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THE RAPID QUANTITATIVE DETERMINATION OF C_{25} EPIMERIC STEROIDAL SAPOGENINS IN PLANTS

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Abstract—A procedure is described for the quantitative estimation of 25α- and 25β-sapogenins individually and together in a crude plant extract. The method is rapid and has an overall error of 3–10 per cent for individual C_{25} epimers and 3–5 per cent for the total sapogenin. The procedure is appropriate for the study of the variation in the C_{25} epimers in morphological parts and with season, as well as for routine screening for steroidal sapogenins of economic importance.

INTRODUCTION

STEROIDAL sapogenin-affording plants are of economic importance as raw material to the pharmaceutical industry for the production of the medicinal steroids and oral contraceptive drugs. Our recent investigations[1] required a rapid and accurate means of determination of C_{25} epimeric steroidal sapogenins at concentrations of 0·1–5·0 per cent in 1–10 g of dried plant material.

The direct estimation of the gravimetric yield of sapogenin from plants[2, 3] is limited to those instances where the whole of the extract, or at least most of it, is sapogenin and where a relatively large amount of plant material is available. Even then an approximate estimation only is obtained.

We have found that mixtures of both the C_{25} epimers, e.g. diosgenin (Δ^5, 25α-spirosten-3β-ol) and yamogenin (Δ^5, 25β-spirosten-3β-ol), normally occur. The ratio of their concentrations in plant tissue is dependent upon a number of factors. Important amongst these are the morphological part and its stage of development; the biochemical significance of these observations are being investigated. The properties of the C_{25} epimers are very similar and they are difficult to separate from one another. Some degree of resolution can be obtained using refined but tedious methods of thin-layer chromatography.[4, 5]

Methods for the quantitative densitometry of steroidal sapogenins have been reported,[6–8] but these are not readily applicable to the rapid screening of samples from different plant sources although they are necessary in certain cases.

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[1] F. R. Y. FAZLI and R. HARDMAN, *Tropical Sci.* **10**, 66 (1968).
[2] J. W. ROTHROCK, P. HAMMES and W. J. MCALEER, *Ind. Eng. Chem.* **49**, 186 (1957).
[3] M. P. MORRIS, B. ROARK and B. CANCEL, *J. Agr. Food Chem.* **6**, 856 (1958).
[4] H. HALPAAP, *Chem.-Ing.-Tech.* **35**, 388 (1963).
[5] R. D. BENNETT and E. HEFTMAN, *J. Chromatog.* **21**, 488 (1966).
[6] G. BLUNDEN, R. HARDMAN and J. C. MORRISON, *J. Pharm. Sci.* **56**, 948 (1967).
[7] G. BLUNDEN and R. HARDMAN, *J. Chromatog.* **34**, 507 (1968).
[8] K. R. BRAIN and R. HARDMAN (in preparation).

Wall *et al.*[9] devised an assay dependent on the highly specific absorption of the spirostan ring in the i.r. region between 1000 and 800 cm^{-1}. The plant material was extracted with ethanol to isolate the crude saponin, which was then purified by repeated extraction with benzene before hydrolysis with 4 N alcoholic hydrochloric acid. The released sapogenin was extracted with cold benzene, acetylated, and further extracted with benzene before removal of the solvent and solution in a known volume of carbon disulphide. This procedure is tedious and requires a fairly high degree of manipulative skill in the repeated extractions. Furthermore, isolation of the crude saponin followed by hydrolysis is frequently unsatisfactory due to the extraction of pigments, the production of tarry material, and decomposition of unsaturated sapogenins to sapogenin dienes.[10] Epimerization of the 25α and 25β forms may also occur in the presence of ethanolic hydrochloric acid.[11]

