Evaluation of SFC/FT-IR for Examination of Hydrogenated Soybean Oil

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Partial hydrogenation of vegetable oils causes isomerization of the unsaturated fatty acids in the triacylglycerol molecules. Some of these isomers have been shown to cause adverse physiological effects in animal feeding studies. The study reported here evaluates the use of supercritical fluid chromatography (SFC) with flow-cell Fourier transform infrared (FT-IR) spectrometry to determine the relative level of unsaturation and the extent of isomerization in partially hydrogenated soybean oil. Free fatty acids (FFAs) from the hydrolysis of soybean oil and the intact triacylglycerols (TGs) of soybean oil were analyzed. These compounds can be analyzed by SFC using low temperatures and similar chromatographic conditions. GC analysis of FFAs and TGs generally requires high temperature and different chromatographic columns. The SFC/FT-IR spectra were compared with the spectra of fatty acid methyl esters by employing gas chromatography/matrix isolation/FT-IR.

INTRODUCTION

Soybean oil is one of the most important edible oils in the United States. Considerable efforts have been made in the modification of soybean oil for food technology applications. Refined soybean oil contains 6-8% linolenic acid, which can also cause off-flavors from oxidation. Selective hydrogenation can reduce the linolenic acid content to 3%, which improves the stability of the product (Erickson et al., 1980). Partial hydrogenation of vegetable oils leads to the formation of numerous positional and geometric fatty acid isomers and a dramatic decrease in total polyunsaturated fatty acids. The *t*9,*t*12-18:2 isomer (linoelaidic acid) is found in soybean oil only after hydrogenation and has been shown to cause adverse physiological effects (Kummerow, 1979). Therefore, analytical methods that permit the identification of individual isomers from the partial hydrogenation of soybean oil are desirable.

Wojtusik et al. (1989) recently reviewed the status of the separation and detection of triacylglycerols (TGs) by HPLC. They believe that the best chromatographic resolution can be obtained by using reversed-phase HPLC. Complete characterization of naturally occurring TGs, although, necessitates the use of several analytical techniques.

Separation of TGs by carbon number (Schmid et al., 1979; Grob et al., 1980; Wakehan and Frew, 1982; Mares and Husek, 1985) and degree of unsaturation (Geeraert et al., 1983, 1987; Geeraert and Sandra, 1985; Kalo et al., 1986; Sandra, 1987; Hinshaw and Ettre, 1989) have been achieved by using GC. Separation of TGs by degree of unsaturation requires high temperatures and very stable polar stationary phases. Grob et al. (1980) listed a variety of requirements when analyzing TGs by GC. These include (a) highly deactivated columns to prevent degradation of the TGs during the analysis and (b) cold on-column injections to suppress discrimination of the higher molecular weight components. Hinshaw and Ettre (1989) recently investigated cold split/splitless injection and cold on-column injection. They used saturated TGs in the study to suppress possible bond migration in unsaturated compounds due to the high temperatures required for separation.

IR detection in lipid analysis is generally used for two reasons. The first reason for using IR detection is that it becomes a selective detector for HPLC by monitoring the carbonyl stretching region (5.75 μm) (Robinson and Macrae, 1984; Hamilton et al., 1987; Parris, 1979). Baseline drift occurs with gradient elution, although it can be minimized with the proper choice of solvents and gradient conditions. The second reason for using IR detection is to determine the amount of conversion of the fatty acid isomers from the cis to the trans configuration during hydrogenation (Allen, 1969; Huang and Firestone, 1971a,b; Madison et al., 1982). Generally, IR methods are used to determine the total trans content without separation from the cis by analyzing the methyl ester derivatives of the fatty acids. For example, a recent GC analysis of fatty acid methyl esters (FAMEs) coupled with matrix isolation/Fourier transform infrared (MI/FT-IR) detection permitted the identification of low levels of trans isomers resulting from hydrogenation of edible oils (Mossoba et al., 1990).

SFC has recently been employed in the analysis of lipids. Separation of lipids as fatty acid esters (Wilsh and Schneider, 1983; Nomura et al., 1989; Gorner and Perrut, 1989), free fatty acids (FFAs) (Chesters, 1984; Markides et al., 1986; Hellgeth et al., 1986; Gumer et al., 1987), and TGs (White and Houck, 1985; Prost et al., 1986; Huopalalahti et al., 1988; Kaillo et al., 1989) has been achieved by using both packed and open tubular columns. Hellgeth et al. (1986) analyzed FFAs using a PRP-1 column with FT-IR detection. Severe chromatographic peak distortion occurred for the C_{18} carboxylic acids found in hydrolyzed soybean oil. Inspection of the FT-IR spectra through the chromatographic peak showed that chromatographic resolution was not achieved between the saturated and

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unsaturated C_{18} fatty acids. Markides et al. (1986) were able to partially resolve the saturated and unsaturated FFAs using a 50% cyanopropylpolysiloxane capillary column. They employed a FID detector and indicated that C_{18} cis and trans isomers as well as isomers differing in the position of the double bonds were separated. Gorner and Perrut (1989) were able to separate unsaturated FAMEs using a packed silica column with 100% CO_2. Their paper did not indicate whether cis and trans isomers were separated under the chromatographic conditions employed. TGs were separated by carbon number with nonpolar columns and were separated by degree of unsaturation with polar columns.

