SEPARATION OF CHLOROPLAST LEAF PIGMENTS BY THIN-LAYER CHROMATOGRAPHY ON CELLULOSE SHEETS*

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One-dimensional paper chromatography is not especially effective for the separation of the chloroplast pigments. With many different wash liquids, pigments from leaf extracts are poorly separated and yield double-tailing zones[1]. Carotenoid pigments in saponified extracts are also incompletely separated by many wash liquids[1,2]. Since cellulose is a very mild adsorbent, systems involving cellulose are useful for the separation and recovery of the chloroplast pigments. Cellulose will not isomerize neoxanthin and violaxanthin as do many siliceous adsorbents[3], nor will it alter the chlorophylls as do siliceous adsorbents as well as alumina, lime and magnesia[3,4].

Because of these important advantages, we decided to evaluate Cellulose Chromagram sheets, which have recently become available, for pigment separations. These sheets contain very pure non-fibrous cellulose in a 160 μ layer on a poly-(ethylene terephthalate) backing without binder. They are thin-layer media which can be used with paper-chromatographic techniques.

Separations of leaf pigments on these sheets by one-dimensional as well as two-dimensional and radial procedures will be compared with those obtained in systems including cellulose paper, conventional cellulose thin layers on glass plates and cellulose columns.

EXPERIMENTAL

Cellulose Chromagram Sheets, 20 × 20 cm, without fluorescent indicator, were obtained from Distillation Products Industries, Rochester, N. Y.

One-dimensional ascending development was carried out as previously described[5] in rectangular TLC tanks (Warner-Chilcott Co.) lined with filter paper and wrapped in aluminum foil to protect the pigments from light. The pigment extract (see below for details) was spotted with micropipettes 1 in. from the bottom of the sheet, the sheet was air-dried and placed on an A-frame and into the tank which had been equilibrated with 250 ml of wash liquid. After the wash liquid had risen 15 cm past the origin, the sheet was removed and the zones were visually observed. Some paper chromatograms were also run in the same way on 20 × 20 cm sheets of Whatman No. 1 filter paper.

Two-dimensional chromatography was performed as above except that the

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initial zone was placed in one corner of the sheet and two solvents were each allowed to migrate 15 cm in transverse directions, with 5 min drying between runs.

Radial chromatography was carried out horizontally in a large covered jar on sheets cut in the shape and dimensions as already described[6].

Cellulose layers of 0.25 mm on 20 × 20 cm glass plates were prepared from Camag Cellulose powder for TLC with Desaga Brinkmann spreading apparatus according to instructions provided from the manufacturer. The layers were air-dried overnight, heated 15 min at 110° and cooled 10 min before use. The plates were developed as above.

Columns were prepared in chromatographic tubes of 1 cm I.D. × 25 cm. The adsorbent was dry-packed in small portions to a height of 20 cm. The sample (200–400 μl of leaf extract) from a micropipette was washed into the dry column with a few 1.0 ml portions of wash liquid before development was begun. Development was stopped when the front reached the bottom of the column.

Typical development times were 30–45 min for cellulose sheets and Whatman No. 1 paper, 15–30 min for layers on glass plates and 90–100 min for columns (without suction).

Separated zones were detected by visual observation and by color reactions with HCl. When developed sheets were exposed to the vapors of concentrated HCl, carotene and lutein remained yellow, violaxanthin turned blue and neoxanthin became blue-green. The pigments eluted from separated zones on sheets or in columns were identified by their spectral absorption properties determined with a Cary 14 recording spectrophotometer.

Leaf extracts were prepared by disintegrating 2.0 g of fresh spinach in a Waring Blendor with acetone. The mixture was centrifuged, and the pigments were transferred to petroleum ether (b.p. 60–110°) by adding this solvent and aqueous NaCl solution. The solvent was evaporated and the residue dissolved in 1 ml petroleum ether (b.p. 60–110°). For isolation of the carotenoid pigments free of chlorophylls and fatty substances, the green acetone extract was saponified with methanolic KOH before transferring the pigments to diethyl ether–petroleum ether (b.p. 60–110°) (1:1,v/v). In each case, one μl of test solution contained the pigments from 2 mg of fresh leaf material. Details of these methods have already been published[1,3,4].

