Journal of Chromatography, 79 (1973) 147–156

CHROM. 6654

PREPARATIVE ARGENTATION COLUMN CHROMATOGRAPHY FOR SEPARATION OF STEROLS FROM ORANGE VESICLES

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(Received December 5th, 1972; revised manuscript received February 13th, 1973)

SUMMARY

Gradient elution, argentation column chromatography was found useful for separating desmethyl sterols present in Valencia orange juice sac lipids. The sterols, as acetates, from fractions off argentation column chromatographic columns can be further resolved and analyzed by conventional thin-layer and gas–liquid chromatographic methods. In addition to the seven sterols previously reported in citrus, ten additional sterols comprising 3.2 % of the total sterols have been isolated from citrus for the first time. These have been characterized as three saturated, three mono-unsaturated and four di-unsaturated desmethyl sterols.

INTRODUCTION

For many years sitosterol, campesterol and stigmasterol have been considered the only plant sterols. With recent improvements in gas–liquid chromatographic (GLC) and thin-layer chromatographic (TLC) techniques the complexity of the plant sterol fraction has been manifested[1,2]. Reports of trace-to-significant amounts of stanols[3] and Δ^7-sterols[4] in higher plants have appeared in the literature. In our study of the desmethyl sterols in orange juice sacs[5], we observed, by GC, several minor peaks occurring as shoulders on the major sterol peaks (sitosterol, campesterol, stigmasterol, 24-ethylidene cholesterol). This led us to investigate further the possibility that these major sterol peaks may be overlapping other minor sterol components. For our first approach to this problem, various liquid phases were studied for their resolving capacity of a complex plant sterol mixture[6]. As a second approach we investigated other forms of chromatography which might enable us to resolve citrus sterols on a preparative scale.

Various investigators have studied the separation of phytosterols by TLC as free sterols[7–10] and as acetate[7,11,12], propionate[13] and trifluoroacetate[14] derivatives. Modified adsorbents have been tested, *e.g.*, use of a non-polar liquid phase for reversed-phase TLC[11,15,16] and impregnation of the silica gel[7,17] or aluminum oxide[10] with silver nitrate. The major objective in these investigations was to devise a procedure for separating certain critical pairs such as campesterol–sitosterol by reversed-phase and cholestanol–cholesterol by argentation TLC. The latter critical pair has also

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been resolved by bromination[11] and oxidation[18]. We were, however, unsuccessful in applying a number of these analytical TLC methods to a preparative scale because of the complexity of the citrus sterol fraction.

Argentation column chromatography (AGCC) of sterols was first proposed by DE VRIES for separation of cholesterol from cholestanol[19]. With AGCC, KNIGHTS[18] separated mono-unsaturated sterol acetates from the di-unsaturated by eluting them with 2 and 30 % ether, respectively, in petroleum ether. Chemical derivatives of the sterols were then prepared for further separation of these two groups of sterols by TLC. VROMAN AND COHEN[12], in a batch-elution column procedure for sterol acetates used increasing percentages of benzene in hexane as eluent. Data were presented on the resolution of synthetic mixtures of critical sterol pairs. Although VROMAN AND COHEN stated that the method had been used in their laboratory for separation of sterols isolated from biological material, no examples were given.

We adapted the AGCC procedures of KNIGHTS[18] and VROMAN AND COHEN[12] to selectively fractionate minor sterol constituents of orange juice sacs from major sterols by eluting silver nitrate-impregnated silica gel columns with gradient amounts of ether in hexane. The initial fractions were resubjected to AGCC for further refinement. Fractions were monitored by TLC, argentation TLC and GLC.

EXPERIMENTAL

Juice sac and standard sterols

Lipids from 10 l of Valencia orange juice sacs were extracted by the Celite method[20,21] and neutral lipids isolated by column chromatography[22]. The neutral lipid fraction was saponified under conditions described for sterol esters[22]. The desmethyl sterols were separated from triterpene alcohols and 4-methyl steroid compounds by preparative TLC on precoated Silica Gel G plates (20 × 20 cm, 500 μm thick, Analtech, Inc.*, Wilmington, Del.) with hexane–ethyl acetate (4:1). For detection, the plates were sprayed with Rhodamine 6G. The desmethyl sterols were restreaked on 500-μm precoated Silica Gel G plates and developed three times (multi-development) in chloroform for further purification. The sterols were acetylated with pyridine–acetic anhydride (2:1) for 1 h at 110° in sealed acetylation tubes and purified by preparative TLC with hexane–ethyl acetate (4:1), yield approximately 500 mg of desmethyl sterol acetates.

