Phytochemistry, 1965, Vol. 4, pp. 587 to 592. Pergamon Press Ltd. Printed in England

STEROID SAPOGENINS—IX

THIN-LAYER CHROMATOGRAPHY

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(*Received* 6 *January* 1965)

Abstract—A thin-layer chromatographic procedure is described for the resolution of mixtures of steroid sapogenins. The method has been applied to an examination of the mixtures of sapogenins isolated from 16 plant specimens.

THE known importance of the steroid sapogenins has stimulated several workers to develop rapid methods for their detection and identification on a micro-scale. Most of the earlier workers used different types of solvent on paper chromatograms. Thus Sannié and Lapin[1] used acidic solvents, Heftmann and Hayden[2] neutral ones while McAleer and Kozlowski[3] and Wall *et al.*[4] favoured systems of the Zaffaroni type. Apart from the failure of most of these systems to resolve stereoisomers, they suffer from other drawbacks such as serious tailing, long time for development and failure to attain equilibrium conditions. For example, although the separation of smilagenin-sarsasapogenin has been reported,[1,2] later observations[5] have not confirmed these findings. A recent modification has also been reported[6] in which silica gel- and alumina-impregnated papers were used with a variety of solvent systems. The application of gas–liquid chromatography[7] has also been attempted, but resolutions between C-25 and some other isomers were not effective.

The difficulties inherent in the nature of these mixtures of stereo- and structural isomers have led more recently to the development of several thin-layer chromatographic systems. Among the earliest was the use of morin-impregnated alumina plates with combinations of several solvents in a procedure[8] for separating steroidal sapogenins, alkaloids, androstanes, pregnanes and cholestanes. Silica gel[9] and kieselguhr[10] plates have been used for the detection of steroid sapogenins in *Solanum* plants and also in mixtures containing phytosterols.[11]

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[5] T. OKANISHI, A. AKAHORI and F. YASUDA, *Ann. Rept. Shionogi Res. Lab.* **8**, 927 (1958); R. K. CALLOW, D. H. W. DICKSON, J. ELKS, R. M. EVANS, V. H. T. JAMES, A. G. LONG, J. F. OUGHTON and J. E. PAGE, *J. Chem. Soc.* 1966 (1955).
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[9] H. SANDER, H. HAUSER and R. HÄNSEL, *Planta Med.* **9**, 8 (1961); H. SANDER, *Z. Naturforsch.* **16b**, 144 (1961).
[10] H. SANDER and G. WILLUHN, *Flora (Jena)* **151**, 150 (1961); H. SANDER, *Naturwiss.* **48**, 303 (1961).
[11] K. SCHREIBER, O. AURICH and G. OSSKE, *J. Chromatog.* **12**, 63 (1963).

The chromatoplate technique has also been used for the study of diosgenin biogenesis.[12] Records of the R_f values of steroid sapogenins have also been given by Smith and Foell.[13] Heřmánek *et al.*[14] and by Takeda *et al.*[15] Perhaps the most detailed study of steroid sapogenins on thin-layers is that made by Bennett and Heftmann[16] who used several solvent systems on both silica gel and kieselguhr plates and used 50% sulphuric acid as spray reagent. They reported separations between sapogenins differing in the number of polar groups as well as in configuration at both C-5 and C-25. When applied to mixtures of natural origin, containing stereo- as well as structural isomers, however, these methods were found by the present authors to be of little help. The present communication reports a thin-layer chromatographic procedure which can be applied both to mixtures of intimately related sapogenins, and for the routine analysis of plant material. Since no single set of conditions could possibly completely resolve a complex natural mixture of sapogenins according to both hydroxyl content and isomeric variation the process of identification has been separated into a sequence of operational stages.

EXPERIMENTAL

Adsorbents

Alumina G (Merck) and kieselguhr impregnated with sodium acetate were used. Plates of the latter adsorbent were prepared by using a 1% solution of sodium acetate in making the slurry with a G-grade material prior to spreading.

Solvent Systems

I. *n*-hexane:ethanol (10:1); II. cyclohexane:ethanol (20:1); III. *n*-hexane:ethylene chloride (10:2); IV. *n*-hexane:ethylene chloride (10:1); V. *n*-hexane:butanol (10:1); VI. *n*-hexane:toluene (2:1) saturated with water, organic layer:ethyl acetate (10:1); VII. cyclohexane:*n*-propanol (10:3).

