Quantitation and Structure Elucidation of the Positional Isomers in a Triacylglycerol Mixture Using Proton and Carbon One- and Two-Dimensional NMR

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An interesterified triacylglycerol mixture prepared from an 11/1/1 mole ratio of triacetylglycerol/tripropionylglycerol/hydrogenated canola oil was quantitatively characterized using ^1H and ^{13}C NMR experiments. Resonance assignments were facilitated by the combination of ^1H–^1H and ^1H–^{13}C two-dimensional NMR experiments, and species present at 1 mol % or greater were identified. Because of the multiplicity of triacylglycerol species present in the mixture, it was necessary to perform experiments in mixtures of perdeuteriobenzene and deuteriochloroform to resolve overlapping resonances. The isomer distribution determined by NMR is consistent with random interesterification of the reaction mixture.

INTRODUCTION

SALATRIM 23CA is one of a family of triacylglycerol mixtures prepared from natural oils by interesterification with short-chain triacylglycerols. The composition resulting from these modifications determines the textural properties and nutritional value of the material. In this paper we report the use of ^1H and ^{13}C nuclear magnetic resonance (NMR) for the identification and quantitation of the major positional isomers in SALATRIM 23CA.

The ^1H NMR spectrum provides an immediate quantitative measure of the mole percent of the glycerol species present as mono-, di-, and triacyl derivatives. It is also possible to determine the levels of olefinic species present and the relative amounts of short- and long-chain fatty acids. However, the high degree of spectral overlap that occurs in the ^1H spectrum interferes with the identification of the isomeric forms of the glycerol esters.

The greater spectral resolution afforded by ^{13}C NMR spectra has led to its use as an established tool for the characterization of triacylglyceride mixtures (Shoolery, 1977; Ng and Ng, 1983; Bonnet et al., 1990; Mazur et al., 1991). The positions of ^{13}C resonances are determined primarily by the identity of the functional group but can be very sensitive to molecular conformation and inductive effects from seemingly remote groups. It has been shown that olefinic acyl groups in the 2-glycerol position can be distinguished from those on the 1- and 3-positions on the basis of subtle differences in the olefinic and carbonyl resonances (Ng, 1984, 1985; Bonnet et al., 1990; Wollenberg, 1990, 1991). Mazur et al. (1991) have found a similar long-range inductive effect to be useful in identifying positional isomers with saturated acyl chains up to 10 carbons long.

The resonance assignments for triacylglycerol mixtures have generally been established by comparison with known compounds (Shoolery, 1977; Ng 1984, 1985; Ng and Ng, 1983; Bonnet et al., 1990; Wollenberg, 1990, 1991; Mazur et al., 1991).

With a growing interest in compositional modification of natural oils, the syntheses of the required model compounds can become a laborious endeavor. Fortunately, the battery of one- and two-dimensional NMR experiments routinely employed by synthetic chemists makes it unnecessary to rely on reference compounds or empirical rules to establish the covalent structure of a given molecular species, even in a mixture. The set of resonances belonging to a single chemical component can be determined by way of the proton–proton, proton–carbon, and even carbon–carbon scalar couplings. No other analytical method provides such unambiguous information about molecular structure.

In this work, identification and quantitation of the major positional isomers in SALATRIM 23CA were accomplished using a series of one- and two-dimensional ^1H and ^{13}C NMR experiments. Resolution of crucial resonances was found to depend on solvent; consequently, experiments were performed in both deuteriochloroform ($CDCl_3$) and perdeuteriobenzene (C_6D_6). The composition of SALATRIM 23CA was found to be consistent with a random interesterification reaction.

MATERIALS AND METHODS

The triacylglycerol mixture SALATRIM 23CA was produced from a random interesterification of triacetin, tripropionin, and hydrogenated canola oil (HCO) in 11:1:1 mole ratios, respectively (Klemann et al., 1994). All components in the mixture have at least one long-chain fatty acid attached. Samples were prepared by first melting the material to ensure a homogeneous mixture. Proton NMR experiments were performed with 20 mg of material/mL of solvent, either deuterated chloroform (99.8%) or perdeuterated benzene (99.6%). Carbon NMR experiments were performed using 300 mg of material/mL of solvent (C_6D_6 or $CDCl_3$). All solvents were purchased from Merck Isotopes Inc. and used as received.

All experiments were performed on a Varian VXR-400 NMR spectrometer operating at 400 MHz for proton and 100 MHz for carbon. Spectra were collected with a 5-mm proton/carbon dual probe holding the sample at 30.0 °C.

Quantitative Proton Spectra. Spectra were obtained using a 3.6-kHz (9 ppm) spectral width and a pulse-cycle time of 15 s (5 times the longest proton T_1). Longitudinal relaxation times of the proton resonances were measured to establish the total time between pulses to be used in the quantitative analysis. A standard inversion recovery pulse sequence was used, with 14 spectra collected to define the recovery. Decay curves were analyzed as single exponentials. The transmitter offset and pulse width were adjusted to give the most reliable integrals possible in a given spectral region. In so doing, it was found that relative

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integrals for resonances within 2.0 ppm of the transmitter offset deviated by less than 1% from the proton mole ratios expected for those resonances. The chemical shifts are reported relative to the residual proton resonance for the deuterated solvent (7.27 ppm for deuteriochloroform and 7.15 ppm for the deuteriobenzene).

Proton COSY Experiments. Resonance assignments were confirmed through the use of the two-dimensional ^1H–^1H homonuclear correlated (COSY) experiment (Bax et al., 1981) with 256 intervals consisting of 1K data points over a spectral width of 3.5 kHz. The delay between pulse cycles was 2 s, and the spectra were processed with a sinebell weighting function and zero-filling to 2K in each dimension.

