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Colorimetric Determination of Steroidal Sapogenins. II.

Tameto Okanishi and Masatoshi Togami

Shionogi Research Laboratory, Shionogi & Co., Ltd.[1]

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A microdetermination method for quantitative analysis of the steroidal sapogenin content of plants was established. The sapogenins possessing slightly different *Rf* values were separated completely on thin-layer plates according to the method reported by Peereboom and Beekes. Because of the low recovery rate of the sapogenins after extraction procedures the Kieselgel layer containing the sapogenins was directly heated with anisaldehyde, then again heated with phosphoric acid and centrifugated. The absorbances of the clear supernatants were measured at 540 mμ. The effect of the Kieselgel on the absorption spectra of the sapogenins was not observed. The recovery curves were linear between 10 and 60 μg.

The importance of steroidal sapogenins as source materials for synthesis of steroidal hormones had led investigators to develop analytical methods for determination of the sapogenins. The methods reported involve measurement of the bands detected in the infrared spectra of the sapogenines,[2,3] colorimetric determination after reaction with sulfuric acid,[4] perchloric acid[5] or antimony trichloride,[6] and application of the densitometer to thin-layer chromatograms.[7] The present authors previously undertook the development of a new method in order to eliminate such defects as prolonged incubation time and incomplete separation of the sapogenins which are found in the above mentioned methods and reported a reaction with anisaldehyde and phosphoric acid.[8] Although this method afforded satisfactory results for the anlysis of sapogenins contained in mature *Dioscorea tokoro* Makino,[8] the complete separation of tokorogenin from isodiotigenin, a new sapogenin recently isolated from the seedlings of this plant,[9] by ordinary paper or thin-layer chromatography was found to be imperfect.

While a better separation of compounds can be expected generally by thin-layer chromatography than by paper chromatography, several modified methods[10–13] are preferred for the separation of compounds having close *Rf* values. On the other hand, the low recovery rate of isodiotigenin and tokorogenin from Kieselgel G plates made these methods unsuitable for quantitative anlysis of the sapogenins. These defects have again led the authors to reexamine the analytical methods for determination of steroidal sapogenins.

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Materials and Methods

Reagents——Phosphoric acid (Wako Pure Chemical Industries, Ltd. JIS special grade) 0.5% anisaldehyde solution: 50.0 mg of anisaldehyde (Wako Pure Chemical Industries, Ltd.), distilled before use, were dissolved in 10 ml of 99% ethanol.

Thin-Layer Plates——Kieselgel G (E. Merck) layers of 250 μ thickness were prepared on 20×20 cm glass plates according to the method of Stahl[14] and activated at 110° for 45 min. The ends of the plates were immersed into a mixture of chloroform–acetone–acetic acid (80:20:5, v/v), left in the chamber until the solvent reached the upper ends of plates, then air-dried and prepared as shown in Fig. 3-b.

Procedure——Five milligrams of the sapogenins were dissolved in 10 ml of a mixture of methanol and chloroform (1:1, v/v). Of these solutions 0.01—0.06 ml were exactly pipetted, spotted on the starting line 2 cm apart from one end of the thin-layer plates and developed with chloroform–acetone–acetic acid (80:20:5, v/v). After air-drying, the plates were sprayed with water and the sapogenins could be detected as white spots on a light gray background. These were marked with a pencil. The plates were dried in an oven at 110° for 30 min and each marked spot was then scraped from the plates and put into separate test tubes. As blanks, equal amounts of Kieselgel obtained from areas which did not contain the sapogenins were used. To each test tube 0.1 ml of anisaldehyde solution was added and the reaction mixtures were warmed on a boiling water bath for 15 min, then cooled in ice-cold water for 10 min. Again to each tube 5 ml of phosphoric acid were added and the tubes warmed on a boiling water bath for 70 min. During this period the tubes were shaken every 15 min. By these procedures a complex of the sapogenin, anisaldehyde and phosphoric acid is formed.

