

THE NATURE OF THE STEROLS IN COTTONSEED OIL

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Cottonseed oil has recently engaged the attention of chemists as a convenient source of vitamin E (α -tocopherol). Little is known, however, about the other constituents in the unsaponifiable fraction. In the earlier papers the various investigators who have reported upon the composition of the unsaponifiable matter usually have described their products by giving the melting points of the sterols, and in some cases of their acetates. Bömer and Winter¹ isolated a sterol having a melting point of 136–137°. Siegfeld² described a product which melted at 138.8–139.8°, and which gave an acetate melting at 131.5–132.5°. König and Schluckebier³ obtained a sterol which melted at 137–138°. Its acetate after five recrystallizations melted at 125–128°.

That the unsaponifiable matter in cottonseed oil contains no stigmasterol was first shown by Heiduschka and Gloth⁴ who employed the method of Windaus and Hauth⁵. From the dibromoacetate a sterol was obtained which melted at 136°. Wagner and Clement⁶ later confirmed the observation that cottonseed oil contains no stigmasterol. They did claim, however, to have been able to separate the sterol residues into two fractions, the one melting at 139°, and its acetate melting at 125°; the other at 130–131°, giving an acetate of a melting point of 120°. Similar results have been reported by Anderson and Moore.⁷ By fractional crystallization these investigators also claimed to have been able to separate the phytosterols of cottonseed oil into two fractions that differed slightly in melting point and optical rotation. According to them one fraction melted at 134–135°, and had a specific rotation $[\alpha]_D^{20} = -33.61$. Its acetate melted at 119°. From the mother liquors there was isolated what

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¹ BÖMER AND WINTER, *Z. Nahr. Genuss.*, **4**, 865 (1901).

² SIEGFELD, *ibid.*, **7**, 581 (1904).

³ KÖNIG AND SCHLUCKEBIER, *ibid.*, **15**, 653 (1908).

See also MATHES AND HEINZ, *Archiv Pharm.*, **247**, 161 (1909).

⁴ HEIDUSCHKA AND GLOTH, *Pharm. Zentr.*, **49**, 863 (1908).

⁵ WINDAUS AND HAUTH, *Ber.*, **39**, 4378 (1906); *ibid.*, **40**, 3682 (1907).

⁶ WAGNER AND CLEMENT, *Z. Nahr. Genuss.*, **17**, 266 (1909).

⁷ ANDERSON AND MOORE, *J. Am. Chem. Soc.*, **45**, 1944 (1923).

was considered to be another sterol of melting point 138–139°; $[\alpha]_D^{20} = -34.19$. This product gave an acetate which melted at 124°. They doubted, however, the purity of their products, and from their experiments concluded that it was doubtful whether these two compounds could “ever be separated into homogeneous substances by simple recrystallization.” It should be pointed out that apparently no saturated sterol was obtained by them.

With the increase in our knowledge of sitosterol mixtures since the above investigations were made, it seemed desirable to reinvestigate the nature of the phytosterols of cottonseed oil. The present investigation was also undertaken in connection with previously published work from this laboratory on α -sitosterol from wheat-germ oil⁸ in which it was shown that the α -sitosterol of Anderson⁹ is a mixture of at least two sterols, both of which are doubly unsaturated and precipitable with digitonin; α_1 -sitosterol being an isomer of stigmasterol, and α_2 -sitosterol presumably being a homologue, $C_{30}H_{50}O$. A richer source of these two sterols was greatly desired in order to obtain sufficient amounts for a complete determination of their structures. The observation of Thompson¹⁰ that the phytosterols of cottonseed oil are free from γ -sitosterol suggested that one might be able to get these two sterols with less difficulty from this source rather than from the sterol fraction of wheat-germ oil which is known to contain both β -, and γ -sitosterols. Accordingly, a very intensive systematic fractionation of the crude sterols from cottonseed oil and certain of their derivatives was carried out for the purpose of obtaining the sterols present in a pure and homogeneous state.