The present method uses the preferred extraction procedure[6,12] of an *in situ* hydrolysis of the saponin by aqueous hydrochloric acid, followed by hydrocarbon extraction of the free sapogenin. Much time is saved and some sources of error are eliminated by the use of the free sapogenins rather than the acetates. These are freely soluble in chloroform (but not in carbon disulphide). Wall *et al.*[9] assumed that samples contained sapogenin of only one C_{25} configuration and used only the 980 cm^{-1} band for estimation. Our work has extended and improved the measurement of the specific spirostan absorption and calculation of the absorbance of the bands at 915 cm^{-1} and 900 cm^{-1} enables the determination of the 25β and 25α forms separately. Wall *et al.* estimated that two workers could assay forty to fifty samples in a 40 hr week; our method has been operated at two to three times that rate. It is the first reported procedure for the accurate determination of the proportions of 25α and 25β sapogenins in a crude mixture and it has been applied to a range of problems which will be reported shortly. The standard deviation of the determination of total sapogenin in crude extracts was 3–5 per cent and of the individual C_{25} isomers 3–10 per cent depending upon the plant source and the 25α:25β ratio.

RESULTS AND DISCUSSION

The bands in the i.r. spectrum characteristic of the spirostan ring, and hence of sapogenins, are near 980, 915, 900, 865, and/or 840 cm^{-1}. Absorption at 980, 915 and 900 cm^{-1} is the most pronounced and has the least variable background. The 980 cm^{-1} band is strong for both the 25α and 25β sapogenins and the prominence of the bands at 915 and 900 cm^{-1} is diagnostic for the 25β and 25α epimers, respectively, although both bands are present in the spectrum of each pure isomer (Fig. 1). The calibration graphs for each pure isomer showed a linear relationship between the corrected absorbance (at 980 and 900 cm^{-1} for 25α, and at 980 and 915 cm^{-1} for 25β sapogenins) and the concentration of sapogenin. The ratio graph obtained from mixtures of diosgenin with sarsasapogenin yielded a smooth curve over the range from 1 part of 25α sapogenin with 4 parts of 25β sapogenin to 3 parts of 25α sapogenin with 1 part of 25β sapogenin (Fig. 2).

Each test sample was run and the corrected absorbance at each of the above frequencies calculated. Two methods of calculation were devised for the following two situations: (1)

[9] M. E. WALL, R. C. EDDY, M. I. MCLENNAN and M. E. KLUMPP, *Anal. Chem.* **24**, 1337 (1952).
[10] W. PEAL, *Chem. Ind.* **44**, 1451 (1957).
[11] C. W. SHOPPEE, *Chemistry of the Steroids*, 2nd edition, p. 403, Butterworths, London (1964).
[12] G. BLUNDEN, R. HARDMAN and W. R. WENSLEY, *J. Pharm. Pharmacol.* **17**, 274 (1965).

where either the 25α or the 25β form is markedly predominant with the other epimer as a minor constituent, and (2) where the sample contained considerable amounts of both epimers.

If the spectrum showed a 25α:25β absorbance ratio of less than 1:4 or greater than 3:1 then method of calculation No. 1 was applied (the direct method) and the total sapogenin concentration was calculated from the 980 cm^{-1} calibration graph. The concentration of

