The Unsaponifiables of *Vernonia anthelmintica* Seed Oil[1]

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ABSTRACT

The unsaponifiable fraction of *Vernonia anthelmintica* seed oil has been studied in detail. Preparative thin layer chromatography (TLC) on argentated plates has revealed that the hydrocarbon fraction contains squalene as well as normal odd chain alkanes among which C_{31} predominates. A small fraction containing C_{18}-C_{28} *n*-alcohols was also found. The sterol fraction made up the bulk of the unsaponifiables (66%). This fraction contained at least six sterols but two of these accounted for over 90% of the total. The sterols occur in the oil in the esterified form; gas liquid chromatography shows that they are preferentially esterified to linoleic acid. These esters have been isolated by column chromatography on silica gel and have been separated by preparative TLC. Purification by preparative TLC followed by high resolution mass spectrometry has shown that empirical formulas of the two most abundant sterols are $C_{29}H_{46}O$ and $C_{29}H_{48}O$. The latter corresponds to the already reported Δ^7-avenasterol. The former is a new sterol having three double bonds and a stigmastane backbone.

INTRODUCTION

During the past four years our laboratory has studied the effects of epoxide-containing glycerides on rats (1,2). The source of these naturally occurring epoxyglycerides was *Vernonia anthelmintica* seed oil. During the past decade this oil has received considerable attention as a potential commercial source of epoxidized oil (3-5). In view of this interest and of the work done on the glyceride fraction of this oil (6,7 and references cited therein) the information on the unsaponifiable fraction seemed meager, indeed. The early work of Majumdar indicated the presence of stigmasterol and brassicasterol in the unsaponifiables (8). Later Gunstone (9) and Krewson et al. (10) showed that the oil has a substantial (6-8%) unsaponifiable content but did not examine this fraction further. Recently Ward and Frost have isolated and analyzed the sterol fraction and found that it contains mostly Δ^7-avenasterol with smaller amounts of stigmasterol and an unknown C_{29} sterol (11). Our purpose here was to complete the analysis of the unsaponifiable fraction of this oil.

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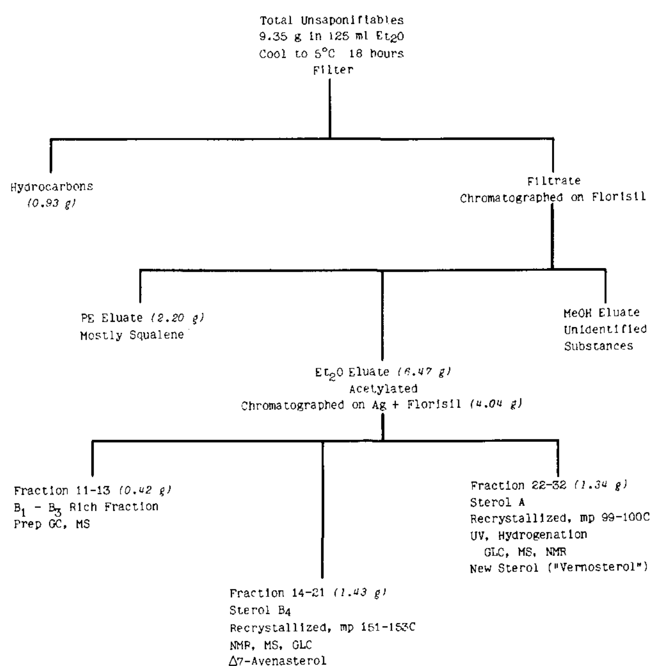

FIG. 1. Isolation scheme of *Vernonia* oil sterols.

EXPERIMENTAL PROCEDURES

Materials

The oil used for this study was obtained from seed grown in this country during 1968 by K. Lessman of Purdue University, Indiana. The oil was extracted and recrystallized once from hexane by R. Hale, Angola Soya Company. The oxirane value of this oil was 2.74% (12), the peroxide value was 1.4 (13), the unsaponifiable content was 5.2% (14) and the free fatty acid content, calculated as oleic acid, was 12.6% (15). The hydrocarbons and alcohols used as standards for gas liquid chromatography (GLC) were purchased from Applied Science Laboratories, State College, Pa. The common sterols used were obtained from Steraloids, Inc., Pawling, New York; campesterol, poriferasterol, 7-dihydrositosterol and chondrillasterol were obtained from M.J. Thompson, U.S. Department of Agriculture, Bethesda, Md. G.W. Patterson of the University of Maryland, College Park, Md., furnished samples of Δ^5 and Δ^7-ergostenol, clionasterol and Δ^7-chondrillastanol. W. Sucrow of the University of Berlin provided samples of Δ^7-stigmastenol, and $\Delta^{7,22,25}$-stigmastatrienol. Fucosterol was isolated from brown algae (16) and α-spinasterol was prepared from the lipids of dried alfalfa (17).

