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I am submitting herewith a thesis written by Thongchai Plerksophon entitled "Plant Sterols in Vegetable Fats and Oils Processed in the United States." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology and Science.

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S. L. Melton, Major Professor

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PLANT STEROLS IN VEGETABLE FATS AND OILS PROCESSED IN THE UNITED STATES

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Thongchai Plerksophon

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ABSTRACT

Campesterol, stigmasterol, and β-sitosterol were quantitated by gas chromatography in corn, coconut, cottonseed, soybean, and palm oils at various steps of processing: in corn oil margarine and in shortening. The latter two also were processed in the United States. Without prior clean-up, the unsaponifiable components were used to form sterol acetates and were chromatographed on a 3% OV-101 (80-100 mesh chromosorb W-HP), 1.83 m x 4.23 mm i.d. glass column at 272.5°C utilizing cholestane as an internal standard. The accuracy and the precision of the method were determined. All the stigmasterol added to hydrogenated palm oil was recovered at lower concentration level (0.5 mg added), and 81.9% of the campesterol, and 92.5% of β-sitosterol added were recovered. Precision of the method determined as the coefficient of variation of each sterol content was $\pm$ 10%. Sterol content decreased progressively as oils were refined, deodorized, winterized, and hydrogenated. Campesterol, stigmasterol, and β-sitosterol content, mg/100g sample, respectively, in fats and oils as consumed were: 28-32, 29-33, and 104-127 for soybean oil; 12-23, 1-5, and 153-347 for cottonseed oil; 2-12, 2-16, and 8-34 for palm oil; 3-4, 6-7, and 39-58 for coconut oil; 58-138, 28-46, and 299-701 for corn oil; 27-90, 14-30, and 120-436 for margarine; and 11-24, 12-27, and 52-105 for shortening. No significant changes were observed in the compositional ratio of the sterols at any stage of processing. Tentatively identified brasscicasterol, relative retention time (RRT, cholestane): 2.70, and Δ^5-avenasterol, RRT (cholestane): 4.25, peaks were detected in small amounts in nearly all of the fat and oil samples.

The presence of peculiar unknown sterol was found in coconut fat and deodorized fully hydrogenated cottonseed oil. Unknown components: RRT (cholestane): 1.44, 1.60, and 1.75, in several fats and oils were tentatively identified as tocopherols.

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CHAPTER I

INTRODUCTION

To the public, atherosclerosis is caused by high levels of serum cholesterol. Although the pathology and the exact contribution of cholesterol to the etiology of the disease has yet to be clarified, much of the evidence leans heavily on serum cholesterol level. Whatever side one takes in the many controversies within the field of atherosclerosis, all agree that a general drop in serum cholesterol level should be beneficial. Indeed, most therapeutic preparations are judged by their hypocholesteremic potency (Beveridge et al., 1958; Kritchevsky, 1958).

Regardless of the questionable degree of effectiveness and role(s) of diet on the blood cholesterol level, one of the most important approaches to lowering serum cholesterol has been the diet. National dietary goals set forth by the U. S. Senate Select Committee on Nutrition and Human Needs in 1977 recommended a reduced intake of dietary cholesterol and increased intake of polyunsaturated fat. The increase in polyunsaturated fat intake can be met only by increasing intake of processed vegetable oils.

Two contributors to the hypocholesteremic potency of vegetable oils are the degree of fatty acid unsaturation and the sterol content of the oils. Several hypotheses for the hypocholesteremic mechanism of plant sterols have been suggested. Plant sterols might inhibit the uptake of cholesterol by lipoproteins or glycoproteins of the intestinal mucosa. Sitosterol might form a nonabsorbable complex with cholesterol

in the intestine. Plant sterols might interfere with cholesterol esterification. However, to date, no direct proof of any of these hypotheses is available (Subbiah, 1973).

The increased intake of vegetable oil will increase consumption of plant sterols which have this hypocholesteremic effect. However, there is limited information concerning sterol composition and content in vegetable oils or fats processed in the United States. The objectives of this investigation were (a) to determine the major sterol (campesterol, stigmasterol and β-sitosterol) content in leading vegetable fats and oils in the United States at different stages of processing and as consumed by the public, and (b) to use gas liquid chromatography for the sterol determination.

CHAPTER II

REVIEW OF LITERATURE

Nature of Plant Sterols

Plant sterols commonly encountered in vegetable oils and the human diet are campesterol, stigmasterol, and β-sitosterol (Subbiah, 1973). They contribute an average of approximately 20% of the total sterols present in the body of adult human beings. These sterols have a structure containing a cyclopentenophenanthrene ring, a $\Delta^{5,6}$ double bond and 3β-hydroxyl group. Table 1 and Figure 1 list the identification of the sterols by common name, number of carbon atoms, double bond location, and IUPAC name, and the projected chemical structure of the sterols, respectively (Heftmann, 1970; Swern, 1979).

Sterol Composition of Vegetable Fats and Oils

Sterol fractions from soybean, cottonseed, palm, coconut, corn, and margarines (made from the oil or blends of oils mentioned) have been analyzed by several researchers in many countries (Eisner and Firestone, 1963; Itoh et al. 1973; Jeong et al., 1974; Parodi, 1973). Percentage distribution of individual sterol and composition of the sterol fraction in the fats and oils differ among the reported data. An excellent review was presented by Itoh et al., (1973). Difference in variety, origin of the samples and the procedures used in extracting the oil from the fruit or seed are the presumable explanations that contribute to the differences. However, three sterols, campesterol, stigmasterol, and β-sitosterol, are reported to be the major sterol components with

Table 1. Identification of sterols by common name, total number of carbon atoms, double bond location and IUPAC name.[a]

Sterol	No. of Carbon Atoms	Position of Double Bond	Abbreviated Structure Identification	Other Structure Characteristic	IUPAC
Cholestane	27	-	-	-	5α-cholestane
Cholesterol	27	C5	FC-27		Δ^5-cholesten-3-ol
Campesterol	28	C5	FC-28	24R-CH_3	24-methyl-Δ^5-cholesten-3β-ol
Stigmasterol	29	C5,C22	FC-29F	24S-C_2H_5	24-ethyl-$\Delta^{5,22}$-cholestadien-3β-ol
β-sitosterol	29	C5	FC-29	24R-C_2H_5	24-ethyl-Δ^5-cholesten-3β-ol

[a]Heftmann, 1970; Swern, 1979.

(a) Cholestane

(b) Cholesterol

HO

(c) Campesterol

HO

(d) Stigmasterol

HO

(e) β-sitosterol

HO

Figure 1. Projected structure of the sterols.

Source: Heftmann, 1970; Swern, 1979.

β-sitosterol being the most predominant. Considerable amounts of cholesterol have been reported in coconut and palm oils (Copius-Peereboom, 1965; Fedeli et al., 1966; Itoh et al., 1973). In addition, occurrence of brassicasterol, Δ^5-avenasterol, Δ^7-stigmasterol, Δ^7-avenasterol and unknown sterols have also been reported in all the types of fats and oils mentioned (Itoh et al., 1973).

Data for the distribution of individual sterols from the fats, oils, and margarines reported by Weihrauch (1977) are shown in Table 2.

Quantitative Changes in Sterol Content in Fat and Oil During Commercial Processing Procedures

The total sterol content in fat and oil is decreased gradually, but progressively, throughout different processing steps (Gutfinger and Letan, 1974b; Kanematsu et al., 1973; Morua Fe, 1971; Nelson and Milun, 1968; Thrope, 1972). Decreases occur especially during the deodorization and hydrogenation processes (Kanematsu et al., 1973) and neutralization and deodorization (Gutfinger and Letan, 1974a).

The change in individual sterol compositional ratios (namely for campesterol, stigmasterol, and β-sitosterol) in the sterol fraction is nearly negligible during the processing steps (Gutfinger and Letan, 1974a; Kanematsu et al., 1973). However, Morua Fe (1971) reported a proportional decrease in campesterol while stigmasterol and β-sitosterol increased during deacidification of coconut oil.

The effects of different processing steps unsaponifiable components in vegetable oils have been investigated by several researchers.

Table 2. Sterol content of fats and oils.

Fats or Oils		Sterol Content[a]								
		Total Sterol	Campesterol	Stigmasterol	β-sitosterol	Δ^5-avenasterol	Δ^7-stigmasterol	Δ^7-avenasterol	Brassicasterol	Cholesterol
		mg/100g Fats or Oils								
Corn Oil	Crude	1390	259	98	989	36	11	tr	tr	
	Refined	952	158	76	690	22	6	tr		
Coconut Oil	Crude	133	8	21	77	18	7		tr	2
	Refined	91	6	14	53	13	5		tr	1
	Refined and Hydrogenated	64	4	10	37	9	3			1
Palm Oil	Crude	117	23	14	72	2			tr	5
	Refined	49	10	6	30	1			tr	2
Soybean Oil	Crude	327	68	64	183	5	5	2		
	Refined	221	47	47	123	1	1	tr		
	Refined and Hydrogenated	132	26	30	76					
Cottonseed Oil	Crude	431	26	tr	400	5	tr	tr	tr	
	Refined	327	20	tr	303	4	tr	tr	tr	
	Refined and Hydrogenated	241	14	tr	223	3	tr	tr	tr	tr
Margarine	Regular, 80% Fat, Stick	483	102	50	331					
	Regular, 80% Fat, Tub	520	95	38	387					
	Diet, 40% Fat, Tub	421	107	38	276					

[a]Weihrauch, 1977.

[b]Tr = trace.

Decolorization. Sterols may either undergo chemical change to form 7-hydroxysterols which, when subjected to heat and oxygen, are converted into hydrocarbons and ketosteroids (Kaufman et al., 1970) or be adsorbed on bleaching clay (Nadirov et al., 1973).

Deacidification and deodorization. Sterols are removed to a great extent, along with by-products (namely, soapstock and deodorizer distillate) obtained from these two processes. Hence, the by-products are good sources for recovery of sterols and tocopherols (Gutfinger and Letan, 1974a; Nelson and Milun, 1968).

Hydrogenation. Campesterol, stigmasterol, and β-sitosterol can be readily hydrogenated in the laboratory. The hydrogenation products can be shown as follows:

campesterol $\longrightarrow$ campestanol

stigmasterol $\xrightarrow[\text{hydrogenation}]{\text{side chain}}$ β-sitosterol

stigmasterol $\xrightarrow[\text{hydrogenation}]{\text{ring}}$ Δ^{22}-stigmastenol

β-sitosterol $\longrightarrow$ stigmastanol

Δ^{22}-stigmastenol $\longrightarrow$ stigmastanol

The presence of these hydrogenation products could not be detected in hydrogenated fats and oils (Kanematsu et al., 1973; Parodi, 1975). A possible explanation for this is that the hydrogenation catalysts used and the conditions employed for selective hydrogenation may not be suitable for sterol hydrogenation (Parodi, 1975). Hence, the decrease as

reported in the literature must be caused by other factor(s) involved in the hydrogenation process.

Gas Chromatography of Phytosterol Mixtures

Since the first application by Beerthuis and Recourt (1960), it has been recognized that gas chromatography is an extremely powerful technique for separating and identifying natural sterols with structures closely resembling each other. However, successful gas chromatography of sterols is rather difficult. The very low vapor pressure, thermal sensitivity and high polarity of these complex organic molecules make special precautions necessary in sample handling, column design and instrumentation.

Column Design

Support material. Kabot and Ettre (1964) reported that the blocking of two active groups (-Si-Si- and -Si-O-Si-) on the surface
OH OH
of the diatomaceous earth type support is necessary to prevent undesirable interaction with sterol. This is normally done by the method called "silanization."

Liquid phase. The liquid phases used for sterol analysis should be selective enough for the individual substances but should not retard the substances too long. At the same time, the liquid phase should have a low vapor pressure at high temperature to minimize "bleeding." The concentration of the stationary phase in the column should be at the minimum loading (<5%) so that a column with required efficiency and

reasonably short retention times can be obtained (Kabot and Ettre, 1964; Rozanski, 1966).

Various types of liquid phases, both polar (selective) and nonpolar (nonselective), have been employed for sterol analysis with varying success. Problems stem from the complexity of the sterol compositions present in plants. Resolution of all the most prominent "critical sterol pairs," stigmastanol-sitosterol, stigmasterol-campesterol, and sitosterol-fucosterol and/or isofucosterol, are not achieved on any single major liquid phases used for sterol analysis; SE-30, QF-1, PMPE, and NGS (Ikekawa et al., 1968; Nordby and Nagy, 1973; Patterson, 1971). Grunwald (1969) reported that OV-101 at the 5% level gave the best overall results as compared with 5% SE-30 and 5% OV-22 in relative retention values (δ), effective plate values (N) and resolution for the biologically important phytosterols, cholesterol, campesterol, stigmasterol, and β-sitosterol, either as their free-OH or TMS-ether derivatives. Nordby and Nagy (1973) presented an evaluation of recent gas liquid chromatographic liquid phases for resolution of the most prominent desmethyl phytosterols and recommended SP-1000 as being superior to all other phases in its ability to resolve the critical pairs.

