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A Thin-layer Chromatographic Method for the Separation and Identification of Phorbic and Piscidic Acids

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Phorbic and piscidic acids were recently found to make up a major part of the nonvolatile organic acids in several plants [1-5] and it was presumed that, in a near future, there would be a need for appropriate analytical methods in connection with investigations of their metabolism. In the present investigations emphasis was placed on separation and identification of the pure acids. Preliminary applications of the methods to plant materials are reported. Detection of piscidic acid (*p*-hydroxybenzyl-tartaric acid) by means of thin-layer chromatography did not involve special difficulties. Being a phenolic acid, piscidic acid can easily be detected by means of spray reagents like Fast blue salt B. This reagent produces a yellow-brown color with piscidic acid.

Detection of phorbic acid, on the other hand, proved to be more difficult. The difficulties are mainly related to the tendency of the acid to form lactones, which give rise to several spots and heavy tailing during the chromatography. This problem was acute in one-dimensional chromatography of the acid and even more evident in two-dimensional chromatography.

a *b*

Fig. 1. a: Phorbic acid dilactone in water. *b:* Phorbic acid dilactone in acetone.

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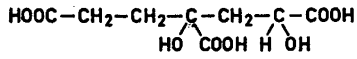
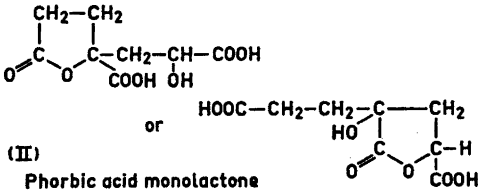
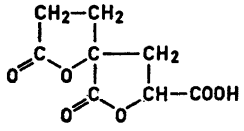
It has been shown [3] that phorbic acid (I) lactonizes very easily, forming a monolactone (II) and a dilactone (III).



(I) Phorbic acid



(II) Phorbic acid monolactone



(III) Phorbic acid dilactone

If phorbic acid dilactone, which is a crystalline, welldefined substance (m.p. 152—154°C), is dissolved in a non-aqueous solvent (like dry acetone) and chromatographed with a nearly waterfree solvent mixture on a cellulose sorbent, only one spot, corresponding to the dilactone, will appear on the chromatogram (*cf.* Fig. 1b). 1-Pentanol-formic acid-water (50:50:2.5), or the upper phase of a water saturated mixture of diethyl ether and conc. formic acid (87:12) were found suitable for chromatography of the dilactone.

Being a lactone as well as an acid, the phorbic acid dilactone can be visualized on the chromatogram by means of the hydroxamic acid reaction and also by means of a pH indicator spray, bromophenol blue. When the dilactone of phorbic acid was dissolved in water and chromatographed in the same solvent as the dilactone dissolved in dry acetone (*cf.* above), three spots appeared on the chromatogram, corresponding to the dilactone, the monolactone, and the non lactonized acid (*cf.* Fig. 1a). The spots could be visualized by means of bromophenol blue, or by the hydroxamic acid reaction (hydroxamate test) upon heating of the plates for half an hour at 100°C.

Finally, if an aqueous solution of the dilactophorbic acid is applied on the plate and heated for one hour at 100°C before chromatographing in the 1-pentanol-formic acid-water mixture (50:50:2.5), only two spots will appear on the chromatogram, corresponding to the two lactones (*cf.* Fig. 2b).

The acids were isolated from the plants (as a mixture) by means of ion exchange resins, but the solvents used for separating the pure acids gave unsatisfactory separation when applied to these extracts. Better results were obtained by utilizing more polar systems, like butanol-acetic acid-water (60:15:25).

In the plant extracts phorbic acid was found mainly in the unlactonized or in the monolactone form. In a few cases the dilactone was also observed. The separation method was tested on extracts of *Euphorbium resinifera* Berg, *Euphorbium*, and *Agave americana* L. which have been found to contain the two acids.[2,3]

Malic and citric acid gave nearly the same hR_F values as phorbic and piscidic acid when chromatographed under the same conditions, but as the two last mentioned acids could be detected by means of the more specific spray reactions mentioned above, a reliable identification was found possible.