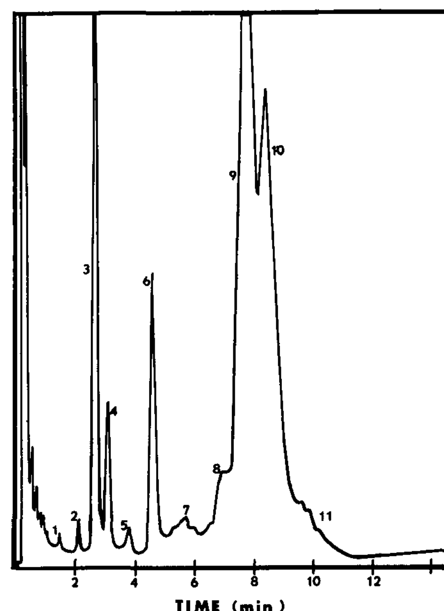

FIG. 2. GLC of *Vernonia* oil unsaponifiables. See Table VI and text for peak identity.

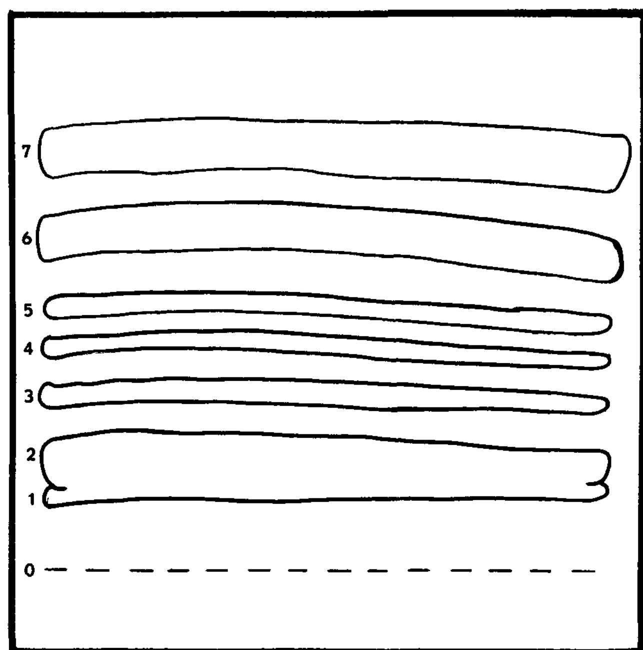

FIG. 3. Preparative TLC of *Vernonia* oil unsaponifiables. (1) Sterol A, (2) B sterols, (3) and (4) unknowns, (5) aliphatic alcohols, (6) squalene and (7) alkanes.

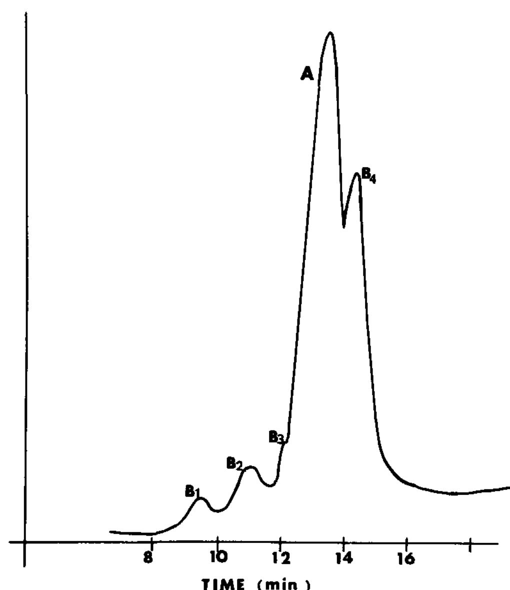

FIG. 4. GLC of *Vernonia* oil sterols. A is the new sterol; B_4 is Δ^7-avenasterol (Fig. 5). See text for details.