High-quality organic solvents were used as wash liquids for the development of the chromatograms. The low boiling (20–40°) fraction of petroleum ether was employed in wash liquids.

RESULTS AND DISCUSSION

Leaf extracts

Typical one-dimensional chromatographic separations and sequences of the chloroplast leaf pigments from a 2 μl loading on cellulose sheets are summarized in Table I. The wash liquids chosen were those most effective for separations on Whatman No. 1 cellulose paper. Some results on paper at the same loading are given for comparison. The most adsorbed pigment is listed first. The results in Table I show that in each chromatographic system, the cellulose sheet was more selective than paper. This increased selectivity always resulted in a better separation of chlorophylls *a* and *b* and in some cases of the carotenoids as well. Besides this increased selectivity,

TABLE I

COMPARATIVE ONE-DIMENSIONAL CHROMATOGRAPHY OF LEAF PIGMENTS ON CELLULOSE

Abbreviations and symbols: Ac = acetone; Bz = benzene; Chl = chloroform; E = ethyl ether; Ip = isopropanol; PE = petroleum ether; Pr = *n*-propanol; a = chlorophyll *a*; b = chlorophyll *b*; C = carotene; L = lutein; N = neoxanthin; V = violaxanthin; () = contiguous, incompletely separated zones.

Form	*Wash liquid*	*Pigments and sequence*
Sheet	PE+1%Pr	N,(V+b),(L+a),C
Paper		(N+V+b),(L+a),C
Layer		N,(V+b),(L+a+C)
Sheet	PE–Chl(3:1)	(b+N+a+V),L,C
Paper		(b+N+a+V+L+C)
Sheet	E–PE(1:1)+0.25%Pr	N,b,(V+a),L,C
Paper		(N+b+V+a+L),C
Sheet	PE–Bz–Chl–Ac–Ip (50:35:10:5:0.17)	(N+b),a,V,L,C
Paper		(N+b+a+V+L+C)
Layer		(N+b+a+V+L+C)

cellulose sheets generally sorbed the pigments more strongly than did Whatman paper (*i.e.*, the *R* values of the pigments were higher on paper). In each system, loadings of 2, 5 and 10 μl were tested. The lightest loading (2 μl) yielded the best separation in

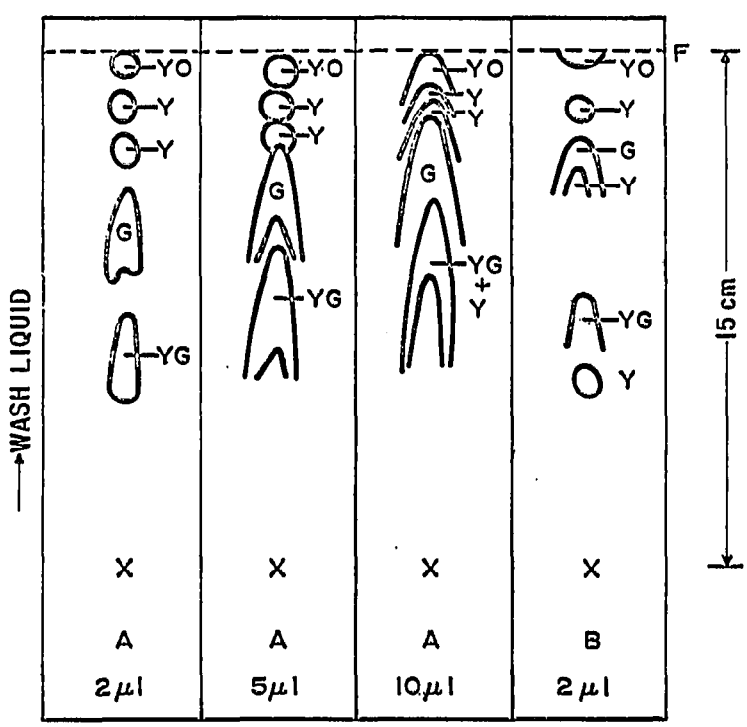

Fig. 1. Pigments in various amounts of leaf extract separated by one-dimensional migration on cellulose sheets with (A) petroleum ether–benzene–chloroform–acetone–isopropanol (50:35:10:5:0.17) and (B) ether–petroleum ether (1:1) plus 0.25% *n*-propanol as the wash liquid. F = Wash liquid front; f = faint; G = green; O = orange; X = starting point; Y = yellow.

all cases. At this loading, however, the presence of minor pigments could not be easily detected[4], and below 2 μl, some of the major zones could no longer be detected. Fig. 1 demonstrates the effect of loading on resolution in a typical system (see Table I) and also graphically emphasizes that a change in the sequence of zones can, and often does, result from a change in wash liquid[1,7,8].

