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194. Nobue Matsumoto: Systematic Analysis of Steroids. II.*[1] Analysis of Steroid Sapogenins by Thin Layer Chromatography.*[2]

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Separation of steroid sapogenins had been effected through liquid column chromatography,[1] paper chromatography,[2,3] and gas chromatography.[4] Walens, Turner and Wall[1] separated sapogenins into mono- and di-hydroxyl derivatives by liquid column chromatography, using alumina or magnesium silicate as the adsorbent, and benzene, benzen-chloroform, chloroform or benzene-ethanol as the developing solvent. There are numerous reports on the application of paper chromatography to separate sapogenins. Sannie and Lapin[2] separated several kinds of sapogenins by paper chromatography, using a solvent system of petroleum ether-chloroform-acetic acid. Recently VandenHeuvel and Horning[4] applied gas-liquid chromatography to separation of steroid sapogenins. By the aid of thin layer chromatography however scant literatures[5~7] are available on the separation of steroid sapogenins. This technique has made a rapid progress during the last few years. The paper reported by Tschesche and Wulff,[5] and that by Černý, Joska, and Lábler[6] who dealt with the spread layer method were concerned with the separation of only several kinds of sapogenins. No systematic, simultaneous analysis has been conducted.

In order to study the distribution of steroid sapogenins in plant kingdom, the author carried out systematic, simultaneous analysis of twenty kinds of pure sepogenins by thin-layer chromatography and examined the relationship between chemical structure and adsorptivity to silica gel.

Experimental

Except as described below, chromatoplates were prepared and developed as in the previous paper.*[1]

Preparation of the Plate——A plate, 20×20 cm., was prepared with Wakogel B-5 (a commercial product of silica gel for thin-layer chromatography containing 5% gypsum, Wako Pure Chemicals Co.). The silica gel was activated prior to use by drying at 130° for 10 min. The activity is indicated by the Rf value of 0.65 for butter yellow and 0.11 for indophenol (solvent system: benzene).

Application of the Sample——Each sample was made into 0.1~1% solution in benzene or benzene-MeOH and spotted with a capillary in 0.1~1 μg. (ca. 10^{-9} mole) at the position 1.5 cm. from the lower end of the plate.

Development——The following 22 kinds of solvent systems were used for development.

1) $CHCl_3$-MeOH (8:2) (v/v)
2) Benzene-EtOH (85:15)
3) $CHCl_3$-Me_2CO (7:3)
4) Benzene-Me_2CO (7:3)
5) $CHCl_3$-MeOH (95:5)
6) Benzene-MeOH (9:1) (v/v)
7) $CHCl_3$-AcOEt (1:1)
8) Benzene-AcOEt (1:1)
9) Benzene-Ma_2CC (8:2)
10) Benzene-MeOH (92:8)
11) CCl_4-EtOH (9:1) (v/v)
12) Hexane-AcOEt (1:1)
13) CCl_4-Me_2CO (8:2)
14) Benzene-Me_2CO (85:15)
15) Benzene-EtOH (92:8)

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*1 This paper constitutes a part of a series entitled 'Systematic Analysis of Steroids' by Shoji Hara. Part I: This Bulletin, **11**, 1183 (1963).

*2 A part of this work was presented at the Kanto Local Meeting of the Pharmaceutical Society of Japan, November 23, 1962. A brief account of the work appeared as a Communication in J. Chromatog., in press.

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1) H.A. Walens, A. Turner, Jr., M.E. Wall: Anal. Chem., **26**, 325 (1954).
2) C. Sannie, H. Lapin: Bull. Soc. chim. France, **1952**, 1080.
3) E. Heftmann, A.L. Layden: J. Biol. Chem., **197**, 47 (1952).
4) W.J.A. VandenHeuvel, E.C. Horning: J. Org. Chem., **26**, 634 (1961).
5) R. Tschesche, G. Wulff: Chem. Ber., **94**, 2019 (1961).
6) U. Černý, J. Joska, L. Lábler: Collection Czech. Chem. Commun., **26**, 1658 (1961).
7) R.D. Bennett, E. Heftmann: J. Chromatog., **9**, 353 (1962).