Quantitative Carbon Spectra. Quantitative carbon spectra were obtained using a standard pulse sequence with NOE suppression to avoid differences in enhancement factors among the resonances. Waltz broadband decoupling was used during the acquisition period with the decoupler frequency set at roughly the center of the proton resonances. Data were collected with a 25-kHz spectral width and 131K data points. The time between acquisitions was at least 40 s, i.e., 5 times the longest longitudinal relaxation time as determined from a standard inversion recovery experiment.

To improve resolution in selected regions of the ^{13}C spectrum, it was necessary to work with both deuteriochloroform and perdeuteriobenzene as solvents. Spectra were collected using several different mole ratios of deuteriochloroform and perdeuteriobenzene to establish the positions of the resonances in the two solvents. The solvent resonance is often used as a chemical shift reference in ^{13}C spectra, but in these solvent mixtures, the resonances shift from their usual positions. Consequently, the long-chain methyl resonance was chosen as a chemical shift reference for all ^{13}C spectra since it undergoes only small shifts with solvent and was common to all samples.

Proton–Carbon Heteronuclear Two-Dimensional Experiments. The two-dimensional ^1H–^{13}C heteronuclear correlated (HETCOR) pulse sequence (Bax, 1983; Wilde and Bolton, 1984) and the two-dimensional long-range ^1H–^{13}C heteronuclear correlated (LRHETCOR) pulse sequences (Salazar et al., 1988; Krishnamurthy and Nunlist, 1988) were used to establish the one-, two-, and three-bond proton–carbon connectivities. Both sequences use 90° observe pulse widths and 90° decoupler pulse widths which were found to be 14.7 and 15.3 μs, respectively. A one-bond ^1H–^{13}C scalar coupling constant of 140 Hz was used for the HETCOR pulse sequence, and a long-range ^1H–^{13}C coupling constant of 4 Hz was used in the LRHETCOR experiment. The one-bond coupling values were estimated from fully coupled ^{13}C spectra of SALATRIM 23CA, and the long-range coupling constant was estimated from two- and three-bond couplings identified by selective decoupling experiments.

RESULTS

The composition of SALATRIM 23CA will be discussed in terms of acetic, propionic, and long-chain acyl species. The term "long-chain" encompasses the major fatty acid species found in the hydrogenated canola oil (stearic, palmitic, oleic, and behenic acyl chains).

Solvent Dependence of Carbon and Proton Spectra. It was necessary to run experiments in both deuteriochloroform and perdeuteriobenzene to attain adequate resolution of the resonances in all of the spectral regions of interest. Most of the triacylglycerol ^{13}C resonances are well resolved in $CDCl_3$, whereas the α-methylene ^1H resonances are better resolved in C_6D_6. For this reason, the COSY and HETCOR spectra were obtained using C_6D_6 to facilitate assignment of the ^1H and ^{13}C propyl resonances. LRHETCOR data were obtained in $CDCl_3$ since it was found that the carbonyl region was best resolved in that solvent.

Proton Resonance Assignments. Regardless of solvent, all of the proton resonances for the saturated triacylglycerols fall between 5.5 and 0 ppm, as shown in Figure 1. Table 1 provides a list of the major resonances

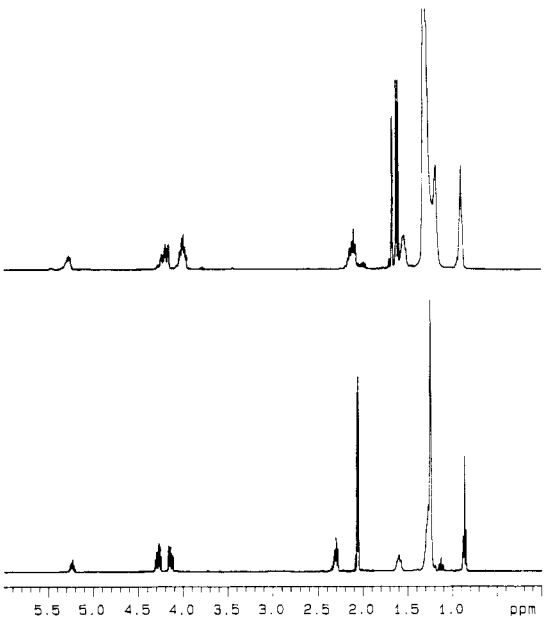

Figure 1. 400-MHz ^1H NMR spectrum of the SALATRIM 23CA mixture in C_6D_6 (top) and $CDCl_3$ (bottom). Chemical shifts are referenced to C_6H_6 at 7.15 ppm or to $CHCl_3$ at 7.28 ppm. Propyl methyl protons and long-chain β-CH_2 protons are resolved at 1.1 and 1.6 ppm, respectively, in $CDCl_3$ (bottom) and the propyl β-CH_2 are better resolved in C_6D_6 (top).

Table 1. Assignments for Major Proton Resonances for SALATRIM 23CA

δ^a in $CDCl_3$	δ^a in C_6D_6	T_1,[b]	assignment
5.25	5.28	2.4	2-glycerol methine
4.14, 4.28	4.0, 4.2	1.0	1,3-glycerol methylene
2.3	2.12	1.1	long-chain α-methylene
2.3	2.0	2.5	propionyl α-methylene
2.05	1.65	2.3	acetyl methyl
1.6	1.55	1.0	long-chain β-methylene
1.25	1.2, 1.3	1.2	long-chain methylenes
1.15	0.9	0.9	propionyl methyl
0.85	0.9	2.7	long-chain methyl

[a] The chemical shifts are in parts per million using the solvent as a chemical shift reference: 7.27 ppm for deuteriochloroform and 7.15 ppm for perdeuteriobenzene. [b] Longitudinal relaxation times were measured from an inversion recovery experiment using a single-exponential decay for analysis.

in each solvent with assignments. Resonance assignments were confirmed by the ^1H–^1H scalar coupling patterns observed in a two-dimensional COSY experiment.

