Results and discussion

The R_F values of the parent amines, the hydroxylamines, and the stable nitroxides are given in Table I. Good separations were achieved. Oxidation products of hydroxylamines were best separated with solvent system (B), but (A) gave similar results. Mixtures of hydroxylamines or of nitroxides were separated best by (C) (see Fig. 1). The systems outlined were quick (25–35 min), convenient methods for separating hydroxylamines and nitroxides, and for determining the purities of the various preparations. Preparative thin layer chromatography was satisfactory for obtaining larger amounts of these products. Up to 10 mg could be streaked on each plate.

Acknowledgements

The author would like to acknowledge the help of Dr. BARBARA BECK in purifying the hydroxylamines. This work was supported in part by grant No. UI 00238-01 from the National Institute of Health.

Institute of Marine Resources, Department of Nutritional Sciences, University of California, Berkeley, Calif. (U.S.A.) JAIR T. WEIL

1 J. T. WEIL, *Ph. D. Thesis*, University of California, Berkeley, Calif., 1968.
2 C. SCHÖPF, A. KOMZAK, F. BROWN AND E. JACOBI, *Ann. Chem.*, 559 (1948) 40.
3 E. R. BLOUT, S. G. COHEN AND M. GREEN, *U.S. Patent*, 2,843,481 (1958).
4 R. A. HENRY AND W. M. DEHM, *J. Am. Chem. Soc.*, 72 (1950) 2280.
5 R. BRIERE, H. LEMAIRE AND A. RASSAT, *Bull. Soc. Chim. France*, (1965) 3273.
6 G. A. SNOW, *J. Chem. Soc.*, (1954) 2588.

Received April 19th, 1968

CHROM. 3553

Two-dimensional thin-layer chromatography of polyphenols from *Dryopteris* species

The medicinally-important *Dryopteris* ferns contain a large number of interrelated phloroglucinol derivatives and the chromatographic separation of these is important both in taxonomic studies of this complex genus and in the evaluation of Male Fern and related drugs. Previous chromatographic studies have been reported[2–5], including two-dimensional paper[6] and thin-layer[7] methods. In these latter, however, the same solvent was used in both directions.

We now present a two-dimensional, thin-layer method which provides differential separation in the two directions of development and in which decomposition of the labile polyphenols is prevented by incorporation of an anti-oxidant in the chromatoplate layer. A further advantage is that visualisation of the compounds is possible, without recourse to spraying, by examination under ultraviolet light.

Experimental

Plant material

Rhizomes, with attached frond bases, of *Dryopteris filix-mas* (L.) Schott. and *D. dilatata* (Hoffm.) A. Gray were collected in the West of Scotland in October 1966 and September 1967, respectively. After thorough washing and removal of roots and dead portions, the samples were dried in a forced-draught oven at not more than 35° for about three weeks.

The dried material was then powdered (moderately coarse powder) and immediately percolated to exhaustion with ether. The solvent was removed, care being taken to avoid overheating the residual oleo-resin: this was packed in well-filled, well-closed containers and stored in the dark until required. At all stages in handling the material was exposed to light as little as possible.

Reference compounds

Filicic acid. The crude ether extract (450 g) of *Dryopteris filix-mas*, on standing for several weeks, deposited a yellowish brown crystalline mass from which the supernatant liquid was removed and reserved for the separation of flavaspidic acid. The crystals (approx. 5 g) were washed with a minimal amount of acetone to remove adherent resin and then recrystallised several times from ethyl acetate. Yellow, tabular crystals m.p. 183.5–184°.

Flavaspidic acid. This (2 g) was obtained from the supernatant above, following the method of WIDEN[8]. Recrystallised from methanol, yellow rosettes m.p. 90°, remelting at 156°. (Melting points were sharp only on recently crystallised material.)

Aspidin. The crude ether extract (400 g) from *Dryopteris dilatata*, treated by the method of BUCHI[9], yielded yellow needles (3.5 g), m.p. 124–125°, from ethanol.

Aspidinol. Further treatment[9] of the residual solutions, after separation of aspidin, yielded aspidinol, m.p. 143°, from benzene, as either pale yellow needles or yellow rectangular prisms, (0.7 g).

Desaspidin. A specially purified sample was bought from Medica Ltd., Helsinki, Finland.

Chromatography

Plates. All plates were 20 × 20 cm, spread to a depth of 250 μ, and used within 24 h of preparation.