Cholestanol, campesterol, stigmasterol, and sitosterol were obtained from Applied Science Laboratories, State College, Pa.; coprostanol acetate (5β-cholestan-3β-ol acetate), 7,(5α)-cholesten-3β-ol, cholesterol acetate, and 5,7-cholestadien-3β-ol acetate from Steraloids, Inc., Pawling, N.Y., and ergosterol from Eastman Organic Chemicals, Rochester, N.Y. Fucosterol was isolated from *Fucus* and *Ascophyllum*[23] obtained from Carolina Biological Supply Co., Burlington, N.C. The free sterol standards were acetylated and purified by preparative TLC with hexane–ethyl acetate (4:1).

Argentation column chromatography

10 g of non-activated Adsorbosil-CABN (25 % silver nitrate on 140–200 mesh

* Mention of brand names does not imply endorsement by the U.S. Department of Agriculture.

silica gel, Applied Science Laboratories) were poured, as a slurry in hexane, into 0.9 × 30 cm columns. AGCC columns, numbered 1–4, were used separately to fractionate 100 mg of Valencia desmethyl sterol acetates. Fractions rich in saturated sterols from columns 1–4 were combined and resubjected to further separation on column 5. The diene fractions from columns 1–4 were combined and further separated on an additional column (column 6). Column 7 was used to separate a standard mixture of saturated (cholestanol, coprostanol), monene (cholesterol, Δ^7-cholestenol, campesterol, sitosterol), diene ($\Delta^{5,7}$-cholestadienol, stigmasterol) and triene (ergosterol) sterol acetates. AGCC columns 1–4, 6 and 7 were eluted with increasing percentages of ether (0.5 to 100 %) in hexane. To determine optimum elution parameters, the ether percentages were varied for each of the four preparative columns (numbered 1–4). These columns were eluted in 25-ml fractional aliquots. Column 5 was eluted in 15-ml fractional aliquots with increasing increments of benzene in hexane. Fractional aliquots from all columns were reduced to dryness and dry weights recorded. All samples were stored in heptane at 4° prior to TLC and GLC analyses.

Thin-layer chromatography

Fractions from preparative AGCC columns were monitored on 20 × 20 cm, 250 μm thick, precoated Silica Gel G plates developed with solvent A (hexane–ethyl acetate, 4:1), solvent B (hexane–benzene, 5:3) or solvent C (petroleum ether–chloroform–acetic acid, 75:25:0.5) (ref. 11). For detection of sterols, plates were sprayed with 50 % H_2SO_4, $SbCl_3$ in $CHCl_3$[24] or ceric sulphate–sulphuric acid[24], heated for 15 min at 130°, and viewed under visible and UV light (366 nm). Migration times (R_S) relative to cholesterol acetate were recorded. Fraction AGCC-2-6, which contained a steroid group that stains blue with $CeSO_4$, was purified by preparative TLC on a Silica Gel G plate in a continuous developing chamber (Shandon Scientific Co., Inc., Sewickley, Pa.) for 2 h with solvent B.

For argentation TLC, 250-μm precoated Silica Gel G plates were impregnated by the $AgNO_3$–methanol development method previously described[21] and activated 1 h at 110° before use. Plates were developed with either solvents B or C for 1 h to a height of 19 cm in the continuous developing chamber. Plates were sprayed with 50 % H_2SO_4 for detection of sterols.