An advantage of SFC over GC analysis is that TGs can be chromatographed at temperatures below 200 °C. An advantage of SFC over HPLC is that SFC is readily interfaced to FID and IR detection. On-line FT-IR detection using SFC is not limited to monitoring only the carbonyl region (Hellgeth et al., 1986), which suggests that an opportunity exists to determine the extent of unsaturation and isomerization in a single analysis. TGs and FFAs can be chromatographed under similar SFC conditions (Calvey and Taylor, 1988), thereby making the analysis of complex mixtures containing both types of compounds relatively easy. The purpose of this study was to evaluate the ability of SFC with on-line FT-IR to detect changes in the chemical composition of vegetable oil due to processing. Refined soybean oil and soybean oil that was partially hydrogenated were chosen for the study because of their dietary importance. Supporting SFC/FID and SFC/FT-IR data were also obtained with lipid standard solutions. A comparison of several characteristic FAME bands observed in the GC/MI/FT-IR (Mossoba et al., 1990) and SFC/FT-IR spectra is also included.

EXPERIMENTAL PROCEDURES

A Model 501 supercritical fluid chromatograph (Lee Scientific, Inc., Salt Lake City, UT) with a flame ionization detector (FID) set at 350 or 375 °C was used. The SFC pump and the injector were cooled to <5 °C by using a recirculating bath. Chromatographic separation was achieved with Coleman grade or SFC grade CO_2 (Matheson, Dorsey, MD) as the mobile phase. Restriction was achieved by employing a 100 μm i.d. frit restrictor. The chromatographic column was directly attached to the injection employing a polyimide ferrule previously described (Roach et al., 1989). The chromatographic conditions varied with the components analyzed and are given in the figure captions. All chromatographic columns were obtained from Lee Scientific. A 1-s timed-split injection employing a 200-nL rotor was used. FT-IR spectra were obtained by using a 740SX FT-IR spectrometer (Nicolet Instrument Corp., Madison, WI) equipped with a 0.6 mm i.d. × 5 mm path length (1.4 μL) high-pressure flow cell in-line with an FID. All spectra were obtained in real time at 8-cm^{-1} resolution. Spectra were acquired by collecting 1.1 files/s, 8 scans/file. FT-IR data acquisition was started 20 min into the chromatographic run for the FFA and the TG separations. The transfer line was maintained at or near the oven temperature. The flow cell was maintained at 35 °C. The GC/MI/FT-IR data were previously reported (Mossoba et al., 1990).

Refined soybean oil and soybean oil that was partially hydrogenated with a Ni or Ni–S catalyst were analyzed. The soybean oil was hydrogenated with 0.1% Ni (100 °C; 138-kPa hydrogen pressure, 80 min) or 0.25% Ni–S (200 °C; 207-kPa hydrogen pressure, 40 min) catalysts in a 1-gal pressure vessel reactor (Autoclave Engineers, Erie, PA). The FFAs from the unhydrolyzed soybean oil samples were obtained by hydrolyzing an aliquot of the oil with 0.5 N aqueous NaOH with continuous stirring and addition of heat overnight. The nonhydrolyzed TGs were removed from the NaOH solution by washing three times with 4 mL of CH_2Cl_2. The aqueous layer was then acidified with 1 N H_2S_4, and the FFAs were extracted from the aqueous layer with three 4-mL hexane washes. The solutions were concentrated to approximately 1 mL. TG standard mixture containing tristearin, triolein, trilinolein, and trilinolenin at 25 mg each was obtained from Sigma (St. Louis, MO). The standard mixture was dissolved in a 100-mL volume of approximately 30% toluene and 70% isooctane. Other lipid (Sigma) standard solutions were prepared in the following concentrations: methyl palmitate, 5 mg mL^{-1} MeOH; 17:0, *t*9-18:1, *t*9,*t*12-18:2, methyl esters, 0.18 mL^{-1} isooctane; linoleic acid (*c*9,*c*12-18:2), 2 mg mL^{-1} CH_2Cl_2; elaidic acid (*t*9-18:1), 6.5 mg mL^{-1} hexane.

