THE CYTOGENETICS OF *LOTUS*

XI. THE USE OF THIN-LAYER CHROMATOGRAPHY IN THE SEPARATION OF SECONDARY PHENOLIC COMPOUNDS IN *LOTUS* (LEGUMINOSAE)*

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INTRODUCTION

Secondary phenolic compounds have recently been found to have taxonomic significance in several plant genera[1–6] and chromatography has been the most important technique used in their separation.

In the Leguminosae, secondary phenolics have been shown to be particularly useful in the elucidation of taxonomic problems. Extensive studies have been carried out on the mapping of species specific compounds in *Baptisia*[1] and interspecific hybrids have been shown to contain compounds from both parents[3]. Paper chromatographic analyses by HARNEY AND GRANT have indicated that most of the species of *Lotus* can be identified by their phenolic content alone[4] and that interspecific diploid hybrids generally possess all of the compounds found in the leaves of both parental species[7]. Since considerable time is required for the separation of the compounds by paper chromatography and the spots obtained are in many cases rather diffuse, it was decided to investigate thin-layer chromatography to see if this technique was an improvement and whether it could be used as an adjunct to, or even instead of, paper chromatography in the study of secondary phenolics of *Lotus*.

MATERIALS AND METHODS

Samples of both fresh and dried leaves of North American species of *Lotus* were prepared by weighing out 0.08 g fresh leaves, or taking a sample of approximately the same quantity of dried leaves, and leaving them in 0.5 ml of 1 % hydrochloric acid in methanol at room temperature, in the dark, overnight.

A. Chromatographic procedure

The separation of the compounds contained in unhydrolysed extracts of *Lotus* leaves were tested on three different coating materials, namely, silica gel G, cellulose, and polyamide powder, with different solvents using both one- and two-dimensional methods.

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Shandon thin-layer chromatographic equipment was used throughout. Standard plates 20 × 20 cm or 5 × 20 cm were coated with layers of 250 μ thickness, and the quantities given in the experimental section below for the preparation of the slurries are sufficient to coat five 20 × 20 cm plates. The coated plates were either oven dried (silica gel) or air-dried (cellulose and polyamide) and then stored in a desiccator until required. The plates were prepared for development by applying approximately a 7 μl spot of sample solution with a micropipette at a distance of 1.5 cm from the base. Two spots were run for each sample to check the reproducibility of the separation, and the spots were applied 1 cm apart using the Shandon template. The position of the solvent front was marked off by drawing a line across the layer at the required height. Ascending development was carried out at room temperature and the tanks were made air tight by sealing with masking tape. The solvent was always added to a tank at least half an hour before a plate was developed to allow the atmosphere to become saturated. If this was not done there was a marked drop of from 0.5 to 1.0 in R_F value, the difference being more marked with the more volatile solvents. Because of this drop in R_F value, it was also found necessary to let the atmosphere become fully saturated again after the removal of one plate before a second plate could be developed. Solvents were renewed frequently.

B. Solvents

A preliminary screening experiment was used to test a number of solvents for their suitability. Several spots of *Lotus* extract were applied in rows to a plate coated with the required layer. The spots were applied at 4 cm intervals, then each one was treated with a different solvent fed to the spot with a micropipette. The solvent was allowed to spread out in a circle to 1.5 cm diameter, and from the positions of the concentric rings formed by the components of the extract one could decide by visual inspection, after some experience, whether or not the solvent was going to be of value.

The following solvents were used and are referred to later in the text by abbreviations which are given in brackets after each solvent.

Single component solvents. Petroleum ether (PE), cyclohexane (C), chloroform (Ch), ethyl acetate (EA), butyl acetate (BA), acetone (Ac), butanol (B), ethanol (E), methanol (M), acetic acid (A).