In the experimental part of this paper experiments are described which show that, unlike wheat-germ oil, not only does cottonseed oil contain no γ -sitosterol as was observed by Thompson¹⁰, but both α_1 - and α_2 -sitosterols also appear to be absent. Examination of the bottom fraction for the more soluble α_1 - and α_2 - sitosterols by the method of Wallis and Fernholz⁸ failed to disclose the presence of these latter sterols even in small amounts. It was possible to isolate from this fraction only the dinitrobenzoate of β -sitosterol. No α_1 - and α_2 - sitosteryl dinitrobenzoate could be obtained. In this fraction we did observe the presence of appreciable amounts of a low-melting wax. It was not a sterol, however, but seemed to be of the nature of a hydrocarbon. As yet it has not been further investigated.

Since previous investigators did not observe the presence of saturated sterols in cottonseed oil it seemed advisable to examine the unsaponifiable

⁸ WALLIS AND FERNHOLZ, *ibid.*, **58**, 2446 (1936).

⁹ ANDERSON, SHRINER, AND BURR, *ibid.*, **48**, 2987 (1926).

¹⁰ See SPRING, *J. Chem. Soc.*, **1930**, 2664.

material for such sterols. For this purpose the top fraction was used, and the method of Schoenheimer¹¹ was employed. Decomposition of the digitonide thus obtained gave a sterol which on further purification with acetic anhydride and sulfuric acid had the physical properties of stigmastanol. It melted at 136–137°, and gave an acetate of melting point 129–130°; $[\alpha]_D^{25} = +14.9$. The saturated sterol content in this top fraction is 0.9%.

Thus, from our experiments it is to be concluded that β -sitosterol constitutes the greater part of the sterols in cottonseed oil. Since it contains only small amounts of a saturated sterol, and no detectable amounts of α_1 -, α_2 -, or γ -sitosterol the unsaponifiable matter of cottonseed oil is an excellent source for pure β -sitosterol. The physical constants of the β -sitosterol so obtained together with those of certain of its derivatives are listed in Table I. The melting points recorded were taken with a standard thermometer calibrated by the Bureau of Standards. No corrections for stem exposure were made.

TABLE I

TYPE OF COMPOUND	MELTING POINT, °C.	SPECIFIC ROTATION $[\alpha]_D^{25}$
Free sterol	136–137	–36.6
Acetate	125–126	–41.0
Benzoate	146–147	–13.8
<i>m</i> -Dinitrobenzoate	202–203	–10.4

EXPERIMENTAL

Five hundred grams of the crude sterols† isolated from cottonseed oil (m.p. 130–133°) were subjected to an intensive systematic fractional crystallization (triangle scheme) from a mixture of benzene and alcohol in such a manner as to obtain eight fractions. Approximately one hundred crystallizations were made. The melting points and specific rotations of the eight fractions so obtained are listed in Table II.

Analysis of fraction 2. Calc'd for $C_{29}H_{50}O$: C, 83.98; H, 12.16.

Found: C, 83.89, 83.85; H, 12.25, 12.16.

Isolation of the saturated sterols from fraction 1.—Thirteen grams of sterol was dissolved in one liter of hot ethyl alcohol. The solution was then placed in a freezing mixture of ice and salt. When the solution reached 20° a cold, normal alcoholic solution of bromine was added drop by drop. The addition was continued (with shaking) until the solution acquired a permanent yellow color. At 5° a precipitate formed but this disappeared on standing at room temperature. After about 2.5 hours an excess of 1% solution of digitonin (1.29 grams) was added. The mixture was kept in the

¹¹ SCHOENHEIMER, *Z. physiol. Chem.*, **192**, 81–84 (1930).

† The crude cottonseed oil sterols used in these experiments were furnished by the Merck and Company, Inc., Rahway, New Jersey, and were obtained from the unsaponifiable matter in cottonseed oil.

dark for two days until precipitation of the digitonide was complete. Occasionally small amounts of an alcoholic solution of bromine were added to keep the color of the supernatant liquid faintly yellow. The digitonide so formed was filtered, washed with alcohol, ether, and dried at 80°. It was then decomposed in the usual manner with pyridine. The yield of saturated sterol was 0.124 g., corresponding to 0.9%. Since the Liebermann reaction was not entirely negative this sterol was further purified by treatment with acetic anhydride and concentrated sulfuric acid. The acetate so obtained melted at 129–130°, $[\alpha]_D^{25} = +14.8$ (20.3 mg. in 2 cc. of chloroform solution gave $\alpha_D^{25} +0.15$, 1 dm. tube).