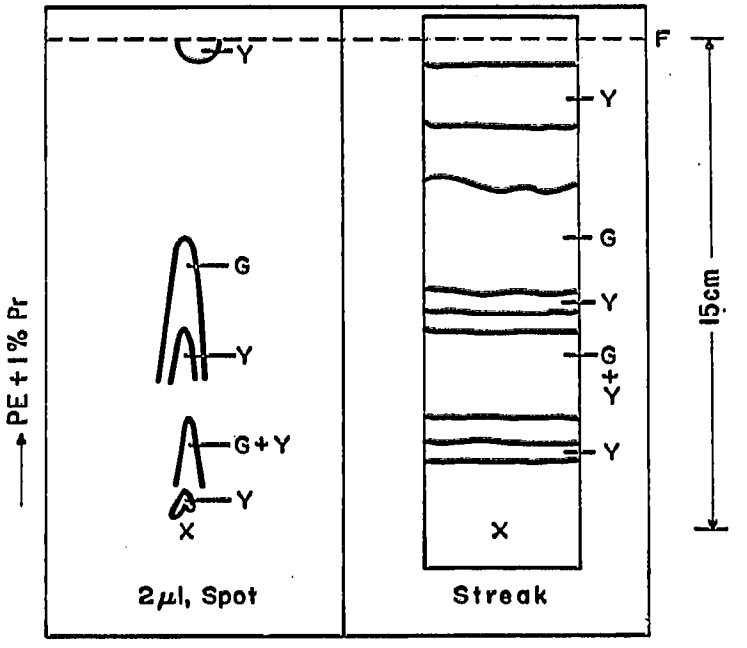

Fig. 2. Pigments in leaf extract separated by one-dimensional migration on cellulose sheets from initial zones formed as a spot and a streak. (See the legend to Fig. 1 and Table I for symbols and abbreviations).

The zones developed on cellulose sheets had double-tailing portions[1] characteristic of separations in paper. When a streak instead of a spot was initially applied across a 2-in. wide sheet and developed with petroleum ether plus 1 % *n*-propanol, the zones occurred in the same sequence as that from spots (Table I), but the tailing was eliminated (Fig. 2).

Several runs were made to compare sandwich development apparatus with the lined development tanks used throughout this work. In the Eastman sandwich apparatus, designed specifically for use with Chromagram media, development of leaf extract on cellulose sheets with petroleum ether plus 1 % *n*-propanol required 49 min compared to 29 min in the conventional tank. The resolution was identical to that obtained in the tank (Table I). With ether–petroleum ether (1:1) plus 0.25 % *n*-propanol in the Eastman sandwich tank, the solvent front seemed to stop on cellulose sheets after it had risen only 12 cm past the origin (in 80 min). This partial development yielded diffuse, poorly separated zones compared with the run in the tank. Sandwich apparatus fashioned from a Mallinckrodt Chroma-Kit was also found to be less effective than tanks with several wash liquids from Table I. In each case the wash liquid did not rise the required 15 cm, and the separations from these partial runs were poor.

Two-dimensional chromatograms obtained by development of leaf extract with petroleum ether plus 1 % *n*-propanol followed by petroleum ether–chloroform (3:1)

in the transverse direction were quite similar to those obtained on Whatman No. 1 paper[9]. Multiple zoning of the xanthophylls[9] occurred at loadings of 3–10 μl. The pattern of zones was slightly different than on paper because of the different selectivity and sorptive properties of the cellulose layer mentioned above. Only violaxanthin and chlorophyll *b* were contiguous at low loadings. A superior two-dimensional system for pigment separations is described in another report[10].