Gas chromatography

All fractions from column 7 were analyzed on a 6 ft. × 4 mm I.D., U-tube glass column. The column was packed with 1 % SP-1000 (Supelco, Inc., Bellefonte, Pa.) coated on 100–120 mesh Gas-Chrom Q. Sterol acetates in heptane were injected on-column in a Hewlett-Packard Model 7610A gas chromatograph equipped with flame ionization detectors. Oven temperature was 240°, detector and injection port temperature 270° and helium flow-rate 80 ml/min. All retention times were recorded relative to cholesterol acetate (RRT). Quantitation was obtained with the aid of a disc integrator. For verification of sterol structures by GLC retention times, representative saturate, monoene, and diene sterol fractions from AGCC columns were analyzed on four GLC liquid phases, *viz.*, HI-EFF 8 BP, OV-210, OV-101 and PMPE under conditions previously reported[6]. For confirmation of saturated sterol structures, acetates of cholesterol, campesterol, sitosterol and a portion of fraction 9 from AGCC column number 6 were hydrogenated with 10 % Pd–C catalyst (1 h,

TABLE I

ELUTION SOLVENTS AND FRACTIONAL WEIGHT PERCENTAGES FROM FOUR PREPARATIVE ARGENTATION COLUMNS

Fraction No.[a]	*Column 1*		*Column 2*		*Column 3*		*Column 4*	
	Ether[b]	%	*Ether*	%	*Ether*	%	*Ether*	%
1	0		0		0		0	
2	1.0		0.5		0.5		0.5	
3	1.0		0.5		0.5		0.5	
4	1.0		0.5		0.5		0.5	
5	1.0	43.80	0.5		0.5		0.5	
6	1.0	30.90	0.5	0.55	0.5	0.03	0.5	0.27
7	1.0	8.07	0.5	15.10	0.5	6.27	0.5	7.77
8	1.0	2.49	0.75	26.01	0.5	33.03	0.5	27.38
9	1.0	1.27	0.75	25.34	0.5	25.31	0.5	25.48
10	5.0	2.35	0.75	12.99	1.0	13.13	0.5	14.13
11	5.0	6.86	0.75	4.46	1.0	7.31	0.5	6.82
12	5.0	1.14	1.0	2.16	1.0	2.00	0.5	3.28
13	10.0	1.29	1.0	2.55	1.0	1.70	0.5	1.38
14	100.0	1.83	5.0	3.08	1.0	1.70	1.0	1.23
15			5.0	4.11	1.0	1.14	1.0	1.24
16			10.0	2.23	5.0	1.36	5.0	3.95
17			10.0	0.41	5.0	3.89	5.0	3.57
18			100.0	1.01	10.0	2.15	10.0	2.51
19					100.0	0.98	100.0	0.99

[a] Each fraction is 25 ml.
[b] Values are % ether in hexane.

50 lbs./in.^{2}, room temperature, Parr apparatus); their GLC relative retention times were compared with those of cholestanol acetate and the saturated fractions from the AGCC columns.

RESULTS AND DISCUSSIONS

Table I lists the eluent compositions and the weight percentages of the various fractions from the four preparative columns. These data show that at least a trace of ether is needed to effect elution of desmethyl sterols. With column 1, elution began with a noticeably large sample weight in the first detectable fraction (fraction 5). Examination of this fraction by GLC did not reveal any detectable amounts of saturated sterols. With smaller fractional volumes the saturates undoubtedly could have been detected.

By initiating the elution of sterol acetates from columns 2–4 with 0.5 % ether in hexane, separation of saturates from monoenes was improved slightly in the first initial fractions. Table II shows the sterol composition of the initial sterols eluted from columns 2–5. These initial fractions contain essentially nine different sterols; three of these were "$CeSO_4$-blue-reacting" sterols. The "$CeSO_4$-blue-reacting" area in fraction 6 of column 2 was separated from the other sterol bands by preparative TLC. When analyzed by GLC this area was observed to contain three sterols. From their colors with three TLC detection reagents (see Table VI), they appear to be Δ^5 monoenes, however, an instant blue color developed without heating the plate.

TABLE II

WEIGHT-PERCENT COMPOSITION OF SATURATED, MONOENE AND UNIDENTIFIED "$CeSO_4$-BLUE-REACTING" STEROL ACETATES FROM INITIAL COLUMN FRACTIONS OF TABLES I AND IV

