Purification of Sitosterol[1]

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ABSTRACT

Sitosterol and stigmastanol are obtained from the Raney nickel catalyzed hydrogenation of stigmasterol. The sitosterol was separated from stigmastanol by silica gel column chromatography of its dibromide. Sitosterol was also obtained from the steam deodorizer distillate of crude cottonseed oil and purified by manifold recrystallizations of the free sterol from isopropanol and its acetate from alcohol-acetic acid mixtures at a low temperature.

INTRODUCTION

The isolation of pure sitosterol in quantity has always been a problem (1-4). Although it is the most widespread sterol in the plant kingdom, unlike the animal sterol cholesterol or the fungal sterol ergosterol, it usually occurs mixed with substantial quantities of similar compounds such as campesterol, stigmasterol and brassicasterol in mixtures that are readily separable on an analytical basis (5-7) but not on a preparative scale (8). The Sitosterol, N.F. available commercially is a 40:60 mixture of campesterol and sitosterol that is probably derived from crude soybean sterols after removal of stigmasterol (9).

Our work with insects requires pure sitosterol for itself and as a starting material for the preparation of 5,7-stigmastadien-3β-ol and 7-stigmasten-3β-ol (10). Sitosterol has been obtained by the partial hydrogenation of stigmasterol (4) or its 3,5-cyclo derivative (1), by chemical synthesis from pregnenolone (3), by degradation of impure material and resynthesis (2) and from natural sources by manifold crystallizations of the benzoate and acetate from ethyl acetate and alcohol (8). We report here the purification of sitosterol from two sources: (a) the mixture obtained by the selective hydrogenation of stigmasterol over Raney nickel (11); and (b) the steam distillate obtained from the deodorizer process in the commercial refining of cottonseed oil.

The steam deodorization process concentrates the sterols in vegetable oils. Crude oils derived from the olive, cottonseed, soybean, peanut, palm kernel, rapeseed, sunflower seed and linseed contain 0.1-0.45% sterols (12-14) and are in themselves poor sources of these compounds. During the deodorization process, however, the sterols are selectively removed from the mass of triglycerides in the oil by superheated steam in a vacuum so that their concentrations in these steam distillates are greatly augmented (15,16). A search of the literature (13,17) suggested that the steam distillates from olive and cottonseed oils would be the best sources of sitosterol. These oils have 98 and 96%, respectively, of their sterol fraction as sitosterol, the remainder being campesterol.

Deodorizer distillates of olive oil obtained from California and Greece proved to be poor sources of sitosterol. The sterol concentrations in these products were low and the squalene concentrations were very high, so that it was impractical to isolate sitosterol from them. We accordingly turned to the deodorizer distillate from the processing of cottonseed oil and found it to be an excellent source of sitosterol.

MATERIALS, METHODS AND RESULTS

Analysis

The analyses for campesterol, sitosterol, stigmasterol and stigmastanol were performed by gas liquid chromatography (GLC) and thin layer chromatography (TLC) as described in a following communication (18). In addition, sterols were separated from steryl esters by TLC on silica gel with cyclohexane-ethyl acetate 60:40.

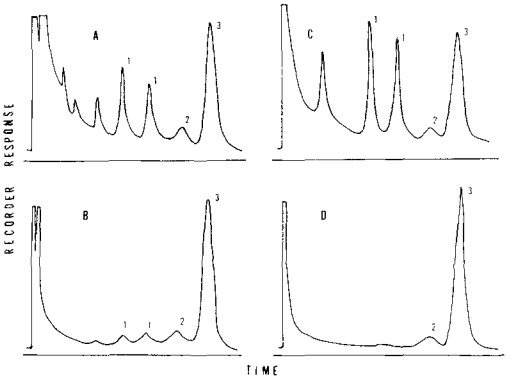

FIG. 1. Gas liquid chromatography diagrams: (1) tocopherols, (2) campesterol, (3) sitosterol. A. Total steam deodorizer distillate from cottonseed oil, B. solids from A, C. filtrate from A, D. crystals from B after crystallization from ethanol.

