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QUANTITATIVE ESTIMATION OF MONOHYDROXY SATURATED STEROIDAL SAPOGENINS IN PLANT MATERIALS BY DENSITOMETRIC THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A thin-layer chromatographic technique using Silica Gel G as the adsorbent and six-fold development with *n*-hexane–ethyl acetate (12:1, v/v) has been devised for the separation and analysis of monohydroxy, saturated sapogenins in *Asparagus*, *Smilax* and *Yucca* species. It was shown that a linear relationship exists between the logarithm of the weight of sapogenin 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 %. Smilagenin and sarsasapogenin were found to be insufficiently separated by the described method and were estimated together. Similarly, tigogenin and neo-tigogenin were not separated and were estimated together. Equal spot intensities were produced by equal weights of sarsasapogenin, smilagenin, neo-tigogenin and tigogenin. The results obtained using the densitometric assay procedure showed close correlation with results obtained by an infra-red spectroscopic method.

Steroidal sapogenins are of economic importance as precursors of many of the medicinally useful steroids. They occur in plants as glycosides and are of somewhat restricted distribution. Economically, steroidal sapogenins are isolated mainly from species in the genera *Dioscorea*, *Agave* and *Yucca*, although they are to be found in several other genera, including *Smilax*, *Asparagus*, *Strophanthus*, *Digitalis* and *Trigonella*. The individual sapogenins of primary industrial value are diosgenin (Δ^5-25α-spirosten-3β-ol) from *Dioscorea* species, hecogenin (5α,25α-spirostan-3β-ol-12-one) from *Agave* species and sarsasapogenin (5β,25β-spirostan-3β-ol) from *Yucca* species. In addition to the genus *Yucca*, sarsasapogenin is found in *Asparagus officinalis* L[1] and *Smilax* species[2], the latter including the commercially used sarsasaparillas.

After hydrolysis of the naturally occurring saponins and extraction of the liberated sapogenins with a hydrocarbon solvent, the sapogenins are usually assayed by gravimetric[3,4] or infra-red techniques[5,6]. The gravimetric methods are applicable

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to plant materials with high sapogenin yields, but are inaccurate when the proportion of contaminants extracted is high. Infra-red spectroscopic methods are more suitable, as they are based on the spirostan structure of the sapogenin and are thus specific for steroidal sapogenins. It has been found, however, that non-sapogenin compounds extracted by the hydrocarbon solvent may interfere with the infra-red assay method and cause high results. To overcome these disadvantages it was felt that a convenient analytical technique, suitable for small quantities of plant material, was required. Densitometric thin-layer chromatography has been studied and its use for the estimation of diosgenin in *Dioscorea* species reported[7]. Extension of the method for sarsasapogenin and other monohydroxy, saturated sapogenins is reported in this paper. This method was developed for future studies on the genus *Yucca*, in particular *Y. glauca* Nutt., a plant found growing in the U.S.A. from Iowa and South Dakota to Montana, south to Missouri, Texas and Arizona[8]. From the leaves of *Y. glauca*, sarsasapogenin has been isolated[9,10], but in addition to this sapogenin they contain smilagenin (5β,25α-spirostan-3β-ol), neo-tigogenin (5α,25β-spirostan-3β-ol) and tigogenin (5α,25α-spirostan-3β-ol)[11]. As a result, this paper reports the development of an assay procedure for the estimation of monohydroxy, saturated sapogenins.

EXPERIMENTAL

Glass plates, 15 × 20 cm, were spread with a layer of Silica Gel G (Merck) to a thickness of 250 μ, according to the method of STAHL[12] and air-dried before use. Known quantities of sarsasapogenin, smilagenin, neo-tigogenin and tigogenin were applied to the plates by micropipettes and developed with *n*-hexane–ethyl acetate (12:1, v/v), using the super-saturated method of STAHL[13]. After the solvent had advanced 15 cm from the starting line, the plates were removed from the tank, dried and re-developed five more times with the same solvent system. Following development, the plates were dried and sprayed uniformly with a solution of antimony trichloride in concentrated hydrochloric acid (3:1, w/w)[14]. The sapogenins were visualised by heating the plate at 100° in a circulating air-stream until the spots were a blue-black colour. This normally took 30 min.

The plates were allowed to cool before the absorbances of the spots were estimated by a Vitatron densitometer* using a slit width of 20 mm by 2 mm and either a tungsten lamp or 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 sarsasapogenin, smilagenin, neo-tigogenin and tigogenin, dissolved in chloroform, were applied to thin-layer plates in concentrations ranging from 18 μg to 80 μg. After development, the spots produced were scanned and a linear relationship between weight applied and absorbance was established for all four sapogenins within the range tested.

