Fate of Dietary Sterols in Hydrogenated Oils and Fats

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ABSTRACT

Samples of table margarines, so-called polyunsaturated table margarines, hydrogenated vegetable oils, and so-called polyunsaturated hydrogenated vegetable oils were shown by infrared spectroscopy to contain hydrogenated components. Examination of the sterols from these oils by argentation thin layer chromatography and gas liquid chromatography did not reveal campestanol, stigmastanol, or Δ^{22}-stigmastenol, the expected hydrogenation products of the natural sterols. The sterol compositions of the above samples, animal fats, and blends of hydrogenated vegetable oils and animal fats were determined. The compound 24-methyl cholest-7-en-3β-ol was identified tentatively in sunflower and safflower oils.

INTRODUCTION

In 1951, Peterson (1) reported that the addition of phytosterols to a diet rich in cholesterol prevented the increase in plasma cholesterol which usually occurs after cholesterol feeding in chicks. Following Peterson's report, other workers reported on the hypocholesterolemic effect of phytosterols in the rabbit (2) and in the rat (3). Kritchevsky (4) has summarized the reported studies on man, most of which resulted in lowered plasma cholesterol levels. The hypocholesterolemic effect of phytosterols and their effect on cholesterol levels has been reviewed (5-7).

In a recent, critical examination of the literature of saturated fat in the diet and plasma cholesterol concentration, Reiser (8) considered that the experimental data suggesting a hypercholesterolemic effect of hydrogenated oils were weak and susceptible to alternative interpretations. A possible explanation for the reported differences in plasma cholesterol concentrations after ingestion of hydrogenated or natural vegetable oils, is the effect of hydrogenation on the phytosterols. Reiser considered it reasonable to assume that unsaturated phytosterols are hydrogenated along with the fatty acids and lose their hypocholesterolemic properties. This paper reports on the examination of margarines and shortenings for evidence of sterol hydrogenation.

EXPERIMENTAL PROCEDURES

Samples

Samples of 1 pound cubes of table margarine, so-called polyunsaturated table margarine, hydrogenated vegetable oil, so-called polyunsaturated hydrogenated vegetable oil, animal fat, and blends of hydrogenated vegetable oil and animal fat, were purchased at regular intervals from supermarkets in Brisbane, Australia, from August, 1973, to May, 1974. Fat was extracted from the margarines by melting at 45 C, centrifuging, and filtering through filter paper to dry the samples. Oil and fat samples were melted and used without further treatment. All samples were stored under nitrogen at -20 C until required for analysis.

Check for Hydrogenation

The infrared (IR) spectra between 2000cm^{-1} and 650cm^{-1} were recorded using a rapid thin film technique (9). The intensity of the band at 967cm^{-1}, due to isolated *trans* unsaturation, was used to indicate if part or all of the oils and fats had been hydrogenated. A Perkin-Elmer Model 237 Infrared Spectrophotometer was used in this study.

Preparation of Campestanol and Stigmastanol

Campestanol and stigmastanol were prepared from campesterol and stigmasterol, respectively, by dissolving the sterols in ethyl acetate which contained a trace of acetic acid and hydrogenating at room temperature with a slight pressure of hydrogen. Platinum dioxide was used as a catalyst. The mixture was stirred by a magnetic stirrer and allowed to react overnight. The catalyst was removed by transferring the mixture to a small chromatography column containing Florisil, and eluting the hydrogenated component with chloroform.

Preparation of Δ^{22}-Stigmastenol (24-Ethyl Cholest-22-en-3β-ol)

Two g sodium was added in small pieces to a boiling solution of 200 mg 24-ethyl cholest-4,22-dien-3-one (Koch-Light Laboratories, Colnbrook, England) in 20 ml dry n-pentanol. The solution was refluxed for 30 min after the sodium had dissolved. Water was added, and the n-pentanol layer was separated, dried over sodium sulphate, and distilled under reduced pressure. The reaction product then was chromatographed on 10 g neutral alumina (Brockman Activity 111), with gradient amounts of benzene in petroleum ether. Fractions were monitored by GLC. The fraction containing Δ^{22}-stigmastenol was impure, containing possibly the 3α-ol isomer (10). This fraction was dissolved in 95% ethanol and reacted with an excess of digitonin in 95% ethanol, and allowed to stand overnight. The resulting digitonide was collected and cleaved using dimethyl sulphoxide (11). The liberated sterol, having a 3β-ol configuration, was found to be better than 99% pure by GLC analysis. The retention time on the 3 phases, OV-17, QF-1, and SP-1000 was less than that of stigmastanol. From published data (12,13), it is seen that in the C24 alkyl substituted series, the Δ^{22}-bond shows an anomalous effect in causing a reduction in retention time as compared to that of the saturated compound. The sterol had a mp of 160 C; Δ^{22}-stigmastenol is reported to have a mp of 159 C (10). The IR spectrum of a 1% solution in carbon disulphide was typical of a sterol with the 3β-ol configuration. In addition, the spectrum showed a strong absorption band at 972cm^{-1} which was attributed to the Δ^{22}-*trans* disubstituted double bond (14). No absorption bands were found at 840cm^{-1} and 800cm^{-1}, which indicated the absence of Δ^{5}-unsaturation.

