Analytical ^{13}C NMR: A Rapid, Nondestructive Method for Determining the *cis,trans* Composition of Catalytically Treated Unsaturated Lipid Mixtures

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ABSTRACT AND SUMMARY

High resolution natural abundance ^{13}C Fourier transform nuclear magnetic resonance (NMR) has been found to be an effective tool for the rapid and direct determination of the *cis/trans* composition in partially hydrogenated and isomerized unsaturated lipids. With the *cis* and *trans* allylic carbon resonances as representative probes for double bond stereochemistry. Evaluation of the *cis/trans* composition of complex, positionally isomerized mixtures can be made without the necessity of carrying out detailed analyses of multishift olefinic carbon resonances. Migration of double bonds in monoenes and polyenes and formation of conjugated unsaturation in catalytically treated fats are discussed and assessed as possible sources of error in the evaluation of *cis/trans* isomer ratios. Carbon spin lattice relaxation times T_1 were measured for both *cis* and *trans* allylic resonances in isomeric mixtures of varying composition to assure quantitative intensity relationships. ^{13}C NMR compositional analysis of complex mixtures is demonstrated.

INTRODUCTION

During the hydrogenation processing of fats and oils an appreciable percentage of the unsaturated moieties are positionally isomerized and converted from the naturally occurring *cis* to the *trans* form (1-3). Since the *trans* forms have a different spacial configuration than the *cis*, it has been hypothesized that the former's natural biological functions may vary (4-7). The percentage of *trans* unsaturation also has an important effect on the physical characteristics of various food fats, e.g., melting point, crystallization, and solubility. Compositions high in *trans* unsaturation tend to have higher melting profiles which are often desirable for certain applications. It is for these various reasons that the *cis/trans* ratio in fats has become an important diagnostic value.

Several methods including infrared (IR) analysis (8), ^{1}H nuclear magnetic resonance (NMR) analysis of mercuric acetate derivatized esters (9), and gas liquid chromatography (GLC) of epoxidized esters (10) have been described for determining the *cis/trans* ratio in partially hydrogenated fats. All of these methods require chemical modification prior to the measurement of geometric isomer composition, i.e., transesterification of the triglycerides, while the last two also require chemical reaction at the points of unsaturation. Furthermore, the second method can be used only to measure geometric isomer ratios of specific positional isomers (9) while the third is effective only for the analysis of monoenes (10). Recently, more sophisticated use of long packed (11) and open tubular capillary gas chromatography columns (12) as well as HPLC (13) have shown promise for the analysis of *cis,trans* isomers; yet in complex positional isomer mixtures satisfactory resolution is still a problem.

The AOCS standard method (8) furnishes only weight percent *trans* unsaturation values through measurement of the *trans* double bond IR absorption. However, the *cis/trans* ratio may be readily calculated after methyl esters have been examined by GLC to determine the relative amounts of saturates and unsaturates. The IR measurements must be performed on the methyl esters since the double bond absorption values are subject to errors introduced by other absorbing groups of the triglyceride molecule (14).

Using natural abundance Fourier transform (FT) ^{13}C spectroscopy, Barton and co-workers (15) observed that the *cis/trans* ratio could be determined from mixtures of methyl oleate and methyl elaidate by comparing the *cis* and *trans* C_9 and C_{10} olefinic carbon resonances. Although this technique is effective in its application to position specific unsaturated lipids, it cannot be applied to the analysis of randomly isomerized unsaturates that are most often encountered in partially hydrogenated fats and oils (3). Bus and Frost (16) examined the ^{13}C spectra of a series of C_{18} *cis* and *trans* monoenoic methyl esters having unsaturation extending from positions 8 through 12. Their findings indicated that the chemical shifts of the allylic carbon resonances were strongly dependent upon the stereochemistry of the adjacent double bond, i.e., those allylic carbons adjacent to a *cis* double bond absorbed at higher fields than those adjacent to a *trans* double bond. More importantly, it was observed that theses respective resonance positions were independent of double bond location from C_8-C_{12}. In this work we describe the determination of the *cis/trans* composition of various complex mixtures of partially unsaturated lipid materials by the measurement of the respective allylic ^{13}C resonances and compare the results with those obtained by the conventional IR-GLC procedure. We also demonstrate the use of ^{13}C NMR for the compositonal analysis of all the major lipid components.

EXPERIMENTAL PROCEDURES

High resolution, natural abundance ^{13}C NMR spectra were obtained at 20.0 MHz and 22.6 MHz with Varian CFT-20 (8K data) and Bruker WH-90 (Nicolet BNC-20 8K data) spectrometers with complete proton decoupling at 31 C and 34 C, respectively. Each 0.50 g sample was dissolved in 1.3 ml of $CDCl_3$, and the resulting solution was placed in a 10 mm NMR tube. The spectra were taken with 90° pulse angles and 5-7 sec repetition rates except for those solutions containing 0.04-0.1 N chromium acetyl acetonate. The spectra of the latter samples were obtained with no added delay time between pulses. In most cases, the spectra were obtaned with no more than 300 transients; however, in some instances accurate measurement of very minor components required as many as 10,000 transients and repetition rates as high as 20 sec. Each analytical spectrum of the alkyl carbon region was obtained by pulsing at 1,200-2,000 Hz spectral widths and displayed at a sweep width of 500 Hz. The full spectrum of each sample was obtained by pulsing at 6,000 Hz spectral width and was displayed at ca. 3,000 Hz. Spectra for determining com-

