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Chromatography of fat-soluble chloroplast pigments on preformed kieselguhr layers

In an earlier report[1], separations of chloroplast pigments from leaves and algae by chromatography on preformed flexible thin layers of cellulose, silica gel, alumina and polyamide were described. Precoated kieselguhr TLC sheets have very recently become available, and these are now evaluated for the separation of pigments extracted from leaves, algae and photosynthetic bacteria.

Experimental

Precoated Kieselguhr F_{254} TLC sheets with a 0.25-mm layer on aluminum backing are manufactured by E. Merck AG, Darmstadt, G.F.R. and distributed by Brinkmann Instruments, Inc., Westbury, N.Y., U.S.A. For adsorption chromatography, the sheets are dried for 15 min at 105° and cooled in air just before spotting. For reversed-phase partition chromatography, the sheets are soaked in a 7% (v/v) solution of Wesson Oil (a mixture of cottonseed plus soybean oils; Hunt-Wesson Foods, Inc., Fullerton, Calif., U.S.A.) in petroleum ether (60–110°), air dried a few minutes, and then oven dried at 75° for 30 min. After cooling, the sheet is spotted and developed with methanol–acetone–water (20:4:3) which has been equilibrated with the oil in a separatory funnel.

Ascending development for a distance of 15 cm was carried out by standing the sheets in the solvent held in the bottom of paper-lined, saturated, rectangular chambers covered with aluminum foil to retard the photo-decomposition of the pigments. Development times ranged from about 20 min for some solvents with unimpregnated sheets to about 50 min for the reversed-phase system.

Identification of the separated pigments was based on their colors, chromatographic sequence, absorption spectra in the visible region, and reaction to the vapors of concentrated HCl.

Pigments were extracted from spinach leaves by a method employing a blender as previously described[2]. The method used to extract pigments from *Chlorella pyrenoidosa* suspension has also been described[3]. For the extraction of the bacteria, this procedure was modified by using methanol–diethyl ether–petroleum ether (5:2:1) as the extraction solvent and dissolving the final residue in a small volume of diethyl ether–acetone (1:1) (for Chlorobium) and diethyl ether–petroleum ether (1:1) for Rhodospirillum. The treatment with boiling water was not required to increase the extractability of the pigments from Rhodospirillum.

Results and discussion

Separations by adsorption chromatography. Despite reports by other workers of successful separations of the pigments in extracts of leaves[4], algae[5] and bacteria[6], no solvent system was found in the present study which yielded a good separation of the chlorophylls and carotenoids from spinach or *Chlorella pyrenoidosa.* (These unsuccessful results were also obtained earlier[2,7,8] on home-made layers of Kieselguhr G.) All solvents employed by other workers were tested, plus many modifications which had proven sucessful with other types of preformed adsorbent layers[1]. In no case were

chlorophylls *a* and *b* cleanly separated, and streaked chlorophyll and carotenoid zones and extra green zones were usually obtained.

Many different solvents were tested for the separation of the four carotenoids in saponified[2] spinach and Chlorella extract, but only petroleum ether (20–40°) plus 10% acetone gave a complete separation, and then only at low loading (1–2 μl of saponified extract). The sequence of the separated zones was carotene (R_F 1.0), lutein (0.82), violaxanthin (0.68) and neoxanthin (0.39). The separated pigments were immediately cut from the sheet, eluted with ethanol, and their absorption spectra recorded in this solvent. The shapes and maxima of the spectra[2,8] indicated no rearrangement of the carotenoids. If the chromatogram is exposed to the vapors of HCl, neoxanthin and violaxanthin turn bluish green while carotene and lutein remain yellow.

With petroleum ether plus 1% *n*-propanol as the solvent, the major portions of the four carotenoids are well separated, but the lutein and violaxanthin zones have faint streaking tails.

Separations by partition chromatography. Development of spinach or Chlorella extract on Wesson Oil-impregnated kieselguhr sheets with methanol–acetone–water (20:4:3) resulted in the complete separation of the six major zones at loadings from 1–10 μl of extract: carotene (R_F 0.0), chlorophyll *a* (0.067), chlorophyll *b* (0.20), lutein (0.53), violaxanthin (0.80), neoxanthin (0.93). Visible absorption spectra of the eluted zones matched those reported for the pure pigments. Saponified extracts were also completely separated in this system.

If Wesson Oil is replaced with olive oil and oil-saturated methanol–acetone (20:1) is used as the developing solvent, R_F values are higher and the separation, although complete at the lowest loadings, is not so good.

The pigments of cultures of the green sulfur bacteria *Chlorobium thiosulfatophilum* and *Chlorobium limicola* yielded reversed-phase chromatograms with major spots at R_F 0.0 (orange) and R_F 0.35 (green). These spots were eluted with diethyl ether and their spectra identified the pigments as chlorobactene (491, 461, 435 nm)[9] and Chlorobium chlorophyll 660 (660, 431 nm)[10]. In addition to these, the following minor spots were noted: red-orange and green zones just above chlorobactene and a purple-grey zone at R_F 0.90 in the chromatograms of both organisms, and a blue zone, which was undoubtedly bacteriochlorophyll (see below), just below Chlorobium chlorophyll in *Chl. limicola* only.

The pigments of a culture of the non-sulfur purple bacterium *Rhodospirillum rubrum* separated on Wesson Oil-impregnated kieselguhr yielded a chromatogram with only two spots: spirilloxanthin (reddish pink, R_F 0.0; 528, 493, 463 nm[11], in ether) and bacteriochlorophyll (blue, R_F 0.30; 770, 576, 391, 358 nm[8], in ether). There was absolutely no indication of the presence of any other pigments as separated by STRAIN AND SVEC[12] from other cultures of *R. rubrum* in columns of powdered sugar. To check this, our extract was developed on a Baker preformed silica gel thin layer with petroleum ether plus 30% acetone and on unimpregnated kieselguhr with petroleum ether plus 10% acetone. In each case only two zones were resolved; spirilloxanthin streaked in both systems and bacteriochlorophyll formed a zone with double tails on the unimpregnated kieselguhr.

Attempts to reproduce the reversed-phase partition separations of EGGER[13] were unsuccessful, as they had been[7] on home-made layers of Kieselguhr G.

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