Results of radial chromatography with petroleum ether plus 1.5 % *n*-propanol were identical to those reported earlier on paper[6], the separation being represented by: N,(V+b),L,a,C.

Except for the occasional appearance of a faint grey zone less sorbed than chlorophyll *a*, no minor zones were detected in any of these systems.

Saponified leaf extracts

Table II shows the sequence and separation of pigments in saponified leaf extract from a 2 μl loading on cellulose sheets with various wash liquids. Some results on Whatman No. 1 paper at the same loading are also included.

TABLE II

COMPARATIVE ONE-DIMENSIONAL CHROMATOGRAPHY OF SAPONIFIED LEAF PIGMENTS OF CELLULOSE

Form	*Wash liquid**	*Pigments** *and sequence*
Sheet	PE–Chl(3:1)	(N+V),L,C
Paper		(N+V),L,C
Layer		N,V,(L+C)
Sheet	E–PE(1:1)+1 %Pr	N,(V+L+C)
Paper		N,(V+L+C)
Sheet	PE–Bz–Chl–Ac–Ip (50:35:10:5:0.17)	N,V,L,C
Paper		N,V,L,C
Layer		N,V,L,C
Sheet	PE+1 %Pr	N,(V+L),C
Paper		(N+V+L),C
Sheet	CCl_4+0.25 %Pr	N,V,L,C
Sheet	Bz–PE(2:1)	N,V,L,C

* For abbrevations, see Table I.

In the first three systems, results are similar on the sheet and paper. The last two wash liquids listed do not completely resolve the principal carotenoid pigments on paper[2], so that cellulose sheets have an advantage with the last three wash liquids. Fig. 3 shows the separations obtained on cellulose sheets with the three best wash liquids.

The zones formed by the development of five 10 μl spots with the benzene–petroleum ether wash liquid were cut out and eluted with ethanol. The wavelengths of the maxima of the spectral absorption curves in each case corresponded to those of the pigments[7,11] named in Fig. 3. Consequently, the cellulose sheets had not caused

rearrangement of any of the yellow pigments as has been observed with acids and on some siliceous adsorbents[3].

There was no indication of additional major pigments being present in leaves as reported by other workers[12-15]. Only in the system employing the petroleum ether–benzene–chloroform–acetone–isopropanol wash liquid were minor yellow zones ob-

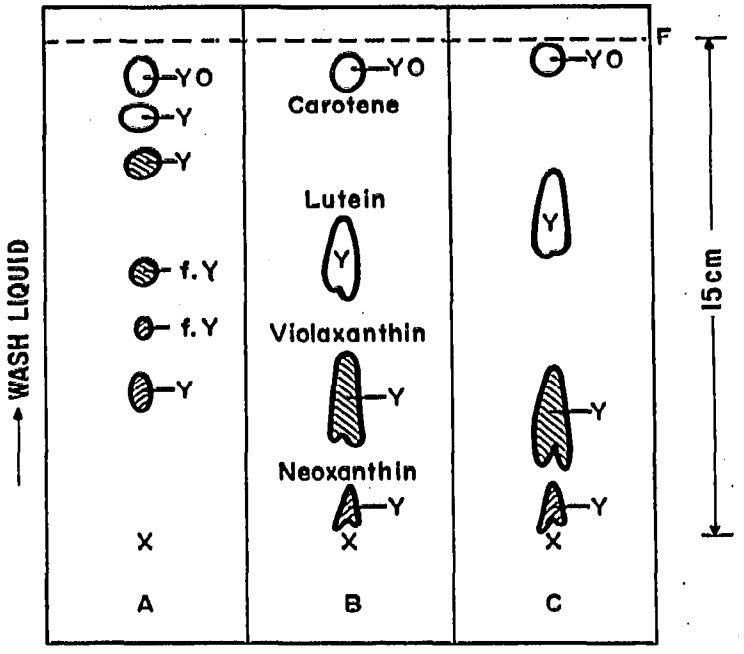

Fig. 3. Pigments in a 2 μl of saponified leaf extract separated by one-dimensional migration on cellulose sheets with (A) petroleum ether–benzene–chloroform–acetone–isopropanol (50:35:10:5:0.17), (B) carbon tetrachloride plus 0.25% *n*-propanol and (C) benzene–petroleum ether (2:1) as the wash liquid. (See the legend to Fig. 1 for symbols and abbreviations). ▨, blue by HCl vapor; ▨, blue-green by HCl vapor.

