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Thin-layer chromatographic separation of flavonoids in *Medicago* (Papilionaceae)

Biochemical systematics which has developed very rapidly within the last decade, has revealed the significance of flavonoids as favored taxonomic markers among many secondary constituents of plants. Significant correlations between flavonoid distribution patterns and morphological features of various plant species, have been reported in literature[1] and therefore the present study on the distribution of flavonoids in different species of *Medicago* (Papilionaceae), was taken up. Although paper chromatography has been successfully used for the separation of flavonoids, thin layer chromatography (TLC), due to its greater sensitivity and shorter developing time, has recently been widely used in the separation of anthocyanins[2], flavonoids[3] and secondary phenolic compounds[4,5]. In the present communication, a simple, rapid and a very sensitive technique for separation of the flavonoids of *Medicago* species, by means of commercially produced Silica Gel and Cellulose Eastman Chromagram Sheets, is briefly discussed.

Materials and methods

Petals from fifty flowers of perennial and annual species of *Medicago*, weighing approximately 0.05 g, were dried at room temperature (70–80°F). The dried petals were ground in 1 ml petroleum ether (boiling range 37.8°–58.2°) and thus the carote-

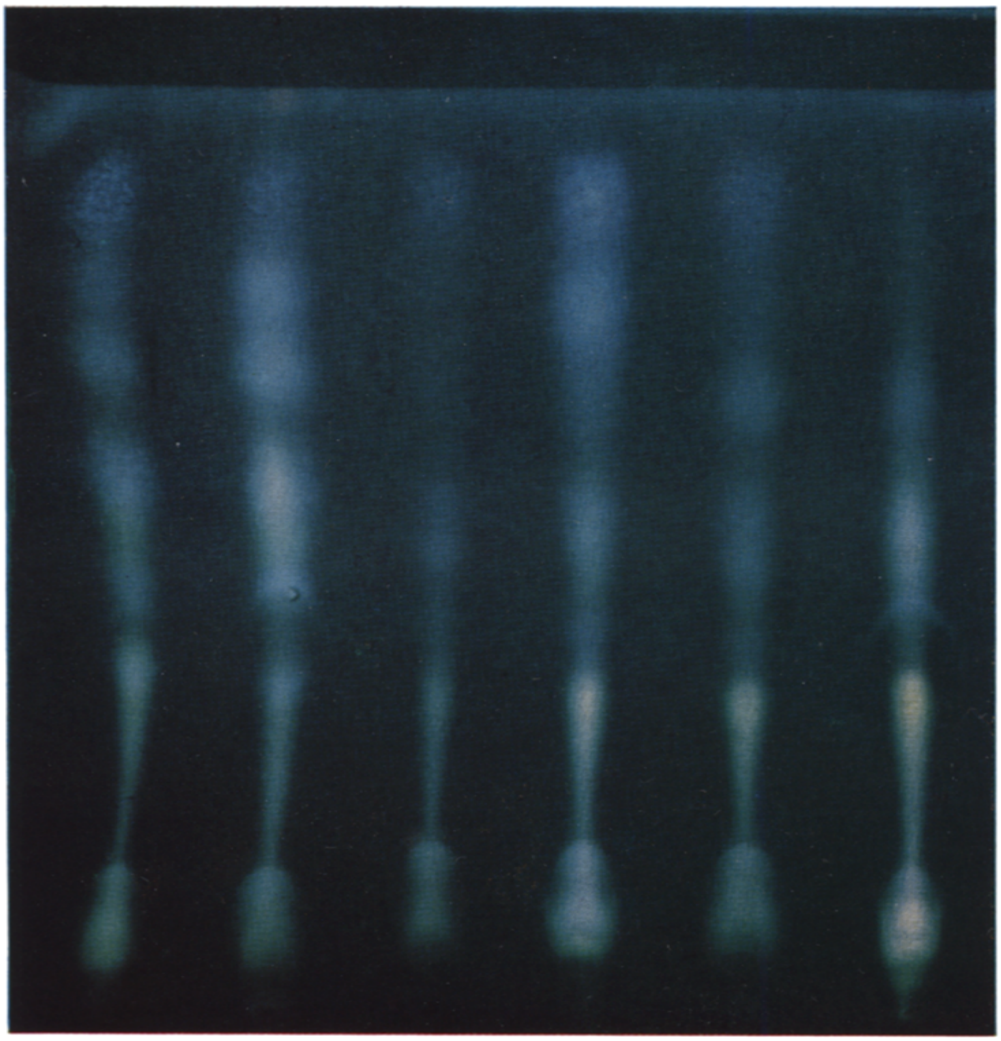

Fig. 1. Chromatogram of flavonoids from flower petals of five different accessions of *Medicago murex* (from left to right 1–5 spots) and *Medicago constricta* (left to right spot 6), developed with *n*-amyl alcohol–acetic acid–water (2:1:1) and sprayed with 1% sodium borohydride in isopropanol (day light Agfachrome).

noids were first removed by centrifugation. If the petals showed presence of more yellow carotenoids, washing with petroleum ether was repeated. The residue was then dried and 0.5 ml of 0.1 *N* HCl was added to the samples which were thoroughly stirred by a glass rod and left inside a refrigerator over night. Next day, samples were centrifuged and a pale yellow supernatant containing flavonoids was collected in narrow vials.