Acetate	*GLC-RRT*[a]	*Column fraction*								
		2–6	*2–7*	*3–7*	*4–6*	*4–7*	*5–8*	*5–9*	*5–10*	*5–11*
Cholestanol	0.95	3.2	0.1	0.5	3.6	0.4	1.1	1.2	0.4	T
Cholesterol	1.00	T[b]	0.5	0.8	T	0.8	0.3	0.3	0.6	0.1
Unknown C_{28}[c] ($CeSO_4$-blue)	1.19	T	—	—	—	—	6.7[d]	6.1	2.3	
Ergostanol	1.23	21.0	0.2	3.1	21.4	2.4				1.1
Campesterol	1.30	1.6	10.6	9.5	T	9.9	1.1	4.8	7.9	10.8
Stigmastanol	1.53	51.6	0.1	4.9	53.6	4.9	28.1	18.5	4.7	
Unknown C_{29}[c] ($CeSO_4$-blue)	1.55	T	—	—	—	—	20.4[e]	60.0	83.0	
Sitosterol	1.60	12.9	88.0	79.1	14.3	80.6				88.0
Unknown C_{30}[c] ($CeSO_4$-blue)	1.90	9.7	0.5	2.1	7.1	1.0	42.3	9.1	1.1	—

[a] GLC retention times relative to cholesterol acetate on SP-1000.
[b] T = Trace.
[c] Carbon number determined by mass spectrometry.
[d] Unresolved mixture of "C_{28}-$CeSO_4$-blue" and ergostanol.
[e] Unresolved mixture of "C_{29}-$CeSO_4$-blue" and sitosterol.

Their R_s values with solvent A indicate that they may be 4-monomethyl sterols. However, TLC separation of this fraction with solvent B showed these steroids to be less polar than the 4-monomethyl sterols and the triterpene alcohols. Although these unidentified "$CeSO_4$-blue-reacting" steroids elute with the saturated and monoene sterols, they can readily be separated as a group from the latter two sterols by TLC in solvent B.

Table II shows that in the initial column fractions (*e.g.* 2–6 and 4–6) most saturates eluted ahead of their respective monoene homologs. Approximately 75 % of the sterols in column fractions 2–6 and 4–6 are saturates. GLC analysis of the saturated-rich fractions from column 5, notably fractions 8–11 are also shown in Table II. The C_{30} "$CeSO_4$-blue-reacting" steroid from column fractions 5–8 accounts for 42.3 % and stigmastanol for 28.1 %. The ratio of these two compounds in this fraction is 4:3. The ratio of these steroids in succeeding fractions, *i.e.*, 5–9 and 5–10, are 1:2 and 1:5, respectively. Apparently at least under the hexane–benzene gradient elution system, "$CeSO_4$-blue-reacting" steroids elute ahead of saturates on silver nitrate columns.

Table III lists the percentages of the three major monoene sterols and of stigmasterol in various fractions from columns 2 and 4. For column 2 elution is maximal for cholesterol acetate in fraction 11, for campesterol in fraction 10, and for sitosterol in fraction 7. This decrease in polarity with increase in methyl substitution at C-24 has been generally accepted[24]. Stigmasterol, with a double bond in the side-chain (C-22), forms a weaker $AgNO_3$ complex than fucosterol or isofucosterol [double bond at 24 (28)] and thus elutes somewhere between the monoenes and the more strongly retained dienes on AGCC. The major portion of stigmasterol elutes

from columns with 0.5 to 1.0 % ether in hexane while the last trace comes off at 5.0 %. The minimum percentage of ether necessary for complete elution of this di-unsaturated sterol was not determined. Tables I and III show that the three major monoene sterols and stigmasterol comprise approximately 92 % of the total

TABLE III

PERCENT COMPOSITION OF SITOSTEROL, CAMPESTEROL, CHOLESTEROL AND STIGMASTEROL IN FRACTIONS OFF COLUMNS 2 AND 4

Fraction No.	% *Composition*							
	Column 2				*Column 4*			
	Sitosterol	*Campesterol*	*Cholesterol*	*Stigmasterol*	*Sitosterol*	*Campesterol*	*Cholesterol*	*Stigmasterol*
6	12.9	1.6	T[a]		14.3	T	T	
7	88.0	10.6	0.5		80.6	9.9	0.8	
8	87.4	11.4	1.2		85.8	12.8	1.4	
9	81.5	16.8	1.7	T	81.0	17.1	2.1	
10	75.4	20.0	2.3	2.3	77.0	19.3	2.7	1.0
11	67.3	19.5	2.6	10.6	70.6	23.6	3.5	2.3
12	30.9	8.2	1.2	59.7	70.0	22.4	3.2	4.4
13	3.8	1.4	T	94.8	62.7	22.1	3.5	11.7
14	2.6			97.4	45.8	16.3	2.2	35.7
15				0.7	10.8	3.2	T	86.0
16				0.4	3.7	0.7		95.6

[a] T = Trace.

