Notes

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Itsuo Nishioka,[*1] Nobuo Ikekawa,[*2] Akira Yagi,[*3] Toshio Kawasaki,[*1] and Takeo Tsukamoto[*3] : Studies on the Plant Sterols and Triterpenes. II.[*4] Separation of Stigmasterol, β-Sitosterol and Campesterol, and about So-called "γ-Sitosterol."

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The sterol components of many plants have been investigated for long time and various kinds of sterols were reported to be isolated. However, until a few years ago, the method of identification was based on the comparison of either melting points, specific rotations or infrared spectra, which are not so much different in some of the plant sterols and then in some cases, different physical properties had been reported for the same sterol. A recent development of gas chromatography exhibit its high resolution even for the closely resembling compounds, and it has become necessary to reinvestigate sterol problems in a few years back. However, it is still difficult to obtain a pure sterol because of the formation of mixed crystal and no suitable method have been reported. From these reasons, the plant sterols, reported by our laboratory several years ago, were reinvestigated and the separation methods for obtaining pure substance of stigmasterol, β-sitosterol and campesterol were established by using the gas chromatography.

Purification of Stigmasterol

The sterol, isolated from the fruit of *Xanthium Strumarium* L. (Xanthii Fructus), was a mixture of 55% stigmasterol and 45% β-sitosterol, as reported in a previous report.[*4] The pure stigmasterol was obtained from such a sterol mixture by the following method. The benzoate of the sterol was recrystallized from ethyl acetate, repeatedly (15~20 times) and less soluble fraction was saponified. Further recrystallization of the acetate from ethanol gave pure acetate of m.p. 142~143°. The free sterol, m.p.167~168°, showed one peak on gas chromatogram and no depression on admixture with the standard sample.

Separation of β-Sitosterol and Campesterol

It was found by the gas chromatographic analysis that the sterol from Oenothera sp.[1] was a mixture of 95% β-sitosterol and 5% campesterol. Gas chromatographically pure β-sitosterol was obtained easily by the several times recrystallizations of either the acetate or benzoate. This plant may be one of the best source to obtain the pure β-sitosterol.[*5]

In previous papers,[2,3] it was reported that sterol-A and sterol-B, in addition to 7-dehydrostigmasterol, were isolated from the bark of *Phellodendron amurense* Rupr.

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*3 Nanakuma, Fukuoka, Japan (八木 晟, 塚本赳夫).
*4 Part I. T. Tsukamoto, A. Yagi, K. Mihashi : Japanese J. Pharmacognosy, **17**, 11 (1963).
*5 The sterol contents of various plants will be published in a forthcoming paper.

1) T. Tsukamoto, I. Nishioka : Yakugaku Zasshi, **74**, 1199 (1954); T. Tsukamoto, I. Nishioka, N. Kinoshita : *Ibid.*, **75**, 1019 (1955).
2) T. Tsukamoto, I. Nishioka, K. Mihashi, H. Miyahara : Yakugaku Zasshi, **78**, 1099 (1958).
3) I. Nishioka : *Ibid.*, **78**, 1432 (1958).

(Phellodendri Cortex) and these sterol-A and -B were assigned as γ-sitosterol and β-sitosterol, respectively, by means of the melting points and specific rotations of the sterols and their derivatives. However, gas chromatographic analysis showed that both sterols were a mixture of campesterol and β-sitosterol consisting of 1:1 for sterol-A (γ-sitosterol) and 1:3 for sterol-B. In order to comfirm the established fact, an isolation of both compounds was achieved from the mixture by the following methods.

After a removal of the 7-dehydrostigmasterol by the method[3] reported previously, the benzoate of the sterol was recrystallized from ethyl acetate. The less soluble portion (Fraction 1), m.p. 143~144°, abounded with β-sitosterol and the easily soluble portion (Fraction 2), m.p. 140~141°, abounded with campesterol. Fraction 1 could be concentrated to about 95% pure benzoate of β-sitosterol, m.p. 150~151°, by a repeated fractional recrystallization (15~20 times) from ethyl acetate. After a saponification of the benzoate, recrystallization of the acetate from ethanol gave pure acetate, m.p. 120~121.5°, and gas chromatographically pure β-sitosterol, m.p. 137~138°, was obtained from the acetate. Fraction 2 was converted to the acetate and a repeated recrystallization (10~15 times) from ethanol gave acetate of about 90% purity, m.p. 138~141°, and a repeated recrystallization (10~15 times) of free sterol from acetone gave pure campesterol, m.p. 158~159°, which showed identical melting point and retention time with the standard sample.*6 For the purification of β-sitosterol, a recrystallization of benzoate was effective and for campesterol those of acetate and free sterol were effective. The physical properties of these sterols were summarized in Table I.

