DIOSCOREA BELIZENSIS LUNDELL AS A SOURCE OF DIOSGENIN

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Received November 28, 1962

Twenty-nine species of wild *Dioscorea* from British Honduras were screened for saponins. The tubers of *D. belizensis* Lundell afforded yields of steroids of about 2 per cent of the dry weight, calculated as the acetate of the predominant genin, diosgenin. A routine assay for diosgenin in these tubers is given and the effects of comminution, chemical disintegration, fermentation, refrigeration, autoclaving, and drying of the tubers are described. The yield is controlled by an endogenous enzyme system.

DIOSCOREA tubers are an important source of steroids for the pharmaceutical industry. In 1959, Dr. S. S. Bampton of the Tropical Products Institute, London collected 29 species of wild yams from British Honduras. These were subsequently grown in the hot house at Nottingham and screened for steroids. One species, *Dioscorea belizensis* Lundell, identified and described by Blunden, Hardman and Trease (1963) was chosen because it had a steroid content of potential commercial interest and was readily propagated (Blunden and Hardman, unpublished) for future selection of high yielding strains.

Steroidal assays of yams are usually done by acid hydrolysis of the glycosides *in situ* and extraction of the sapogenins by a hydrocarbon solvent. The gravimetric method of Morris, Roark and Cancel (1958) is of this kind. It was modified to give a reliable routine procedure appropriate to the form and lignified nature (Blunden, Hardman and Trease, 1963) of the tuber of *D. belizensis* and enabled us to study the effect of various preliminary laboratory treatments of this species on its yield of diosgenin.

EXPERIMENTAL

It was unnecessary to extract the yams to screen them for saponins. A fragment of fresh or dried tuber was placed on a blood agar plate at about 19° and any haemolysis noted in 3 to 6 hr., before the influence of any contaminants was evident.

Tubers of *D. belizensis*, received by air mail from British Honduras, deteriorated rapidly unless they were repacked on arrival, in vermiculite in well filled sealed tins and kept at 17 to 20°. They then remained sound for at least 1 year.

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Assay: Standard Procedure

The tuber branches occur in lengths of up to 1 metre and from 2·5 to 5 cm. in diameter. A clean (free of soil and fungal attack) piece (70 g.) was sliced transversely, two thick (about 9 mm.) slices being taken for the sapogenin estimation and the next thin (about 3 mm.) slice for the moisture content. This sampling was continued for the entire length of the tuber. It was arranged that about 30 g. of the tuber was used for each of two sapogenin determinations and about 5 g. for each of two moisture values. The latter were obtained by drying the slices at 95–105° for 12 hr. The tuber slices for the sapogenin determination were cut into thin strips before being disintegrated for 5 min. in a Townson and Mercer top drive macerator in the presence of 100 ml. water. The mixture, with water rinsings (100 ml.), was incubated at 25 or 37° for 5 days in a plugged flask. After concentrated hydrochloric acid had been added to make the acid concentration 2N, the mixture was boiled for 2 hr., and cooled. The acid-insoluble material collected at the pump, was neutralised by washing with water, 20 per cent sodium carbonate solution and again with water.

TABLE I

DUPLICATE DETERMINATIONS, (*a*) AND (*b*) OF SAPOGENIN ACETATE BY THE STANDARD ASSAY PROCEDURE

Fresh tuber Piece No.	Yield*		Fresh tuber Piece No.	Yield*	
	(*a*)	(*b*)		(*a*)	(*b*)
1	2·26	2·21	4	0·43	0·42
2	1·40	1·42	5	2·31	2·33
3	2·07	2·07	6	1·62	1·64

* Per cent of moisture free tuber calculated as diosgenin acetate from infra-red spectra.

The residue was dried at 80° overnight, powdered and the sapogenins extracted with light petroleum (b.p. 40–60°) in a Soxhlet apparatus for 24 hr. The petroleum-soluble material (0·1–0·2 g.) was acetylated using 2 ml. acetic anhydride and the procedure of Wall, Eddy, McClennan and Klumpp (1952) and the benzene-soluble material assayed in carbon disulphide at a concentration of 0·7 to 2 per cent in the 1·0 mm. cells of a Hilger H 800 infra-red spectrophotometer. The estimation was based on the band at 982 $cm.^{-1}$ at which pure diosgenin acetate had the extinction value of 7·685.

This assay procedure was used to study the effect on the sapogenin yield of various pre-assay treatments of the tubers. The paper chromatography method of Sannié and Lapin (1952) was used to examine the light petroleum-soluble extract for sapogenins, the latter being detected by spraying with antimony trichloride in hydrochloric acid (Nakao, Hirai and Yoshizawa, 1958).

