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Synthesis and Analysis of Symmetrical and Nonsymmetrical Disaturated/Monounsaturated Triacylglycerols

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Symmetrical disaturated triacylglycerols of the structure SUS, where S is stearic acid (18:0) and U is an unsaturated fatty acid, either oleic (O; 9*cis*-18:1), linoleic (L; 9*cis*,12*cis*-18:2), or linolenic (Ln; 9*cis*,12*cis*,15*cis*-18:3), are important components providing functionality to interesterified fat blends and structurally modified oils. Nonsymmetrical triacylglycerols of the structure SSU can significantly change melting point and solid fat content profiles. To characterize the physical properties of pure and symmetrical and nonsymmetrical triacylglycerol mixtures, the same reaction sequence has been used to prepare multigram quantities of triacylglycerols SUS and SSU. Tristearin was converted to a mixture of mono-, di-, and triacylglycerols, and the 1,3- and 1,2-diacylglycerol fraction was isolated by silica column chromatography. The 1,3-diacylglycerols were removed by crystallization from acetone and esterified with the appropriate fatty acid to form the symmetrical triacylglycerols with >99% SUS structure. The more difficult to obtain 1,2-diacylglycerols were prepared by esterification of the enriched 1,2-diacylglycerol fraction (80−86% 1,2-diacylglycerols) remaining after removal of much of the 1,3-isomer by crystallization, but silver resin or silver nitrate impregnated silica gel chromatography was required to isolate the nonsymmetrical triacylglycerols. SSL and SSLn were prepared in purities of >98% by this procedure, but not SSO. Silver ion HPLC was found to be as accurate as, and more rapid than, lipolysis/gas chromatography for the determination of the isomeric purities of the synthesized triacylglycerols.

KEYWORDS: Triacylglycerol; symmetrical; nonsymmetrical; synthesis; analysis; silver

INTRODUCTION

The amounts and types of triacylglycerols in the oil phase of margarines and spreads are considered to be responsible for such properties as spreadability, resistance to water/oil loss, and melting at body temperature (*1*). Hydrogenation of vegetable oils has traditionally been used to reduce the unsaturation of the oil triacylglycerols and to produce oils containing triacylglycerols with the sharp melting points required for their use as base feedstocks. Over the past 25 years, a number of economic, health, and consumer-driven factors have stimulated research aimed at reducing the levels of *trans* fatty acids formed during the hydrogenation of vegetable oils (*2*). Alternatives to hydrogenation include interesterification, blending of tropical and liquid vegetable oils, fractionation, and, more recently, development of structurally modified oils by transgenic or conventional plant breeding methods (*3*). Random, base-catalyzed interesterification of liquid oils consisting largely of triunsaturated and diunsaturated triacylglycerols with trisaturated triacylglycerols yields mixtures of the mono- and disaturated triacylglycerols with the high, steep, sharp melting points and solid fat content (SFC) profiles required to produce both shortening and margarine/spread oils (*3*).

Before interesterification, disaturated triacylglycerols in vegetable oils have the symmetrical structure (*4*) according to the 1(3)-position of the saturated fatty acids. In our example, S is stearic acid (18:0) and U is an unsaturated fatty acid, either oleic (O; 9*cis*-18:1), linoleic (L; 9*cis*,12*cis*-18:2), or linolenic (Ln; 9*cis*,12*cis*,15*cis*-18:3) fatty acid. After interesterification, as much as 50% of the triacylglycerols of the structure SUS are converted to triacylglycerols of the structure SSU, a change that could account for the observed increases in melting point and solid fat content profiles (*5*). (Fatty acids in the 1- and 3-positions on the glycerol backbone of chemically synthesized triacylglycerols are considered to be equivalent, so the nonsymmetrical triacylglycerols of the structure SSU may be written SSU or USS.) Preparation of pure samples of symmetrical and nonsymmetrical disaturated/monounsaturated triacylglycerols (of the structures SUS and SSU, respectively) was therefore undertaken to characterize the physical properties of these compounds and to study their functional properties in food oil systems.

