*Journal of Chromatography*, 121 (1976) 329–334
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CHROM. 9025

# A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE BENZOATE ESTERS OF SAPOGENINS ISOLATED FROM *AGAVE*

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(Received December 3rd, 1975)

## SUMMARY

The major sapogenins (hecogenin, Δ9(11)-dehydrohecogenin and tigogenin) occurring in the *Agave* species are analyzed by reversed-phase high-performance liquid chromatography after derivatization with benzoyl chloride. Results of the analysis of crude sapogenins from various species and geographic locations are presented. Application of this procedure in the analysis of plant juice hydrolysis material is illustrated. Sarsasapogenin, Δ9(11)-dehydrotigogenin and diosgenin are also analyzed by this rapid and accurate method.

## INTRODUCTION

The analysis of sapogenins derived from species of the Agavaceae family is of prime interest to the steroid manufacturer who desires to utilize hecogenin as a starting material for corticosteroid production. The routine analytical methods are numerous and have been outlined previously[1]. One of the most recent approaches[2] and its modifications[1,3] have involved the use of densitometric thin-layer chromatography (DTLC). This method has been suggested as an improvement on previous IR and gravimetric analyses, which are inaccurate, require large samples and are not specific owing to interfering substances.

Blunden and Hardman[2] showed that DTLC has an experimental error of approximately 7%, and Lockwood *et al.*[1] suggested the use of an internal standard to minimize this experimental error. With this improvement, a calibration graph is still required for each TLC plate. The calibration graphs must be constructed from numerous standard concentration solutions, owing to the non-linearity of sample peak area ratios to sample weights. The antimony trichloride reagent produces colours that are not stable and fade with time, thus requiring the measurement to be made at an approximate asymptotic value. In order to obtain adequate spot resolution for quantification purposes, as many as six TLC plate developments were necessary.

Fitzpatrick and Siggia[4] have demonstrated the utility of forming the benzoate esters of non-UV-absorbing hydroxy steroids for use in high-pressure liquid chromatography (HPLC). In this paper, we report a method for the HPLC analysis of 3-hydroxysapogenins after simple and efficient formation of the benzoate derivative.

The method is accurate and rapid. Thirty sapogenin samples can easily be assayed in a single working day, rendering determinations that have 95% confidence intervals of better than ±2%.

## EXPERIMENTAL

### *Sample preparation*

A sample equivalent to approximately 25 mg of sapogenin was accurately weighed and transferred into a 40-ml screw-capped test-tube. A 2.0-ml volume of pyridine (Merck, Darmstadt, G.F.R.; analytical grade) was added, the sides of the tube being washed down so as to remove any powder that might have adhered. The tube was not shaken in an attempt to dissolve the sample completely. Then 0.1 ml of benzoyl chloride (Schuchardt, Munich, G.F.R.; reagent grade) was added to the mixture and the tube was tightly sealed with a PTFE-lined screw-cap. The sample was heated in a water-bath at 80°C for 30 min and, after cooling to room temperature, 10.0 ml of distilled dichloromethane were added, followed by 10 ml of distilled water and 2.0 ml of concentrated hydrochloric acid. The sealed tube was shaken for 15 sec. After phase separation, the aqueous layer was aspirated off and the washing was repeated once with 10 ml of distilled water.

### *Preparation of standards*

Amounts of 5, 10, and 25 mg of hecogenin, 2.5, 5 and 10 mg of $\Delta$9(11)-dehydrohecogenin and 5, 10 and 20 mg of tigogenin were benzoylated by the method described above. Hecogenin (Joba, Amsterdam, The Netherlands) and tigogenin (Merck) were used as obtained from the suppliers. $\Delta$9(11)-Dehydrohecogenin was synthesized from hecogenin by bromination and dehydrohalogenation according to the Glaxo method[5].

### *Preparation of hydrolysate from plant material*

Approximately 1 g of the dried acid-hydrolysis product of the plant juice[6] was accurately weighed into a 22 × 80-mm thimble and placed in a suitable Soxhlet extractor. A 70-ml volume of heptane (Welka, Milan, Italy) was added to a 100-ml round-bottomed flask of known tare weight. A condenser was attached to the Soxhlet and flask and the sample was extracted for 16 h. The heptane solution was evaporated to dryness on a rotary evaporator and the flask was then dried for 3 h at 100 °C in an oven. After determining the weight of the dry heptane extract, approximately 30 mg were weighed into a 40-ml screw-capped test-tube and treated as described under *Sample preparation*.

