Thin-Layer Chromatography of Rotenone and Related Compounds

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A mixture of rotenone, deguelin, elliptone, tephrosin, sumatrol, and toxicarol was chromatographed on silica gel G. Both rotenone and sumatrol were separated as their mercuric acetate derivatives with n-propyl alcohol–acetic acid (100 to 1). Sumatrol was precipitated at the origin by 85% phosphoric acid–5N potassium iodide–ethyl alcohol–water (10:1:10:30). Chloroform-ether (95 to 5) separated sumatrol and tephrosin. Heptane–cyclohexanone–ethyl acetoacetate–water (1200:200:800:3) was used for tephrosin and elliptone. Hexane–ethyl acetate–water (600:400:1) separated sumatrol plus toxicarol (one spot) and gave a partial resolution of the others. Plates prepared with 12.5% silver nitrate solution and developed with chloroform–acetic acid (199:1) were useful for rotenone; those with 16% ferric chloride, for sumatrol plus toxicarol. Hydriodic acid spray (1 volume of 5N potassium iodide plus 30 volumes of 45% phosphoric acid) turned rotenone blue, elliptone reddish violet, and sumatrol grayish blue immediately; and deguelin, tephrosin, and toxicarol pink after 20 minutes at 120° C.

ROTENONE, an insecticide and fish poison of relatively low toxicity to warm-blooded animals (7), is found in various species of plants along with closely related rotenoids. Leaves of the tropical legume *Tephrosia vogelii* Hook f. are a promising commercial source of rotenone for insecticide manufacture. A breeding program at the Federal Experiment Station in Mayaguez, Puerto Rico, has resulted in the development of superior lines of this plant which are to be made available for commercial evaluation. Studies now in progress indicate that extracts from *T. vogelii* leaves contain mainly rotenone and deguelin in varying proportions, with minor amounts of tephrosin. Since deguelin reacts to some extent in both the modified Goodhue red-color test (4) and guppy toxicity test (5) used at this station for rotenone evaluation, a rapid means of separating rotenone from deguelin and other rotenoids is highly desirable.

The separation of rotenoids by classical chemical methods is a long and arduous process requiring many extractions, precipitations, and crystallizations (7). A paper chromatography system for rotenone reported by Chen and Tsai (2) has been found useful in this laboratory for separating rotenone and elliptone from one another (3), but not for other mixtures. None of the other techniques reviewed in the latter publication was suitable.

Thin-layer chromatography, well known as a rapid and selective means of separating related compounds, proved capable of resolving rotenoid mixtures. This report describes the separation of rotenone, elliptone, tephrosin, and suma-

trol from one another, and from deguelin and toxicarol through the use of thin-layer chromatography on silica gel G.

Experimental

Silica Gel G Plates. Silica gel G was mixed with twice its weight of distilled water and the resulting slurry was spread in a layer 0.25 mm. thick on 50 × 200 mm. or 200 × 200 mm. glass plates. The plates were activated by heating at 120° C. for 90 minutes and were cooled and stored in a desiccator over silica gel.

Silver Nitrate Plates. The silver nitrate plates were prepared as above, except that the absorbent was mixed with a 12.5% aqueous solution of silver nitrate instead of water. The plates darken upon exposure to light, but they may be reactivated by spraying with nitric acid and heating at 120° C. for 30 minutes with occasional ventilation. Since silver nitrate attacks stainless steel, it is best to coat the spreader lightly with a plastic spray and readjust the spreader opening before use. After each application, the spreader should be disassembled and thoroughly washed with water.

Ferric Chloride Plates. The ferric chloride plates were prepared by mixing the silica gel G with twice its weight of 16% aqueous ferric chloride solution. Unlike the silver nitrate plates, they are stable under normal storage conditions and do not require reactivation. Ferric chloride does attack stainless steel, however, and the precautions given for silver nitrate should be followed.

Preparation of Mercuric Acetate Derivatives. The mercuric acetate reagent was prepared by dissolving 1.4 grams of mercuric acetate in 25 ml. of methyl alcohol, 0.25 ml. of water, and 0.1 ml. of acetic acid. One hundred

microliters of this reagent were added to 100 μl. of chloroform containing 0.5 mg. of rotenoid. The mixtures were stored for 24 hours at room temperature in the dark. The solvent was removed by evaporation over concentrated sulfuric acid under reduced pressure in a desiccator. The residue was taken up in 500 μl. of chloroform and washed twice with 100 μl. of water. The chloroform solution was dried over sodium sulfate. Preliminary trials showed that the removal of excess mercuric acetate by water washing was necessary for good separations; the drying step was desirable but not vital.