MATERIALS AND METHODS

Chemicals. Stearic, oleic, linoleic, and linolenic acids were purchased from Nu-Chek-Prep (Elysian, MN). Sodium methoxide was obtained from Harshaw Chemical Co. (Cleveland, OH). *N*,*N*-Dicyclo-

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hexylcarbodiimide and *p*-toluenesulfonic acid were from Sigma-Aldrich (St. Louis, MO), 4,4-(Dimethylamino)pyridine was from Eastman Fine Chemicals (Rochester, NY), and silica gel was from J. T. Baker Chemical Co. (Phillipsburg, NJ). All solvents were of either HPLC grade (acetone, acetonitrile, and methanol) or ACS grade (benzene, petroleum ether, carbon tetrachloride, and ethyl ether) and were used as received.

Liquid Chromatography. Silver resin chromatography on a 2.5 × 45 cm glass Michael-Miller column packed with 100/200 mesh silver ion-saturated Rohm and Haas XN1010 macroreticular sulfonic acid resin was utilized for preparative purification of nonsymmetrical disaturated/monounsaturated triacylglycerol isomers. [For silver ion incorporation and packing of the glass columns see Adlof and Emken (*6*).] A Waters 510 HPLC pump and an R403 refractive index detector (Waters Associates, Milford, MA) were used. The elution solvent was acetone, 5.0 mL/min, isocratic, 23 °C. Samples (1−4 g per injection) were applied by needle-tipped syringe through a septum to the top of the column. Fractions were isolated and, if necessary, rechromatographed until the desired triacylglycerol purities (>98% SSU) were achieved.

Silver Ion HPLC (Ag-HPLC). A P2000 solvent delivery system (Spectra-Physics Analytical, San Jose, CA), a 7125 injector (Rheodyne, Inc., Cotati, CA) with a 20 μL injection loop, and a V4 absorbance detector (ISCO, Inc., Lincoln, NE) at a wavelength of 206 nm were used. The 250 × 4.6 mm i.d., 5 μm, Chromspher Lipids silver-ion impregnated columns were purchased from Varian-Chrompack International, Middelburg, The Netherlands, and used as received.

(i) Purity Determination (%SUS versus % SSU) of Synthesized Triacylglycerols. Two Ag-HPLC columns connected in series were utilized. Sample size was 50−100 μg. The elution solvent was 0.5% ACN in hexane at a flow rate of 1.0 mL/min. UV detection was made at 206 nm.

(ii) Semipreparative Application. Four Ag-HPLC columns were connected in series; an elution solvent of 1.5% acetonitrile in hexane and a sample size of 2 mg (20 μL injection of 100 mg of sample/mL of hexane) were used. Other conditions were the same as in (*i*), above.

Thin-Layer Chromatography (TLC). Formation of the distearoylglycerol intermediate(s) and the final triacylglycerol(s) was followed by TLC. Samples (5−10 mg) were removed from the reaction vessel, dissolved in 1.0 mL of hexane, and applied (~10 μL) to 1 × 3 in. silica gel TLC [K6] plates (Whatman, Inc., Clifton, NJ). The elution solvent was benzene/ethyl ether (80:20 v/v); visualization was by I_2 vapor or by spraying with 10% $CuSO_4$ in 8% H_3PO_4/heating to120 °C by hot plate (*7*, *8*).

Gas Chromatography (GC). The triacylglycerol or 2-monoacylglycerol fractions obtained by lipolysis of triacylglycerol samples were converted to fatty acid methyl esters (5% HCl in methanol) and analyzed on a model 3400 gas chromatograph (Varian Instruments, Palo Alto, CA) equipped with a 100 m × 0.32 mm i.d. SP2380 capillary column (Supelco, Inc., Bellefonte, PA) with a flame ionization detector (FID) and utilizing helium as carrier gas. Operating conditions were as follows: injector, 240 °C; split ratio, 100:1; oven temperature programmed from 155 to 220 °C at 3 °C /min with an initial hold time of 15 min; detector, 280 °C (*9*).