Spray Reagents

A mixture of *p*-anisidine (0·5 g) and *o*-phosphoric acid (3 ml) in 80% methanol (100 ml) was used for sugars, and a mixture of chlorosulphonic acid and acetic acid (1:3) for the steroid sapogenins. The plates were inspected under short-wave u.v. light after they were sprayed.

Natural Mixtures

The plant material was processed by the micro method described in detail by Wall *et al.*[17] and the i.r. spectrometric assays[17] were made on a Perkin-Elmer "Infracord 137B" instrument in carbon disulphide solution. The acetylated material remaining after the i.r. examination was freed from solvent, dissolved in a small volume of benzene and then refluxed with 5% ethanolic potassium hydroxide for 1·5 hr. Working up in the usual manner, with separation of layers through centrifugation, gave residue of saponified material which was examined on the "exploratory" and then on the "analysing" plates (*cf.* Discussion).

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[16] R. D. BENNETT and E. HEFTMANN, *J. Chromatog.* **9**, 353 (1962).
[17] M. E. WALL, C. R. EDDY, M. L. MCCLENNAN and M. E. KLUMPP, *Analyt. Chem.* **24**, 1337 (1952).

RESULTS AND DISCUSSION

Differentiation of the common steroid sapogenins into monohydroxy and dihydroxy types was best obtained on alumina plates using solvent system I (Table 1). The ambiguity which may arise from the similarity in R_f values of hecogenin, markedly more polar than other monohydroxylic sapogenins, and samogenin can be resolved by inspection of the plates sprayed with the chlorosulphonic acid reagent[18] under u.v. light. The fluorescence under u.v. light (*cf.* Table 1), were found to be more useful for discriminating than colours in daylight; they were generally more brilliant with ketonic sapogenins than with the others. System I, however, did not satisfactorily resolve mixtures of closely related compounds within either of these groups. For this purpose, the use of system II, also on alumina plates,

TABLE 1

Sapogenin	R_f values in system*							Fluorescence (u.v.), intensity§
	I†	II†	III‡	IV‡	V‡	VI‡	VII‡	
Diosgenin	0·72	0·42	0·61					Orange red, m
Yamogenin	0·73	0·43	0·58					Brick red, ww
Tigogenin	0·75	0·44	0·67					Orange yellow, s
Neotigogenin	0·75	0·44						Lemon yellow, ss
Smilagenin	0·80	0·56	0·94	0·63				Yellow, s
Sarsasapogenin	0·80	0·57	0·94	0·56				Brownish yellow, s
Hecogenin	0·59	0·27						Sky blue, sss
Chlorogenin	0·19				0·17			Orange, m
Samogenin	0·55				0·78			Brown, w
Ruscogenin	0·19				0·39			Yellowish brown, s
Gitogenin	0·17				0·11	0·31		Sky blue, ss
Yuccagenin	0·15				0·11	0·65		Brownish violet, w
Manogenin	0·10				0·05		0·20	Yellow, sss
Kammogenin	0·10				0·05		0·24	Brownish yellow, sss

* Solvent systems: I, *n*-hexane: ethanol (10:1); II, cyclohexane: ethanol (20:1); III, *n*-hexane: $CH_2H_4Cl_2$ (10:2); IV, as III (10:1); V, *n*-hexane: butanol (10:1); VI, *n*-hexane: toluene (2:1) satd. with H_2O; organic layer: ethylacetate (10:1); VII, cyclohexane: propanol (10:3).

† On alumina.

‡ On kieselguhr impregnated with sodium acetate.

