Results and discussion

Table I shows the colors of the twelve compounds, and their approximate R_F values in the four solvent systems used. The detection limit with UV light or the unaided eye was about 0.5 μg or less for compounds 2, 3, 6, 8, 9, 10 and 11, and about 1.0 μg or less for the compounds 1, 4, 5, 7, and 12.

Fig. 1 illustrates the results of the separation obtained using the two-dimensional technique. When radioactive Planavin® herbicide was used, the separated compounds were isolated or eluted and the radioactivity quantified using liquid scintillation counting or scanning methods. In instances where the concentration was below the visible detection limits, the radioactive spots were located by exposing the chromatogram to X-ray film for a specified time, after which the film was developed. The areas were then located and eluted for quantification.

The author is indebted to R. D. SKILES and K. D. ZWAHLEN, who prepared the compounds used in this study, and to T. D. HOEWING and N. H. POONAWALLA for technical assistance.

Shell Development Company, Biological Sciences Research Center, Modesto, Calif. 95352 (U.S.A.) W. B. BURTON

1 W. J. HUGHES AND R. H. SCHIEFERSTEIN, *Proc. Ann. Mtg. Southern Weed Conf., 19th, 1966*, p. 170.

Received July 31st, 1970

J. Chromatog., 53 (1970) 397–399

CHROM. 4947

The separation of plant glycosides and aglycones using thin-layer chromatography and electrophoresis

Thin-layer chromatographic techniques have been used previously to investigate the phenolic glycosides[1–4] and their hydrolysed aglycones[5] present in plants. In a taxonomic study of the distribution of these compounds in the genus *Coprosma* (Rubiaceae), it became apparent that such chromatography was inadequate. One-dimensional separations failed to separate all the compounds present and two-dimensional separations were difficult to analyse because of the increased spot sizes and concomitant lack of definition (a difficulty also with paper chromatography).

Since a number of methods have been developed to separate organic mixtures by thin-layer electrophoresis[6] it was decided to investigate this technique for these phenolic compounds. Its principal advantage over chromatography is the speed of separation of any ionized compounds formed, thereby decreasing zone distortion and increasing definition.

Materials and methods

Extraction. Leaves from both freshly collected and herbarium specimens of

Coprosma species and hybrids were analysed. Unhydrolysed glycosidic extracts were prepared by soaking 1 g fresh weight or 0.5 g dry weight of leaves for 24 h in 5 ml of 1% hydrochloric acid in methanol (all solvents were analytical reagent grade, and proportions v/v or v/v/v). The leaf debris was then removed and the extract stored at 4° until required. To prepare samples of hydrolysed aglycones, 1 g fresh weight or 0.2 g dry weight of leaves was immersed in 7 ml of 2 *N* HCl. This was heated in a boiling water bath for 30 min, cooled, and the debris centrifuged off. To the supernatant was added 0.4 ml of isoamyl alcohol, and this mixture was shaken vigorously for 30 sec. The two layers were then allowed to separate, the alcohol layer pipetted off the top, and stored at 4°.

Chromatography. Shandon equipment was used to prepare the thin-layer plates and for chromatographic separations. For five 20 × 20 cm plates, 15 g of cellulose powder MN300 (Macherey, Nagel & Co) was dispersed in 90 ml distilled water using a Virtis "45" homogeniser, and spread to a wet depth of 0.25 mm. The layer was dried at 60° overnight. Using Microcap pipettes (Drummond Scientific Co.), 5–10 μl quantities of the extracts were applied 3 cm from the edge of the plate (and for two-dimensional separations, 5 cm from the cathodic end). After drying, 1 μl of catechol (50 mg/10 ml ethanol) was added as a reference compound.

A number of solvents were tested for their ability to separate the compounds in the leaf extracts. They were: acetic acid–hydrochloric acid–water (30:3:10), formic acid–hydrochloric acid–water (10:1:3), amyl alcohol–acetic acid–water (AAW, 2:1:1), AAW (10:6:5), *n*-butanol–acetic acid–water (BAW, 4:1:5, top phase), BAW (6:1:2), BAW (8:1:2), isopropanol–ethyl acetate–water (7:1:2), 40% aq. acetic acid, and 6% aq. acetic acid. Only 6% aq. acetic acid gave an even distribution of both glycosides and aglycones over the length of the solvent run.