Melting Points. *(i) Diacylglycerol melting points* were determined with a Thomas-Hoover melting point apparatus (Arthur H. Thomas Co., Philadelphia, PA).

(ii) Melting points of synthesized triacylglycerols (symmetrical and nonsymmetrical) and of commercial fats (**Table 1**) were determined on a dropping point apparatus (Mettler Instruments, Hightstown, NJ).

Triacylglycerol Structure Determination/Lipolysis. A method previously described (*10*) was used with modification. The triacylglycerol sample (30 mg) was placed in a 1.6 × 12 cm screw-capped glass test tube with 2 mL of 1 M Tris buffer, 120 μL of 22% calcium chloride, 70 μL of 0.1% sodium cholate solution, and 30 mg of porcine lipase (70 units/mg). The solution was stirred by vortex for 20 s, and the samples were incubated, under inert gas and with shaking, at 37 °C. After 15 min, the mixture was cooled to room temperature and the reaction stopped by the addition of 10 mL of ethyl ether. After stirring by vortex, the layers were allowed to separate over 15 min. The organic layer, a mixture of triacylglycerols, diacylglycerols, monoacylglycerols, and free fatty acids, was transferred to a test tube and evaporated to dryness under a stream of nitrogen. Acetonitrile (~1 mL) was added and evaporated under a stream of nitrogen gas to remove residual water. The lipolysate was transferred to a 3 mL microsilica column (Bond Elut, Varian, Inc., Walnut Creek, CA) that had been prerinsed with hexane. A hexane/ethyl ether (9:1 v/v; 20 mL) solution was used to elute triacylglycerols and free fatty acids. Diacylglycerols were eluted with 3 mL of ethyl ether/hexane/acetic acid (50:50:1, v/v/v) and monoacylglycerols with methanol (4 mL). Fraction compositions were confirmed by TLC on Polygram Silica G, 4 cm × 8 cm plates (Alltech Associates, Deerfield, IL) developed with ethyl ether/hexane (6:4, v/v) and I_2 visualization. The triacylglycerols and monoacylglycerols (from lipolysis) were converted to fatty acid methyl esters (*11*) and analyzed by GC as described above. Triacylglycerol structures were determined from the fatty acid composition at the 2-position of the monoacylglycerols (*12*, *13*).

Table 1. Melting Points of Structured Lipids and Commercial Fats

fat/lipid	mp[a] (°C)
SOS	44.3
SLS	37.9
SlnS	36.5
SSL	42.3
coconut oil	26.7[b]
cocoa butter	29.2[b]
palm oil	41.4[b]
lard	42.6[b]
hydrogenated soybean oil IV 81	29.3[b]
hydrogenated soybean oil IV 65 41.2	41.2[b]

[a] AOCS Method CC-16-60 (*18*). [b] Unpublished data.

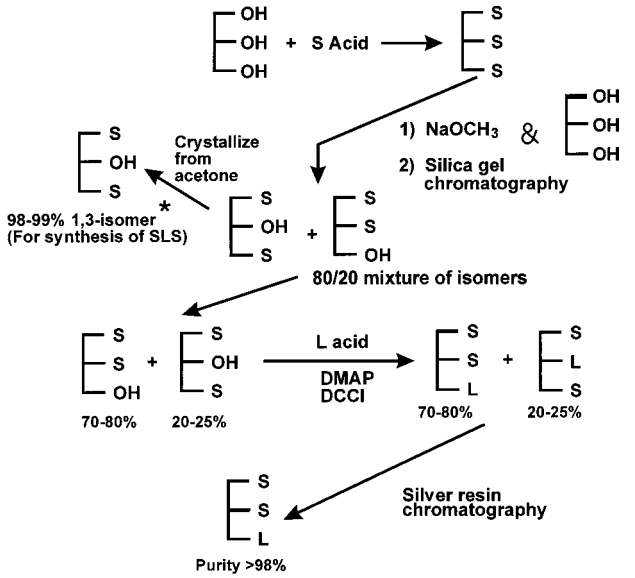

Figure 1. Flow diagram for synthesis of nonsymmetrical triacylglycerol SSL, where S = 18:0 and L = 9*cis*,12*cis*-18:2. The 1,3-distearoylglycerol fraction marked with an asterisk (*) may be used to prepare the symmetrical triacylglycerol of structure SLS.