Two component solvents. Petroleum ether–ethyl acetate 1:1 v/v (PE.EA 1:1) and 1:3 (PE.EA 1:3), cyclohexane–ethyl acetate 1:1 (C.EA 1:1) and 1:3 (C.EA 1:3), chloroform–ethyl acetate 1:1 (Ch.EA 1:1), hydrochloric acid–methanol 1:99 (HCl.M 1:99), methanol–chloroform 10:90 (M.Ch 10:90), 20:80 (M.Ch 20:80) and 30:70 (M.Ch 30:70), acetic acid–water 15:85 (A.W 15:85), formic acid–water 2:98 (F.W 2:98).

Three (or more) component solvents. Butanol–acetic acid–water 6:1:2 (B.A.W 6:1:2), 4:1:2.2 (B.A.W 4:1:2.2), and 4:1:5 (B.A.W 4:1:5), *n*-amyl alcohol–acetic acid–water 20:12:10 (nAm.A.W 20:12:10), acetic acid–hydrochloric acid–water 20:6:10 (Forestal), water–hydrochloric acid–formic acid 8:4:1 (W.HCl.F 8:4:1), butanol–hydrochloric acid–water 15:3:6 (B.HCl.W 15:3:6), toluene–acetic acid–water 4:1:5 (T.A.W 4:1:5), butanone–ethyl acetate–formic acid–water 3:5:1:1 (Bu.EA.F.W 3:5:1:1), water–ethanol–butanone–acetylacetone 13:3:3:1 (W.E.Bu.A. acet 13:3:3:1).

After development, the plates were dried and all traces of the solvents removed

with a warm air jet. The separation of the spots on each plate was examined in daylight, in long and short wave ultraviolet (U.V.) light by means of a Chromato-Vue (Model C-3, Ultra-Violet Products, Inc., San Gabriel, Calif., containing a 3660 Å long wave, and a 2537 Å short wave, U.V. lamp) and in ultraviolet light after fuming with ammonia vapor. Generally, no special procedure was carried out for the improvement of spot visibility as none was found satisfactory, though the usefulness of some techniques is commented on in the Experimental section. The results were recorded by mapping the separations on paper and also photographing the plates through the viewing window of the Chromato-Vue. Both black and white (Ilford FP3 film, exposure 30 sec/f8 at a distance of 20.3 cm from the plate) and color (Kodak High Speed Ektachrome film, exposure 25 sec/f8 at a distance of 20.3 cm from the plate) were used. A yellow filter (Kodak Filter No. 2A) was used with both films to cut down reflected blue light. Color film gave the best results and the color renderings on the slides were excellent. In the black and white photographs blurring around the edge of each spot was intensified and without the contrast of color it was difficult to identify spots which were close together.

EXPERIMENTAL

A. Silica gel G

Layers were prepared by weighing out 28 g silica gel G (E. Merck, Darmstadt, Germany) into a conical flask, adding 60 ml distilled water and shaking for 90 sec before applying the slurry to the plates. The flask must be absolutely dry before the water is added, and the slurry must be applied immediately after preparation since it hardens rapidly. An amount of 28 g was used instead of the recommended 30 g since this improved the spreading consistency of the slurry. The coated plates were left to set for 5 min, then dried at 100° for a minimum period of 20 min. It was found that further heating made no difference to the chromatographic properties of the layers. Care had to be taken when applying the extracts to the layers, as they were quite soft and it was easy to make a hole right through the silica gel.

The *Lotus* extracts contained two different sets of chemical compounds, one of which (designated the pink spots since the majority of the spots were pink) moved considerably faster in most solvents than did the other (designated the origin spots, containing spots of all colors and moving generally well behind the pink spots).

Solvents

The following solvents were tried:

PE and C — There was no movement from the origin.

Ch — Only the pink spots moved from the origin. These were well separated, but there was some spot tailing.

EA — The pink spots all moved near the solvent front but there was good resolution of the origin spots.

BA — The pink spots massed at the solvent front and there was very little movement of the origin spots.

Ac — Fairly good separation of both the pink spots and the origin spots, but there was a lot of tailing, particularly near the origin.

B — Good general spot movement but too much tailing for the solvent to be useful.