After the chromatogram had been thoroughly dried by a hot-air fan and left to stand overnight, new margins were scribed through the layer, 1.5 cm under the origin and 1 cm below the solvent front. These were made to reduce electrophoretic distortions near the original edges.

Electrophoresis. Thin-layer electrophoresis was carried out using the equipment and techniques described by BIELESKI[7]. The buffers tested were (1) pH 5: 675 ml of 95% ethanol, 206 ml of water, 32 g of boric acid, 16 g of sodium acetate, adjusted to pH 5 with glacial acetic acid; (2) pH 5.3: 25 ml of pyridine, 8 ml of acetic acid, made up to 2.5 l with distilled water; (3) pH 8.04: phosphate buffer; (4) pH 10: 6 g of boric acid, 3 g of sodium hydroxide, made up to 2 l with distilled water; (5) pH 9.2: 38.1 g of borax (disodium tetraborate) dissolved in 2.5 l of distilled water.

The buffer was sprayed evenly onto the thin layer taking care not to spray directly the origin or the line of chromatographically separated compounds. The surface was then blotted with clean filter paper so that it appeared matte under reflected light, thus giving an even depth of buffer over the layer. To prevent evaporation, the plate was then immediately immersed in the coolant of the electrophoresis tank with the origin at the cathode. Wicks of Miracloth (Calbiochem) covered with dialysis tubing were placed so that one end dipped into the buffer, the other being laid carefully upon the layer with a 1-cm overlap. Glass rods held these in position. A potential difference of 1000 V was then applied to the system for 20 min. After electrophoresis, the plate was carefully removed from the coolant and dried with a hot-air fan.

Detection. Both glycosides and aglycones were visualised with short-wave UV light followed by a spray of diazotised benzidine[8]. The distance moved by each compound in both dimensions was calculated with respect to catechol, *i.e.*

$$R_{Cat} = \frac{\text{distance moved by spot}}{\text{distance moved by catechol}} \times 100$$

Results and discussion

Movement in buffer 1 was very slow and extensive heating occurred as the resistance of the system increased. Some of the compounds appeared to be insoluble in buffers 2 and 3 as indicated by their diffusion from the origin with no electrophoretic movement, and buffer 4 was unstable, bubbles of gas arising from the layer during the course of the run. The heat produced by these four buffers at this potential also tended to dry the layer so that the wick–layer contact dried out and electrical arcing carbonised the layer, destroying the contact. By contrast, the borax buffer (5) maintained a relatively steady current with therefore negligible coolant heating, and gave excellent separations. These are achieved after 17–20 min at 1000 V and 20° (*i.e.* approximately 55 V cm^{-1} length, 1.1 mA cm^{-1} width, 0.06 W cm^{-2} or 4.8 cal sec^{-1}). Furthermore, if buffer polarity is reversed after each run, at least 25 separations can be made without