A. Chromatographic procedure

Precoated Silica Gel Eastman Chromagram sheet type 6061, 6065 and Cellulose Eastman Chromagram sheet 6064 (Eastman organic chemicals, Distillation Products Industries, Rochester, New York) were tested for thin layer chromatographic separation of unhydrolysed flavonoids. Various developing solvent mixtures were kept inside capped reagent bottles and the atmosphere inside the bottles was made saturated by frequent shaking. Then a 4 cm wide strip of the chromagram sheet having two sample spots, was left inside for developing and the bottle was capped. In this way a preliminary screening of useful solvents was carried out and finally a 20 cm × 20 cm sheet was spotted with 15 μl of sample extract at a distance of 2 cm from the base as well as from each other. The Eastman Chromagram Developing Apparatus caused non-uniform movement of different spots due to irregular movement of the solvent front. Therefore the chromagram sheets were developed (one dimensional, ascending) inside the Desaga Chromagraphy tank, lined with filter paper for maintaining a saturated atmosphere of the solvent inside. The solvent was added at least 2 h before the sheets were developed and the solvent front was allowed to move up to a height of 15 cm.

B. Solvents

A variety of solvents which have been reported to be suitable for a paper chromatographic separation of flavonoids[1,6,7], were chosen. The following developing solvents were tested and are referred to through out the text. (1) *n*-Butanol–acetic acid–water (4:1:5 v/v, upper phase; (2) *n*-butanol–2 *N* HCl (1:1), upper phase; (3) *n*-propanol–acetic acid–water (1:1:1); (4) toluene–ethylformate–formic acid (5:4:1); (5) ethyl acetate–methyl ethyl ketone–formic acid–water (5:3:1:1); (6) *n*- or isoamyl alcohol–acetic acid–water (2:1:1); (7) water–HCl–formic acid (8:4:1); (8) ethyl acetate–formic acid–water (8:2:3) upper phase; (9) formic acid–HCl–water (5:2:3); (10) water–acetic acid–HCl (42:8:3); (11) 5 % formic acid; (12) 5 % phosphoric acid.

Visualisation techniques. After development, the sheets were dried and examined in visible light, long wave ultra violet (U.V.) light (350 mμ) and short wave U.V. light (254 mμ) by means of universal U.V. lamp Camag type TL900. They were examined in U.V. light after exposing the chromatograms to various chromogenic sprays namely 5 % ethanolic aluminium chloride, 5 % aqueous sodium carbonate, 1 % sodium borohydride in isopropanol and 1 % methanolic tetraphenyl boroxide ethanolamine complex (K & K Laboratories Inc., Plain View, New York). Chromatograms were also examined after fuming with ammonia. The spots were directly recorded on the sheet by a soft pencil. Color slides, with Kodak High Speed Ektachrome film exposing for 20 min/f 5.6 at a distance of 50 cm from the sheet in 3660 Å long wave U.V. light (ultra violet lamp chromatovue, Model C-3, Ultra violet Products Inc. San Gabriel, California) or with Daylight Agfachrome exposing for 8 min under the same conditions, were prepared. A Kodak Wratten 2B filter was used.

Experimental

Silica Gel Eastman Chromagram Sheet type 6061 and 6065

All the developing solvents which are mentioned before, were tested and only the two solvents namely upper phase of *n*-butanol–2 *N* HCl (1:1 v/v) and isoamyl alcohol–acetic acid–water (2:1:1), were found suitable for the separation of flavonoids.

Although all the chromogenic sprays described earlier were found satisfactory, 1% sodium borohydride in isopropanol was preferably used because the spots showed less diffusion in isopropanol.

Cellulose Eastman Chromagram Sheet 6064

Twelve different solvents whose reference has been made earlier were tested. Only three solvents *n*-butanol–2 *N* HCl (1:1 v/v), *n*-amyl alcohol–acetic acid–water (2:1:1 v/v) and ethylacetate–formic acid–water (8:2:3), were found suitable. One dimensional ascending chromatograms were developed and sprayed with the chromogenic sprays.

Discussion and conclusion

Both Silica Gel type 6061, 6065 and Cellulose type 6064 Eastman Chromagram sheets were found suitable for one dimensional ascending separation of unhydrolysed flavonoids of *Medicago* species. A micro amount of sample could easily be analysed in 1–2 h by the above technique while paper chromatographic separation took 12–14 h. Spraying with the chromogenic agents was found essential for good visibility and color photography.

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