Determination of the Positional Distribution of Fatty Acids in Butterfat Triacylglycerols

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Triacylglycerol (TAG) standards were separated by analytical high-performance liquid chromatography (HPLC) with laser light-scattering detection (LLSD). A high sensitivity for TAGs was observed with LLSD whereas poor sensitivity was observed with ultraviolet detection. The HPLC-LLSD analytical separation of butterfat TAGs showed that the TAGs were eluted according to increasing carbon number. Preparative HPLC-LLSD was used to characterize butterfat TAGs that contained hypercholesterolemic fatty acids (laurate, myristate, palmitate) with carbon chainlengths of 12 or greater. These TAG fractions accounted for 29.2% of the total butterfat TAGs. Analysis of the positional distribution of fatty acids of selected butterfat TAGs containing hypercholesterolemic fatty acids showed the presence of positional isomers in each of these fractions. These butterfat TAGs also showed the predominant presence of hypercholesterolemic fatty acids at the *sn*-2 position. The characterization of the positional distribution of hypercholesterolemic fatty acids in butterfat TAGs is the first step for the determination of the metabolic role of the positional distribution in the hypercholesterolemic effects of butter.

KEY WORDS: Butter, fatty acids, HPLC, laser detection, light-scattering, positional distribution, triacylglycerols.

Analysis of the positional distribution of fatty acids in triacylglycerols (TAGs) of fats and oils has become increasingly important to lipid biochemists due to their possible role in cholesterol metabolism (1,2). The separation of the molecular species of TAGs has generally been performed by high-performance liquid chromatography (HPLC) in the reversed-phase mode. TAGs are eluted in an ascending order of chainlength, although a double bond in the chain reduces the retention time by approximately 2 carbon atoms (3). The development of gradient elution systems has facilitated the separation of complex TAG mixtures, such as those encountered in butter (4–7). Detection and identification of TAGs, however, still remains a problem (8).

Robinson and Macrae (9) indicated that a commercially available light-scattering detector (LSD) connected with a mass detector was a sensitive HPLC method for the detection of TAGs. The available literature on LSD (10) and on the laser light-scattering detector (LLSD) (4,5) has suggested that, in addition to instrumental factors, optimization of such factors as temperature and gas flow is also important for sensitivity.

Butterfat, which contains a large number of different fatty acids, has presented a particular challenge to analysts in terms of identification and separation of TAGs, due to the complex variety of molecular species of TAGs (4,8,9,11,12). Stolyhwo *et al.* (4) obtained an HPLC-LLSD profile of butterfat TAGs, but no peaks were identified. Christie (3) reported a preliminary HPLC profile of bovine milk TAGs obtained from mass detection. No attempt was made to identify any of the detected peaks, although Christie (3) suggested that mass spectroscopy coupled to HPLC would be needed for proper peak identification. In addition, a limited number of TAG and diacylglycerol components of bovine and goat milkfat have been identified by HPLC-mass spectrometry (MS) (13,14).

The objective of the present study was to separate TAGs from butterfat and to determine the positional distribution of selected TAGs. The present paper describes a method for the separation of butterfat TAGs by HPLC-LLSD. Preparative HPLC separation of butter TAG fractions was carried out as a first step in the identification of individual TAGs. The analysis of the positional distribution of fatty acids in individual butter TAGs took place *via* enzymatic hydrolysis of the isolated HPLC TAG fractions, coupled with gas liquid chromatographic (GC) analysis. As far as the authors are aware, no previous studies have reported HPLC preparative analysis of butter TAGs followed by analysis of the positional distribution of fatty acids.

