Alteration of Sterols and Steryl Esters in Vegetable Oils during Industrial Refining

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Steryl esters of fatty acids have been isolated from vegetable oils by rapid chromatography on a short column of silica gel and quantitatively determined by HPLC on RP-18 columns using an evaporative light scattering detector that was calibrated with β-sitosteryl oleate as external standard. The steryl esters isolated according to the above technique from corn, soybean, and rapeseed oils at different stages of industrial refining have been analyzed for their constituent sterols and fatty acids by GC. Moreover, the total sterols were analyzed by GC. In all three oils a substantial part of the sterols occurred as steryl esters. Some alterations in the composition of the constituent sterols and fatty acids of the steryl esters were observed during industrial processing.

Keywords: *Industrial fat refining; HPLC; sterols; steryl esters; vegetable oils*

INTRODUCTION

The acylglycerols of most seed oils amount to 95% or more of the oils by weight. The remaining nonacylglycerol fraction consists of a mixture of classes of compounds including hydrocarbons, tocopherols, free sterols, and steryl esters.

The classical approach for isolating and quantifying nonacylglycerol compounds involves conversion of acylglycerols to fatty acid salts (soaps) by saponification followed by extraction of the unsaponifiable fraction with an organic solvent. The unsaponifiable fraction may then be subjected to procedures appropriate for isolation and quantitation of the classes of compounds of interest (Worthington and Hitchcock, 1984). Especially sterols, both free and esterified, constitute a major portion of the nonacylglycerol fraction of vegetable oils. Reports on the distribution of sterols in vegetable oils usually give values that are obtained after saponification of oils (Kochar, 1983). Sterols have been used as an aid in the identification of vegetable oils and detection of adulteration; however, only limited data are available on the distribution patterns of sterols in free sterols and steryl esters. Such data may be useful in identifying questionable oils.

The content and composition of the steryl ester fraction in edible oils vary with the identity of the oil. The steryl ester fraction is a complex mixture consisting of a variety of sterols esterified to various fatty acids, and analysis of such mixtures is hindered by the presence of high proportions of triacylglycerols and other components of oils (Gordon and Griffith, 1992). Methods used so far for the analysis of steryl esters generally involve their isolation by preparative thin-layer chromatography or column chromatography followed by gas chromatography (GC) (Gordon and Griffith, 1992; Kuksis et al., 1986), high-performance liquid chromatography (HPLC) (Gordon and Griffith, 1992; Perkins et al., 1981), or coupled HPLC/GC (Grob et al., 1990). Recently, preparative HPLC has been used for the isolation of steryl esters, which is followed by their analysis using GC (Miller and Gordon, 1995). Since steryl esters containing di- and tri-unsaturated fatty acyl moieties have previously been reported to degrade during GC analysis (Kuksis et al., 1986), HPLC should be the method of choice for the analysis of steryl esters.

This paper reports the use of rapid nondestructive techniques for the separation of steryl esters from edible oils and their analysis by HPLC. Recently, a rapid column chromatographic technique has been applied for prefractionation of steradienes from vegetable oils for their further analysis by HPLC (Schulte, 1994). On the basis of the above principle we have carried out initial separation of steryl esters from oils by rapid chromatography on a short silica gel column, which was followed by quantitative HPLC on RP-18 columns using an evaporative light scattering mass detector. Moreover, the composition of the constituent sterols and fatty acids of the steryl esters as well as those of the whole oils was determined by GC. This technique of analysis of steryl esters has been applied to follow the alteration in the content and composition of steryl esters in vegetable oils during industrial refining.

MATERIALS AND METHODS

Materials. Three different types of oils were used: corn, soybean, and rapeseed. The oils were obtained from processing lines in commercial factories in Brazil. Samples were taken before and after each stage of chemical refining process. Samples of corn oil were from Rafinacoes de milho Brasil Ltda., Mogi-Guacu, Brazil, and those of soybean and rapeseed oils from Cocamar Ltda., Maringá, Brazil.