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16) $CHCl_3$-EtOH (95:5) (v/v)
17) Benzene-AcOEt (7:3)
18) $CHCl_3$-Me_2CO (9:1)
19) Hexane-Me_2CO (8:2) (v/v)
20) Benzene-MeOH (95:5)
21) Benzene-AcOEt (8:2)
22) $CHCl_3$-Me_2CO (95:5) (v/v)

The solvent system was contained in a height of 1 cm. in the developing vessel. In order to accelerate saturation, a piece of filter paper soaked in the solvent was pasted on the inside wall of the vessel. The vessel was closed and the air inside the vessel was saturated with the solvent vapor, and the thin layer plate was placed inside the vessel. When the solvent front reached 15 cm. from the starting point, the development was discontinued, the plate was taken out of the vessel and the solvent front was marked in the layer with a capillary. The time required for development was usually 30~60 min. The solvent on the silica gel was dried with an IR lamp and submitted to detection by coloration.

Detection——For developing the color of the compounds, the following 4 kinds of reagents were used:

1) Conc. H_2SO_4
2) Ac_2O-conc. H_2SO_4 (Ac_2O is sprayed over the silica gel layer, which is dried under an IR lamp, and H_2SO_4 is sprayed over it)
3) HSO_3Cl-AcOH (1:2)[8]
4) Satd. $CHCl_3$ solution of $SbCl_3$[9]

One of these reagents was sprayed and the plate was dried at 80~90° for 10~15 min. This procedure produced a color spot characteristic to each sapogenin. The colored spots were examined under ordinary light as well as UV ray.

Results and Discussion

The steroid sapogenins in the present series of experiments differed in the number, position, and configuration of the hydroxyl groups. The chemical structures of these sapogenins are given in Table I. For the separation of these sapogenins by adsorption chromatography, adsorbents with activity of grade II to III according to Brockmann, and solarsolvent systems with median polarity such as benzene and chloroform seemed to be suitable. The developing solvent systems used in the present work consisted of non-polal solvent (chiefly benzene and chloroform) and small amount of polar solvent (ethanol, methanol, acetone). Whereas these solvent combinations were of similar polarity as well as eluting power, their separability for sapogenins differed greatly depending upon the constituents. Some solvent systems possessing best separability are shown in Table II

Table I. List of Compounds Studied

Expt. No.	Compounds	m.p. (°C)	C-25	A/B	OH	CO
1	Luvigenin	183~184	D	$\Delta^{1,3,5}$, 4-CH_3	—	—
2	Neometeogenin	177~178	L	$\Delta^{1,3,5}$, 1-CH_3	11α	—
3	Meteogenin	157~158	D	〃	〃	—
4	Sarsasapogenin	193	L	*cis*	3β	—
5	Diosgenin	204~205	D	Δ^5	〃	—
6	Tigogenin	205	〃	*trans*	〃	—
7	Pennogenin	235~237	〃	Δ^5	3β, (17α?)	—
8	Gentrogenin	214~216	〃	〃	3β	12
9	Hecogenin	247~255	〃	*trans*	〃	12
10	Convallamarogenin	258~260	—	*cis*	1β, 3β	—
11	Isorhodeasapogenin	238~240	D	〃	〃	—
12	Rhodeasapogenin	284~287	L	〃	〃	—
13	Nogiragenin	201~203	D	〃	3β, 11α	—
14	Heloniogenin	212~213	〃	Δ^5	3β, 12α	—
15	Yonogenin	241~243	〃	*cis*	2β, 3α	—
16	Gitogenin	269	〃	*trans*	2α, 3β	—
17	Tokorogenin	265~266	〃	*cis*	1β, 2β, 3α	—
18	Metagenin	273~275.5	〃	〃	2β, 3β, 11α	—
19	Kitigenin	291~296	〃	〃	1β, 3β, 4β, 5β	—
20	Kogagenin	315~316	〃	〃	1β, 2β, 3α, 5β	—

8) R. Tschesche: J. Chromatog., **5**, 217 (1961).
9) E. Stahl: Chemiker-Ztg., **82**, 323 (1958).

