fully hydrogenated with platinum (IV) oxide and an excess of hydrogen gas (8). The acetyl-(pooled from several TLC plates) and the butyryl-containing acylglycerols were recovered separately and subjected to partial Grignard degradation as previously described (10). The *sn*-1,2(2,3)- and the *X*-1,3-diacylglycerols were resolved by normal-phase TLC on boric acid-containing silica gel G (11). For chiral-phase HPLC, the diacylglycerol fractions (10–400 μg) recovered from the TLC plate were converted to 3,5-dinitrophenylurethane (DNPU) derivatives by reaction with 3,5-dinitrophenylisocyanate (Sumitomo Chemical Co., Osaka, Japan) for 60 min at room temperature (12). The DNPU derivatives were purified by normal-phase TLC (12) with petroleum ether/1,2-dichloroethane/ethanol (40:10:3) as the developing solvent. Chiral-phase HPLC was performed on a Hewlett-Packard Model 1084 liquid chromatograph equipped with a column (25 cm × 4.6 mm i.d.) containing (*R*)-(+)-1-(1-naphthyl)ethylamine polymer as the chiral phase (YMC-Pack A-KO3; YMC Co., Kyoto, Japan) and a variable wavelength detector set at 226 nm (13). The DNPU derivatives (10–20 μg) were resolved with hexane/1,2-dichloroethane/ethanol (40:10:1) as the mobile phase at a flow rate of 1 mL/min. The column and solvent temperature were 25°C. Fatty acid composition and carbon number distribution of the original triacylglycerols and the derived diacylglycerol fractions were determined by gas–liquid chromatography (GLC) on polar (14) and nonpolar (15) capillary columns, respectively. The identification of the molecular species of the diacylglycerols was confirmed by chiral-phase liquid chromatography/mass spectrometry (LC/MS) of the DNPU derivatives (16).

RESULTS AND DISCUSSION

We had shown earlier (8) that acetyl acylglycerols make up at least 2% and butyryl acylglycerols about 36% of the R-4 molecular distillate of butteroil, where they occur at the frequency of one short-chain residue per triacylglycerol molecule in association with the more common long-chain fatty acids. The occurrence of acetyl-containing triacylglycerols in whole bovine milk fat had been previously reported by Parodi (9).

TLC separation. Figure 1 shows the TLC resolution of the partial Grignard degradation products of the hydrogenated acetyl and butyryl (and higher) diacylglycerol fractions of the butteroil distillate. A series of diacylglycerol bands was obtained for the products of the acetyldiacylglycerol fraction (Sample A). These diacylglycerol

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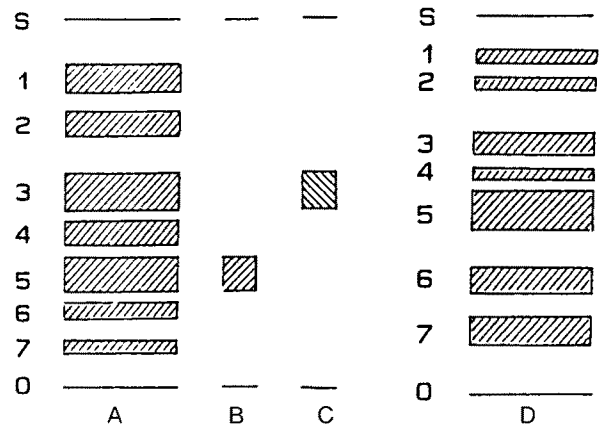

FIG. 1. Thin-layer chromatographic (TLC) resolution of partial Grignard degradation products of the hydrogenated acetyl- (A) and butyryl- (D) diacylglycerols from R-4 butteroil distillate. B, Standard *rac*-1,2-acetylpalmitoylglycerol; C, standard *rac*-1,2-dipalmitoylglycerol. S, Solvent front; 1, residual triacylglycerols; 2, tertiary alcohols from Grignard reaction; 3, *X*-1,2-diacylglycerols (long-long) in A and *X*-1,2-diacylglycerols (long-long) plus *X*-1,3-diacylglycerols (long-short) in D; 4, *X*-1,3-diacylglycerols (long-short) in A and *X*-1,2-diacylglycerols (long-medium) in D; 5, *X*-1,2-diacylglycerols (long-short); 6, 2-monoacylglycerols; 7, *X*-1-monoacylglycerols; O, origin. Borate-TLC with chloroform/acetone (94:6) as developing solvent. Other TLC conditions are given in text.

