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Chromatography of leaf pigments on silica gel and aluminum hydroxide loaded papers

Earlier papers in this series compared the separation of fat-soluble chloroplast pigments from leaves, algae and photosynthetic bacteria on cellulose paper[1], ion-exchange papers[2], pre-formed cellulose thin layers[3,4] and silica[5,6], magnesia[7], alumina[6,7], saccharide[7], polyamide[6] and kieselguhr[8] thin layers. Reports of synergistic effects on media composed of cellulose plus an active adsorbent (*e.g.*, CHANG AND CHAN's study of the separation of organic acids on silica gel–cellulose layers[9]) plus the desire to compare further thin-layer and paper chromatography, prompted the present extension of our work on pigment separations to papers loaded with silica gel and aluminum hydroxide.

Experimental

Whatman SG-81 chromatography paper loaded with 22% SiO_2 and AH-81 paper loaded with 7.5% Al_2O_3 were used as received from H. Reeve Angel Co., Clifton, N. J. Occasionally the papers were activated by heating in a chromatography oven for 1 h at 110°.

Ascending development was carried out as previously described in Unikit tanks (Shandon Co.) lined with Whatman No. 1 paper and wrapped in aluminum foil. Sheets of paper, 20 × 20 cm, were spotted 1 in. from the bottom with pigment, air-dried, formed into a cylinder, fastened with tongued clips, and developed in a tank which had been equilibrated with 100 ml of wash liquid for at least 15 min. After the solvent had risen 15 cm past the origin in the machine direction, the paper was removed and the zones observed.

Separated zones were detected and identified by visual observation and by color reactions with hydrochloric acid. Confirmation of identity was obtained by eluting the pigments and determining their spectral absorption properties with a Beckman DK-2A recording spectrophotometer, by the chromatographic sequence of the zones, and by comparison with authentic pigments.

Leaf extracts were prepared by disintegrating 2.0 g of fresh spinach in a blender with acetone, centrifuging and salting the pigments into petroleum ether (20–40°). The solvent was evaporated and the residue dissolved in 1 ml of petroleum ether (60–110°) to prepare the final extract sample. Alternatively, the green acetone extract was saponified with methanolic potassium hydroxide before the pigments were transferred to ether–petroleum ether (20–40°) (1:1). The solvent was then evaporated and the residue dissolved in 1 ml of ether–petroleum ether (60–110°) (1:1)[10]. This procedure removed the green pigments and some colorless material so that the separation of the carotene and xanthophylls could be studied.

The wash liquids tested were those found earlier to be most effective in the separation of leaf extracts and saponified leaf extracts on cellulose papers and layers and silica gel and alumina layers. Their compositions are expressed in volume percentages. Low-boiling (20–40°) petroleum ether was used to prepare the wash liquids containing that component.

Results

Separation of pigments in leaf extract. The best solvent found for the separation of the six major leaf pigments on silica gel paper was petroleum ether–acetone (7:3). On unheated paper the separation of the pigments with this solvent was complete at the lowest loading (2 μl of final extract) except for the chlorophyll zones which were contiguous. R_F values were as follows: carotene 0.98, chlorophyll *a* 0.95, chlorophyll *b* 0.91, lutein 0.84, violaxanthin 0.68, and neoxanthin 0.35. A faint trace of green pigment was evident at the origin after the run, indicating a slight amount of chlorophyll decomposition in this system. Improvement of this separation was not obtained by using petroleum ether–acetone (8:2) because with this less-polar solvent the chlorophylls were badly streaked, extending back to the origin through the xanthophyll zones. Complete separation was obtained, however, with petroleum ether–acetone (7:3) on heated silica gel paper with the following R_F values: carotene 0.98, chlorophyll *a* 0.91, chlorophyll *b* 0.85, lutein 0.75, violaxanthin 0.57, and neoxanthin 0.31. Heating caused an increase in adsorption (lower R_F values) and selectivity, but also slightly increased the amount of green pigment remaining at the origin.

Petroleum ether–acetone (7:3) yielded badly streaked chlorophyll zones and a heavy green residue at the origin on aluminum hydroxide paper, and similar results were obtained for all other solvents tested on both papers. In all cases carotene was separated from the pigment mixture, but the other carotenoids were mixed with chlorophyll.

Separations of pigments in saponified extract. Various solvents provided complete separations of the four leaf carotenoids at lowest loadings (1–5 μl of final extract) on both papers in the sequence carotene (least sorbed), lutein, violaxanthin, and neoxanthin (most sorbed). When exposed to vapors of concentrated HCl, the violaxanthin zone becomes blue and the neoxanthin zone blue-green. The most compact zones were obtained with isooctane–acetone–diethyl ether (3:1:1) on both papers, the respective R_F values being 0.76, 0.38, 0.27, and 0.13 on silica gel paper and 0.80, 0.47, 0.33, and 0.13 on aluminum hydroxide paper. These values, and those obtained with petroleum ether–acetone (7:3), which also completely separates the carotenoids on both papers, indicate that the strength of adsorption of these papers for the carotenoids is quite similar. Heating the aluminum hydroxide paper had little effect on the separation obtained with this latter solvent.

Carotenoids separated on both papers with isooctane–acetone–ether (3:1:1) were eluted immediately with ethanol and their spectra determined in this solvent. Elution of the pigments was easily obtained and seemed to be complete by visual inspection. When compared with spectra[10] of authentic carotenoids, no rearrangement was evident.

Discussion

The complete one-dimensional separation of the six major leaf pigments is not possible on pure cellulose paper or layers. The best separations on these media are obtained with petroleum ether–benzene–chloroform–acetone–isopropanol [50:35:10:0.5 (paper) or 5.0 (layer):0.17], which separates all pigments except chlorophyll *b* and neoxanthin[3]. The complete one-dimensional separation of leaf pigments is possible on silica gel paper with petroleum ether–acetone (7:3) as described above, as it is on silica gel layers with this same solvent and with isooctane–acetone–ether (3:1:1)[6].

The separation of the four carotenoids from saponified extract can be carried out on pure cellulose paper and layers with the five-component solvent defined above, but it is also obtained with more compact zones on silica gel and alumininum hydroxyde paper as described above with much simpler solvents of the type which give complete separations on silica and alumina layers[6]. It is not surprising that solvents of the type required for successful work on silica and alumina layers rather than those necessary for the more weakly adsorbent pure cellulose media were found to be best for sorbents composed of mixtures of cellulose and these stronger adsorbents. The double-tailing chlorophyll zones characteristic of pure cellulose paper and thin-layer systems[3] were not formed on loaded papers.

Chromatography on loaded papers is more convenient than with home-made thin layers and much less expensive than with commercially precoated layers on plastic, glass or aluminum backing. Development times for the systems tested in this study ranged from 40–65 min, not significantly different from the times required to develop thin layers with the same solvents[6]. Elution of the pigments from the loaded papers was no problem, and no carotenoid rearrangements were noted. Aluminum hydroxide paper, like alumina and kieselguhr layers, caused alteration and streaking of the chlorophylls. The same effect, but to a smaller degree, was found on silica gel paper, but not on most silica thin layers studied earlier[6]. No significant amounts of chlorophylls *a'* or *b'* or pheophytins were formed during chromatography on the loaded papers.

Chemistry Department, Lafayette College, Easton, Pa. 18042 (U.S.A.) JOSEPH SHERMA

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