Quantitative Estimation of Diosgenin in *Dioscorea* Tubers by Densitometric Thin-Layer Chromatography

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Thin-layer chromatography was employed to separate diosgenin from other plant constituents present in *Dioscorea* tuber extracts. The diosgenin was rendered visible by antimony trichloride and the colored spot estimated by scanning with a photoelectric densitometer attached to an integrating logarithmic recorder. Diosgenin could be estimated with an experimental error of approximately 7 per cent when applied in concentrations between 10 and 75 mcg. Assay results obtained for diosgenin by this method were slightly lower than those obtained by conventional infrared spectrophotometry, since the 25 D-spirosta-3,5-diene formed during the extraction of diosgenin was not estimated in the chromatographic procedure.

STEROIDAL SAPOGENINS are of considerable economic importance as precursors of the pharmacologically active steroids, including the oral contraceptives and corticosteroids. The most important steroidal sapogenin is diosgenin, which is obtained commercially from the tubers of several *Dioscorea* species.

Diosgenin is usually assayed by gravimetric (1, 2) or infrared techniques (3, 4); the naturally occurring saponin is acid-hydrolyzed and the sapogenin then extracted with a hydrocarbon solvent. The gravimetric methods are applicable to plant material with high sapogenin content containing little contaminating matter, but are less satisfactory where the proportion of contaminants is high, or when a sensitive method is required for studying the factors affecting the yield of sapogenin from a given source, or screening botanical specimens for steroidal sapogenins. In these circumstances infrared spectrometric methods are more suitable. Based on the spirostan structure of the sapogenin, they are specific for steroidal sapogenins, but take no account of the nature of the steroidal nucleus; the 3,5-diene of diosgenin, for example, being assayed as diosgenin. Furthermore, the authors have found that nonsapogenin compounds extracted by the sapogenin solvent, both saponifiable and nonsaponifiable, can also interfere with the infrared assay method and cause high results. Consequently, it was felt that a convenient analytical technique, suitable for small quantities of plant material and overcoming some of the disadvantages of the existing methods, would be valuable. The authors report the development of such a technique and its application, in the first instance, to the assay of diosgenin in tubers of species of *Dioscorea*. Extension of the method to other species and morphological parts, such as seeds, will be reported.

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EXPERIMENTAL

Development and Treatment of the Chromatogram—Glass plates, 15 × 20 cm., were spread with a layer of Silica Gel G to a thickness of 250 μ according to the method of Stahl (5) and air-dried before use.

Known quantities of diosgenin, dissolved in chloroform, were applied to the plates by micropipets and the chromatogram developed with *n*-hexane–ethyl acetate (4:1 v/v) using the supersaturated method of Stahl (6). When the solvent had advanced 15 cm. from the starting line, the plates were removed, dried, and double developed with the same solvent system. Following development, the plates were dried and sprayed uniformly with a solution of 3 Gm. of antimony trichloride in 1 ml. of concentrated hydrochloric acid (7). The sapogenins were then located by heating the plates evenly at 100° in a circulating air stream until the diosgenin spot was developed to a green-black color. This normally took 30 min.

After the plates had cooled, the absorbances of the spots were estimated by a Vitatron densitometer,[1] using a slit width of 20 mm. by 2 mm. and a mercury lamp source. The values so obtained were calculated by an automatic integrator. Spots were scanned in the direction opposite to that of solvent flow and erratic readings from extraneous light were eliminated by the use of a light-tight box.

Preparation of the Standards—Pure diosgenin was applied to thin-layer plates in concentrations ranging from 5 mcg. to 100 mcg. Following development, the spots produced were scanned and a linear relationship between concentration and absorbance was established for concentrations of diosgenin between 10 mcg. and 75 mcg.

Preparation of Plant Material—Diosgenin was extracted from *Dioscorea* tubers of known weight and moisture content (4). This involved maceration of the fresh tuber, incubation of the disintegrated material, acid-hydrolysis of the saponins by refluxing with 2 *N* hydrochloric acid for 2 hr., and extraction of diosgenin from the dry, acid-insoluble

[1] Fison's Scientific Apparatus Ltd., Loughborough, Leicestershire, England.

residue with petroleum ether (40–60°). After evaporating to dryness, the extract was dissolved in chloroform and made to volume. Dried, powdered tuber was first incubated with water before being treated by the above procedure.