E — Little movement from the origin.
M — More movement than with E, but still only to R_F 0.5 and there was bad spot tailing.
A — Good separation of spots from the origin to the solvent front, but groups of spots tended to run into one another so that spot resolution within the group was not satisfactory.
PE.EA 1:1 — The pink spots moved too near the solvent front, and there was little movement of the origin spots.
PE.EA 1:3 — Good separation of the origin spots but the pink spots still moved too near the solvent front.
C.EA 1:1 — Gives a good resolution of the pink spots and a fair resolution of the origin spots. The latter is improved considerably if the solvent is run two or three consecutive times (multiple-pass development, see below).
C.EA 1:3 — The pink spots travelled too near the solvent front.
Ch.EA 1:1 — The pink spots travelled faster and the origin spots slower, decreasing the resolution of both sets as compared with C.EA 1:1.
HCl.M 1:99 — Not enough movement from the origin and there was considerable spot tailing.
M.Ch 10:90, 20:80, and 30:70 — Movement from the origin increased as the proportion of methanol in the solvent was increased. M.Ch 30:70 gave the best resolution of the origin spots, which separated from R_F 0 to R_F 0.9.
AA.H_2O 15:85 — Practically all the spots moved with the solvent front. Since phenols are hydrophylic this was most likely due to the high water content of the solvent.
B.A.W. 6:1:2 — Spots were badly blurred and the resolution was poor.

The single component solvents were tried first to give some idea of their suitability. None of these alone gave a satisfactory separation of both sets of spots, so mixtures were tried including two solvents (B.A.W. 6:1:2 and A.W 15:85) which had been found suitable for the separation of secondary phenolics in both *Lotus*[4], and other genera (*Baptisia*[1,3], *Haworthia*[5] and *Zinnia*[6]). These two solvents were found to be useless for silica gel layers; in A.W 15:85, the spots moved almost with the solvent front and there was bad tailing in B.A.W 6:1:2. One of the two-component solvents, C.EA 1:1, gave a good separation of the pink spots, and another, M.Ch 30:70, gave a good separation of the origin spots. Therefore, combinations of these two solvents were tried. The best separation was obtained in two stages since some spots only became separated after a second development in a solvent. C.EA 1:1 was used as the first solvent and allowed to pass twice up the layer to a height of 15 cm, the plate being dried and the positions of the spots being noted after each run. By this technique all the pink spots were moved above R_F 0.5. The second solvent, M.Ch 30:70, was then allowed to run up to R_F 0.5 only. This gave a good separation of the origin spots from R_F 0 to R_F 0.5 (Fig. 1). Occasionally a second run of the second solvent was carried out if some of the origin spots were still overlapping. This procedure, using multiple-pass development, gave an excellent separation of the *Lotus* extracts (Figs. 1, 2). A total of 50 spots have been found in all the species examined, of which about 25 were present in any single extract. Generally the pink spots showed little variation except quantitatively (judging by spot size and color intensity alone) whereas there was extensive variation from species to species in the origin spots.

Two-dimensional separations were tried, but the spots became too diffuse during the second run, and tended to tail into one anotber hadly.