MATERIALS AND METHODS

Separation of individual TAGs of butterfat. The complex mixture of butterfat TAGs was separated by gradient elution in a nonaqueous reversed-phase HPLC system (Beckman Model 126, Beckman Instruments, Inc., San Ramon, CA) with LLSD (Varex Corporation, Burtonsville, MD) and computerized integration and data handling. A Beckman analog interface Model 406 was used to transfer data from the mass detector to the HPLC system. Mass detection was performed at 72°C in the presence of an inert gas (N_2) with a flow rate of 30 mL/min. The dehydrated butterfat (100 mg) was solubilized in 10 mL chloroform. Manual injection was carried out from a Rheodyne valve 7125 with a 20-μL loop (200 μg of dehydrated butterfat) onto two Spherisorb-ODS-2 columns (150 × 4.6 mm i.d., pore size 5 mm) (Altech Associates, Inc., Deerfield, IL). The HPLC columns were kept at 25°C by means of a column heater Model 7960 (Jones Chromatography Ltd., Hingoed, Wales, United Kingdom). Elution was performed with chloroform/acetonitrile at a linear gradient ranging from 20 to 50% chloroform (omnisolv grade, BDH Inc., Poole, United Kingdom) at a flow rate of 1.0 mL/min for 45 min. The weight of fractionated TAGs ranged from 20 to 40 μg.

For semi-preparative HPLC, a Spherisorb-ODS-2 column (300 × 10 mm i.d., pore size 5 mm) (Altech Associates, Inc.) was used at room temperature. The dehydrated butterfat (100 mg) was solubilized in 1 mL chloroform. The loop size for manual injections was 100 μL (10 mg of dehydrated butterfat). Elution was performed isocratically with chloroform and acetonitrile (30:70, vol/vol) at a flow rate of 3.0 mL/min for 60 min. The weight of fractionated TAGs ranged from 225 to 475 μg. Two to three preparative runs of scale fractionations were performed

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to obtain a sufficient quantity of a fractionated TAG for positional distribution studies.

Positional distribution of fatty acids. The positional distribution of fatty acids in butterfat TAGs was determined according to the procedure described by Kuksis (15) for the analysis of phospholipids. The procedure involves pancreatic lipase action on TAGs to yield *sn*-2 monoacylglycerols, from which the fatty acid composition of the *sn*-2 position can be determined by GC analysis. Pancreatic lipase-mediated breakdown of the TAG substrate also gives essentially random mixtures of *sn*-1,2- and *sn*-2,3-diacylglycerols. In the present work, additional stereospecific analysis of the *sn*-2,3-diacylglycerols with stereospecific lipases was carried out to determine the complete positional distribution of fatty acids of the TAG molecules studied. The *sn*-2,3-diacylglycerols were converted to phosphatidylcholines (16) by the action of Grignard reagent. This was performed (17) by the solubilization of 5 mg butterfat TAGs into 0.4 mL diethyl ether mixed with 0.1 mL of 1 M ethyl magnesium bromide. The mixture was vortexed for 25 s. The mixture was acidified, and the aqueous phase was removed. The organic phase was washed once with 2% $NaHCO_3$ and two times with water. The *rac*-1,2- and 1,3-diacylglycerols were extracted and purified before subsequent analysis. The derived phosphatidylcholines were then acted upon sequentially by phospholipase A_2 and phospholipase C to yield *sn*-1-monoacylglycerophosphatidylcholine and *sn*-1,2-diacylglycerol, respectively. Following analysis of the fatty acid composition of the total TAG molecule and the monoacylglycerol and diacylglycerol products generated from enzymatic reactions described above, the composition of fatty acids in the *sn*-1, *sn*-2 and *sn*-3 positions of the TAG molecules was calculated.

Preparation of fatty acid methyl esters. Preparation of fatty acid methyl esters from TAG fatty acids was performed according to Badings and De Jong (18). TAGs (100 mg) were dehydrated with sodium sulfate and solubilized in 6.0 mL pentane. This was followed by treatment with 0.06 mL sodium methoxide (2M). After centrifugation (1,000 × *g*, 5 min), the supernatant was taken for GC analysis.

Free fatty acid methylation was also performed according to Badings and De Jong (18) by using a mixture of methanol and 20% hydrochloric acid (80:20, w/w) as the methylating reagent. The methylation was carried out in a shaking water bath at 85°C for 15 min. The mixture was centrifuged (1,000 × *g*, 5 min), and the supernatant was analyzed by GC for fatty acid content.