Synthesis of SUS and SSU Triacylglycerols (Figure 1). *(i) Preparation of 1,3- and 1,2-Distearoylglycerol Precursors. (a) Preparation of Tristearoylglycerol.* Stearic acid (62.4 g, 0.22 mol) was combined with glycerol (6.08 g, 0.66 mol) and 1.4 g of *p*-toluenesulfonic acid in a 200 mL, three-neck round-bottom flask fitted with an inert gas inlet and thermometer. The contents were stirred magnetically and heated by oil bath to 115 °C. Water, which condensed on the flask walls during the reaction, was removed by use of a heat gun. Reaction progress was measured by TLC. After 6 h, the reaction mixture was cooled and dissolved in 800 mL of toluene, and the precipitate was removed by filtration. The tristearoylglycerol was crystallized from the toluene at 4 °C, isolated by vacuum filtration, and crystallized from ~800 mL of acetone at −20 °C. After isolation by filtration, the final

product was dried under vacuum (21 in. of Hg) at 40 °C for 2 h to yield 55 g of tristearoylglycerol (88% yield) with a melting point (mp) of 73.5 °C.

(b) Preparation of 1,2- and 1,3-Distearoylglycerol. Tristearoylglycerol (53.4 g, 0.06 mol) and glycerol (2.76 g, 0.03 mol) were combined as described above, and 0.33 g of sodium methoxide was added. The mixture was heated under inert atmosphere to 115 °C and stirred magnetically. Samples were removed and analyzed by TLC. When the distearoylglycerol concentration was maximized (~4 h), the mixture was cooled to room temperature. The crystalline residue was dissolved in a minimum volume (50 mL) of benzene and eluted through a large glass column (35 cm × 5 cm i.d.) packed with 400 g of Baker 60/200 mesh silica gel. Residual triacylglycerols were eluted with benzene, the 1,3- and 1,2-distearoylglycerols (27.6 g; ~50% yield of distearoylglycerol) with ethyl ether/benzene (1:9), and monoacylglycerols/free fatty acids with 100% ethyl ether. [Note: 5% ethyl ether in petroleum ether and 20% ethyl ether in petroleum ether may be substituted for benzene and ethyl ethee/benzene (1:9), respectively.]

The predominant 1,3-distearoylglycerol (80% of total 1,3-/1,2-distearoylglycerols) was removed by low-temperature crystallization/filtration from acetone to yield the 1,3-isomer in >96% purity. A second crystallization from acetone yielded 1,3-distearoylglycerol of >99% purity (mp 78−79 °C). The isolated 1,3-distearoylglycerol was utilized for the synthesis of the symmetrical triacylglycerols SOS, SLS, and SLnS. The acetone fractions were combined, and the acetone was removed under vacuum by rotary evaporator to yield a 1,2-distearoylglycerol fraction (~80−86% 1,2-isomer; mp 68−69 °C). This fraction was utilized to prepare the nonsymmetrical triacylglycerol isomers SSO, SSL, and SSLn.

Synthesis of Symmetrical Triacylglycerols. The symmetrical triacylglycerols (SOS, SLS, and SLnS) were synthesized from 1,3-distearoylglycerol in yields of 82−86% as described for the nonsymmetrical triacylglycerols below.