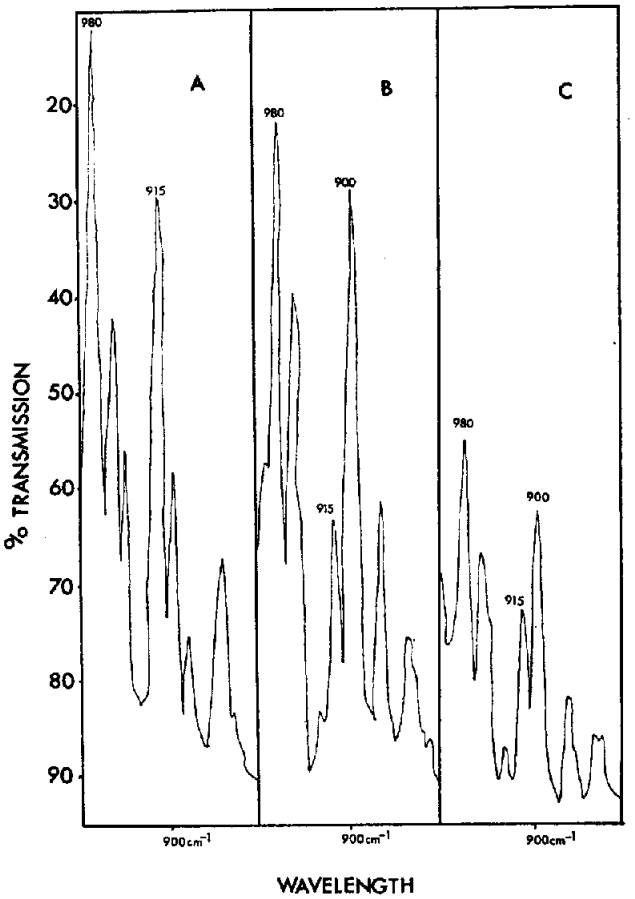

FIG. 1. INFRA-RED SPECTRA OF (A) SARSASAPOGENIN (25β), 1·0% w/v IN $CHCl_3$ IN A 1·0 mm CELL; (B) DIOSGENIN (25α), 1·0% w/v IN $CHCl_3$ IN A 1·0 mm CELL; (C) A NATURAL MIXTURE OF DIOSGENIN AND YAMOGENIN (25β) ISOLATED FROM *Trigonella foenumgraecum* SEED, 0·5% w/v IN $CHCl_3$ IN A 1·0 mm CELL.
In each case the spectrum was scanned from 1000 to 800 cm^{-1}.

the 25α or 25β sapogenin affording the predominant band at 915 or 900 cm^{-1} was derived directly from the appropriate calibration graph and the minor epimer obtained by subtraction of the value for the major epimer from the total value. If the result from the 915 or 900 cm^{-1} band was equal to, or greater than that for total sapogenin from the 980 cm^{-1} band, then it was assumed that the material contained sapogenin of a single configuration, and the result was expressed as the mean of the values obtained from 980 and 915 or 900 cm^{-1}.

If the 25α:25β absorbance ratio fell between the limits defined above then the sample contained considerable amounts of both epimers. The influence of each epimer on the other must be taken into account and an alternative method of estimation, No. 2 (the ratio method), is necessary. Table 1 gives a comparison of the results for the total sapogenin as determined

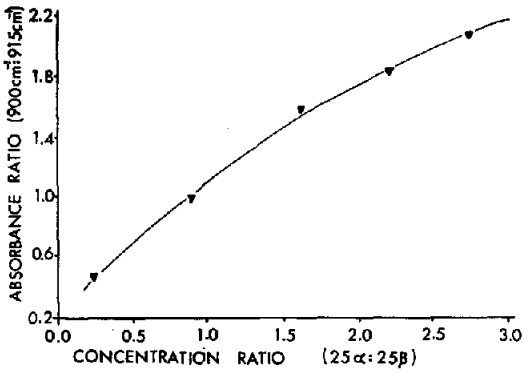

FIG. 2. THE RATIO GRAPH FOR MIXTURES OF DIOSGENIN WITH SARSASAPOGENIN.

TABLE 1. COMPARISON OF THE RESULTS FOR THE TOTAL SAPOGENIN DETERMINED BY THE RATIO AND THE DIRECT METHODS

Sapogenins	Actual total (mg)	Found	
		Ratio method (mg)	Direct method (mg)
Sarsasapogenin + hecogenin	29·8	28	23
Sarsasapogenin + tigogenin	30·6	29	28
Sarsasapogenin + rockogenin*	35·1	36	21†
			29·5‡

* 5α,25α-spirostan-3β, 12β-diol.
† Calculated as sarsasapogenin.
‡ calculated as diosgenin.

by the direct and ratio methods and indicates the errors which can occur if this interaction between epimers is ignored. The predominant isomer concentration was read directly from the appropriate calibration curve. The ratio of the absorbance of 25α:25β was calculated and the ratio of the concentrations of 25α:25β was determined from the ratio graph. Thus, the amount of the predominant isomer and the ratio of the concentration of it and its epimer was known and hence the minor isomer could be derived by simple proportion.