TABLE IV

ELUTION SOLVENTS FOR COLUMNS 5–7

Fraction No.[a]	*Column 5*[b]	*Column 6*[c]	*Column 7*[c]
1	10.0	5.0	0.5
2	10.0	5.0	0.5
3	10.0	5.0	0.5
4	10.0	5.0	0.5
5	15.0	5.0	0.5
6	15.0	5.0	0.5
7	15.0	5.0	0.75
8	20.0	5.0	0.75
9	20.0	100.0	0.75
10	20.0		0.75
11	20.0		1.0
12	20.0		2.0
13	20.0		2.0
14	20.0		5.0
15	20.0		5.0
16	20.0		30.0
17			30.0
18			100.0

[a] Each fraction is 15 ml for column 5 and 25 ml for columns 6 and 7.
[b] Values are % benzene in hexane.
[c] Values are % ether in hexane.

TABLE V

PERCENT COMPOSITION OF STEROL ACETATES IN FRACTIONS FROM COLUMN 6

Sterol acetate	*Tentative structure of unknown*	*RRT*[a]	*Weight % in fraction*							
			2	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Brassicasterol		1.12	T[b]							
Stigmasterol		1.39	32.9	2.1	1.3					
24-Methylene cholesterol		1.45					1.8	6.9	8.0	15.0
Unknown A	$\Delta^{5,25}$-Stigmastadienol	1.70	19.4	17.8	14.1	10.8	3.5			
Unknown B	"24-Methylene-C_{29} sterol"	1.77				9.3	18.6	41.5	64.7	75.3
Isofucosterol		1.92	39.4	76.9	83.4	79.9	76.1	51.6	23.7	8.7
Unknown C	Δ^{7}-Stigmastenol	2.03	7.9							
Unknown D	$\Delta^{7,24(28)}$-Stigmastadienol	2.29		3.2	1.2					
Total weight (mg) in fraction			6.9	13.3	6.8	2.0	0.9	0.6	0.2	1.6

[a] RRT to cholesterol acetate on SP-1000.
[b] T = Trace.

desmethyl sterols and are, for most practical purposes, effectively eluted by the first 25 ml of 5 % ether in hexane. Stigmasterol, 95 % purity, can be obtained in two fractions with 1 % ether in hexane.

Interesting elution patterns are obtained when the percentage of ether is increased from 5 to 10 %. From 8 to 11 % (Table I) of citrus sterol acetates elute from columns in this region. When analyzed by GLC, the major peak in this diene region had an RRT on SP-1000 of 1.92. This sterol on 13 other GC stationary phases[6] had RRTs corresponding to values reported in the literature for isofucosterol[2,25]. For further resolution of isofucosterol from other dienes, the diene fractions (includes stigmasterol fractions) from columns 1–4 were combined, resubjected to AGCC (column 6) and eluted with an ether-in-hexane mixture (Table IV). The major unknown sterol acetates eluted from column 6 had RRTs on SP-1000 of 1.70, 1.77, 2.03 and 2.29 and were labeled unknowns A–D, respectively (Table V). A minor sterol was identified tentatively from its RRTs on four other liquid phases, as brassicasterol. Table V shows that as the elution progresses there is a decrease in the weight percentage of stigmasterol and unknowns A, C and D and a concomitant build-up in 24-methylene cholesterol and unknown B. Isofucosterol increases to a maximum percentage at fraction 4 and thereafter declines.

TABLE VI

R_s AND COLORS OF STEROL ACETATES ON TLC AND ARGENTATION TLC

A = Hexane–ethyl acetate (4:1); B = hexane–benzene (5:3); B′ = hexane–benzene (5:3), $AgNO_3$ impregnated plate; C = pet. ether–$CHCl_3$–acetic acid (150:50:1), $AgNO_3$ impregnated plate; D = 50% H_2SO_4, solvent system A–vis.; E = acetic anhydride–H_2SO_4, solvent system A–vis.; F = acetic anhydride–H_2SO_4, solvent system A–UV; G = $SbCl_3$–$CHCl_3$, solvent system B–vis.; H = $CeSO_4$–70% H_2SO_4, solvent system B–vis.