Fatty acid analysis by GC. Gas-liquid chromatographic analysis of fatty acid methyl esters was performed in a Varian Model 3400 gas chromatograph (Varian Associates, Sunnyvale, CA). The system was equipped with an on-column injector and a flame-ionization detector (FID) with a capillary column (30 m × 0.32 mm i.d. coated with polyethylene glycol, Supelco Canada Ltd., Oakville, Ontario). The flow rates for carrier gas (helium), hydrogen and air were, respectively, 1.8, 30.0 and 300.0 mL/min. The detector temperature was 230°C and the injector temperature was 220°C. Injection was performed at 35°C, and, after an isothermal period of 5 min, the column temperature was raised at a rate of 8°C/min to a final temperature of 200°C.

RESULTS AND DISCUSSION

Figure 1 shows the HPLC profiles for the separation of a mixture of TAG standards obtained by LLSD detection. Gradient elution was required to separate the large range of molecular weights of the TAGs found in butterfat. The results clearly demonstrate the sensitivity of LLSD (Fig. 1). The present findings also show separation by LLSD of the more volatile TAGs such as tributyrin. Other workers have demonstrated that the mass detector is sensitive to TAG separations with no base-line drift, even for lengthy gradient elutions (9,12,19–21).

The HPLC-LLSD analytical separation of the TAGs of butterfat showed an excellent resolution (Fig. 2). The resolution observed is comparable to the HPLC-LLSD analytical separations reported by Christie (3) but markedly higher resolution than the HPLC-LLSD chromatograms observed by Robinson *et al.* (8).

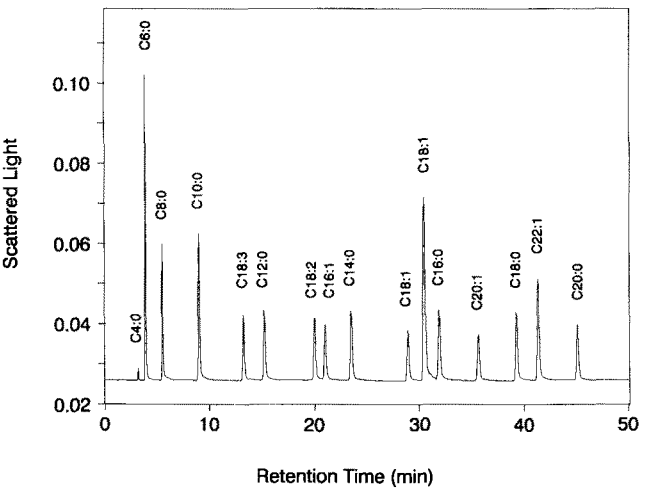

FIG. 1. High-performance liquid chromatographic analysis of standard triacylglycerols with a laser light-scattering detector.

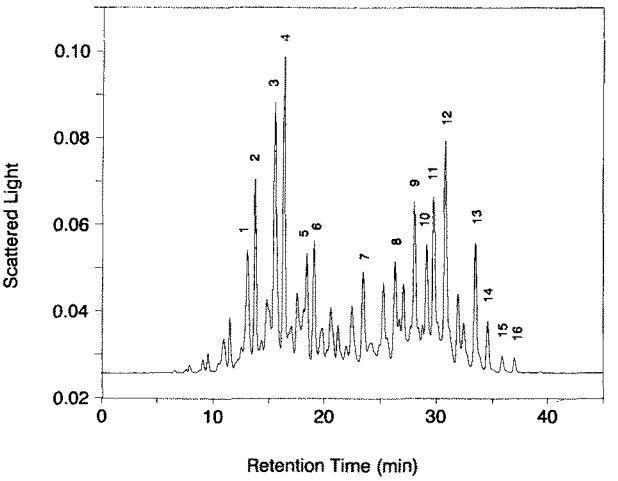

FIG. 2. Analytical separation of triacylglycerols of butterfat by high-performance liquid chromatography with laser light scattering detection.