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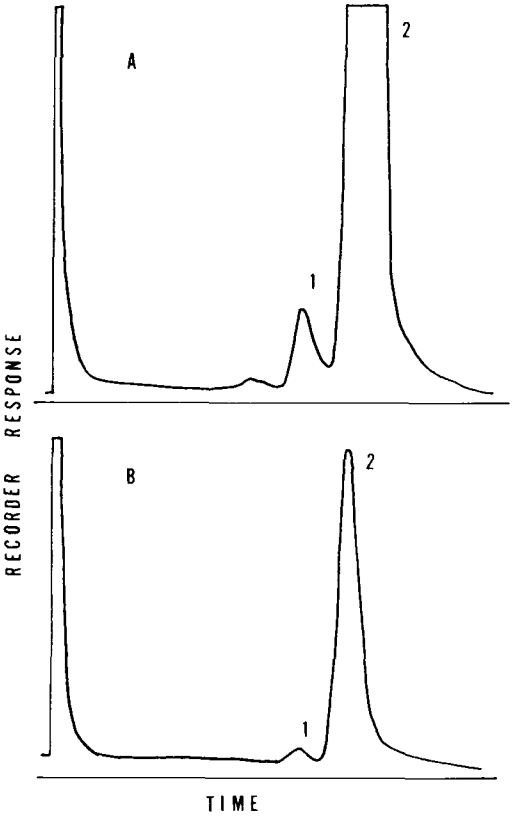

FIG. 2. Gas liquid chromatography diagrams: (1) campesterol, (2) sitosterol. A. Fraction Z, 5 μl (50 mg/ml), B. fraction Z, 1.4 μl (50 mg/ml).

Melting Points

Melting points were taken in vacuo; corrected.

Deodorizer Distillates

Deodorizer distillates from cottonseed were obtained from Ranchers Cotton Oil Co., Fresno, Calif., and those from olive from Wilsey Foods, Inc., Lindsay, Calif., and from Elais Oleaginous Products, S.A., Neon Faliron, Pireaeus, Greece.

Sitosterol from the Selective Hydrogenation of *Stigmasterol*

A typical hydrogenation of stigmasterol over Raney nickel (11) gave a product having the composition: 67% sitosterol, 31% stigmastanol, 2% stigmasterol (18). Twelve grams of this mixture in 200 ml ether was treated dropwise with 4.0 g Br_2 in 50 ml ether at room temperature and then placed in a freezer at -20 C for 3 days. After removal of 1.7 g stigmastanol, the filtrate was concentrated in vacuo without heat and the residue chromatographed on a 400 g silica gel column with ether-low boiling petroleum ether 1:4. The first four fractions (150 ml each) contained nothing, the next six contained 5.5 g sitosterol dibromide and the last six were composed of the dibromide and stigmastanol. The latter were rechromatographed in the same way to yield more of the dibromide free of the stanol. The combined dibromides were refluxed 4 hr in 250 ml ethanol-acetic acid 1:1 over 5 g Zn dust to yield 5.1 g sitosterol after recrystallization from acetone. This product, mp 136-7 C, was completely free of stigmastanol and showed only a trace of stigmasterol by GLC (Fig. 3A). The overall yield of purified sitosterol was 63% of that present in the original hydrogenation mixture.

Sitosterol from Cottonseed Oil Steam Deodorizer Distillate

Five gallons of the semisolid distillate (Fig. 1A) were filtered through three large, coarse porosity, sintered glass funnels with an oil pump vacuum. The filtration, which took several weeks, was hastened by daily scraping of the filter cake from the surface of the sintered glass with a large, flat-ended spatula. When the filter cake had been reduced to 6 liters, it was removed, melted on a steam bath and allowed to recrystallize. These crystals were filtered again for a week to remove another 3 liters of liquid. The resulting solid (2840 g, Fig. 1B) was saved for purification; the combined filtrates (Fig. 1C) were discarded. The solid was crystallized from 4 liters ethanol to remove remaining tocopherols to yield 2 kg of a mixtue of campesterol, sitosterol and steryl esters (Fig. 1D). In our system, the esters were not readily eluted from the GLC column. They were detected by a high R_f spot on TLC.