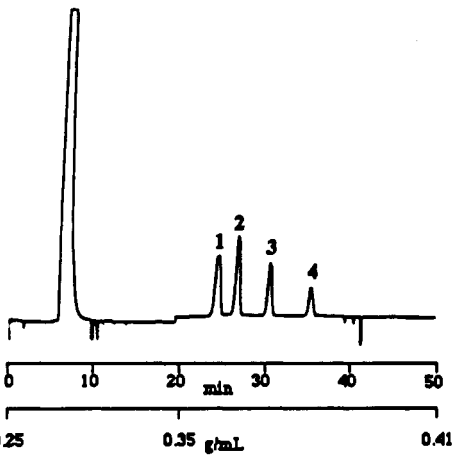

Figure 1. SFC chromatogram of triacylglycerol standard mixture on 50% cyanopropylpolysiloxane–50% methylpolysiloxane open tubular column (10 m × 100 μm i.d., 0.25-μm film thickness). (1) Tristearin; (2) triolein; (3) trilinolein; (4) trilinolenin. Oven temperature: 140 °C. Density program: 0.25–0.35 g mL^{-1} at 0.005 g mL^{-1} min^{-1}, 0.35–0.45 g mL^{-1} at 0.002 g mL^{-1} min^{-1}. FID is directly connected

RESULTS AND DISCUSSION

Lipid Standard Analysis. The TG standard mixture was chromatographed by using three different stationary phases. The SFC separation of the TG standard mixture on a 30% biphenylpolysiloxane open tubular column resulted in tristearin and triolein coeluting with a total analysis time of 50 min. Baseline resolution was achieved in under 42 min with a 25% cyanopropylpolysiloxane open tubular column. To date, the best separation for the triacylglycerol standard mixture has been obtained by using a 50% cyanopropylpolysiloxane capillary column (Figure 1). Due to the low FT-IR absorbance of TGs, the amount needed for detection usually led to column overload, as evidenced by the peak fronting.

The on-line SFC/FT-IR spectra of FAMEs, TGs, and FFAs are similar (Figure 2). The absorbances between 2800 and 3000 cm^{-1} are due to methylene and methyl symmetric and asymmetric stretching. Absorbances above 3000 cm^{-1} are due to unsaturation in the hydrocarbon chain. An absorbance at 3018–3016 cm^{-1} indicates the presence of at least two double bonds in the cis, cis configuration. A single double bond in the cis configuration has an absorbance at 3012–3010 cm^{-1}. The C=O stretching vibration shifts from 1747 cm^{-1} in the methyl ester and TG to 1763 cm^{-1} in the free acid. The C—O stretching vibration also shifts from 1160–1175 cm^{-1} in the methyl ester and TG to 1133 cm^{-1} in the free acid. If one examines the carbonyl region of a FFA standard (Figure 2b) in SC–CO_2, two peaks (1762 and 1717 cm^{-1}) are readily observed. The absorbance at 1762 cm^{-1} is caused by the FFA in the monomer state, while the absorbance at 1716 cm^{-1} is caused by the presence of the FFA in the dimer state (Hellgeth et al., 1986).

Comparison of SFC/FT-IR and MI/FT-IR Spectra. The FAME bands observed by SFC/FT-IR and MI/FT-IR for the *c*9,*c*12-18:2 and *t*9,*t*12-18:2 isomers are listed in