All analytical grade reagents, adsorbents, and solvents for HPLC were obtained from E. Merck, Darmstadt, Germany. Sterol standards were purchased from Sigma, Deisenhofen, Germany, and fatty acid methyl ester mixtures from NuChek

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Prep, Elysian, MN. β-Sitosteryl oleate, used for calibration of the HPLC detector, was prepared according to the method of Gupta et al. (1977).

Isolation of Steryl Esters by Column Chromatography. The total steryl esters were isolated from the oil by chromatography on a short silica gel column. Glass columns used were of 10 mm i.d. and 150 mm length with a 25 mL reservoir and funnel on top, in one piece, without stopcock. Silica gel, particle size 0.063−0.200 mm (70−230 mesh ASTM, Merck No. 7734) was adjusted to water content of 5% (Waltking and Wessels, 1981). The end of the glass column was plugged with a little piece of defatted cotton wool that was compressed with a glass rod. Silica gel, 5 g, was filled into the column and packed by tapping the column softly on a wooden block. Oil sample, 500 mg, was weighed into a small beaker and dissolved in 2 mL of petroleum ether, and the solution was poured onto the column. The beaker was rinsed twice with 2 mL of the same solvent. As soon as the solvent had drained to the top of the column packing, the nonpolar substances were eluted with 20 mL of petroleum ether. A second 15 mL fraction was eluted with a mixture of petroleum ether/dicloromethane (85:15, v/v), and a final fraction containing steryl esters was obtained by eluting with 20 mL of a mixture of petroleum ether/dicloromethane (85:15, v/v). Composition of the fractions was monitored by thin-layer chromatography (TLC) using silica gel H as adsorbent and hexane/diethyl ether/acetic acid (95:5:1, v/v/v) as developing solvent; the lipid fractions were stained by exposing the chromatograms to iodine vapor.

The second and third fractions were combined, the solvent was evaporated to dryness in a rotary evaporator, and the residue was transferred to a Pasteur pipet containing 0.6 g of silica gel; the steryl ester fraction was eluted with 10 mL of a mixture of petroleum ether/dicloromethane (85:15, v/v). This second cleanup was carried out to remove traces of triacylglycerols that can be eluted together with steryl esters from the silica gel column. The steryl ester fraction was evaporated to dryness with a stream of nitrogen, dissolved in 500 μL of dichloromethane, and analyzed by HPLC.

HPLC of Steryl Esters. HPLC analysis was conducted using four 125 × 4 mm Lichrospher 100 RP-18 (5 μm) columns and a Merck-Hitachi L-6200 pump using a 20 μL sample loop (Rheodyne, Berkely, CA, type 7125). An evaporative light scattering detector (ACS Model 750/14 Mass Detector, Applied Chromatography Systems, Macclesfield, U.K.) was set at attenuation range 4, time constant 5 s, photomultiplier sensitivity 4, evaporator 50 °C, and air pressure 1.38×10^4 Pa. A Merck-Hitachi D-2500 Chromato-Integrator was used. The eluent was acetonitrile/methanol/dichloromethane (1:1:1, v/v/v) at a flow rate of 1 mL/min; solvent mixtures were degassed by immersing the flask in an ultrasonic bath under vacuum of water jet pump. The absence of wax esters in the samples was checked by capillary GC as given below.

The areas of all individual peaks in the range of steryl esters were summed, and the total steryl ester content was calculated by using β-sitosteryl oleate as external standard. The detectors were calibrated using solutions of β-sitosteryl oleate in a concentration range of 1.13−11.30 mg/mL in dichloromethane.