Five gram samples of the solid mixture were crystallized from 40 different solvents to determine which solvent would be the best for purification of sitosterol. The results were assayed by GLC and TLC as well as weight of solid recovered. Ten solvents (pyridine, dioxane, 2-butanone, ethyl acetate, tetrahydrofuran, *n*-amyl alcohol, 2-methyl-2-butanol, acetophenone and triethylamine) selectively preciptiated 0.1-0.5 g of the steryl esters from the 5 g mixture. These precipitates were combined (3 g) and recrystallized three times from *t*-butanol to yield a material, mp 85.5-87 C, that showed only one high R_f spot on TLC. It was hydrolyzed with alcoholic KOH to give only campesterol and sitosterol in the neutral fraction and palmitic acid in the acid fraction (GLC methyl ester, mp methyl ester 32-33 C, lit [19] 30 C).

A number of solvents (acetic acid, dimethyl-

formamide, chloroform, isopropyl alcohol, 2-ethoxyethanol and di-isobutyl ketone) appeared to precipitate sitosterol with some enrichment over its concentration in the original mixture. Of these, isopropyl alcohol was chosen for large scale work because of the yield of crystals (1.45 g at room temperature from 5 g in 100 ml solvent) and the ease of concentration and recovery when working with this alcohol.

The 2 kg of crude sterol were then saponified in and extensively crystallized and recrystallized from isopropyl alcohol. Twenty times as much solvent as sterol was routinely used. The sterol was dissolved on a steam bath in 20 liter cylindrical Pyrex water bath jars and allowed to crystallize at room temperature. Filtrates were concentrated and second and third crops obtained. The crystalline precipitates were assayed by GLC: 50 mg was dissolved in 1 ml benzene; 5 μl of this solution was injected, and the size of the campesterol peak measured in arbitrary units (Fig. 2A, B).

As the crystallizations, combination of similar fractions, and recrystallizations proceeded, the size of the campesterol peak gradually diminished until further crystallizations from isopropyl alcohol caused no decrease in its size relative to the large sitosterol peak. At this point the purified sitosterol fractions were acetylated. They were mixed with equal weights of acetic anhydride and of benzene and evaporated to dryness in large crystallizing dishes on a steam bath in the hood. The acetates were then crystallized and recrystallized from absolute ethanol, isopropanol and mixtures of the two alcohols with acetic acid.

It was more advantageous to recrystallize the sitosteryl acetates from dilute solution in the cold room at 4 C (ethanol-isopropanol-acetic acid, 1:1:1, 80-100 ml solvent/gram acetate) than from a more concentrated solution (20-50 ml/gram) at room temperature. The solubility vs. temperature curves of campesteryl and sitosteryl acetates are apparently not parallel lines.

When the process was arbitrarily halted after a total of 160 recrystallizations of the free sterols and steryl acetates, the six products shown in Table I were on hand. The purities of the sitosteryl acetates were estimated by GLC. The sizes of their campesteryl acetate peaks were compared to those of standards that were prepared by addition of 0.5, 1, 2, and 3% campesteryl acetate to samples of fraction 42.

A portion of fraction 42 was hydrolyzed to sitosterol, melting point 138-139 (Fig. 3B). Recent reported values for the melting point of sitosterol are 139-140 C (1,2); 136-137 C (4); 137-138 C (8).

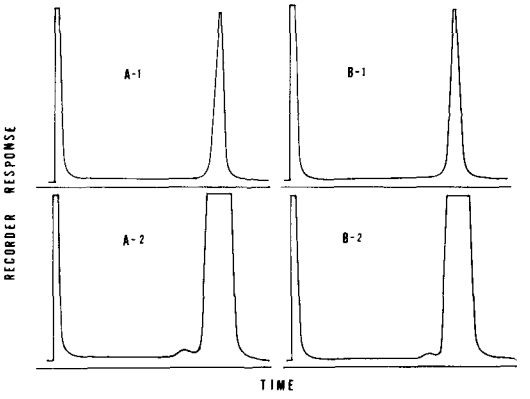

FIG. 3. Gas liquid chromatography diagrams. Purified sitosterol from: A. selective hydrogenation of stigmasterol (1. 1.4 μl [50 mg/ml]; 2. 5 μl [50 mg/ml]); B. cottonseed oil steam deodorizer distillate (1. 1.4 μl [50 mg/ml]; 2. 5 μl [50 mg/ml]).