positional information were run using either 20 sec repetition rates or no delay time with added chromium acetyl acetonate (0.04 N). The NOE (nuclear Overhauser enhancement) suppressed spectra (gated decoupling without NOE) were used exclusively for these determinations and took ca. 1 to 2 hr to complete using the above sample concentration. All shifts are reported relative to internal tetramethylsilane (TMS). Using the Bruker WH-90, the area of the carbon resonances of each sample was measured with an intensity and integral digital printout and checked by cutting out each peak and weighing it. For triglyceride mixtures, good agreement in the *cis/trans* values were found between the intensity measurements and cutouts while the ester mixtures showed better agreement between the integral values and the cutout values. These differences were presumably due to the greater variation in line shapes among resonances noted in the ester spectra. The spectra obtained with the Varian CFT-20 instrument were obtained by pulsing with a spectral width of 4,000 Hz; integrals were measured by digital readout of each stepped integral. Each measurement was made a minimum of six times and the standard error was calculated (see tables).

High purity *rac*-1,3-dioleoyl-3-stearoyl-glycerol (SOO) and *rac*-1,2-dioleoyl-3-palmitoyl-glycerol (POO) were synthesized by R.J. Jensen, University of Connecticut, Storrs, CT. High purity glycerol trielaidate (EEE) was obtained from Applied Science Laboratories, Inc., State College, PA. The purity of the component fatty acids of triglycerides was in excess of 99% as determined by GLC analyses of the corresponding methyl esters. All samples (standard triglycerides and mixtures, partially hydrogenated tallow and shortening) were converted to their corresponding fatty acid methyl esters by the method of Luddy et al. (17). Weight percent *trans* unsaturation in each sample was determined by the standard AOCS IR method (8) without corrections using a Perkin-Elmer Model 21 IR spectrometer. The degree of unsaturation in each sample was determined by GLC analysis on an Aerograph 1522 gas chromatograph equipped with dual hydrogen flame ionization detectors. The column used was 9 ft x 1/8 in. OD coiled stainless steel packed with 15% EGS on 60/80 mesh Gas Chrom P and maintained isothermally at 190 C. The *cis,trans* analyses were made on a 20 ft x 1/8 in. OD 15% OV-275 gas chromatography column. Butter oil methyl esters were separated on Silica Gel G (Analtech) precoated 250 μ plates which were previously treated with 10% silver nitrate in acetonitrile solution. The mixture was eluted with 1:1 hexane-benzene. The spots were subsequently scraped off the plate, the *cis* isomers were made up to a volume of 0.5 ml in CS_2, and the *trans* isomers to a volume of 100 μl. The ratio of these isomers was determined by injecting known volumes of each solution onto an 8 ft-1/4 in. OD EGA GLC column. The area under the peak of each component was measured by an Infotronics Model CRS-11HSB electronic integrator coupled to a digital printout. Areas and percentages thus obtained were in good agreement with mixtures of methyl esters of similar but known compositions.

RESULTS AND DISCUSSION

cis/trans Analysis of Methyl Oleate-Methyl Elaidate and Isomerized Monoene Systems

The ^{13}C NMR spectrum of a typical binary mixture of methyl oleate and methyl eladiate is shown in Figure 1. The unsaturated carbon resonances *cis* C_9, C_{10} and *trans* C_9, C_{10}, appear at δ 129.8, 130.0 and δ 130.3, 130.5, respectively, while the alkyl allylic resonances C_8, C_{11}, representing the *cis* and *trans* isomers, are observed at δ 27.2, 27.3, and δ 32.5, 32.6, respectively. Comparison of the sum of

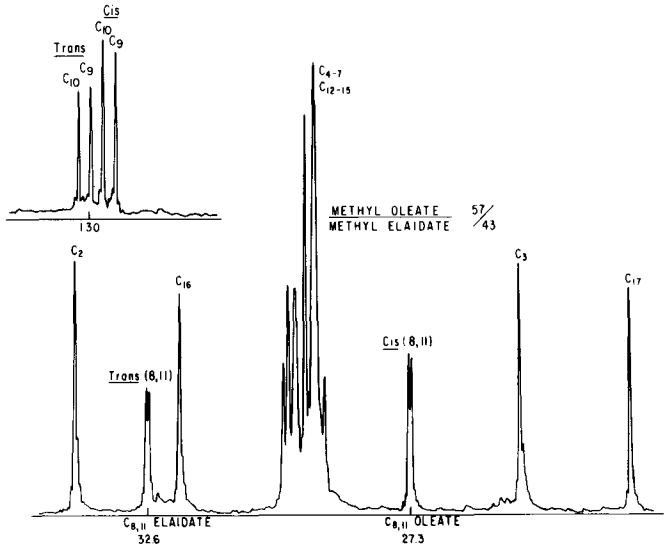


FIG. 1. ^{13}C spectrum of a mixture of 57% methyl oleate, 47% methyl elaidate taken in $CDCl_3$; the methoxy group is omitted. Insert shows olefinic carbons at ca. 130 ppm.