Synthesis of Nonsymmetrical Triacylglycerols (Figure 1). *(a) Preparation of SSL.* SSL and the other nonsymmetrical triacylglycerols (SSO and SSLn) were synthesized from 1,2-distearoylglycerol according to the method of Kodali (*14*). 1,2-Distearoylglycerol (6.26 g, 0.01 mol) in 150 mL of carbon tetrachloride was transferred to a heat-dried, three-neck, round-bottom flask equipped with a mechanical stirrer, thermometer, and argon inlet. Linoleic acid (3.2 g, 0.011 mol; 10% excess) in 10 mL of carbon tetrachloride was added via glass syringe. 4-(Dimethylamino)pyridine (1.22 g) was added in one batch and 1.34 g of *N*,*N*′-dicyclohexylcarbodiimide dropwise over a 30 min period. The reaction was stirred at 28 °C for 2 h, during which time a precipitate formed. The precipitate was removed by vacuum filtration and the solvent by rotary evaporator. The SSL/SLS triacylglycerol residue was purified by elution through a 1 in. diameter glass column packed with 70 g of silica gel. Triacylglycerols were eluted with 5% ethyl ether in petroleum ether, whereas diacylglycerols, monoacylglycerols, and residual free fatty acids were eluted with 100% ethyl ether. SSO and SSLn were prepared in a similar fashion, with final yields of triacylglycerols averaging 82−86%. The triacylglycerol mixture was fractionated by silver resin chromatography (**Figure 2**) and the structure determined by lipase hydrolysis and by Ag-HPLC (**Figure 3**).

RESULTS AND DISCUSSION

Higher yields of diacylglycerols were obtained when tristearoylglycerol and glycerol rather than glycerol and stearic acid were utilized as starting reagents for their preparation, and a single reaction sequence was developed to prepare the isomeric diacylglycerol precursors in amounts sufficient to yield the 4−6 g samples of highly pure (>98%) symmetrical and nonsymmetrical triacylglycerols required for SFC, DSC, and other analyses. Unlike the 1,3-diacylglycerols, which could be readily isolated (or purchased in multigram quantities from commercial sources), to produce symmetrical triacylglycerols SUS in purities of 99%, the isolation of highly pure 1,2-diacylglycerols was difficult. Removal of the remaining 1,3-isomers from the desired 1,2-isomers by repeated crystallization resulted in removal of

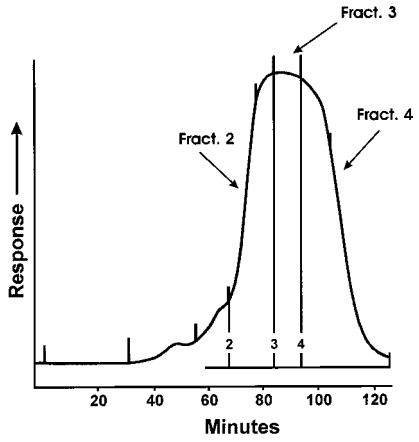

Figure 2. Fractionation of SSL/SLS mixture by silver resin chromatography.

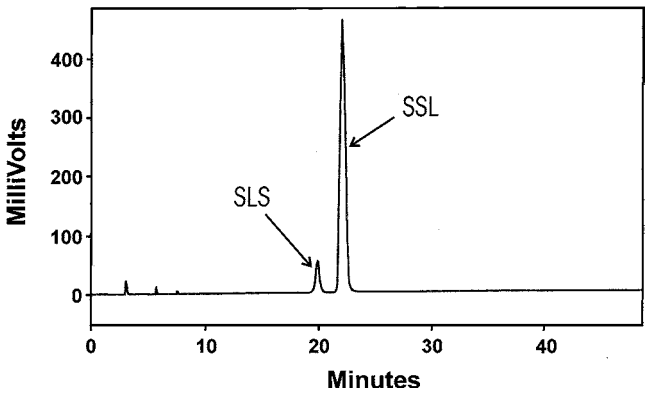

Figure 3. Analysis of SLS/SSL fraction isolated by Ag-HPLC.

~50% of the desired 1,2-isomer. (A dual-column Ag-HPLC system and 1% acetonitrile in hexane as solvent could be utilized to analyze the diacylglycerol intermediates, but some 1,2-/1,3-diacylglycerol isomerization was noted during analysis.)