Quantitative Determination of Fatty Acid Ratios. In quantitative comparison of resonance integrals, the accuracy varies systematically across the spectrum. It depends on the frequency difference between two resonances being compared, the position of the transmitter frequency relative to these resonances, and the power spectrum for the instrument and probe being used. With this instrument, the resonance area per mole of protons varies by 1% or less for ^1H resonances within 800 Hz (2 ppm) of the transmitter frequency. The proton spectrum is particularly useful in determining the acetyl/propionyl/long-chain fatty acid ratios because the methyl resonances for each are well resolved but within 1.5 ppm of one another.

The fatty acid mole ratios were calculated from combinations of integrals for the α-methylene, β-methylene, and methyl ^1H resonances using spectra collected in $CDCl_3$ and a 1:1 mole ratio of $CDCl_3$, and C_6D_6. The 1:1 solvent mixture was used to improve the resolution of the propionyl methyl resonances. The average mole percents are 56.4 ± 0.6 acetyl, 5.3 ± 1.3 propionyl, and 38.3 ± 1.7 long chain. These values are consistent with 58.6% acetyl, 5.3% propionyl, and 36.1% long-chain acyl expected from a

Table 2. Prediction of the Number and Relative Intensities of the Acetyl ^{13}C Resonances for a Random Interesterification Reaction

species[a]	degen[b]	wt[c]	1,3-A[d]	2-A[d]
ALA	1	$X_A^2X_L$	$2X_A^2X_L/N$ = 29.3%	
AAL + LAA	2	$X_A^2X_L$	$2X_A^2X_L/N$ = 29.3%	$2X_A^2X_L/N$ = 29.3%
ALL + LLA	2	$X_AX_L^2$		$X_AX_L^2/N$ = 1.3%
LAP + PAL	2	$X_AX_PX_L$		$2X_AX_PX_L/N$ = 2.7%
LPA + APL	2	$X_AX_PX_L$	$2X_AX_PX_L/N$ = 2.7%	
ALP + PLA	2	$X_AX_PX_L$	$2X_AX_PX_L/N$ = 2.7%	

[a] Triacylglycerol species considered, where A, P, and L stand for acetyl, propionyl, and long-chain acyl groups, respectively, and the ordering is indicative of position on the glycerol. The species AAA, AAP, APA, PAA, PPA, PAP, APP, and PPP are not present in the mixture (Klemann et al., 1994). [b] Degeneracy of the species given that the 1- and 3-positions are not distinguished in the NMR experiments. [c] Unnormalized statistical weight for forming the species in the reaction mixture, where X_A = 0.8462, X_P = 0.0769, and X_L = 0.0769 are, respectively, the acetyl, propionyl, and long-chain fatty acid mole fractions used in the reaction. [d] Relative intensity of the resonance corresponding to the acetyl in the 1- or 3-glycerol position (1,3-A) or 2-glycerol position (2-A). The normalization factor is with respect to the sum of all acetyl resonances for a given carbon type, e.g., all methyl resonances: $N = 6X_A^2 + 3X_AX_L^2 + 6X_AX_PX_L$.

random interesterification with a reaction mixture of 11 mol of triacetin/1 mol of tripropionin/1 mol of HCO. All species containing only acetyl and propionyl chains are removed by distillation at the end of the reaction. The various isomers present in the mixture cannot be identified from the proton spectrum because the resonances are not sufficiently resolved. There are olefinic and allylic resonances near 5.35 and 2.0 ppm, respectively, which were found to be connected by a cross peak in the COSY spectrum. Given that oleic acid is the principal unsaturated fatty acid in SALATRIM 23CA (Huang et al., 1994), these resonances correspond to 4.6 mol % of the triacylglycerols containing an unsaturated fatty acid; 3 mol % oleic acid was found chromatographically (Huang et al., 1994). Resonances near 5.1 and 3.7 ppm were assigned by comparison with reference compounds as glycerol methine and methylene protons, respectively, for a 1,2- or 2,3-diacylglycerol. These resonances correspond to 2.2 mol % of the total glyceride species which, again, is consistent with the 2.8 mol % found in the chromatographic studies (Huang et al., 1994).

Prediction of the Number and Intensity of ^{13}C Resonances for Selected Spectral Regions. Because SALATRIM 23CA is composed primarily of acetyl, propionyl, and saturated long-chain fatty acid esters of glycerol, analysis of the ^{13}C spectrum will be limited to a few key regions of the spectrum: carbonyl (175–169 ppm), 2-glycerol methine (70–68 ppm), 1,3-glycerol methylene (63–62 ppm), propionyl α-methylene (28–27 ppm), and the acetyl (22–20 ppm), long-chain (15–14 ppm), and propionyl (10–9 ppm) methyl carbons. The number and intensities of the resonances in each of these regions are readily predicted if the interesterification reaction used to prepare the mixture is random. Table 2 lists the relative intensities expected for the acetyl resonances for SALATRIM 23CA, and Table 3 presents the distribution of resonances that can be expected in either the complete carbonyl region or the glycerol region of the ^{13}C spectrum. The ^{13}C spectrum should exhibit up to eight resonances for the acetyl carbonyls (171–169 ppm) and for the acetyl methyl carbons (22–20 ppm). Likewise, there could be 19 distinct resonances for species present at greater than 1 mol % in both the carbonyl region (175–169 ppm) and the glycerol region (70–62 ppm). Species that contain no acetyl residues are expected to be present at roughly 0.1 mol % or less, which is effectively beyond the detection limit of these ^{13}C experiments. Justification for the assignments of these spectral regions is given in Tables 4–6. Some of these regions are expected to exhibit similar distributions of resonance intensities.