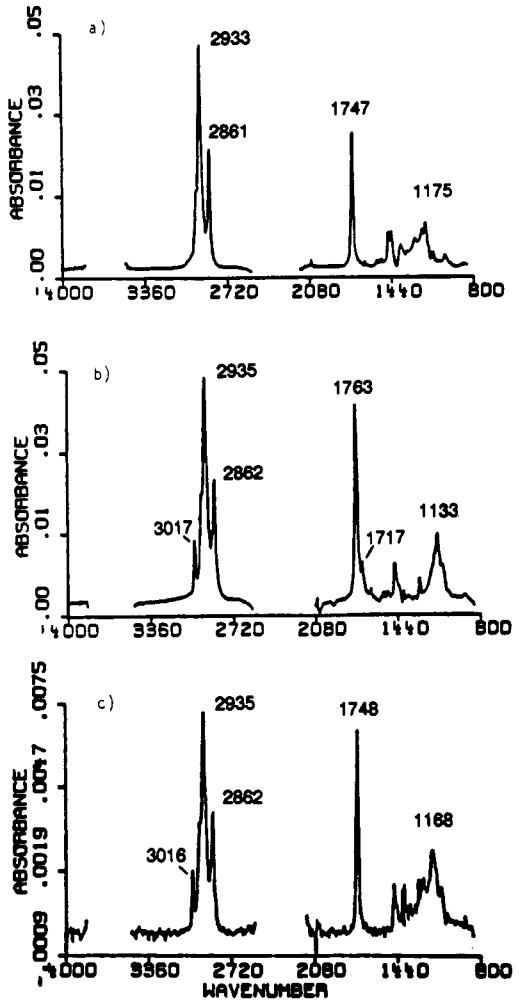

Figure 2. On-line SFC/FT-IR chromatographic file spectra of standards (8 scans/file). (a) Methyl palmitate (14 files coadded); (b) linoleic acid (20 files coadded); (c) trilinolein (23 files coadded).

Table I. FAME Bands (cm^{-1}) Observed by SCF/FT-IR and MI/FT-IR

	*c*9,*c*12-18:2		*t*9,*t*12-18:2	
IR mode	SFC[a]	MI[b]	SFC[a]	MI[b]
=C—H stretch trans			3032	3035
			3005	3005
=C—H stretch cis	3016	3018		
CH_3 asym str	2962	2961	2962	2961
CH_2 asym str	2935	2935	2933	2935
CH_34 sym str	2880[c]	2880	2880[c]	2880
CH_2 sym str	2863	2863	2832	2836
ester C=O str	1741	1754	1747	1754
CH_2 scissors	1459	1463	1459	1463
CH_3 sym scissors		1381		1381
ester sym C—O str	1176	1176	1176	1176
CH def, trans			972	972
CH_2 rock	*d*	730	*d*	730

[a] 8-cm^{-1} resolution. [b] 4-cm^{-1} resolution. [c] Shoulder on CH_2 sym str absorbance. [d] Cannot observe bands below 800 cm^{-1} due to absorbance by CO_2.

Table I. The CH_3 symmetric stretch was observed as a shoulder on the CH_2 symmetric stretch (Figures 3 and 4). The SFC data were collected at 8-cm^{-1} resolution, while the MI data were collected at 4-cm^{-1} resolution. The CH_2 rocking frequency was not observed in the SFC spectra because of CO_2 absorbance below 800 cm^{-} The FT-IR spectra (Figures 3a, 4a) of *t*9,*t*12-18:2 have an absorbance at 972 cm^{-1} (CH deformation) which indicates that the double bonds are in the trans configuration.

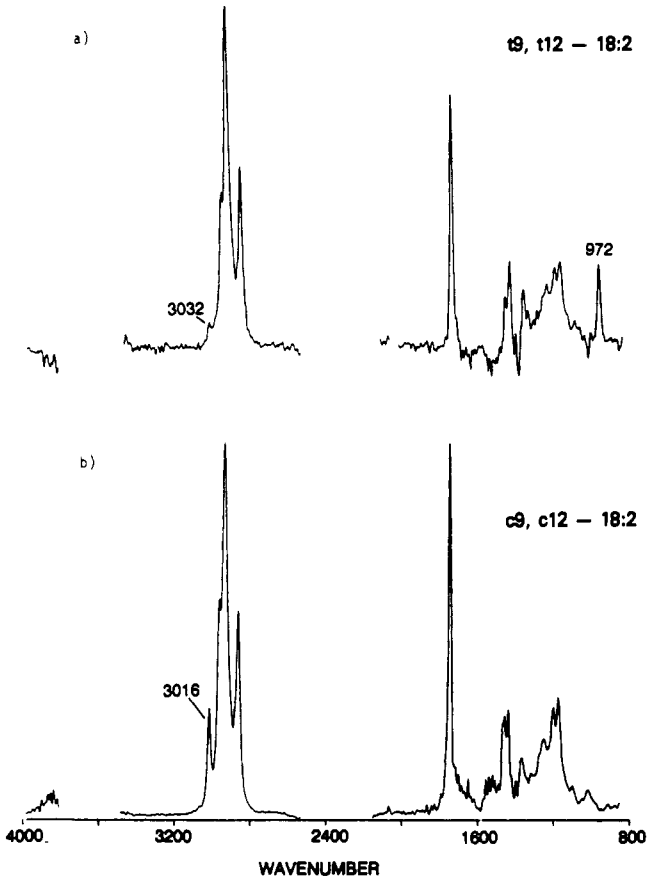

Figure 3. SFC/FT-IR spectra of FAMEs. (a) *t*9,*t*12-18:2 (136 scans); (b) *c*9,*c*12-18:2 (96 scans).