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Table II. Rf Values in Thin layer Chromatography

Expt. No.	Solvent system 2	10	12	14	15	16	18	19
1	0.93	0.85	0.80	0.80	0.72	0.87	0.76	0.78
2	0.85	0.66	0.64	0.63	0.48	0.81	0.52	0.48
3	0.85	0.66	0.64	0.63	0.48	0.81	0.52	0.48
4	0.67	0.51	0.51	0.46	0.31	0.65	0.39	0.42
5	0.57	0.42	0.46	0.41	0.25	0.59	0.35	0.34
6	0.53	0.39	0.46	0.39	0.23	0.59	0.35	0.29
7	0.50	0.42	0.35	0.30	0.27	0.53	0.26	0.22
8	0.42	0.32	0.26	0.25	0.24	0.49	0.24	0.16
9	0.50	0.37	0.21	0.22	0.25	0.46	0.22	0.16
10	0.42	0.30	0.28	0.24	0.22	0.39	0.21	0.22
11	0.47	0.28	0.28	0.25	0.24	0.39	0.18	0.22
12	0.47	0.32	0.28	0.25	0.22	0.37	0.18	0.22
13	0.37	0.20	0.18	0.15	0.18	0.27	0.11	0.15
14	0.30	0.16	0.16	0.13	0.18	0.26	0.07	0.11
15	0.30	0.17	0.13	0.11	0.18	0.21	0.11	0.09
16	0.35	0.16	0.09	0.11	0.15	0.19	0.07	0.11
17	0.27	0.12	0.03	0.02	0.10	0.09	0.02	0.04
18	0.23	0.09	0.02	0.01	0.07	0.06	0.01	0.01
19	0.20	0.10	0.00	0.01	0.00	0.04	0.01	0.01
20	0.23	0.08	0.00	0.01	0.05	0.03	0.01	0.00

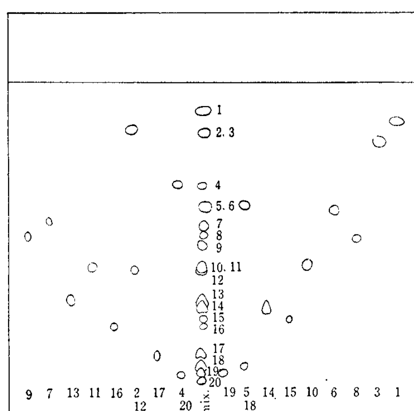

Fig. 1. Thin layer Chromatogram of Steroid Sapogenins

Solvent system : $CHCl_3$-EtOH (95:5)

with Rf values of steroid sapogenins. These solvent systems were capable of separating sapogenins depending upon the difference of hydrophilic substituents, and simultaneous analysis of these compounds was successfully carried out with few exceptions. As example of the chromatogram is shown in Fig. 1.

As for the relationship between the chemical structure and adsorptivity to silica gel, the following conclusions were drawn. i) Adsorptivity of a substance is enhanced as the number of hydroxyl groups is increased, thus there is a definite difference in the Rf values according to the number of hydroxyl groups present, *e.g.* No. 4, sarsasapogenin (3β-OH), No. 10, convallamarogenin (1β-, 3β-OH), No. 17, tokorogenin (1β-, 2β-, 3α-OH), NO. 20, kogagenin (1β-, 2β-, 3α-, 5β-OH). ii) The Rf values of Δ^5-ene and 5α derivatives are similar, *e.g.* No. 5, diosgenin and No. 6, tigogenin; No. 8, gentrogenin and No. 9, hecogenin. iii) The separation of sapogenins differing in the configuration of methyl group in C-25 (neo- and iso-isomers) is difficult in a mixture, even by the use of modified procedures such as gradual development[10] and wedged-tip technique.[11] Separation of Δ^5-ene and its saturated one, and separation of two isomers

10) E. Stahl: Arch. Pharm., **292/64**, 411 (1959).
11) *Idem*: Parfüm. Kosmetik, **39**, 564 (1958).

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with different configuration of 25-methyl group must be carried out based on the difference in the lipophilic nature, and partition chromatography using a lipophilic liquid as the stationary phase seems adequate. iv) The sequence of Rf values varies depending upon the solvent system used, *e.g.* No. 8, gentrogenin, No. 9, hecogenin, and No. 10, convallamarogenin. This variation, as observed in Part I,*1 is likely to be intimately related to solvation effect.