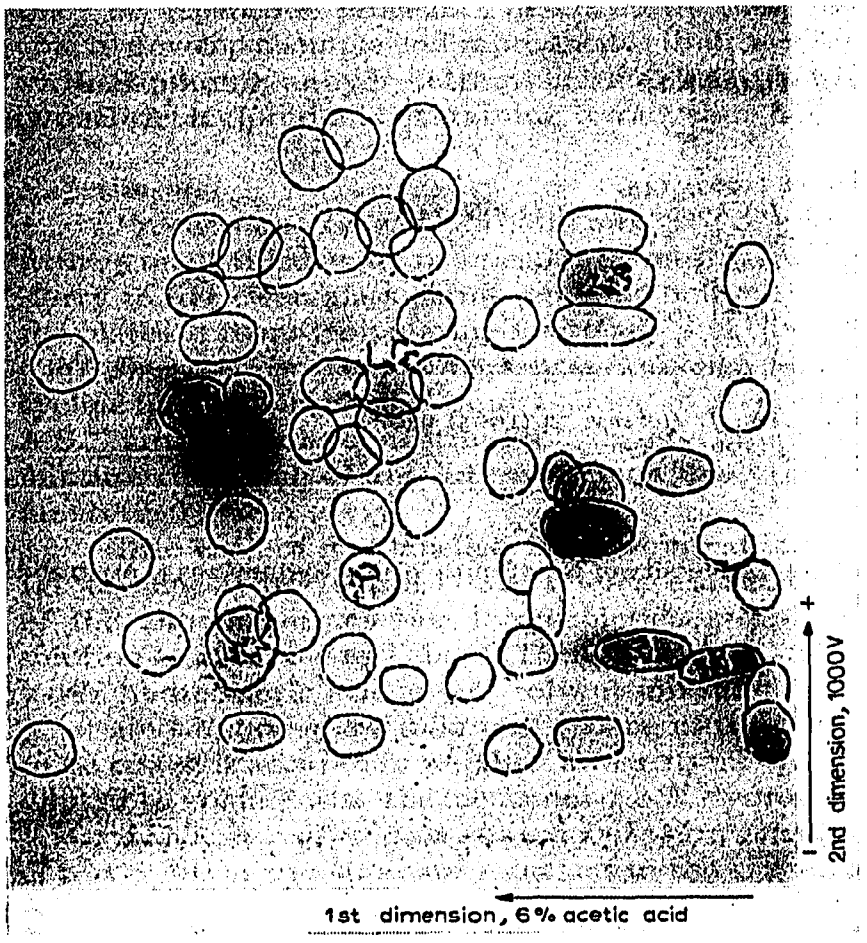

Fig. 1. A two-dimensional separation of the aglycones present in the leaves of a specimen of *Coprosma spathulata* (after UV inspection and spraying with diazotised benzidine). C = catechol.

having to renew the buffer. Residual borax cannot be removed from the thin layer so the chromatographic separation must be performed first. The majority of glycosides and aglycones act as anions under these conditions. A typical separation is shown in Fig. 1.

The aglycones were found to be more amenable to chemotaxonomic analysis than the glycosides. Leaves gathered in different seasons, of different ages, from differing light intensities, and—as most species of the genus *Coprosma* are dioecious—from different sexes, showed much less variation of the aglycone components than of the glycosides. The latter proved to be especially variable quantitatively. Also, under UV light, yellow glycosides at high concentration effectively quenched those compounds with similar R_{Cat} values which were less fluorescent. Thus some glycosides were very difficult to detect.

In the final analysis of 115 *Coprosma* specimens representing some 45 species, a total of 192 aglycones were recognised from the two-dimensional patterns of these individuals. It was apparent that specimens collected in the 1880's were relatively no different from later acquisitions of the same species (as has been noted previously for the genera *Lotus*[2] and *Artemisia*[9], and the family Rosaceae[10]). However, the final numerotaxonomic analysis utilised all an individual's spots or attributes, whether present in all or only one of the specimens of a species. This study is in the process of preparation for press.

Botany Department, University of Auckland,
Auckland 1 (New Zealand)

CEDRIC E. MAY*
J. M. A. BROWN

1 S. BOSE AND S. FROST, *Hereditas*, 58 (1967) 145.
2 W. F. GRANT AND J. M. WHETTER, *J. Chromatog.*, 21 (1966) 247.
3 S. B. GUPTA, *J. Chromatog.*, 36 (1968) 258.
4 H. JAWORSKA AND N. NYBOM, *Hereditas*, 57 (1967) 159.
5 K. BRUNSBERG, *Botan. Notiser*, 118 (1965) 377.
6 V. STEFANOVICH, *J. Chromatog.*, 31 (1967) 466.
7 R. L. BIELESKI, *Anal. Biochem.*, 12 (1965) 230.
8 D. E. HATHWAY, in I. SMITH (Editor), *Chromatographic and Electrophoretic Techniques*, Vol. I, Heinemann, London, 1960, p. 324.
9 H. R. HOLBO AND H. N. MAZINGO, *Am. J. Botany*, 52 (1965) 970.
10 E. C. BATE-SMITH, *Phytochemistry*, 4 (1965) 535.

Received July 24th, 1970

* Present address: Botany School, University of New South Wales, Sydney 2033, Australia