Methods

Gas Liquid Chromatography. The methyl esters were analyzed on a 10 ft, 1/8 in. O.D. aluminum column containing 10% stabilized DEGS on 80/100 mesh Anakrom ABS at 200 C. The helium flow rate was 75 ml/min; the injector and detector temperature was 230 and 275 C, respectively. The sterols and other unsaponifiables present were run on a 10% UC-W98 column as already described (18) and on a 6 ft, 1/8 in. O.D. SS column packed with 10% OV-225 on 80/100 mesh Chromosorb W HP (Supelco, Inc.) at 265 C. An F&M Model 810 (Hewlett Packard Corp.) and a Perkin Elmer Model 900, both equipped with dual flame ionization detectors were used interchangeably in this study, although the latter instrument was favored when free sterols were being analyzed. Both instruments were connected to a Model CRS-104 Digital Integrator (Infotronics Corp.) which permitted quantitation of all samples run. Preparative GLC was run as already described (18).

Preparation and Fractionation of the Total Unsaponifiables. Vernonia oil (400 g) was saponified for 48 hr at room temperature in 2 liters of 1 N KOH in 95% ethyl alcohol. The mixture was shaken occasionally until a single phase was achieved. Most of the alcohol was evaporated on the steam bath under N_2. The soaps were dissolved in 3 liters of water and extracted with ethyl ether (E) for 65 hr in a liquid-liquid extractor. The ether extract (about 1 liter) was washed two times with 0.5N KOH, water-washed four times (neutral pH) and dried over anhydrous $MgSO_4$. The weight of the isolated unsaponifiables was 22.8 g.

About 25 mg of this material was streaked on an argentated plate, developed, visualized, scraped and eluted with E. Each of the seven bands was analyzed by GLC; bands 1-5 (Fig. 3) were also subjected to the Fitelson Test (19).

Isolation of the Sterol Esters. The sterol esters were isolated from the oil by means of silicic acid column chromatography. In the first (coarse) fractionation 20.0 g of oil was dissolved in 20-40 C petroleum ether (PE) and loaded onto a large (5x70 cm) column made up of 400 g Davison 923 100/200 mesh silica gel (Grace Davison Chemicals) which had been previously packed and thoroughly washed with the same solvent. Consecutively, 800 ml each of PE, 1% E in PE, 4% E in PE and finally 2 liters 10% E in PE were passed through the column. The 200 ml fractions taken were analyzed by GLC and thin layer chromatography (TLC). The unknown esters were concentrated in fractions 13 and 14 which contained 1.38 g of material which was rechromatographed on a more efficient column (6). In this case the eluting sequence was approximately 300 ml each of 1, 2, 4 and 10% ether in PE. GLC and TLC analysis of the 25 ml aliquots taken showed that the desired esters were in fraction 25-29. The esters were further purified by partitioning in 40 ml 50% PE in methanol and cooling to 5 C. The lower phase (1.15 g), which was analyzed by GLC on a short column normally used for triglycerides, contained 90% pure esters. The esters were further purified and fractionated into two bands by preparative TLC. About 100 mg of the esters were applied to four 20 x 20 cm, 750 μ thick plates prepared by mixing 5.0 g $AgNO_3$ and 40 g silica gel G. The plates were developed with 50% E in PE, visualized by spraying with 2,7-dichlorofluorescein in ethanol. The two bands obtained were scraped off and extracted with E. They were analyzed before and after transmethylation with 0.4 N $KOCH_3$ in methanol.

Large Scale Preparation and Identification of Sterol Fraction. A 9.35 g sample of unsaponifiables, prepared as described above, was dissolved in 125 ml E and fractionated according to the scheme in Figure 1. After removal of most of the saturated hydrocarbons by low temperature crystal-

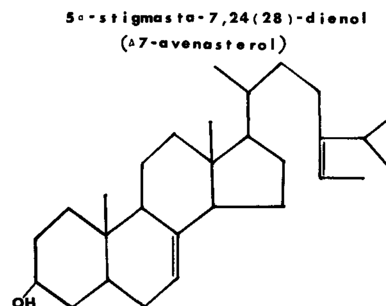

FIG. 5. Δ^7-Avenasterol. Identified in this and other *Vernonia* seed oils (11).