Coloration reagents used in this study include antimonous chloride which is widely used for steroid sapogenins resolved by paper chromatography, concentrated sulfuric acid, concentrated sulfuric acid-acetic anhydride and chlorosulfonic acid-acetic acid. The spots of steroid sapogenins appeared in characteristic color when these reagents were sprayed and warmed under the conditions described in Experimental part. Specificity and sensitivity of the color reaction were highly increased when the spot was examined under an ultraviolet lamp. Thus under an ultraviolet lamp, it is possible to detect an amount as small as 10^{-1} to 10^{-3} μg. of a sample under ultraviolet ray as compared with about one microgramm under day light. Such characteristic coloration allow to use this thin-layer chromatography for separatory detection and qualitative analysis, and quantitative analysis might be possible by the use of a densitometer.

Table III. Developing Color of the Compounds

Expt. No.	Coloration reagent 1		2		3		4	
	V	UV	V	UV	V	UV	V	UV
1	lt. Y R	v. lt. Y R	wk. Y R	v. lt. R	lt. Y R	v. lt. R	lt. Y R	v. lt. R
2	lt. R	lt. R	v. lt. Y R	v. lt. Y R	Y R	lt. R	v. lt. Y R	v. lt. P
3	lt. R	lt. R	lt. Y R	lt. Y R	Y R	lt. P R	v. lt. Y R	v. lt. P R
4	Y R	Y	lt. Y R	lt. Y	wk. Y	v. lt. Y	v. lt. Y R	v. lt. Y R
5	v. dk. G	v. dk. Y	dk. Y R	wk. Y R	v. dk. Y	v. dk. Y	wk. G	wk. R
6	dk. Y	Y R	v. lt. Y R	Y	dk. Y R	dk. Y	v. lt. Y R	v. lt. Y
7	dk. Y R	dk. Y R	dk. R	dk. P R	v. dk. Y R	dk. Y R	lt. P R	lt. R
8	dk. R	dk. R	dk, Y R	lt. P R	v. dk. Y	v. dk. Y R	lt. R	lt. P R
9	Y	v. lt. B	lt. Y	lt. P B	wk. Y R	v. lt. R	v. lt. Y R	lt. P B
10	dk. Y R	dk. Y R	wk. Y R	lt. Y	wk. R	wk. R	wk. R	v. lt. R
11	lt. Y G	v. lt. G	wk. Y R	v. lt. Y R	wk. Y R	wk. Y R	wk. Y R	v. lt. P R
12	dk. Y	Y	dk. Y R	lt. Y	wk. Y R	v. lt. P	wk. R	v. lt. P R
13	dk. Y	dk. Y	v. dk. Y R	wk. Y G	wk. Y G	wk. G	wk. Y	v. lt. P B
14	dk. Y R	dk. R	dk. Y R	wk. P R	v. dk. R	v. dk. Y R	v. dk. R	dk. R
15	dk. R	lt. R	v. lt. P	lt. P B	wk. Y R	wk. G	v. lt. Y R	v. lt. P
16	dk. R	lt. R	v. lt. P	lt. P B	dk. Y R	wk. Y G	v. lt. Y	v. lt. P B
17	dk. Y R	lt. R	dk. Y	lt. Y G	v. dk. Y R	v. dk. R	v. lt. Y	v. lt. P
18	dk. R	v. lt. R	wk. Y R	v. lt. Y	wk. Y R	wk. P B	v. lt. Y	lt. P
19	dk. Y	lt. R	lt. P	v. lt. P B	wk. R	v. lt. P	v. lt. Y R	v. lt. P
20	dk. P R	lt. R	lt. Y G	wk. Y R	wk. Y R	wk. P R	v. lt. Y R	v. lt. P R

Color tone; P: purple, B: blue, G: green, Y: yellow, R: red, lt.: light, v.: very, dk.: dark, wk.: weak, V: visible ray, UV: ultraviolet ray.

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Summary

Thin layer chromatography was applied to systematic, simultaneous analysis of twenty kinds of steroid sapogenins. Examining into twenty-two solvent systems and four coloring reagents, simultaneous, sensitive analysis was successfully carried out.

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