Table 3. Prediction of the Number and Intensities of the Carbonyl and Glycerol Resonances for a Random Interesterification Reaction[a]

species	1,3-A	1,3-P	1,3-L	2-A	2-P	2-L
LLL			0.1%			0.06%
LLP + PLL		0.1%	0.1%			0.06%
LPL			0.1%		0.06%	
LPP + PPL		0.1%	0.1%		0.1%	
PLP		0.1%				0.06%
LLA + ALL	1.5%		1.5%			1.5%
LAL			3.1%	1.5%		
LAA + AAL	16.9%		16.9%	16.9%		
ALA	16.9%					8.5%
ALP + PLA	1.5%	1.5%				1.5%
APL + LPA	1.5%		1.5%		1.5%	
PAl + LAP		1.5%	1.5%	1.5%		

[a] Refer to Table 2 for the general approach in the calculations. Again, the labels A, P, and L correspond to acetyl, propionyl, and long-chain acyl residues, respectively. [b] The labels 1,3-A, 1,3-P, and 1,3-L refer to resonances corresponding to the glycerol 1- and 3-positions occupied by acetyl, propionyl, and long-chain residues, respectively. Likewise, 2-A, 2-P, and 2-L are for those residues in the glycerol 2-position. The elements of the table are the intensities of the resonances relative to the sum of intensities for all resonances represented in the table. The table can refer to either all of the triacylglycerol resonances in the carbonyl region or all of the glycerol methylene plus methine resonances.

Table 4. Carbonyl ^{13}C Resonances for SALATRIM 23CA

label[a]	δ in $CDCl_3$[b]	δ in C_6D_6[b]	% int[c]	evidence[c]	assignment
C1	174.028	173.16	1.8	a′, b′, c′	1,3-P
C2	174.015	173.149	1.8	a′, b′, c′	1,3-P
C3	173.977	173.101	1.6	ref comp[f]	diglyc-L
C4	173.668	172.964	1.8	regional[g]	2-P
C5	173.429	172.591	19.3	d′, e′, f′	1,3-L
C6	173.416	172.591	1.7	regional[h]	1,3-L
C7	173.407	172.579	3.1	regional[h]	1,3-L
C8	173.063	172.375	9.4	g′, h′, i′	2-L
C9	173.047	172.362	1.8	regional[h]	2-L
C10	173.028	172.362	2.9	regional[h]	2-L
C11	171.094	170.132	0.9	ref comp[g]	diglyc-A
C12	170.610	169.660	0.6	regional[h]	1,3-A
C13	170.600	169.660	15.6	j′, k′	1,3-A
C14	170.587	169.648	19.1	j′, k′	1,3-A
C15	170.578	169.648	0.6	regional[h]	1,3-A
C16	170.198	169.421	16.4	m′, l′	2-A
C17	170.189	169.421	0.6	regional[h]	2-A
C18	170.167	169.421	0.9	regional[h]	2-A

[a] The labels correspond to the sequential order in which the resonances appear in the CDCl spectrum. [b] Chemical shift is relative to the stearic acid methyl resonance set to 14.361 ppm. [c] Resonance intensities estimated from peak heights; percentages calculated from the sum of all carbonyl resonances. [d] The primed lower-case letters refer to cross peaks labeled in the LRHETCOR plot in Figure 4, which are evidence of two- or three-bond scalar coupling of the carbonyl carbon with glycerol protons or the α- and β-protons of the acyl chain. [e] The letters A, P, and L refer to acetyl, propionyl, and long-chain residues, respectively, and the numerals refer to the position on the glycerol backbone. The prefix diglyc refers to a diacylglycerol species. [f] Distearylglycerol has carbonyl resonances at 174.110, 173.960, and 173.640 ppm in $CDCl_3$ at 30 °C with the stearyl methyl resonance set to 14.361 ppm. [g] Assignment was based on the observation that the 2-carbonyl resonance for acetyl and long-chain residues is consistently 0.2 ppm upfield from the 1,3-carbonyl resonances of the same acyl species. The 1,3-propionyl resonances were assigned by LRHETCOR, and the 0.2 ppm upfield shift was assumed for the 2-propionyl resonance. [h] Assignment was based on proximity to unambiguously assigned resonances.