If one examines the SFC and MI spectra of the 18:2 isomers, the absorbance of the ester carbonyl stretch (1754 cm^{-1}) is larger compared to the absorbance of the aliphatic C–H stretch (2935 cm^{-1}) in the MI spectra than in the SFC spectra. One would anticipate a difference in the ester carbonyl region. Dissolution in supercritical CO_2 has been observed to shift the carbonyl stretching vibration as well as alter the intensity of absorbance. Also of note is the ratio of the ester symmetric C–O stretch relative to the 1754-cm^{-1} band. The absorbance of 1176 cm^{-1} is more intense in the MI spectrum than in the SFC spectrum. The differences in absorbance intensity in this region (1400–1200 cm^{-1}) may be due to the need to subtract the absorption of CO_2 caused by Fermi resonance. Although the SFC spectra were collected at 8-cm^{-1} resolution, one is still able to observe clearly in the 18:2 *t*9,*t*12 isomer spectrum the weak absorbance bands slightly above 3000 cm^{-1} due to the trans =C—H stretch which compared well with those found by MI/FT-IR (Figure 5).

Soybean Oil Analysis. Although the standard C54 TGs were readily resolved on the 50% cyanopropylpolysiloxane column (Figure 1), the TGs from refined soybean oil were not completely resolved. This can be explained in part by the distribution of TGs in soybean oil. There are at least six TGs containing 54 carbons with the number of double bonds ranging from two to seven and at least three C_{52} TGs with two to four double bonds. The FID profiles change significantly upon hydrogenation (Figure 6). The decreases in unsaturation as measured by the iodine value (from 131 to 98–100) of the Ni and Ni–S samples were similar. Loss, in the hydrogenated samples (Figure 6b,c), of the later eluting components found in the refined soybean oil (Figure 6a) is indicative of increased saturation of the fatty acids. Although the iodine values indicated similar levels of total saturation, the FID profiles

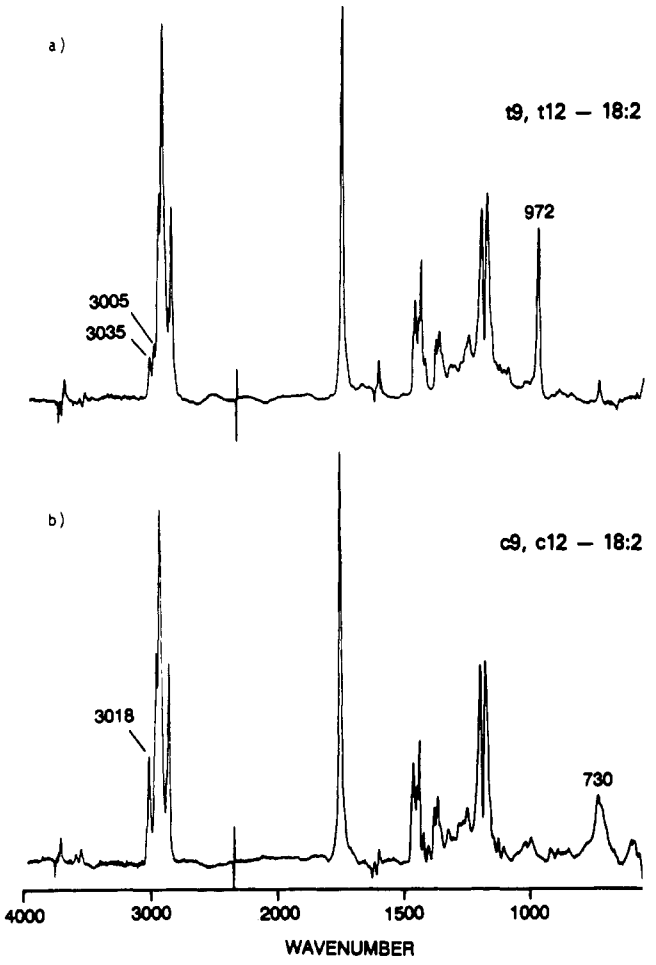

Figure 4. MI/FT-IR spectra of FAMEs. (a) *t*9,*t*12-18:2 (300 scans); (b) *c*9,*c*12-18:2 (300 scans).