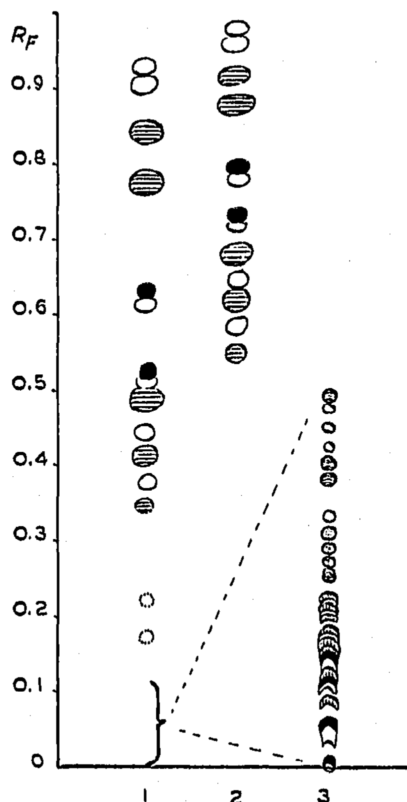

Fig. 1. Chromatograms of unhydrolysed leaf extracts of *Lotus* illustrating the one-dimensional ascending multiple-pass development technique employing two two-component solvents. The first solvent [cyclohexane–ethyl acetate (1:1 v/v)] was allowed to pass twice up the silica gel layer to a height of 15 cm (columns 1 and 2). The second solvent [methanol–chloroform (30:70)] was then allowed to run up to R_F 0.5 to separate the large number of closely associated spots proximal to the origin (column 3). Two dotted spots one on either side of R_F 0.2 were very faint and were not observed after the second run with the first solvent. Spot colors: horizontal lines, pink; vertical lines, yellow; dotted, blue; clear, white; solid, black.

Visualization techniques

Three visualization techniques were tried to see if any improvement could be found over the use of U.V. light: NYBOM's[8] aluminum chloride spray technique, *p*-nitroaniline spray and iodine crystals. The aluminum chloride spray was used by NYBOM to intensify the colors of the spots and render them more permanent when viewed under U.V. light. Under the present conditions it made very little difference to the color intensity and so was not generally used. The *p*-nitroaniline spray was not sufficiently sensitive to react with the small quantities present in the separations. Iodine crystals were used by letting the vapor from the crystals come into contact with a developed chromatoplate in a closed container for half an hour. The spots took on a dark coloration, but since all spots were colored the same it was not possible to identify as many spots as under U.V. light. The method, though not as sensitive as U.V. light, would be useful for marking the positions of separated spots.

Variability in Lotus extracts

The *Lotus* extracts were tested for possible sources of variability using the standard separation technique described above.

Dried and fresh material

Extracts were made of fresh material, and of dried material which varied from one month to eighty years old. The results showed that perfectly good separations could be made from dried material even up to eighty years old. There appeared to be no difference between dried and fresh material except that the concentrations of the chief pink spots were always higher in the dried material. In a further experiment two identical samples of fresh leaves were weighed, one set being sampled immediately and the other set being pressed and dried for one month before being sampled. The two chromatographic separations were identical except for the pink spots, which were present in higher concentrations in the dried sample. Since the samples were the same weight and were taken from the same position on a single *Lotus* plant, this difference must have been caused in some way by the drying.

The effect of light and dark

To test whether the phenolic content of fresh samples varied with the time of day at which they were taken, two *Lotus* species, *L. purshianus* and *L. pedunculatus*, maintained in a growth chamber under growing conditions of 21° and 70–80% relative humidity, were sampled after (1) a dark period of 16 hours and (2) a light period of 3 hours following the dark period. The separations of *L. purshianus* and *L. pedunculatus* under both conditions were identical as to the number of spots. In the case of the latter species, four spots appeared darker on the plates after the dark period. From these results it was assumed that the time of sampling made no difference to the content of the samples with the exception of certain of the U.V. absorbing spots which decreased in concentration in the light.

The position of the leaves on the plant

One plant of *L. oblongifolius* var. *nevadensis* (B-157) was used. Samples of young leaves were taken from the growing point and immediately behind it, and old leaves from the base of the stem. No differences were found between the two samples.

B. Cellulose

Layers were prepared by weighing out 14 g cellulose MN 300 (Macherey, Nagel & Co., Düren, Germany), adding 90 ml distilled water and shaking the mixture in a Waring Blendor set at "high" for 90 sec. The coated plates were air-dried overnight, then stored in a desiccator. To eliminate the fibrous surface 14 g of cellulose were used instead of the recommended 15 g. Cellulose was easier to use than silica gel. The cellulose layer adhered to the glass more firmly and the surface was hard enough for the extracts to be applied without the danger of making a hole in the layer with the pipette. The surface was also hard enough to write on, facilitating labelling the layers.