Type 1. Silica Gel G (Merck, 25 g) slurried with McIlvaine buffer (citric acid–phosphate), pH 6.0 (55 g) and dried at 105° for 30 min.

Types 2, 3, and 4. As type 1 except that ascorbic acid (100, 150, and 250 mg, respectively) was dissolved in each 55 g amount of buffer solution the resultant pH values for which were 5.8, 5.7, and 5.6.

Solvents. Petroleum ether (40–60°) redistilled and the 48–50° fraction used. Cyclohexane purified by passing through an alumina column. Ethanol and ethyl acetate purified by standard techniques[10].

Developing solvents. (1) Petroleum ether–ethanol (95:5); (2) cyclohexane–ethyl acetate (50:50).

Development. This was performed, in the dark, at a controlled temperature of 25°. Each plate, carrying oleo-resin (0.3–1.2 mg) suitably diluted in ether for application (10 mg/ml), was developed, in a Shandon Universal tank, with solvent 1 (100 ml) to a distance of 15 cm (60 min, approx.) then carefully dried *in vacuo*, in the

dark, for 10–15 min. Development in the second direction, with solvent 2, took approx. 70 min for a run of 15 cm. Plates were allowed to dry in the dark.

Detection of compounds. (a) When examined in U.V. light (366 mμ) types 2, 3, and 4 plates exhibited a pale blue fluorescence against which the phloroglucides showed as dark areas. (b) Fast Blue salt B (Merck) in aqueous solution (0.1 %), applied as a spray, gave blue to red colours with most of the phenols, although in some cases the colour was yellow (aspidin) or brown (filicic acid). Where colour development was poor, due to very low concentrations, overspraying with sodium hydroxide solution (0.1 *N*) intensified the colours.

Results and discussion

Fig. 1 shows a typical chromatogram obtained from a prepared mixture of the five reference compounds separated on plates (Type 3) impregnated with ascorbic

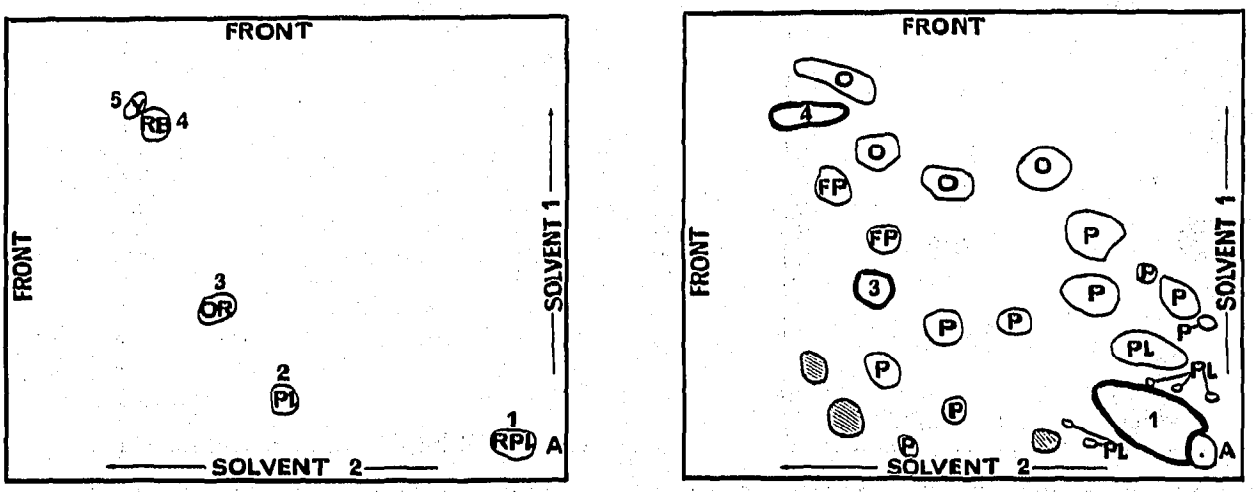


Fig. 1. Two-dimensional thin-layer chromatograph, on a type 3 plate, of five reference compounds: (1) flavaspidic acid; (2) aspidinol; (3) desaspidin; (4) filicic acid; (5) aspidin. All spots were detectable by fluorescence quenching. The letters refer to the colours obtained after spraying with Fast Blue salt B. Y = yellow; RB = red brown; OR = orange red; Pl = purple; RPl = reddish purple.