the areas of the *cis* olefinic resonances with those of the *trans* olefinic resonances leads to the direct determination of the *cis/trans* ratio. Similar treatment of the allylic resonances area associated with the *cis* and *trans* isomers yields comparable compositional values. Table I lists the percent *trans* values obtained from these measurements, the actual composition of the mixtures (prepared gravimetrically), and the values obtained by the IR procedure. The percent *trans* values from both the allylic and olefinic carbon resonances agree well with the actual compositions and the values of the weight percent *trans* determined by IR. Note that in mixtures containing only monounsaturates the weight percent *trans* determined by IR corresponds directly with the *trans* values (i.e., percent *trans* of the total unsaturates only) obtained from the ^{13}C measurements. However, in mixtures containing saturates, the weight percent *trans* values had to be corrected for the percent saturates. This was accomplished by dividing the weight percent *trans* values by the fraction of unsaturation in the sample as determined by GLC. These packed column GLC analyses do not distinguish between *cis* and *trans* isomers.

Obviously, since the double bond position is restricted to the 9,10 position in these pure standard mixtures, the low field olefinic carbon resonances are observed as two sets of sharp C_9 and C_{10} resonances (Fig. 1). This is also true for triglycerides whose double bonds are restricted to a single carbon chain position. However, for both simple esters and triglycerides whose double bonds have migrated under catalytic conditions, the low field olefinic carbon resonances become difficult to analyze. This is due primarily to the strong chemical shift dependence of unsaturated carbons on the chain position. Figure 2 illustrates this point with the spectrum showing the poorly defined olefinic resonances of a partially hydrogenated and isomerized monoenoic tallow oil. Nevertheless, the high field portion of the spectrum displays two sharp singlets representing the allylic $C_{8,11}$ type carbons for *cis* and *trans* moieties irrespective of chain position. In the future when we mention $C_{8,11}$ type carbons, we are referring specifically to the allylic type carbons which are "external" to a double bond as in the case of methyl oleate or methyl elaidate, although the $C_{8,11}$ type carbon terminology does not imply that these carbons are necessarily found at the $C_{8,11}$ positions, i.e.; for a double bond migrated to position 6, the $C_{8,11}$ type carbons would correspond to carbons 5 and 8. Table II lists the percent *trans* values

TABLE I

Analysis of Methyl Oleate-Methyl Elaidate Mixtures Expressed as Percent *trans* in Sample

(Gravimetric)	^{13}C (Olefinic)	^{13}C (Allylic)	IR (965 cm^{-1})
43.6	43.6 ± 0.5	43.7 ± 0.5	43.5 + 0.7
23.2	23.4 ± 0.5	23.9 ± 0.5	22.6 ± 0.3
4.4[a]	3.9 ± 1[b]	3.7 ± 1.0[b]	2.0

[a]Determined by gas liquid chromatography on a silver sulfobenzyl silica column.

[b]6,500 Transients and gated decoupling, average of three measurements.

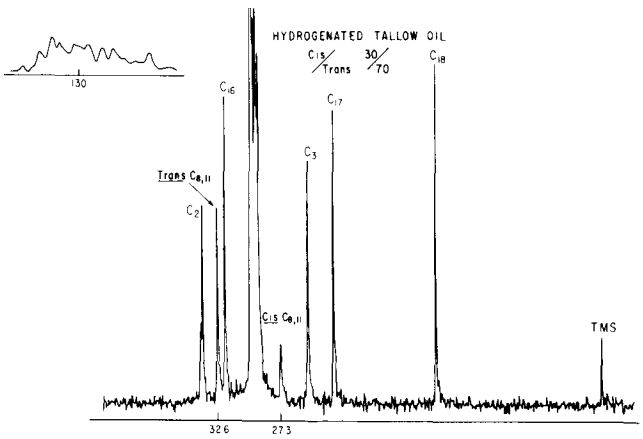


FIG. 2. ^{13}C spectrum of hydrogenated tallow oil taken in $CDCl_3$; insert shows the plot expansion of the olefinic region centered at ca. 130 ppm.

determined for a number of both catalytically treated and untreated mixtures of methyl esters and triglycerides. The values were obtained through measurement of the *cis* and *trans* allylic carbon absorptions. Previous analyses of mixtures of partially hydrogenated isomerized fats by epoxidation-GLC have shown less than 2% double bond migration beyond C_{13} and less than 1% beyond C_8 (10). These findings are supported by the ^{13}C data which show little deviation in the position of the allylic resonance chemical shift as predicted by Bus (16).

Obtaining quantitative data from ^{13}C NMR spectra has usually been described as being difficult because one of the problems inherent in the repetitive-pulse FT method is the possible nonlinearity of the ratios of the peak intensities to the number of nuclei contributing to those resonances. This situation is apt to arise unless the time interval between pulses is several times larger than the longitudinal relaxation times (T_1) for the nuclei involved. Otherswise each nucleus undergoes a steady-state magnetization which is less than tht which occurs when the time interval between pulses is sufficiently long to allow complete repolarization of the nuclei. A time interval of >4 T_1 is required for this latter condition to be met. Since T_1 values for the protonated carbons in fatty acids are in the range of 0.1-5 sec and pulse repetition times of 0.5-1.0 sec are normally utilized, less than maximum steady-state magnetization is usually encountered. The magnitude of steady-state magnetization varies with T_1; hence, the peak intensity ratios will not usually reflect the ratio of the numbers of nuclei responsible for each resonance peak. A further factor which may distort these ratios is that the nuclear Overhauser enhancement for the various carbons may be different. For quantitative studies involving pulsed ^{13}C spectroscopy, one must know both the relaxation times, T_1, and the relative nuclear Overhauser enhancement (NOE) associated with each resonance being measured (15) or operate under conditions which assure that the nuclear spin populations for the various carbons are affected equally.