Fatty Acid Composition of Steryl Ester Fraction and of Whole Oil. A small aliquot (25−100 μL) of the total steryl ester fraction or 5−10 mg of the whole oils was dried in a stream of nitrogen, dissolved in 500 μL of petroleum ether, and transmethylated with 20 μL of sodium methoxide solution (30%, w/v) for 2 h at room temperature (Schulte and Weber, 1989). The methyl esters of fatty acids were analyzed by GC (Varian Model 3700, Varian Analytical Instruments, Darmstadt, Germany). The separations were carried out on a DB-23 column, 25 m × 0.25 mm, film thickness 0.25 μm (J&W Asschem, Bad Homburg, Germany), that was programmed from 150 to 230 °C at 5 °C/min using H_2 at 3.5×10^4 Pa inlet pressure as carrier gas. Other conditions of GC were as follows: injector, 280 °C; flame ionization detector, 250 °C; and split ratio, 1:15.

Detection of Wax Esters in the Steryl Ester Fraction. For the detection of wax esters in the steryl ester fraction an aliquot of this fraction was analyzed in the above gas chromatograph using a Restek MXT-1 capillary column, 15 m × 0.28 mm, 0.1 μm film thicknes (Restek GmbH, Sulzbach, Germany). Column temperature was programmed from 250 to 350 °C at 5 °C/min using H_2 at 4.5×10^4 Pa inlet pressure as carrier gas. Other conditions of GC were as follows: injector, 340 °C; flame ionization detector, 360 °C; and split ratio, 1:5.

Sterol Composition of Steryl Ester Fractions and Total Sterols. The product obtained after transmethylation of the steryl esters or whole oils also contained the sterols in addition to the methyl esters. This mixture was heated for 10 min with 100 μL of the silylating agent, consisting of 1 mL of *N*-methyl-*N*-(trimethylsilyl)heptafluorobutyramide (MSHFBA, Macherey Nagel, Düren, Germany) and 50 μL of methylimidazole, and the resulting trimethylsilyl derivatives of the sterols were analyzed by capillary GC according to the method of Arens et al. (1990).

RESULTS AND DISCUSSION

Fractionation of the total lipids of the oils by rapid chromatography on a short silica gel column yielded, upon elution with petroleum ether, a fraction of nonpolar lipids that can be used for the analysis of steradienes by HPLC or GC (Schulte, 1994). The second and third fractions eluted with a mixture of petroleum ether/dichloromethane (85:15, v/v) yielded the entire steryl ester fraction with a trace of triacylglycerols. A rapid cleanup of this fraction in a Pasteur pipet, packed with silica gel, yielded the pure steryl ester fraction, which was analyzed by HPLC using an evaporative light scattering mass detector.

Recovery of the β-sitosteryl oleate standard after silica gel chromatography was between 97 and 102%. Calibration of the HPLC system, equipped with an evaporative light scattering mass detector, using β-sitosteryl oleate as external standard (retention time = 42.0 min), showed good correlation between β-sitosteryl oleate concentration (1.13−11.30 mg/mL) and the area of the resulting peak; the correlation coefficient was 0.9955. Relative standard deviation ranged from 2.83% to 2.86% ($n = 4$). Instead of β-sitosteryl oleate a commercially available cholesteryl stearate can also be used as an external standard to calibrate the HPLC detectors.

Figure 1 shows the typical HPLC chromatograms of steryl ester fractions of crude corn, soybean, and rapeseed oils monitored with an evaporative light scattering mass detector. It is evident from these chromatograms that each oil has a characteristic pattern of distribution of steryl esters that might be useful for the identification of such oils. Further research is done to explain the identity of the different HPLC peaks.

Analysis of the three oils before and after industrial refining using the method reported here showed that crude corn oil before refining had the highest level of steryl esters (1090 mg/100 g), followed by crude rapeseed oil (759 mg/100 g) and crude soybean oil (121 mg/100 g) (Ferrari et al., 1996). It was also noteworthy that in both crude corn and rapeseed oils a major part of the sterols occurs as steryl esters; even in soybean oil a substantial part of the sterols occurred as steryl esters (Ferrari et al., 1996). A minor decrease (5−10%) in the level of steryl esters was observed after complete refining.