Table 5. Glycerol ^{13}C Resonance Assignments for SALATRIM 23CA

label[a]	δ in $CDCl_3$[b]	δ in C_6D_6[b]	% int[c]	evidence[d]	assignment[e]
G1	69.489	69.709	0.9	regional[f]	2-A
G2	69.479	69.658	1.8	regional[f]	2-A
G3	69.431	69.613	18.4	a, n′	2-A
G4	69.230	69.505	2.0	regional[f]	2-A, P, or L
G5	69.109	69.447	2.3	regional[f]	2-A, P, or L
G6	69.097	69.400	1.5	regional[f]	2-A, P, or L
G7	69.036	69.346	7.8	b	2-L
G8	68.408	68.383	1.7	ref comp	2-diglyc
G9	65.506	65.376	1.4	ref comp	1,3-diglyc
G10	65.248	65.165	1.5	ref comp	1,3-diglyc
G11	62.620	62.492	1.6	regional[f]	1,3-A, P, or L
G12	62.607	62.460	1.6	regional[f]	1,3-A, P
G13	62.581	62.454	15.0	c	1,3-L
G14	62.575	62.416	1.4	regional[f]	1,3-A, P, or L
G15	62.543	62.394	16.7	c, o′	1,3-A
G16	62.422	62.336	1.6	regional[f]	1,3-A, P, or L
G17	62.368	62.308	1.6	regional[f]	1,3-A, P, or L
G18	62.326	62.263	1.8	regional[f]	1,3-A, P, or L
G19	62.275	62.228	1.5	regional[f]	1,3-A, P, or L
G20	62.253	62.215	1.5	regional[f]	1,3-A, P, or L
G21	62.253	62.193	1.5	regional[f]	1,3-A, P, or L
G22	62.231	62.193	15.5	d	1,3-A

[a] The labels correspond to the sequential order in which the resonances appear in the $CDCl_3$ spectrum. [b] Chemical shift is relative to the stearic acid methyl resonance set to 14.361 ppm. [c] Resonance intensities estimated from peak heights; percentages calculated from the sum of all glycerol resonances. [d] The lower-case letters refer to cross peaks labeled in the HETCOR plot in Figure 5, which are evidence of one-bond scalar coupling of the glycerol carbon to the hydrogens directly attached. Since the hydrogen resonances are unambiguously assigned as methine or methylene glycerol resonances, the cross peaks provide assignment of the glycerol carbons. The primed lower-case letters refer to cross peaks labeled in the LRHETCOR plot in Figure 4, which are evidence of two- or three-bond scalar coupling of the glycerol carbon with the α-protons of the acyl chain. The prefix diglyc refers to a diacylglycerol species; these resonances were assigned by comparison with spectra of distearylglycerol and the results from Mazur et al. (1991). [e] The letters A, P, and L refer to acetyl, propionyl, and long-chain residues, respectively, and the numerals refer to the position on the glycerol backbone. [f] Assignment based on proximity to the unambiguously assigned 2-A resonance, with the expectation that LAL and PAL + LAP are present at the predicted levels.

Carbon-13 Resonance Assignments. The ^{13}C spectral regions discussed above are presented in Figure 2 for SALATRIM 23CA in deuteriochloroform and perdeuteriobenzene. The multiplicity of resonances in each of these regions changes drastically with solvent. To relate the resonances in deuteriochloroform with those in perdeuteriobenzene, samples were prepared with three different mixtures of these two solvents. All of the resonances studied followed clear trends in chemical shift as the mole percent perdeuteriobenzene increased, as exemplified in Figure 3. The carbonyl chemical shifts are linear in mole percent perdeuteriobenzene, while similar plots for all of the other resonances studied exhibited distinct curvature. Resonances for which the curves crossed were easily distinguished by their relative intensities.

Table 4 lists the carbonyl resonances observed in deuteriochloroform and perdeuteriobenzene. A total of 18 resonances are observed in deuteriochloroform at roughly 1 mol % or greater. This suggests that at least 1 of the 19 resonances predicted in Table 3 is buried beneath another resonance. There is also evidence in the proton spectra for 2.2 mol % diacylglycerol in the sample, as discussed previously. The carbonyl resonances of the diacylglycerol can be significantly shifted from the equivalent triacylglycerol (Mazur et al., 1991); consequently, at least two more resonances must be coincident with other resonances in this region.

Table 6. Acetyl Methyl and Propionyl ^{13}C Resonnces for SALATRIM 23CA

label[a]	δ in $CDCl_3$[b]	δ in C_6D_6[b]	% int[c]	evidence[d]	assignment[e]
A1	21.083	20.488	2.8	g	1,3- or 2-A
A2	21.083	20.453	30.1	g, j′	1,3-A
A3	20.994	20.284	1.6		1,3- or 2-A
A4	20.889	20.192	3.9	h, m′	1,3- or 2-A
A5	20.889	20.182	29.4	h, m′	1,3- or 2-A
A6	20.889	20.154	32.1	h, m′	1,3- or 2-A
α-P1	27.752	27.545	32.3	e, b′, p′	2-P[g]
α-P2	27.599	27.330	33.4	f, b′, p′	1,3-P[g]
α-P3	27.599	27.305	34.2	f, b′, p′	1,3-P[g]
b-P1	9.282	9.124	33[f]	i, c′, q′	2-P[g]
b-P2	9.270	9.116	33[f]	i, c′, q′	1,3-P[g]
b-P3	9.270	9.108	33[f]	i, c′, q′	1,3-P[g]

[a] The labels correspond to the sequential order in which the resonances appear in the $CDCl_3$ spectrum. [b] Chemical shift is relative to the stearic acid methyl resonance set to 14.361 ppm. [c] Resonance intensities estimated from peak heights; percentages calculated from the sum of all resonances in the region of interest. [d] The lower-case letters refer to cross peaks labeled in the HETCOR plot in Figure 5, and the primed lower-case letters refer to the cross peaks in the LRHETCOR plot in Figure 4. [e] The letters A, P, and L refer to acetyl, propionyl, and long-chain residues, respectively, and the numerals refer to the position on the glycerol backbone. [f] Too poorly resolved for a clear measure of intensities; all three resonances appear to be of equal amplitude. [g] In deuteriochloroform only two distinctive resonances are observed with intensities of 1:2 for both the α- and β-carbons. Given a random interesterification, the downfield resonance in both regions can be assigned as the 2-propionyl of APL + LPA and the upfield resonances represent the PAL + LAP + ALP + PLA species.