bands were identified as *X*-1,2-diacylglycerols containing two long acyl chains (Band 3), *X*-1,3-diacylglycerols containing one acetyl and one long-chain acyl chain (Band 4) and *X*-1,2-diacylglycerols containing one acetyl and one long acyl chain (Band 5). The diacylglycerols from the butyryldiacylglycerol fraction (Sample D) were distributed among three zones. The upper zone (Band 3) contained *X*-1,2-diacylglycerols having two long acyl chains, and the lower zone (Band 5) contained *X*-1,2-diacylglycerols having one long and one short (C_4) acyl chain. The middle zone (Band 4) was a heterogeneous fraction containing diacylglycerols having one short acyl chain (C_6 or higher) in combination with medium and long acyl chains.

Chiral-phase HPLC. We investigated the resolution of enantiomeric diacylglycerols containing short-chain fatty acids by chiral-phase HPLC of the DNPU derivatives of synthetic standards of acetyl- and butyrylpalmitoylglycerols. The *sn*-1-acetyl-2-palmitoylglycerol emerged as a symmetrical peak before the *sn*-2-palmitoyl-3-acetylglycerol, as established for the long-chain diacylglycerols (13). The *sn*-1-palmitoyl-3-acetylglycerol elutes just beyond the *sn*-1,2-enantiomer with a similar retention time. This elution order was confirmed by separate injection and coinjection analyses of the *rac*-1,2-, *sn*-2,3- and *sn*-1,3-acetylpalmitoylglycerols. A similar chiral-phase resolution was obtained for the standard *sn*-1-palmitoyl-3-butyryl- and *rac*-1-butyryl-2-palmitoylglycerols as the DNPU derivatives; the *sn*-1,3-enantiomer emerged first, followed by the *sn*-1,2- and the *sn*-2,3-enantiomer, as observed for the long-chain diacylglycerols (13). Resolution of the *sn*-1,3-isomers from the *sn*-1,2-enantiomers of acetyl- and butyrylpalmitoylglycerols is poor, as indicated by a low separation factor ($\alpha = 1.05$) between the *sn*-1,3- and *sn*-1,2-butyrylpalmitoylglycerols (see Fig. 2). On the other hand, the *X*-1,3-dipalmitoylglycerol is resolved from the corresponding *sn*-1,2-enantiomer ($\alpha = 1.22$), even with a shorter retention time under the same conditions. The enantiomer resolution of the short-chain diacylglyerols is also poorer than that of the long-chain diacylglycerols. Thus, the α values between the enantiomers of *rac*-1-acetyl-2-palmitoyl-, *rac*-1-butyryl-2-palmitoyl- and *rac*-1,2-dipalmitoylglycerols were 1.27, 1.36 and 1.44, respectively.

Figure 2 shows the chiral-column resolution of the *X*-1,2-diacylglycerols with two long acyl chains (Band 3) and the *X*-1,2-diacylglycerols with one acetyl and one long acyl chain (Band 5) derived from the acetyldiacylglycerol fraction. The long-chain *X*-1,2-diacylglycerols (Band 3) contain the *sn*-1,2-enantiomers (Fig. 2A). The acetyl long-chain *X*-1,2-diacylglycerols (Band 5) contain mainly *sn*-2,3-enantiomers with lesser amounts of *X*-1,3-isomers