RESULTS

Tubers of four of the 29 species gave a positive test for saponins. One of these was *D. belizensis*; its root, stem, and leaf also caused haemolysis. The fresh tuber contained 73 to 85 per cent moisture. The sapogenin

content is expressed as diosgenin acetate per cent of the moisture free tuber. Chromatographic examination (Sannié and Lapin, 1952; Nakae and others, 1958) of the petroleum-soluble material obtained in the above assay of the tuber, disclosed four sapogenins with diosgenin predominating.

TABLE II

YIELD OF SAPOGENIN. COMPARISON OF YIELD BY INFRA-RED ANALYSIS OF LIGHT PETROLEUM-SOLUBLE MATERIAL AFTER ACETYLATION, WITH THE GRAVIMETRIC YIELD OF THIS MATERIAL BEFORE AND AFTER ACETYLATION

Fresh tuber Piece No.	Infra-red*	Gravimetric†	
		Acetylated	Unacetylated
7	1·91	2·92	2·80
8	2·26	3·58	3·46
9	1·40	3·50	3·25
10	1·82	4·11	3·88

* Per cent of moisture free tuber calculated as diosgenin acetate from infra-red spectra.
† Per cent of moisture free tuber.

The latter crystallised from the light petroleum solution when it was concentrated and on re-crystallisation from ethanol had m.p. and mixed m.p. 204–206°; its acetate, from methanol, had m.p. and mixed m.p. 198°. Moreover the infra-red spectrum obtained from the acetate of the predominant sapogenin was identical with that obtained from diosgenin acetate.

TABLE III

THE EFFECT OF CONTAMINANTS ON THE YIELD OF DIOSGENIN ACETATE WHEN DETERMINED FROM THE INFRA-RED SPECTRA

Weight of contaminant, g.	Weight of diosgenin acetate added, g.	Yield of diosgenin acetate, per cent
0·1622	0·0278	102·3
0·0814	0·0508	100·9
0·0812	0·0493	98·4
0·0972	0·0411	100·1
0·1102	0·0436	100·4

The nature of the tuber of *D. belizensis* is such that it is difficult to remove it from the ground, free it of soil and transport it, without the branches of the tuber being broken. Small pieces (70 g.) were commonly received, for example pieces Nos. 1–23 (see Tables); large pieces (165 g.) for example Nos. 24 and 25 (Table V), were rare. This limited the design of the experiments.

The validity of the method of subsampling of the tuber branches was demonstrated by the close agreement of duplicate determinations of sapogenins (Table I). An infra-red determination was necessary as seen from a comparison of the value so obtained with the gravimetric yield of crude sapogenin acetate (Table II). The contaminants did not interfere with the infra-red assay; known weights of pure diosgenin acetate were mixed with known weights of benzene-soluble material obtained by

extracting the powdered dry tuber with benzene (Table III). Addition of known weights of diosgenin to the disintegrated tuber at the onset of the standard assay resulted in the expected yield.

The yields of sapogenin obtained from duplicate subsamples of tuber, one of which had received the standard assay procedure and the other had not been fermented, are shown in Table IV. Small increases were

TABLE IV

COMPARISON OF SAPOGENIN YIELDS* FROM FRESH TUBER WHEN UNFERMENTED AND WHEN FERMENTED

Fresh tuber Piece No.	Unfermented	Fermented (standard assay procedure)	Increase, per cent
11	1·92	2·21	15
12	2·07	2·27	10
13	1·37	1·44	5
14	2·23	2·39	7
15	0·86	0·92	7

* Per cent of moisture free tuber calculated as diosgenin acetate from infra-red spectra.

always achieved by fermentation; the results were similar whether the temperature was 25 or 37°. In a single experiment the deliberate addition of soil (air dried, 2 g.) received on the tubers from British Honduras, to soil-free fresh tuber (30 g.) resulted in a marked loss of diosgenin on fermentation at 25° for 5 days (diosgenin; 1·39 per cent compared with 2·02 per cent without soil).

TABLE V

SAPOGENIN YIELD* AFTER DRYING AT 80°

Fresh tuber Piece No.	Standard assay procedure (fermented)	Sliced, dried, and powdered		Homogenised, dried, and powdered (unfermented)		Increase (per cent) on fermentation (relative to column 3)
		Unfermented	Fermented			
16	0·76	0·57				33
17	1·23	0·85				45
18	0·87	0·41				112
19	2·63	1·24				112
20		1·12	2·59			131
21		0·43	1·33			209
22		0·63	1·82			189
23		0·80	1·77	(*a*)	(*b*)	121
24	2·27	1·82	2·21	2·20	2·19	23
25	1·51	0·78	1·53	1·48	1·47	95

* Per cent of moisture free tuber calculated as diosgenin acetate from infra-red spectra.