TABLE 1

Fatty Acid Composition of Butter Triacylglycerol Fractions Containing Fatty Acids with a Chainlength of 12C or Greater

Triacylglycerol fraction[b]	Fatty acid composition (%)[a]													
	C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0
8	—[c]	—	0.6	14.5	15.4	22.4	0.5	28.1	0.9	8.2	8.1	1.1	0.2	—
9	—	0.5	0.6	3.1	7.1	26.4	2.1	27.0	3.8	5.3	21.6	1.4	1.1	—
10	—	—	—	3.9	9.5	25.9	—	34.3	0.1	10.3	15.4	0.6	—	—
11	—	—	—	—	0.4	5.0	—	33.3	0.9	5.7	54.0	0.7	—	—
12	—	—	—	—	0.4	20.4	—	47.1	0.9	6.4	24.5	0.3	—	—
13	—	—	—	—	—	1.8	—	35.9	0.2	32.3	29.8	—	—	—
14	—	—	—	—	—	12.4	1.5	53.6	—	29.2	3.3	—	—	—
15	—	—	—	—	—	2.8	—	13.4	—	57.8	26.3	—	—	—
16	—	—	—	—	0.3	4.2	0.2	—	35.0	52.3	6.7	—	—	1.3

[a]Percent of each fatty acid relative to the total fatty acids content in the triacylglycerol fraction.
[b]Triacylglycerol fractions of butterfat as obtained by preparative high-performance liquid chromatography analysis.
[c](—) Not detected.

An examination of the fatty acid composition of the isolated analytical HPLC fractions of butterfat TAGs showed that they were eluted according to increasing carbon number (Fig. 1, Table 1). The characterization of butterfat TAGs containing fatty acids with carbon chainlengths of 12 or greater is of great metabolic interest because these TAGs contain the hypercholesterolemic (*i.e.,* laurate, myristate and palmitate) and hypocholesterolemic (*i.e.,* oleate) fatty acids. The TAG fractions that contained the greatest proportion of these fatty acids of interest were found by GC analysis to correspond to peaks 8 to 16 of the analytical HPLC separation of the butterfat TAGs (Fig. 2, Table 2). Although preliminary GC analyses (data not shown) demonstrated that peaks 1 to 7 also contained C12 and C14 fatty acids, longer-chain saturated (*i.e.,* palmitate and stearate) and unsaturated (*i.e.,* oleate) fatty acids were not present. Because peaks 1 to 7 were also of lesser quantitative importance relative to peaks 8 to 16, further analysis was focussed upon a selected subsample of these latter peaks.

Preparative HPLC-LLSD was used to isolate TAG fractions 8 to 16 for the determination of the positional distribution of the fatty acids in a portion of these TAGs (Fig. 3). These TAG fractions accounted for 29.2% of the total TAG content of butterfat (Table 2).

The positional distribution of fatty acids of TAGs in HPLC fractions 9, 11 and 12 was determined. As illustrated in Table 1, these fractions were the major subsamples of the TAGs found in fractions 8 to 16 that contained oleate with either myristate or palmitate and were chosen to provide information related to the role of positional distribution of these fatty acids on the hypercholesterolemic properties of butterfat. The degradation of the TAG fractions 9, 11 and 12 by hydrolysis with pancreatic lipase yielded *sn*-2-monoacylglycerols and random mixtures of *sn*-1,2- and *sn*-2,3-diacylglycerols. The fatty acid composition of the *sn*-2-monoacylglycerol fractions of the original TAG fractions 9, 11 and 12 was determined by GC and is shown in Table 3. The results showed a different predominance of fatty acids present at the *sn*-2-position in the different TAG fractions. There was a near equivalent distribution of myristate, palmitate and oleate present at the *sn*-2-position of TAGs found in fraction 9. On the other hand, palmitate and oleate were the predominant fatty acids in the *sn*-2-position of fraction

TABLE 2

Absolute and Relative Content of Butter Triacylglycerol Fractions Containing Fatty Acids with a Chainlength of 12C or Greater

Triacylglycerol fraction[a]	Triacylglycerol content (%)	
	Absolute[b]	Relative[c]
8	3.2	11.4
9	4.5	14.1
10	3.1	16.3
11	4.5	14.4
12	7.2	24.2
13	4.3	13.0
14	1.5	4.3
15	0.5	1.2
16	0.4	1.1
Total	29.2	100.0

[a]Triacylglycerol fractions of butterfat as obtained by preparative high-performance liquid chromatography analysis.
[b]Absolute percent of each triacylglycerol relative to the total triacylglycerol content in butterfat.
[c]Relative percent of each triacylglycerol relative to the total triacylglycerol content in triacylglycerol fractions 8 to 16.