Table 1 shows the relative proportion of individual sterols in the steryl esters during the course of refining of the three oils. In both corn and soybean oils, the distribution of sterols in steryl esters was somewhat

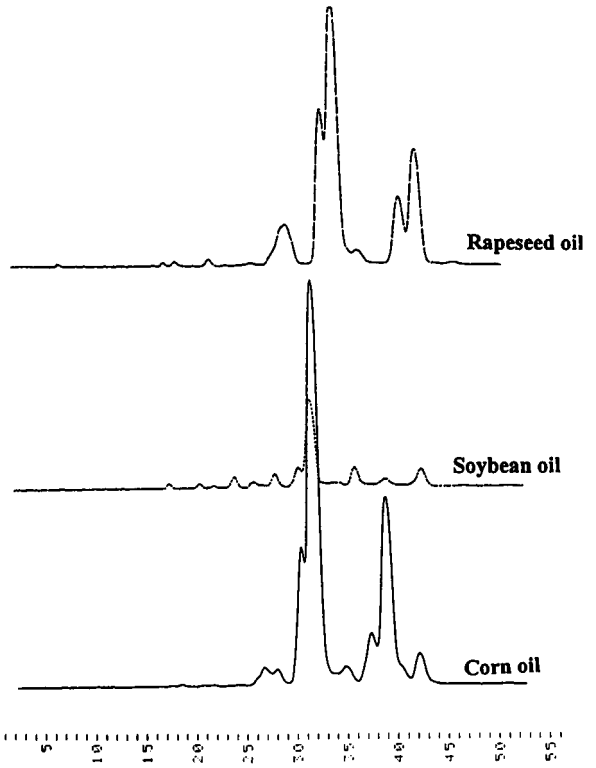

Figure 1. Chromatograms showing the patterns of steryl esters of corn, soybean, and rapeseed oil isolated by rapid liquid column chromatography and fractionated by HPLC on a RP-18 column. The column effluent was monitored using an evaporative light scattering detector. The scale represents elution time in minutes.

different from that in total sterol fractions (Table 1). The total sterol fraction of corn oil contained less stigmasterol and Δ^7-stigmasterol than did the steryl ester fraction (Table 1). In soybean oil the total sterol fraction contained less Δ^7-stigmasterol and Δ^7-avenasterol and more campesterol and stigmasterol than did the steryl ester fraction (Table 1). There were no distinct differences in the distribution of sterols in total sterols and steryl esters of rapeseed oil (Table 1). The data presented in Table 1 show that the relative proportion of individual sterols in steryl esters is not substantially altered in different refining stages.

The distribution of fatty acids esterified with sterols differed considerably from those esterified in total lipids of corn, soybean, and rapeseed oils (Tables 2−4). In each case steryl esters contained lower levels of oleic acid than the corresponding whole oils (Tables 2−4). Steryl esters of corn and rapeseed oils contained higher levels and those of soybean oil lower levels of linoleic acid than the corresponding whole oils (Tables 2−4). Corn oil contained a higher level of lignoceric acid in steryl esters than in acylglycerols (Table 2). Steryl esters of soybean and rapeseed oils also contained higher levels of linolenic acid than the corresponding whole oils (Tables 3 and 4). In this context it is interesting to note that Johansson and Appelqvist (1978) also found higher levels of linoleic and lower levels of oleic acid in the steryl ester fraction of rapeseed oil than in fatty acids of whole oil. The steryl esters of soybean oil contained higher levels of arachidic, behenic, and lignoceric acids than the whole oil (Table 4). The data presented in Tables 2−4 show that the fatty acid composition of the steryl esters is not substantially altered during industrial refining of the three oils.