A large piece of tuber (165 g.) (pieces Nos. 24 and 25 of Table V) was cut into alternate thick and thin transverse slices. The thin slices afforded the moisture content in duplicate. Each thick slice was cut in turn into four approximately equal segments and each segment was allocated in rotation to one of five samples. One sample was used for the assay by the standard procedure. Two samples were bulked, dried at 80° for 16 hr., and powdered in a hand-mill until all the particles passed through a No. 60 sieve. After mixing the powder well and removing portions for moisture determinations, the rest was divided into two equal parts. One part was mixed with water (250 ml.) and acid hydrolysed, and the other

was fermented in water (250 ml.) before acid hydrolysis as in the standard assay procedure. The remaining two samples were treated separately as follows (assay in duplicate): The segments were first cut into small pieces and then homogenised with water portionwise, in a Potter-Elvehjem homogeniser during 5 hr. and using up to 400 ml. water. The resultant suspension was dried at 80° for 24 hr. and the residue assayed without being fermented. The results of this and related experiments are given in Table V. In such an experiment chromatographic examination (Sannié and Lapin, 1952) (Nakao and others, 1958) of the petroleum-soluble material from the fermented tuber via the standard assay method and that from tuber dried at 80° and not fermented, gave the same four sapogenins.

TABLE VI

COMPARISON OF SAPOGENIN YIELDS* FROM DISINTEGRATED (STANDARD ASSAY PROCEDURE) AND HOMOGENISED TUBER, BEFORE AND AFTER FERMENTATION

Fresh tuber Piece No.	Standard assay procedure (fermented)	Homogenised	
		Unfermented	Fermented
26	1·31	1·29	1·27
27	0·89	0·86	0·89

* Per cent of moisture free tuber calculated as diosgenin acetate from infra-red spectra.

Similar subdivision of single pieces of tuber afforded this result: homogenised tuber when fermented at 37° for 5 days gave the same sapogenin yield as when the tuber was homogenised, dried and assayed without being fermented (Table VI).

The yield by the standard assay procedure was also compared with the yield obtained when the tuber slices were first autoclaved at 115° for 30 min. before subjecting to the standard procedure, and when the fermentation stage of this procedure was omitted after autoclaving (Table VII).

TABLE VII

COMPARISON OF SAPOGENIN YIELDS* BEFORE AND AFTER AUTOCLAVING

Fresh tuber Piece No.	Standard assay procedure (fermented)	Autoclaved, then disintegrated		Increase (per cent); column 2 relative to column 4
		Unfermented	Fermented	
28	2·27	0·88	0·95	139
29	2·26	1·40	1·37	65
30	2·59	1·83	1·76	41
31	1·65	0·88	0·89	85

* Per cent of moisture free tuber calculated as diosgenin acetate from infra-red spectra.

The results obtained after storage of the tuber slices at 5° for 1 week before the standard assay procedure and such storage and assay but without fermentation, are shown in Table VIII.

The sliced tuber when disintegrated by treating with boiling 5 per cent potassium hydroxide solution for 20 min. gave a low sapogenin yield

(0·95 per cent) when compared with slices given the standard assay process (1·60 per cent).

None of the specimens of acid insoluble material in the above experiments gave a positive haemolysis test on blood agar, thus showing absence of saponins.

TABLE VIII

COMPARISON OF SAPOGENIN YIELDS* BEFORE AND AFTER STORAGE AT 5° FOR 7 DAYS

Fresh tuber Piece No.	Standard assay procedure (fermented)	Stored at 5°, then disintegrated		Increase (per cent) on fermentation (Column 4 relative to column 3)
		Unfermented	Fermented	
32	1·65	0·56	1·66	196
33	0·53	0·38	0·57	50
34	2·41	1·36	2·39	76

* Per cent of moisture free tuber calculated as diosgenin acetate from infra-red spectra.

DISCUSSION

The tubers of species of saponin-holding yams vary markedly in their texture, content of petroleum-soluble material, and the ease with which they afford diosgenin.

Rothrock, Hammes, and McAleer (1957) and Morris, Roark and Cancel (1958) have described gravimetric assays of yams for diosgenin. Their procedures were modified to give a reliable routine assay of small weights of *D. belizensis* tuber. Its moisture content was about 80 per cent; the method of this estimation, as described by Morris and others (1958), was found unsatisfactory on the weight of tuber involved. When water alone was used for washing the acid-insoluble material it was found that the filtrate could be neutral to indicator paper but the particles when squeezed between the paper still gave an acid reaction. A loss of diosgenin occurred when the acid-insoluble material was dried in an acid state and then stored for 2 months, but not when the material was left in a slightly alkaline condition. The final light petroleum extract of the acid-insoluble residue contained sufficiently high and variable quantities of contaminants to make a gravimetric procedure unreliable.