Preparation of plant material

The sapogenins were extracted from plant material of known weight and

* Fison's Scientific Apparatus Ltd., Loughborough, Leic., Great Britain.

moisture content by the method of BLUNDEN, HARDMAN AND WENSLEY[10]. This involved maceration of the fresh material, incubation of the distintegrated material, acid hydrolysis of the saponins by refluxing with 2 *N* hydrochloric acid for 2 h, and extraction of the sapogenins from the dry, acid-insoluble residue with light petroleum (40–60°). Dried, powdered plant material was incubated first with water before being treated by the above procedure. The petroleum extract was evaporated to dryness, re-dissolved in chloroform and made up to a known volume. These extracts were applied to thin-layer plates along with standard sapogenin solutions. In the case of *A. officinalis* and *Smilax* species, only a standard solution of sarsasapogenin was necessary, but with *Y. glauca*, standard solutions of sarsasapogenin and tigogenin were applied. 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 sarsasapogenin and tigogenin contents were calculated relative to the standard solution, and the resultant value obtained from the mean of six plates, each containing two spots of the plant extract.

To determine the variation in spot intensity produced by equal weights of sarsasapogenin, smilagenin, neo-tigogenin and tigogenin, 20 μg and 60 μg of each compound were applied to thin-layer plates and the spot intensities produced were compared.

The extent of breakdown during extraction of each of the four sapogenins, sarsasapogenin, smilagenin, neo-tigogenin and tigogenin was determined by refluxing 20 mg samples of each compound with 50 ml of 2 *N* hydrochloric acid for 2 h and subsequently extracting them with chloroform. These solutions were assayed and compared with appropriate standards of untreated compounds.

Samples of *A. officinalis*, *S. aristolochaefolia*, *S. regellii*, and *Y. glauca* were assayed by the thin-layer chromatographic and infra-red spectroscopic methods for purposes of comparison. The infra-red spectroscopic determinations of *Asparagus* and *Smilax* samples were performed by dissolving the sapogenin extracts in chloroform and assaying them using a Hilger H 800 spectrophotometer. The total sapogenin concentration was estimated from the intensity of the band near 980 cm^{-1} and the

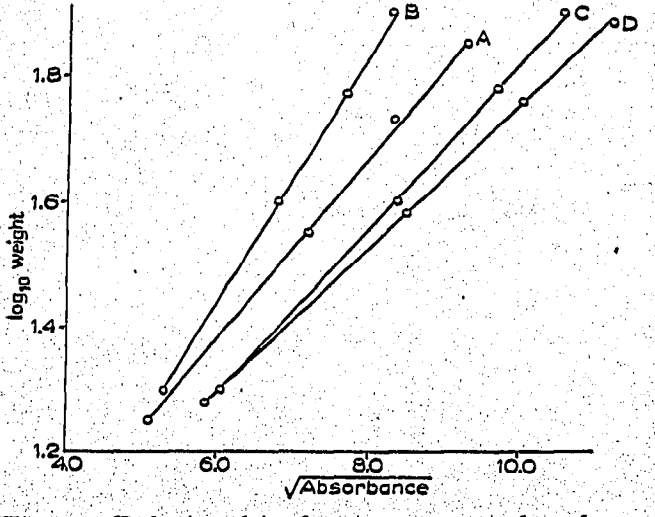

Fig. 1. Relationship between spot absorbance and weight of sarsasapogenin, smilagenin, neo-tigogenin and tigogenin. A = Sarsasapogenin; B = smilagenin; C = neo-tigogenin; D = tigogenin.

TABLE I

RELATIONSHIP BETWEEN WEIGHTS OF SARSASAPOGENIN, SMILAGENIN, NEO-TIGOGENIN AND TIGOGENIN APPLIED AND THE ABSORBANCIES OF THE SPOTS PRODUCED, EXPRESSED AS A READING FROM THE AUTOMATIC INTEGRATOR

Sapogenin	*Wt. (μg)*	*Log_{10} wt.*	*Integrator[a] reading*	*$\sqrt{}$Integrator reading*
Sarsasapogenin	17.9	1.25	26	5.1
	35.8	1.55	52	7.2
	53.7	1.73	69	8.3
	71.6	1.85	86	9.3
Smilagenin	19.7	1.29	28	5.3
	39.4	1.60	46	6.8
	59.1	1.77	59	7.7
	78.8	1.90	69	8.3
Neo-tigogenin	20.0	1.30	38	6.2
	40.0	1.60	70	8.4
	60.0	1.78	94	9.7
	80.0	1.90	111	10.5
Tigogenin	19.0	1.28	34	5.8
	38.0	1.58	72	8.5
	57.0	1.76	100	10.0
	76.0	1.88	126	11.2

[a] Mean result from six plates.