Solvents

The following solvents were tried: F.W 2:100, nAm.A.W 20:12:10, Forestal, A.W 15:85, W.HCl.F 8:4:1, B.A.W 6:1:2, B.A.W 4:1:5, B.HCl.W 15:3:6, T.A.W

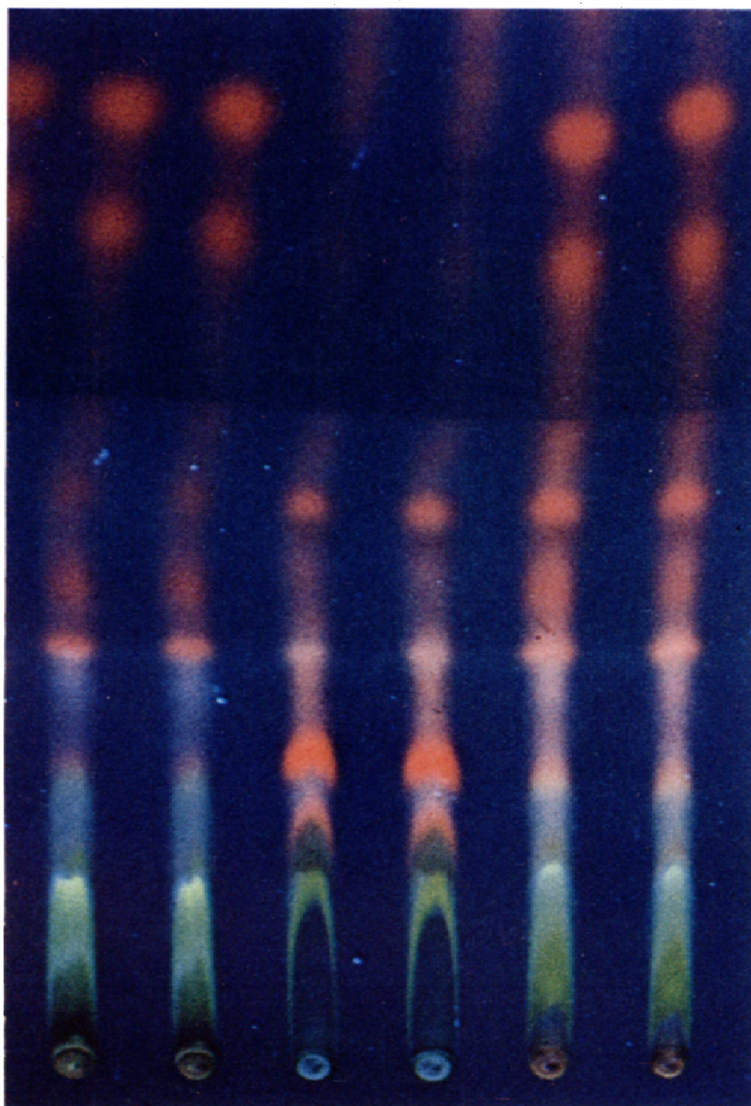

Fig. 2. Photograph of chromatograms of *Lotus* species. Extracts are run in pairs from one plant. From left to right, Pair 1, *Lotus purshianus*; Pair 2, unidentified species; Pair 3, *L. micranthus*. Dark line across plate represents R_F 0.5.

4:1:5. Of these, only F.W 2:100, Forestal and A.W 15:85 were of any use and though they gave a reasonable one-dimensional separation, there was only about 25% of the number of spots obtained in comparison with those obtained on silica and these showed some tailing. In both nAm.A.W 20:12:10 and W.HCl.F 8:4:1 all the spots moved too near the solvent front, and in all the butanolic solvents and T.A.W 4:1:5 there was excessive tailing and very little spot definition. NYBOM's[8] two-dimensional method for phenolic "finger-printing" was tried, using F.W 2:98 followed by nAm.A.W 20:12:10 and the results of an extract of *Rubus* (with which NYBOM was working) were compared with those of *Lotus*. Although the separation of spots was good for *Rubus*, in *Lotus* the concentration of spots in certain regions made this method unsatisfactory. Other combinations of solvents were tried two-dimensionally, including the three most suitable solvents already mentioned, but in all cases there was insufficient spot differentiation.