To determine the relative NOE contribution to each allylic carbon the *cis/trans* ratios were measured using gated decoupling (suppressed NOE conditions and 20 sec repetition times). *cis/trans* Ratios determined under these conditions were the same within experimental error as those measured with full NOE indicating comparable Overhauser enhancements for each allylic carbon resonance. This was confirmed through the determination of the NOE values obtained by comparison of the peak intensities for the gated decoupled and continuously decoupled spectra. Table III gives the NOE values of pertinent resonances to be considered in this system. Note that there is some variation among these resonances. However, the allylic resonances appear to have the same values for oleate and elaidate. We discuss methods for dealing with the differences in NOE values when we consider the quantitative measurement of ^{13}C signals from carbon atoms in a variety of chemical environments in the last section concerned with composition studies.

In an earlier ^{13}C study of *cis/trans* composition, Barton et al. (15) suggested that measurements of the olefinic resonances be made with 20 sec repetition rates so that sufficient time would be given to allow complete nuclear relaxation and quantitative intensity relationships. From the results of inversion recovery experiments (18), we

TABLE II

Percent *trans* in Unsaturated Moieties

Sample	^{13}C[a]	IR-GLC[b]	GLC[c]	Composition (gravimetric)
SOO + EEE[d]	23.5 ± 0.6	22.6 ± 0.4	–	23.3
Methyl ester derived from SOO + EEE	24.1 ± 0.5	22.6 ± 0.4	–	23.2
Mixture of methyl esters, 16:0, *cis* + *trans* - 18:1, *cis* - 18:2	23.1 ± 0.5	24.5 ± 0.3	–	21.5
Hydrogenated tallow oil I	72.3 ± 0.5	69.2 ± 0.4	73.0	–
Methyl esters of tallow oil I	71.9 ± 0.6	69.2 ± 0.4	–	–
Hydrogenated tallow oil II	70.2 ± 0.6	67.5 ± 0.1	70.4	–
Shortening	15.8 ± 0.4	20.6 ± 0.4	16.8	–
Butter oil	11.2 ± 0.5	–	12.0[e]	–

[a]Average of a minimum of six determinations for each triglyceride mixture.

[b]Analysis performed on the derived methyl esters. IR-GLC = infrared-gas liquid chromatography.

[c]Determined by the *cis/trans* analysis using 1/8 in. x 20 ft OV-275 on the derived methyl esters, total unsaturation was 48%.

[d]SOO = *Rac*-1,2-dioleoyl-3-stearoyl-glycerol. EEE = Glycerol trielaidate.

[e]Total unsaturation was 28%.

TABLE III

T_1 and Nuclear Overhauser Enhancement (NOE) Vaues of the Saturated Carbon Resonances of Methyl Oleate, Methyl Elaidate, Methyl Linoleate, and POO[a]

	T_1[b] (NOE)						
	C_2	C_3	$C_{8,11}$	C_{11}	C_{16}	C_{17}	C_{18}
Methyl oleate	2.0 (2.8)	2.0 (2.9)	1.7 (2.8)	--	3.5 (2.8)	3.5 (2.6)	3.9 (2.5)
Methyl elaidate	2.5 (2.8)	2.0 (2.9)	1.5 (2.8)	--	3.5 (2.8)	3.8 (2.6)	4.0 (2.5)
Methyl linoleate	2.7	1.5	2.8	2.3	4.2	4.8	5.0
POO	0.9	0.7	1.3	--	3.2	3.5	3.4

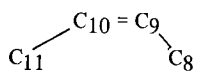
[a]Thirty percent solutions in $CDCl_3$ at 31 C. POO = *Rac*-1,2-dioleoyl-3-palmitoyl-glycerol.
[b](Sec). T_1 = Longitudinal relaxation time.

calculated by a least squares plot a T_1 of 1.7 sec for methyl oleate and 1.3 sec for methyl elaidate allylic carbon resonances in 30% v/v $CDCl_3$ solutions at 31 C (Fig. 3). These results confirm that only 6-7 sec repetition rates are necessary for measurements of the *cis/trans* ratios of esters in this concentration and temperature range. Table III lists the T_1 values for many of the carbon resonances in these esters. Interestingly, none of these respective values appears to change when the oleate and elaidate mixtures are varied in different proportions keeping the total concentration constant. For triglycerides, the T_1 values for both *cis* and *trans* allylic carbons are less than 1.4 sec because of the higher molecular weight and consequently longer reorientational correlation times. Thus, measurement of the isomer distribution in triglycerides allows somewhat shorter repetition rates (5-6 sec) than those of simple esters. The T_1 values for the $C_{8,14}$ resonances in skipped diene methyl esters are somewhat longer than their monoene counterparts; however, these T_1's also shorten in the corresponding polyunsaturated triglyceride allowing for reliable quantitation within a 6-7 sec repetition rate.