Column tubing. A silanized glass column and on-column injection are preferred to avoid any possible catalytic effect accelerating the decomposition of the sterols, particularly at higher temperatures (Kabot and Ettre, 1964).

Since column capacity is related to its length and size (inside diameter), the number of theoretical plates are studied as a criterion

for judgement of column efficiency. Rozanski (1966) reported that about 3000 theoretical plates were required for essentially complete separation of equal size peaks of the closest two components, campesterol and stigmasterol.

Also, the potential use of glass capillary column has been investigated since it was found to be quick, efficient and to give a better determination of small quantities of individual sterols when compared with the filled column (Mordret et al., 1977).

Instrumentation

Injection block and column heating. Horning et al. (1963) emphasized the importance of uniform heating. Wide variation in temperature along the column may result in "hot spots" which leads to localized deterioration of the column packing. In turn, the alteration of the packing leads to decomposition or adsorption of the sample components.

Detector. Horning et al. (1963) emphasized the essential high sensitivity of ionization detectors in sterol analysis. The flame ionization detector (FID) is favored compared to the argon ionization detector because its response is more independent of the molecular species; its linear range is larger; it is much less temperature sensitive; it allows a large variation in flow rates; it is insensitive to water, and it has reduced sensitivity for the solvents commonly used. Preliminary studies were reported by Tateo (1973) on the optimum operating conditions for the use of gas chromatography with electron capture detector (ECD) to determine the sterols as heptafluorobutyrate

derivatives. However, the ECD does not have as large a linear range as the FID.

Sample Handling

Kabot and Ettre (1964) reported that four factors particularly warrant the use of derivatives for sterol analysis. The derivatives have a greater volatility and generally a higher thermal stability. They can be separated better and at the same time, some detectors might have a higher sensitivity for the derivatives.

Sterols are normally chromatographed as free sterols, methyl ether derivatives (Clayton, 1962), trimethylsilyl ether derivatives or acetate derivatives with the latter two forms being more widely used (Patterson, 1971).

There were no significant qualitative or quantitative differences in the analysis of sterols between the following two forms of derivatives analyzed: TMS-ether derivative and free-OH form on two stationary phases of different polarity, JXR and OV-17 (Mannino and Amelotti, 1975), or the TMS-ether derivative and acetate derivative on SE-30, OV-17, JXR, XE-60, DEXIL-300, and OV-225 (Mordret et al., 1977).

Derivatizing methods. Since the exact derivatization procedures differ among reported investigations, general procedures are reviewed for the conversion of organic hydroxy compounds into TMS-ether and acetate derivatives.

TMS-ether. The hydroxyl group is substituted by the silyl ether group as shown in the general reaction:

$$3ROH + Me_3SiNHSiMe_3 + Me_3SiCl \longrightarrow 3ROSiMe_3 + NH_4Cl$$

(HMDS) (TMCS)

Hexamethyldisilazane (HMDS) is the silyl donor while trimethylchlorosilane (TMCS) serves as a catalyst. An excellent review with specific information can be found in the Pierce Hand Book and General Catalog, (Pierce, 1977-78).

Acetate derivative. The chemical reaction demonstrating the substitution of the hydroxyl group with the acetate group is shown as follows:

$$CH_3CO\text{-}O\text{-}OCCH_3 + ROH \xrightarrow[\Delta]{C_5H_5N} R\text{-}O\text{-}\underset{\underset{O}{\|}}{C}\text{-}CH_3 + CH_3COOH$$

Acetic anhydride serves as the acetate donor. The reagent can either be used alone or with a solvent, pyridine. There are some slight variations in the amount of reagent used and heating time employed in the derivatizing procedures among the reported investigations (Applied Science Laboratories, Inc., 1979; Nordby and Nagy, 1973; Patterson, 1971; Supelco, Inc., 1979; Thrope, 1972).

Identification of Phytosterols

A correct identification of the various types of sterols related to cholesterol (C27-series) such as ergosterol (C28-series), β-sitosterol (C29-series), and lanosterol (C30-series), by gas chromatographic analysis is rather difficult. Although reports of many investigations in this field have been published (Clayton, 1962; Ikekawa et al., 1968; Knight, 1964a; Patterson, 1971; Vandenheuvel and Horning, 1962), the

chemical structure of many compounds in the chromatograms of phytosterols from edible oils remains unknown.

Prior to 1964, several investigators (Clayton, 1962; Knight, 1964a; Vandenheuvel and Horning, 1962) studied the molecular structure of sterols analyzed by gas liquid chromatography. Awareness of the presence of the minor sterol components which were obscured by major sterol peaks or disregarded completely marked the necessity of further elucidating analysis of phytosterols by GLC. Copius-Peereboom (1965) applied combinations of thin-layer chromatography (TLC) and gas chromatography in which the relationship between chromatographic behavior and structure of sterols were based upon different principles for identification of unknown sterol types. Ikekawa et al. (1968) reported the relationship between the separation factors for double bond isomers of free sterols on various polar and nonpolar liquid phases. Nordby and Nagy (1973) and Patterson (1971) reported the gas chromatographic characteristics of sterol acetates and their retention times on several liquid phases.

With the increasing availability of more accurate techniques, the application of gas chromatography combined with mass spectrometry has been employed to accomplish a more precise identification of sterols (Itoh et al., 1973; Fedeli et al., 1976; Schiller, 1973).

Quantitative Determination

Relative response and linearity. Based on the similarity in the sterol structures, the relative weight responses of sterols in the flame ionization detector in several reports (Eisner and Firestone, 1963; Jeong et al., 1974; Nelson and Milun, 1968) are assumed to be equal.

However, in exact analysis of sterols (campesterol, stigmasterol and β-sitosterol) the relative weight responses of all the components must be established, and the peaks under consideration should not be assumed to constitute the whole sample. Also, the ratio of the partition coefficients in gas chromatographic analysis for these sterols, especially for campesterol and stigmasterol, is close to unity, and unless high efficiency columns are used and peak tailing is eliminated, the overlap of the peaks is considerable and the accuracy is low (Rozanski, 1966).

Furthermore, the response of the flame ionization detector has been shown to vary with the quantity of sterol injected and with different sterols. Hence the linearity of the detector for the range of weight under investigation must also be substantiated to attain high accuracy (Gracian, 1976; Nelson and Milun, 1968; Punwar, 1975; Rozanski, 1966).

Standard addition method. The use of an internal standard, required in exact quantitative GLC to allow for variations in the volume injected, met with two difficulties (Rozanski, 1966). Firstly, in GLC of phytosterols, such as soybean sterols, a variety of minor impurities were found, and it would seem necessary to correct for any overlap with the internal standard. Secondly, the variability in the relative retention time and the standard deviation of the peak area ratio greatly increased with the separation of the standard and the compound being analyzed.

With the exception of Punwar (1975), Nelson and Milun (1968), Rozanski (1966), and Zürcher et al. (1976), other researchers eliminated

interference problems from the unsaponifiable components. Less polar compounds (hydrocarbons, aliphatic alcohols, etc.), triterpene alcohols and 4-methylsterols were isolated from the sterol fraction either by column chromatography on silica, alumina or florisil or by thin layer chromatography on silica G prior to subsequent GLC analysis. In spite of the interference problems and without the application of the clean-up procedures, Zürcher et al. (1976) reported recoveries of 99.8% and 100.3% for cholesterol in rapeseed oil and in coconut oil, respectively, analyzed on an OV-17 column.

Internal standard. Cholestane, squalane, and cholesterol have been used as internal standards in sterol analysis (Patterson, 1971). Cholestane, however, is the most popular internal standard for sterol analysis by GLC on a variety of columns and for acetate derivatives.

CHAPTER III

MATERIALS AND METHODS

Nature and Source of Samples

A total of 62 samples of fats, oils, and blends in the form of shortening and margarine was analyzed. Samples were obtained from two oil processing plants in Tennessee and were purchased from local supermarkets. Samples were categorized as follows:

Soybean oil. (a) Refined, deodorized, and slightly hydrogenated to an iodine value (I.V.) consistent with flavor stability in a salad oil, and winterized, and (b) refined, deodorized, and hydrogenated to an I.V. of 65-70 (a base stock).

Cottonseed oil. (a) Refined, bleached, deodorized, and winterized as a salad oil, and (b) refined, bleached, deodorized, and hydrogenated to a hard stock for use in production of shortening and margarine.

Coconut oil. Refined, bleached, and deodorized.

Palm oil. (a) Refined palm oil, and (b) refined, bleached, deodorized, and hydrogenated to a hard stock for use in production of shortening and margarine.

Corn oil. (a) Refined and deodorized as a salad oil, and (b) refined, deodorized, hydrogenated, and blended with refined and deodorized corn oil to produce corn oil margarine.

Blend of fats and oils. Soybean oil and cottonseed oil, and soybean oil and palm oil obtained as shortening and/or margarine.

Storage and Sample Preparation

Upon receipt, all the samples were stored at 5-6°C until the time of analysis. Oils were analyzed without further treatment and fats were melted prior to further treatment.

The fat portion in margarines was separated, after melting, from the water layer (bottom) which was discarded, and the fat was dried by filtration through anhydrous sodium sulfate. The foam layer, found in melted whipped margarines, was scraped off the top of the melted fat and discarded.

Characteristics of Fat and Oil Samples

A: Iodine value. The two methods presented below were employed for the analysis.

1. Refractometric Method: The A.O.C.S. (1970) Official Method Cc 7-25 was followed. The procedure was applicable to all of the samples except coconut oils. The refractive index was measured at 20°C, 40°C, and 60°C according to the nature of the samples. Temperature corrections were made by the following equation:

$$R = R' + K(T' - T)$$

where

R = refractive index at temperature T (°C)

R' = refractive index at T' (°C)

T = the standard temperature (25°C)

T' = the temperature (°C) at which the reading R' was made

K = 0.000365 for fats and 0.000385 for oils

The iodine value was calculated from the following equation recommended for linseed and soybean oils (Swern, 1979):

$$n_D^{25} = 1.45765 + 0.0001164\ (I)$$

where

n_D^{25} = R = the refractive index at 25°C

I = Iodine Value

2. Wijs' Method: The A.O.C.S. (1970) Official Method Cd 1-25 was employed for coconut oils. Approximately 1.000 gm of sample was used.

B: Isolated trans-isomer content. The analysis was performed according to the A.O.C.S. (1970) Official Method Cd 14-61. If the trans-isomer content of the samples were less than 15%, the sample was converted to its methyl ester derivative according to the A.O.C.S. (1970) Official Method Ce 1-62 and reanalyzed. Measurements were made on a Pye-Unicam model SP-1100 infrared spectrophotometor, using matched KBr cells with a path length of 0.5000 mm.

Gas Liquid Chromatographic Analysis of Sterols

Materials and instruments.

1. Sterol standards:

Cholestane, campesterol, stigmasterol, and β-sitosterol were purchased from Applied Science Laboratories, Inc., State College, PA, or Supelco, Inc., Bellefonte, PA.

2. Silanizing reagents:

Dimethyldichlorosilane (DMCS), Pierce Chemical Co., Rockford, IL.

Toluene, Fisher Scientific Company, Fair Lawn, NJ.

3. Derivatizing reagents:

Acetylation reagents: pyridine-acetic anhydride, Applied Science Laboratories.

TMS-ether derivatizing reagent: Hexamethyldisilazane (HMDS)—trimethylchlorosilane (TMCS)—pyridine; "Tri-Zil," Pierce Chemical Company.

4. Saponifying reagents:

Potassium hydroxide, reagent grade, Fisher Scientific Company.

Reagent alcohol: ethanol-methanol-isopropanol (90+5+5), reagent grade, redistilled in glass, Fisher Scientific Company.

Benzene, reagent grade, Fisher Scientific Company.

n-heptane, analytical grade, Eastman Kodak, Rochester, NY.

5. Column packing:

Silanized glass wool, Applied Science Laboratories.

1% SP-1000 on 100/120 supelcoport, Supelco, Inc.

3% OV-101 on 80/100 chromosorb W(HP), Pierce Chemical Company.

3% OV-17 on 80/100 chromosorb W(HP), Supelco, Inc.