Yields of diosgenin are reported to be increased when comminuted tubers of dioscorea are fermented (Schering Corporation, 1956). Krider, Cordon and Wall (1954) and Rothrock Stoudt, and Garber (1955) have shown that fungi are capable of hydrolysing steroidal saponins to sapogenins. Only clean tubers, free of mould attack and soil, were normally used in our experiments. When a large amount of soil was deliberately introduced a low yield of diosgenin was obtained after fermentation.

Diosgenin yield may be expected to vary with tissue maturity. This was taken into account in the method of subsampling in the individual experiments. The results fall into two groups: Low yield of genin as given by the autoclaved tuber and high yield as obtained when the tuber is homogenised. Low yields of similar quantities were obtained when the fresh tuber slices were autoclaved; or dried at 80°, powdered and not

fermented; or stored at 5° for 1 week, disintegrated and not fermented; or treated with boiling 5 per cent potassium hydroxide solution. High yields of similar quantities were obtained when the fresh slices were disintegrated and fermented; or dried at 80°, powdered and fermented; or homogenised; or homogenised and dried; or stored at 5° for 1 week, disintegrated and fermented. The high yield was 40 to 200 per cent more than the low one. The results indicate the presence of an endogenous enzyme system capable of producing much of this increase in diosgenin during only 5 min. of cell disintegration of the fresh tuber. While the optimum cell damage for this increase may be that caused by the homogenisation procedure used, this took 5 hr. to achieve with 30 g. fresh tuber. Both the vascular tissue which ramifies throughout the tuber, and the cork, are lignified. Five min. distintegration afforded only about 10 per cent less diosgenin which could be made up by fermentation of the aqueous tuber mash containing particles with edges up to 6 mm. Dried sliced tuber if powdered and fermented gave the same yield as the homogenised tuber. The results after chilling (5°) or drying (80°), common procedures for glycoside studies on medicinal plants, show an apparent loss of genin if, before the assay, the appropriate conditions are not given for the formation of steroids. This is of obvious importance where dried yam is being assayed for diosgenin or is the starting material for its isolation.

Unknown to us, Roark and Morris (1961) and Roark, Cruzado, Delpin and Morris (1961) made similar experiments using the tubers of *Dioscorea composita* and *D. floribunda*. Our results with *D. belizensis*, the tuber of which has a different form from that of the other two yams, are in agreement with and extend those of Roark and his colleagues. Our chemical studies on *D. belizensis* begun in 1960 were completed before the work of Roark and others (1961) was seen and before our studies on the morphology and propagation of *D. belizensis* were made. All of our work on *D. belizensis*, including that reported in this paper, was published by one of us in March, 1962 in thesis form (Blunden, 1962). No doubt had Roark and Morris (1961) fermented their tubers which had been "sliced, dried at 85° and ground to a fine dust" they would have got the high steroid yield afforded by homogenising the tubers. They did not study the effect of chilling of the tubers.

Heftman, Bennett, and Bonner (1961) report unexpected and very low (0·013 per cent) incorporation into diosgenin of ^{14}C from labelled acetate and even less from labelled mevalonic acid, when these precursors of sterols, were used with homogenised tubers of *D. floribunda* under their conditions. While they did not say how the tubers were stored before homogenisation the latter step was done in the presence of cracked ice. This might have contributed to the low uptake of acetate since diosgenin is not immediately synthesised when the tuber of *D. belizensis* is disintegrated in water at 17–20° after the tuber has been stored at 5°. Roark and Morris (1961) have also used 1-^{14}C-labelled acetate with homogenised tubers of *D. floribunda* and obtained labelled sapogenins. Unfortunately, they give no indication of the amount of acetate so incorporated.

Much of the diosgenin which can be obtained from the tubers of *D. belizensis* is not present as such nor as its glycoside in the harvested tuber but is very rapidly produced by an endogenous enzyme system on disintegration of the sound tissue. All sapogenin yielding yams may behave in this manner and plants which are outside the family Dioscoreaceae but yield steroidal or triterpenoid compounds may also exhibit the phenomenon. This is being investigated.

Acknowledgements. We wish to thank the Tropical Products Institute, London, for the Department of Scientific and Industrial Research Studentship (to G. B.), Dr. S. S. Bampton, of the Institute for his expedition to British Honduras, Mr. H. J. Dothie and Mr. T. Coomes, also of the Institute, for the infra-red spectra, and Mr. R. Waters, Conservator of Forests, Belize, for the air-mail shipments of tubers of *D. belizensis*.

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