Table I. Physical Properties of Campesterol, β-Sitosterol and Stigmasterol

	m.p. (°C)	$[\alpha]_D$[a]	RRT[b]	IR $\nu_{cm^{-1}}^{Nujol}$ (cm^{-2})
Campesterol	158~159	−37	1.31	959
Acetate	144~145	−44	1.71	959, 1280, 1750
Benzoate	157~158	−16	—	—
β-Sitosterol	137~138	−39	1.61	959
Acetate	120.5~121.5	−43	2.30	—
Benzoate	148.5~149	−14.1	—	—
Stigmasterol	167~168	−50	1.40	959, 970
Acetate	142~143	−55.6	1.80	—
Benzoate	165	—	—	—

a) Solvent: $CHCl_3$, c=0.3~0.4, Temp. 20~25°.
b) Relative retention time to cholesterol.

So-called "γ-Sitosterol"

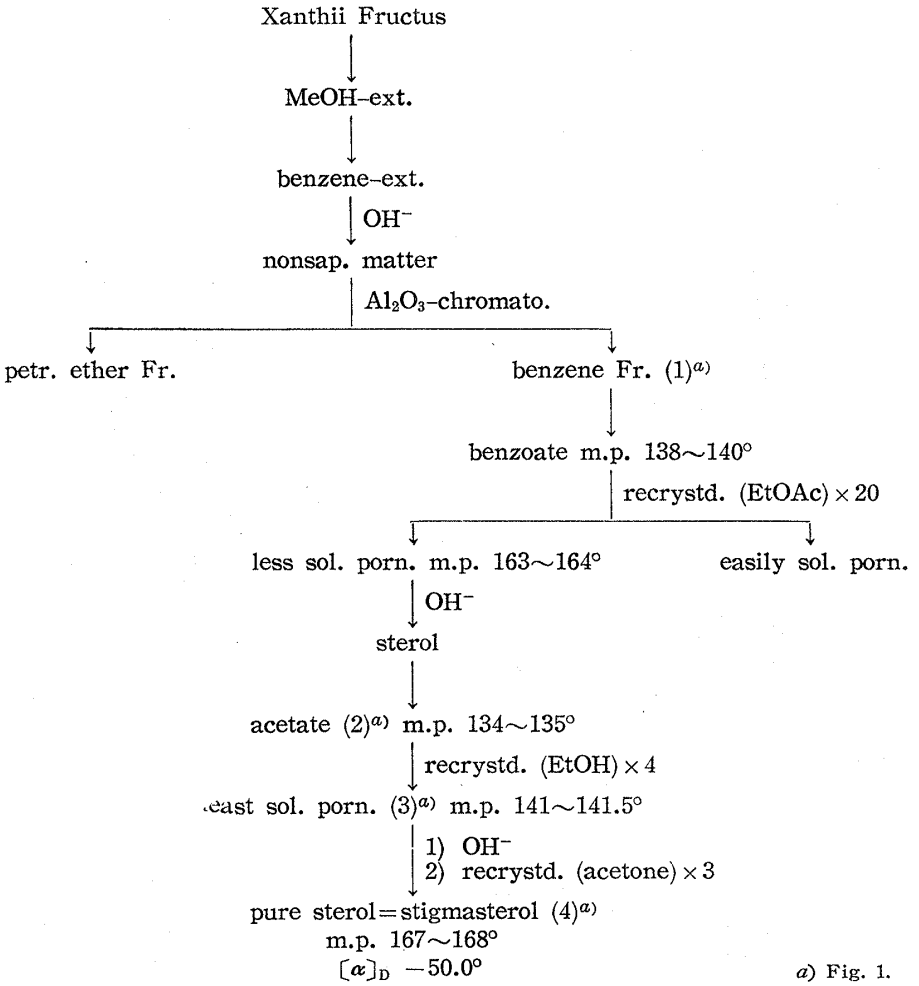
From the above results, it was strongly suggested that the so-called "γ-sitosterol" might be a mixture.*7 γ-Sitosterol was isolated by Anderson[4] from corn oil, by Bonstedt,[5] Bengtsson[6] and Dierscherl[7] from soybean and by Gloyer[8] from rye germ oil. Identification methods which described in these papers were all based on the comparison of melting points and specific rotations. However, somewhat different physical preperties were reported for this sterol and γ-sitosterol was the name of the sterol which showed the melting point of 143~148° and $[\alpha]_D$ −41~−45°. Although the structure of