### *Calculations*

The sapogenins present were calculated from the following equation:

$$\text{Sapogenins in hydrolysate } (\%) = \frac{\text{mg sapogenin observed}}{\text{mg heptane extract analyzed}} \times \frac{\text{total mg of heptane extract}}{\text{mg hydrolyzate extracted}} \times 100$$

### *Chromatography*

The chromatograph used was a Hewlett-Packard Model 1010A (Grötzingen,

G.F.R.) modified with a high-pressure sample injection valve having a 10-$\mu$l sample injection loop (Altex, Model 905-01). The detector was a variable-wavelength type (Schoeffel, Model 770) set at 235 nm and 1.0 O.D. A strip-chart recorder (Beckman, Model 1005) was used with a chart speed of 0.2 cm/min. Sample and standard injections were made on to a 0.25 m $\times$ 4 mm stainless-steel column (Hewlett-Packard) packed with 10-$\mu$m LiChrosorb RP8 (Merck), an 8C-modified lipophilic-surface silica gel. The mobile phase consisted of acetonitrile (Merck) plus distilled water (8:2), and was pumped through the column at a flow-rate of 3.9 ml/min, resulting in a column pressure drop of 120 atm. In all instances, chromatography was conducted, at room temperature. The amounts of individual sapogenins present in the sample were interpolated from comparisons of peak heights with the calibration graph constructed from the standard solutions.

## RESULTS AND DISCUSSION

Fig. 1 illustrates a chromatographic separation of the three major sapogenins that occur in various species of *Agave*. The capacity factors ($k'$) are 6, 7 and 21, respectively, for $\Delta$9(11)-dehydrohecogenin, hecogenin and tigogenin benzoates. There is satisfactory resolution between the $\Delta$9(11)-dehydrohecogenin and hecogenin benzoate peaks, which permits accurate quantification. The elution time of the least polar derivative (tigogenin benzoate) is 10 min, resulting in a relatively short analysis time.

The dichloromethane and residual solvents from the derivatization step are completely eluted within two column volumes and therefore do not interfere with the sapogenin determinations. The nature of the sample-solvent system appears to have no adverse effects on the LiChrosorb RP8 column life. A single column has been in daily use for over 7 months and there has been no change in resolution or peak symmetry during this period.

### *Linearity and precision*

The calibration graphs for the three standard sapogenins are linear within the concentration range of interest (0–3.0 mg/ml) and all have zero intercepts.

The precision of the benzoylation procedure was determined by repeatedly assaying a single batch of hecogenin sample material. The results were found to have a 95% confidence interval of $\pm$1.56%. The precision of the chromatographic separation itself was evaluated by repeatedly injecting a single sample of hecogenin benzoate, and a 95% confidence interval of $\pm$0.5% was found.

### *Analysis of hecogenin derived from different Agave species from various geographical locations*

Depending upon factors such as species, climate, soil and plant age, the relative proportions of the various sapogenins obtained from the plant source can vary considerably[7]. Table I shows the results of the application of the method to the analysis of samples of diverse origins and species. It can be seen that the relative proportions of the three major sapogenins vary over a wide range. An accurate estimation of the purity of hecogenin is important in establishing purification methods or variations in synthetic procedures to be used in the eventual production of corticosteroids.