3% SE-30 on 100/120 gas chrom Q, Supelco, Inc.

Apparatus.

1. Derivatizing Vial: Teflon-lined screw-capped vial, 5 ml (Pierce Chemical Co.). Tubes were silanized as follows. Clean tubes (acid-cleaned with potassium dichromate-sulfuric acid cleaning solution) were rinsed with anhydrous methanol and dried. The tubes were filled with a 10% solution of dimethyldichlorosilane in toluene and let stand for approximately one hour. They were rinsed thoroughly with anhydrous methanol, dried in a 100°C oven and stored in a desiccator until used.

2. Gas Chromatographic Column: A 1.83 m long, 4.23 mm internal diameter, U-shaped glass column was silanized prior to packing. The column was silanized by the same procedure used for the vial, but was dried by air-flow. With the aid of a vibrator and aspirator, the column was filled with packing material until the level was approximately 5 cm from the inlet end and 2 cm from the outlet end. Finally, the ends of the column were plugged with silanized glass wool. The packed column was conditioned overnight at 300°C for OV-17, SE-30, and OV-101, and at 250°C for SP-1000, with a nitrogen flow rate of 15-20 ml/min.

3. Gas Chromatograph: A Bendix gas chromatograph (model 2500) equipped with hydrogen flame ionization detector, a Leeds and Northrup recorder with an adjusted speed of 0.25 in/min. and a Shimadzu data processor (model Chromatopac-E1A) was used for the sterol analysis in the present study.

4. Chromatographic Conditions for OV-101 Column and Quantitation: Temperature (°C): injection port 300°C, detector, 300°C and column 272.5°C. Gas flow rates (ml/min): nitrogen ∿ 60 ml/min, hydrogen ∿ 60 ml/min, and air ∿ 300 ml/min.

Derivatization of Sterol Standards and Samples

1. The acetates of sterols were prepared by heating the sterols for one hour at 75°C in a water bath with the pyridine-acetic anhydride acetylation reagent (2:1; V:V) according to Nordby and Nagy (1973). The excess reagent was removed by evaporating under a stream of nitrogen and the residue was dissolved in 0.2 ml n-heptane.

2. The TMS-ether derivatives were prepared by adding 1 ml of "Tri-Zil." According to Parodi (1973), the derivatives were ready to be chromatographed within two minutes.

Column Packing and Sterol Derivative Selection for Quantitation of Sterols

To obtain the most efficient method for quantitation of sterols, both acetate derivatives and TMS-ether derivatives of standard sterol solutions were chromatographed on four columns, each containing a different liquid phase loading: SP-1000, a modified carbowax 20M; OV-101, dimethyl silicone; OV-17, 50% phenyl-methyl silicone and SE-30, methyl silicone. Separation of the critical sterol pair (campesterol-stigmasterol) as judged by the peak separation (δ) and the peak resolution (R) calculated according to Ettre (1963) was used as criterion for selection of derivative and column packing.

Chromatographic conditions for analysis are listed in Table 3. Based upon results which are presented and discussed later, the OV-101 and acetate derivative were selected as the method for quantitation of sterols in fats and oils.

Sterol Standard Solutions and Mixtures

Stock solutions of cholestane, cholesterol, stigmasterol, campesterol, and β-sitosterol were prepared to contain 1 mg of each compound per ml of solution in n-heptane. From these stock solutions, a series of standard mixtures were prepared that had the composition shown in Table 4. Each mixture was prepared twice. The concentrations in each mixture were attained by pipeting the correct volume of the respective stock solution by a calibrated pipet into a derivatization vial. The solvent was evaporated from the mixture by heating the mixture under a stream of nitrogen in a 40°C water bath. The dried residue was derivatized as acetates.

Table 3. Chromatographic conditions for selection of column packing and sterol derivative.

Liquid Phase	TMS-ether		Acetate	
	Oven Temp. °C	Nitrogen Flow ml/min.	Oven Temp. °C	Nitrogen Flow ml/min.
OV-101	262.5	60	272.5	60
SE-30	265.0	50	265.0	50
OV-17	270.0	60	270.0	50
SP-1000	-	-	250.0	20

Table 4. Concentration of sterols in mixtures used to determine relative response factors.

Mixture	Cholestane	Cholesterol	Campesterol	Stigmasterol	β-sitosterol
			mg in 0.2 ml n-heptane		
1	0.2	0.5	0.55	0.33	1.71
2	0.2	0.5	0.66	0.40	2.04
3	0.2	0.5	0.77	0.47	2.38
4	0.2	0.5	0.88	0.54	2.72
5	0.2	0.5	0.99	0.61	3.06

The weight ratios among the sterols in the mixtures were representative of sterol composition in palm oil. The amount and ratio of sterols in palm oil are in the mid-range of sterols in the fats and oils investigated. [By adjusting the weight of oil analyzed and determining the response factor for each sterol to cholestane and the linear range of the FID for each sterol in the mixtures, it was possible to determine sterol content of all fats and oils investigated within the linear range of the instrument.]

Determination of Relative Response and Ratios

Each sterol standard mixture derivative (approximately 3 µl) was analyzed in duplicate by GLC on OV-101 column. Y, the ratio of each sterol peak area to the normalized peak area of each of two internal standards, cholestane or cholesterol, was calculated by means of the following equation:

$$Y = \frac{A_s}{A_{is}} \times W_{is}$$

where

A_s = area of sterol peak

A_{is} = area of cholestane or cholesterol peak

W_{is} = weight in mg of cholestane or cholesterol

Analysis of variance was run on the variable, Y, for each sterol to partition the variance into concentration effect, and error. Significant concentration effects were separated by orthogonal polynomials into linear, quadratic, and cubic effects. Relative response factors (Rf) of each sterol to cholestance and cholesterol were calculated by the following equation:

$$R_f = \frac{A_{is} \times W_s}{A_s \times W_{is}}$$

where

W_s = weight of sterol

Isolation of Sterols from Fats and Oils

One ml cholestane solution (0.2 mg/ml) was pipeted into a 300 ml Erlenmeyer flask with ȿ 24/40 ground glass top and evaporated to dryness under a stream of nitrogen. Fat or oil in a given weight range (Table 5) was weighed into the flask. A stirring bar was added, and the flask was placed on a magnetic stirring hot plate. Ten ml of 10% aqueous KOH and 40 ml reagent alcohol were added to the flask, and the sample was dissolved by gentle stirring. A condenser was attached and the solution was heated and refluxed for one hour. The heat was turned off and 60 ml reagent alcohol was added through the condenser to the saponified sample. The sample was cooled while stirring until it ceased refluxing. The condenser was removed and 100 ml benzene was added to the sample while it was being stirred slowly. The stirring bar was removed and the flask was stoppered and shaken vigorously for 30 seconds. The sample was transferred to a 500 ml separatory funnel, and 200 ml of 1 N aqueous KOH was added. The funnel was stoppered and shaken vigorously for 10 seconds. The layers were allowed to separate and the lower turbid phase was discarded. The upper benzene layer was washed with 40 ml 0.5 N aqueous KOH and transferred to a 250 ml separatory funnel. The benzene solution was back-washed with distilled water (4-6 times) until the pH of the last water wash was approximately 7. The washed benzene extract was transferred to a 125 ml glass-stoppered Erlenmeyer flask containing

Table 5. Weight of fat or oil samples used for sterol analysis.

Sample	Weight in grams
Soybean Oil	2.0-2.5
Coconut Oil	5.0
Cottonseed Oil	4.0
Palm Oil	5.0
Corn Oil	2.0
Margarine	1.5-2.0
Shortening	3.0-3.5

30 g anhydrous sodium sulfate. The flask was shaken vigorously and let stand for 15 minutes.

A 50 ml aliquot was pipeted into a 100 ml round-bottom flask with ȝ 24/40 ground glass neck and evaporated to dryness on a rotary evaporator at 40°C. Three ml of n-heptane was added to the flask to dissolve the residue and a 2 ml sample was removed to derivatizing vial. The sample was evaporated to dryness under nitrogen and stored at 4-6°C under nitrogen until derivatization and GLC analysis.

Analysis of Samples

Approximately 1.5-3.0 µl of the acetate-derivatized sample (the lower volume was for corn oil) was injected into the gas chromatograph. Retention time (RT) and area for each peak were obtained from the data processor (method 41 mode). Identification of the sterols was made by comparing relative retention times (RRT) with those of standards. Cholestane with a RT of 5.76 min. had a RRT of 1.00; cholesterol RRT, 2.40; campesterol RRT, 3.10: stigmasterol RRT, 3.37; and β-sitosterol RRT, 3.87.

The quantity of each sterol in the sample was obtained from the data processor programmed in Method 43 which calculates the quantity according to the equation:

$$\text{mg sterol/100 g sample} = R_f \times \frac{A_s}{A_{is}} \times \frac{W_{is}}{W_{spl}} \times 100$$

where

R_f = response factor or relative response

W_{spl} - sample weight in gram

W_{is} = internal standard weight in mg

Precision and Accuracy of Sterol Quantitation

The precision of the method for the sterol quantitation was determined as the standard deviation for campesterol, stigmasterol, and β-sitosterol analyzed in five samples of stock hydrogenated palm oil. Sterols were isolated from the palm oil and derivatized as previously described. Each sample was injected into the GLC five times. Precision of analysis within each sample was determined as the standard deviation of mean sterol content calculated from the five injections of each sample. The precision among samples was calculated by determining the standard deviation of the mean sterol content across five samples, where the estimate of the sterol content in each sample was the mean across five injections.

Accuracy of the method was determined by spiking hydrogenated palm oil samples with 0.5 to 2.0 mg stigmasterol at 0.5 mg intervals. Two oil samples were spiked at each concentration of stigmasterol. In one of the oil samples, 0.80 mg β-sitosterol and 0.55 mg campesterol also were added. Sterols were isolated, derivatized, and measured in the oil and spiked oil samples as previously described. Percentage recovery, a measure of the accuracy of the method, was determined by dividing the difference in sterol content between the spiked sample and the oil alone by the amount of sterol added and multiplying the answer by 100.

CHAPTER IV

RESULTS AND DISCUSSION

Iodine Value Determination

In general, the relationship between refractive indices and the iodine values (I.V.) of natural fats are related linearly (Zeleny and Neustadt, 1940). These investigators reported that the relationship is identical for most oils, especially those that are freshly prepared and contain only traces of unsaturated fatty acids other than oleic, linoleic and linolenic acids. Measuring iodine value by refractive index is very rapid. The general equation for calculating iodine value initially was proposed for linseed and soybean oils (Swern, 1979), but it was extrapolated to determine I.V. of all the fats and oils in the present study. One of the ways to assess the accuracy of a new method of analysis is to compare its results with those obtained by other methods that have been established to give accurate results (Pomeranz and Meloan, 1971). Wijs' method has been established by the American Oil Chemists Society to be accurate. Hence, an experiment was conducted to compare the I.V. determined by refractive index with I.V. determined by conventional Wijs' method (A.O.C.S., 1970; Official Method Cd 1-25). The results are reported in Table 6. The I.V. determined by the two methods for any one fat were close together. Also, the repetition of the refractive index measurement for any oil was good with only small difference occurring between duplicates.

Of all the samples analyzed, however, the iodine value of coconut oil could not be measured by its refractive index. For this oil, the

Table 6. Comparison of iodine values determined by refractive index and Wijs' method.

Sample	Oil Components	I.V.[a] (Wijs' Method)	I.V.[a] Refractometric Method
Shortening I	pHSB[b] & pHP[c]	90.8	89.8
Shortening II	pHSB & pHCS[d]	88.6	90.2
Soybean Oil	pHSB	117.6	112.1
Corn Oil	-	127.9	128.4
Winterized Cottonseed Oil	-	112.8	110.4

[a]Average of two determinations.

[b]pHSB, partially hydrogenated soybean oil.

[c]pHP, partially hydrogenated palm oil.

[d]pHCS, partially hydrogenated cottonseed oil.

refractive index was so low that upon substitution into the equation for iodine value calculation, a negative value was obtained. A possible explanation could be that the oils contain high levels of short chain fatty acids such as lauric acid and are very saturated (I.V. = 9-11, Wijs' method).

Gas Liquid Chromatographic Analysis of Sterols

Liquid phase selection. Two major criteria, the best resolution (as judged by peak resolution value) of the campesterol-stigmasterol pair and the shortest retention time for β-sitosterol were utilized to select the liquid phase of the GLC column and the appropriate derivative form for the sterol analysis in this study. Four liquid phases: SP-1000, OV-101, OV-17, and SE-30, and two derivative forms: acetate and TMS-ether were investigated. The descriptive values of the sterol behavior on these liquid phases are shown in Table 7. A peak resolution, R, of 1.5 corresponds to peak separation, δ, of 95%.