*6 The sample of 90% pure campesterol was supplied kindly from Dr. M. J. Thompson, Beltsville, Maryland, U. S. A.
*7 A doubtful existence of γ-sitosterol in soybean as the pure form was pointed out by N. Ikekawa at the 6th Meeting of Kanto Branch of Pharm. Soc. Japan, Nov. 1962, Tokyo.
4) R. J. Anderson, R. L. Shriner: J. Am. Chem. Soc., 48, 2976 (1926); R. J. Anderson, R. L. Shriner, G. O. Burr: *Ibid.*, 48, 2987 (1926).
5) K. Bonstedt: Z. Physiol. Chem., **176**, 269 (1928).
6) B. E. Bengtsson: *Ibid.*, **237**, 46 (1935).
7) W. Dierscherl, H. Nahm: Ann., **555**, 57 (1944); **558**, 231 (1947).
8) S. W. Gloyer: J. Am. Chem. Soc., **61**, 1901 (1939).

the sterol isolated from soybean was determined by Diersherl[7] as the configuration isomer of C-24 ethyl group of β-sitosterol, equivocal relation with clionasterol[9] has still been remanined unclear.

Then γ-sitosterol,m.p. 143~144°, isolated from soybean according to the method of Bonstedt,[5] was investigated by gas chromatography. The chromatogram and infrared spectra were completely identical with that of sterol-A from *Phellodendron amurense* RUPR. and suggested to be a mixture of 50% campesterol and 50% β-sitosterol. Further recrystallization of this substance from acetone gave a substance with a higher melting point and a content of campesterol was increased step by step. After ten times recrystallization, about 85% campesterol was obtained, but the crystal form was most fine at the stage of m.p. 143~144°. These two components were isolated from this sample by the same way as mentioned above. Recrystallization of a mixture of both campesterol and β-sitosterol in same amount gave a substance which showed completely the same melting point and infrared spectra as the so-called "γ-sitosterol."

During the course of this work, Thompson, *et al.*[10] have reported the non-homogeneity of γ-sitosterol by gas chromatographic evidence and they arrive completely to the same conclusion.



a) Fig. 1.

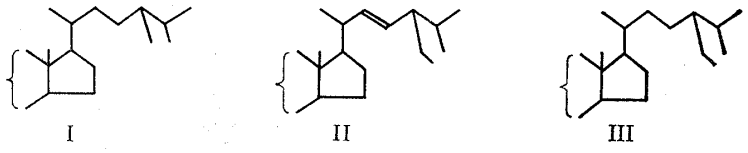
Chart 1. Purification of Stigmasterol

9) Bergmann and Low have determined by measuring molecular rotation that β-sitosterol and clionasterol were C-24 epimers, and they also suggested that the samples of γ-sitosterol which have been described so far might contain some impurities. E. Bergmann, E. M. Low: J. Org. Chem., 12, 67 (1947).

10) M. J. Thompson, W. E. Robbins, G. L. Baker: Steroids, 2, 505 (1963).

Even if soybean contains both C-24 ethyl isomers, these isomers may not be separated by gas chromatography as described by Thompson,[10] Tsuda[11] and also as shown from our experimental results, and so there will be no proof about the non-existence of C-24 β isomer in soybean. However, methyl or ethyl group of campesterol (I),[12] stigmasterol (II)[13] and β-sitosterol (III)[7,14] has been determined as C-24 α configuration and therefore biogenetically it might not be likely to contain C-24 β isomer in the same plant.

In the reports of Eisner,[15] the peak eluted before that for stigmasterol was assigned to γ-sitosterol on the gas chromatograms of some plant sterols, but this should be assigned to campesterol.



