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Note

Determination of phorbic acid in *Echeveria elegans* by gas–liquid chromatography

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Previously the content of phorbic acid [(1R,3R)-1,3-dihydroxypentane 1,3,5-tricarboxylic acid] in plant material was evaluated by isolation and weighing of the pure dilactone[1–3]. The purpose of this investigation was to devise a method for the determination of phorbic acid in plants, and to apply it to the determination of this acid in *Echeveria elegans* Rose (Crassulaceae). Recently, a method for the determination of pure phorbic acid by the gas–liquid chromatography (GLC) of its open-chain trimethylsilyl (TMS) derivative was described[4].

For the determination of organic acids in biological material, usually basic and neutral substances must be removed from the extract before derivatization and GLC. Plant acids, including phorbic acid, are frequently isolated by either cation- or anion-exchange chromatography[3,5–8]. Preliminary investigations showed that the conditions applied earlier in ion-exchange chromatography for the isolation of phorbic acid[3] resulted in a considerable loss of the acid. For this reason, several chromatographic systems for isolation of the acid were tested (see Table I). It was found that phorbic acid shows properties towards ion-exchange chromatography that is important with respect to future work on this acid.

In the determination of phorbic acid in *Echeveria elegans*, it was found possible to purify the extract simply by passing it through a column of Dowex 50 [H^+], before derivatization and GLC. This was unexpected as removal of neutral substances is usually required in the determination of acids by the GLC of their TMS derivatives.

EXPERIMENTAL

Plant material and chemicals

Fresh plant material of *Echeveria elegans* Rose was supplied by the Botanical Garden, University of Oslo, Norway. Powdered dry material was used for the investigation.

The dilactophorbic acid was isolated from *Euphorbia resinifera* L.[1]. D-Glucaric acid 1,4-lactone (Sigma, St. Louis, Mo., U.S.A.) served as the internal standard.

The silylation was performed with trimethylchlorosilane (Koch-Light, Colnbrook, Great Britain) and 1,1,1,3,3,3-hexamethyldisilazane (Merck, Darmstadt, G.F.R.). Pyridine (Merck), freshly distilled and kept over pellets of sodium hydroxide, was used as a solvent.

The ion exchangers employed were Dowex 50 W, 20–50 mesh (Fluka, Buchs, Switzerland), Dowex 1-X8, 20–50 mesh (Fluka) and Amberlite IR 45 (analytical grade, BDH, Poole, Great Britain). All chemicals (ammonia, ammonium carbonate, formic acid, hydrochloric acid and sodium hydroxide) used in connection with the ion exchangers were of analytical-reagent grade.

Gas–liquid chromatography

A Varian 1400 gas chromatograph with a flame-ionization detector (FID) was used. The column was a glass coil (3 m × 2 mm I.D.), filled with 10% OV-17 on Gas-Chrom Q (80–100 mesh). The following conditions were used: carrier gas (nitrogen) flow-rate, 40 ml/min; detector temperature, 300°; injector temperature, 250°; column temperature, 202° (isothermal). Peak area integration was carried out with an Autolab minigrator (Spectra-Physics) and the gas chromatograms were printed out by an Omniscribe recorder (Houston Instruments). The peak-area ratios reported here are mean values of three different injections of the same TMS preparations.

Test solutions

The acids were dissolved in water to give solutions with the same concentrations as earlier described[4]: solution A, dilactophorbic acid (1 mg/ml) and glucaric acid 1,4-lactone (0.5 mg/ml); solution B, dilactophorbic acid (0.25 mg/ml); solution C, glucaric acid 1,4-lactone (0.25 mg/ml).

Open-chain TMS derivatives

The TMS derivatives were prepared from the sodium salts of the acids as earlier reported[4].

Ion-exchange chromatography

A 10.0-ml volume of solution A was applied to different ion-exchange chromatography systems. In Table I, the types, forms and amounts of the anion exchangers used are listed. The amounts of the ion exchangers required were determined by analysis of the aqueous eluates from the columns for phorbic and glucaric acids.

The acids were eluted with different solvents (see Table I). After removal of the solvents by repeated evaporation, the residues were dissolved in water (10 ml). From these solutions the open-chain TMS derivatives of phorbic and glucaric acids were prepared via their sodium salts. The reference chromatogram (blank) was obtained with the open-chain TMS derivatives of phorbic and glucaric acids prepared from 10 ml of solution A (not pre-treated with ion exchangers). All data in Table I are made with reference to this chromatogram[4].

Calibration graph

Aliquots of solutions A, B and C were mixed in such a way that the ratio between dilactophorbic acid and glucaric acid 1,4-lactone varied from 1.33 to 2.25 and the total amount of the acids was about 20 mg (ref. 4). The acid mixtures were passed through columns containing 10 ml of Dowex 50 (H^+). The eluates and washings (50 ml of water) from each column were combined, evaporated to dryness and the residue was dissolved in 10 ml of water. The open-chain TMS derivatives were prepared from

these solutions. After GLC, the ratios of the peak areas were plotted against the weight ratios of the acids (dilactophorbic acid and glucaric acid 1,4-lactone).

Extraction of plant material

Samples of 1.000 g were extracted three times for 5 min with 50 ml of boiling water. The extracts were combined and the volume was adjusted to 200.0 ml. Two aliquots (25.0 ml) from each extraction were treated as described for the acid mixtures (see under *Calibration graph*). Glucaric acid 1,4-lactone (internal standard) and dilactophorbic acid (for recovery tests) were added to the plant material before extraction.