The results presented pertain to the sterol behaviors at equal concentrations and are obtained under GLC conditions to achieve an acceptable peak resolution for quantitation. Sterol behavior on these highly-rated liquid phases are in agreement with the published literature (Grunwald, 1969; Nordby and Nagy, 1973). The nonpolar phases (OV-101 and SE-30) which exhibit "non-selective mechanism" resolve campesterol and stigmasterol adequately for both qualitative and quantitative analysis. OV-101, however, had better resolution, and gave more symmetrical peaks than SE-30 for both acetate and TMS-ether derivatives. OV-17 which is a moderately polar phase (McReynolds constant, 119) also exhibited good

Table 7. Retention time of β-sitosterol and peak separation and resolution of campesterol and stigmasterol of two sterol derivatives on different GLC column liquid phases.

Liquid Phase	Phase Structure	x', McReynolds Constant	β-sitosterol Net RT		δ^a = Peak Separation		R^a = Peak Resolution	
			Acetate	TMS-ether	Acetate	TMS-ether	Acetate	TMS-ether
			Minutes		%			
SE-30	Methyl-silicone	15	54.25	40.25	79.07	67.42	1.05	0.86
OV-101	Dimethyl-silicone	17	24.50	30.45	92.24	86.21	1.26	1.15
OV-17	50%, phenyl-methyl-silicone	119	47.00	40.10	88.31	81.08	1.17	1.02
SP-1000	Modified-carbowax-20M	332	32.00	-	25.00	-	0.69	-

[a] δ and R are calculated according to Ettre (1963).

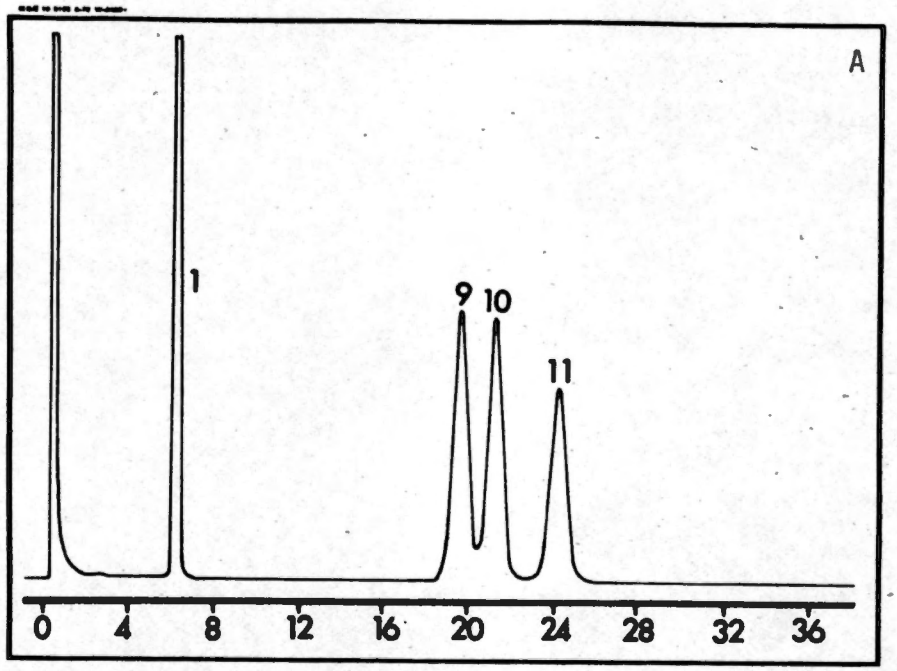
resolution due to its 50% phenyl moiety and compared to nonpolar phases, OV-17 had better resolution than SE-30 but not OV-101. Nordby and Nagy (1973) reported that SP-1000 had exceptional resolution for campesterol and stigmasterol in the acetate form; however, it was found to have the least resolution of the liquid phase investigated in the present study.

The sterol acetate derivatives were resolved better than the TMS-ether derivatives by any liquid phase. Furthermore, TMS-ether derivatives are deposited as an oxidation product on the flame ionization detector of the GLC, and the detector must be cleaned frequently during such analysis. Analysis of the acetate derivative does not present this problem. Under the same chromatographic conditions (same column and oven temperature), acetate derivatives have longer retention times than TMS-ether derivatives, but by increasing oven temperature, the retention times can be shortened without sacrificing much resolution. Hence, the acetate derivative was chosen for sterol analysis in the present study.

Figure 2-A illustrates the separation of an equal concentration mixture of campesterol, stigmasterol, β-sitosterol, and cholestane (0.5 mg each in 0.2 ml n-heptane) on the OV-101 column. Figure 2-B illustrates the separation of an unequal concentration mixture of the same sterols (0.55 mg campesterol, 0.33 mg stigmasterol, 1.70 mg β-sitosterol, 0.5 mg cholesterol, and 0.2 mg cholestane each in 0.2 ml n-heptane).

Relative Response and Ratios for Sterol Quantitation

Analysis of variance for Y with cholestane as the internal standard or with cholesterol as the internal standard are shown in Table 8. Linear effects were significant at the $P<0.001$ level for each sterol with either cholestane or cholesterol as the internal standard. Only quadratic effect



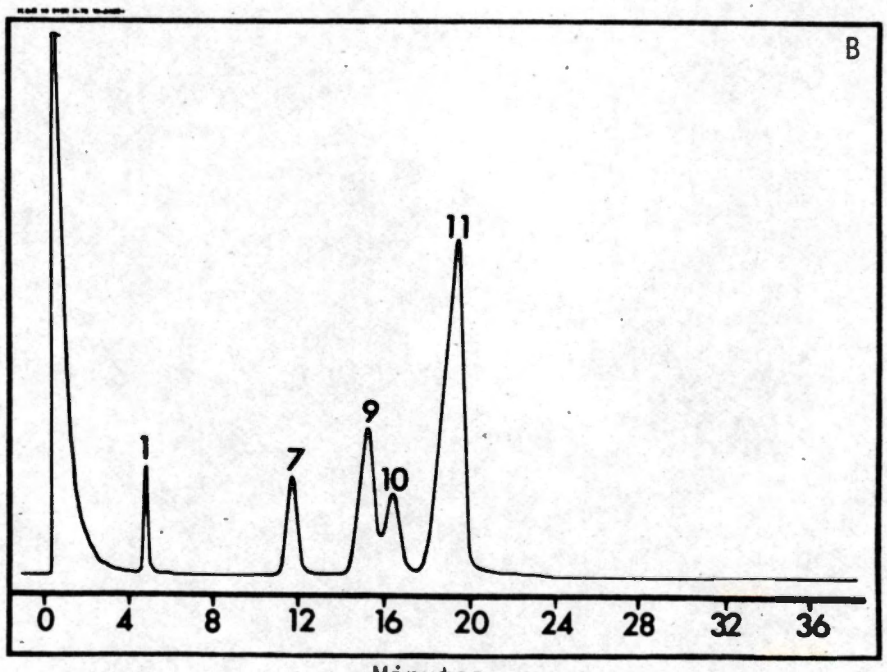


Figure 2. Representative chromatograms of standard sterol mixtures: (A) chromatogram of sterols of equal concentration (0.5 mg/ 0.2 ml) on OV-101, and (B) chromatogram of sterols of unequal concentration on OV-101, cholestane (1), cholesterol (7), campesterol (9), stigmasterol (10), and β-sitosterol (11).

Table 8. Analysis of variance for Y, ratio of sterol peak area to normalized internal standard peak area.

Source	DF	Mean Square Values for Y[a] Sterol: Campesterol	Stigmasterol	β-sitosterol
Y, Cholestane[b]				
Sterol Concentration	4	0.1286***	0.0386***	0.9685***
Linear	1	0.5118***	0.1538***	3.8445***
Quadratic	1	0.0009	0.0002	0.0045
Cubic	1	0.0001	0.0001	0.0037
Error (Replication and Replication Concentration)	5	0.0004	0.0003	0.0053
Y, Cholesterol[c]				
Sterol Concentration	4	0.1414***	0.0427***	1.0536***
Linear	1	0.5650***	0.1705***	4.2111***
Quadratic	1	0.0005*	0.0001	0.0020
Cubic	1	0.0000	0.0000	0.0015
Error (Replication and Replication Concentration)	5	0.0001	0.0001	0.0010

[a]Y, the ratio of each sterol peak area to the normalized peak area of the internal standard.

[b]Y, calculated by using normalized peak area of cholestane.

[c]Y, calculated by using normalized peak area of cholesterol.

*P<0.005, ***P<0.001.

was significant at the $P<0.05$ level for campesterol with cholesterol as the internal standard. Average values for Y (cholestane) and Y (cholesterol) in each sterol mixture are given in Table 9. A linear regression was run for each sterol with sterol concentration as a function of Y (cholestane) or Y (cholesterol). Coefficient of determination, intercept and reciprocal of the slope of each line (relative response factor, R_f) are shown also in Table 9. Since palm oil contained cholesterol and the relative response of campesterol, stigmasterol, and β-sitosterol with cholestane as the internal standard were greater than with cholesterol as the internal standard. Cholestane was chosen as the internal standard for quantitation of sterols.

Precision of Sterol Quantitation

Standard deviations or precision of measurement of sterol quantitation within and among samples are shown in Table 10. Precision within samples expressed as a coefficient of variation [C.V. = (standard deviation/mean) x 100] varied from 2% in the case of stigmasterol and β-sitosterol in sample 5 to as high as 15% in the case of β-sitosterol in sample 1. An average C.V. across sterols for each sample is approximately 7% and represents the precision within each sample. If five samples were taken from each oil and each sample was injected five times and the mean of sterol content per sample determined for each sterol, the mean across samples can be determined. The standard deviation of this mean across samples would be an estimate of precision among samples and is shown in Table 10. To prepare five samples from one oil sample and inject each sample five times is not practical because of time of analysis and cost of materials. It was decided for

Table 9. Mean values for Y[a] for standard sterol mixtures and estimates of intercept, reciprocal of slope (relative response), and coefficient of determination for linear regression of sterol concentration as function of Y.

Sterol Mixture	Campesterol Y[a]		Stigmasterol Y[a]		β-sitosterol Y[a]	
	Cholestane	Cholesterol	Cholestane	Cholesterol	Cholestane	Cholesterol
1	0.5473	0.6138	0.2837	0.3181	1.7140	1.9218
2	0.6994	0.7711	0.3657	0.4030	2.1563	2.3768
3	0.8470	0.9360	0.4517	0.4988	2.5515	2.8181
4	1.0082	1.1031	0.5342	0.5845	2.9798	3.2604
5	1.1928	1.2882	0.6379	0.6890	3.4944	3.7743
Coefficient of Determination	0.9996	0.9992	0.9979	0.9988	0.9979	0.9991
Intercept	-0.2190	-0.2267	-0.1341	-0.1212	-0.4898	-0.3817
Relative Response	0.7189	0.6581	0.7983	0.7582	0.7755	0.7409

[a]Y, the ratio of each sterol peak area to the normalized peak area of the internal standard, cholestane or cholesterol.

Table 10. Standard deviation within and among samples as a measure of precision of sterol quantitation.

Sample	Sterol		
	Campesterol	Stigmasterol	β-sitosterol
	mg/100 g hydrogenated palm oil		
1	10.27 ± 1.30[a]	14.64 ± 2.11[a]	30.98 ± 4.38[a]
2	11.82 ± 0.68[a]	16.30 ± 0.90[a]	33.43 ± 1.92[a]
3	12.79 ± 0.72[a]	16.72 ± 1.27[a]	36.79 ± 3.28[a]
4	11.13 ± 0.28[a]	15.19 ± 0.56[a]	33.79 ± 0.78[a]
5	11.84 ± 0.49[a]	15.92 ± 0.32[a]	35.59 ± 0.58[a]
Overall	11.57 ± 0.94[b]	15.75 ± 0.84[b]	34.15 ± 2.22[b]

[a]Mean is based upon five injections/sample and is a measure of precision within the sample.

[b]A measure of precision among samples; mean across five samples.

quantitation of sterols to make the acetate derivative one time for each oil sample and to analyze the derivative in triplicate by the GLC. If instrument response gave a coefficient of variation of more than 10% for any sterol, another derivative of the oil was prepared and analyzed again.

Variation in instrument response is measured by the precision within samples. The variability of the precision may be due to the fact that oven temperature control in the GLC Bendix (model 2500) is very dependent on line voltage. Variations in this voltage caused changes in temperatures which were reflected in actual retention times of sterols from one run to another. The relative retention time for any one sterol remained fairly constant but total time of analysis of all sterols varied from 27 to 31 minutes. These variations could possibly cause the differences in precision within samples.