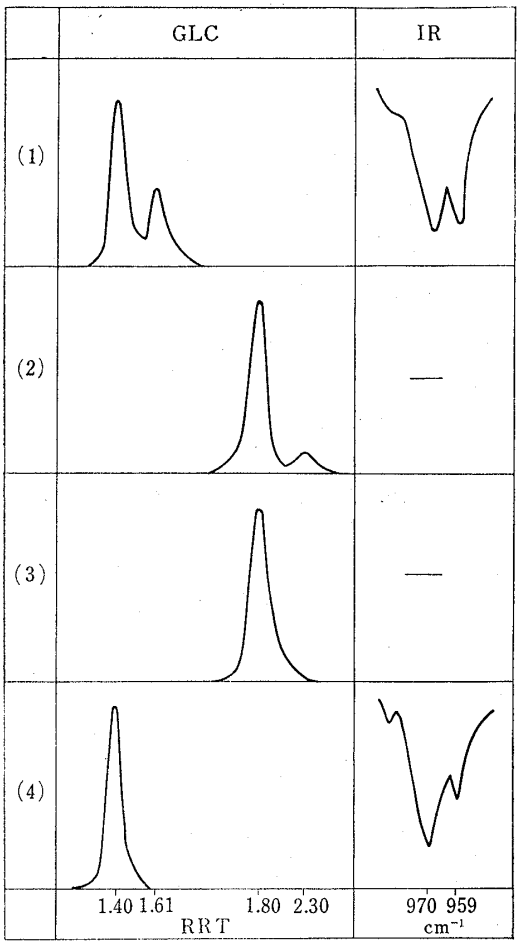


Fig. 1. Gas-liquid Chromatograms and Infrared Spectra

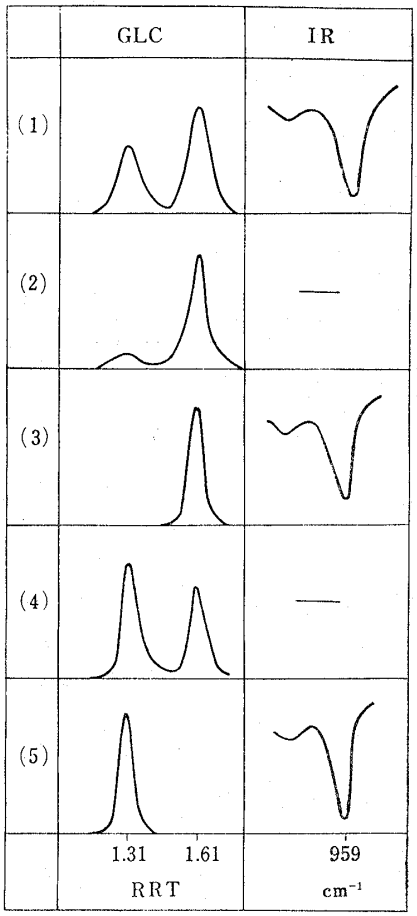


Fig. 2. Gas-liquid Chromatograms and Infrared Spectra

11) K. Tsuda, K. Sakai, N. Ikekawa : This Bulletin, **9**, 835 (1961).
12) E. Fernholz, H. B. MacPhillamy : J. Am. Chem. Soc., **63**, 1155, 1157 (1941).
13) K. Tsuda, R. Hayatsu, Y. Kishida : Chem. & Ind. (London), **1959**, 1411; Y. Kishida : *Ibid.*, **1960**, 465.
14) W. Dierscherl, H. Nahm : Ber., **76B**, 635 (1943).
15) J. Eisner, N. P. Wong, D. Firestone, J. Bond : J. Assoc. Offi. Agri. Chem., **45**, 337 (1962); J. Eisner, D. Firestone : *Ibid.*, **46**, 542 (1963).

Experimental

Gas Chromatographic Analysis——A Shimadzu Seisakusho Gas Chromatograph Model GC-1B with hydrogen flame detector was used in this study. The U-shape stainless steel column, 150 cm. × 4 mm. i. d., was packed with 1.5% SE-30 on Chromosorb W, 80~100 mesh, acid washed and siliconized. The packing was prepared by the filtration technique. The standard conditions were as follows : column temp. 235°, detector temp. 240°, sample heater temp. 280°; carrier gas, N_2, 60 ml./min.; retention time of cholesterol, 7.2 min.