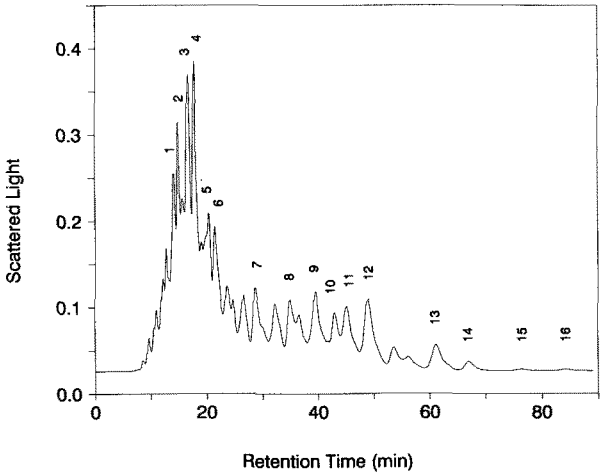

FIG. 3. Preparative separation of triacylglycerols of butterfat by high-performance liquid chromatography with laser light scattering detector.

TABLE 3

Fatty Acid Composition of 2-*sn*-Monoacylglycerols Obtained from the Hydrolysis of Butter Triacylglycerol Fractions 9, 11 and 12

	2-*sn*-Monoacylglycerol fraction[a]		
	9	11	12
Fatty acid	Percent composition[b]		
C4:0	—[c]	—	—
C6:0	—	—	—
C8:0	0.4	0.4	0.9
C10:0	—	—	1.0
C12:0	1.3	0.1	1.4
C14:0	37.0	3.7	10.3
C14:1	1.5	0.5	0.5
C16:0	24.2	47.2	71.1
C16:1	1.0	0.8	1.0
C18:0	—	—	1.2
C18:1	32.9	46.1	11.2
C18:2	1.7	1.2	1.2
C18:3	—	—	—
C20:0	—	—	0.2

[a]2-*sn*-Monoacylglycerol fractions obtained by the specific hydrolysis of selected butter triacylglycerol fractions.
[b]Percent of each fatty acid relative to the total fatty acids content in the individual diacylglycerol fraction.
[c](—)Not detected.

TABLE 4

Fatty Acid Composition of 2,3-*sn*-Diacylglycerols Obtained from the Hydrolysis of Butter Triacylglycerol Fractions 9, 11 and 12

	2,3-*sn*-Diacylglycerol fraction[a]		
	9	11	12
Fatty acid	Percent composition[b]		
C4:0	—[c]	—	—
C6:0	—	—	—
C8:0	—	—	—
C10:0	—	0.2	—
C12:0	—	4.9	9.3
C14:0	70.3	2.6	7.7
C14:1	—	0.7	8.1
C16:0	14.5	36.5	46.3
C16:1	—	4.6	6.7
C18:0	—	13.0	8.2
C18:1	15.2	35.0	8.7
C18:2	—	1.4	1.0
C18:3	—	0.8	—
C20:0	—	0.3	—

[a]2,3-*sn*-Diacylglycerol fractions obtained by the specific hydrolysis of selected butter triacylglycerol fractions.
[b]Percent of each fatty acid relative to the total fatty acids content in the individual diacylglycerol fraction.
[c](—)Not detected.

11, and palmitate was the predominant fatty acid present in the *sn*-2-position of fraction 12.