After cooling in ice-cold water for 10 min, the reaction mixtures were transferred into centrifuge tubes and centrifugated for 10 min at 3500 rpm. After 30 min the absorbances of the clear supernatants were recorded at 540 mμ against the blank solution with a Hitachi Photoelectric Spectrophotometer Model EPU-2A.

Results

Effect of Kieselgel on the Absorption Spectra of Sapogenins

As demonstrated in Fig. 1-a, the absorption spectra of anisaldehyde–phosphoric acid complexes formed with pure samples of yonogenin, tokorogenin and isodiotigenin have tow absorption maxima at 420 mμ and 540 mμ. Fig. 1-b shows identical absorption spectra of pure and plant derived isodiotigenin.

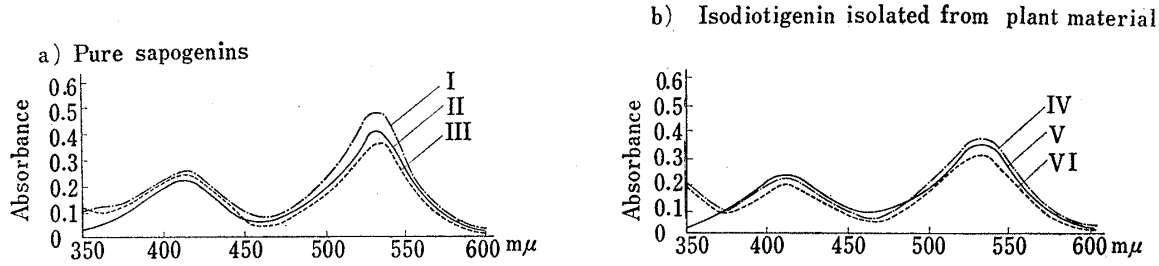

Fig. 1. Absorption Spectra of Sapogenin–Anisaldehyde–Phosphoric Acid Complexes

I: yonogenin (50 μg) II: tokorogenin (50 μg) III: isodiotigenin (50μg) IV: pure isodiotigenin
V: free. VI: saponin-type isodiotigenin isolated from the seedlings of *D. tokoro*.
Sapogenins are heated with 0.5 mg of anisaldehyde at 100° for 15 min, then heated again with 5 ml of phosphoric acid at 100° for 70 min.

Extraction of Tokorogenin from the Thin-Layer Plates

Tokorogenin (50 μg) was spotted on a thin-layer plate and developed as above. From the Kieselgel of the area corresponding to the spot of tokorogenin, the sapogenin was first extracted with chloroform for 30 min under reflux and after filteration, the filtrates were evaporated *in vacuo* and treated with anisaldehyde and phosphoric acid. The absorbance of the reaction mixture was equivalent to that of 22.9 μg of tokorogenin (recovery rate, 45.8%).

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Table I. Absorbances at 540 mμ and Recovery Rates of Yonogenin, Tokorogenin and Isodiotigenin–Reagent Complexes

a) Yonogenin						
Amount of sapogenin (μg)	10.0	20.0	30.0	40.0	50.0	60.0
Absorbance of pure sapogenin solution	0.117	0.226	0.303	0.407	0.497	0.600
Absorbance of sapogenin recovered from thin-layer plate	0.109	0.219	0.293	0.407	0.470	0.572
Recovery rate (%)	93.1	96.9	96.7	100.0	94.6	95.3
b) Tokorogenin						
Amount of sapogenin (μg)	10.0	20.0	30.0	40.0	50.0	60.0
Absorbance of pure sapogenin solution	0.094	0.181	0.273	0.342	0.404	0.502
Absorbance of sapogenin recovered from thin-layer plate	0.088	0.175	0.259	0.352	0.422	0.492
Recovery rate (%)	93.6	96.7	94.9	103.5	104.5	98.0
c) Isodiotigenin						
Amount of sapogenin (μg)	10.0	20.0	30.0	40.0	50.0	60.0
Absorbance of pure sapogenin solution	0.077	0.155	0.241	0.305	0.377	0.441
Absorbance of sapogenin recovered from thin-layer plate	0.066	0.145	0.232	0.289	0.375	0.451
Recovery rate (%)	85.7	93.5	96.3	94.8	99.5	102.3

Solutions containing various amounts of a sapogenin are prepared.
One part of the solution is measured directly and the other part is measurd often recovery from a thin-layer plate.

