♣ Octadecatrienoic Fatty Acid Isomers of Partially Hydrogenated Soybean Oil

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The octadecatrienoic fatty acids of partially hydrogenated soybean oil (PHSBO) were concentrated, isolated and analyzed. The results indicated that the 18:3 acids present in PHSBO are composed of four isomers. The isomer present in the largest amount (2.7%) is the all *cis* isomer, c9,c12,c15-18:3 linolenic acid, and comprises 68.60% of all the isomeric 18:3 acids of PHSBO.

The remaining three 18:3 isomers found were t9,t12,c15-, t9,c12,c15- and c9,c12,t15-18:3, which in total accounted for 1.2% of the total fatty acids of PHSBO.

Soybean oil is a major vegetable oil in the world market. However, one disadvantage in its use as an edible oil is its relatively high (7–8%) linolenic acid content. Linolenic acid (c9,c12,c15-18:3) is highly susceptible to autoxidation, which results in the production of undesirable flavor and odor components in soybean oil.

It is common practice to subject liquid soybean oil to partial and selective hydrogenation which serves to lower the linolenic acid content to ca. 3%, thereby improving the oxidative stability of the oil. Aside from functioning to saturate the double bonds, partial hydrogenation also produces geometric and positional isomers of the unsaturated fatty acids present in the oil (1,2).

Linolenic acid is now thought to function as an essential fatty acid in the human diet (3); it may have some further function in nervous tissue (4) and acts as a prostaglandin precursor (5). Soybean oil is a natural source of linolenic acid. Other dietary sources of linolenic acid are less readily available.

It is therefore of practical importance to know the amount of linolenic acid (c9,c12,c15-18:3) as well as the quantity and types of trienoic isomers formed from linolenic acid via partial hydrogenation of soybean oil (PHSBO).

In the present study the content and types of trienoic acids present in a representative sample of PHSBO were determined. This (PHSBO) is typical of that produced for use as both a salad and a cooking oil (1,2).

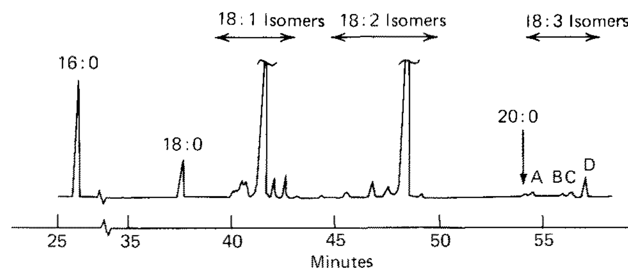

FIG. 1. Gas liquid chromatogram of the fatty acid methyl ester of PHSBO. The 18:3 isomers are designated A, B, C and D. Column, SP2560 100 m × 0.25 cm; temperature, 150–200 C at 0.4 C/min; injector temperature, 250 C; detector temperature, 250 C; carrier gas, H_2; linear flow rate, 19 cm/sec; column pressure, 19 psi; volume, 3 μl; sample concentration, 7 mg/ml.

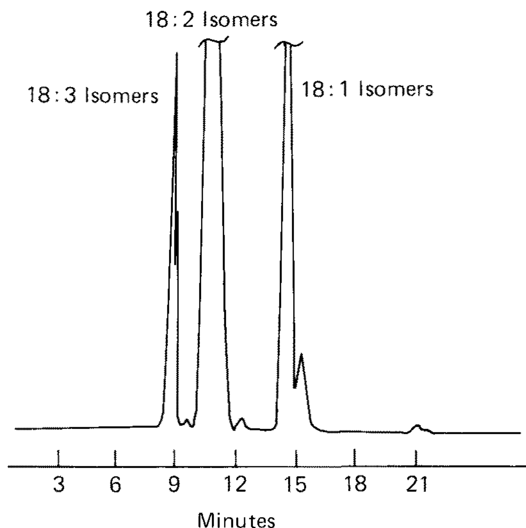

FIG. 2. Reverse phase high performance liquid chromatography separation of the tri-, di- and monoenoic acid methyl esters of PHSBO. Column, LC-18, 25 cm × 4.6 mm; mobile phase, acetonitrile; temperature, 30 C; flow rate, 0.7 ml/min; detector, differential refractometer; sample volume, 20 μl; sample concentration, 250 mg/ml.

