A Method for the Separation of Seed Oil Steryl Esters and Free Sterols: Application to Peanut and Corn Oils

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ABSTRACT

A method for separating and quantitating seed oil steryl esters and free sterols was developed using a combination of preparative column, thin layer (TLC), and gas liquid chromatography (GLC). Cholesteryl heneicosanoate and cholesterol served as internal standards. The method was applied to corn-oil samples (Mazola, Kroger) obtained from the local market and peanut-oil samples prepared in the laboratory from commercial varieties of peanuts (Florunner, Starr). Concentration (mg/100 g oil; mean ± SD) of steryl esters and free sterols in the 4 oils were: Mazola, 1420 ± 40 and 370 ± 8; Kroger, 950 ± 40 and 320 ± 4; Florunner, 74 ± 0.5 and 150 ± 3; and Starr, 51 ± 0.5 and 130 ± 2. Sitosterol was the major sterol in both the free sterol and steryl ester fractions of all oils and together with campesterol, stigmasterol and Δ^5-avenasterol made up 90-95% of all sterols. Steryl esters of peanut oil contained higher proportions of linoleic acid and long-chain acids (C_{20}-C_{24}) than did whole oil. Corn-oil steryl esters also contained a higher proportion of linoleic acid than did whole oil. Squalene was the major hydrocarbon of all oils with the remaining hydrocarbon fraction consisting of a mixture of compounds.

INTRODUCTION

The acylglycerols of most seed oils make up 95% or more of the oils by weight. The remaining nonacylglycerol fraction consists of a mixture of classes of compounds including hydrocarbons, tocopherols, steryl esters, free sterols, glycolipids and others. Individual compounds within a class are usually present in trace amounts and therefore may be difficult to isolate and quantitate.

The classical approach to isolating and quantitating nonacylglycerol compounds involves converting acylglycerols to fatty acid salts (soaps) by saponification followed by extracting the organic nonsaponifiable fraction with an organic solvent (1). The nonsaponifiable fraction may then be subjected to procedures appropriate for isolating and quantitating the classes of compounds of interest.

Saponification effects changes not only in acylglycerols but in other alkali and heat-labile compounds as well. In order to avoid the harsh conditions of saponification, other procedures have been devised for the separation of nonacylglycerols from the acylglycerol matrix. Johansson and Appelqvist (2) and Johansson (3) recently applied low temperature crystallization to rapeseed, sunflower and poppy seed oils and obtained enrichment of several classes of compounds, including sterols and steryl esters, in the noncrystallizable fraction.

Gravity flow adsorption column chromatography has been used extensively in preparative fractionation of oils but this technique requires long development times and is subject to significant variation in column-to-column performance. An inverse relationship between column diameter and efficiency of separation is an additional disadvantage of this technique because it limits column size, thereby limiting the amount of sample that can be effectively separated per column run.

Reports on the distribution of sterols in vegetable oils usually give values that are obtained after oil saponification (4). Sterol values have been used as an aid in the identification of vegetable oils and to detect adulteration (5,6), however only limited data are available on the distribution patterns of sterols in the free sterol and steryl ester forms (2,3,7,8). Such data may aid in identifying questionable oils.

This paper reports the use of nondestructive techniques to separate steryl esters and free sterols from seed oils and the application of these techniques to peanut and corn oils. Appropriate steryl ester and free sterol internal standards were incorporated into the oil samples, after which initial separations were made with a preparative liquid chromatograph. Total elution time for steryl esters and free sterols was ca. 15 min. Final separations and quantitations were made by thin layer (TLC) and gas liquid chromatography (GLC).

MATERIALS AND METHODS

Oil Samples

Samples of commercial corn oil (Kroger, Mazola) were obtained from the local market. Peanut-oil samples were prepared in the laboratory from sound mature seed of 2 commercial varieties of peanuts (Florunner, Starr). The peanut oil was expressed with a laboratory Carver press (Sterling Inc., Milwaukee, WI), filtered and analyzed without further purification.

Reagents

The internal standards cholesterol and cholesteryl heneicosanoate (NuChek Prep, Elysian, MN), were greater than 99% pure. Solvents for high performance liquid chromatography (HPLC) were obtained from commercial suppliers and checked for impurities before use. Samples of sitosterol, stigmasterol and campesterol were purchased from Supelco, Inc., Bellefonte, PA, and a fatty acid methyl ester mixture (21A) representative of peanut oil was purchased from NuChek Prep, Elysian, MN. Sodium methoxide (1 M) was prepared in the laboratory from anhydrous methanol and metallic sodium.