Effect of Variation in the Amounts of Sapogenins

From 10 to 60 μg of sapogenins were spotted on thin-layer plates individually and treated as above. Absorbances of the sapogenin–reagent complexes at 540 mμ were compared with those of the pure sapogenins. Standard and recovery curves obtained were linear between 10 and 60 μg with almost constant recovery rate as shown in Table 1 and Fig. 2.

The amounts of the sapogenins are calculated as follows:

Yonogenin	$x=110.011y-2.948$
Tokorogenin	$x=122.699y-1.546$
Isodiotigenin	$x=131.062y+0.983$

x is the amount of sapogenin (μg) spotted on the thin-layer plate and y is the absorbance ($-\log T$) at 540 mμ.

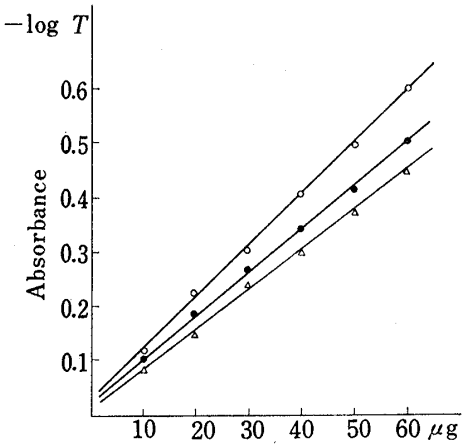

Fig. 2. Standard Curves of Yonogenin, Tokorogenin and Isodiotigenin

—○—○— yonogenin standard
—●—●— tokorogenin standard
—△—△— isodiotigenin standard

The Separation of the Sapogenins by modified Thin-Layer Chromatography

Isodiotigenin and tokorogenin were spotted at the same place on a modified thin-layer plate and treated as above. The amounts of the two sapogenins recovered from the thin-layer

Table II. Separation of Isodiotigenin and Tokorogenin by Modified Thin-Layer Chromatography according to Peereboom and Beekes

Amounts of sapogenins spotted (μg)		Amounts of sapogenins recovered (μg)	
Tokorogenin	Isodiotigenin	Tokorogenin	Isodiotigenin
14.2	16.4	14.0	17.0
28.0	16.4	29.4	17.0
14.2	30.5	14.7	30.2
28.0	30.5	28.1	31.7

plates are summarized in Table II. It is evident that these two sapogenins are separated completely at least in amounts lower than 30 μg.

Variation in the Sapogenin Content of the Spots

Numerous spots (30 μl each) of a solution containing a mixture of sapogenins were spotted on a thin-layer plate and developed. The Kieselgel from one to three spots each of yonogenin, tokorogenin and isodiotigenin were collected and treated as above. The collective amounts of each sapogenin recovered were proportional to the number of spots collected (Table III).