Rotenoid Application and Development. The rotenoids were dissolved in acetone at a concentration of 1 mg. per ml. One microliter of each solution was applied to the plate 50 mm. from one end. The developing chambers were lined with filter paper which dipped into the solvent mixture, and were equilibrated with the solvent before the plates were inserted. The solvent layer was 10 mm. deep both in the cylinders used for the small plates and in the tanks used for the large plates. The development distance was 100 mm. from the point of rotenoid application.

Solvent System. The solvent mixtures, their compositions, and their average development times are:

A. Chloroform–diethyl ether (95 to 5), 37 minutes.

B. Chloroform–acetic acid (199 to 1), 22 minutes.

C. n-Propyl alcohol–acetic acid (100 to 1), 110 minutes.

D. Hexane – ethyl acetate – water (600:400:1), 19 minutes.

E. Heptane – cyclohexanone – ethyl acetoacetate–water (1200:200:800:3), 65 minutes.

F. 80% phosphoric acid–5N potassium iodide solution (aq.)–ethyl alcohol–

water (10:1:10:30), 150 minutes.

All solvents except the phosphoric acid and potassium iodide solutions were redistilled. Mixture F was prepared fresh just before use. All solvents, plates, and chromatography chambers were brought to 25° C. before development. After development, the plates were heated at 120° C. until free of solvent odor.

Hydriodic Acid Spray Reagent. One volume of 5 N potassium iodide was mixed with 30 volumes of 45% orthophosphoric acid just before use. This is essentially the reagent reported earlier (3) for use on paper chromatograms, with water added to reduce the viscosity of the mixture. The reagent is sprayed until the plate appears slightly camp, the position of any colored spots appearing within a few minutes is noted, and the plate is heated at 120° C. for 20 minutes to intensify the colors and produce spots with additional rotenoids.

Results and Discussion

Although a number of general reagents such as iodine vapor and sulfuric acid plus heat can be used to locate rotenoids on thin-layer chromatograms, the most generally useful reagent is the hydriodic acid reagent described above. This reagent is sensitive and selective for rotenoids. There are two major differences between its action on paper and on silica gel G chromatograms: On silica gel G, rotenone, elliptone, and sumatrol give colors almost immediately; and deguelin, tephrosin, and toxicarol can be visualized by heating the thin-layer chromatogram. The paper could not be heated because of discoloration.

The colors given by the various rotenoids following treatment with the hydriodic acid reagent are shown in Table I. The colors originally produced with rotenone, elliptone, and sumatrol disappear when the plate is first heated and reappear along with the colors of the other rotenoids after 20 minutes at 120° C. They fade slowly upon standing when the plates absorb moisture from the air. With silica gel G layers 0.25
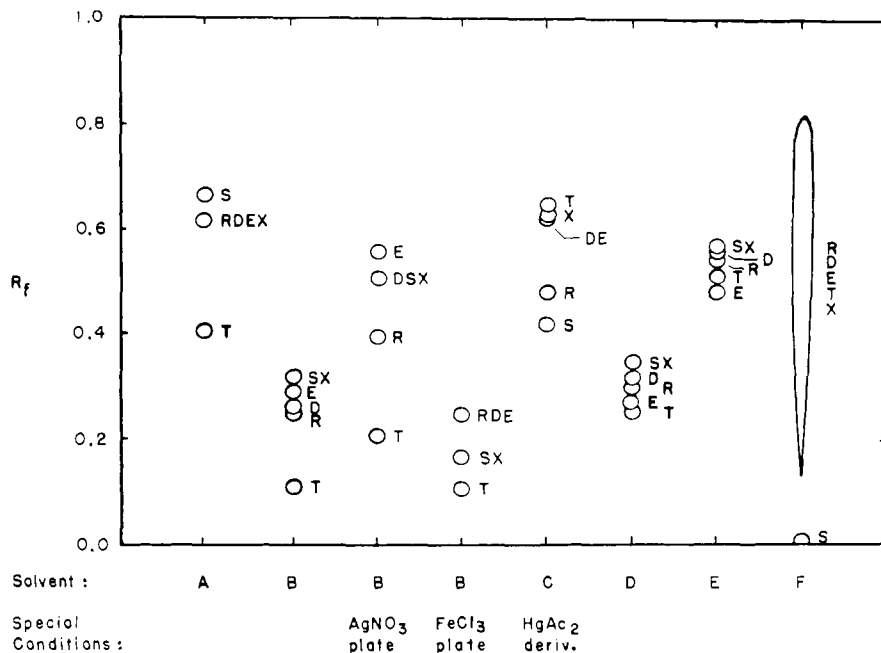
mm. thick, as little as 0.2 μg. of rotenoid per spot (1 μg. per sq. cm.) can be detected, but 0.5- to 1.0-μg. applications per spot are generally preferable.