Accuracy of Sterol Quantitation

The saponification and extraction procedure employed in this study was the one recommended by Punwar (1975) with some modifications. One important change was that the internal standard was added prior to saponification instead of prior to derivatization. This was an attempt to compensate for any lost and/or change of the sterol due to the procedure.

The accuracy of a method is how close the estimate is to the true value. For a method to be absolutely accurate, a 100% recovery must be obtained. Table 11 shows the percentage recoveries for stigmasterol added at different concentrations to palm oil and for one sample spiked with campesterol and β-sitosterol. Recoveries of stigmasterol at lower

Table 11. Sterol recovery from spiked palm oil samples.

Sterol	Weight (mg) of Sterol Added to 100 g Palm Oil	Weight (mg) of Sterol in 100 g Palm Oil	Weight (mg) of Sterol in 100 g Spiked Palm Oil	% Recovery[a]
Stigmasterol	9.98	12.52	22.65	101.5
	9.92	12.52	22.51	100.7[b]
	15.55	12.52	28.43	102.3
	19.40	12.52	30.98	95.2
	29.96	12.52	39.28	89.3
	29.93	12.52	37.59	83.8
	39.87	12.52	52.88	101.2
	39.98	12.52	45.58	82.7
Campesterol	10.96	13.02	22.00	81.9
β-sitosterol	15.95	34.99	49.75	92.5

[a]Mean of two measurements.

[b]Mean of three measurements.

concentrations (1.0 mg and below) were essentially complete; however, recoveries at higher concentrations (above 1.0 mg) with one exception were low (82.7%). The recovery of campesterol was also low (81.9%).

Sterol Content

Tables 12 through 18, respectively, show iodine values, trans-isomer content, and sterol (campesterol, stigmasterol, and β-sitosterol) content in soybean oil, cottonseed oil, palm oil, coconut oil, corn oil, corn oil margarines, and shortenings from different oil and fat blends.

Soybean oil samples which had not been hydrogenated (I.V. = 132.5) or had been slightly hydrogenated (I.V. = 105.9 to 115.8) and which had trans-isomer contents ranging from 10% to 18% contained in 100 g oil; 27 to 41 mg campesterol, 27 to 37 mg stigmasterol, and 104 to 149 mg β-sitosterol. Soybean oil samples with an I.V. 93.9 and 95.1 and 29.5% and 24.9% trans-isomer content, respectively, had in 100 g oil; 22 and 26 mg campesterol, 21 and 26 mg stigmasterol, and 95 and 118 mg of β-sitosterol. Soybean oil samples with an I.V. range from 62.9 to 73.7, and a trans-isomer content from 44.5% to 53.8%, contained in 100 g oil: 18 to 25 mg campesterol, 19 to 25 mg stigmasterol, and 70 to 88 mg β-sitosterol. Weihrauch (1977), in a review, reported refined soybean oil and hydrogenated soybean oil contained, respectively, in 100 g oil, 47 and 26 mg campesterol, 47 and 30 mg stigmasterol, and 123 and 76 mg β-sitosterol. These values are in the same range as were found in this study. The only direct comparison which can be made is with the refined soybean oil, which in this study had, in 100 g oil, 30 mg campesterol, 27 mg stigmasterol, and 128 mg β-sitosterol (Table 12).

Table 12. Iodine value, trans-isomer content and sterol content of soybean oil.

Source	Sample	Iodine Value	Trans-Isomer Content	Sterol Content[a]		
				Campesterol	Stigmasterol	β-sitosterol
		gI/100g Oil	%	mg/100g Oil	mg/100g Oil	mg/100g Oil
Company A	Hydrosoy 65 I.V.					
	#40044	63.8	44.5	19	21	74
	#40045	62.9	46.7	20	20	73
	#40046	64.2	51.3	20	20	70
	#40047	62.9	51.9	25	23	88
	#40048	62.9	51.1	22	25	82
Company A	Hydrosoy 106 I.V.					
	#40049	106.3	19.9	30	32	120
	#40050	105.9	20.1	31	29	114
	#40051	105.9	16.2	31	30	135
	#40052	105.9	17.4	27	29	106
	#40053	106.3	18.9	35	33	129
Company A	Hydrosoy 109 I.V.					
	#40054	106.7	18.3	41	39	142
	#40055	106.7	15.4	40	38	149
	#40056	107.6	16.9	38	38	143
	#40057	106.7	16.9	41	37	140
	#40058	108.0	18.0	41	37	142

Table 12 (continued)

Source	Sample	Iodine Value	Trans-Isomer Content	Sterol Content[a]		
				Campesterol	Stigmasterol	β-sitosterol
		gI/100g Oil	%	mg/100g Oil	mg/100g Oil	mg/100g Oil
Company B	Soybean Oil 70 I.V.	73.7	53.8	18	19	81
Company B	Refined Soybean Oil	132.5	-	30	27	128
Company B	Slightly Hydrogenated Soybean Oil	95.1	24.9	26	26	118
Company B	Slightly Hydrogenated and Winterized Soybean Oil	93.9	29.5	22	21	95
Company C	Brand C, pHSB[b]	111.0	15.6	31	30	104
Company D	Brand D, pHSB[b]	109.8	12.6	28	29	127
Company E	Brand E, pHSB[b]	115.8	10.8	32	33	118

[a]Average of three measurements.

[b]pHSB, partially hydrogenated soybean oil.

Table 13. Iodine value, trans-isomer content and sterol content of cottonseed oil.

Source	Sample	Iodine Value	Trans-Isomer Content	Sterol Content[a]		
				Campesterol	Stigmasterol	β-sitosterol
		gI/100g Oil	%	mg/100g Oil	mg/100g Oil	mg/100g Oil
Company A	Winterized Cottonseed Oil #43779	111.5	-	20	5	347
Company A	Winterized Cottonseed Oil #43783	113.2	-	23	1	315
Company B	Winterized Cottonseed Oil	107.2	-	12	3	153
Company A	Hydrogenated Cottonseed Oil #43780	60.4	45.2	5	-	71
Company B	Deodorized Fully Hydrogenated Cottonseed Oil	18.7	-	-	88[b]	55

[a]Average of three measurements.

[b]Intensity of the peak with RRT (cholestane): 3.53.

Table 14. Iodine value, trans-isomer content and sterol content of palm oil.

Source	Sample	Iodine Value	Trans-Isomer Content	Sterol Content[a]		
				Campesterol	Stigmasterol	β-sitosterol
		gI/100g Oil	%	mg/100g Oil	mg/100g Oil	mg/100g Oil
Company A	Refined Palm Oil #43778	53.9	-	3	3	10
Company A	Refined Palm Oil #43783	56.5	-	2	2	8
Company B	Refined Palm Oil	67.7	-	7	7	24
Company A	Hydrogenated Palm Oil	26.0	15.6	3	2	12
Company B	Hydrogenated Palm Oil	25.6	-	12	16	34

[a]Average of three measurements.

Table 15. Iodine value, trans-isomer content and sterol content of refined, bleached, and deodorized coconut oil.

Source	Sample	Iodine Value[b]	Trans-Isomer Content	Sterol Content[a]		
				Campesterol	Stigmasterol	β-sitosterol[c]
		gI/100g Oil	%	mg/100g Oil	mg/100g Oil	mg/100g Oil
Company J	Coconut Oil	9.3	-	3	6	39
Company J	Coconut Oil	9.7	-	4	7	46
Company J	Coconut Oil	10.8	-	3	6	42
Company J	Coconut Oil	9.6	-	3	6	57
Company J	Coconut Oil	11.0	-	3	6	58

[a]Average of three measurements.

[b]Determined by Wijs' method.

[c]A combination of two sterols.

Table 16. Iodine value, trans-isomer content and sterol content of corn oil.

Source	Sample	Iodine Value	Trans-Isomer Content	Sterol Content[a]		
				Campesterol	Stigmasterol	β-sitosterol
		gI/100g Oil	%	mg/100g Oil	mg/100g Oil	mg/100g Oil
Company D	Refined Corn Oil	126.0	-	78	31	390
Company F	Refined Corn Oil	128.2	-	122	44	629
Company F	Refined Corn Oil	129.9	-	128	46	679
Company F	Refined Corn Oil	128.7	-	138	42	701
Company G	Refined Corn Oil	123.9	-	58	28	299

[a]Average of three measurements.

Table 17. Iodine value, trans-isomer content and sterol content of margarine.

Source	Type of Margarine	Oil Components	Iodine Value	Trans-Isomer Content	Sterol Content[a]		
					Campesterol	Stigmasterol	β-sitosterol
			gI/100g Oil	%	mg/100g Margarine		
Company D	Regular, ~79% Fat, Stick	C[b] & pHC[c]	95.1	22.5	27	18	120
Company F	Diet, ~42% Fat, Tub	C & pHC	114.9	16.4	37	14	193
Company F	Diet, ~42% Fat, Tub	C & pHC	114.5	15.3	39	14	201
Company H	Regular, ~79% Fat, Stick	C & pHC	102.9	24.9	35	18	193
Company H	Regular, ~79% Fat, Stick	C & pHC	101.6	25.6	40	17	208
Company H	Regular, ~79% Fat, Stick	C & pHC	101.6	20.5	40	17	209
Company I	Regular, ~79% Fat, Stick	C & pHC	100.7	27.1	66	25	331
Company I	Regular, ~79% Fat, Stick	C & pHC	100.7	25.5	70	25	338
Company I	Whipped, ~77% Fat, Tub	C & pHC	99.4	28.9	90	28	432
Company I	Whipped, ~77% Fat, Tub	C & pHC	99.4	26.3	75	30	436

[a]Average of three measurements.

[b]C, liquid corn oil.

[c]pHC, partially hydrogenated corn oil.

Table 18. Iodine value, trans-isomer content and sterol content of shortening.

Source	Sample	Oil Components	Iodine Value	Trans-Isomer Content	Sterol Content[a]		
					Campesterol	Stigmasterol	β-sitosterol
			gI/100 Oil	%		mg/100g Oil	
Company C	Brand C	pHSB[b] & pHP[c]	89.6	17.4	21	21	74
Company C	Brand C	pHSB & pHP	88.7	16.9	23	20	70
Company C	Brand C	pHSB & pHP	88.7	15.7	23	20	74
Company C	Brand C	pHSB & pHP	89.6	17.1	24	23	78
Company D	Brand D	pHSB & pHP	92.6	25.9	11	12	52
Company D	Brand D	pHSB & pHP	95.1	25.5	11	12	53
Company E	Brand E	pHSB & pHCS[d]	90.9	25.9	24	27	105
Company E	Brand E	pHSB & pHCS	90.0	27.0	24	26	98
Company F	Brand F	pHSB & pHP	92.1	20.7	18	21	72
Company F	Brand F	pHSB & pHP	92.1	19.0	18	21	72

[a]Average of three measurements.

[b]pHSB, partially hydrogenated soybean oil.

[c]pHP, partially hydrogenated palm oil.

[d]pHCS, partially hydrogenated cottonseed oil.

Winterized cottonseed oil in Table 13, page 45, (I.V. 107.2 to 113.2) contained small amounts of campesterol (12-23 mg/100 g oil) and very small amounts of stigmasterol (1-5 mg/100 g oil) in comparison with β-sitosterol (153 to 347 mg/100 g oil). Weihrauch (1977) reported refined cottonseed oil contained, in 100 g oil, 20 mg campesterol, traces of stigmasterol and 303 mg β-sitosterol. Hydrogenated cottonseed oil samples with I.V. of 60.4 and 18.7, respectively, had 71 and 55 mg β-sitosterol, respectively, in 100 g oil (Table 13, p. 45). These values are low compared to 223 mg β-sitosterol/100 g refined, hydrogenated cottonseed oil reported by Weihrauch (1977). He reported no data concerning the level of saturation of the cottonseed oil.

Refined palm oil (Table 14, p. 46) with I.V. from 53.9 to 67.7 had very small amounts of sterols. For example, 8 to 24 mg of β-sitosterol/100 g oil, the most abundant sterol, were found in refined palm oil samples. These values compare well with 30 mg/100 g oil in refined palm oil reported by Weihrauch (1977). Hydrogenated palm oil samples (I.V. 25.6 and 26.0) contained 12 and 34 mg β-sitosterol, respectively.