In tests of accuracy of the method, recovery of added sapogenin, calculated as diosgenin and sarsasapogenin, was 90–103 per cent for the separate isomers and 94–102 per cent for the total sapogenin content (Table 2). However, the total concentration of sapogenin had some effect on the instrumental accuracy (Table 3) and a total concentration of 0·5–1·0 per cent w/v was preferred. The greatest variation was in the 915 cm^{-1} band due to the 25β

TABLE 2. RECOVERY OF ADDED SAPOGENIN*

Configuration		Concentration g/100 ml			
		1	2	3	4
25α	Originally present	0·12	0·04	0·30	0·24
25β		0·24	0·03	0·33	0·71
Both		0·36	0·07	0·63	0·95
25α	Added	0·80	0·80	—	0·28
25β		0·20	0·20	0·40	0·28
Both		1·00	1·00	0·40	0·28
25α	Total calculated	0·92	0·84	0·30	0·52
25β		0·44	0·23	0·73	0·71
Both		1·36	1·07	1·03	1·23
25α	Total found	0·83	0·83	0·29	0·52
25β		0·47	0·22	0·68	0·73
Both		1·30	1·05	0·97	1·25
25α	Per cent recovery	90	99	97	100
25β		94	96	93	103
Both		96	98	94	102

* Calculated by the ratio method.

TABLE 3. EFFECT OF THE TOTAL SAPOGENIN CONCENTRATION ON THE ERROR OF THE ABSORBANCE DETERMINATION

Sapogenin concentration	% Standard deviation of the absorbance determination at		
(% w/v)	980 cm^{-1}	915 cm^{-1}	900 cm^{-1}
0·25	2·9	13·6	3·1
0·50	0·9	9·2	1·5
0·75	2·0	5·5	1·2
1·00	1·3	6·1	1·5

configuration. The ratio of the absorbances of 25α:25β had little effect on the accuracy of the absorbance determination (Table 4) and again the 915 cm^{-1} band was the most variable.

The absorbance data for pure sapogenins in chloroform solution (Table 5) shows that, whilst the values at 900 cm^{-1} for the 25α and at 915 cm^{-1} for the 25β sapogenin are reasonably uniform, much greater variation occurs in the 980 cm^{-1} band. The range of recovery encountered using artificial mixtures of various sapogenins, other than those used for the calibration, with the ratio method is given in Table 6. In the most extreme case, calculation of hecogenin as diosgenin and neotigogenin as sarsasapogenin gave recoveries of 83 per cent and 113 per cent respectively, but it is interesting to note that the total recovery in this case was 105 per cent. As a general rule it is best to calibrate the instrument with the particular sapogenins under study when these are available. In practice pure 25β sapogenins, apart from sarsasapogenin, are difficult to obtain.

Sterols, including cholesterol often co-occur with sapogenins[13] in the plant extracts submitted for analysis but the recovery of sapogenin in the presence of added cholesterol

[13] G. BLUNDEN, C. J. BRIGGS and R. HARDMAN, *Phytochem.* 7, 453 (1968).

TABLE 4. EFFECT OF THE ABSORBANCE RATIO ON THE ERROR OF THE ABSORBANCE DETERMINATION

Absorbance ratio	% Standard deviation of the absorbance determination at		
(25α:25β)	980 cm^{-1}	915 cm^{-1}	900 cm^{-1}
1·55	1·8	7·2	2·6
3·30	0·9	9·2	1·5

TABLE 5. ABSORBANCE DATA FOR PURE SAPOGENINS IN CHLOROFORM SOLUTION IN A 1·0 mm CELL

Sapogenin	Absorbance of a 1·0% w/v solution at		
	980 cm^{-1}	915 cm^{-1}	900 cm^{-1}
Hecogenin (5α,25α-spirostan-3β-ol,12-one)	0·660	0·119	0·477
Tigogenin (5α,20α,22α,25α, spirostan-3β-ol)	0·498	0·109	0·410
Smilagenin (5β,25α-spirostan-3β-ol)	0·765	0·073	0·428
Diosgenin (Δ^5,25α-spirosten-3β-ol)	0·488	0·119	0·423
Sarsasapogenin (5β,25β-spirostan-3β-ol)	0·721	0·437	0·122