When measurements are made at higher temperatures and under more dilute conditions, T_1 becomes longer because fof increased thermal motions and consequently shortened correlation times. For example, at 42 C the oleate and elaidate allylic resonances have T_1 values of 1.9 and 1.7 sec, respectively. If quantitative relationships are to exist between the allylic carbon and other resonances having longer T_1 values, longer delay times will be necessary. Alternatively, this inconvenience can be overcome by the presence of 0.04 N chromium acetyl acetonate, a paramagnetic relaxation reagent, which alleviates the need for long pulse repetition rates by shortening the T_1 values for all carbon nuclei to about 1.0 sec. Use of this chelate gives good quantitative results in dilute solutions.

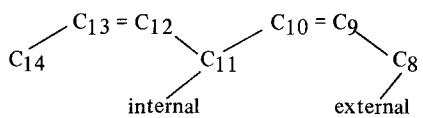
cis/trans Analysis of Double Bonds in Dienes and Mixtures of Dienes, Saturates, and Monoenes

The *cis/trans* analysis of a pure diene or diene-saturate mixture may be simply performed as indicated above by directly measuring the relative amounts of *cis* and *trans* $C_{8,14}$ external type allylic resonances found at the same shift position given above for monoene *cis* and *trans* $C_{8,11}$. However, in mixtures containing both monoene and diene the *cis/trans* analysis requires additional manipulation. When measuring isomeric double bonds in monoenes, we count the area of two carbons of represent one double bond, i.e., the $C_{8,11}$ type external flanking carbons. However, for diene species, the allylic $C_{8,14}$ type resonances represent two double bonds.



Monoene (2 allylics/1 double bond)

vs



Diene (2 allylics/2 double bonds)

Clearly, in order to evaluate monoene-diene mixtures we must compensate for these differences. This is easily accomplished by adding twice the area of a single carbon resonance unique to the diene species to the area of its $C_{8,14}$ resonance thereby putting the diene double bond count on par with the monoene. Aside from the olefinic region of the ^{13}C spectrum of methyl linoleate, there are two important spectral differences which set the *cis,cis*

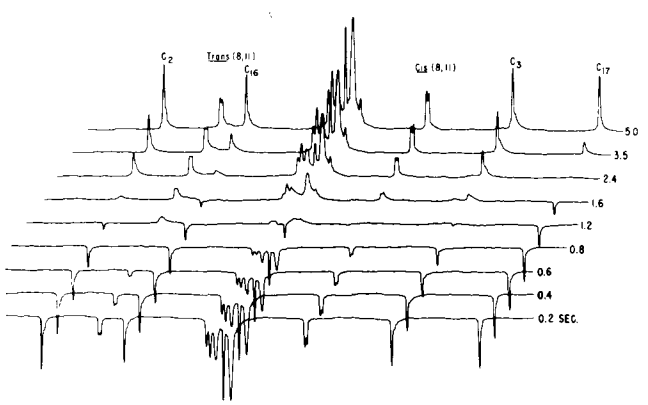


FIG. 3. Inversion recovery experiment for determining T_1 values for a 50:50 mixture of methyl oleate-methyl elaidate in 30% v/v $CDCl_3$ at 31 C.

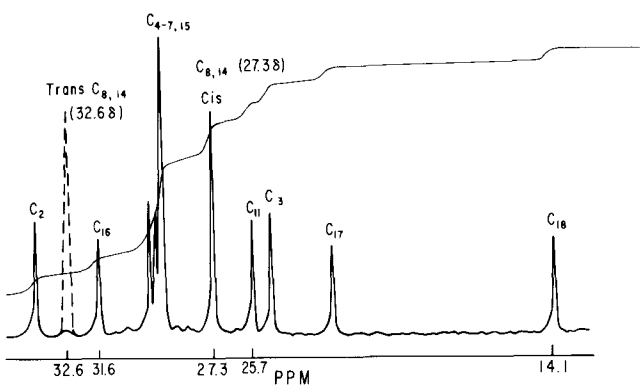


FIG. 4. ^{13}C spectrum of methyl linoleate (0.5 g in 1.3 ml of $CDCl_3$) 300 transients, 6 sec repetition rate, spectrum taken at 2,000 Hz displayed at 700 Hz.

skipped diene system apart from the *cis* monoene. These are the resonance associated with the C_{11} type (internal) allylic carbon of the *cis,cis* diene (*cis,cis* C_{11}) at δ 25.7 and the resonance of the C_{16} carbon at δ 31.64 (Fig. 4). When the stereochemistry of the skipped diene compounds in a diene-monoene mixture is predominantly *cis,cis*,, the total *cis* composition is determined by adding twice the area of the *cis,cis* C_{11} type absorption or twice the area of the C_{16} resonance to the combined area of the *cis*-$C_{8,11}$, *cis,cis* -$C_{8,14}$ type resonance at δ 27.3. The *cis/trans* composition is subsequently calculated using this area and the area of the low field allylic resonance at δ 32.6 which represents almost exclusively the $C_{8,11}$ carbons of *trans* mononene, as follows:

$$\%\ trans \text{ in Monoene, Diene Mixture} = 100 \times \frac{trans\ C_{8,11}}{[cis\ C_{8,11} + cis,cis\ C_{8,14}] + trans\ C_{8,11} + 2\ cis,cis\ C_{11}} \quad (I)$$