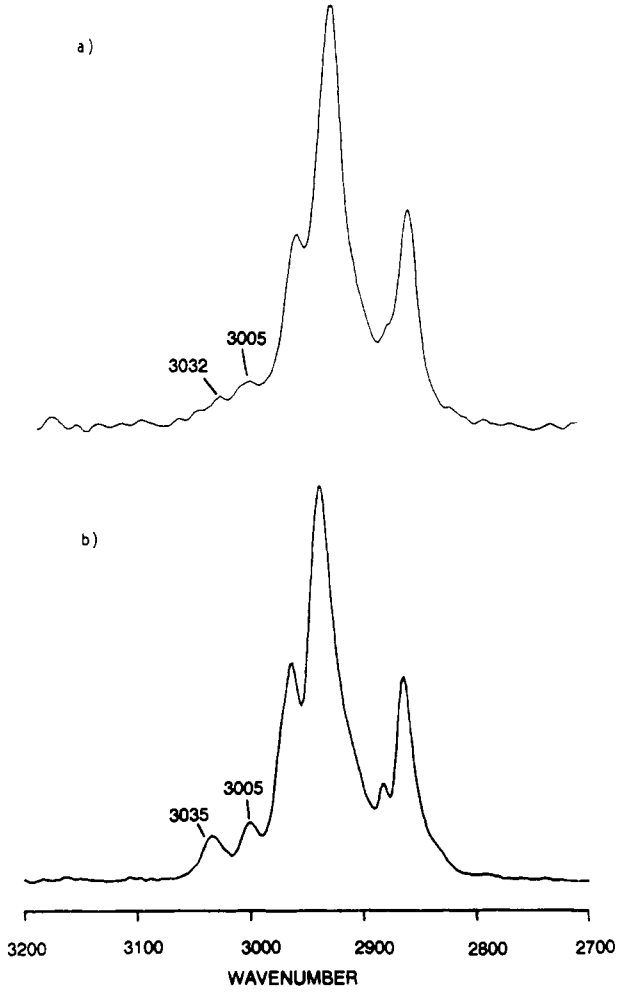

Figure 5. Expanded FT-IR spectra of FAME *t*9,*t*12-18:2. (a) SFC spectrum; (b) MI spectrum.

were significantly different. The nature of the Ni–S catalyst surface appears to favor more complete hydrogenation of the highly unsaturated TGs found in soybean oil. This is shown in the FID profile (Figure 6c) by the loss of the later eluting components found in the refined soybean oil and the Ni hydrogenated soybean oil (Figure 6a,b). Okonek (1987) reported that the Ni–S catalyst promotes the formation of high levels of trans isomers during hydrogenation. This greater conversion to trans isomers can also account for the broad peak in the SFC/FID chromatogram.

An oven temperature of 60 °C was used for the elution of the FFAs on the basis of a paper by Markides et al. (1986). While slight improvement in resolution was obtained with a slower gradient rate, this improvement came with an analysis time of over 1 h. The density gradient program chosen for the FFA separation resulted in an analysis time of approximately 40 min. The density program used resulted in the elution of trans 18:1 between 18:0 and cis 18:1. The FID profiles (Figure 7) of the FFA separation shows more clearly than the TG chromatograms the loss of unsaturated components as well as the isomerization of the fatty acids. In the Ni hydrogenated soybean oil profile the presence of the trans isomers is shown by the broader 18:1 peak and the front shoulder on the 18:2 fatty acid peak. A more concentrated solution of the Ni hydrogenated sample more readily shows that some 18:3 is still present. In the Ni–S hydrogenated soybean oil, almost complete conversion to trans 18:1 occurs as demonstrated by the shift in retention times. Comparison of peak heights between the chromatograms is difficult because FID response factors for cis and trans isomers were not determined.

Capillary GC analyses of these samples confirm the trends observed under SFC conditions. The changes due to hydrogenation in the FID were futher demonstrated. Figure 8 shows representative on-line FT-IR chromatographic file spectra of TGs from refined soybean oil and hydrogenated soybean oil. The spectra represent chromatographic peaks with similar retention times observed in the three samples. The spectra indicate a change in the configuration of some of the double bonds from cis to trans. This isomerization was demonstrated by the loss of absorbance at 3016 cm^{-1} and the presence of absorbance at 972 cm^{-1}. The FT-IR spectra of the TGs of the Ni–S hydrogenated soybean oil indicate that there was greater conversion to trans isomers than with the Ni hydrogenated soybean oil. This conversion to trans isomers accounts for the broad peak in the FID trace. Due to the low isomer content of the Ni hydrogenated soybean oil, the presence of the absorbance at 972 cm^{-1} was difficult to observe in the intact TGs but became more evident in the spectra of the FFAs. The highest S/N of the trans absorbance at 972 cm^{-1} in the TG spectra from the Ni hydrogenated soybean oil was 2. The highest S/N in the FFA spectra was 3.5. The noise was measured as the background peak-to-peak absorbance between approximately 950 and 850 cm^{-1}. The intensity of the absorbance at 972 cm^{-1} is less in the TGs because the trans fatty acid is distributed among several of the TGs.