The results obtained with the various solvent systems are shown in Figure 1 by means of circles of uniform size. The R_f was measured from the point of origin to the front of each spot.

Figure 2 shows the structures of the rotenoids included in this study. Since they differ from one another in polarity, they should be easily separable into three classes: those with no hydroxyl group (rotenone, elliptone, and deguelin); those with an aliphatic hydroxyl group (tephrosin); and those with an aromatic hydroxyl group (sumatrol and toxicarol). With untreated silica gel G plates, however, toxicarol, and especially sumatrol, had R_f values as high as, or higher than, compounds such as rotenone which lack a hydroxyl group (Figure 1, solvents A, B, D, and E). This probably is due to hydrogen bonding between the hydroxyl group and the adjacent carbonyl group, and is also found with other compounds, such as the nitrophenols (6).

Rotenone can be separated from the other compounds tested either by chromatography on silver nitrate-treated plates with solvent B, or by chromatography of the mercuric acetate adduct on silica gel G with solvent C. The silver nitrate system has the advantage of simplicity, but localization of the compound is more difficult. The hydriodic acid

reagent cannot be used because it turns the plate black. Likewise, iodine vapor and sulfuric acid are unsuitable.

For the silver nitrate plates, either of two methods was useful. In the first method the plate is sprayed with concentrated nitric acid and heated at 120° C. for 15 minutes. One or more such treatments with nitric acid may be necessary. At first the rotenoids are visible as dark absorbing spots under short-wave ultraviolet light, but after adequate nitric acid treatment, they change to fluorescent spots. In the second method the plate is exposed to the air and to subdued light for several hours. The rotenoids slowly become visible as dark spots on a light background. Of course, neither means of visualizing the rotenoids permits differentiation of the rotenoids except by position, and neither is very sensitive. Comparison of this system to the same solvent with untreated silica gel G shows that the absorption of rotenone, sumatrol, and toxicarol is enhanced compared to deguelin and elliptone.

The preparation of the mercuric acetate derivatives involves more work than the use of the silver nitrate system, but the separation of rotenone is equally clean and the visualization of the compounds is much simpler; the derivatives show the characteristic colors of the parent compounds when sprayed with the hydriodic acid reagent.

Deguelin presents a problem. It is not separated from the complete mixture

Figure 1. Separation of rotenoids on silica gel G and modified silica gel G plates

R. Rotenone
D. Deguelin
E. Elliptone
T. Tephrosin
S. Sumatrol
X. Toxicarol. For solvent systems, see text

OCH₃ CH₃O [chemical structure]

R = H, Rotenone
R = OH, Sumatrol

OCH₃ CH₃O [chemical structure]

Elliptone

OCH₃ CH₃O [chemical structure]