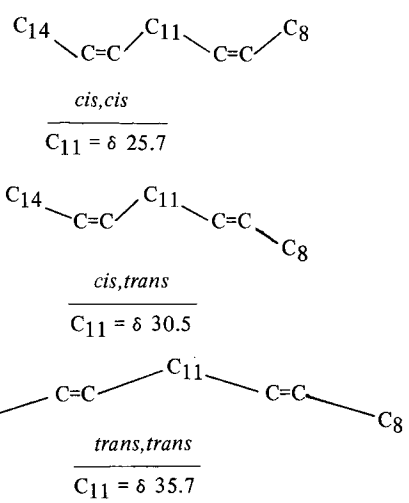
An example of the calculation of the % *trans* among the unsaturated moieties in a mixture of methyl esters containing saturates, monoenes and dienes (Table II, example 3) is illustrated:

Shift and Identity	Area
δ 27.3 – *cis*$C_{8,11}$ + *cis,cis* $C_{8,14}$	= 174.0
δ 32.6 – *trans* $C_{8,11}$	= 81.3
δ 25.7 – Internal allylic *cis,cis* C_{11}	= 50.0

$$\%\ trans = 100 \times \frac{81.3}{174.0 + 81.3 + 2(50.0)} = 23.0$$

It is generally observed that in most mixtures of partially hydrogenated fats little skipped *cis,trans* diene (∿ 3%) and, at most, traces of *trans,trans* skipped diene are present (3). However, to account for the quantities of these isomers, it would be necessary to observe a resonance associated with the intervening C_{11} type carbon of each stereoisomer. In the case of the *trans,trans* diene, the C_{11} type resonance,

Allylic Carbons of Polyunsaturates



All *cis* $C_{8,14}$ = δ 27.3
All *trans* $C_{8,14}$ = δ 32.6

as found in methyl linoelaidate, is observed at δ 35.7, a shift far removed from all other alkyl carbon resonances and readily observable. Inclusion of this isomer into the *cis,trans* double bond count would then be easily accomplished by addition of twice the area of the δ 35.7 resonance to the area of the exterior *trans* allylic absorptions at δ 32.6. In all hydrogeanted mixtures examined, we have not found any evidence of the *trans,trans* skipped double bond species. For the case of the skipped *cis,trans* moiety, the C_{11} type resonance is not readily observable since it overlaps severely with the resonances of carbons 4, 5, and 6 in complex mixtures; however, it can be observed at ca. δ 30.5 in pure samples of methyl 9-*cis*,12-*trans*-octadecadienoate. Even though it is impossible to accurately account for this isomer in mixtures, we can readily evaluate the error associated with the failure to completely measure it. The following equation gives Δ% *trans*, the difference between the actual *trans* composition and the *trans* composition as measured by NMR, neglecting the *cis,trans* C_{11} absorption:

$$\Delta\%\ trans = \left[\frac{trans\ C_{8,11} + trans\ C_{8,14} + cis,trans\ C_{11}}{trans\ (C_{8,11}, C_{8,14}) + 2\ cis,trans\ C_{11} + cis\ (C_{8,11} + C_{8,14}) + 2cis,cis\ C_{11}} - \frac{trans\ C_{8,11} + trans\ C_{8,14}}{trans\ (C_{8,11} + C_{8,14}) + cis\ (C_{8,11} + C_{8,14}) + 2\ cis,cis\ C_{11}}\right] \times 100 \quad (II)$$

Using the composition values found for a typical shortening (3), i.e., *trans* monoene 16.1%, *cis* monoene 25.1%, and diene 27.4%, we varied the *cis,trans* diene composition from 0-28% and calculated the the Δ% *trans* for this range. Figure 5 illustrates the least squares fit for the plot of percent *cis,trans* diene concentration vs. the Δ% *trans* values. A maximum deviation of 3% occurs at 19% *cis,trans* diene concentration but this error drops off to 1% with mixtures containing the normally encountered quantities of 3% (3) *cis,trans* diene. The error profile for conjugated *cis,cis*, *cis,trans*, and *trans,trans* diene should be similar to the one derived above, and since conjugation is generally limited to less than 1% in most samples studied, its contribution to errors is minimal. Table II lists the *trans* composition of an ester mixture containing saturates, monoenes, and dienes calculated using the *cis,cis*, C_{11} resonance as shown in Equation I. For an average of eight determinations agreement between the IR-GLC and ^{13}C method is within 0.6%.