DISCUSSION

Two simple techniques have been described for the isolation of sitosterol from inexpensive starting materials. The first uses the selective hydrogenation of stigmasterol to sitosterol over Raney nickel under mild conditions (11) with subsequent separation of sitosterol from stigmastanol by bromination and silica gel column chromatography. This method is appropriate for the preparation of gram quantities of sitosterol and is thereby more convenient than the preparative TLC plate separation of the mixture of sterol propionates obtained by the Pd-catalyzed hydrogenation of stigmasterol (4).

The second method, perhaps more useful in the preparation of centigram quantities of the sterol, takes advantage of the large amount of sitosterol present in the steam deodorizer distillates from cottonseed oil processing and the selectivity of isopropyl alcohol for the purification of sitosterol from contaminating campesterol. Although both methods are time consuming, they are easy to carry out with simple apparatus and they have provided us with the sitosterol that we need for insect feeding studies and further chemical modification.

The presence of steryl esters in the cottonseed oil deodorizer distillate was unexpected. Although the ratio of campesterol to sitosterol in the esters was the same as that found as free sterols, the absence of fatty acids other than palmitate was surprising. Possibly sterol esters of oleate and linoleate, two other acids found in cottonseed oil, were eliminated in the early filtration processes and only the sterol palmi-

TABLE I

Fractions of Sitosteryl Acetate Obtained from the Steam Distillate of Cottonseed Oil

Fraction no.	Weight, g	Melting point, C	Estimated purity
42	100	120.8-121.5	99.5%
61	99	121 -122	99.0%
64	141	121 -122	98.5%
65	102	121.5-122	98.0%
66	127	122 -123.2	97.5%
67	114	122.5-124	96.0%

tates were sufficiently high melting and insoluble to be carried over in our sterol fraction.

Another unexpected result was the increase in the melting point of sitosteryl acetate with an increase in its campesteryl acetate content (Table I). Similar results were obtained with the artificial mixtures of the two steryl acetates used to estimate the purities of the products in Table I. Addition of 0.5, 1, 2 and 3% campesteryl acetate to fraction 42 raised its melting point to 121-121.6, 121-121.7, 121-122 and 121-122.3 C, respectively. In these cases, the presence of an impurity raised rather than lowered the melting point of a compound. The reasons for this may be the close structural similarity between the two sterol acetates and the higher melting point (158-159 [8]) of the campesterol derivative. The two acetates appear to form an ideal solid solution in the concentration range that we investigated.

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REFERENCES

1. Steele, J.A., and E. Mosettig, J. Org. Chem. 28:571 (1963).
2. Fujimoto, G.I., and A.E. Jacobsen, Ibid. 29:3377 (1964).
3. Ikan, R., A. Markus and E.D. Bergmann, Ibid. 36:3944 (1971).
4. Allais, J.P., and M. Barbier, Qual. Plant. Mater. Veg. 16:215 (1968).
5. Patterson, G.W., Anal. Chem. 43:1165 (1971).
6. Tu, C., W.D. Powrie and O. Fennema, J. Food Sci. 35:601 (1970).
7. de Souza, N.J., and W.R. Nes, J. Lipid Res. 10:240 (1969).
8. Nishioka, I., N. Ikekawa, A. Yagi, T. Kawasaki and T. Tsukamoto, Chem. Pharm. Bull. (Tokyo) 13:379 (1965).
9. Poulos, A., J.W. Greiner and G.A. Fevig, Ind. Eng. Chem. 53:949 (1961).
10. Heed, W.B., and H.W. Kircher, Science 149:758 (1965).
11. Kircher, H.W., and F.U. Rosenstein, Lipids 8:101 (1973).
12. Karleskind, A., Rev. Fr. Corps Gras 14:251 (1967); 15:379 (1968).
13. Eisner, J., and D. Firestone, J. Ass. Off. Agr. Chem. 46:542 (1963).
14. Fedelli, E., A. Lanzani, P. Capella and G. Jacini, JAOCS 43:254 (1966).
15. Hoffman, R.L., C.D. Evans and J.C. Cowan, Ibid. 41:116 (1964).
16. Nelson, J.P., and A.J. Milun, Ibid. 45:848 (1968).
17. Karleskind, A., F. Audiau and J.P. Wolff, Rev. Fr. Corps Gras 13:165 (1966).
18. Rosenstein, F.U., and H.W. Kircher, Lipids 8:107 (1973).
19. "Handbook of Chemistry and Physics," 48th Edition, 1968.

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