Because the more difficult to purify (and more expensive to purchase) 1,2-isomer was present at only 20−25% (vs the 1,3-isomer) in the original diacylglycerol mixture, distearoylglycerol precursors containing 80−85% of the 1,2-isomer were utilized for synthesis of the nonsymmetrical SSU isomers, with final purification by silver resin chromatography (**Figure 2**). Acetone was used as elution solvent due to its ability to solubilize triacylglycerols. The mesityl oxide formed by condensation of acetone catalyzed by residual sulfonic acid groups on the resin (*15*) was removed by crystallization of the triacylglycerols from acetone, and triacylglycerols of the structures SSL and SSLn were prepared and purified in this manner. Although the XN1010 resin we used is no longer commercially available, columns packed with silver nitrate (8−15%) on silica gel may also be used to isolate SSL and SSLn from SLS and SLnS, respectively.

Unfortunately, the nonsymmetrical triacylglycerol of structure SSO could not be isolated in multigram quantities from its SOS isomer by this method. Silver ion high-performance liquid chromatography (Ag-HPLC), a versatile procedure (*16*) for the analysis of geometric and positional fatty acid esters and triacylglycerol isomers (*9*, *17*), was applied in the semipreparative mode to fractionate an SOS/SSO mixture (see Materials and Methods). A 10−15 mg sample of the desired SSO isomer (>98% pure) was isolated by repeated injections of ~2 mg samples (**Figure 4**) of the SOS/SSO mixture.

Ag-HPLC was also applied to determine the structural purity of the synthesized symmetrical and nonsymmetrical triacyl-

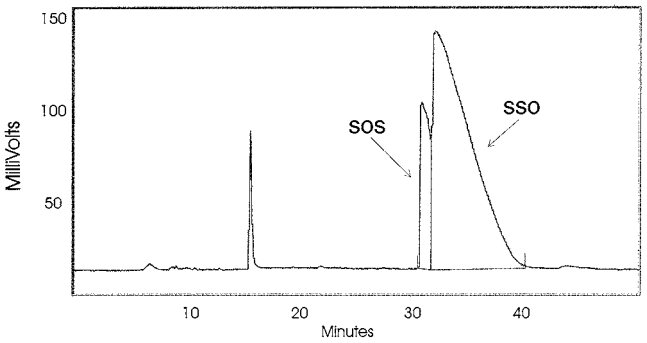

Figure 4. Semipreparative isolation of SSO from SOS/SSO mixture by Ag-HPLC.

glycerols. As has been noted (*17*), fatty acids in the 1- or 3-position of triacylglycerols exert a greater influence on triacylglycerol retention than the fatty acid in the 2-position. SLS therefore elutes before SSL (**Figure 3**) and SOS before SSO (**Figure 4**). We found Ag-HPLC with UV detection at 206 nm to be a rapid (25 min or less) and reproducible method to analyze the isolated symmetrical and nonsymmetrical triacylglycerols, with a detection limit of <0.5% for the "undesired" isomer. A single column was sufficient to achieve the desired separation(s), but two columns connected in series resulted in improved sample capacity and peak-to-peak resolution (*9*). Ag-HPLC and lipolysis/gas chromatography (*4*) were both used to analyze the percentage of each isomer in an SLS/SSL mixture fractionated by silver resin chromatography (**Figure 2**). Percent composition (SLS/SSL; Ag-HPLC vs lipolysis) was calculated for (a) the starting SLS/SSL mixture (12.5/87.5 vs 9.7/90.3%) and (b) fraction 4 (1.9/98.1 vs 1.4/98.6%). We found the percent composition results obtained by Ag-HPLC to be comparable to results achieved by lipolysis/gas chromatography.

The melting points of the structured lipids prepared in this study are shown in **Table 1** along with those of some commercial fats including coconut and palm oil, lard, cocoa butter, and hydrogenated soybean oils. The latter are used to formulate shortenings and margarines/spreads. The melting points of the structured triacylglycerols as pure materials are in the range observed for the natural fats and oils, all of which melt near body temperature of 37 °C. Further characterization of the pure, isolated triacylglycerols and of nonsymmetrical/symmetrical triacylglycerol mixtures in food oil systems by DSC and NMR is underway and should provide additional information on the behavior of functional triacylglycerols in these systems.

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