cis/trans Analysis of Complex Mixtures Containing Monoene, Diene, and Triene

Also to be considered in mixed lipid systems are the trienoic molecules which represent as much as 6% of all double bonds. There are four important types of chemical shifts associated with triene moieties: a unique C_{17} at δ 20.6, a C_8 external allylic carbon adjacent to a *cis* double bond at δ 27.3, the C_8 associated with the corresponding *trans* double bond at δ 32.6, and the *cis,cis,cis* $C_{11,14}$ internal allylic carbon resonances at δ 25.7(Table IV). Since only one carbon is represented in the external allylic chemical shift position per three double bonds, we must compensate for this deficiency by adding an area equivalent to five additional carbons that are representative of the triene moiety. By adding twice the area of the $C_{11,14}$ resonance, we account for a total area of five carbons, and the addition of the unique C_{17} brings the total to six carbons or two carbons per double bond as used for monoene and diene. Note that we consider only the *cis* configuration for the triene system. However, if we were to assume *trans* isomerization, we would expect only small overall errors in the total *cis/trans* analysis because of the innately small amount of triene present (3) and the small perturbation the *trans* isomer produces in the calculation (see above section on *cis,trans* diene error). Table II gives the percent *trans* content of a shortening containing saturates, monoene,

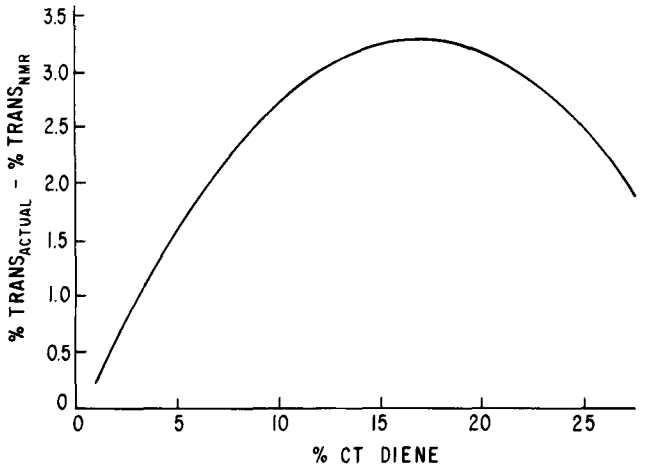
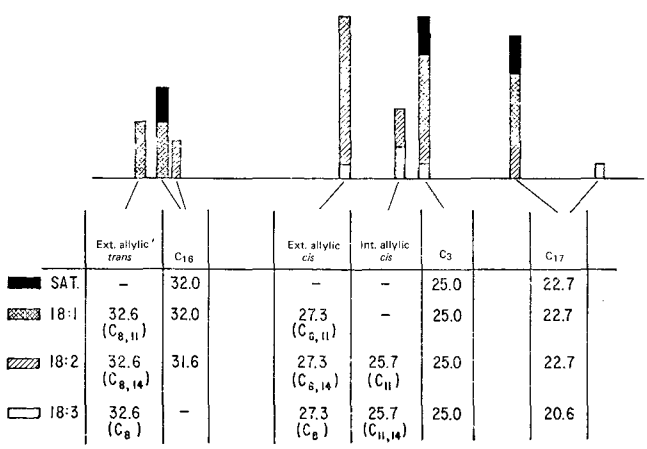


FIG. 5. Least squares plot of *trans* error due to the uncertainty in the nuclear magnetic resonance (NMR) measurement of *cis,trans* diene vs. percent *cis,trans* diene in sample. The composition of the fat is 16% *trans* monoene, 25% *cis* monoene, and 27% diene.

TABLE IV

Schematic Representation of Pertinent Akyl Carbon Shifts of Various Lipid Components as Foud in a Complex Partially Hydrogenated Shortening[a]



[a]Shifts expressed in ppm relative to internal tetramethylsilane (TMS), overlapping shifts in the range of δ 29.0-30.0 were omitted for simplicity.

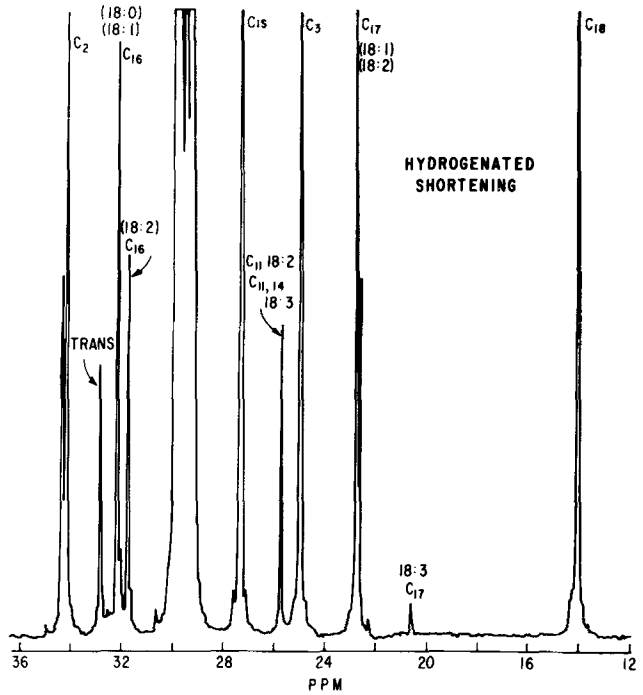


FIG. 6. ^{13}C spectrum of the alkyl carbon region of a partially hydrogenated shortening, ca. 30% in $CDCl_3$, 6,000 transients. Solution contained 0.04 N chromium acetyl acetonate and was run with a 1 sec repetition rate.