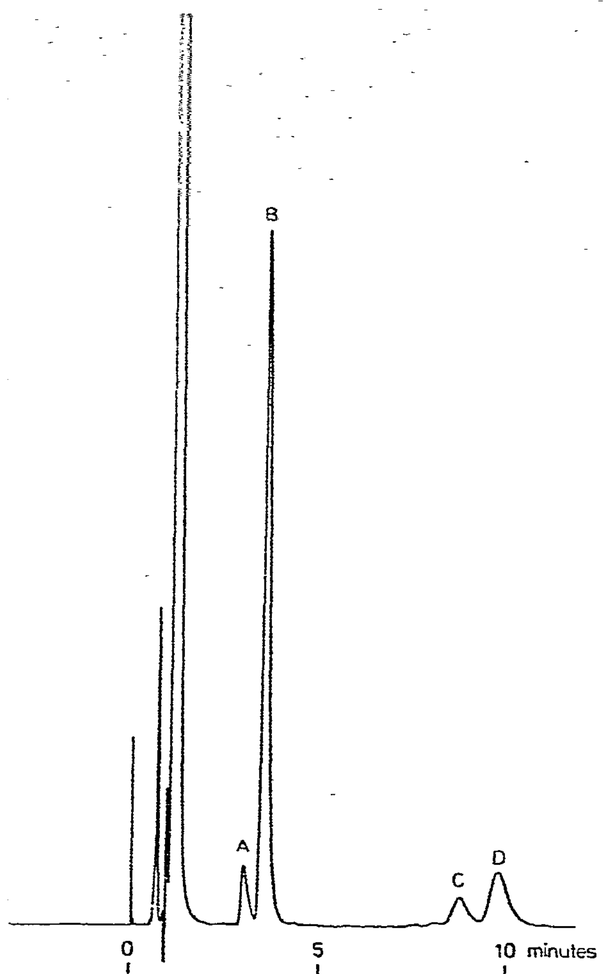


Fig. 1. Typical chromatogram from analysis of hecogenin raw material. A = Δ9(11)-dehydrohecogenin; B = hecogenin; C = unknown; D = tigogenin.

TABLE I

RELATIVE PROPORTIONS OF THE THREE MAJOR SAPOGENINS FROM *AGAVE* SAMPLES OF VARIOUS ORIGINS AND SPECIES

| *Species* | *Origin* | *Hecogenin* | *Tigogenin* | *Δ9(11)-Dehydrohecogenin* |
|---|---|---|---|---|
| *A. vera-cruz** | India | 83.7 | 4.7 | 11.6 |
| *A. sisalana* | Angola | 91.3 | 0.0 | 8.7 |
| *A. sisalana* | Haiti | 80.9 | 16.3 | 2.8 |
| *A. sisalana* | Haiti | 81.4 | 14.7 | 0.8 |
| *A. fourcroydes* | Mexico | 86.5 | 0.0 | 13.5 |
| *A. americana* | Italy | 68.3 | 0.0 | 31.7 |
| *A. americana* | Italy | 84.5 | 0.0 | 15.5 |

* Contained numerous other unidentified hydroxysapogenins.

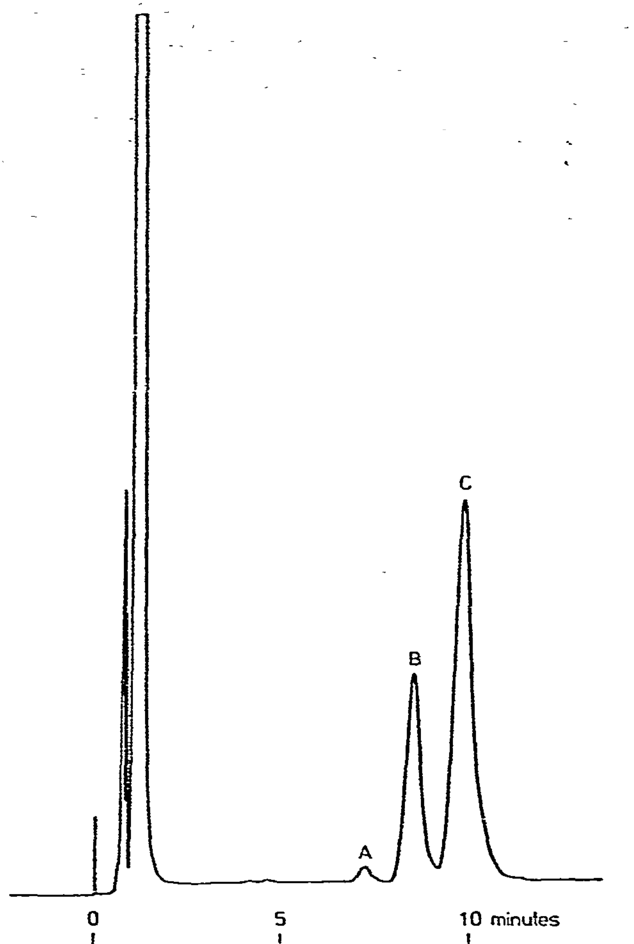


Fig. 2. HPLC separation of benzoate esters of sarsasapogenin (C), diosgenin (B) and an unidentified impurity (A) from sarsasapogenin.