Hydrolysis of the acetate with a 5% alcoholic potassium hydroxide solution gave a purified sterol which melted at 135.5–137°. The sterol and its acetate did not give depressions of the melting point when mixed with the corresponding stigmasterol derivatives. Depressions of the melting point were observed with β -sitosterol and its acetate.

Isolation of β -sitosterol from fraction 1.—The alcoholic filtrate from the precipitation of the digitonide described above was poured into a large amount of water and the dibromositosterol was filtered. Debromination with zinc in acetic acid solution

TABLE II

FRACTION	MELTING POINT, °C.	SPECIFIC ROTATION $[\alpha]_D^{25}$
1	136–136.5	–34.9
2	136–137	–36.6
3	135–136	–35.9
4	135	–34.5
5	136–136.5	–36.3
6	135.5–136.5	–34.0
7	136.5–137	–35.2
8 (residue)	128–131 (Softened at 120, waxy, contained coloring materials)	–26.2

gave a product which after four recrystallizations from ethyl acetate melted at 136–137°.

β -Sitosterol from fraction 2.—Attempts were made at further purification of fractions 2 and 5 by fractional crystallization of their acetates, benzoates, and *m*-dinitrobenzoates. No evidence was found that these fractions contained any other sterol than β -sitosterol. A description of one of several experiments is given here. Twenty grams of sterol from fraction 2 was dissolved in acetic anhydride and heated for two hours on the water bath. The solution was then boiled for 15 minutes and allowed to cool. The product was worked up in the usual manner, and the acetate so obtained was recrystallized from alcohol several times, and from acetone and methyl alcohol with no detectable change in melting point and in optical rotation. The crystals melted at 125–126°; $[\alpha]_D^{25} = -41$ (19.1 mg. in 2 cc. of chloroform solution gave $\alpha_D^{25} -0.39$).

Anal. Calc'd for $C_{31}H_{52}O_2$: C, 81.51; H, 11.48.

Found: C, 81.49, 81.60; H, 11.59, 11.55.

The mother liquors from the above crystallizations were evaporated to dryness. The product so obtained without a recrystallization melted at 119°–122°. Since Anderson and Moore⁷ from their experiments believed that cottonseed oil contained

a second sterol whose acetate melted at 119° this product was further examined as described below.

Preparation of β -sitosteryl benzoate.—Five grams of β -sitosteryl acetate (m. p. 125–126°) obtained in the above experiment was hydrolyzed with a 5% alcoholic solution of potassium hydroxide. The free sterol so obtained (m. p. 136–137°) was converted into the benzoate by treatment with benzoyl chloride in pyridine solution. Crystallization from benzene and alcohol gave a product which melted at 146–147°; $[\alpha]_D^{25} = -13.8$ (23.1 mg. in 2 cc. of chloroform solution gave $\alpha_D^{25} -0.16$).

Anal. Calc'd for $C_{36}H_{56}O_2$: C, 83.33; H, 10.50.

Found: C, 83.36, 83.54; H, 10.65, 10.46.

The acetate residues from the mother liquors (m. p. 119–122°) were also hydrolyzed in a wholly similar manner, and the free sterol so obtained (m. p. 135–136°) was converted into the benzoate. Crystallization of this benzoate from benzene and alcohol gave crystals in well defined leaflets which melted at 146°, and showed no appreciable difference in optical rotation; $[\alpha]_D^{27} = -13.3$.

A sample of the benzoate was also prepared from the free sterol of fraction 2. It melted at 146–146.5°.