Several of the carbonyl resonances were assigned to acetyl, propionyl, or long-chain acyl groups through the use of the LRHETCOR experiments (Figure 4). The α-proton resonances for the acetyl, propionyl, and long-chain residues are well resolved in the ^1H spectrum, and two-bond couplings were observed between these resonances and several of the carbonyl ^{13}C resonances. The propionyl resonances are generally shifted 0.5–0.6 ppm downfield from the equivalent carbonyl resonances for the long chains, while the acetyl resonances are shifted 3.4 ppm upfield.

Three-bond coupling between glycerol hydrogens and the carbonyl carbons was also observed in the LRHETCOR experiments for several carbonyl resonances, allowing assignment of the carbonyl to the 2-glycerol position or the 1,3-positions. The latter are indistinguishable in these NMR experiments.

Table 3 also suggests that there should be four resonances at roughly 17 mol % and one at half that intensity, i.e., the resonances for the LAA + AAL and ALA species. In Table 4, the resonances labeled C5, C8, C13, and C14 are consistent with this expectation. The acetyl resonances presented in Table 6 are also in very good agreement with the predictions in Table 2. Because of the low abundance of propionyl residues in this mixture, only triacylglycerols that contain both a propionyl and an acetyl residue will be present at greater than 1 mol %. Since these triacylglycerols must also contain a long chain to have been retained during distillation, they must be the three sets of isomers listed at the end of Table 2. Thus, there should be two distinct species with propionyl in the 1- or 3-position and a third species with 2-propionyl, all at equal mole percents. This is supported by the list of propionyl resonances in Table 6.

In the glycerol region of the spectrum, 22 individual resonances were observed at 1 mol % or greater (Table 5); this is 3 more than would be predicted with the assumptions used in generating Table 3. The pattern of major components in Table 5 is roughly consistent with that expected, i.e., resonances G3, G13, G15, and G22 corre-

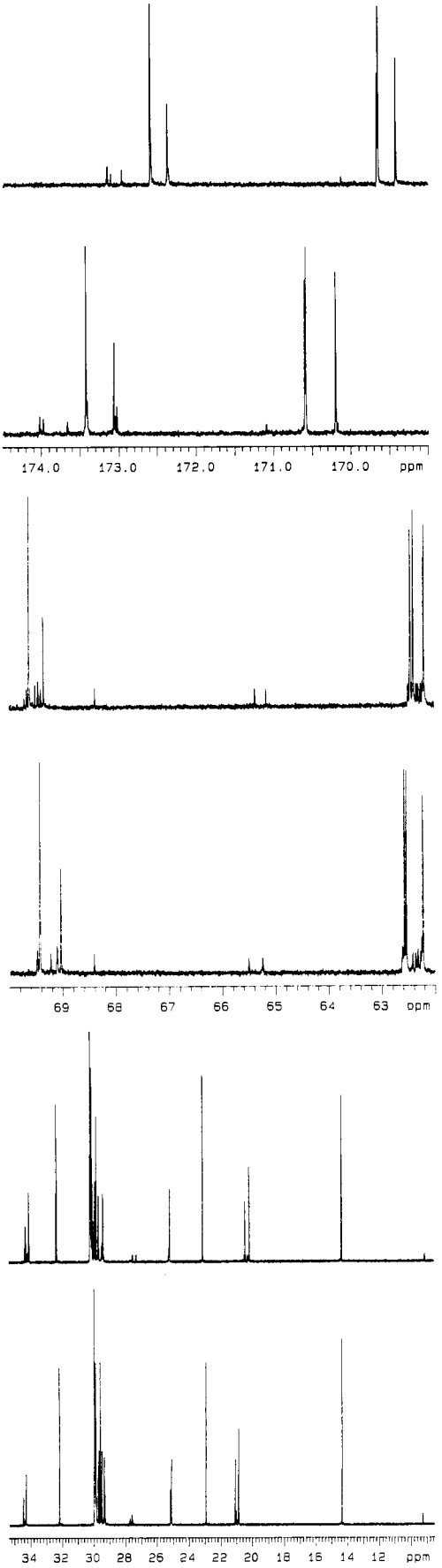

Figure 2. 100-MHz ^{13}C NMR spectra of the SALATRIM 23CA mixture in $CDCl_3$ (lower spectra) and C_6D_6 (upper spectra). (a, top) Carbonyl region from 169 to 174.5 ppm; (b, middle) glycerol methylene and methine resonances (62–70 ppm); (c, bottom) fatty acid aliphatic resonances (9–34.5 ppm). Chemical shifts are referenced to the stearic methyl carbon at 14.361 ppm in both solvents.