§ Intensity expressed as: w (weak), ww (very weak), m (medium), s (strong), ss (very strong), sss (very brilliant). Spray reagent: chlorosulphonic acid-acetic acid (1:3).

was found to afford reasonable differentiation between the various monohydroxylic sapogenins. Thus hecogenin, a very common sapogenin, was clearly separated from both the sarsasapogenin–smilagenin group and the diosgenin–yamogenin–tigogenin–neotigogenin group neither of which were well resolved by this system. The dihydroxy sapogenins had zero R_f in this system. For the finer resolution of the monohydroxy isomers, plates of kieselguhr impregnated with sodium acetate were found to give excellently shaped spots with no tailing, which otherwise occurs if plain water were used. Using this adsorbent with solvent system III, it was possible to distinguish clearly between the various monohydroxy sapogenins of the diosgenin-group. The 5β-products, smilagenin and sarsasapogenin, were

[18] This reagent[10] was preferred to all other previously used spray reagents such as trichloroacetic acid[2], vanillin–phosphoric acid–ethanol[3] and phosphomolybdic acid–ethanol.[19] The latter, for example, gave a non-discriminating blue colour with all sapogenins.

[19] D. KRITCHEVSKY and M. R. KIRK, *Arch. Biochem. Biophys.* 35, 346 (1952).

better resolved with the same solvent combination having a lower polarity (system IV). For resolution of the dihydroxylic sapogenins, system V afforded the best results; gitogenin and yuccagenin being separately resolved with system VI, and manogenin and kammogenin with system VII on the same adsorbent.

It is common experience that mixtures of steroid sapogenins isolated from plants are invariably contaminated with other substances which give rise to difficulties when the chromatoplates are treated with the common spray reagents. The spots due to the contaminants are then difficult to distinguish from the genuine sapogenins. This difficulty was overcome in an indirect manner. By spraying the chromatoplates first with a mixture of *p*-anisidine and *o*-phosphoric acid in 80% methanol, followed by heating at 120° for *no longer than* 2 min, most of the contaminants developed yellow fluorescent spots under the u.v. light while those of the steroid sapogenins were invisible. The positions of the steroid sapogenins were subsequently revealed by spraying with the chlorosulphonic acid reagent.

For the final confirmation of identity of the sapogenins, the spots appearing on the "exploratory" plate (system I) at the characteristic zones, was brushed off the unsprayed plate with the guidance of sprayed narrow marginal strips. Extraction of the removed adsorbent with chloroform–methanol was followed by examination on a selected succession of specific systems described above. In this manner it was possible to differentiate and identify all the steroid sapogenins, indicated in Table 1, in all possible combinations found in natural mixtures.

The usefulness of the new technique was examined by undertaking an investigation of the leaves of several steroid sapogenin-containing plants. The isolation method recommended by Wall *et al.*[17,20] was employed using small amounts of plant material (2–5 g, dry weight). This afforded several milligrams of a crude acetylated sapogenin mixture which was first examined by i.r. spectroscopy[17,21] in order to obtain an approximate estimate of the sapogenin content, as well as an indication of the predominating configuration at C-25. The crude material was then saponified and examined by thin layer chromatography. The results obtained are given in Table 2.

The 16 plants examined include the commonest *Agave*, *Furcraea* and *Yucca* species grown locally, of which 12 have previously been examined in this laboratory[22–27] by the usual macro-isolation procedures, and the reported sapogenin content is also included in Table 2. The results obtained by the micro technique reported here largely agree with previous data.[22–27] A marked difference appears, however, in both *Yucca aloifolia* specimens which were collected[23] at a different season. This is not after all surprising for it appears from the studies of Marker *et al.*[28] that the steroid sapogenin content changes in *Yucca* plants both in quantity and quality in a regular cycle around the year with the flowering cycle. The agaves, which flower only once towards the end of their life, exhibit one sapogenin cycle which begins with the "infant" plant. This might explain the discrepancies observed between these and previous findings in *Agave angustifolia* var. *marginata* and *A. attenuata* where in the latter a

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[21] C. R. EDDY, M. F. WALL and M. K. SCOTT, *Analyt. Chem.* **25**, 266 (1953).
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[24] A. A. DAWIDAR and M. B. E. FAYEZ, *Arch. Biochem. Biophys* **92**, 420 (1961).
[25] M. S. BEDOUR and M. B. E. FAYEZ, *UAR J. Chem.* **4**, 257 (1961).
[26] M. S. BEDOUR and M. B. E. FAYEZ, *UAR J. Chem.* **4**, 265 (1961).
[27] M. B. E. FAYEZ and L. A SALLAM, *UAR J. Chem.* (in press).
[28] R. E. MARKER and J. LOPEZ, *J. Am. Chem. Soc* **69**, 2375, 2403 (1947).