TABLE I

Triglyceride Analysis of *Vernonia* Oil[a]

Retention time, min	Identity	Composition, %
4.4	Diglyceride	0.2
5.6	Diglyceride	0.6
8.0	C_{42} (IS)[b]	---
11.2	Unknown ester	7.5
12.6	C_{50}	5.0
13.7	C_{52}	4.2
14.8	C_{54}	10.0
15.8	VPP + C_{56}	0.2
16.7	VSP	0.7
17.4	VSS	1.0
19.0	VVP	6.9
20.0	VVS	9.3
21.0	VVA	7.1
22.8	VVV	47.3

[a]See reference (18).
[b]Internal Standard.

lization, the sample was applied to 200 g acid-treated Florisil (Supelco, Inc.) which had been previously activated 90 min at 125 C. The column was 3 x 60 cm and was water cooled to minimize cracking during solvent change. After application of the sample, 400 ml PE, 250 ml 40% E in PE, 700 ml E and 400 ml methanol were passed through the column. The 50 ml fractions collected were analyzed by GLC and pooled into three main fractions. The second one, the ether eluate, was acetylated with acetyl chloride in dry pyridine (30% v/v, 24 hr at room temperature). One half of this acetylated samples (4.04 g) was rechromatographed on the same column used above, this time packed with argentated florisil (20). The elution order in this case was the following: 300 ml PE; 400 ml each 2, 5, 10, 25, 50 ether in PE; 100% ether and ethyl acetate. The fractions collected were again analyzed by GLC and combined according to content (Fig. 1).

Melting points were measured on a Kofler hot stage. UV work was performed using a Model 14 Cary Spectrophotometer. High resolution mass spectrometry (MS) was done using a CEC 21-110C spectrometer and direct introduction probe technique has already been reported (18). Nuclear magnetic resonance (NMR) was carried out using a Varian Associates A60 spectrometer and in a few instances a Jealco HR-100 instrument.

RESULTS AND DISCUSSIONS

Due to a short growing season, the oil content of the *Vernonia* seed used was low, and the oxirane content (2.74%), even after a crystallization, was substantially lower than previously encountered (3,6). The high free fatty acid content suggests that a good portion of the oil triglycerides had been hydrolyzed by the enzyme, known to be present in the seed, during processing (10). It is also indicative of the fact that the seed was immature at the time of harvest. TLC of the oil followed by the picration technique (6) showed the presence of common as well as mono- and diepoxy triglycerides. A quantitative analysis of the oil appears in Table I. The oxirane moiety in these glycerides was converted to the 1,3-dioxolane derivative of cyclopentanone prior to GC analysis (7). Here V represents vernolic, P, palmitic, S, stearic and A, arachidic acid. The 7.5% unknown sterol ester present (not corrected for flame response) had a retention time equivalent to a C_{47} triglyceride. It was of interest because it is a major component of the unsaponifiables of, not only this oil, but other *Vernonia* oils examined in this and other laboratories and can account for as much as 11% of the oil (18 and I.A. Wolff, private communication). It is the prominence of this peak that first attracted our attention to the unsaponifiables of *Vernonia* oil.

TABLE II

The *n*-Alcohol Fraction of *Vernonia* Oil

Retention time, min	Identity	Relative Composition, %
1.7	C_{17}	0.5
2.3	C_{18}	23.6
2.7	C_{19}	Trace
3.2	C_{20}	18.8
4.0	C_{21}	Trace
4.9	C_{22}	10.4
6.1	C_{23}	1.0
7.1	C_{24}	18.7
9.4	C_{25}	Trace
11.9	C_{26}	16.8
15.0	C_{27}	Trace
18.9	C_{28}	13.4

TABLE III

The Hydrocarbon[a] Fraction of *Vernonia* Oil

Retention time, min	Identity	Relative Composition, %
2.0	C_{25}	0.8
2.4	C_{26}	Trace
2.9	C_{27}	2.9
3.5	C_{28}	0.5
4.2	C_{29}	21.3
5.1	C_{30}	3.2
6.1	C_{31}	59.7
7.4	C_{32}	1.5
9.0	C_{33}	7.1
10.9	C_{34}	0.4
13.2	C_{35}	0.6
15.9	C_{36}	Trace
19.1	C_{37}	0.2

[a]*n*-Paraffins.

The total unsaponifiables, prepared as described above, were analyzed by GLC on the 10% UC-W98 column. The results are shown in Figure 2. Peaks 1, 2, 4-8 and 11 represents *n*-odd-chain paraffins. Peak 3 is squalene. Peaks 9 and 10 comprise the sterols of *Vernonia* oil and will be discussed in greater detail below.

