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Note

Reversed-phase thin-layer chromatography of chloroplast pigments on chemically bonded C_{18} plates

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As traditionally performed, reversed-phase thin-layer chromatography (TLC) is a laborious, inconvenient, and messy separation procedure, which has been mostly neglected. Silica gel, kieselguhr, or cellulose layers must be impregnated with an oil or wax, and the mobile phase saturated with the stationary phase so it is not stripped from the layer during development. When used for preparative separations, the impregnation agent could be eluted during recovery of the separated compounds.

Chemically bonded C_{18} silica gel plates for reversed-phase TLC have recently become commercially available. These are efficient layers of high capacity, which would seem ideal for simplified analytical and preparative separations. Since adsorptive sites are chemically bonded to the stationary phase, the chromatographic systems should be mild for labile compounds.

In this article the preliminary evaluation of C_{18} layers for plant pigment separations is reported. Classical reversed-phase procedures have been applied to pigment separations using oil-impregnated paper[1] and kieselguhr[2], but these systems are usually rejected[3] because of the problems stated above. Although some separations on impregnated kieselguhr have yielded unaltered pigments[2], other kieselguhr systems have caused pigment alteration[2,4]. The chlorophylls and carotenoids from plants were good compounds to test the new layers because of their recognized lability[5].

EXPERIMENTAL

Pigments were extracted from fresh spinach by acetone in a blender using previously[1] detailed conditions known to cause no alterations. The extract was applied to 20 × 20 cm Nano/Gram C_{18} layers as received from Quantum Industries (Fairfield, N.J., U.S.A.) without pre-treatment, using wooden applicator sticks. Approximately 5 μl from the final extract solution of 1 ml, containing essentially all the pigments in 2 g of spinach, was applied.

Development was carried out in rectangular glass TLC tanks lined with filter paper and equilibrated with solvent for 15 min. After development for a distance of 10 cm past the origin, the layer was air dried in the dark and held over HCl vapors if confirmation of the location of neoxanthin (turns from yellow to blue-green) and violaxanthin (yellow to blue) was required.

RESULTS AND DISCUSSION

Development of the pigment mixture with methanol–acetone–water (20:4:3) provided the best overall separation which could be achieved (Fig. 1). The sequence of the pigments was indeed reversed compared to separations on untreated silica gel, alumina, or cellulose, but was the same as the order on chromatography paper or thin layers impregnated with Wesson Oil to prepare a reversed-phase medium[1]. The hR_F values were: N 56, V 50, L 32, b 14, a 5, c 0 (for abbreviations, see the legend of Fig. 1). A very similar separation and the same sequence was obtained with methanol–water (97:3) as solvent.

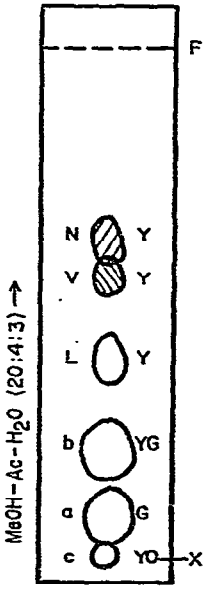

Fig. 1. Pigments in 5 μl of leaf extract separated by development on Quantum C_{18} reversed-phase layers. N = Neoxanthin; V = violaxanthin; L = lutein; b = chlorophyll *b*; a = chlorophyll *a*; c = carotene; F = solvent front; X = origin; Y = yellow; O = orange; G = green; /// = blue-green over HCl vapors; \\\ = blue over HCl vapors; MeOH = methanol; Ac = acetone. The elution time was 32 min.

The sequence on the C_{18} layer was, however, different than that obtained by high-performance column liquid chromatography[6], whereby lutein was eluted between chlorophylls *a* and *b* using methanol–water (98:2) as solvent and a Waters Bondapak C_{18}–Porasil B column (37–75 μm). This discrepancy may be due to different partitioning of the solvent on the coarse, more porous reversed-phase column packing compared to the microparticulate reversed-phase layer. The separation on a microporous C_{18} column might well be comparable to the TLC results.

The resolution of the two upper and two bottom zones is seen to be not complete in Fig. 1. The application of a lower initial zone load would improve these separations; the use of a different solvent can emphasize these resolutions at the expense of the overall separation.

For example, neoxanthin (hR_F 23) and violaxanthin (hR_F 18) were separated completely from each other and from lutein (hR_F 9) and the other pigments by devel-

opment for 35 min with methanol–acetone–water (2:2:1). Carotene and chlorophyll *a* were well resolved with butanol–methanol (8:7) (Fig. 2). The hR_F values were: N + V 82, L 73, b 62, a 51, c 36. Figs. 1 and 2 emphasize the rule that a more non-polar solvent increases migration distances in reversed-phase systems.

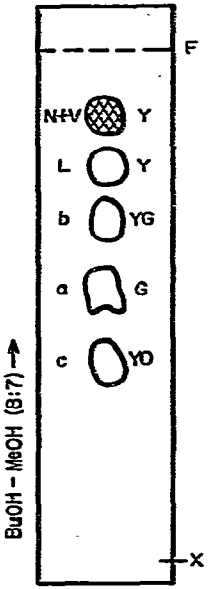

Fig. 2. Same as Fig. 1 except development with a different solvent system. BuOH = *n*-butanol. The elution time was 30 min.

A streak of extract was applied with a pipet guided by a ruler across a C_{18} layer and developed with methanol–acetone–water (2:2:1) to achieve maximum separation of violaxanthin from neoxanthin and lutein. The neoxanthin band was scraped off, collected in a small beaker, and the pigment eluted with methanol. The visible absorption spectrum had maxima at 440 and 470 nm and an overall shape that matched unaltered neoxanthin[5]. Thus, the C_{18} layer proved to be a mild medium which did not alter the most labile carotenoid pigment in the leaf-extract mixture. In no separations were zones of chlorophylls *a'* or *b'* or pheophytins observed, indicating there was no alteration of chlorophylls as well.

Further work is under way with other classes of compounds to optimize separation conditions and detections and to achieve quantitation by densitometry on these reversed-phase layers, which have proven to be a selective and mild medium for resolution of chloroplast pigments.

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