Gas Liquid Chromatography (GLC) Analysis of Sterols

Sterols were isolated from the unsaponifiable fraction of the oils and fats by preparative thin layer chromatography (TLC) (15). The sterol composition was determined quantitatively by the GLC method outlined previously (16), except that the sterols were chromatographed in the free form and on a 1.83 m x 2 mm internal diameter (ID) glass column packed with 3% OV-17 on 100-120 mesh Gas-Chrom Q.

GLC sterol profiles of the oils and fats were studied using 1.83 m x 2 mm ID glass columns packed with 3% OV-17 on 100-120 mesh Gas-Chrom Q; 3% QF-1 on 100-120 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, PA), and 1% SP-1000 on 100-120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA). GLC analyses were carried out using a Varian-Aerograph Model 1840 Gas Chromatrograph. Quantitative results were obtained with the aid of a Varian-Aerograph Model 480 Digital Integrator. Cholesterol (99+% pure), campesterol (99% pure), stig-

TABLE I

Relative Retention Times of Sterols

Sterol	Liquid Phase		
	OV-17	SP-1000	QF-1
Cholesterol	1.00	1.00	1.00
Cholestanol	1.00	0.91	0.91
Campesterol	1.34	1.34	1.34
Campestanol	1.33	1.20	1.52
Δ22-Stigmastenol	1.46	1.29	1.50
Stigmasterol	1.47	1.41	1.39
Stigmastanol	1.69	1.49	1.77
β-Sitosterol	1.68	1.64	1.63
Δ7-Stigmastenol	1.98	2.00	1.89.

masterol (99.5% pure), and β-sitosterol (95% pure) were obtained from Applied Science Laboratories. Cholestane and cholestanol were from Sigma Chemical Co., (St. Louis, MO). Δ7-Stigmastenol was isolated from sunflower seed oil.

Preparative Thin Layer Chromatography

To separate Δ5-sterols from 5α-stanols, the isolated sterols, dissolved in chloroform, were applied as a band to 20cm x 20cm glass TLC plates coated to a thickness of 0.3 mm with Kieselgel G (E. Merck, Darmstadt, Germany) containing 25% (w:w) silver nitrate. The plates were developed using the solvent system chloroform:ethanol (99.8:0.2). Bands were visualized under UV light after being sprayed with 0.05% ethanolic 2',7'-dichlorofluorescein and scraped into small chromatography columns containing a 3 cm plug of Florisil. The components of the bands were eluted with 100 ml chloroform.

RESULTS AND DISCUSSION

The vegetable oils most likely to be used for the manufacture of margarine and hydrogenated vegetable oil contain 3 major sterols: campesterol, stigmasterol, and β-sitosterol. Sunflower and safflower oils, used in polyunsaturated products because of their high content of linoleic acid, contain an additional major sterol, Δ7-stigmastenol (16-19). On hydrogenation, campesterol (24-methyl cholest-5-en-3β-ol) would be converted to campestanol (24-methyl-5α-cholestan-3β-ol). Stigmasterol (24-ethyl cholest-5,22-dien-3β-ol) on hydrogenation may produce 2 isomers. If the side chain is hydrogenated, β-sitosterol (24-ethyl cholest-5-en-3β-ol) would be produced. Hydrogenation of the ring would produce Δ22-stigmastenol (24-ethyl cholest-22-en-3β-ol). β-Sitosterol and Δ22-stigmastenol on hydrogenation both would produce stigmastanol (24-ethyl-5α-cholestan-3β-ol). If either sunflower or safflower oil were hydrogenated, Δ7-stigmastenol (24-ethyl cholest-7-en-3β-ol) would also be converted to stigmastanol. IR spectra of the oils from the table margarines, so-called polyunsaturated table margarines, hydrogenated vegetable oils, and so-called polyunsturated hydrogenated vegetable oils showed an absorption band at 967cm^{-1} due to isolated *trans* unsaturation. The GLC sterol profile of these oils showed they were solely of vegetable origin. Common vegetable oils do not contain acids with a *trans* configuration (20,21). Thus the *trans* unsaturation present in these oils can be considered to result from hydrogenation of part or all of the oils. The animal fats and blends of hydrogenated vegetable oils and animal fats also exhibited an absorption band at 967 cm^{-1}. In these 2 groups of oils, *trans* unsaturation could be due to the animal fat component, as the depot fat of ruminants contains *trans* fatty acids (22).