Refined coconut oil samples (I.V., 9.3 to 11.0) contained in 100 g oil, 3 to 4 mg campesterol, 6 to 7 mg stigmasterol, and 39 to 58 mg β-sitosterol (Table 15, p. 47). These values compare well with 6 mg campesterol, 12 mg stigmasterol, and 53 mg β-sitosterol per 100 g refined coconut oil reported by Weihrauch (1977).

The sterol content of refined corn oil samples (I.V. 123.9 to 129.9) had wide variation in 100 g oil: 58-138 mg campesterol, 28-46 mg stigmasterol, and 299-701 mg β-sitosterol (Table 16, p. 48). The higher

values compare well with those in refined corn oil reported by Weihrauch (1977): 158 mg campesterol, 76 mg stigmasterol, and 690 mg β-sitosterol per 100 g oil.

Corn oil margarines which contained blends of liquid corn oil and partially hydrogenated corn oil had iodine values ranging from 95.1 to 114.9 and trans isomer content ranging from 15.3 to 28.9% (Table 17, p. 49). Again, wide ranges in sterol content were found per 100 g margarine; 27 to 90 mg campesterol, 14 to 30 mg stigmasterol, and 120 to 436 mg β-sitosterol. The type of margarines had some effect on sterol level. Large differences in sterol content were found among margarines from different companies. For example, margarines from company I had 338-436 mg β-sitosterol/100 g margarine compared with 193-209 mg in margarines from company H and 120 mg in margarine from company D. Also, in margarine from the same company, tub margarines had more sterols than stick margarines (company I, Table 17, p. 49). Weihrauch (1977) reported corn oil margarines (regular stick and tub) had 95-130 mg campesterol, 38-65 mg stigmasterol, and 331-392 mg β-sitosterol per 100 g margarine.

Shortenings containing a blend of partially hydrogenated soybean oil (pHSB) with partially hydrogenated palm oil (pHP) or partially hydrogenated cottonseed oil (pHCS) had iodine values ranging from 88.7 to 95.1 and trans-isomer content from 15.7 to 27.0% (Table 18, p. 50). Shortenings containing pHSB and pHP had in 100 g oil: 11-24 mg campesterol, 12-23 mg stigmasterol, and 52-78 β-sitosterol. Shortenings containing pHSB and pHCS had higher sterol content than the shortenings containing pHSB and pHP.

Quantitative Changes in Sterols During Oil Processing

As expected, a decrease in sterol content was found with each additional processing step. For example, from company B (Table 12, p. 43), refined soybean oil had 128 mg β-sitosterol/100 g oil. After being slightly hydrogenated, this amount dropped to 118 mg/100 g oil; and after being slightly hydrogenated and winterized the oil contained 95 mg β-sitosterol/100 g oil. If the oil were hydrogenated to an I.V. of 73.7, it contained only 81 mg β-sitosterol/100 g oil. Cottonseed oil (Table 13, p. 45) also lost sterols during hydrogenation process.

There was as wide a variation in sterol content among brands of corn oil (Table 16, p. 48) and corn oil margarine (Table 17, p. 49) as there was between refined corn oil and corn oil margarine which contained hydrogenated corn oil blended with corn oil. Corn oil margarines, however, generally had the lowest sterol content compared with refined corn oil.

Identification of Some Minor Sterol Components in the GLC Chromatogram

Chromatograms representative of soybean oil sterols are shown in Figure 3. Several peaks were observed besides the sterols quantitated. The peaks with retention relative to cholestane and identification are:

Peak No.	Identification	Relative Retention Time
1	Cholestane	1.00
2		1.20
3		1.29
4		1.44
5		1.60
6		1.75
7	Cholesterol	2.40
8	Brassicasterol	2.70
9	Campesterol	3.10
10	Stigmasterol	3.37

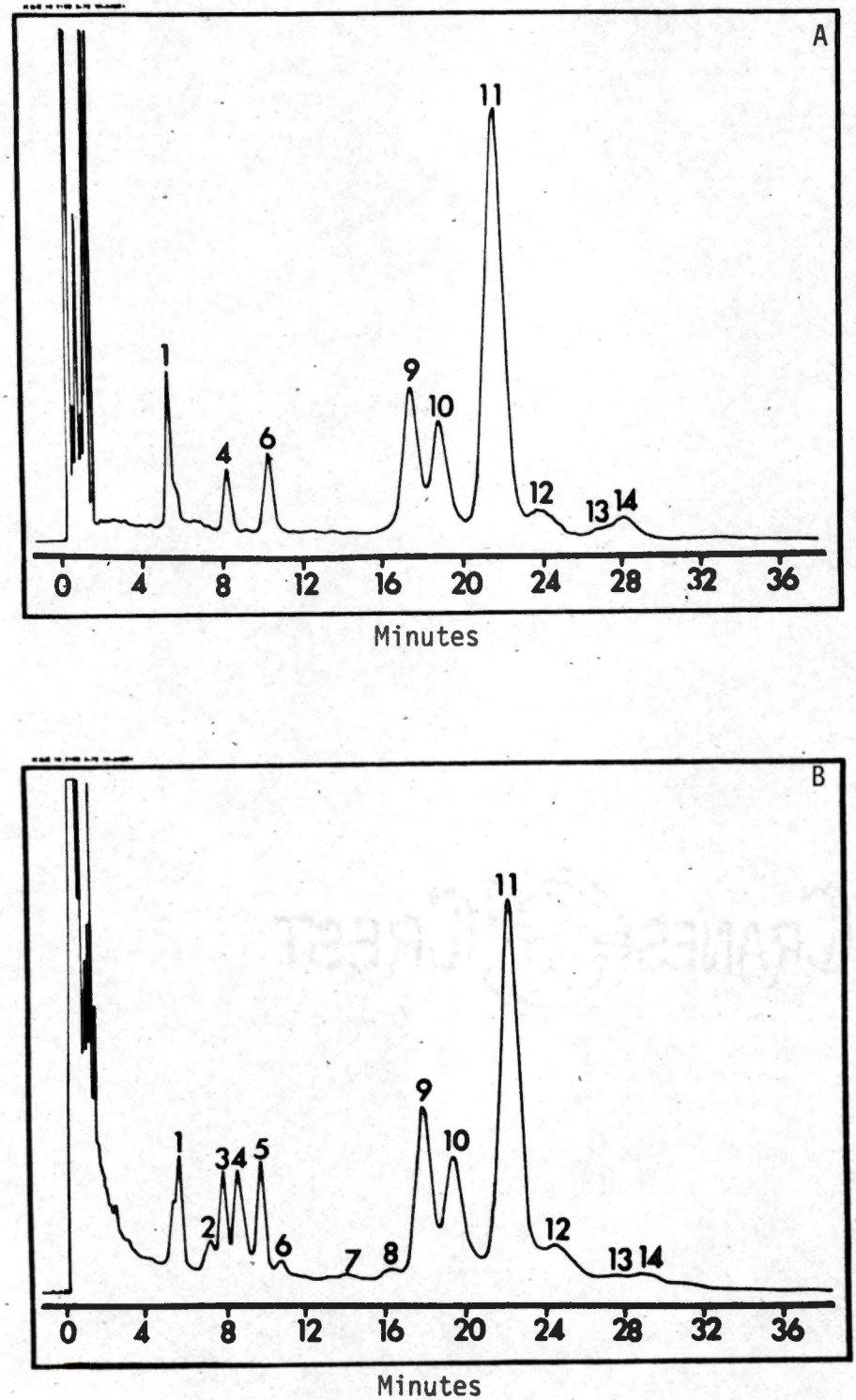


Figure 3. Representative chromatograms of soybean sterols: (A) refined soybean oil, and (B) winterized and partially hydrogenated soybean oil.

Peak No.	Identification	Relative Retention Time
11	β-sitosterol	3.87
12	Δ^5-avenasterol	4.25
13		4.63
14		4.83

The unidentified peaks 2 through 6 in Figure 3-B (brand C soybean oil) were found also in brand D soybean oil and soybean oil I.V. 106 from company A. Other soybean oils had the same chromatogram as the refined soybean oil in which only unidentified peaks 4 and 6 were present. These peaks most likely are tocopherols. Nelson and Milun (1968) identified several tocopherols (α-, β-, δ- and Δ-tocopherols) while investigating sterols in soybean residues. These tocopherols had shorter retention times than campesterol on a nonpolar column, and Nelson and Milun (1968) took special precaution to prevent their oxidation. Since no special precaution was taken to prevent oxidation in the present investigation, it is reasonable to assume that the lack of peaks in several of the soybean oils is because of the oxidation prior to or during analysis.

Peak 8 in Figure 3-B is tentatively identified as brassicasterol. This peak has a RRT (cholestane) of 2.70 or RRT (cholesterol) of 1.15. These relative retention times are comparable with the RRT (cholesterol) of 1.12 for brassicasterol on an OV-101 column (Nordby and Nagy, 1973). This column was the same as that used in the present study.

Chromatograms for sterols in cottonseed oil are shown in Figure 4. Deodorized fully hydrogenated cottonseed oil (Figure 4-C) had a peak with a RRT (cholestane) of 3.53 which is between that of stigmasterol and β-sitosterol. This peak would mask any stigmasterol content (which is low in cottonseed anyway) and was not identified. The

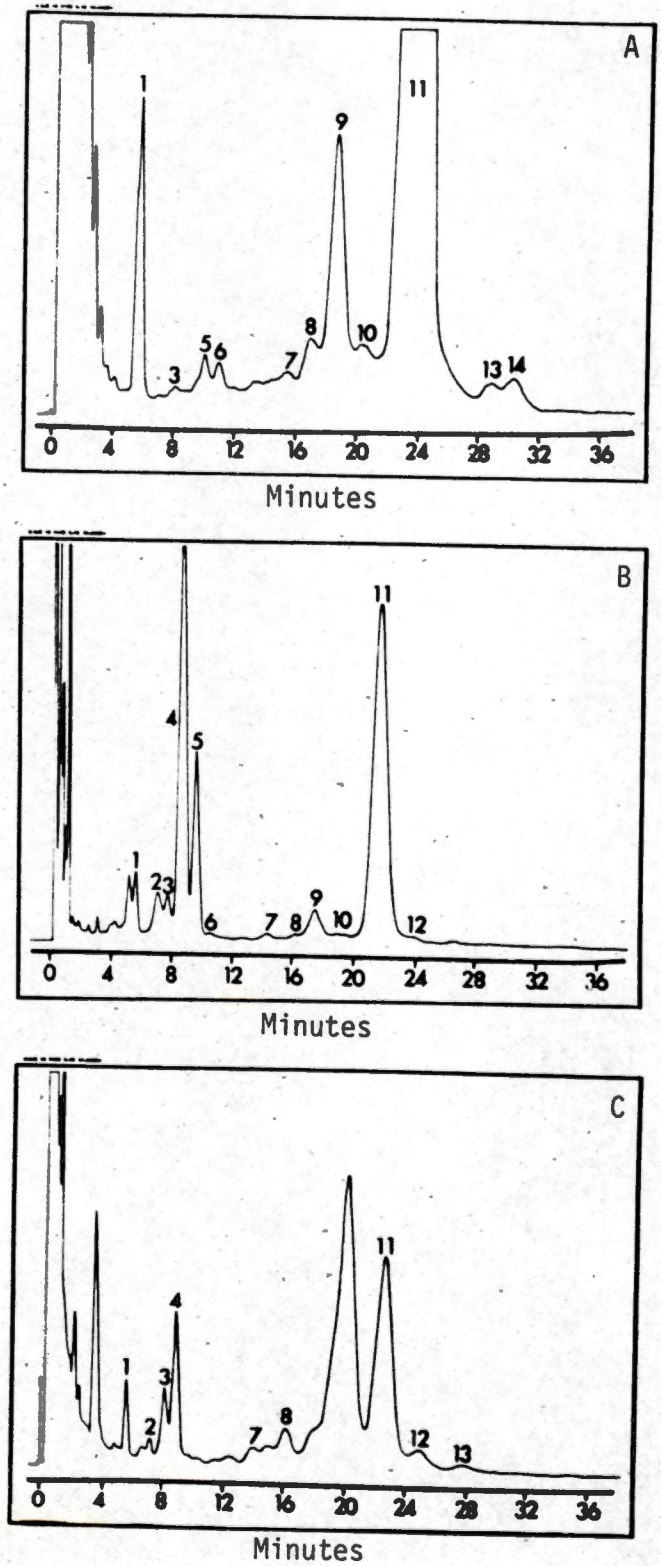


Figure 4. Representative chromatograms of sterols in (A) winterized cottonseed oil, (B) partially hydrogenated cottonseed oil, and (C) fully hydrogenated, deodorized cottonseed oil.

sample was hydrogenated under low selectivity conditions (high hydrogen pressure, 10 atmospheres, and low temperature, 120°C) since it had no measurable trans isomers (Coenen, 1976). Under these conditions, it is possible that stigmasterol was hydrogenated to Δ^{22}-stigmasterol which could be the sterol interferring with the stigmasterol peak. Other peaks (2 through 6) in Figure 4 had the same retention times as those peaks found in soybean oil and are most likely tocopherols. Also, small amounts of brassicasterol (tentatively identified as peak 8) were found in winterized and fully hydrogenated cottonseed oil.