TABLE II

QUANTITATIVE RESULTS FOR SARSASAPOGENIN, SMILAGENIN, NEO-TIGOGENIN AND TIGOGENIN RECOVERIES

Sapogenin	*Wt. applied (μg)*	*Recovered[a]*		*No. of estimations*
		μg	*%*	
Sarsasapogenin	17.9	17.4 ± 1.0	97.0 ± 5.5	40
	35.8	35.5 ± 2.5	99.2 ± 7.0	40
	53.7	53.3 ± 2.4	103.0 ± 4.3	40
Smilagenin	19.7	19.6 ± 1.3	99.7 ± 6.6	40
	39.4	39.2 ± 2.6	99.6 ± 6.7	40
	59.1	58.2 ± 4.1	98.7 ± 7.0	40
Neo-tigogenin	20.0	20.1 ± 1.4	100.3 ± 6.8	40
	40.0	40.5 ± 2.0	101.3 ± 4.9	40
	60.0	60.2 ± 3.7	100.3 ± 6.1	40
Tigogenin	19.0	19.1 ± 1.1	100.3 ± 5.8	40
	38.0	38.2 ± 2.3	100.3 ± 5.9	40
	57.0	58.1 ± 4.7	101.9 ± 8.1	40

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

major components, if 25β-epimers, are estimated from the intensity of the band near 915 cm^{-1}, or if 25α-epimers, from that of the band near 900 cm^{-1}. The minor component was obtained by difference. This technique is applicable only when the sample is predominantly of one configuration and there is not much contamination from non-sapogenin materials. For the assay of *Y. glauca* samples it was necessary to ignore the 980 cm^{-1} peak owing to the appreciable 25α-components and to use a ratio method, based on the ratio of the intensities of the 915 and 900 cm^{-1} peaks[15].

RESULTS AND DISCUSSION

PURDY AND TRUTER[16] found that there was a linear relationship between the logarithm of the weight of substance applied and the square root of the area of the spot produced. BLUNDEN, HARDMAN AND MORRISON[7] found that this linear relationship held for the logarithm of the weight of diosgenin applied and the absorbance of the spot produced. Four monohydroxy, saturated steroidal sapogenins, sarsasapogenin, smilagenin, neo-tigogenin and tigogenin have now been studied and a similar linear relationship found to apply, from approximately 18 μg to 80 μg. The limits of the linear relationship were not determined (Fig. 1 and Table I). These results showed that densitometric thin-layer chromatography provides a suitable method of assay for these sapogenins.

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

The solvent system used for the separation of diosgenin from the other constituents in *Dioscorea* extracts was *n*-hexane–ethyl acetate (4:1, v/v)[7]. Double development of each chromatogram was necessary to effect sufficient separation of the sterol and diosgenin spots to enable accurate densitometric estimations to be made. The same solvent system when applied to the plant extracts in the present study did not separate the sarsasapogenin from the sterol sufficiently for the quantitative estimation of the genin. A number of other solvent systems commonly used for sapogenins[17,18] were tried, but without success. Finally, six-fold development in *n*-hexane–ethyl acetate (12:1, v/v) proved satisfactory. When β-sitosterol was added to previously assayed samples of *A. officinalis*, *S. aristolochaefolia* and *Y. glauca* in quantities equal to the sarsasapogenin yield and the mixtures assayed again, results were obtained which were not significantly different from previously obtained figures, showing that the added sterol had been effectively separated from the sapogenin spots and did not interfere with the assay.

S. aristolochaefolia, *S. regellii* and *Y. glauca* extracts contained smilagenin in addition to sarsasapogenin. Six-fold development in the *n*-hexane–ethyl acetate solvent system did not effectively separate these two compounds, although smilagenin was clearly visible as a cap over the sarsasapogenin spot. As a result, smilagenin present was estimated along with sarsasapogenin. In addition to sarsasapogenin and smilagenin, *Y. glauca* contains tigogenin and neo-tigogenin. These latter two sapogenins were not separated from each other on the thin-layer chromatograms, but were well separated from the combined smilagenin/sarsasapogenin spot (Fig. 2). As a result, tigogenin and neo-tigogenin were also estimated together and calculated as