MATERIALS AND METHODS

Soybean oil. A representative sample of partially hydrogenated soybean oil in the form of a liquid salad oil was purchased from a commercial grocer.

Preparation of free fatty acids. Free fatty acids were prepared by heating 10 g of partially hydrogenated soybean oil (PHSBO) in 100 ml of 2.5% potassium hydroxide in ethanol under reflux conditions. After heating for 2 hr the solution was acidified to pH 2 with cold 1% H_2SO_4 in water (v/v). The free fatty acids were extracted using 3- to 100-ml portions of hexane:ethyl ether (50:50). The organic extract was washed with water, dried over sodium sulfate and the solvent removed under vacuum with a rotary evaporator.

Methylation. Methyl esters were prepared from free fatty acids using boron trifluoride-methanol according to AOCS method Ce 2-66 (6).

Hydrazine reduction. Methyl esters (3–5 mg) were dissolved in 10 ml of 96% methanol. The solution was heated to 50 C and oxygenated (ca. 1–2 ml/min) for the time of the reaction. One ml of anhydrous hydrazine was added, followed by heating for 2 hr. The reaction was terminated by an addition of 3N HCL to pH 4–5 (7). The methyl esters were extracted with 3 × 7 ml hexane as described in the preparation of fatty acids.

Fractional crystallization. A 5% solution of fatty acids in acetone was cooled at −40 C in an acetone/dry ice bath

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TABLE 1

Component Peaks in the Isolated 18:3 FAMEs of PHSBO

Relative		A	B	C	D
Peak	x	15.44	4.65	10.10	68.60
Area%	s%	0.78	2.50	3.70	0.91

x, Average of 3 runs (GLC); s%, relative standard deviation.

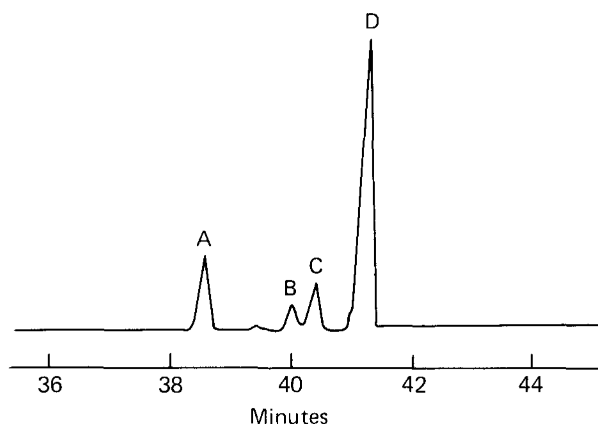

FIG. 3. Gas liquid chromatogram of the 18:3 isomers of PHSBO. Column, SP2560; temperature, 170 C; sample volume, 3 μl; sample concentration, 2 mg/ml; further conditions in Fig. 1.

for 30 min. The −40 C filtrate was collected along with two cold acetone rinses (−67 C) of the −40 C crystals, and again cooled to −54 C. The −54 C filtrate and acetone rinses were collected, combined, solvent evaporated and resultant fatty acids recovered.

Reverse phase high performance liquid chromatography. The system consisted of a dual piston Tracor 995 isocratic pump (Tracor Instrument, Austin, Texas); a Rheodyne loop injector (20 l) (model 7120); a Waters R401 differential refractometer (Waters Assoc., Milford, Massachusetts), and a Hewlett Packard 3390A reporting integrator (Hewlett Packard, Palo Alto, California). The column was a stainless steel column of octadecyl bonded spherical silica (LC-18, Supelco, Supelco Park, Bellefonte, Pennsylvania), 25 cm × 4.6 mm and 5μ particle size.

Elution with methanol as the mobile phase at 0.7 ml/min effected separation of methyl esters according to degree of unsaturation. The trienoic methyl esters were separated with acetonitrile:water (4:1) at 2.0 ml/min as the eluting solvent.