TABLE V

Lipid Composition by ^{13}C and Gas Liquid Chromatography (GLC)

Mixture	Distribution	^{13}C[a]	GLC[b]	Composition (gravimetric)
SOO + EEE[c]	SAT	27.2	27.0	27.9
	18:1	72.8	73.0	72.1
Methyl ester mixture	SAT	37.0	35.4	32.7
mixture	18:1	40.5	40.5	42.8
	18:2	22.6	23.9	24.5
Shortening[d]	SAT	26.6	25.4	—
	18:1	44.7	46.8	—
	18:2	25.9	25.7	—
	18:3	2.7	2.0	—

[a]Performed on triglyceride mixtures.

[b]Performed on derived methyl esters.

[c]SOO = *Rac*-1,2-dioleoyl 3-stearoyl glycerol. EEE = Glycerol trielaidate.

[d]Although this analysis can be made routinely within 20-30 min, the values obtained for triene composition require 2-3 hr to acquire acceptable accuracy.

dienes, and trienes. Figure 6 shows a spectrum of the alkyl region of a partially hydrogenated shortening from which these values were derived. The *cis,trans* diene contribution to this mixture is less than 3% (3). Although the precision for both the IR-GLC and ^{13}C measurements is comparable to that shown for the less complex samples, i.e., unisomerized standard mixtures, the agreement in the geometric isomer composition values of the shortening determined by these two methods is not as close. Better agreement is shown between the *cis,trans* resolving GLC analysis (1/8 in. 20 ft OV-275) and ^{13}C method. The analysis of butter oil illustrates the power of the ^{13}C method for analyzing complex mixtures containing less than 3% total *trans* unsaturation. Here again very acceptable agreement is observed for the NMR method and the standard analytical techniques.

Composition Analysis

Composition analysis of each sample provides a means of verifying the internal consistency of the observed *cis/trans* values and simultaneously allows for the identification and quantitation of most sample components (19). Table IV lists the pertinent chemical shifts that can be used to determine each specific kind of compound in lipid mixtures.

Triene and diene: Since each fatty acid contains a C_3 carbon and since the T_1 for C_3 in triglycerides is relatively short, we prefer to use the area of the C_3 resonance at δ 25.0 to represent one carbon for all aliphatic chains. Dividing the area of the characteristic triene C_{17} resonance at δ 20.6 by the area of C_3 yields the fraction of triene in the sample. Likewise, comparing the area of the unique shift of the C_{16} of the diene species at δ 31.6 with the C_3 area gives the fraction of diene, i.e., diene other than conjugated types.

Monoene and saturates: The monoene composition is determined by adding the areas of the external *cis* (δ 27.3) and *trans* (δ 32.6) allylic carbons (two carbons per chain for monoene and diene, and one carbon per chain in

triene), subtracting the area equivalent to one carbon per chain of triene, i.e., the area of C_{17} of triene at δ 20.6, followed by subtraction of twice the area of the diene C_{16} resonance at δ 31.6. Thus, the contribution of diene is removed. This leaves an area corresponding to two carbons for each monoene chain. Dividing this area by two and then by the C_3 area gives the fraction of monoene. This is summarized in the following equations:

$$1 \text{ Carbon/monoene chain} = \frac{[cis + trans \text{ external allylics}] - C_{17} \text{ triene} - 2\,C_{16} \text{ diene}}{2}$$

$$\% \text{ Monoene} = \frac{1 \text{ carbon/monoene chain}}{C_3} \times 100$$

The percent saturates may be easily obtained by difference from 100% (since we have already calculated the triene, monoene, and diene) or may be calculated by subtracting the area of one carbon per monoene chain (as obtained above) from the area of the C_{16} resonance at δ 32.0 associated with monoene and saturate. The resulting area will represent the area of one carbon per saturated chain. Dividing by the C_3 area gives the fraction of saturates. This calculation is made:

$$\% \text{ Saturates} = \frac{C_{16} \text{ of [monoene + saturates]} - 1 \text{ carbon/monoene chain}}{C_3} \times 100$$

In most instances, because of the long T_1 values of C_{16} and C_{17} resonances, it is advisable to adjust the areas of these peaks before performing the above calculations. This can be done by taking the sum of the C_{16} resonance areas at δ 31.6 and δ 32.0 and that of the C_{17} associated with triene at δ 20.6 and normalizing it to the area of the standard C_3 whose T_1 is short relative to the pulse repetition rate. Such a manipulation obviates the need for long delay times between pulses making analysis possible within a short time. Alternatively, almost identical results may be obtained from the spectra using short pulse repetition times in the presence of paramagnetic chromium acetyl acetonate (0.04 N) (Fig. 6). With the addition of this compound relaxation is dominated by the electron-^{13}C dipole-dipole mechanism thus effectively suppressing the nuclear Overhauser enhancement effects which arise only when the dipole-dipole mechanism is contributing to the relaxation. Furthermore, at 0.04 N the carbon-electron longitudinal relaxation times are less than the time interval between pulses giving further assurance of quantitative absorption relationship.

Table V compares the compositions for some standard mixtures and a complex shortening as determined by GLC and ^{13}C NMR. It is evident that the two methods compare favorably.

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