Known volumes of the *Dioscorea* extract were applied to prepared thin-layer plates along with a standard diosgenin solution. The applied spots of the plant and standard solutions were alternated on the plate to minimize the effects of variation in thickness and opacity of the adsorbent layer. The diosgenin content of the *Dioscorea* extract was calculated relative to the standard solution, and the resulting value obtained from the mean of six plates, each containing two spots of the plant extract.

To determine the recovery of diosgenin added to a plant extract, 5 Gm. of a powdered edible *Dioscorea* tuber was extracted and assayed as above. To this extract of known volume, 40 mg. of diosgenin was added and the chromatographic assay carried out to estimate the recovery of the added diosgenin. The assay was repeated following the addition of 40 mg. cholesterol to the *Dioscorea* extract containing the added diosgenin, to show that this sterol did not interfere with the recovery of diosgenin. Cholesterol is a fairly common plant sterol (8) which occurs in *Dioscorea* tubers (9) and co-chromatographs with the common phytosterols β-sitosterol and stigmasterol in the silica gel system used. These procedures were then repeated using an assayed sample of dried, powdered *D. floribunda* Mart. et Gal. tuber.

Similarly, to determine the extent of breakdown of the sapogenin during extraction, 40 mg. of diosgenin was refluxed with 100 ml. of 2 *N* hydrochloric acid for 2 hr. and subsequently extracted with chloroform. This solution was then assayed and compared with an appropriate standard of untreated diosgenin.

Samples of the extracted *Dioscorea* tubers were assayed by the thin-layer chromatographic and infrared spectroscopic methods for purposes of comparison.

RESULTS

Relationship Between Spot Absorbance and Weight of Diosgenin—The relationship between the logarithm of the weight applied and the square root of the absorbance of the spot produced was found to be linear for weights of diosgenin from 10 mcg. to 75 mcg. (Fig. 1 and Table I).

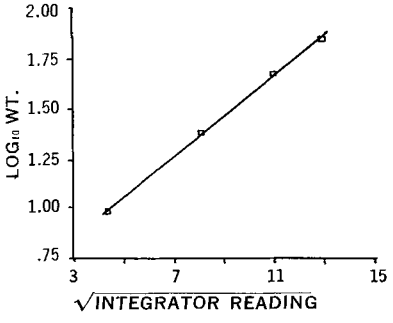

Fig. 1—Relationship between spot absorbance and weight of diosgenin.

TABLE I—RELATIONSHIP BETWEEN WEIGHT OF DIOSGENIN APPLIED AND ABSORBANCE OF THE SPOT, EXPRESSED AS A READING FROM THE AUTOMATIC INTEGRATOR

Diosgenin Wt.[a], mcg.	Log_{10} Wt.	Integrator Reading	Integrator √Reading
5	0.6990	13.0	3.61
10	1.0000	18.0	4.24
25	1.3979	64.0	8.00
50	1.6990	117.0	10.82
75	1.8751	163.0	12.77
100	2.0000	250.0	15.81

[a] Mean results from 6 plates.

TABLE II—QUANTITATIVE RESULTS FOR DIOSGENIN RECOVERY

Applied mcg.	Diosgenin Recovered mcg.	Diosgenin Recovered %[a]	No. of Estimations
20	20.32 ± 1.4	101.6 ± 6.9	40
40	40.08 ± 2.9	100.2 ± 7.3	40
60	58.2 ± 4.4	97.0 ± 7.4	40

[a] Recovered per cent is expressed as the mean of all determinations ± one standard deviation.

TABLE III—COMPARISON OF DENSITOMETRIC AND INFRARED ASSAY PROCEDURES

Plant Source	% Diosgenin m.f.b.[a] Densitometric	% Diosgenin m.f.b.[a] Infrared
D. floribunda sample *1*	3.91	3.95
D. floribunda sample *2*	4.25	4.55
D. floribunda sample *3*	2.38	2.40
D. sylvatica sample *1*	2.50	2.80
D. sylvatica sample *2*	3.65	3.85
D. deltoidea sample *1*	3.06	3.40
D. deltoidea sample *2*	3.14	3.35

[a] Moisture free basis.

The percentage error in the method was calculated by applying 20, 40, and 60 mcg. of diosgenin to thin-layer plates and estimating the variation observed in spot intensity. Ten chromatograms each of four spots were prepared in these determinations (Table II).