The pancreatic lipase also releases *sn*-2,3-diacylglycerols as intermediates in the enzyme hydrolysis. An analysis of these moieties of the TAGs in fractions 9, 11 and 12 was performed enzymatically with stereospecific lipases to obtain a stereospecific positional analysis of these diacylglycerols (Table 4). The results of the fatty acid analysis of the 2,3-*sn*-diacylglycerols demonstrated a preponderance of myristate in fraction 9, palmitate and oleate in fraction 11 and palmitate in fraction 12 (Table 4).

Following analysis of the fatty acid composition in the diacylglycerol, monoacylglycerol and TAG fractions, the positional distribution of fatty acids in TAGs in the different fractions was determined. Positional analysis of the TAGs in fractions 9, 11 and 12 demonstrated the presence of positional isomers in each of these fractions (Table 5). In fraction 9, the *sn*-1-position is esterified with palmitate and oleate in equal proportions, while *sn*-2- and *sn*-3-positions are occupied by myristate, palmitate and oleate. Oleate occupies the *sn*-1-position, whereas palmitate is interchanged with oleate at the *sn*-2- and *sn*-3-positions in the two positional isomers in fraction 11. Oleate is present at the *sn*-1-position, and palmitate is esterified at the *sn*-2- and *sn*-3-positions in the predominant TAG in fraction 12. The minor TAG in fraction 12 is a positional isomer of the major TAG with palmitate interchanged with oleate at the *sn*-1 position.

The characterization of the positional distribution of hypercholesterolemic fatty acids in butterfat TAGs is the first step for the determination of the metabolic role of the positional distribution in the hypercholesterolemic effects of butterfat. A number of human (22–24) and animal (25,26) studies have suggested that the capability of dietary saturated fats to increase serum cholesterol is dependent on the presence of a saturated fatty acids at the *sn*-2 position of the TAG. For example, despite the

TABLE 5

Positional Distribution of Fatty Acids of Triacylglycerol Isomers Obtained from High-Performance Liquid Chromatography Preparative Separation of Butter Triacylglycerol Fractions 9, 11 and 12

	Triacylglycerol	
Fraction	Isomer	Percent[a]
9a	αC16:0 β¢14:0 α′C18:1	20
9b	αC18:1 β¢14:0 α′C16:0	20
9c	αC18:1 β¢16:0 α′C14:0	25
9d	αC16:0 β¢18:1 α′C14:0	35
11a	αC18:1 β¢16:0 α′C18:1	50
11b	αC18:1 β¢18:1 α′16:0	50
12a	αC18:1 β¢16:0 α′C16:0	85
12b	αC16:0 β¢18:1 α′C16:0	15

[a]Percent of each triacylglycerol isomer relative to the total isomers in the triacylglycerol fraction.

predominant presence of saturated fatty acids in cocoa butter, the high oleate content of the *sn*-2 position of cocoa TAGs is thought to be partly responsible for the neutral effects of this dietary fat on serum cholesterol (26).

Moreover, recent findings have indicated that the substitution of saturated fatty acids for oleate in the *sn*-2 position of dietary model TAGs slows the metabolic clearance of chylomicrons (1). The present study indicates the predominant presence of hypercholesterolemic fatty acids (myristate and palmitate) in the *sn*-2 position of a major fraction of butterfat TAGs (*i.e.,* 65%, 50%, 85% of TAGs in fractions 9, 11 and 12, respectively). It is possible that the hypercholesterolemic properties of butterfat arise in part from the proponderance of palmitate and myristate at the *sn*-2 position. Nutritional and metabolic studies will be needed to determine the functional significance of the positional distribution of these TAGs in terms of the hypercholesterolemic properties of butterfat.

The present study has demonstrated the utility of HPLC-LLSD to provide excellent separation and resolution of the complex mixture of TAGs found in butterfat. Moreover, the results have demonstrated that preparative HPLC in combination with enzymatic degradation of the TAG fractions can be used to determine the positional distribution of fatty acids in selective TAG fractions. The characterization of the positional distribution of hypercholesterolemic fatty acids in butterfat TAGs obtained in the present study can be used to determine the metabolic significance of these positional distributions in terms of the hypercholesterolemic properties of butterfat.

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