TABLE 6. ASSAY OF ARTIFICIAL MIXTURES OF 25α AND 25β SAPOGENINS*

Sapogenin	Configuration	Present (mg)	Found (mg)	% Recovery
Smilagenin	25α	7·9	8	101
Sarsasapogenin	25α	7·4	7	98
	Both	15·3	15	99
Hecogenin	25α	15·0	17	113
Neotigogenin	25β	6·0	5	83
	Both	21·0	22	105
Hecogenin	25α	8·6	8	93
Sarsasapogenin	25β	21·2	20	95
	Both	29·8	28	94
Tigogenin	25α	20·8	20	96
Sarsasapogenin	25β	9·8	9	92
	Both	30·6	29	95
Rockogenin	25α	16·9	17	101
Sarsasapogenin	25β	18·2	19	104
	Both	35·1	36	103

* Calculated as diosgenin and sarsasapogenin by the ratio method.

(Table 7) was 100–101 per cent for both the total and individual values, indicating that the presence of this compound does not affect the absorbance in this region. Addition of geraniol, a biogenetic precursor of steroids,[14] did not affect the recovery of sapogenin. These results indicate the high specificity of the procedure.

[14] D. J. BAISTED, *Phytochem.* 6, 93 (1967).

The errors involved in the whole estimation procedure, for three different plant sources and two morphological parts, are given in Table 8. *Dioscorea deltoidea* tuber contains the 25α epimer (diosgenin) only with a trace of oil. *Trigonella foenumgraecum* seed contains approximately equal quantities of diosgenin and yamogenin together with 10 per cent of oil, *Balanites aegyptiaca* seed contains large amounts of both diosgenin and yamogenin together

TABLE 7. RECOVERY OF SAPOGENIN* IN THE PRESENCE OF ADDED STEROL AND TERPENE

Configuration		% Sapogenin present	
		+ Cholesterol	+ Geraniol
25α	Originally present	0·92	0·96
25β		0·98	1·14
Both		1·90	2·01
25α	After addition	0·93	0·97
25β		0·98	0·99
Both		1·90	1·96
25α	% Recovery	101	101
25β		100	98
Both		100	98

* Calculated by the ratio method.

TABLE 8. DETERMINATION OF THE TOTAL ERROR OF THE SAPOGENIN ASSAY

Plant source	% Standard deviation of the final sapogenin determination for		
	25α	25β	Total
**Trigonella foenumgraecum* seed	4·4	7·2	4·6
**Balanites aegyptiaca* defatted seed	5·0	10·4	4·7
†*Dioscorea deltoidea* tuber	2·6	—	2·9

* Calculated by the ratio method.
† Calculated by the direct method.

with 50 per cent oil. This seed requires to be crushed and defatted by soxhlet extraction with light petroleum (b.p. 40–60°) to reduce its oil content to about 10 per cent prior to acid hydrolysis. This is necessary because large amounts of oil cause spurious absorption in the relevant spectral region.[15] The results indicate an overall error of 4–5 per cent for 25α and total sapogenin, and of 7–10 per cent for 25β sapogenin using the "ratio method" and 2–3 per cent for the 25α epimer and the total sapogenin using the "direct method".

Recently the derivation of the final results from the corrected absorbance values has been further facilitated by data processing on an Elliot 803 computer (School of Mathematics, Bath University of Technology).

[15] E. A. SOFOWORA, Ph.D. Thesis, University of Nottingham (1967).

EXPERIMENTAL

Instrument

A Hilger H 800 double-beam recording i.r. spectrometer with rock-salt prism was used to produce the spectra, under the following conditions: 1 mm path length cell, Slit 550 μ at 900 cm^{-1}, Autoslit 25, Gain 7, Damping 4, Scan speed 3 min/rev.