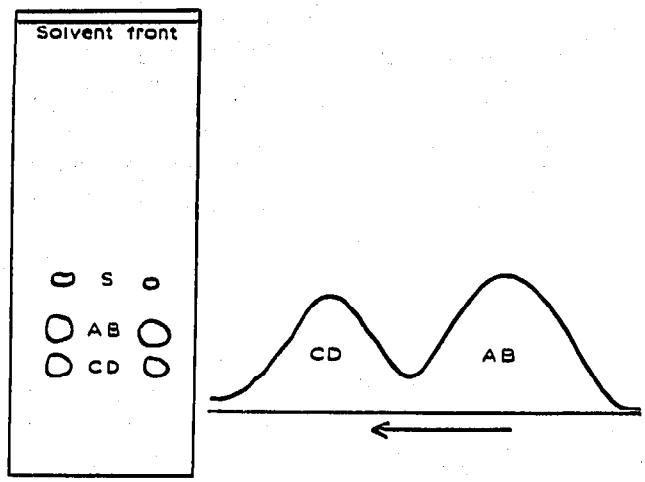

Fig. 2. Thin-layer chromatogram of *Y. glauca* extract and integrator tracing of the sapogenin spots. S = Sterol; AB = sarsasapogenin/smilagenin; CD = tigogenin/neo-tigogenin.

tigogenin. It was shown that equal weights of sarsasapogenin, smilagenin, neo-tigogenin and tigogenin give equal spot intensities with the antimony trichloride reagent. It was concluded, therefore, that no error was involved in estimating smilagenin with sarsasapogenin and neo-tigogenin with tigogenin.

To determine the sensitivity of the assay procedure, sarsasapogenin was added to previously assayed samples of *A. officinalis* rhizomes, *S. aristolochaefolia* roots and *Y. glauca* leaves. To each of the different extracts of known volume, sarsasapogenin was added equivalent to approximately 25 % and 50 % of the total sarsasapogenin yield of the extracts and the chromatographic assay carried out on the mixture to determine the recovery of the added sapogenin. Similar recovery tests were performed

TABLE III

RECOVERY OF ADDED SAPOGENINS TO ASSAYED SAMPLES OF PLANT EXTRACTS

Sapogenin added	*Amount added as % of quantity in extract*	*Recovery (%)*
Sarsasapogenin	25[a]	97.4[c]
	50[a]	99.7[c]
Smilagenin	25[a,b]	99.8[c]
	50[a,b]	100.2[c]
Tigogenin	25	107.3[d]
	50	96.0[d]
Neo-tigogenin	25	104.1[d]
	50	100.0[d]

[a] Amount in extract taken as the sum of sarsasapogenin and smilagenin yields.
[b] Recovery of added smilagenin calculated as sarsasapogenin.
[c] Mean of results from *S. aristolochaefolia*, *A. officinalis* and *Y. glauca*.
[d] Only applicable to *Y. glauca*.

TABLE IV

COMPARISON OF DENSITOMETRIC AND INFRA-RED ASSAY RESULTS

Species	*Morphological part*	*Infra-red results (%)*			*Densitometric results (%)*[a]		
		25β-Sapogenins	*25α-Sapogenins*	*Total*	*5β-Sapogenins*	*5α-Sapogenins*	*Total*
A. officinalis	Rhizome						
Sample 1		1.00	—	1.00	0.99	—	0.99
Sample 2		0.78	—	0.78	0.75	—	0.75
S. aristolochaefolia	Root	0.43	0.02	0.45	0.46	—	0.46
S. regellii	Root	0.05	0.16	0.21	0.19	—	0.19
Y. glauca	Leaf						
Sample 1		0.86	0.39	1.25	0.84	0.38	1.22
Sample 2		1.15	0.29	1.14	1.44	0.26	1.40
Sample 3		1.12	0.27	1.39	1.14	0.28	1.42

[a] Moisture-free basis.

on extracts of the same three species to determine the recovery of added smilagenin, calculated as sarsasapogenin. The recovery from an extract of *Y. glauca* of added neo-tigogenin and tigogenin was also carried out. In all cases, within the limits of experimental error, quantitative recoveries of the added sapogenins were obtained (Table III).

Close correlation was found between the assay results obtained using the infra-red and densitometric methods (Table IV). In the assay of diosgenin it was shown that the results obtained by the infra-red spectroscopic method were higher, as it estimated diosgenin and 25α-spirosta-3,5-diene together, whereas the two compounds were separated by thin-layer chromatography, resulting in the estimation of diosgenin only by the densitometric method. Sarsasapogenin, smilagenin, neo-tigogenin and tigogenin are saturated compounds and it was shown that refluxing with 2 *N* hydrochloric acid for 2 h did not produce any significant decomposition of the sapogenins, although solutions prepared from the acid-treated compounds were yellow in colour. The unsaturated compound, diosgenin, however, was found to decompose by between 5.1 and 7.3 %[7].

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