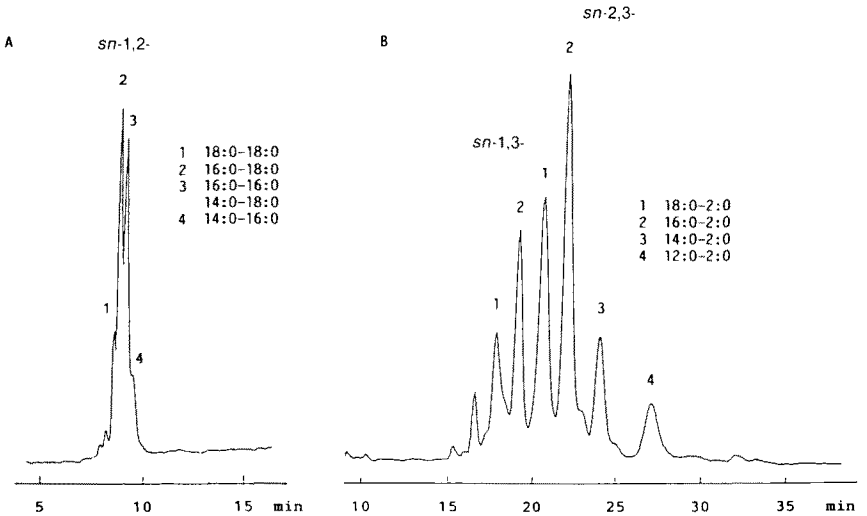

FIG. 2. Chiral-phase high-performance liquid chromatography (HPLC) resolution of the 3,5-dinitrophenylurethanes of the diacylglycerols derived from the hydrogenated acetyldiacylglycerols of R-4 butteroil distillate. A, *sn*-1,2-Diacylglycerol fraction (long-long acyl chains); B, *sn*-2,3-acetylacylglycerol fraction. HPLC conditions are given in text.

(Fig. 2B), which were inadvertently generated from the *sn*-2,3-enantiomers during extraction. The acetyl groups were also associated with the *X*-1,3-isomers in Band 4 (chromatogram not shown). In addition to complete enantiomer resolution and partial resolution of *X*-1,3-isomers from the corresponding *sn*-1,2-enantiomers, the chiral column also gives resolution based on equivalent carbon number (ECN) of diacylglycerols (13). Thus, each diacylglycerol fraction is resolved into several component peaks. The individual peaks were identified on the basis of the agreement of retention volumes with those of synthetic standards, the linear relationship between ECN and retention volumes (13) and knowledge of the fatty acid composition. The identification of the early peaks as *X*-1,3-isomers was confirmed by the presence of an ion at *m/z* 325 in the mass spectrum obtained by chiral-phase chloride-attachment LC/MS (16). This $[M-RCOOH]^-$ ion resulting from the loss of the long-chain fatty acid occurs in the mass spectra of the *X*-1,3-isomers, but not in the spectra of the *sn*-1,2- or *sn*-2,3-isomers. These findings establish the location of the acetyl residue at the *sn*-3-position of the original triacylglycerol molecule. Table 1 lists the composition of the major *sn*-1,2- and *sn*-2,3-diacylglycerols derived from the hydrogenated acetyldiacylglycerol fraction of the butteroil distillate. The major molecular species of the acetyldiacylglycerols contained the C_{18}–C_{18}, C_{16}–C_{18}, C_{16}–C_{16}, C_{14}–C_{18} and C_{14}–C_{16} fatty acid pairs in the *sn*-1,2-diacylglycerol moieties.