The intensity of the CH_2 asymmetric stretching absorbance decreased relative to that of the ester carbonyl

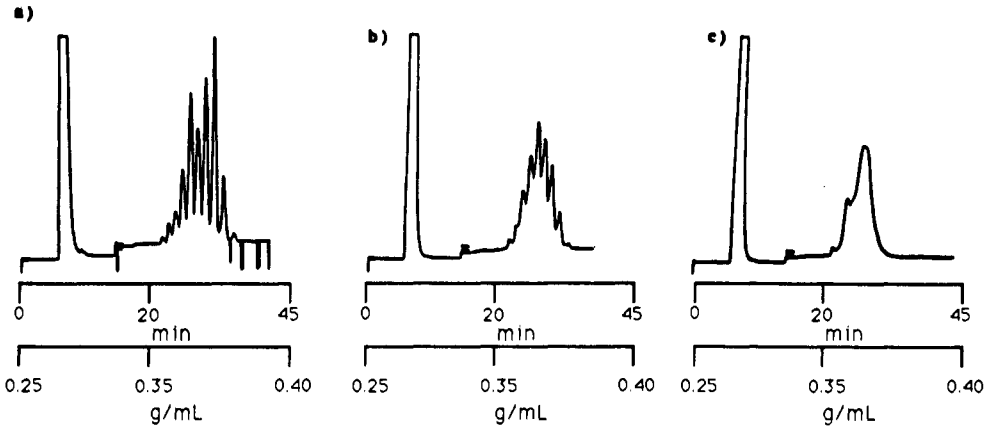

Figure 6. SFC chromatograms of triacylglycerols. (a) Refined soybean oil; (b) Ni hydrogenated soybean oil; (c) Ni–S hydrogenated soybean oil. See Figure 1 for chromatographic conditions.

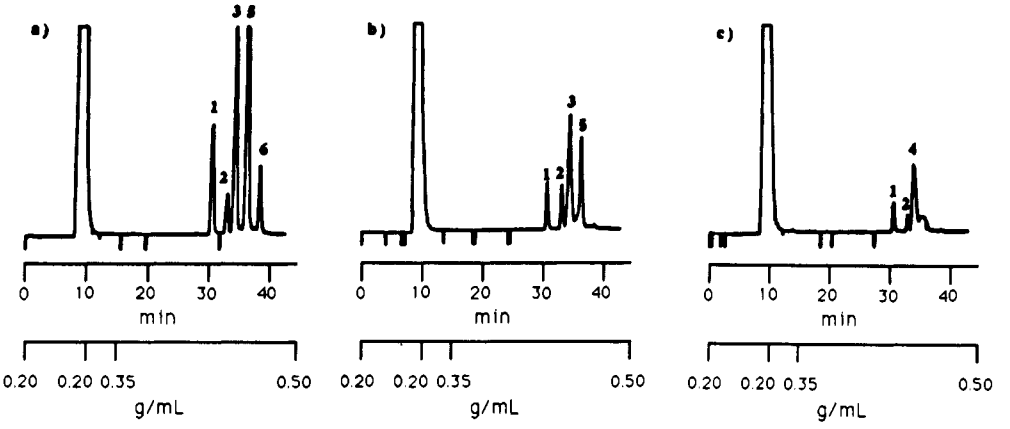

Figure 7. SFC chromatograms of FFAs from (a) refined soybean oil, (b) Ni hydrogenated soybean oil, and (c) Ni–S hydrogenated soybean oil. Column 50% cyanopropylpolysiloxane–50% methylpolysiloxane (10 m × 100 μm i.d., 0.25-μm df). Oven temperature: 60 °C. Density program: 0.20 g mL^{-1}, hold 10 min, 0.20–0.35 g mL^{-1} at 0.025 d mL^{-1} min^{-1}, 0.35–0.50 g mL^{-1} at 0.005 g mL^{-1} min^{-1}. (1) C16:0; (2) C18:0; (3) *c*-C18:1; (4) *t*-C18:1; (5) *c*-C18:2; (6) *c*-C18:3.

Table II. Normalized Intensity of the Cis =C—H and Aliphatic C—H Stretching Bands for C_{18} FAMEs,[a] C_{18} FFAs,[b] and C_{54} TGs[b] Relative to the Ester Carbonyl Stretching Vibration

	=C—H stretch (cis)[c]			aliphatic C—H stretch[d]		
	FAMEs	FFAs	TGs	FAMEs	FFAs	TGs
C18:0				1.60	2.16	2.06
C18:1	0.17	0.19	0.15	1.21	1.62	1.59
C18:2	0.26	0.30	0.28	0.89	1.17	1.08
C18:3	0.44	0.41	0.38	0.74	0.79	0.75

[a] Data from MI/FT-IR. [b] Data from SFC/FT-IR. [c] 3009–3018 cm^{-1}. [d] 2932–2936 cm^{-1}.