Table 1. Sterols in Steryl Esters and Total Sterols of Vegetable Oils

	sterols in steryl esters (%)							sterols in total sterols (%)						
sample	cholesterol	brassicasterol	campesterol	stigmasterol	β-sitosterol	Δ5-avenasterol	Δ7-stigmasterol	Δ7-avenasterol	cholesterol	brassicasterol	campesterol	stigmasterol	β-sitosterol	Δ5-avenasterol	Δ7-stigmasterol	Δ7-avenasterol
corn oil																
crude	0.6		17.6	8.4	71.4	tr[a]	1.4	0.6	0.3		18.5	6.7	64.1	9.3	0.5	0.6
neutralized	0.3		16.0	12.0	62.7	4.8	4.1	0.1	0.2		19.0	6.4	65.8	6.5	0.8	1.3
bleached	0.6		15.7	13.0	58.8	6.0	5.2	0.6	0.1		19.8	6.6	66.2	5.4	0.9	1.1
winterized	0.5		15.4	11.9	61.3	4.3	5.1	1.5	0.2		18.7	6.5	68.7	3.9	0.8	1.2
deodorized	0.5		16.5	14.7	55.8	4.4	6.9	1.1	0.3		18.0	6.5	65.8	6.7	1.1	1.5
soybean oil																
crude	tr		9.3	14.9	58.0	3.1	9.5	5.1	0.3		20.5	18.1	53.7	2.2	3.3	1.8
degummed	tr		12.6	14.4	60.0	2.0	7.1	3.9	0.2		20.3	17.4	54.1	2.5	3.5	2.0
neutralized	tr		10.3	14.3	53.9	5.2	11.5	4.8	0.4		20.1	17.4	53.9	2.6	3.5	1.9
bleached	tr		11.3	15.2	52.9	5.2	10.7	4.6	0.3		19.8	17.4	53.8	2.7	3.9	2.0
deodorized	tr		15.3	11.3	62.9	3.5	4.0	3.0	0.4		19.1	16.7	55.1	2.7	4.0	2.1
rapeseed oil																
crude	tr	7.7	34.0	0.4	57.0		0.6	0.4	0.4	10.8	30.0	0.5	54.5	3.0	0.5	0.3
degummed	tr	9.0	32.6	1.9	53.9		1.7	0.9	0.3	10.6	31.7	0.4	54.5	2.1	0.4	tr
neutralized	tr	9.2	33.0	tr	57.8		tr	tr	0.4	10.3	31.5	0.3	56.9	0.5	0.2	tr
bleached	tr	11.3	29.5	3.0	53.5		2.7	tr	0.3	9.4	32.4	0.4	56.1	1.0	0.4	tr
deodorized	tr	7.9	34.4	1.2	55.4		1.1	tr	0.3	9.3	33.4	0.4	54.8	1.5	0.2	tr

[a] Traces.

Table 2. Composition (Percent) of Fatty Acids in Whole Oil and Steryl Ester Fraction of Corn Oil at Different Steps of Refining

	% fatty acid in sample									
	crude		neutralized		bleached		winterized		deodorized	
fatty acid[a]	oil	steryl ester	oil	steryl ester	oil	steryl ester	oil	steryl ester	oil	steryl ester
16:0	13.1	11.2	12.4	9.7	12.8	10.7	12.0	13.3	12.5	11.8
16:1	0.2	0.8	0.2	tr[b]	0.2	1.0	0.2	tr	0.2	tr
18:0	2.1	2.1	2.2	1.2	2.3	1.5	2.3	1.8	2.3	1.3
18:1	34.1	28.7	34.6	28.1	35.5	28.2	35.0	30.5	34.8	29.7
18:2	48.0	53.4	48.0	57.5	46.6	56.3	47.9	52.0	47.7	56.1
18:3	0.9	0.7	0.9	1.0	0.8	tr	0.9	tr	0.9	tr
20:0	0.6	0.8	0.6	tr	0.6	tr	0.6	1.2	0.6	tr
22:0	0.2	0.9	0.2	0.8	0.2	0.8	0.2	tr	0.2	tr
24:0	0.2	1.3	0.2	1.5	0.2	1.5	0.2	1.2	0.2	1.1

[a] Fatty acids are designated by number of carbon atoms:number of *cis*-double bonds. [b] Traces.