Figure 3 shows the chiral-column resolution of the diacylglycerols derived from the butyryldiacylglycerol fraction. The long-long acyl chain *X*-1,2-diacylglycerols (Band 3) were not resolved from the long-short acyl chain *X*-1,3-diacylglycerols on borate TLC (see Fig. 1). The DNPU derivatives of the diacylglycerols, however, were effectively separated by TLC on plain silica gel with R_f values of 0.55 and 0.49, respectively. However, minor cross-contamination is observed in each diacylglycerol fraction (Fig. 3, A and B). As for the acetylacylglycerols, the long-long chain *X*-1,2-diacylglycerols (Band 3) from the butyryldiacylglycerols contained only *sn*-1,2-enantiomers (Fig. 3A). The long-chain fatty acids of the *X*-1-monoacylglycerols recovered from Grignard degradation (see Fig. 1) exclusively contained the *sn*-1-enantiomer (chromatogram not shown). The long-short chain *X*-1,2-diacylglycerols (Band 5) contained only the *sn*-2,3-enantiomers (Fig. 3D). The butyryl group was also associated with the long-short chain *X*-1,3-isomers (Band 3, Fig. 3B). These findings establish the location of the butyric acid residue at the *sn*-3-position of the original triacylglycerol molecule. The long-medium chain diacylglycerols (Band 4) were resolved into two fractions, which represent *sn*-1,2- and *sn*-2,3-enantiomers (Fig. 3C). This fraction consisted of C_6, C_8 and C_{10} fatty acid-containing diacylglycerols, which were effectively resolved from the butyric acid-containing diacylglycerols on borate TLC (Fig. 1). The chiral-phase HPLC elution pattern shows that some of the medium-chain fatty acids also are found in the *sn*-1-position, although most are located at the *sn*-3-position. (Fig. 3C). Table 2 lists the carbon number distribution for the *sn*-1,2- and *sn*-2,3-diacylglycerols derived from the hydrogenated butyryl-(and higher) diacylglycerols of the R-4 distillate. The major *sn*-1,2-diacylglycerol species are the C_{16}–C_{18},

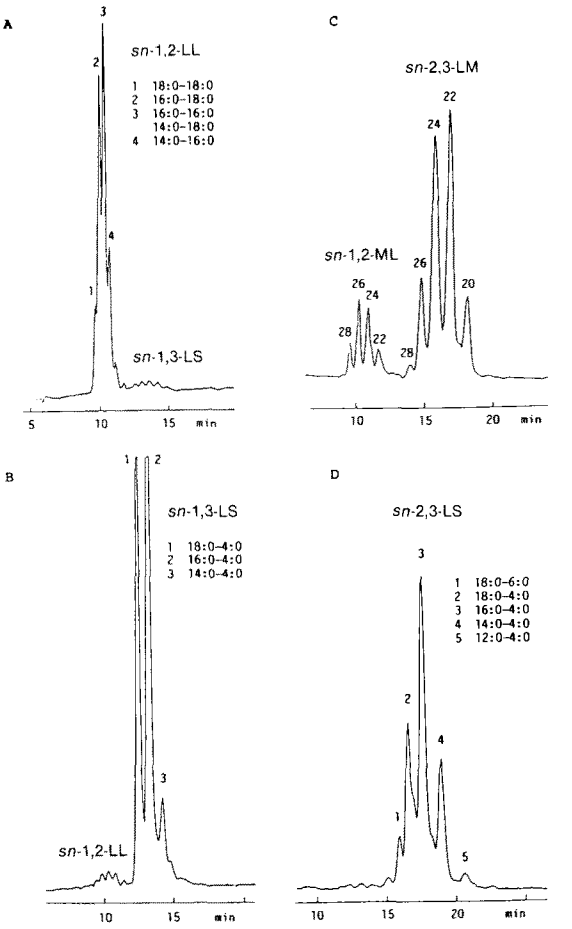

FIG. 3. Chiral-phase high-performance liquid chromatography (HPLC) resolution of the 3,5-dinitrophenylurethanes of the diacylglycerols derived from the hydrogenated butyryldiacylglycerol fraction of R-4 distillate of butteroil. A, *sn*-1,2-Diacylglycerol fraction (long-long acyl chains); B, *sn*-1,3-butyrylacylglycerol fraction; C, *sn*-2,3-diacylglycerol fraction (long-medium acyl chains); D, *sn*-2,3-butyrylacylglycerol fraction. L, long; S, short (C_4); M, medium (C_6 and above). The number above each peak in chromatogram C represents the number of total acyl carbons in the diacylglycerols. HPLC conditions are given in text.