stretch as the number of double bonds increased (Table II). This intensity characteristic was used as a simple identification criterion based on degree of unsaturation for the FAME analysis by GC/MI/FT-IR (Mossoba et al., 1990). It cannot be readily applied to the TG analysis by SFC/FT-IR. When the ratios were determined for the TGs in refined soybean oil, small changes in the ratio values (1.14–1.06) occcurred. There was a trend to lower values at longer retention times, indicating increased unsaturation in the compounds. Further evidence of unsaturation was indicated by the increased value for the ratio of the cis =C—H stretch to the carbonyl stretch (0.14–0.36). Less dramatic changes in these ratio values compared with the values obtained for the TG standards (Table II) may result from incomplete resolution of the TGs in refined soybean oil under the SFC conditions employed in the study. The distribution of the different fatty acids (C_{16} as well as C_{18} fatty acids) among the TGs would result in averaging the absorbances of the fatty acids moieties so that identifiable ratios may not be possible with mixed fatty acid moieties.

CONCLUSION

SFC with on-line FT-IR detection shows promise in the ability to monitor changes in the chemical composition of vegetable oils due to processing. A major advantage of SFC over LC is that on-line detection using SFC permits the monitoring of the C—H deformation region (1000–900 cm^{-1}) in *trans*-$R_1HC{=}CHR_2$ groups, the C—H stretching region (3020–2800 cm^{-1}), and the carbonyl region (1800–1700 cm^{-1}). Therefore, an opportunity to determine the extent of unsaturation and isomerization in a single analysis exists. The on-line FT-IR spectra of the TGs and FFAs from the partially hydrogenated soybean oil showed that although similar levels of unsaturation were achieved with both catalysts during hydrogenation, the Ni–S catalyst produced higher levels of trans isomers. This higher trans isomer content was confirmed by the increased absorbance at 972 cm^{-1} and loss of absorbance at 3010 cm^{-1} in the spectra obtained from the Ni–S samples as opposed to the Ni samples. One area of the IR spectra that is lost due to CO_2 absorbance is the C—H out-of-plane deformation vibrations of the *cis*-$R_1HC{=}CHR_2$ groups. This absorbance occurs at 730 cm^{-1}. Use of SFC/FT-IR with solvent elimination would provide this information. A major advantage of SFC over GC is that FFAs and TGs can be analyzed by using the same chromatographic column. Although refined soybean oil

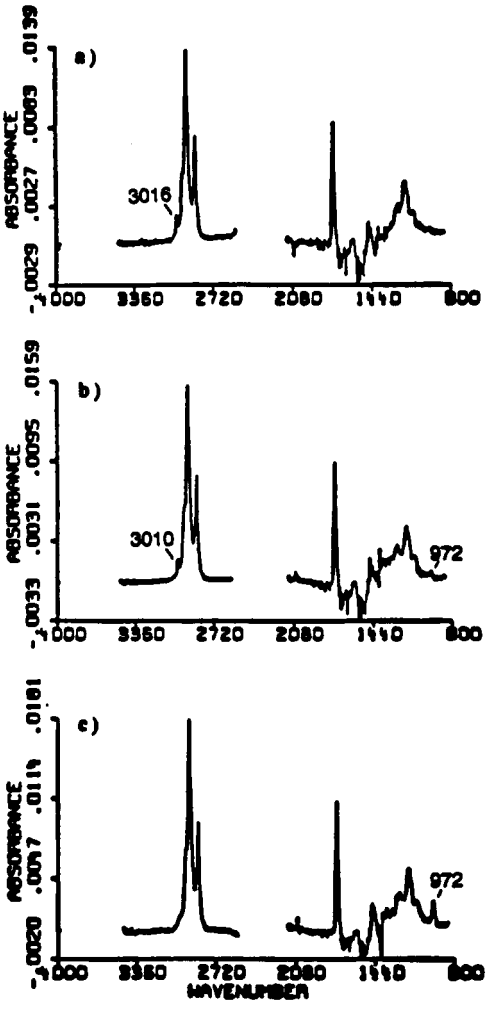

Figure 8. On-line FT-IR chromatographic file spectra (8 scans/file) of triacylglycerols found in (a) refined soybean oil (23 files coadded), (b) Ni hydrogenated soybean oil (27 files coadded), and (c) Ni–S hydrogenated soybean oil (21 files coadded).

consists primarily of TGs, a single analysis of complex mixtures containing both types of compounds would be possible. A major disadvantage of the study is that complete resolution of the TGs and of the geometric (cis and trans) isomers was not obtained. This problem may be addressed as stationary phase technology improves and additional phases become available that will provide improved resolution of lipids.

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