Preparation of β -sitosteryl dinitrobenzoate.—Two grams of β -sitosteryl benzoate (m. p. 146–147°; $[\alpha]_D^{25} = -13.8$) was hydrolyzed in the usual manner. The free sterol so obtained was dissolved in pyridine and treated with 2 grams of *m*-dinitrobenzoyl chloride. The mixture was kept on a boiling water bath for one hour. The dinitrobenzoate was taken up in ether, washed free of pyridine, and decolorized with charcoal. Evaporation of the solvent gave a crystalline residue which crystallized from ethyl acetate in the form of white leaflets with a yellowish tint; m. p. 202–203°; $[\alpha]_D^{25} = -10.4$ (25.0 mg. in 2 cc. of chloroform solution gave $\alpha_D^{25} -0.13$).

Anal. Calc'd for $C_{36}H_{52}N_2O_6$: C, 71.02; H, 8.61.

Found: C, 71.12, 71.14; H, 8.56, 8.71.

In a similar manner the benzoate (m. p. 146°; $[\alpha]_D^{27} = -13.3$) and the residue from its mother liquor, prepared from the acetate of m. p. 119–123°, were hydrolyzed and converted into the *m*-dinitrobenzoate. A product was obtained which on recrystallization from ethyl acetate also melted at 202–203°. Evaporation of the filtrate to dryness gave a residue which melted at 198–200°.

Before concluding the description of this experiment it should be pointed out that a similar experiment was carried out on fraction 5 with the result that here again we could obtain no evidence for the presence of sterols other than β -sitosterol.

Analysis of fractions 7 and 8 for the presence of α_1 - and α_2 -sitosterols.—These two fractions were examined for the presence of these more soluble sterols by the method used by Wallis and Fernholz³ in their examination of the sterols in wheat-germ oil. Thirty-five grams of material was dissolved in 150 cc. of pyridine and treated in the usual manner with benzoyl chloride. The benzoate so formed was taken up in ether, and crystallized from a mixture of benzene and alcohol. Twenty-eight grams of β -sitosteryl benzoate was obtained; it melted at 145–146°. The filtrate was evaporated to dryness and hydrolyzed with a 5% alcoholic potassium hydroxide solution. The free sterol was converted into the *m*-dinitrobenzoate, and after decolorization of the ether solution with charcoal it was evaporated. Crystallization from ethyl acetate gave a product which melted at 201–202°; $[\alpha]_D^{25} = -9.9$ (20.4 mg. in 2 cc. of chloroform solution gave $\alpha_D^{25} = -0.10$, 1 dm. tube).

Anal. Calc'd for $C_{36}H_{52}N_2O_6$: C, 71.02; H, 8.61; N, 4.60.

Found: C, 70.94, 70.89; H, 8.48; 8.58; N, 4.68.

An examination of the mother liquors failed to give any evidence of the presence of α_1 - or α_2 -sitosterol.

The above-described *m*-dinitrobenzoate was hydrolyzed with 5% alcoholic potassium hydroxide solution and portions of the free sterol so obtained were converted into the acetate and benzoate. Their melting points and specific rotations are listed below.

Derivative	Melting Point	$[\alpha]_D^{25}$
Free sterol.....	136.5-137.1°	-35.6
Acetate.....	126-127°	-42.0
Benzoate.....	144-145°	-14.0

We wish to express our thanks to the American Philosophical Society for a grant-in-aid for this work, to Merck and Company, Inc., Rahway, New Jersey, for all the analyses in this article, and for the crude sterols obtained from cottonseed oil which were used in this investigation, and to Mr. E. Gilmore Ford, who helped us in the determination of some of the physical constants.

SUMMARY

An examination has been made of the nature of the sterols present in cottonseed oil.

Evidence is submitted which shows that the chief part of the sterols present is β -sitosterol. No evidence for the presence of a second phyto-sterol could be obtained.

The absence of γ -sitosterol and stigmasterol is confirmed.

The absence of both α_1 - and α_2 -sitosterols is demonstrated.

An analysis of the saturated sterols by the method of Schoenheimer¹¹ shows that stigmastanol is present to an extent of less than 1%.

Cottonseed oil is an excellent source for β -sitosterol.