Recovery of the methyl esters from the acetonitrile: water eluent was achieved by adding a volume of both CH_2Cl_2 and H_2O (equivalent to the volume of ACN:H_2O) to the eluate. The organic phase was removed and the remaining aqueous phase extracted twice more with CH_2Cl_2. Recovery of the methyl esters from the combined organic phases was as outlined in preparation of free fatty acids.

Argentation thin layer chromatography. Thin layer chromatography was carried out on glass plates coated with 10% silver nitrate impregnated silica gel (10μ) of 0.5 mm thickness (Supelco Redi Coats-AG). The plates were activated at 110 C for 30 min and stored over saturated calcium chloride (8). Methyl esters were applied in solution with hexane (1 mg/ml). The plates were then serially developed in chloroform 4 times, sprayed with 0.2% dichlorofluorescein in EtOH and viewed under UV light (254 nm) (9).

The methyl esters were recovered by scraping a band into 40 ml of 1% aq. HCL. Hexane (20 ml) was added, the mixture was warmed for 1-2 min under reflux conditions, cooled and extracted with diethyl ether:hexane (1:1). The combined extracts were rinsed twice with water (20 ml) then saturated NaCl solution (to remove any remaining dye) and finally with water. The organic extract was dried, filtered and evaporated in the usual manner.

Gas liquid chromatography. Analytical gas liquid chromatography (GLC) of fatty acid methyl esters (FAMEs) was carried out with a Hewlett Packard 5790 gas chromatograph fitted with a flame ionization detector (FID) and Hewlett Packard 3390A integrator. The column used was a +75% cyano propyl coated fused silica capillary column: SP2560 (Supelco Inc.), 100 m × 0.25 cm, with a 0.2 μm film thickness. The gas chromatographic conditions were a split mode inlet system at 1:100 split ratio, hydrogen as the carrier gas at 19 cm/sec linear gas rate and column pressure of 19 psi. Injector and detector temperatures were 250 C.

The concentration of sample injected onto the column was based on the concentration of a single component of interest; the concentration range for a single component was 0.5-2.0 μg/μl. The sample concentration ranged from 2.0-5.0 μg/μl. The volume injected ranged from 2.0-4.0 μl.

The equivalent chain length values (ECL) of the unsaturated fatty acids were calculated according to the method described by Miwa et al. (10) and modified by Scholfield (11).

RESULTS AND DISCUSSION

The GLC fatty acid profile of PHSBO fatty acid methyl esters is illustrated in Figure 1. Several unknown peaks designated A, B, C and a major peak D appear in the area associated with trienoic FAMEs. Component D, which is 2.7% of the total oil (by GLC), has the retention time of pure methyl linolenate (c9,c12,c15-18:3).

Fractional crystallization at −40 C was used to remove from solution a major portion of the 16:0 and 18:0 saturated fatty acids. Recrystallization of the −40 C filtrate at −54 C resulted primarily in removal of monoenoic fatty acids. The −54 C filtrate consisted of a mixture of mono-, di- and trienoic 18 carbon FAME of relative proportions 40.6%, 55.6% and 3.7%, respectively, as monitored by GLC.

Reverse phase high performance liquid chromatography (RPHPLC) of the FAMEs resulting from fractional crystallization effected separation of these methyl esters based on equivalent carbon number or unsaturation. This technique allowed the isolation of the trienoic acid fraction from the mono- and dienoic acids (Fig. 2). Subsequent GLC analysis indicated the presence of four components (peaks A, B, C and D, Fig. 3) located in the trienoic acid region and which are identical in retention time to those previously detected in the PHSBO. The relative area percent of each component peak is given in Table 1.