*Analysis of plant material hydrolyzate for sapogenin content*

In practice, the juice obtained from the leaves of the *Agave* plant is hydrolyzed with acid, producing a dark brown precipitate commonly refered to as "coffee grounds". This procedure has been found to be useful in the determination of the sapogenin content of this hydrolyzate material. It was shown that the sapogenin content of the material investigated varied between 5 and 30%.

*Separation of other natural sapogenins and synthetic intermediates*

Other plant genera, such as *Yucca* and *Dioscorea*, have been found to contain sapogenins such as sarsasapogenin[8] and diosgenin[9], which are important to the steroid industry. $\Delta$9(11)-Dehydrohecogenin and $\Delta$9(11)-dehydrotigogenin are two intermediates that can be encountered in the production of corticosteroids from hecogenin[10]. Figs. 2 and 3 illustrate the separations of the benzoate esters of these sapogenins.

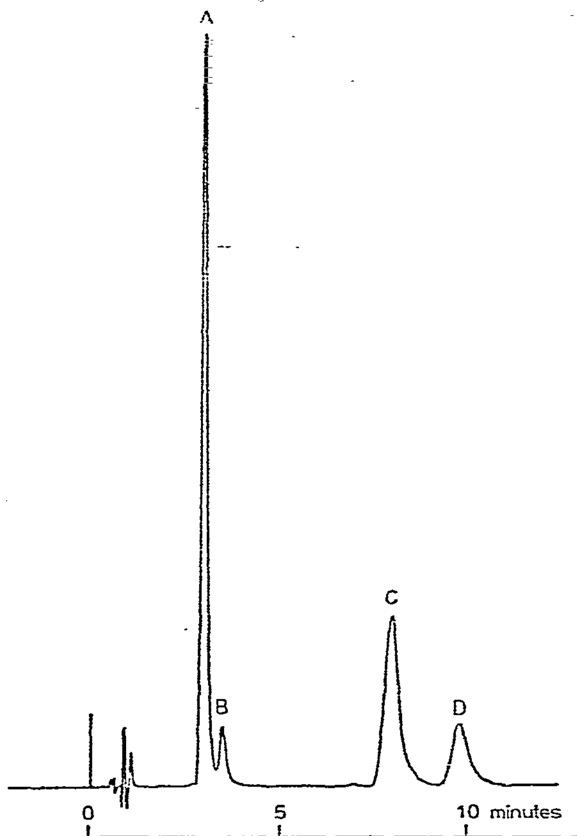


Fig. 3. Separation of intermediates in the synthesis of sapogenins from the starting materials as benzoate esters. A = $\Delta$9(11)-dehydrohecogenin; B = hecogenin; C = $\Delta$9(11)-dehydrotigogenin; D = tigogenin.

## REFERENCES

1 G. B. Lockwood, K. R. Brain and T. D. Turner, *J. Chromatogr.*, 95 (1974) 250.
2 G. Blunden and R. Hardman, *J. Chromatogr.*, 34 (1968) 507.
3 G. Blunden, Yi Yi and K. Jewers, *Lloydia*, 37 (1974) 10.
4 F. A. Fitzpatrick and S. Siggia, *Anal. Chem.*, 45 (1973) 2310.
5 Glaxo Laboratories Ltd., *Brit. Pat.*, 936,964 (1964).
6 R. K. Callow, J. W. Cornforth and P. C. Spensley, *Chem. Ind. (London)*, (1951) 699.
7 R. E. Marker and J. Lopez, *J. Amer. Chem. Soc.*, 69 (1947) 2375.
8 R. E. Marker, R. B. Wagner, P. R. Ulshafer, E. L. Wittbecker, D. P. J. Goldsmith and C. H. Ruof, *J. Amer. Chem. Soc.*, 69 (1947) 2167.
9 R. E. Marker, D. L. Turner and P. R. Ulshafer, *J. Amer. Chem. Soc.*, 62 (1940) 2542.
10 C. Djerassi, H. Martinez and G. Rosenkranz, *J. Org. Chem.*, 16 (1951) 1278.