The chromatogram of sterols in coconut fat (Figure 5) exhibits an unexpected sterol type which interferes with the analysis of the β-sitosterol. Copius-Peereboom (1965) observed this peak in coconut oil sterols and suggested that this so-called "third band sterol type" had the tentative structure of $\Delta^{5,24}$(28)-ergostene-3β-ol. This investigator separated sterols on a SE-30 column and found that the sterol eluded slightly before β-sitosterol. In the present study, it appeared to elude slightly after β-sitosterol on OV-101 column. Because of the presence of this sterol, the quantitation of β-sitosterol (Table 15, p. 47) must be questioned since the area of peak 11 (β-sitosterol) in Figure 5 is due to two sterols.

Chromatograms of palm oil sterols are shown in Figure 6. Palm oil had cholesterol present (peak 7) and traces of peaks 2 through 6 which most likely are tocopherols.

Chromatograms of refined corn oil are shown in Figure 7. Corn oil besides having traces of brassicasterol (peak 8) also had some peaks (2 through 6) which were present in some brands but not in others.

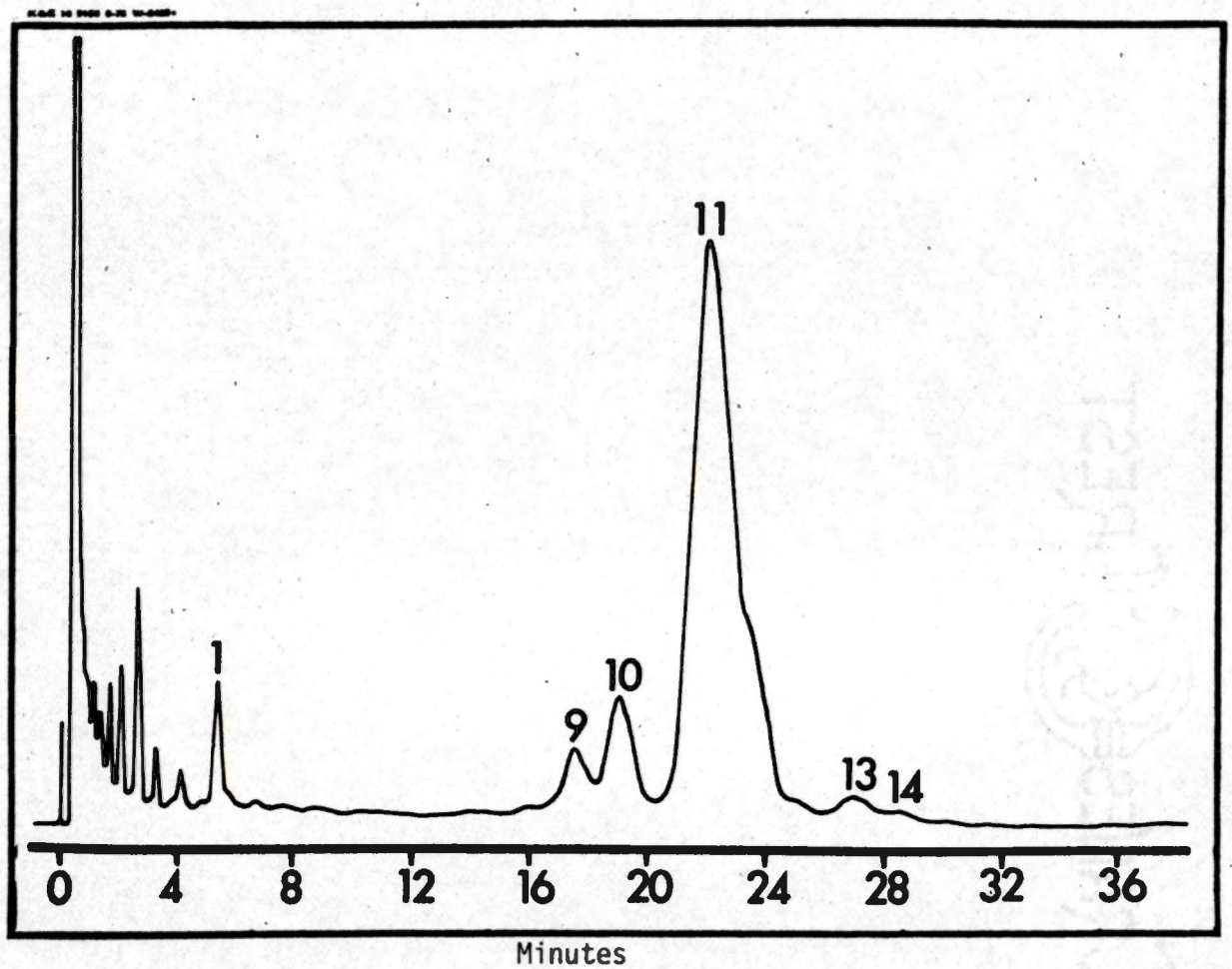
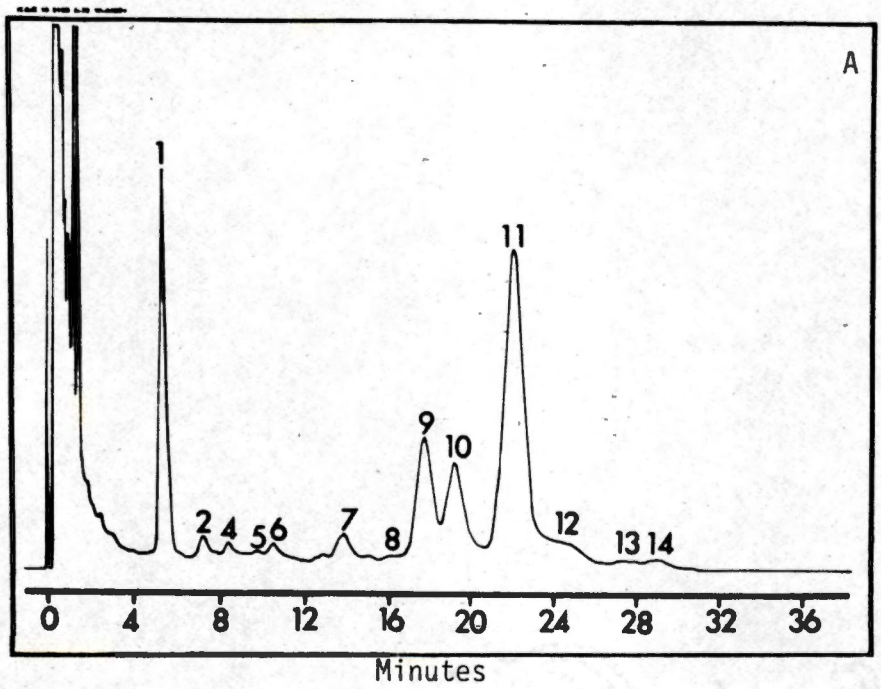


Figure 5. Representative chromatogram of coconut oil sterols.



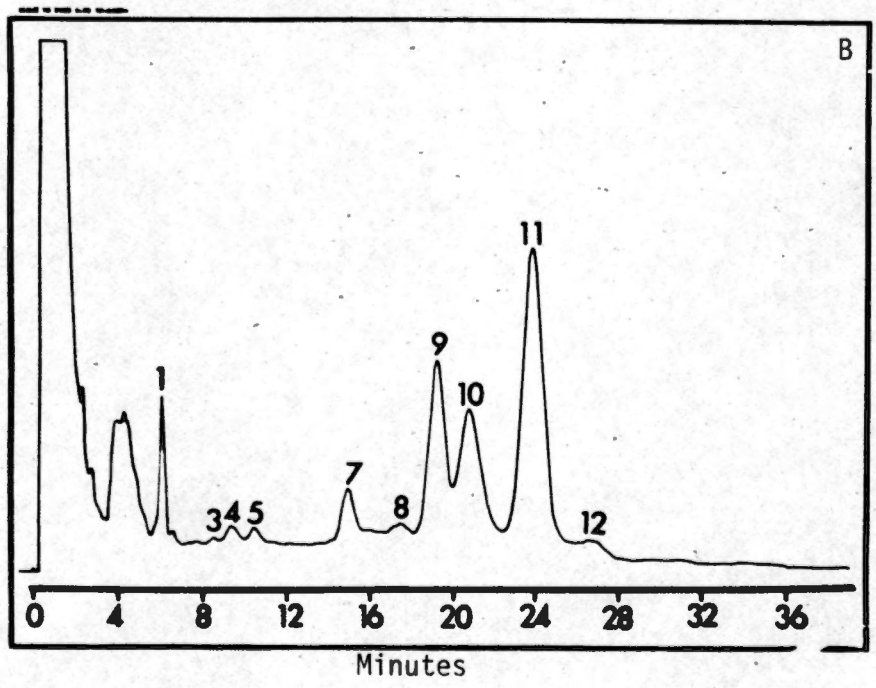
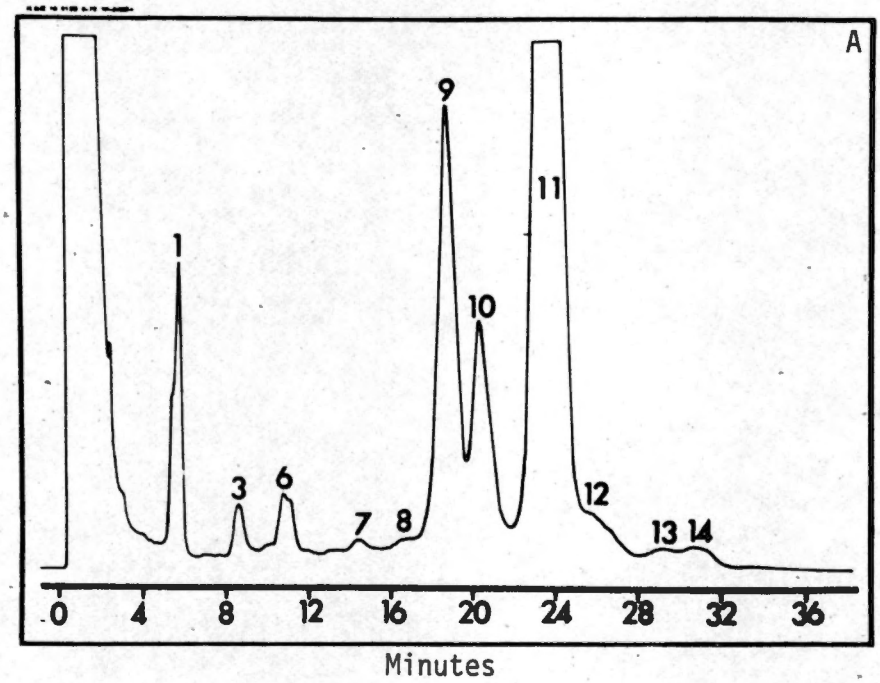


Figure 6. Representative chromatograms of sterols in (A) refined palm oil, and (B) refined, hydrogenated palm oil.



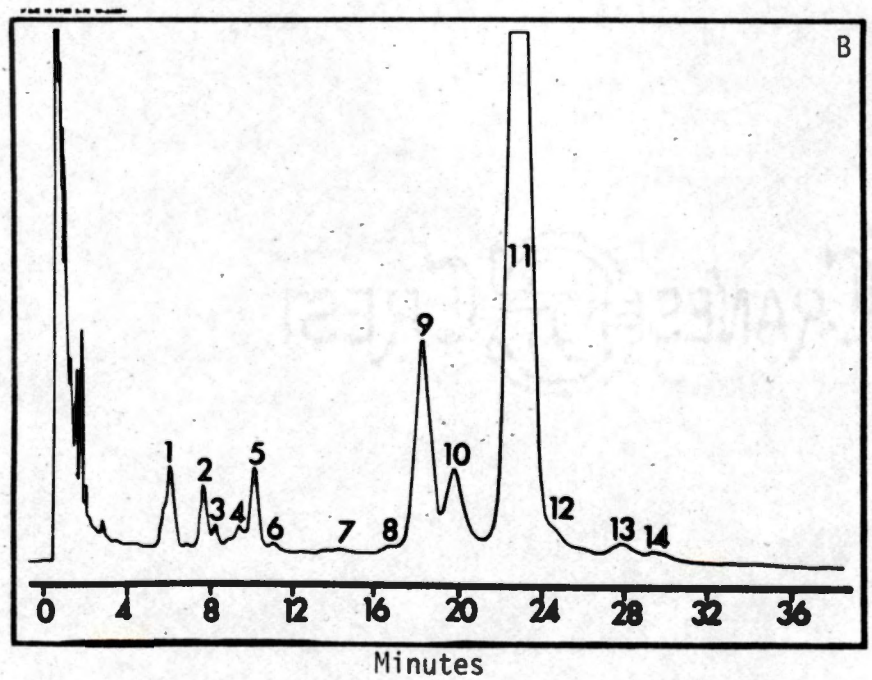


Figure 7. Representative chromatograms of sterols in (A) brand D corn oil, and (B) brand G corn oil.