Scholfield (11) has reported elution orders and equivalent chain length (ECL) values for the geometric isomers of methyl linolenate separated on a 100-m glass capillary Silar 10C column (75% cyano propyl) (Table 2). The Silar 10C coated column is only slightly less polar

TABLE 2

Comparison of the 18:3 ECLs of PHSBO to the ECLs of Linolenate Isomers

Peak[a]	ECL_c[b]	ECL[c]	ΔECL	18:3 isomer[d]
		19.94		t9,t12,t15
		20.12		c9,t12,t15
		20.15		t9,c12,t15
A	20.08	20.22	0.14	t9,t12,c15
		20.31		c9,t12,c15
B	20.24	20.38	0.14	c9,c12,t15
C	20.28	20.42	0.14	t9,c12,c15
D	20.37	20.52	0.15	c9,c12,c15

[a]Peak identification per Fig. 4.
[b]ECL_c, equivalent chain length data of 18:3 isomers from PHSBO.
[c]ECL data from Scholfield (11).
[d]Isomer identification from Scholfield (11).

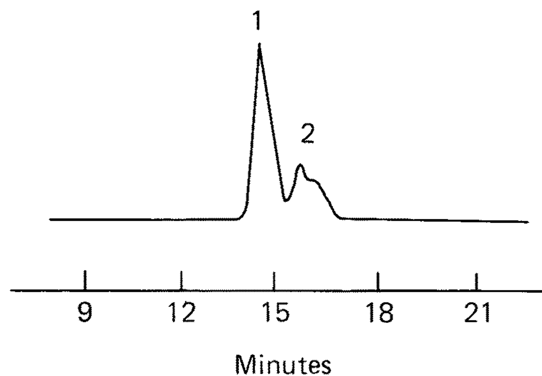

FIG. 4. Reverse phase high performance liquid chromatogram of the 18:3 methyl esters of PHSBO. Column, LC-18, 25 cm × 4.6 mm; mobile phase, acetonitrile:water, 4:1; temperature, 30 C; flow rate, 2 ml/min; detector, differential refractometer; sample volume, 20 μl; sample concentration, 4 mg/ml.

than an SP2560 coated column; therefore, the ECL data is comparable. The difference in ECL values for known compounds (e.g. methyl linolenate) between the two columns can be expressed as ΔECL. It is reasonable to assume that within a family of compounds the ΔECL will remain constant.

The first step taken to analyze a set of peaks of unknown identity in a family of unsaturation is to determine the ΔECL for that family of compounds. Then standard methyl esters are co-chromatographed with the set of unknown peaks to determine which peak or peaks from the set of unknown peaks demonstrates retention times equivalent to the standard methyl esters. For those peaks that co-chromatographed with a standard methyl ester, their tentative identity is set as such. The ECL relationship is then used to determine the identities of the remaining unknown peaks.

The component designated as peak D (Table 1, Fig. 3) is tentatively identified as the c9,c12,c15-18:3 FAME because it coelutes with added methyl linolenate. There is a difference in ECL (ΔECL) of ca. 0.14 between the ECL data for methyl linolenate (all *cis*) reported by Scholfield on a Silar 10C column and the ECL data obtained from the SP2560 column. Using the difference in ECL between the two columns and of the three other peaks separated: A, B, and C, can be tentatively identified as the ttc-isomer, the cct-isomer, and the tcc-isomer of methyl linolenate, respectively (Table 2).

The octadecatrienoic FAMEs of PHSBO which were isolated by RPHPLC were further separated into two fractions using a second RPHPLC system (Fig. 4). The first and second peaks were collected as fractions 1 and 2, respectively. Fraction 1 contained component D, and fraction 2 contained components A, B, C and D, as indicated by GLC.

The same fractionation procedure was carried out with the aid of argentation TLC (AgTLC). Argentation chromatography of the isomeric FAME trienes separated by HPLC resulted in two bands of Rf 0.36 and 0.56. Subsequent GLC analysis of the two bands revealed the presence of components designated A, B and C in band 1 (Rf = 0.36) and component D in band 2 (Rf = 0.56).