When these unsaponifiables were separated by TLC on argentated plates, seven bands appeared as shown schematically in Figure 3. Here O is the origin; bands 1 and 2 (not completely resolved) contain sterols. As shown by charring on a TLC plate and by GLC flame response, bands 3 and 4 represent considerably less than 1% of the total unsaponifiables. Both bands gave a slightly positive Fitelson test (19) and would be expected to contain tocopherols and triterpene alcohols including lanosterol. In connection with the latter, it was noted that the commercial samples gave two peaks on GC analysis perhaps due to the presence of Δ^8-lanostenol in the lanosterol. This sample also gave a strongly positive Fitelson test which supposedly is specific for an α-side chain configuration (21). Whether the positive test was due to the presence of contaminants from the euphane series, e.g., butyrospermol, or to the lanosterol-lanostenol itself, was not ascertained. At any rate, the levels in these two TLC bands were too low to permit detailed analysis and confirmation.

The fifth band contains alcohols and the analysis of it appears in Table II. After the plate had been scraped, these were eluted, acetylated and compared to known standards on a DEGS and UC-W98 column. As shown by GLC flame response, these alcohols represent less than 1% of the total unsaponifiables. Their structure was not confirmed by other tests. Band 6 contains squalene which, as Table VI shows, is the third most abundant component of *Vernonia*

TABLE IV

Fatty Acid Analysis of *Vernonia* Oil Sterol Esters

	Per Cent Relative Composition		
Identity	A Sterol	B Sterols	Combined Sterols
C_{12}	---	---	0.96
C_{14}	---	0.30	$<$0.10
C_{16}	0.49	0.22	2.53
$C_{16:1}$	---	---	$<$0.10
$C_{18:0}$	6.98	---	0.24
$C_{18:1}$	6.08	1.70	5.62
?	6.87	0.71	---
$C_{18:2}$	79.58	97.06	90.65
$C_{18:3}$	---	---	Trace

oil unsaponifiables. The last eluted band (number 7) contains the paraffin hydrocarbon already mentioned above. These are shown in Table III and represent about 15% of the oil under scrutiny. They were analyzed by GLC and NMR. The latter shows no unsaturation and a $-CH_2-$ to $-CH_3$ ratio which is normal for an alkane series of this type. This does not exclude the presence of small amounts of branched chain isomers, however. Worthy of note is the fact that the odd chain homologs predominate and that C_{31} makes up almost 60% of this fraction.

Most of the time in this phase of our work was devoted to analyzing the sterol fraction of *Vernonia* oil (Fig. 3, band 1 and 2). As mentioned earlier, these sterols are found esterified to long chain fatty acids so that on a short silicone GC column they appear as a broad C_{47} triglyceride. In order to determine the exact nature of the acid moiety the pure sterol esters had to be isolated. Coincidentally, we noted that the separation of these sterols on a $AgNO_3$ impregnated plate was easier when the hydroxyl group was esterified.

The large preparative silicic acid column was used to obtain 1.38 g of an ester rich concentrate which was further purified on the more efficient column. On the latter, the desired esters eluted in 4% E in PE with the bulk of it appearing in fractions 25-29. These fractions were combined and found to contain about 10% squalene. When standing at 5 C these fractions gave a chloroform soluble, acetone and ether insoluble white crystalline precipitate. The melting point of this precipitate was 77-79 C. GLC showed seven peaks equivalent to odd chain C_{41}-C_{53} triglycerides. Since it weighed only about 10 mg this fraction was not further characterized. It is believed, however, that these are natural waxes made up of normal alcohols esterified to fatty acids. The PE-methanol partition at 5 C lowered the squalene content of the sterol rich fraction to 4%. The residual squalene was removed and the sample was separated into two fractions by $AgNO_3$ preparative TLC. Each fraction, as well as the starting mixture, was transformed to methyl esters and free sterols by alkaline transesterification. The methyl ester composition of these three samples is shown in Table IV. The "B" sterols are those eluted from the higher R_f band; i.e., those having less unsaturation. The $AgNO_3$ separation is effective only because of the remarkably high level of linoleic acid.

The combined sterols analysis on an OV-225 column appears in Figure 4. This shows two major and at least three minor peaks. Sometimes a fourth one (B_5) is visible as a shoulder of B_4. Peak A represents about 60% of the sterols and is by far the largest component in the oil; it is the only sterol found in the lower R_f fraction of the sterol esters. MS shows a 410 MW with $C_{29}H_{46}O$ as the empirical formula. B_4 dominates the less saturated, higher R_f fraction (B) sterols; it accounts for another 35% of the total sterols. It has a MW of 412 with an empirical formula $C_{29}H_{48}O$. The remaining 5% is made up of B_1-B_3 and B_5 sterols.