R₁ = H
R₂ = H   Deguelin

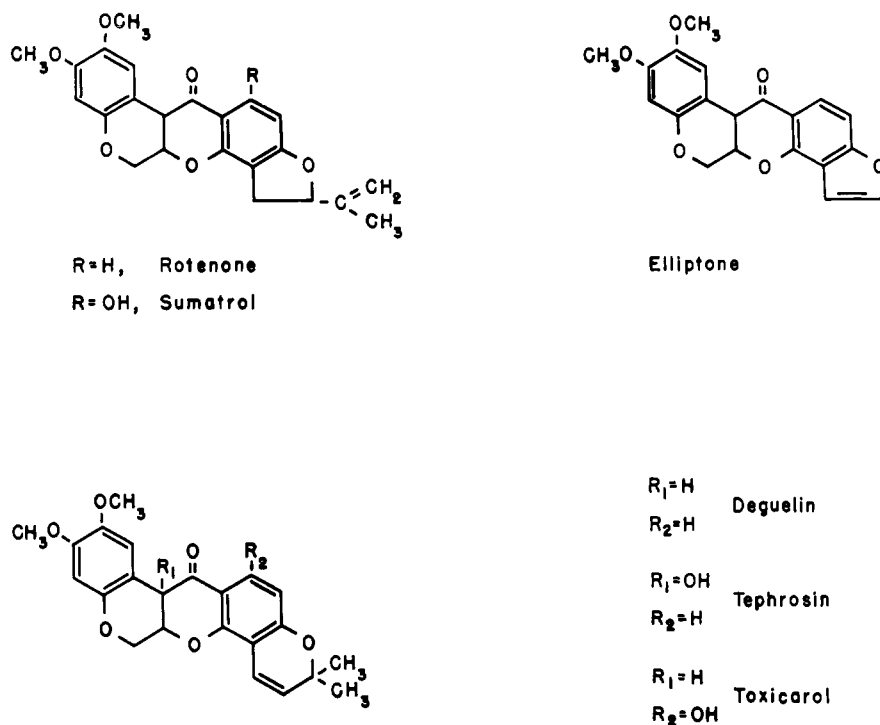R₁ = OH
R₂ = H   Tephrosin

R₁ = H
R₂ = OH   Toxicarol

Figure 2. Structure of rotenoids used in this study

of rotenoids by any of the techniques presented above. A combination of systems in a two-dimensional chromatogram might be successful if the isolation of deguelin were necessary.

Elliptone is separated by solvents B and E, and by solvent B with silver nitrate plates. The first two are preferable to the latter because of the difficulty of visualizing the results on silver nitrate plates, as discussed earlier.

Tephrosin is the easiest of the rotenoids to separate. With the exception of solvents C and F, any solvent system presented here can be used. Solvents A and B are preferable.

Sumatrol can be separated with sol-

vent A without resorting to chemical alteration of the molecule. As the mercuric acetate derivative, sumatrol is easily separated from the other compounds with solvent C. Solvent F precipitates sumatrol at the origin, whereas the other rotenoids give intermediate R_f values, but exhibit extensive tailing. Solvent F could be used to advantage as a second solvent in two-dimensional chromatography to separate sumatrol and toxicarol, for example.

Toxicarol is not completely separated from complex mixtures of rotenoids by any single solvent system used in this report. Two-dimensional chromatography might be used, or toxicarol and

sumatrol could be separated first from the nonphenolic rotenoids by extraction with sodium hydroxide solution and then from one another by chromatography with solvent A, C (mercuric acetate derivatives), or F.

The absorption of sumatrol and toxicarol, both phenols, is enhanced in silica gel G layers impregnated with ferric chloride. Presumably, this is due to the complexing of the phenol with the ferric chloride in the plate. This technique has apparently not been used before. It may find application in the chromatography of other phenols. The plates are reddish brown after being heated for activation, but turn white when heated with the hydriodic acid spray reagent. The rotenoids give their customary colors with this reagent.

The techniques presented in this paper have been useful in separating and qualitatively estimating the amounts of rotenoids in *T. vogelii* leaf extracts at this station. Further studies are under way to develop a quantitative assay for rotenone based on thin-layer chromatography.

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RESIDUE IN TISSUE

Esterase Inhibition in Pheasants Poisoned by O,O-Diethyl S-(Ethylthiomethyl)-phosphorodithioate (Thimet)

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THE HAZARDS involved, especially to wildlife, in the use of persistent organochlorine pesticides, are widely appreciated. This has led to a search for less persistent pesticides, and organophosphorus esters are now being used in increasing quantities. Although they are less likely to have chronic effects, many of these compounds are acutely poisonous, and methods are needed for examining wild birds and possibly other

animals thought to have been killed by these compounds.

There has been much research in the detection, identification, and determination of organochlorine residues in animal tissues, and methods for the routine examination of wildlife relicta are now well established (*20*, *21*). However, the interpretation of the figures obtained is difficult, since residues found after death even in test-fed birds

are not consistent (*23*), and acute poisoning may be aggravated by a number of factors, including residues accumulated over a long period or sudden physiological changes. No comparable analytical methods exist for organophosphorus pesticides in animal tissue, and indeed they would be difficult to set up since most of these compounds are readily broken down in the body even after death, thereby rendering res-