RESULTS AND DISCUSSION

Ion-exchange chromatography

None of the systems used in the anion-exchange chromatography was found to be satisfactory (Table I). The phorbic acid was apparently strongly retained by the anion-exchange material, and therefore incompletely eluted by the acidic solvents. The glucaric acid was destroyed (multiple peaks in GLC) when the Dowex 1-X8 material was applied in the hydroxide form. Phorbic acid seems to be completely eluted from the weak anion exchanger Amberlite IR 45 by means of ammonia or ammonium carbonate. Unfortunately, such conditions destroyed the glucaric acid. When the test solution was passed only through a column of Dowex 50 (H^+), the

TABLE I

ION-EXCHANGE CHROMATOGRAPHY OF A MIXTURE OF DILACTOPHORBIC ACID AND GLUCARIC ACID 1,4-LACTONE EVALUATED BY GAS–LIQUID CHROMATOGRAPHY

A 10-ml volume of solution A was used for the investigation.

Type, form and amount of ion exchanger applied	*Type, concentration and amount of eluation solvent applied*	*Peak-area ratio (phorbic acid to glucaric acid) and/or comments*
Dowex 1-X8		
(carbonate), 10 ml	5 *N* ammonium carbonate, 30 ml	0.4; incomplete elution of phorbic acid
(formate), 18 ml	2 *N* formic acid, 60 ml	0.5; incomplete eluation of phorbic acid
	3 *N* formic acid, 70 ml	0.7; incomplete elution of phorbic acid
(hydroxide), 15 ml	2 *N* formic acid, 40 ml	Multiple peaks of glucaric acid
Amberlite IR 45		
(carbonate), 15 ml	2 *N* ammonia, 40 ml	Multiple small peaks of glucaric acid
(formate), 15 ml	2 *N* formic acid, 70 ml	No peaks
(hydroxide), 15 ml	1 *N* ammonia, 70 ml	Multiple small peaks of glucaric acid
	3 *N* ammonia, 50 ml	Multiple small peaks of glucaric acid
	5 *N* ammonium carbonate, 50 ml	Multiple small peaks of glucaric acid
	2 *N* formic acid, 70 ml	No peaks
	2 *N* hydrochloric acid, 70 ml	No peaks
Dowex 50 (H^+), 10 ml	Water, 50 ml (washings)	1.74
Blank		1.74

peak-area ratio of the open-chain TMS derivatives of phorbic and glucaric acids was the same as for the blank. For this reason, the last mentioned procedure was chosen for the determination.

Calibration graph

The slope of the calibration graph (0.88) prepared by the method described here was the same as for the graph obtained when the test solution were derivatized and chromatographed without previous treatment with Dowex 50 (H^+)[4]. For a weight ratio of 2, the peak-area ratio was 1.74 $\pm$ 0.05 (mean $\pm$ standard deviation of double determinations on eight different preparations of test solution A). For the graph published earlier[4], the standard deviation of the individual values of the ratios was 0.09; in that instance the peak areas were calculated manually.

Dowex 50 extract of plant material

A gas chromatogram of the open-chain TMS derivatives of the extract of *Echeveria elegans*, after passing through a column of Dowex 50 (H^+), is shown in Fig. 1. Peak 6 has the same retention time as the peak of the open-chain TMS deriva-

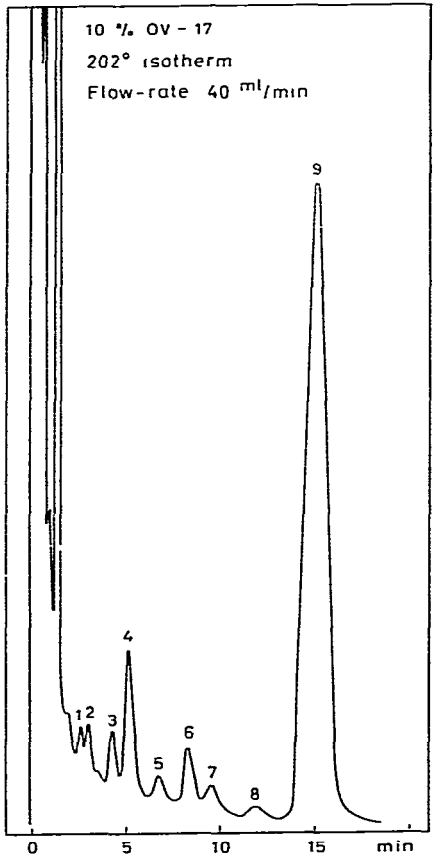

Fig. 1. Chromatogram of the open-chain TMS derivatives of an extract of *Echeveria elegans*. Before derivatization, the extract was passed through a column of Dowex 50 (H^+). Peak 9 is due to phorbic acid.

tive of glucaric acid. The ratio of the areas of peaks 6 and 4 was 0.32 ± 0.04 (mean ± S.D. for two determinations on each of five different extracts). Peak 6 is small compared with that originating from phorbic acid (peak 9) and also with that produced by addition of glucaric acid 1,4-lactone. The area of the peak due to added glucaric acid 1,4-lactone was calculated by subtraction of the area of peak 4 × 0.32.

Phorbic acid in Echeveria elegans

The recovery of pure dilactophorbic acid added to the plant material was found to be 100% (Table II). The content of phorbic acid in *Echeveria elegans* has earlier been estimated to be 3–4% of the dry weight[3]; this work gave a value of 15.5% (see Table II). Both investigations were performed with the same plant material.

TABLE II

DETERMINATION OF PHORBIC ACID IN POWDER OF DRIED *ECHEVERIA ELEGANS* WITH RECOVERY TEST

A 1.000-g amount of the plant material was used for the determination.

Glucaric acid 1,4-lactone added (mg)	*Dilactophorbic acid added (mg)*	*No. of determinations*	*Mean (± S.D.) amount of phorbic acid found* (mg)*
75	—	2	
100	—	3	155 (± 5)
100	25	5	180 (± 9)

* Phorbic acid is calculated as dilactophorbic acid.

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