Numerous selective liquid phases were investigated to obtain the best GLC separation of the stanols and Δ22-stigmastenol from the sterols present in unhydrogenated oils. SP-1000 and QF-1 proved to be the most satisfactory. The relative retention times for the various sterols, stenols, and stanols, using these 2 phases, plus the nonselective phase OV-17, are given in Table I. The GLC sterol profiles of all the oils and fats collected for this study did not reveal the presence of campestanol, stigmastanol, or Δ22-stigmastenol. In many cases, however, there was not a complete return to base line between peaks, and this may have concealed small amounts of campestanol, stigmastanol, and Δ22-stigmastenol.

It has been demonstrated that 5-α-stanols can be separated from Δ5-sterols by argentation TLC (23). The sterols from the oils and fats were chromatographed on silver nitrate impregnated TLC plates. Sterols from all the so-called polyunsaturated table margarines, so-called polyunsaturated hydrogenated vegetable oils, and 1 sample of table margarine separated into 2 spots. One spot corresponded to a reference standard of a Δ5-sterol, the other more mobile spot to a reference standard of 5-α-stanol. Those samples exhibiting a spot corresponding to 5-α-stanol were fractionated by preparative argentation TLC, and the band corresponding to 5-α-stanol was examined by GLC on SP-1000 and QF-1 phases. Two peaks were observed; a major one having the same retention time as Δ7-stigmastenol, and a minor one having a retention time slightly less than that of β-sitosterol. Peaks corresponding to campestanol and stigmastanol were not detected in any of the samples. The band corresponding to Δ5-sterol was similar in sterol composition to the unfractionated samples, except that the peak representing Δ7-stigmastenol was absent.

It has been shown recently (19) on the evidence of combined gas chromatography-mass spectrometry data, that both sunflower and safflower oils contain Δ7-avenasterol (24-ethylidene cholest-7-en-3β-ol). Based on reported retention time, Δ7-avenasterol was found to be present in those samples which exhibited a spot by argentation TLC corresponding to 5α-stanol. It has been demonstrated that cholesterol and Δ7-cholestenol could be separated on silver nitrate impregnated plates (24,25). In this study Δ7-avenasterol chromatographed with the Δ5-sterols, and not with Δ7-stigmastenol. it would appear that only the Δ7-monounsaturated sterols are not strongly complexed with silver nitrate and migrate with 5-α-stanols. The minor component accompanying Δ7-stigmastenol in the band corresponding to 5α-stanol had a relative retention time (RRT) on OV-17 of 1.56 (cholesterol = 1.00) and 0.95 (β-sitosterol = 1.00). This component was also present in oil, freshly extracted in the laboratory, from both sunflower and safflower seeds. An unidentified sterol in safflower oil with RRT = 0.95 on OV-17 (β-sitosterol = 1.00) has been reported (19). A component with a RRT = 0.95 on OV-17 (β-sitosterol = 1.00) has been found in the sterol fraction of *Theaceae* and some other unusual vegetable oils, and identified on the basis of combined gas chromatography-mass spectrometry as 24-methyl cholest-7-en-3β-ol) (26). A sterol found in the Echinoderm *Asteries rubens* with a RRT = 1.58 on OV-17 (Cholesterol = 1.00) was found to be 24-methyl cholest-7-en-3β-ol (27).