TABLE 1

Molecular Species Composition (mole %) of the Major *sn*-1,2- and *sn*-2,3-Diacylglycerols Derived from the Hydrogenated Acetyldiacylglycerols of R-4 Distillate of Butteroil

Carbon number	*sn*-1,2-Diacylglycerols	*sn*-2,3-Diacylglycerols
18:0-2:0	0	31.78
16:0-2:0	0	45.61
14:0-2:0	0	12.67
12:0-2:0	0	9.94
18:0-18:0	5.67	0
16:0-18:0	34.35	0
16:0-16:0 14:0-18:0	45.04	0
14:0-16:0	14.94	0

TABLE 2

Molecular Species Distribution (mole %) of *sn*-1,2- and *sn*-2,3-Diacylglycerols Derived from the Hydrogenated Butyryl- (and higher) Diacylglycerols of R-4 Distillate of Butteroil

	sn-1,2-Diacylglycerols		*sn*-2,3-Diacylglycerols	
Carbon number	Long-Long	Long-Short[a]	Short-Long	Long-Medium[b]
16		1.36	0.85	
16			2.48	
17		0.38	0.14	
18		8.13	2.17	0.14
18			17.65	0.57
19		0.71	1.60	0.15
19		1.88	1.90	
20		45.04	6.28	1.79
20			36.10	5.61
21		1.24	1.75	0.60
21		1.73	1.25	0.94
22		39.49	5.85	26.32
22			17.28	1.70
23			0.24	1.02
23				0.90
24	1.06		0.45	16.39
24			1.30	11.01
24			1.30	
25				0.62
25				1.05
26	3.06		0.32	12.57
26			0.05	
27	0.16			0.37
27	0.27			0.39
28	6.37		0.12	6.22
29	0.40			0.33
29	0.75			
30	0.32		0.30	4.60
30	15.76			
31	1.31			0.34
31	2.17			
32	0.58		0.40	3.53
32	30.10			
33	1.74			0.19
33	2.72			
34	0.34		0.24	1.40
34	24.16			
35	0.63			
35	1.01			
36	6.84			0.27
37	0.09			
38	0.15			

[a]*X*-1,3-Diacylglycerols (long-short) recovered from borate thin-layer chromatography (TLC), along with *X*-1,2-diacylglycerols (long-long), but resolved as the 3,5-dinitrophenylurethane derivatives on plain silica TLC.
[b]Mainly *sn*-2,3-diacylglycerols (long-medium) with some *sn*-1,2-diacylglycerols (long-medium).

TABLE 3

Distribution of Short- and Medium-chain Acids in the Triacylglycerols of R-4 Distillate of Butteroil (mole%)[a]

	sn-Glycerol Position		
Acid	*sn*-1-Position	*sn*-2-Position	*sn*-3-Position
Acetic	0	0	100
Butyric	0	0	100
Caproic	0	0	100
Caprylic	0	15	85
Capric	9	41	50

[a]The data for caproic, caprylic and capric acids were calculated from a previous report (Ref. 2).

C_{16}-C_{16}, C_{14}-C_{18} and C_{14}-C_{16} acyl chain combinations. The *sn*-2,3- and *sn*-1,3-diacylglycerols represent combinations of butyric acid in the *sn*-3-position with C_{14} and C_{18} fatty acids in the *sn*-2- or *sn*-1-position, respectively. Table 3 lists estimates of the relative proportion of the short- and medium-chain fatty acids found at the *sn*-1-, *sn*-2- and *sn*-3-positions of the short- and medium-chain triacylglycerols of the R-4 molecular distillate. The C_8 and C_{10} acids start appearing in the *sn*-2-position before showing up in the *sn*-1-position.

In conclusion, chiral-phase HPLC of the DNPU derivatives of diacylglycerols provides a convenient and reliable method for the determination of the stereospecific positional distribution of short-chain fatty acids in bovine, and presumably other ruminant, milk fat triacylglycerols.

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