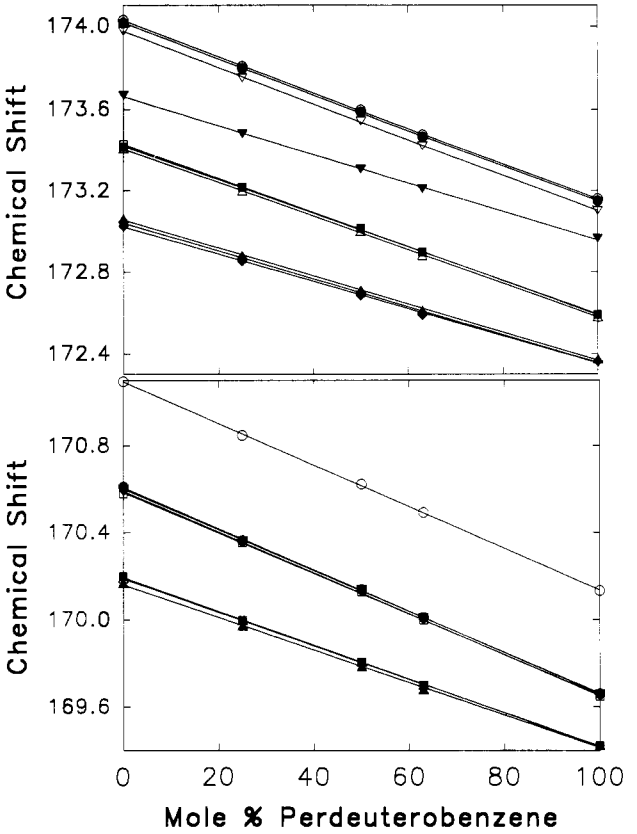

Figure 3. Carbonyl ^{13}C chemical shift changes (ppm) as a function of mole percent perdeuteriobenzene (C_6D_6) in deuteriochloroform ($CDCl_3$). The carbonyl resonances for SALATRIM 23CA follow a linear upfield trend with increasing mole percent C_6D_6. These resonances are clearly best resolved in 100% $CDCl_3$.

spond to the four resonances expected at 17 mol % and resonance G7 corresponds to that expected at 8.5 mol %. All other glycerol resonances are at 2 mol % or lower. The three unexpected resonances are likely to be G8, G9, and G10, which fall in the region for 1,3-diacylglycerols (Mazur et al., 1991). These carbon resonances are present at 1.4–1.7 mol % each, which is in good agreement with the 2.2 mol % diacylglycerol detected in the proton spectra (vide supra).

The glycerol methine and methylene carbons are unambiguously assigned by the one-bond couplings to the corresponding hydrogens, demonstrated in the HETCOR plot of Figure 5. Likewise, the methyl resonances for the acetyl(α-A) and the propionyl(β-P) chains can be assigned from the HETCOR plot in Figure 5 because the corresponding ^1H resonances are resolved and assigned. The α-methylene carbon resonances of the propionyl chains are assigned by two-bond coupling to the attached methyl protons.

Assignment of Resonances to Specific Isomers. Because the relative intensities of the NMR resonances are directly related to the mole fractions of the components, it is possible to deduce the assignments of resonances to particular isomers by comparing intensities. Some precautions must be taken in doing so. The resonance intensities vary systematically across the spectrum in a pulsed experiment because it is usually not possible to deliver the same radio-frequency power over the full 20 kHz of a typical carbon spectrum. In addition, if the decoupler is left on for the entire pulse cycle, the intensities can vary with the efficiency of the proton–carbon NOE enhancement for a given resonance. This was avoided by using the decoupler only during the acquisition period of the pulse cycle and by incorporating a recycle delay that is at least 5 times the T_1 of the protons being decoupled.

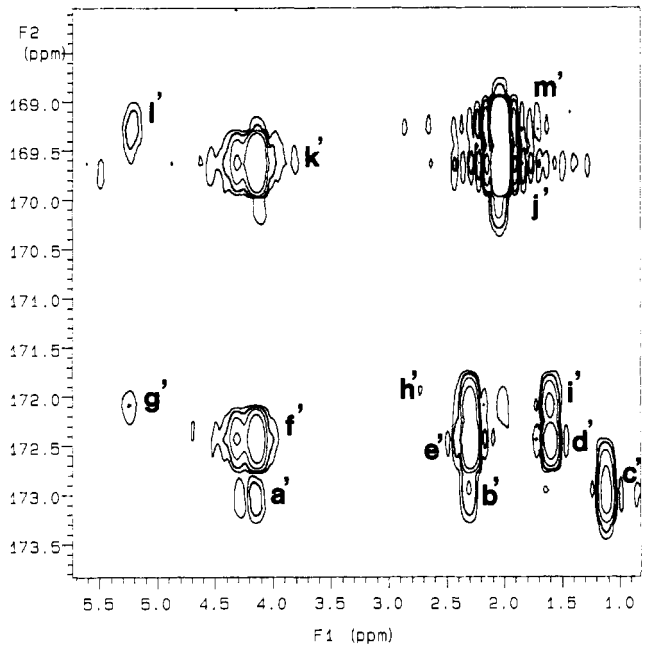

Figure 4. $^{1}H/^{13}C$ long-range HETCOR (LRHETCOR) spectrum of the triacylglycerol mixture obtained in $CDCl_3$ for best spectral dispersion of the carbonyl region. Expansion shows the carbonyl connectivities to the protons of the acetic methyl, propyl β-CH_2 and CH_3, and stearic α-CH_2. Assignments can be found in Table 4. Connectivities not shown in this plot but referenced in Tables 5 and 6 are acetyl methyl proton to glycerol 2-carbon (n'), acetyl methyl proton to glycerol 1,3-carbon (o'), propionyl methyl proton to propionyl α-carbon (p'), and propionyl α-proton to propionyl methyl carbon (q').