Table III. The Proportional Increases in Amount of Sapogenin when Several Spots are Collectively Measured

Numbers of spots	Amounts of sapogenins recovered (μg) 1	2	3
Isodiotigenin	21.3 (1.0)	40.4 (1.9)	60.6 (2.8)
Tokorogenin	13.4 (1.0)	26.4 (2.0)	40.3 (3.0)
Yonogenin	4.8 (1.0)	10.3 (2.1)	14.9 (3,0)

Discussion

In order to determine the concentration of the various sapogenins contained in plant material, especially to define the change in the amounts of individual sapogenins, complete separation of the components is essential. In chromatographic procedures although the sapogenins are generally better and more rapidly separated on thin-layer plates than on filter papers, on Kieselgel G plates with solvents containing *n*-butanol or *n*-propanol marked tailings which are observed in the thin-layer spots of tokorogenin, isodiotigenin and kogagenin make the separation of these three sapogenins impossible. The best result is observed with chloroform–acetone–acetic acid (16:4:1, v/v) as shown in Fig. 3-a. However, even in this method, the spots of these three sapogenins are large and overlap each other when considerable amounts of the sapogenins are spotted. To avoid this overlapping, the Peereboom and Beekes' modified thin-layer chromatography[13] was introduced. By this method the spots of each sapogenin are flattened and well separated (Fig. 3-b). This is supported also by the result summarized in Table II.

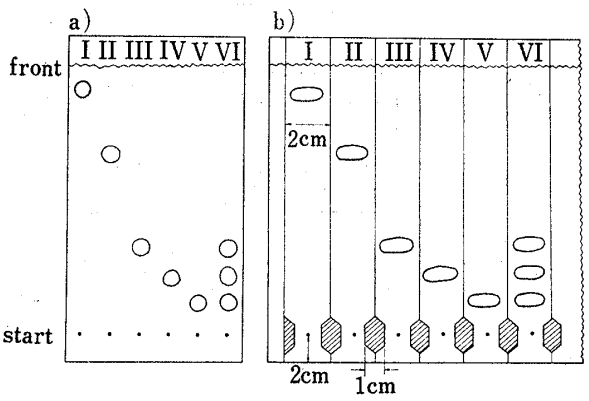

Fig. 3. Thin-Layer Chromatograms of the Sapogenins contained in *D. tokoro*

I: diosgenin II: yonogenin III: tokorogenin IV: isodiotigenin V: kogagenin VI: IIII+V+V
Kieselgel G, 250 μ, 20×20 cm

Developed with chloroform–acetone–acetic acid(16:4:1). Colored with cinnamic aldehyde and $SbCl_3$ in nitrobenzene.
a) ordinary method, *b*) peereboom and Beekes method

One of the difficulties found in the application of thin-layer chromatography to colorimetric quantitative analysis of sapogenins is the interaction of the Kieselgel with the reagent. Another problem is the low recovery rate when the sapogenins are extracted with solvents from the Kieselgel. The first difficulty is excluded by preliminary treatment of the plates with the same developmental solvent before use, and after this pretreatment no change is observed in the absorption spectra of the sapogenins recovered from the Kieselgels as compared with those of the pure sapogenins. The second problem is especially marked in the case of tokorogenin. Therefore, the Kieselgel which

contained sapogenins was treated directly with the reagent. With this method, the recovery rate of the sapogenins was raised to about 95%. However, in the analysis of diosgenin which does not form an efficient complex for the colorimetric determination with anisaldehyde and phosphoric acid[9] the reaction of the Kieselgel with ferric chloride, phosphoric acid and sulfuric acid[9] causes a turbidity in the reaction mixture and makes it unsuitable for colorimetry. Thus diosgenin must be extracted from the Kieselgel before it is treated with this reagent.

Because the amounts of the sapogenins contained in a plant greatly differ, it happens commonly that the amounts of some sapogenins are too small for measurement while the amount of others are suitable for the analysis. In such cases, the amount of these sapogenins contained in small quantities can be determined by preparing several plates, collecting the Kieselgel from several identical spots and measuring the absorbance.

The sapogenins on the thin-layer plates are detected as brown spots in a chamber saturated with iodine vapour or as white spots by spraying with water. Although the effect of iodine on the absorption spectra is not observed, the second method is preferable because of its simplicity. It is said that the modified thin-layer chromatography requires a longer time for development than the ordinary method. However, in this experiment, the overall time needed from the pretreatment to the development is only 90 min.