TABLE V

Relative Retention Time of Plant Sterols

	Relative retention time[a]	
Sterols	10% OV-225	10% UC-W98
Known		
Cholestane	1.00 (1.7)	1.00 (3.5)
Cholesterol	3.44	1.66
Campesterol	4.41	2.00
Poriferasterol	4.53	2.14
Stigmasterol	4.59	2.14
Δ-8 (14)-Stigmasterol	5.18	2.34
β-Sitosterol	5.24	2.40
Spinasterol	5.35	2.34
7-Dehydrositosterol	5.41	2.49
Chondrillasterol	5.47	2.43
Fucosterol	5.59	2.34
Δ-7, 22,25-Stigmastatrienol	5.76	2.31
Δ-7 Stigmastenol	6.06	2.63
Δ-7, 25-Stigmastadienol	6.24	2.54
Vernonia oil		
B_1	4.65	2.16
B_2	5.35	2.41
B_3	6.23	2.70
A	6.29	2.50
B_4	6.88	2.75
B_5	8.35	3.04

[a]Based on the recorded retention time of 5α-cholestane, which is given in parenthesis (min).

At this point we turned to GLC to get some leads as to the identity of these sterols. It was obvious from the start that the main sterols were not the ones commonly found in vegetable oils. We compared the *Vernonia* sterols with some less common C_{29} sterols such as α-spinasterol found in spinach and alfalfa (17) and fucosterol found in brown algae (16). The comparison was made on two columns: UC-W98 is a vinyl silicone and OV-225 a cyanosilicone oil. Table V contains the results of this comparison. The retention time of the main sterol (A and B_4) did not match any of the knowns. It thus became apparent that larger quantities of purified sterols were needed in order to carry out a full identification. Gram quantities of the main sterols were prepared following the scheme in Figure 1. During the course of this work W. Sucrow of Berlin University pointed out to us that Frost and Ward had identified Δ^7-avenasterol as the main sterol in *Vernonia* oil (11). The structure of this sterol is shown in Figure 5. The reported MS, NMR and GLC of this compound (11,22) are exactly the same as that of B_4. It was concluded, therefore, that the second major peak in this *Vernonia* oil was Δ^7-avenasterol. We have since analyzed the unsaponifiables of other *Vernonia* oils grown

TABLE VI

Vernonia Oil Unsaponifiables, Major Components

Peak[a] no.	Component	Retention time	Per Cent of total
1	C_{25}[b]	1.4	Trace
2	C_{27}	2.0	Trace
3	Squalene	2.6	21.6
4	C_{29}	3.0	2.5
5	C_{30}	3.8	Trace
6	C_{31}	4.5	8.6
7	C_{32+}	5.6	Trace
8	C_{33}	7.1	2.0
9	Unknown	7.7	42.3
10	Δ7-Avenasterol	8.3	23.1
11	C_{35}	10.5	Trace

[a]Refer to Figure 2. Peak 9 is the new sterol, "Vernosterol."
[b]C_{25}-C_{35} = *n*-paraffins.

in this country and the ratio of Δ^7-avenasterol to sterol A seems to be fairly constant at about 3:5.

The unknown sterol A was isolated as its acetate in the pure form. The structure of this sterol, to which we have given the name vernosterol, is the subject of another publication (23). This compound had been assigned the structure 5α-stigmasta-8(14),15,24(28)-triene-3β-ol, mostly on the basis of NMR data. Additional information on the chemical shifts of other Δ^{15} sterols (W. Sucrow, private communication) and especially the work of Frost and Ward (24), have convinced us that sterol A is really 8,14,24,(28)-stigmastrienol.

The structure of the minor sterols of *Vernonia* oil (B_1-B_3 and B_5) is still under investigation. Frost and Ward (24) report traces of stigmasterol, spinasterol and β-sitosterol. As shown by Table V this would correspond to B_1 and B_2, but we know B_1 to contain also a minor amount of a 400 MW peak, probably campesterol. Peak B_3 has a 414 MW and most resembles Δ^7-chondrillasterol. Underlying B_2 and B_3 is a smaller component having the molecular formula $C_{29}H_{46}O_2$. This 425 MW peak could be a product of oxidation of the major sterols or a tocopherol with two double bonds in the side chain. Not enough of this material has been isolated to permit further identification. The same is true of B_5.

The last table, Table VI, gives a quantitative analysis of the major unsaponifiable component of the *Vernonia* oil studied. The peak number refers to those in Figure 2. As shown there, the predominant components are alkanes, squalene, Δ^7-avenasterol and the new sterol (unknown) "vernasterol."

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