Fig. 2. Two-dimensional thin-layer chromatograph, on a type 3 plate, of a freshly prepared ether extract of *Dryopteris filix-mas* (L.) Schott. The numbers refer to known reference compounds (see Fig. 1) and the letters to the colour developed with Fast Blue salt B. All spots were detectable by fluorescence quenching before spraying. O = orange; P = pink; F = faint; shaded areas = non-phloroglucinol compounds, probably chlorophylls.

acid. The R_F values obtained in both solvent systems are quoted in Table I. Figs. 2 and 3 illustrate the results of two-dimensional chromatograms obtained with the crude ether extract obtained from *Dryopteris filix-mas* and *D. dilatata*, respectively. Some 25–30 spots were observed on individual plates (Fig. 2) and some of these must represent known homologues[1] of several of the fern constituents which are thus separated by this method. Although filicic acid and aspidin are almost contiguous on plates (Fig. 1), they can be readily distinguished by their colours with Fast Blue salt B. Their separation is rarely a practical problem since co-occurrence in ferns is rare[1,11,12]; they do not occur together in ferns commonly used for medicinal purposes.

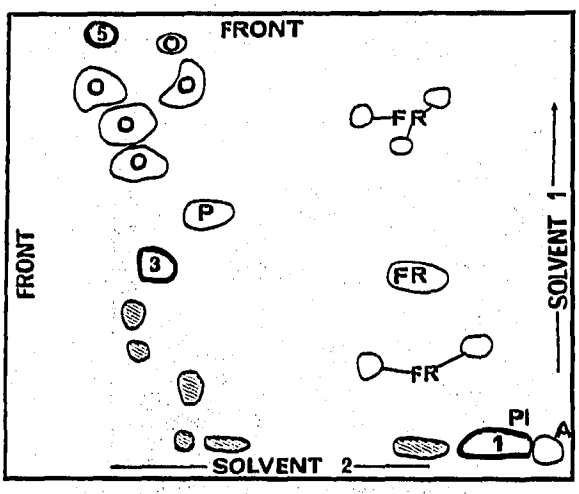
Using type 1 plates (without ascorbic acid) loaded with oleo-resin (1.2 mg), but

TABLE I

R_F VALUES ON BUFFERED, ASCORBIC ACID-IMPREGNATED, TWO-DIMENSIONAL, SILICA GEL CHROMATOPLATES (TYPE III)

Compound	R_{F_1}*	R_{F_2}*
Flavaspidic acid	0.04 – 0.06 – 0.07	0.04 – 0.06 – 0.07
Aspidinol	0.10 – 0.12 – 0.14	0.48 – 0.50 – 0.52
Desaspidin	0.38 – 0.40 – 0.42	0.61 – 0.64 – 0.66
Filicic acid	0.80 – 0.83 – 0.85	0.72 – 0.75 – 0.78
Aspidin	0.85 – 0.88 – 0.90	0.70 – 0.73 – 0.75

* R_{F_1} in petroleum ether–ethanol (95:5); R_{F_2} in cyclohexane–ethyl acetate (50:50).



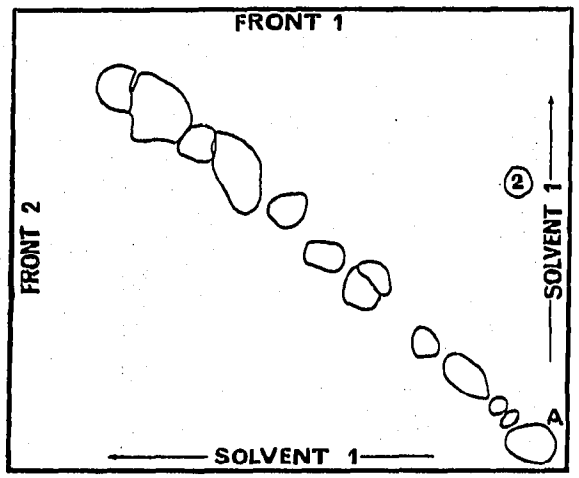


Fig. 3. Two-dimensional thin-layer chromatograph, on a type 3 plate, of a freshly prepared ether extract of *Dryopteris dilatata* (Hoffm.) A. Gray. Labelling as for previous figures. R = red.