Preparative Liquid Chromatography

The methods employed in the separation of hydrocarbons, steryl esters and free sterols are outlined in Figure 1. Initial separations were made with a Waters Prep LC/System 500 liquid chromatograph equipped with a refractive index detector and 30 × 5.7 cm polyethylene columns packed with 75 μm silica gel (9).

Each column was prewashed with 1 L ethyl acetate followed by 1 L 2% ethyl acetate in hexane. A sample of the oil to be analyzed (ca. 10 g) was accurately weighed and diluted with 5 mL hexane containing 3 mg cholesteryl heneicosanoate and 5 mg cholesterol as internal standards. After thorough mixing, 10 mL of the oil and internal standard mixture was injected onto the column. The hydrocarbons and sterol esters were eluted with 1100 mL 2% ethyl acetate in hexane, triacylglycerols were eluted with 1000 mL 10% ethyl acetate in hexane and a final fraction, containing free sterols, was obtained with 1000 mL of ethyl acetate (Fig. 2). All solvent flow rates were 200 mL/min and total elution time for the 3 fractions was ca. 15 min.

Thin Layer Chromatography

Developing solvents were removed from sample fractions with a continuous-feed Buchler flash evaporator. The steryl ester-hydrocarbon fractions were dissolved in hexane and applied to 500 μm silica gel thin layer plates that had been

[1] Presented at the AOCS meeting, Toronto, May 1982.

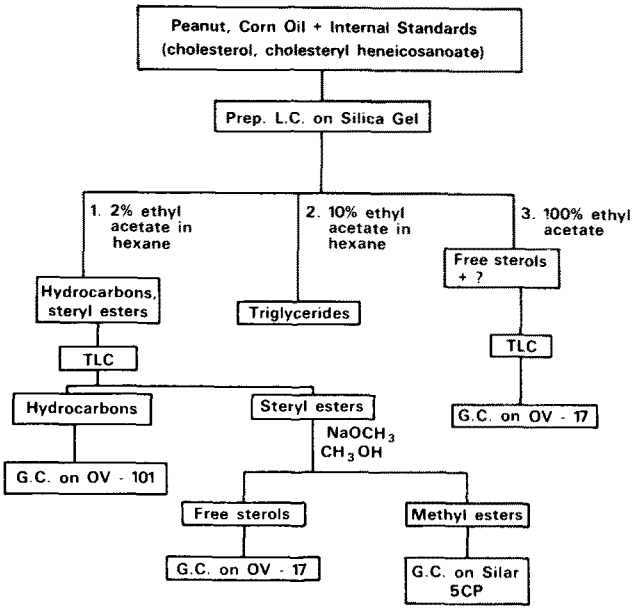

FIG. 1. Flow diagram for separation of hydrocarbons, steryl esters and free sterols.

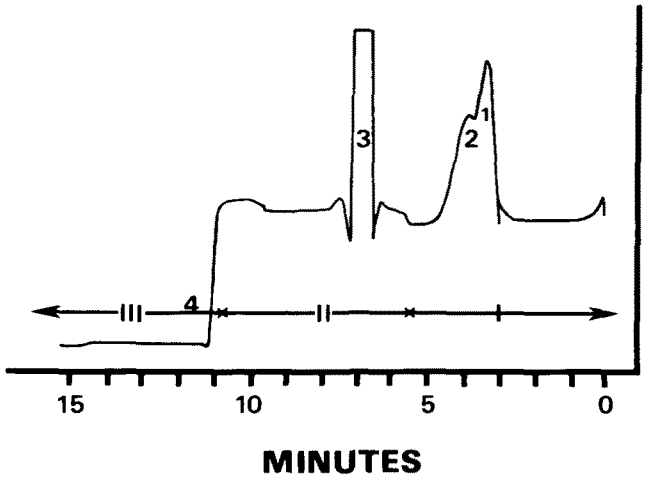

FIG. 2. Preparative column chromatography of oils. I. Fraction eluted with hexane/ethyl acetate (98:2, v/v), II. fraction eluted with hexane/ethyl acetate (90:10, v/v), III. fraction eluted with 100% ethyl acetate; 1. Hydrocarbons, 2. steryl esters, 3. triacylglycerols, 4. fraction containing free sterols.

prewashed in dichloromethane/methanol (1:1, v/v) solvent system. The plates were developed twice in hexane/ethyl acetate (99:1, v/v), sprayed with dye (10) and the steryl ester (R_f ca. .15) and hydrocarbon (R_f ca. .4-.7) bands located by UV light. The bands were scraped from the plate and eluted with dichloromethane.