Although AgTLC effected the same separation as RPHPLC, the latter was preferred. After developing the plate three times to obtain adequate separation, the triene band tailed badly. This indicated that a small amount of sample was distributed over a large surface, making recovery of sample difficult due to inefficient extraction and, more importantly, increased sample oxidation. Only 60% of the fatty acids applied were recovered. Furthermore, analyses using AgTLC required long time periods. The advantage of HPLC over AgTLC is that the column

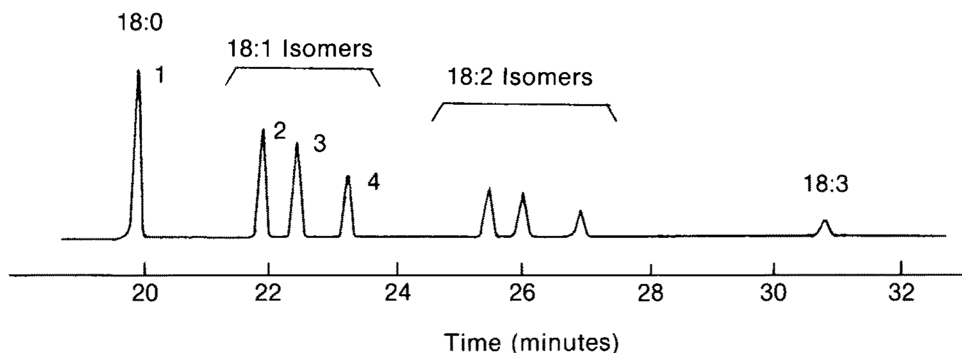

FIG. 5. Gas liquid chromatogram of the methyl ester reduction products of component D, an 18:3 isomer of PHSBO. Column, SP2560; temperature, 180 C; sample volume, 3 μl; sample concentration, 7 mg/ml.

is a reusable resource, sample separations are simple and rapid, analysis time is short, and recoveries are usually over 95%.

Historically, the procedure for analysis of double bond location in an isolated trienoic FAME involves four phases (9): partial hydrazine reduction of the trienoic FAME, isolation of the monoenoic acid products, separation of the *cis* and *trans* monoenes and oxidative cleavage of the monoenoic acids. However, the increased resolution available from capillary GLC columns and the availability of published ECL values for these columns allows for structure determination of trienoic FAME using both the ECL value of the triene and the ECL values of the monoenes produced via partial hydrazine reduction of the trienoic FAME (12,13).

Fraction 1, which contained component D of the isolated 18:3 methyl esters of PHSBO, was partially reduced using hydrazine. Partial hydrazine reduction of an authentic sample of methyl linolenate resulted in the formation of compounds with retention times identical to those formed in the partial hydrazine reduction of component D (Fig. 5). Peak 1 (Fig. 5) co-chromatographed with methyl stearate and was identified as such. Peak 2 co-chromatographed with methyl oleate and was thus identified; even though its ECL value on the SP2560 column is very similar to the ECL value of c8-18:1 on the Silar 10C column (11), it is the ΔECL which must be used when comparing the ECL of one column to another. The ECL_c of peaks designated 2, 3 and 4 were compared to ECL values reported by Scholfield (11) for c9-, c12- and c15-isomers of 18:1 and were found to be in good agreement (Table 3). There is adequate difference in ECL between the relevant c18:1 isomers to distinguish between the positional isomers. Other data indicated that component D of the trienoic acids isolated from PHSBO, when reduced to a saturated compound, produced methyl stearate. Partial hydrazine reduction of component D yielded three monoenoic acids: c9-, c12- and c15-18:1.

TABLE 3

Comparison of the ECLs for the 18:1 Reduction Products of Component D and Some *cis* Positional Isomers of 18:1

Peak[a]	ECL_c[b]	ECL[c]	ECL	18:1 isomer[d]
		18.56		c8
2	18.57	18.61	0.04	c9
		18.64		c10
		18.70		c11
3	18.72	18.75	0.03	c12
		18.84		c13
4	18.92	18.96	0.04	c15

[a]Peak identification per Fig. 5.
[b]ECLs of the 18:1 reduction products of component D, from the 18:3 isomers of PHSBO.
[c]ECL data from Scholfield (11).
[d]Isomer identification from Scholfield (11).

The original structure of component D was, therefore, c9,c12,c15-18:3.