Calibration Procedure

Solutions of pure diosgenin and pure sarsasapogenin, 1·0 per cent w/v, were prepared by dissolving 0·25 g of each in chloroform and diluting each to 25 ml in a calibrated flask. Dilutions of these solutions were used, respectively, to obtain the data for the 25α and 25β sapogenin calibration graphs. The 25α sapogenin calibration graphs were constructed by plotting the absorbance, corrected for background as described by Wall *et al.*,[9] of the 900 cm^{-1} band and of the 980 cm^{-1} band against diosgenin concentration. Similarly the 25β sapogenin calibration graphs were obtained by plotting the 915 cm^{-1} band and the 980 cm^{-1} band against sarsasapogenin concentration.

The ratio graph, Fig. 2, was constructed by plotting the ratio of the absorbance at 900 cm^{-1} to that at 915 cm^{-1} against the ratio of the concentration of diosgenin to the concentration of sarsasapogenin for the diosgenin/sarsasapogenin mixtures.

Isolation of Crude Sapogenin

A known weight (1–10 g) of the sample of plant material, of known moisture content, in whole or in powdered form, was hydrolysed by heating for 2 hr under reflux with 2 N HCl, using 20 ml/g of dried plant material. After cooling and filtering the mixture, the acid-insoluble residue was washed with water, 20 ml/g, before neutralization with 40 ml/g of 5 per cent w/v ammonia solution.

When it had drained, the insoluble residue was dried in a hot-air oven at 60° for 16 hr before it was extracted in a soxhlet with light petroleum (b.p. 40–60°) to exhaustion (normally 24 hr). The solvent was removed on a rotary vacuum evaporator and the residue dissolved in $CHCl_3$ to give a total sapogenin concentration of 0·5–1·0 g/100 ml.

Sapogenin Assay

The spectrum of the crude sapogenin solution was run over the 1000–800 cm^{-1} region. The trace was inspected for the presence of the four or five bands which are characteristic of the spirostan structure, i.e. near 980, 915, 900, 865 and/or 840 cm^{-1}. If all of these were present then sapogenin was confirmed.

Determination of the Accuracy and Specificity of the Method

Four solutions of sapogenin extracts, of varying concentrations and 25α to 25β ratios, were assayed by the proposed ratio method. Known quantities of 25α and/or 25β sapogenins were added and the solutions were re-assayed. Results for the recovery of individual and total sapogenin concentrations are in Table 2.

The effect of the total sapogenin concentration on the error of the absorbance determination was investigated by dissolving a natural mixture of diosgenin and yamogenin in $CHCl_3$ to give solutions of 0·25, 0·50, 0·75, and 1·00 per cent w/v of total sapogenin. Twenty replicate traces were run with each solution, the absorbance values were calculated for each trace, and the per cent standard deviation at each concentration was determined. The results are given in Table 3.

The consequence of variation in the 25α:25β ratio on the accuracy of the absorbance determination was examined as follows. Three natural samples of crude "diosgenin", isolated from three different species of plants and having different absorbance ratios, were separately dissolved in chloroform to give solutions of 0·5 per cent w/v. Twenty replicate traces were run for each solution and the absorbances and per cent standard deviations calculated as above. The results are in Table 4.

To ascertain the effect of variation in the steroidal nucleus the corrected absorbance of a 1·0 per cent w/v solution in chloroform of various 25α and 25β sapogenins was determined at 980, 915 and 900 cm^{-1} for the results in Table 5. Furthermore, artificial mixtures of various 25α and 25β sapogenins, of known concentration, were assayed and the results calculated by the proposed ratio method (Table 6).

A check was made on the specificity of the procedure by addition of possible sapogenin precursors to plant material before extraction. In one experiment a sample of *Trigonella foenumgraecum* seed was extracted and assayed by the ratio method. A second batch of the seed was treated in the same manner except that, before acid hydrolysis, 50 mg of cholesterol was added to the reaction flask. Another sample of *T. foenumgraecum* seed was assayed by the ratio method and then re-assayed with the addition of 2 ml of geraniol before hydrolysis (Table 7).

Over the past 3 years many hundreds of plant samples have been assayed by this method and the per cent standard deviations of the results, calculated from the means of the duplicate determinations, for three different plant materials, are given in Table 8.

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