The free sterol fractions that were eluted from the preparative column with ethyl acetate were applied to preparative silica gel TLC plates and developed in hexane/ethyl acetate (3:1, v/v). A one-inch strip cut from one side of the plate was sprayed with 50% H_2SO_4 in methanol and heated at 100 C for 5 min. The sterols appeared as a pink band contaminated with other materials. The free sterols were scraped from the remaining portion of the plate, eluted with dichlomethane and isolated by TLC using chloroform/benzene (2:1, v/v) as the developing solvent. The sterols were again located as described above and appeared to be completely separated from other constituents.

Gas Liquid Chromatography

The steryl ester fractions were converted to methyl esters and free sterols by treating them with sodium methoxide ($NaOCH_3$) as described by Tuckey and Stevenson (11). Sterols obtained from the steryl ester and free sterol fractions were quantitated by GLC on 1.8 x 4 mm i.d. columns packed with 3% OV 17 and operated isothermally at 270 C with a carrier gas flow rate of 40 cc/min. Identifications were based on retention times of standards and published relative retention times (4). Fatty acid methyl esters were quantitated on 10% Silar 5 CP columns operated isothermally at 230 C with a carrier gas flow rate of 100 cc/min. Hydrocarbons were examined by GLC on a 1.8 x 4 mm i.d. OV 101 3%) column that was programmed for temperatures from 100 C to 300 C at 5 C/min with a helium carrier gas flow rate of 100 cc/min.

RESULTS AND DISCUSSION

Preparative Liquid Chromatography

Hydrocarbons were weakly adsorbed by the preparative columns and were eluted with ca. 1.4 column volumes (700 mL) of hexane/ethyl acetate (98:2, v/v) (Fig. 2). Steryl esters were eluted shortly thereafter and appeared as a shoulder on the hydrocarbon peak. After eluting triacylglycerols with 1000 mL of hexane/ethyl acetate (90:10, v/v), the free sterols and other moderately polar compounds were eluted with 1000 mL of 100% ethyl acetate. At a flow rate of 200 mL/min, all fractions were eluted in ca. 15 min (Fig. 2). The column was then reequilibrated with hexane/ethyl acetate (98:2, v/v) before the next sample was injected.

TABLE I

Steryl Ester and Free Sterol Content of Peanut and Corn Oil

	mg/100 g oil		
	Steryl esters[a]	Free sterols	Total sterols[b]
Peanut oil			
Florunner	74 ± 0.5[c]	150 ± 3	190
Starr	51 ± 0.5	130 ± 2	160
Corn oil			
Mazola	1420 ± 40	370 ± 8	1160
Kroger	950 ± 40	320 ± 4	840

[a]Expressed as cholesteryl heneicosanoate.
[b]Includes steryl ester values converted to cholesterol equivalent.
[c]One standard deviation.

Hydrocarbons

GLC of the hydrocarbon fraction indicated that the major component of this fraction from peanut and corn oil was squalene, a common constituent of vegetable oils (12). The remainder of the hydrocarbon fraction consisted of a complex mixture of compounds that probably included contaminants derived from the solvents or other sources.

Steryl Esters and Free Sterols

The total steryl ester values were calculated as cholesteryl heneicosanoate and are shown in Table I. The peanut oils prepared in the laboratory contained less than 100 mg steryl esters/100 g oil, which is considerably less than the

amount found in a commercial peanut oil (13). The corn oils contained 1420 mg and 950 mg steryl esters/100 g oil. The total sterol content of the peanut oils analyzed in this study are somewhat lower than the content found in an earlier study of a commercially refined peanut oil (13) and are lower than values reported in the literature for other refined peanut oils (4). Total sterol values for the 2 corn oils are within the range of values reported in the literature for corn oil (4).

The steryl ester fraction from peanut and corn oil consisted primarily of esters of 4 sterols (Table II) that have previously been identified in the nonsaponifiable fraction of both peanut and corn oil (4). Sitosterol was the major sterol in all oils and made up 50% or more of the sterols in both the steryl ester and free sterol fractions (Table II).

The 2 peanut oils contained 150 mg and 130 mg free sterols/100 g oil and the corn oils 370 mg and 320 mg (Table I). In both peanut and corn oil, the distribution of sterols differed considerably between the steryl ester and free sterol fractions (Table II). The free sterol fraction of peanut oil contained more stigmasterol and less campesterol than did the steryl ester fraction. In corn oils the free sterol fraction contained less Δ^5 avenasterol and more campesterol than did the steryl ester fraction.