24-Methyl cholest-7-en-3β-ol is the Δ7-isomer of campesterol. In this study, the GLC separation factor on OV-17 for campesterol: unknown sterol = 1.17, while the separation factor Δ7-:Δ5- for Δ7-stigmastenol:β-sitosterol = 1.18. On the basis of the TLC mobility, GLC retention time, and GLC separation factor, the unknown sterol from sunflower and safflower oil is considered to be 24-methyl cholest-7-en-3β-ol.

The sterol composition of the various margarine fats is given in Table II. Δ5-Avenasterol, which is present in small amounts in both sunflower and safflower oils, has a retention time slightly less than that of Δ7-stigmastenol. When both sterols are present, Δ5-avenasterol appears as a small shoulder on the leading edge of the Δ7-stigmastenol peak. Δ5-Avenasterol was not measured in this study, except in 3

TABLE II

Sterol Composition of Margarines Hydrogenated Vegetable Oils, and Animal Fats

Sterol Content (mg/100 g fat)

Classifications	No. of Samples	Cholesterol			Campesterol			Stigmasterol			β-Sitosterol			Δ^5-Avenasterol			Δ^7-Stigmastenol			Δ^7-Avenasterol			Total Sterols		
		Min.	Max.	Avg.	Min.	Max.	Avg.	Min.	Max.	Avg.	Min.	Max.	Avg.	Min.	Max.	Avg.	Min.	Max.	Avg.	Min.	Max.	Avg.	Min.	Max.	Avg.
So-called poly-unsaturated table margarine	30[a]	0	5.0	1.8	19.2	36.8	27.4	11.9	39.4	25.6	156.7	258.9	226.8				38.3	84.9	59.9	3.1	29.8	11.2	234.1	449.6	340.5
Table margarine	4[b]	0	1.7	1.3	14.9	17.6	16.1	4.3	13.9	7.5	106.5	169.9	149.0	5.8	9.7	7.7[c]	16.0	16.0	4.0[d]	2.8	2.8	0.7[d]	156.8	196.6	184.1
So-called poly-unsaturated hydrogenated vegetable oil	4[b]	3.7	7.3	5.0	30.3	39.6	35.2	21.3	28.6	25.6	149.5	179.2	167.1				45.2	55.1	49.3	2.1	6.0	4.2	256.3	308.2	285.4
Hydrogenated vegetable oil	4[b]	0.7	5.4	3.0	10.7	11.8	11.2	3.9	4.2	4.1	35.8	64.6	48.5										51.6	85.8	66.7
Blended hydrogenated vegetable oil & animal fat	12[a]	124.7	204.0	166.9	1.7	6.4	0.9[c]	3.1	3.1	1.0[d]	3.9	43.3	7.7[e]										135.7	210.8	175.8
Animal Fat	4[b]	113.9	202.8	167.5																			113.9	202.8	167.5

[a]Representing 3 brands from 3 different manufacturers.

[b]One single brand.

[c]Present in 3 samples only.

[d]Present in 1 sample only.

[e]Present in 7 samples only.

samples of table margarine where it was present in relatively large amounts. Of the 12 samples labeled as blended vegetable and animal oil, 5 samples did not contain vegetable oil at all, as judged by the absence of phytosterols in the GLC sterol profile. The remaining 7 samples contained little vegetable oil on the evidence of their cholesterol and phytosterol content.

Although stigmasterol, β-sitosterol, and campesterol can be hydrogenated readily in the laboratory, the presence of hydrogenation products of these sterols could not be detected in those oils and fats which, on IR evidence, indicated the presence of an hydrogenated component. A possible explanation for this is that the hydrogenation catalysts used and the conditions employed for selective hydrogenation of polyunsaturated acids to stable monenoic and dienoic isomers with little production of saturated acids may not be suitable for sterol hydrogenation.

Thus, the assumption (8) that the hypercholesterolemic effect of hydrogenated vegetable oils could be due to hydrogenation of the unsaturated sterols is not supported by the above results. However, the contribution of the sterols in so-called polyunsaturated table margarine and so-called polyunsaturated hydrogenated vegetable oil, which average 340.5 mg/100 g fat and 285.4 mg/100 g fat, respectively, would be negligible in reducing plasma cholesterol concentrations. Data on the hypocholesterolemic activity of phytosterols summarized by Kritchevsky (4) show that doses of 5-45g phytosterols daily were needed to affect varying degrees of plasma cholesterol reduction.

Further, it would be expected that from 25% to 80% of the phytosterol present would be in the form of esters (28), and it has been shown that esterification of phytosterols destroyed their ability to lower plasma cholesterol levels (29).

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