served. Two faint yellow zones which turned blue and blue-green with HCl vapors were located between violaxanthin and neoxanthin (Fig. 3). These zones were probably two of the same minor pigments as recently confirmed by large scale column chromatography of extracts of cocklebur (Xanthium) leaves[4]. However, the chromatographic sequence of these minor pigments has not been determined in this wash liquid.

Radial chromatography of saponified leaf extract with petroleum plus 1% *n*-propanol on cellulose sheets completely resolved the major carotenoids as on cellulose paper[6].

Comparisons of cellulose sheets with columns and conventional thin layers

Chromatography of leaf pigments on cellulose layers spread on glass plates resulted in almost every case in separations inferior to those achieved on cellulose sheets and cellulose paper. A great number of wash liquids were examined with both leaf extract and saponified leaf extract, and results of the best runs are included in Tables I and II.

A number of different wash liquids were also used to develop columns of Camag TLC Cellulose. Petroleum ether plus 1% *n*-propanol gave seven principal zones with leaf extract, and petroleum ether–benzene–chloroform–acetone–isopropanol (50:35:

10:5:0.17) gave four principal zones with saponified leaf extract. These zones were in the sequence observed on sugar columns[6] but were not as well defined or separated as those in the sugar columns.

The observations reported above indicate that Eastman Chromagram Cellulose sheets are an effective and mild medium for pigment separations. They are more selective than cellulose paper and conventional cellulose thin layers with most wash liquids. It is also confirmed that leaves contain chlorophylls *a* and *b*, neoxanthin, violaxanthin, lutein and β-carotene as the principal pigments.

SUMMARY

Leaf pigments and saponified leaf pigments were separated by one-dimensional, two-dimensional and radial chromatography on Eastman Chromagram Cellulose sheets. Comparisons with Whatman No. 1 paper and conventional cellulose thin layers on glass plates show the Chromagram sheets to be superior in many systems. Comparative results in sandwich tanks and with columns of cellulose are also presented. The major pigments in leaves were found to be chlorophylls *a* and *b*, neoxanthin, violaxanthin, lutein and β-carotene.

REFERENCES

1 H. H. STRAIN, J. SHERMA, F. L. BENTON AND J. J. KATZ, *Biochim. Biophys. Acta*, 109 (1965) 1.
2 H. H. STRAIN AND M. K. BECK, unpublished results.
3 H. H. STRAIN, J. SHERMA AND M. GRANDOLFO, *Anal. Chem.*, 39 (1967) 926.
4 H. H. STRAIN, J. SHERMA AND M. GRANDOLFO, *Anal. Biochem.*, submitted for publication.
5 J. SHERMA AND L. V. S. HOOD, *J. Chromatog.*, 17 (1965) 307.
6 H. H. STRAIN, J. SHERMA, F. L. BENTON AND J. J. KATZ, *Biochim. Biophys. Acta*, 109 (1965) 23.
7 H. H. STRAIN, *Ann. Priestley Lectures*, (1958) 32.
8 H. H. STRAIN, in E. HEFTMANN (Editor), *Chromatography*, Reinhold, New York, 1961, p. 584.
9 H. H. STRAIN, J. SHERMA, F. L. BENTON AND J. J. KATZ, *Biochim. Biophys. Acta*, 109 (1965) 16.
10 J. SHERMA AND G. ZWEIG, *J. Chromatog.*, 31 (1967) 589.
11 H. H. STRAIN, *Carnegie Inst. Wash. Publ.*, (1938) 490.
12 A. K. KHUDAIRI, *Biochim. Biophys. Acta*, 46 (1961) 344.
13 F. T. WOLF, *Plant Physiol.*, 38 (1963) 649.
14 K. EGGER, *Planta*, 58 (1962) 664.
15 M. H. ANWAR, *J. Chem. Educ.*, 40 (1963) 29.