Steryl Ester Fatty Acids

The distribution of fatty acids esterified to sterols differed considerably from those esterified to glycerol in peanut and corn oil (Table III). All sterol esters contained higher levels of linoleic and lower levels of oleic and stearic acid than the corresponding whole oils. The peanut-oil steryl esters also contained higher levels of myristic, linolenic and behenic acid than whole oil; myristic and linolenic were not detected in measurable quantities (<0.1%) in whole oils. Johansson and Appelqvist (2) also found higher levels of linoleic and lower levels of oleic in the sterol ester fraction of rapeseed oils than in fatty acids of whole oils. Sunflower and poppy seed oils, however, contained higher proportions of linoleic acid in the whole oil than in the steryl ester fraction (3). Thus the limited data on the fatty acid composition of seed-oil steryl esters show considerable differences in fatty acid composition compared with the corresponding whole oils but these data do not show a definite trend toward either higher or lower levels of unsaturation.

ACKNOWLEDGMENTS

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TABLE II

Steryl Ester Sterols and Free Sterols of Peanut and Corn Oil

	Steryl ester sterols (%)[a]				
	Campesterol	Stigmasterol	Sitosterol	Δ^5 avenasterol	Other
Peanut oil					
Florunner	18.7[b]		60.0	16.8	4.7
Starr	21.8[b]		50.0	21.6	3.4
Corn oil					
Mazola	16.7	6.8	66.9	8.0	1.5
Kroger	15.4	6.7	65.9	9.1	3.5
	Free sterols (%)[c]				
Peanut oil					
Florunner	11.8	11.0	63.9	12.1	1.2
Starr	13.5	12.2	51.5	21.6	1.1
Corn oil					
Mazola	22.1	5.7	69.8	2.4	–
Kroger	20.6	6.5	69.2	2.3	1.4

[a]Percentage of total steryl ester sterols.
[b]Incomplete resolution, stigmasterol estimated to be ca. 1.5% of total steryl ester sterols.
[c]Percentage of total free sterols.

TABLE III

Whole Oil and Steryl Ester Fatty Acids of Peanut Oil and Corn Oil (Values Expressed as Percentage of Total Fatty Acids)

	Peanut				Corn			
	Florunner		Starr		Mazola		Kroger	
Fatty acid	A[a]	B[b]	A	B	A	B	A	B
14:0	–	2.2	–	2.9	–	–	–	0.4
16:0	9.8	9.1	11.9	10.5	11.5	5.8	11.0	7.2
18:0	1.6	0.6	2.7	0.9	1.7	0.4	2.0	0.6
18:1	44.6	14.7	40.4	12.3	24.7	14.6	25.7	16.9
18:2	36.2	66.4	37.3	61.7	61.1	78.1	58.8	70.1
18:3	–	0.8	–	0.9	0.6	0.6	1.5	1.2
20:0	1.1	0.8	1.4	1.6	0.2	–	0.4	0.4
20:1	1.6	0.3	1.0	0.6	0.2	–	0.3	0.2
22:0	2.9	3.3	3.3	5.3	–	–	–	0.9
24:0	1.7	1.1	1.4	1.7	–	–	–	1.7
Other	0.5	0.8	0.6	1.7	–	0.5	0.3	0.4

[a]A-Whole oil fatty acids.
[b]B-Steryl ester fatty acids.

REFERENCES

1. Official and Tentative Methods of the American Oil Chemists' Society, AOCS, 3rd edn., AOCS, Champaign, IL, 1970, Method Ca 6a-40.
2. Johansson, A., and L.-A. Appelqvist, Lipids 13:658 (1978).
3. Johansson, A., Ibid. 14:285 (1979).
4. Itoh, T., T. Tamura and T. Matsumoto, JAOCS 50:122 (1973).
5. FAO/WHO Codex Committee on Fats and Oils, Report of 9th Session, 1977, ALINORM 78/17.
6. Vigneron, P.Y., JAOCS 60:738 (1983).
7. Milkova, Ts., N. Marekov, S. Popov, N. Wulfson and I. Bogdanova, Nahrung 21:1 (1977) (Chem. Abstr. 87:4258j [1977]).
8. Milkova, Ts., A. Popov, A. Selva and U. Vettori, Nahrung 21:7 (1977).
9. Little, J.K., R.L. Cotter, J.A. Pendergast and P.D. McDonald, J. Chromatog. 126:439 (1976).
10. Jones, D., D.E. Bowyer and G.A. Gresham, Ibid. 23:172 (1966).
11. Tuckey, R.C., and P.M. Stevenson, Anal. Biochem. 94:402 (1979).
12. Sonntag, N.O.V., in Bailey's Industrial Oil and Fats Products, 4th edn., edited by D. Dwern, John Wiley & sons, New York, 1979, pp. 1-98.
13. Worthington, R.E., and H.L. Hitchcock, Proc. Am. Peanut Res. Ed. Soc. 13:103 (1981).