Again, if these peaks are tocopherols, they could be oxidized to varying degrees either prior to or during analysis.

Figure 8 shows chromatograms of sterols in corn oil margarines. Once again, differences in the number of peaks 2 through 6 were observed among brands.

Figure 9 shows sterol chromatograms for shortening. Differences among brands were observed in the numbers of peaks 2 through 6. These differences could have been because of oil source as well as oxidation. Brand E shortening (Figure 9-A) consisted of partially hydrogenated soybean oil and partially hydrogenated cottonseed oil, and brand C shortening (Figure 9-B) consisted of partially hydrogenated soybean oil and partially hydrogenated palm oil. The other two shortenings (brand K and brand D) had the same type blends as brand C shortening and their chromatograms were similar to Figure 9-B.

In many chromatograms, unidentified peaks 12, 13, and 14 had RRT (cholestane) of 4.25, 4.63 and 4.83, respectively, and these relative retention times were longer than β-sitosterol RRT (cholestane), 3.87. It is reasonable to assume that these peaks represent sterols. Nordby and Nagy (1973) found that fucosterol, isofucosterol, and stigmastanol eluded after β-sitosterol on an OV-101 column. However, the RRT for peak 12 which can be recalculated relative to cholesterol as 1.77 in the present study was higher than the RRT (cholesterol) of 1.66 for isofucosterol or 1.68 for stigmasterol reported by Nordby and Nagy (1973). In comparison, the RRT (cholesterol) for β-sitosterol in the present study is 1.61 compared to 1.65 reported in that same investigation. Itoh et al. (1973) reported the presence of Δ^5-avenasterol (a

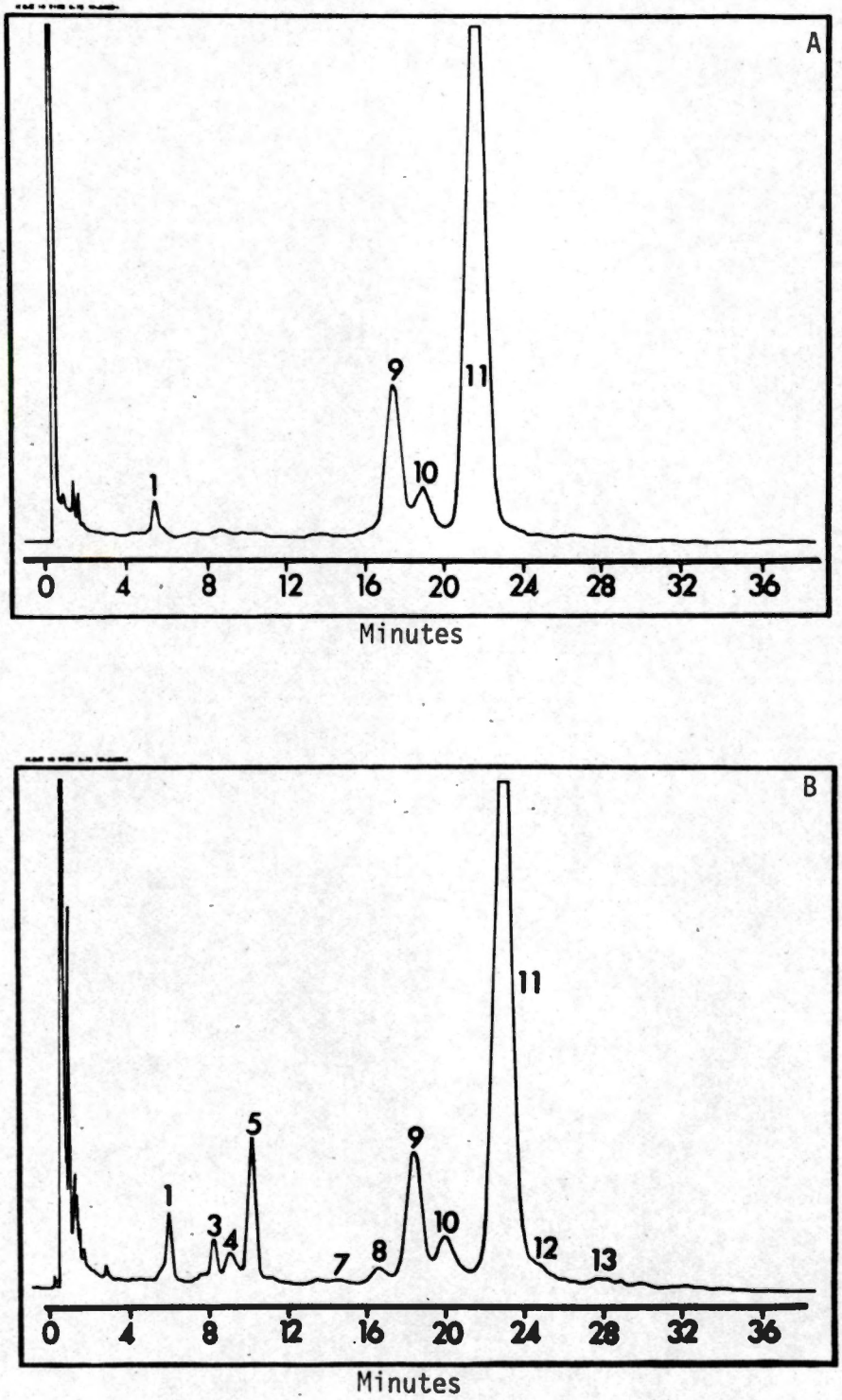


Figure 8. Representative chromatograms of sterols in (A) brand P corn oil margarine, and (B) brand H corn oil margarine.

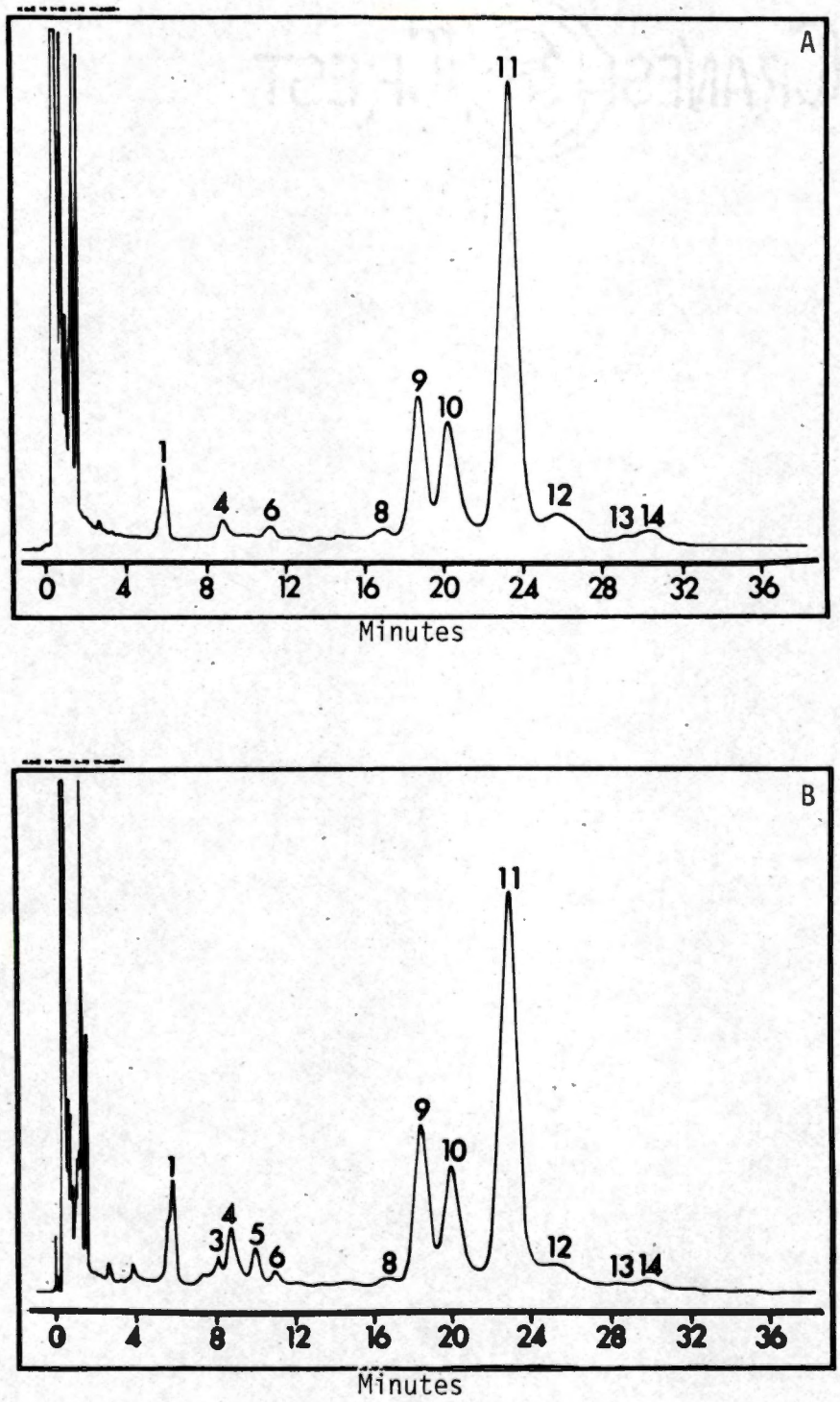


Figure 9. Representative chromatograms of sterols in (A) brand E shortening, and (B) brand C shortening.

C-29 isomer of fucosterol) in the fats and oils and showed that this sterol eluded slightly after fucosterol on an OV-17 column. It is possible that peak 12 is Δ^5-avenasterol. Weihrauch (1977) reported that corn oil, cottonseed oil, soybean oil, coconut oil, and palm oil had measurable amounts of Δ^5-avenasterol and small or trace amounts of Δ^7-stigmastenol and Δ^7-avenasterol. It is possible that peaks 12, 13, and 14, respectively, represent these sterols.

In soybean oil (Figure 3, p. 54), there seemed to be a peak which eluded with cholestane (shouldered cholestane peak). This would increase the area of the internal standard 1 peak (cholestane). If this were true, values determined for campesterol, stigmasterol, and β-sitosterol in soybean oil should have been lower. However, values determined for these sterols in the present study, were in the same range as those reported by Weihrauch (1977). It must be assumed, then, that there was little interference with the internal standard peak. Caution must be taken during analysis of sterols by GLC, however, and more research is needed to identify positively whether the clean-up procedures are needed prior to sterol analysis for any one oil as recommended by Rozanski (1966) and Thorpe et al. (1969).

Conclusions

Under the conditions of this study, it was concluded that:

1. For GLC analysis of the three major sterols; campesterol, stigmasterol, and β-sitosterol.
   a. OV-101 was the most suitable liquid phase when compared with SE-30, OV-17 and SP-1000.

b. Either acetate derivative or TMS-ether derivative can be used satisfactorily for sterol analysis but the acetate derivative gives better resolution for the campesterol-stigmasterol pair and requires less cleaning of the detector during the analysis.

2. Campesterol, stigmasterol, and β-sitosterol content, in mg/100 g sample, respectively, in fats and oils were as follows: 28-32, 29-33, and 104-127 for soybean oil; 12-23, 1-5, and 153-347 for cottonseed oil; 2-12, 2-16, and 8-34 for palm oil; 3-4, 6-7, and 39-58 for coconut oil; 58-138, 28-46, and 299-701 for corn oil; 27-90, 14-30, and 120-436 for corn oil margarine, and 11-24, 12-27, and 52-105 for shortening.

3. Occurrence of tocopherols (tentatively identified) and sterols in minor amounts, tentatively identified as brassicasterol and Δ^5-avenasterol, were detected in all the fats and oils investigated. Difference in tocopherol components were observed among the same type of fat and oil samples.

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