Fraction 2 from HPLC of FAMEs (Fig. 4) which contained components A, B, C and D of the isolated 18:3 methyl esters of PHSBO, was partially reduced using hydrazine. The GLC retention times of peaks 1, 4, 6 and 7 (Fig. 6) were identical to the retention times of 18:0, c9-18:1, c12-18:1 and c15-18:1. Methyl elaidate, t9-18:1, co-eluted with peak 2. A deuterated standard of t12-18:1 eluted slightly before peak 3, but this was expected of a deuterium-containing ester (11). The only other peak of interest for which no standard was available was peak 5 (Fig. 5). The identity of this component was based on correlating its ECL_c with those ECL values reported previously by Scholfield (11) (Table 4).

The ECL of methyl elaidate, t9-18:1, for the SP2560 column, did not correlate well with that reported by

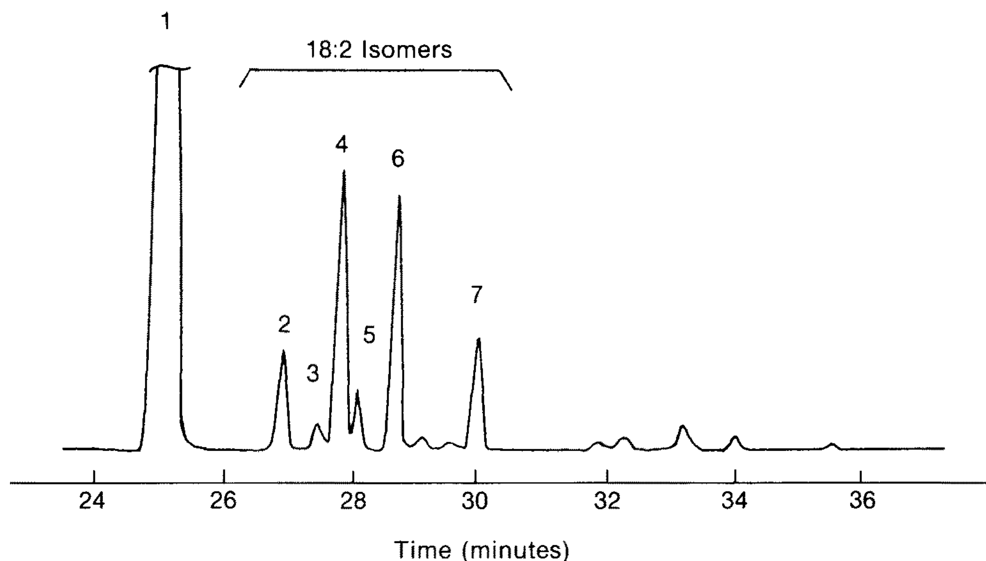

FIG. 6. Gas liquid chromatogram of the methyl ester reduction products of the 18:3 isomers (fraction 2 from HPLC) of PHSBO. Column, SP2560; temperature, 170 C; sample volume, 3 μl; sample concentration, 7 mg/ml; further specifications, Table 2 (sec. 3.4.3.).

Scholfield (Silar 10C). The average ECL obtained for the monoenoic isomers was 0.04, and the ECL for methyl elaidate was 0.01; methyl elaidate eluted later on the SP2560 column than on the SILAR 10C column. Peak 2 was tentatively assigned the identity of methyl elaidate because the two components co-eluted, and the monoene identified by Scholfield with the closest ECL to that of peak 2 was t7-18:1. Since the t7 double bond originated from a *cis* 9-10 double bond it seems unlikely that the t7 monoene would appear in such quantity without the appearance of a peak or shoulder that could be correlated to t9- or t8-18:1. The ECL data reported here for t9- and t15-18:1 agree with that of Scholfield. The components were tentatively identified as indicated in Table 4.