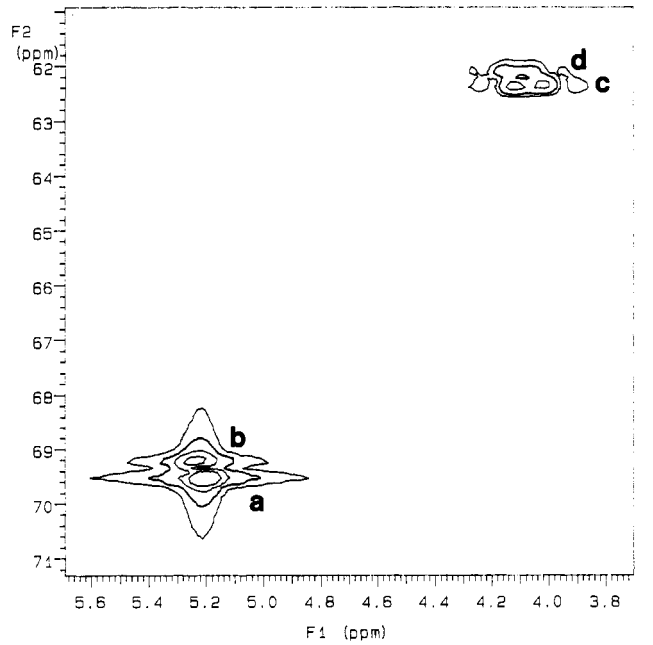

Figure 5. $^{1}H/^{13}C$ HETCOR spectrum of the triacylglycerol mixture showing the one-bond glycerol carbon connectivities to their protons allowing assignment of methylene and methine carbons. One-bond proton–carbon connectivities not shown in this plot but referenced in Table 6 are propionyl α-methylene (e and f), acetic methyl (g and h), and propionyl methyl (i).

As a consequence of the uneven power delivered throughout the carbon spectrum, it is only possible to compare integrals of resonances that fall within a few parts per million of one another. Bonnet et al. (1990) attempted to optimize the power distribution in selected regions of the ^{13}C spectrum through the use of selective excitation centered on that region. This allowed them to use narrower spectral widths than would otherwise be required and avoid fold-over. Their approach was found to be unnecessary in these studies.

Table 7. Isomer Assignments for SALATRIM 23CA

species[a]	% pred[b]	^{13}C resonances[c]	% obsv[d]	% chrom[e]
LAA + AAL	50.7	C16, C13 (default), G3,[g] G22, G13	46.9 ± 1.7	46.7
ALA	25.5	C8, C14,[f] G15,[f] G7	25.8 ± 3.4	25.1
LAL	2.3	C17, G1	2.3 ± 0.6	2.3
ALL + LLA	4.5	C14,[f] G5 or G6	<6	1.3
APL + LPA	4.5	C4, G4	5.7 ± 0.4	4.7
ALP + PLA	4.5	C1 or C2, C14,[f] G5 or G6	5.4	9.2[h]
PAL + LAP	4.5	C1 or C2, C18, G2	5.4	

[a] Species labels are defined in the footnotes of Table 2. [b] The predicted mole percents are with respect to all triacylglycerol species listed in Table 3; the values are obtained from that table by multiplying the percent for the 2-glycerol species by 3. [c] Resonance labels from Tables 4–6. [d] The observed mole percents are with respect to all resonances in Tables 4 and 5, since all triacylglycerols are represented in these regions. Again, the values are those from the tables multiplied by 3. Those values with errors attached are averages over all resonances that ae believed to derive from a single distinguishable species. [e] Values estimated from the chromatographic studies of Huang et al. (1994). [f] Resonances G15 and C14 have been attributed to the ALA and ALL + LLA species on the basis of the near chemical equivalence of the 1,3-A groups in these molecules and the fact that the G15 and C14 resonances are greater in intensity than the G13 and G22, and C13 and C16 resonances, respectively. The relative intensities of these resonances are consistent with the ALL + LLA resonance being coincident with the ALA resonance, given the intensities expected for a random interesterification. [g] Excluded from the calculation of the average because of suspected resonance overlap. [h] ALP + PLA and LAP + PAL are not resolved in the chromatographic studies.

According to Table 3, there should be 7 triacylglycerol species at greater than 1 mol % that can be distinguished by ^{13}C NMR. They are listed in Table 7 with the NMR resonances that have been assigned to them.

Conclusions. Through the use of one- and two-dimensional ^{1}H and ^{13}C NMR experiments performed in solvents chosen to improve resolution in select spectral regions, it was possible to identify the fatty acid resonances for SALATRIM 23CA and assign them to either a 2- or 1,3-glycerol positions. The mole ratios of acetyl, propionyl, and long-chain acyl groups in the mixture were found to be consistent with the values expected from the random interesterification process (Klemann et al., 1994). Olefinic and diacylglycerol species were also detected at levels observed in the chromatographic studies of the same material (Huang et al., 1994).

The relative quantities of the various triacylglycerol isomers were estimated from the carbonyl and glycerol regions of the ^{13}C spectrum in deuteriochloroform. The number and intensities of these resonances at 1 mol % or greater are also consistent with the expectations for the random interesterification and distillation methods used to produce SALATRIM 23CA. Thus, using the distribution of triacylglycerol species predicted from the reaction conditions, it is possible to assign select resonances to particular triacylglycerol species. For example, LAA + AAL are predicted to be present at 10 times the level of any other triacylglycerols containing a 2-acetyl residue. Since only one 2-acetyl carbonyl resonance was found with the appropriate intensity, it was attributed to this combination of isomers. In this way, the relative quantities of the major components in the mixture were determined.

The agreement among these NMR experiments results from chromatographic characterization of the same material (Huang et al., 1994), and the triacylglycerol composition predicted from the reaction conditions supports the hypothesis that the interesterification reaction is random. The methods presented here provide a facile

and versatile approach to characterizing the complex mixtures of triacylglycerols produced when the properties of natural triacylglycerol sources are modified.

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