Experimental. The investigation was carried out with the type of equipment, and according to the standard procedure given by Stahl.[6] As sorbent layer, cellulose Macherey-Nagel MN 300 was used. The thickness of the layers were

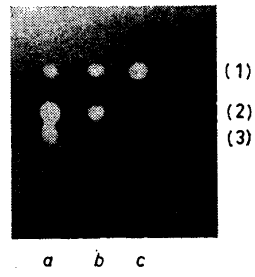


Fig. 2. a: Phorbic acid dilactone in water. *b:* Phorbic acid dilactone in water (100°C, 1 h). *c:* Phorbic acid dilactone in acetone. Developing solvent: 1-Pentanol-formic acid-water (50:50:2.5). hR_F values: (1) dilactone, 68; (2) monolactone, 50; (3) acid, open chain, 40. Length of run 10 cm. Spray reagent: Bromophenol blue, 0.1 % in methanol. $hR_F = 100 \times R_F$.

about 250 μ. 1-Pentanol-formic acid (98 %)-water (50:50:2.5) and butanol-acetic acid (99 %)-water (60:15:25) were selected as solvents. The pure acids or lactones were dissolved in dry acetone or in water (30 mg/ml), and about 1 μl was applied. For detection of the acids the following spray reagents were used:

1) For general detection of acids: Bromophenol blue, 0.1 % in methanol. The acids show up as yellow spots on a blue background.

2) For detection of phorbic acid lactones: Solution A: A filtered solution of 7.0 g hydroxylamine hydrochloride in 100 ml methanol was mixed with an equal volume of 7.2 % (w/v) potassium hydroxide in methanol, filtered, and then applied. Solution B: 1.0 g of ferric chloride dissolved in 100 ml of an 10 % solution of hydrochloric acid in water.

Before spraying the plates were heated at 100°C for 1/2 hour in order to remove the acids from the developing solvent. Upon cooling the plates were sprayed, first with test solution A, and after drying at room temperature for about 10 min with test solution B. Pink spots revealed the presence of the phorbic acid lactones. 3) For detection of piscidic acid: Solution A: 0.5 g Fast blue salt B dissolved in 100 ml of water, freshly prepared. Solution B: A 0.1 N aqueous solution of NaOH. The chromatograms were sprayed, first with solution A and then with B. Yellow-brown spots revealed the presence of piscidic acid.

Preparation of acids from plant materials for testing of the method. 1) *Euphorbia resinifera* Berg. 100 g fresh plant material was homogenized in a Waring blender, mixed with 500 ml of boiling ethanol, and refluxed for half an hour, and filtered. The residue was extracted for one hour with 500 ml of boiling water, pressed, and the extract filtered. The two filtrates were combined, concentrated *in vacuo*, and passed through a column of Amberlite 1 R 45 (OH^-) for isolation of the anions. After washing with distilled water, the acids were eluted from the column with 0.1 N HCl, whereafter the cations were removed on a column of Amberlite IR 120 (H). The isolated acid mixture was evaporated *in vacuo* to dryness, and the residue dissolved in 5 ml of acetone. This solution was used for the thin-layer chromatography.

2) *Euphorbium.* 10 g of the crude drug *Euphorbium* was extracted with 50 ml of boiling water, filtered and the acid isolated in the same way as above. The residue was dissolved in 2 ml of acetone.

3) *Agave americana* L. 100 g of the fresh plant was worked up in the same way as the corresponding sample of *Euphorbium resinifera* Berg. The acid mixture was dissolved in 5 ml of acetone.

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Synthesis of a Pyrite-Type Modification of SiP_2

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The synthesis of orthorhombic SiP_2 has been reported in a recent note.[1] This material was obtained in a low yield by heating a mixture of the elements, silicon powder and red phosphorus, in a sealed evacuated silica tube in a temperature gradient. The reactant sample was held at about 900°C and the product condensed at about 500°C. The stoichiometry was obtained from a single-crystal X-ray study which showed the phase to be isomorphous with $SiAs_2$.

Further studies on the silicon-phosphorus system have revealed the existence of one more intermediate phase. This was collected in low yield from a somewhat higher