Estimation of Diosgenin in Dioscorea Tubers—The diosgenin content of the tuber of the edible *Dioscorea* species was found by the densitometric method to be 0.1%. When diosgenin was added to this extract, a recovery of 99.5% of the added diosgenin was obtained. Similarly, a recovery of 101% of the added diosgenin was obtained when estimated in the presence of an equal quantity of cholesterol. Similar results were obtained with a sample of *D. floribunda* tuber assayed by the same procedure.

Comparison of Densitometric and Infrared Assays—Comparison of assay results obtained from *D. floribunda*, *D. sylvatica* Ecklon., and *D. deltoidea* Wall. using both the densitometric and infrared methods is given in Table III.

DISCUSSION

Purdy and Truter (10) found that the relationship between the logarithm of the weight of substance applied and the square root of the area of the spot produced gave a linear relationship. This linear relationship was found to hold for the logarithm of the weight of diosgenin applied and the absorbance of the spot produced for diosgenin from 10 mcg. to 75 mcg. The results obtained show that densitometric thin-layer chromatography provides a suitable method for the assay of diosgenin in *Dioscorea* tubers.

In this study a number of experimental factors were found to be important in obtaining reproducible results. For example, plates of uniform gel thickness were essential, since variations in the layer produced variations in the amount of light transmitted through the plate, which affected the result recorded by the densitometer (11). Therefore, plates were selected which were uniform in appearance. It was also important to spray the plates evenly and ensure even heating to avoid development of uneven color intensity by the spots. All spots were scanned in the same direction, namely opposite to that in which the plates were developed, since considerable variations were obtained when the spots were scanned in different directions.

Using the solvent syftem *n*-hexane–ethyl acetate, diosgenin was separated from the other constituents in the *Dioscorea* extract. It was, however, observed that the sterol spot of higher R_f value and another spot of lower R_f value were sufficiently close to the diosgenin to interfere with accurate densitometric estimation. This was particularly noticeable when the proportion of sterol to diosgenin was high. This difficulty was easily overcome by double development of the chromatogram in *n*-hexane–ethyl acetate, when the diosgenin spot was well separated from the two interfering spots.

To determine the sensitivity of the procedure, known weights of diosgenin were added to *Dioscorea* tuber extracts of known diosgenin concentration. In other tests, equal weights of cholesterol and diosgenin were added to the *Dioscorea* extracts but in all cases quantitative recoveries of the added diosgenin were recorded, showing that the diosgenin was separated from the other plant constituents which did not interfere with the assay. This was also verified by the close correlation between the infrared assay results and the quantitative thin-layer chromatographic results. The results from the infrared method were always slightly higher than those obtained by quantitative thin layer. This was as expected, as during the extraction procedure of diosgenin, a certain quantity of 25 D-spirosta-3,5-diene is formed (12) on heating diosgenin with 2 *N* hydrochloric acid. The diene is estimated with the diosgenin in the infrared method, but by thin-layer chromatography the diene is well separated from diosgenin and is not estimated. In test experiments, by heating diosgenin with boiling 2 *N* hydrochloric acid for 2 hr., the degree of decomposition was estimated as being between 5.1 and 7.3%.

Using the solvent system described, diosgenin is not separated from its isomer yamogenin, hence the yamogenin present in the samples will be estimated as diosgenin. This is also the case with the infrared method of assay (4).

A major advantage of a quantitative thin-layer method for estimating diosgenin is that the quantity of plant material required is very small. One gram of dry, powdered tuber, of diosgenin content 2.5%, moisture free basis, has been successfully assayed.

SUMMARY

A thin-layer chromatographic technique using Silica Gel G as the adsorbent and hexane–ethyl acetate (4:1 v/v) as the solvent system has been devised for the separation and analysis of diosgenin in *Dioscorea* tubers. It was demonstrated that a linear relationship exists between the logarithm of the weight of diosgenin applied and the square root of the absorbance of the spot produced. Quantitative evaluation was achieved by using a photoelectric densitometer coupled to an integrating logarithmic recorder which computed the absorbance of the spot. The experimental error was found to be approximately 7%. The technique gave quantitative results when diosgenin was applied in concentrations between 10 and 75 mcg. The results were compared with those obtained by infrared spectrometry and were found to be slightly lower due to the nonestimation of 25 D-spirosta-3,5-diene by the quantitative thin-layer method. Several experimental factors which influence the accuracy of the method are discussed.

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