Purification of Stigmasterol——Isolation procedure of stigmasterol from Xanthii Fructus was shown in Chart 1, and the solvents of recrystallization in each step were also listed in the chart. Gas chromatograms and IR spectra of the samples in purification process were shown in Fig. 1. Stigmasterol, $C_{29}H_{48}O$, *Anal.* Calcd. : C, 84.40; H, 11.72. Found : C, 84.28; H, 11.63.

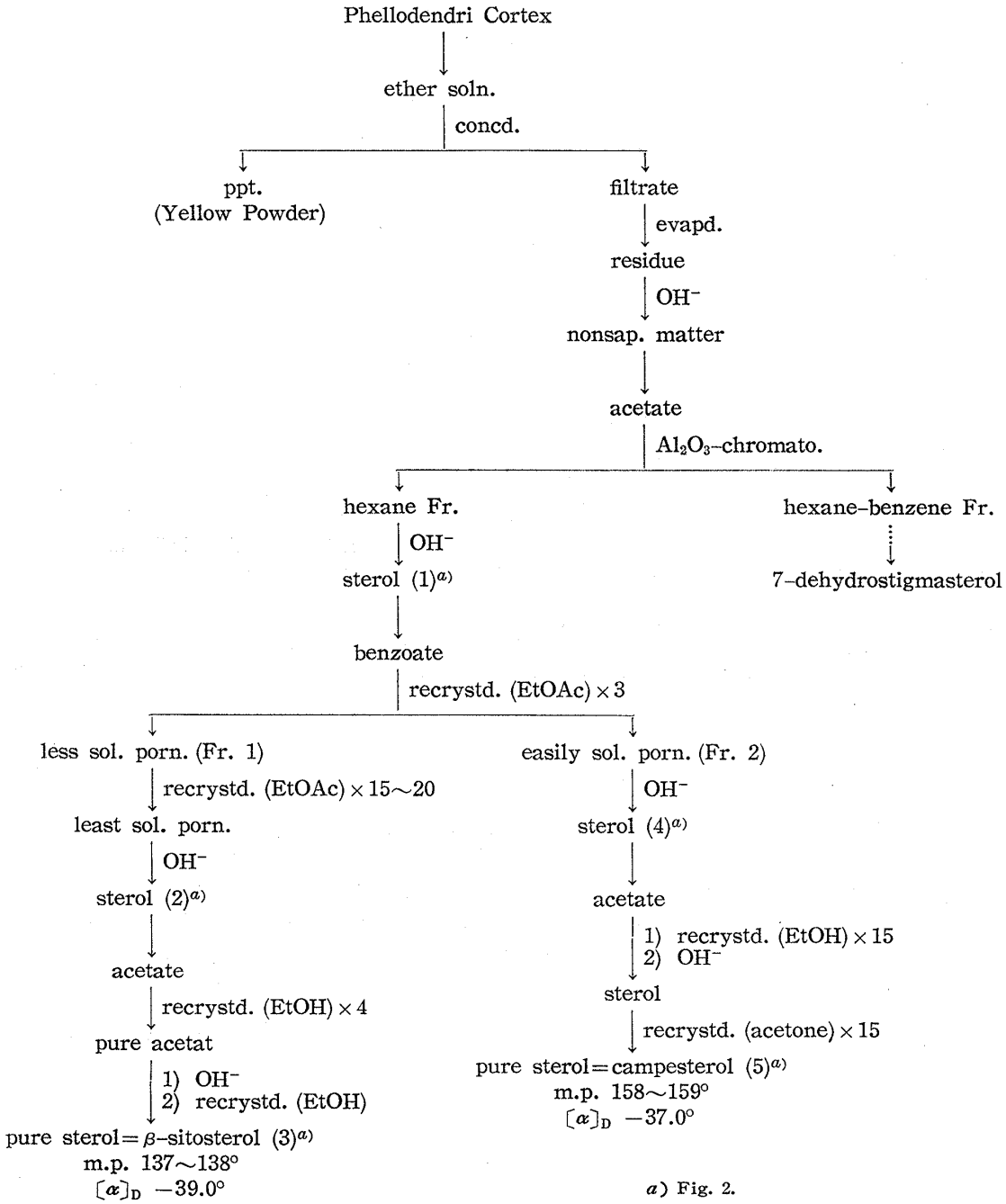


Chart 2. Separation of Campesterol and β-Sitrosterol

Separation of Campesterol and β-Sitosterol——Separation procedure of β-sitosterol, campesterol and 7-dehydrostigmasterol from Phellodendri Cortex was shown in Chart 2, and the gas chromatograms and IR spectra of each step of purifications were shown in Fig. 2. Campesterol, $C_{28}H_{48}O$, *Anal.* Calcd.: C, 83.93; H, 12.08. Found: C, 84.08; H, 12.29. Campesteryl acetate, $C_{30}H_{50}O_2$, *Anal.* Calcd.: C, 81.39; H, 11.38. Found: C, 81.47; H, 11.29. Campesteryl benzoate, $C_{35}H_{52}O_2$, *Anal.* Calcd.: C, 83.28; H, 10.38. Found: C, 83.19; H, 10.65.