TABLE 2

Plant	Estimated sapogenin content* (IR)%	Predominant configuration at C-25 (IR)	Identified sapogenins† (present work, T.L.C.)	Reported sapogenins† (by isolation)
Agave americana var. *marginata*	0·06	D	H, C	H, C[22]
A. angustifolia var. *marginata*	0·18	D	T, H, C	T[26]
A. attenuata	0·46	L	Nt, Sa, Y	Sa[26]
A. ferox	0·27	D	H, K	H, K[27]
A. macroacantha	0·25	D	T, H, G	T, H, G[26]
A. salmiana	0·14	D	H	H[22]
A. sisalana mature leaves	0·07	L+D	Nt, H	Nt, H[24]
A. filifera	2·08	D	T, G, C	—
A. ghiesbrechtii	0·54	D	Sm	—
A. stricta	0·15	L	Nt, Sa	—
A. victoria-regina	0·06	L	Sa	—
Furcraea gigantea var. *medio-picta*	0·11	D	T, H, C	H, C[25]
F. gigantea	0·32	D	T, H, C	T, H, C[25]
Yucca aloifolia	0·06	L	Nt, Sa, H	T, H[23]
Y. aloifolia variegated leaves	0·18	L	Nt, Sa, G	T[23]
Y. filamentosa	0·83	L	Sa	Sa[23]

* Moisture-free basis.

† Abbreviations used: hecogenin-H, chlorogenin-C, tigogenin-T, neotigogenin-Nt, sarsasapogenin-Sa, yuccagenin-Y, kammogenin-K, gitogenin-G, smilagenin-Sm.

very rare sapogenin, yuccagenin, was identified on the chromatoplates. It is not unlikely however that the compounds not hitherto identified were in fact present in previous cases[26] in trace amounts which escaped the macro-isolation procedure. Their detection by the sensitive chromatoplate technique, however, presents no difficulties.

The steroid sapogenin constituents of the remaining 4 *Agave* plants are hereby reported for the first time. *Agave filifera*, affording the highest sapogenin content identified as tigogenin, gitogenin and chlorogenin, has previously been examined by Wall *et al.*[29] who reported the presence of only gitogenin in an American specimen. *Agave ghiesbrechtii* was found to contain only smilagenin; the literature appears to be devoid of any reference to previous work with this plant. *Agave stricta* was found to contain two 25-L-sapogenins, namely neotigogenin and sarsasapogenin. This plant has previously been examined by Wall and his coworkers[29,30] but found to afford no identifiable products; on the other hand Marker *et al.*[31] obtained gitogenin and, in one instance, tigogenin from four varieties of this species. *Agave victoria-regina* was found to have a very low sapogenin content, and only sarsasapogenin was identified. The plant has also been previously examined by Wall *et al.*[29,30] who found no identified sapogenins.

[29] M. E. WALL, M. M. KRIDER, C. F. KREWSON, C. R. EDDY and J. J. WILLAMAN, *U.S. Dept. Agr., Agr. Research Service, circ. AIC*-363.

[30] M. E. WALL, C. R. EDDY, C. S. FENSKE and J. J. WILLAMAN, *U.S. Dept. Agr., Agr. Research Service, circ. AIC*-367; *circ. ARS*-73-4; *J. Am. Pharm. Assoc.* **46**, 653 (1957); *ibid.* **48**, 695 (1959); *ibid.* **50**, 1001 (1961).

[31] R. E. MARKER, R. B. WAGNER, P. R. ULSHAFER, E. L. WITTBECKER, D. P. J. GOLDSMITH and C. H. RUOF, *J. Am. Chem. Soc.* **65**, 1199 (1943).

The results obtained with these 4 *Agave* species have all been confirmed by actual isolations.[32] Coupled with the micro technique developed for the gross estimation of steroid sapogenins in plant tissue,[17] preceded by the selective hemolysis test, a complete analytical method is thus available for the screening of large numbers of plant samples for steroid sapogenins with acceptable reliability and reproducibility.

Acknowledgement—The authors wish to thank Dr. R. Elhalwagy for the identification of plant material.

[32] Unpublished results.