A final step in the analysis is to correlate the tentative identities of the 18:3 acids isolated from PHSBO with the amounts and types of monoenoic acids produced via partial hydrazine reduction of the trienoic acids. Component D has already been identified as methyl linolenate. Components A, B and C (Fig. 2) have been tentatively identified as t9,t12,c15-18:3, c9,c12,t15-18:3 and t9,c12,c15-18:3. *Trans* 9-, t12- and t15-18:1 acids are expected and found in the partial reduction products. Two of the three components, A and C, have a t9 double bond. Only one component contains a t12 double bond (A), and one component contains a t15 double bond (B). Of the *trans* monoenoic acids produced via reduction, t9-18:1 was present in the largest amounts. This is expected as there are two sources for a t9 double bond, components A and C (Fig. 2). Relatively less t12- and t15-18:1 are produced as hydrazine reduction products since only one component contains each double bond type. Therefore, the amounts and types of monoenoic acids (produced via partial hydrazine reduction of the trienoic acids) support the proposed identities of the trienoic acids.

The octadecatrienoic acids present in PHSBO and their relative concentrations are: c9,c12,c15-18:3, 68.60%; t9,t12,c15-18:3, 15.44%; t9,c12,c15-18:3, 10.10%, and c9,c12,t15-18:3, 4.65%. It is notable that the t9 double bond is common to two of the *trans* containing isomers, and the most abundant *trans* isomer contains two *trans* double bonds.

The currently accepted mechanisms of heterogenous hydrogenation indicate that under the selective conditions of partial hydrogenation, the decreased availability of hydrogen makes it thermodynamically easier for a monoadsorbed species to lose a proton and return to a *trans* double bond than add a second proton and become saturated. The *trans* double bond is preferred because it is energetically more stable than the *cis*.

The c15 double bond position in the all *cis* isomer is the most reactive position since it is the least sterically inhibited. Since the c15 double bond is the more reactive of the double bonds, it may be more likely to move from a monoadsorbed species to a saturated species. Those molecules that do not move from a monoadsorbed species to a saturated species instead return to a *trans* double bond. The high reactivity of the c15 double bond accounts for the low concentration of the c9,c12,t15-18:3 isomer.

The absence of the c9,t12,c15-18:3 isomer indicates that the c12 double bond is the least reactive center of unsaturation. This may be due to the steric hindrance and electron-withdrawing properties of the vinylic double bond system.

TABLE 4

Comparison of the ECLs for the *trans* 18:1 Reduction Products of PHSBO and Some *trans* Positional Isomers of 18:1

Peak[a]	ECL_c[b]	ECL[c]	ECL	18:1 isomer[d]
		18.40		t6
		18.43		t7
		18.41		t8
2	18.40	18.41	0.01	t9
		18.48		t10
		18.49		t11
3	18.50	18.54	0.04	t12
		18.59		t13
5	18.61	18.66	0.05	t15

[a]Peak identification per Fig. 6.
[b]ECLs of the *trans* 18:1 reduction products of components A, B, C and D, the trienoic acids of PHSBO.
[c]ECL data from Scholfield (11).
[d]Isomer identification from Scholfield (11).

The c9 double bond appears to be of intermediate reactivity. There are two isomers which contain a t9 double bond. The least common of the two is the t9,c12,c15-18:3 isomer. The t9,c12,c15-18:3 isomer may be formed by adsorption of the c9 double bond of the all *cis* isomer to the catalyst active site and uptake of a hydrogen atom to form the monoadsorbed species. A proton is lost and desorption occurs to form the t9,c12,c15-18:3 isomer. The greater quantity of the tcc isomer versus the cct isomer indicates that the monoadsorbed species at the 9(10) position has a greater tendency to return to the original site of unsaturation in the *trans* form than does a monoadsorbed species at the 15(16) position.

The formation of the t9,t12,c15-18:3 isomer may be due to a sequence of adsorptions and desorptions from the catalytic surface during hydrogenation. Once the t9,c12,c15-18:3 isomer is formed (as explained previously), the tcc isomer becomes diadsorbed at carbons 12 and 13 because the t9 double bond has reduced steric hindrance and/or a catalytic active site is near and available (having been vacated by the 9-10 diadsorbed species). The 12-13 diadsorbed species can alternately gain and lose a proton, thus forming the t9,t12,c15-18:3 isomer, or it can become saturated. That the former may occur is evident from the appearance of the ttc isomer in the octadecatrienoic acid fraction of PHSBO.

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[Received November 12, 1986]