Fig. 4. Two-dimensional thin-layer chromatograph of a commercial Male Fern extract, on a type 1 plate, using solvent 1 in both dimensions; showing aspidinol, resulting from decomposition during chromatography, as out-of-line, purple spot (2).

developed in *both* directions with the same solvent mixture (solvent 1), it was noted that instead of a resultant series of spots in a straight diagonal line, as would be obtained in the absence of decomposition[13], there was an additional out-of-line spot (Fig. 4). This spot corresponded to the common breakdown product aspidinol both in characteristic colour (purple) with Fast Blue salt B and position. A similar result was obtained using solvent mixture 2 when the out-of-line spot again occurred in the position along the second dimension anticipated from the known R_F value of aspidinol (Table I).

Various workers have commented on the poor stability of Male Fern and its preparations[14–17]: a 26 % decrease in the flavaspidic acid content of male fern powder, in five months, has been reported[18] and the ease with which para-aspidin breaks down is known[6]. It has been shown that even though avoidance of exposure to excessive heat or to light and air may improve drug stability, breakdown may be caused during chromatographic procedures thus giving erroneous results. This is especially so under alkaline conditions but occurs also under slightly acid conditions.

The use of ascorbic acid as an anti-oxidant has been described[3] previously in the paper chromatography of fern constituents and we have now shown its advantages in TLC using silica gel. In addition to preventing decomposition of active compounds, ascorbic acid has the advantage of giving a fluorescent layer against which the compounds are easily detected as dark areas when examined in U.V. light (366 mμ). This has obvious advantages in locating compounds prior to their elution for subsequent spectrophotometric estimation. For the detection of most of the compounds fluorescence quenching is as sensitive as colour development with Fast Blue reagent and it is even more sensitive in cases where the reagent gives a yellow colour. Of the various plates used, type 3 provided the optimum conditions for compound detection. The participation of ascorbic acid in fluorescence reactions is well known[19–21] and in the present circumstance, fluorescence appears to result from oxidation of the ascorbic acid since plates dried in an atmosphere of nitrogen were non-fluorescent.

School of Pharmaceutical Sciences, F. FISH
The University of Strathclyde, Glasgow, C. 1 (Great Britain) W. R. KIRK

1 F. FISH AND J. M. CALDERWOOD, *Pharm. Weekblad*, 102 (1967) 515.
2 A. PENTTILA AND J. SUNDMAN, *J. Pharm. Pharmacol.*, 13 (1961) 531.
3 R. KLEVSTRAND, *Medd. Norsk Farm. Selskap*, 23 (1961) 189.
4 M. VON SCHANTZ, *Planta Med.*, 10 (1962) 22.
5 E. STAHL AND P. J. SCHORN, *Naturwissenschaften*, 49 (1962) 14.
6 A. PENTTILA AND J. SUNDMAN, *Planta Med.*, 14 (1966) 157.
7 M. VON SCHANTZ, *Planta Med.*, 10 (1962) 98.
8 B. WIDEN, *Acta Botan. Fenn.*, 37 (1944) 4.
9 J. BUCHI, *J. Pharm. Belg.*, 12 (1957) 391.
10 A. I. VOGEL, *A Textbook of Practical Organic Chemistry*, Longmans, Green and Co., London, 1948, pp. 166 and 173.
11 L. H. FIKENSCHER AND R. HEGNAUER, *Planta Med.*, 11 (1963) 355.
12 L. H. FIKENSCHER AND M. R. GIBSON, *Lloydia*, 25 (1962) 196.
13 P. DECKER, *Naturwissenschaften*, 44 (1957) 305.
14 I. G. ZWIMPFER AND J. BUCHI, *Pharm. Acta Helv.*, 37 (1962) 224.
15 M. KOSTOLOWSKA, *Dissertationes Pharm.*, 13 (1961) 193.
16 R. WASICKY, *Schweiz. Apoth. Ztg.*, 62 (1924) 601.
17 M. ACKERMANN AND H. MUHLEMANN, *Pharm. Acta Helv.*, 21 (1946) 157.
18 R. KLEVSTRAND, *Medd. Norsk Farm. Selskap*, 25 (1963) 191.
19 I. N. CHERNYUK AND I. I. DILUNG, *Dokl. Akad. Nauk S.S.R.*, 156 (1964) 149.
20 W. H. HARRISON, *Biochim. Biophys. Acta*, 78 (1963) 705.
21 H. FREITAG, *Z. Anal. Chem.*, 139 (1953) 263.

Received April 16th, 1968