C. Polyamide

Layers were prepared by weighing out 12 g MN-polyamide (Macherey, Nagel & Co., Düren, Germany) into a conical flask, adding 60 ml methanol and shaking the mixture well. The slurry was very "runny" and was difficult to apply evenly to the plates. When the slurry was passed into the spreader, it was found necessary to provide a stopper of some sort, such as a wad of absorbent paper, to prevent the slurry leaking out before the spreader was moved. The plates were air-dried and then stored in a desiccator.

Solvents

The following solvents were tried: C, Ch, EA, M, E, Ac, W, C.E 1:1, F.W 2:98, W.E.Bu.A.acet 13:3:3:1. The solvents C, EA, C.E 1:1 were not satisfactory. Ch gave a good separation up to R_F 0.5, but above this value there was marked spot tailing. M.E and Ac gave reasonable separations. M was the best solvent but all solvents produced some spots with marked tailing. Neither W nor F.W 2:98 would move up the layer at all—water is obviously sufficiently immiscible with polyamide to prevent any solvent containing a high proportion of water from moving up the layer.

The solvent W.F.Bu.A.acet 13:3:3:1 was tried since EGGER[9] in an analysis of a number of glycosides had used this solvent to separate unhydrolysed phenolics according to the type of attached sugar. From a glycoside mixture he found that the three types of sugar residues, namely, 3,7-diglycosides, 3-biosides and 3-monosides, separated into spots at three distinct R_F regions at 0.58, 0.3 and 0.2 respectively. In an analysis of *Lotus* extracts after development in this solvent, two large spots were found at R_F 0.8 and 0.6 to 0.75, and three smaller spots at R_F 0.25, 0.15 and 0.08. There were no spots corresponding to the 3,7-diglycoside position. Since in impure solutions the R_F value for a given compound is often lowered, the two spots at R_F 0.25 and 0.15 are most likely phenolics with bioside and monoside sugar residues respectively. Since none of the spots from the *Lotus* extracts were analysed in the present study, no further identification of the compounds separated by this solvent was attempted.

DISCUSSION

The one-dimensional technique with silica gel layers was the only one which was suitable for the separation of the unhydrolysed phenolic compounds in *Lotus*. Both

the cellulose and the polyamide layers were unsatisfactory with the solvents which were tried, and no satisfactory two-dimensional separations were achieved. The results with cellulose and polyamide were disappointing since other workers[8,10] had found these layers suitable for phenolic separations. The described technique on silica gel layers gave good separations. The technique would be more valuable if used in conjunction with chemical identifications of the compounds involved, so that a given spot could be identified with certainty from one species to another.

It is hoped that the results described above may be of use to others interested in the separation of unhydrolysed plant phenolics on thin layers. The taxonomic implications of the phenolic constituents in North American species of *Lotus* as determined by the thin-layer one-dimensional multiple-pass development technique described in this paper will be presented elsewhere.

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SUMMARY

The separation of secondary phenolics in *Lotus* by means of thin-layer chromatography was investigated using silica gel G, cellulose, and polyamide as coating materials with a number of different solvents. Cellulose and polyamide layers were found unsatisfactory with the solvents used. Likewise, two-dimensional separations were found unsatisfactory on all three coating layers with the solvents used. The best separation of secondary phenolics in *Lotus* extracts was found to be a one-dimensional ascending multiple-pass development technique employing two two-component solvents on silica gel G. Cyclohexane–ethyl acetate (1:1 v/v) was used as the first solvent and allowed to pass twice up the layer to a height of 15 cm. A second solvent, methanol–chloroform (30:70) was then allowed to run up only to R_F 0.5. The plates were dried between developments. The former solvent separated the spots distal to the origin whereas the latter solvent gave excellent separation of the large number of closely associated spots proximal to the origin.

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