Isolation of So-called γ-Sitosterol from Soybean Sterols——Isolation process of γ-sitosterol was shown in Chart 3 and gas chromatograms and infrared spectra of soybean sterol as well as so-called γ-sitosterol were shown in Fig. 3.

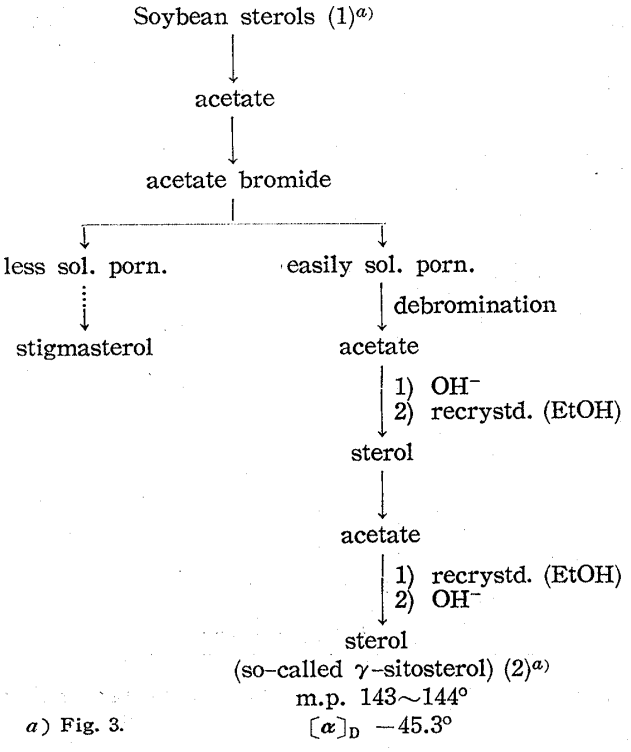


Chart 3. Isolation of So-called γ-Sitosterol

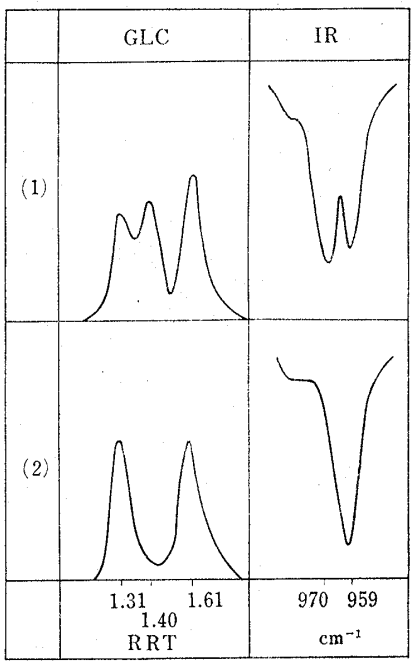


Fig. 3. Gas-liquid Chromatograms and Infrared Spectra

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Summary

The sterols from Xanthii Fructus and Phellodendri Cortex were reinvestigated using gas chromatography. Gas chromatographically pure stigmasterol, β-sitosterol and campesterol were obtained by a repeated recrystallization of either acetate or benzoate of the sterol mixture. γ-Sitosterol isolated from Phellodendri Cortex and also from soybean was found to be a mixture of campesterol and β-sitosterol (1:1).

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