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Oxidation and Halogenation of Jojoba Wax

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ABSTRACT

The oxidation with hydrogen peroxide and permanganate and the allylic bromination, chlorination and chloro-etherification of the olefinic bonds of the liquid wax extracted from beans of jojoba *(Simmondsia chinensis)* were studied. Hydrolytic splitting of the wax into its carboxylic and alcoholic components competed with most reactions carried out in aqueous systems. The use of a suitable phase-transfer catalyst enabled the oxidation of the double bonds to carboxyls using permanganate in aqueous systems. Reaction of the wax with hydrogen peroxide in formic acid yielded formates, which were then hydrolyzed to vicinal glycols. The diglycols obtained by hydrogen peroxide oxidation were benzoylated. Allylic chlorination of jojoba wax with t-butyl hypochlorite in organic solvents was carried out. Conditions were found for the allylic bromination of the wax, yielding mono-, di-, tetra-, or octabromo derivatives.

INTRODUCTION

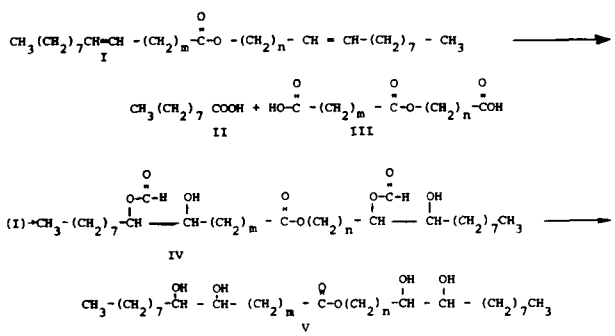
The major constituents of jojoba wax, are straight-chain esters of C_{20} and C_{22} monounsaturated alcohols and carboxylic acids (1). In the course of our investigations of the chemical properties of this wax, we set out to develop syntheses for new basic building blocks that could easily be produced from jojoba wax and to determine the reactivity of the wax to various chemical agents. Knowledge of the latter is, of course, a prerequisite for developing uses for the wax and its derivatives either alone or as components of mixtures.

Oxidation of Jojoba Wax with Potassium Permanganate

Jojoba wax (I) was reacted with a cold solution of potassium permanganate in a 2-phase solvent system consisting of water and dichloromethane.

Almost no reaction occurred, even after prolonged stirring. However, the addition of benzyl triethylammonium chloride, as a phase-transfer catalyst, produced a quantitative yield of the cleavage products, pelargonic acid (II) and an α,ω-dicarboxylic acid (III).

The reaction temperature had to be kept below 39 C to avoid further oxidation to shorter carboxylic acids. We also found that the addition of 1-2 equivalents of acetic acid improved the yield by preventing saponification of the ester group. Attempts to oxidize jojoba wax at a basic pH to produce a tetrahydroxy derivative failed. The only reaction taking place was saponification of the ester group.



Oxidation with Hydrogen Peroxide

Jojoba wax was treated with hydrogen peroxide in a 2-phase system consisting of benzene and formic acid. Nuclear magnetic resonance (NMR) spectral evidence indicated the formation, in the first stage of the reaction, of a formyl ester (IV), which was then hydrolyzed to the glycol (V). The fresh product had a melting temperature range of 30-40 C. After standing for more than a month, the product turned brittle, probably because of polymerization caused by some peroxides formed during the reaction.

The diglycol of jojoba wax was benzoylated with an excess of benzoyl chloride in pyridine. This benzoylated diglycol polymerizes if exposed to visible light but can be kept for months at room temperature in the dark.

Reaction of Jojoba Wax with *t*-Butyl Hypochlorite

t-Butyl hypochlorite reacts with olefins in alcoholic solutions to give saturated branched β-chloroethers (2). When *t*-butyl hypochlorite reacts with olefins in inert solvents in the presence of radical-forming catalysts, e.g., peroxides, good yields of allyl chlorides are obtained as the sole detectable products. Only in cases where no allylic chlorination is possible, a 1:1 addition takes place, even in the presence of free radicals (3).

Jojoba wax was reacted with 2 equivalents of *t*-butyl hypochlorite in benzene in the presence of benzoyl peroxide, yielding the dichloro derivative (VI). The base peak, m/e = 97, is caused by an allylic fragmentation. According