Table 3. Composition (Percent) of Fatty Acids in Whole Oil and Steryl Ester Fraction of Soybean Oil at Different Steps of Refining

	% fatty acid in sample									
	crude		degummed		neutralized		bleached		deodorized	
fatty acid[a]	oil	steryl ester	oil	steryl ester	oil	steryl ester	oil	steryl ester	oil	steryl ester
14:0	1.0	1.0	0.1	0.5	0.1	0.7	0.1	0.5	0.1	0.7
16:0	11.5	11.5	10.8	10.5	10.7	12.6	10.8	11.8	11.2	15.2
16:1	0.1	0.6	0.1	0.4	0.1	0.8	0.1	0.6	0.1	0.9
17:0	0.1	1.6	0.1	0.3	0.1	0.4	0.1	1.0	0.1	0.4
18:0	3.3	3.6	3.5	3.3	3.6	3.7	3.5	3.6	3.5	4.5
18:1	24.2	8.4	24.8	10.0	25.1	10.0	24.8	10.0	24.9	13.7
18:2	52.6	26.4	52.6	27.7	52.6	27.5	52.8	26.9	52.2	32.5
18:3	6.4	19.4	6.4	20.3	6.5	19.0	6.3	19.1	5.9	11.7
20:0	0.4	2.8	0.4	2.4	0.4	2.6	0.4	2.6	0.7	2.3
20:1	0.4	0.9	0.5	1.2	0.5	0.8	0.4	1.3	0.4	0.9
22:0	0.5	12.6	0.5	12.3	0.5	11.9	0.5	11.9	0.5	9.2
24:0	0.2	11.2	0.2	10.9	0.2	9.9	0.2	10.8	0.2	8.0

[a] Fatty acids are designated by number of carbon atoms:number of *cis*-double bonds.

Table 4. Composition (Percent) of Fatty Acids in Whole Oil and Steryl Ester Fraction of Rapeseed Oil at Different Steps of Refining

	% fatty acid in sample									
	crude		degummed		neutralized		bleached		deodorized	
fatty acid[a]	oil	steryl ester	oil	steryl ester	oil	steryl ester	oil	steryl ester	oil	steryl ester
14:0	0.1	0.6	0.1	tr[b]	0.1	tr	0.1	0.3	0.7	0.4
16:0	4.5	3.4	4.4	3.5	4.4	3.6	4.5	3.1	4.5	2.9
16:1	0.3	0.6	0.2	tr	0.3	0.8	0.2	0.6	0.2	tr
18:0	2.4	0.4	2.4	1.0	2.4	0.9	2.4	0.6	2.4	0.7
18:1	64.7	31.3	64.5	3.65	64.6	32.0	64.0	33.7	64.7	34.2
18:2	17.9	51.7	17.9	50.1	17.8	50.0	18.4	55.1	17.7	55.5
18:3	7.9	11.5	7.5	8.8	7.6	11.7	7.5	6.2	7.2	6.0
20:0	tr	0.5	0.7	tr	0.7	1.0	0.7	0.3	0.8	0.3
20:1	1.1	tr	1.2	tr	1.2	tr	1.2	tr	1.3	tr
22:0	0.4	tr	0.4	tr	0.3	tr	0.3	tr	0.4	tr
22:1	0.1	tr	0.1	tr	0.1	tr	0.1	tr	0.1	tr
24:0	0.2	tr	0.2	tr	0.2	tr	0.2	tr	0.2	tr
24:1	0.1	tr	0.1	tr	0.1	tr	0.1	tr	0.1	tr

[a] Fatty acids are designated by number of carbon atoms:number of *cis*-double bonds. [b] Traces.

The method described here for the analysis of steryl esters via precleaning on silica gel followed by HPLC should be applicable for oils that contain only traces of wax esters, because the latter may elute together with steryl esters. For samples containing substantial proportions of wax esters, alternative methods should be sought for the separation of steryl esters from wax esters, such as thin-layer chromatography on magnesium oxide (Kaufmann et al., 1971) or silica gel containing urea (Tiffany, 1982).

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