Sterol acetates	*R_s value in solvent*				*Colors with spray reagents*				
	A	*B*	*B′*	*C*	*D*	*E*	*F*	*G*	*H*
Isofucosterol	1.00	1.00	0.78	0.59	gray	purple	tan	purple	
Unknown A	1.00	1.00	0.78	0.59	gray	purple	tan	purple	
Unknown C	0.96	1.00	0.78	0.65	gray	green	yellow		
Unknown D	0.96	1.00	0.78	0.65	gray	green	yellow		
24-Methylene cholesterol	0.96	1.00	0.54	0.50	gray	purple	tan	purple	
Unknown B	0.96	1.00	0.54	0.50	gray	purple	tan	purple	
Cholestanol	1.02	1.00	1.03	1.14	tan	tan	gray	neg.	
Cholesterol	1.00	1.00	1.00	1.00	red	red	violet	pink	
Δ^7-Cholestenol	0.96	0.95	0.95	1.05	brown	green	yellow	tan	
Stigmasterol	1.00	1.00	0.98	0.92	gray	red	tan	pink	
$\Delta^{5,7}$-Cholestadienol		1.00	0.41	0.46		green	yellow	purple	
Fucosterol	1.00	1.00	0.87	0.60	gray	purple	tan	purple	
Ergosterol	0.96	0.93	0.35	0.47	brown	green	gold	purple	red[a]
"$CeSO_4$-blue" steroids	1.11	1.44	1.30	1.21	red	red	purple	pink	blue[a]

[a] Instant colors upon spraying, after heating, colors same as spray D.

In order to understand more fully the elution sequence of these unknown citrus sterols, a synthetic mixture of nine sterol acetate standards was subjected to AGCC (column 7, Table IV). When 0.5 % ether in hexane was passed through the column, coprostanol and cholestanol eluted first and essentially together. The three Δ^5-monoenes, sitosterol, campesterol and cholesterol, subsequently eluted in their

respective polarity order. Δ^7-Cholestenol eluted from the column essentially one fraction ahead of the Δ^5-monoene, cholesterol. When the percentage of ether was increased to 0.75 %, stigmasterol eluted and when the percentage was 30 %, $\Delta^{5,7}$-cholestadienol and ergosterol emerged.

Table VI shows data obtained when the unknown sterols along with standards were analyzed by TLC. Unknowns A, C and D and isofucosterol are partially resolved into two major spots with solvent C. The lower spot (R_s = 0.59) containing isofucosterol and unknown A showed staining properties characteristic of standard fucosterol and therefore is indicative of Δ^5-sterols. From its Δ^5-staining property and RRTs on four liquid phases[25], unknown A is characterized tentatively as $\Delta^{5,25}$-stigmastadienol. The higher running material (R_s = 0.65) showed staining properties characteristic of Δ^7-sterols. From its Δ^7-staining property and RRTs on four liquid phases[25], unknown C is identified tentatively as Δ^7-stigmasterol. Elution of unknown D slightly before isofucosterol on AGCC strongly indicates that unknown D is $\Delta^{7,24(28)}$-stigmastadienol (Δ^7-avenasterol). Further evidence in favor of this structure is that the compound has Δ^7-staining characteristics and RRTs similar to Δ^7-avenasterol (isolated from oat seed). The structure of unknown B has not been fully determined, however, from AGCC hydrogenation and GLC studies, the compound appears to be a C_{29} sterol with a methylene substituent in the side-chain.

In our previous study[5] five sterols were definitively and two sterols tentatively (24-methylene-cholesterol and brassicasterol) characterized as being present in the neutral sterol fraction of Valencia juice sac lipids. In the present study ten additional compounds have been isolated from the neutral fraction by argentation chromatography. These sterols comprise 3.2 % of the total desmethyl sterols. Unknowns A and B, comprising over 2 % of the total sterols, were previously masked by the sitosterol and isofucosterol areas on GLC analysis. The data presented clearly demonstrate that gradient elution, AGCC can resolve plant sterol mixtures that previously were thought to comprise only one compound. Through use of increasing amounts of a polar solvent (ether) in a non-polar solvent (hexane) steroid compounds such as "$CeSO_4$-blue-reacting" steroids are readily observed. These compounds are not detected when the total desmethyl sterol fraction is analyzed by TLC and/or GLC.

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