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CHROMATOGRAPHY OF CHLOROPLAST PIGMENTS ON PREFORMED THIN LAYERS

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

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SUMMARY

Separations of chloroplast pigments from leaves and algae by chromatography on preformed flexible thin layers of cellulose, silica gel, alumina and polyamide are described. Various brands and types of these media are compared. They are found, in general, to be more convenient and, in some cases, superior to homemade layers of the same adsorbents.

INTRODUCTION

In a recent review[1], it was stated that "pre-coated thin layers cannot be recommended for routine analysis of chloroplast pigments". On the contrary, pre-coated thin layers of cellulose, silica gel, alumina and polyamide on plastic and aluminum backing as well as silica gel and silicic acid stabilized in glass fiber sheets have now proven to be uniform, selective and mild media for effective separations of the pigments of leaves and various algae. Presented below are tabulated results of many of these separations on the popular Eastman Chromagram sheets as well as comparative studies on other commercial layers including "Baker-flex" and Merck aluminum-backed sheets which have become available only very recently. A few of the separations of leaf pigments on Chromagram sheets have been reported in parts of earlier publications[2-4], but, as far as we know, the other sheets have for the most part not been evaluated for pigment separations.

EXPERIMENTAL

Chromagram sheets (designated below as C) were obtained from Distillation Products Industries, Rochester, N.Y. These sheets are composed of 100 μ layers of silica gel (No. 6060), alumina (No. 6062) or cellulose (No. 6064) on a plastic film backing. The silica gel and alumina layers also contain a polymeric resin binder. "Baker-flex" sheets (designated B; J. T. Baker Chemical Co., Phillipsburg, N.J.) contain a 200 μ layer of silica gel with a polymeric resin binder (N, No. 4462) or a

100 μ layer with a starch binder (S, No. 4464), a 200 μ layer of alumina with a polymeric binder (No. 4466), a 100 μ layer of unbound cellulose (No. 4468), or a 100 μ layer of unbound polyamide 6 (No. 4475) on a plastic backing. These layers are manufactured for Baker by the Macherey-Nagel Co. and are very similar to the series of Macherey-Nagel Polygram plastic-backed foils (designated MN) distributed by Brinkmann Instruments Co., Westbury, N.Y.[5]. In addition to these, we tested some new precoated aluminum sheets (designated EM) manufactured by E. Merck AG and also distributed by Brinkmann. These silica gel and aluminum oxide layers are 0.25 mm in thickness while the microcrystalline cellulose and polyamide (type 11) layers are 0.1 mm. The aluminum oxide layers are of two types (T and E) differing only in the nature of the binder. ITLC media (designated G) are glass microfiber sheets impregnated with silica gel (Type SG) or silicic acid (Type SA) manufactured by the Gelman Instrument Co., Ann Arbor, Mich. ChromAR Sheet 500 (designated M) is a 500 μ sheet of 70% silicic acid and 30% glass fiber manufactured by the Mallinckrodt Chemical Co., St. Louis, Mo. All brands of each adsorbent were not necessarily tested with every wash liquid.

All siliceous and alumina layers were heated for 15 min at 105° and cooled just before spotting although in most cases this pretreatment made no difference. Cellulose and polyamide layers were used as received.

Ascending development over a distance of 15 cm was carried out by standing the sheets in wash liquid held in the bottom of paper-lined, saturated, rectangular chambers covered with aluminum foil[2]. Development times ranged from about 20 min on the Gelman SA and SG to 60 min on alumina layers.

Identification of the separated pigments was based upon their colors, chromatographic sequence, absorption spectra[6-8] in the visible region and reaction to the vapors of HCl (neoxanthin turns blue-green, violaxanthin blue)[3,9].

Total pigment or saponified extracts were prepared as previously described[9,10] so as to avoid alterations of the labile pigments. The final solution contained virtually all the pigments present in 2 g of leaves or algal organism dissolved in one ml of petroleum ether (60–110°) or one ml of 1:1 ether–petroleum ether (60–110°) for the saponified extract. Samples were spotted with micropipettes 1 in. from the bottom and at least 1 in. apart on the sheets.

RESULTS AND DISCUSSION

Leaf extract

Table I shows various combinations of wash liquids and layers which served to separate the major pigments in extracts of cocklebur *(Xanthium)* and spinach leaves by one-way development. Table II contains the abbreviations used in this and later tables.

Chromagram and Merck cellulose were found to be stronger sorbents for the pigments than were either MN or Baker cellulose (or cellulose paper[2,11]) and gave good separations of all the pigments except neoxanthin and chlorophyll *b* with petroleum ether–benzene–chloroform–acetone–isopropanol (50:35:10:5:0.17). This wash liquid carried all the pigments near the solvent front without separation on the latter layers. Petroleum ether–diethyl ether (1:1) plus 0.25% *n*-propanol resolved four zones on Chromagram cellulose but also carried all the pigments near the solvent

TABLE I

SEPARATIONS OF LEAF EXTRACT

No.	*Adsorbent*	*Brands*	*Wash liquid*	*Order of separated spots of pigments from the solvent front*
1	Cellulose	C, EM	PE–Bz–Ch–Ac–Ipr (50:35:10:5:0.17)	C-L-V-a-(b + N)
2	Cellulose	C	PE–E(1:1) + 0.25% *n*-Pr	C-L-(a + V)-(b + N)
3	Cellulose	C	PE + 1% *n*-Pr	C-(a + L)-(b + V)-N
		B		C-a-(b + L)-V-N
4	Alumina	C	PE–Ac (4:6)	C-L,a,V,(b + N)
5	Silica Gel	C	Io–Ac–E (3:1:1)	C-p-a-L-b-V-N
		B-P,B-S, MN-N,MN-S		C-a-b-L-V-N
		EM		C-(a + b + L + V + N)
6	Silica Gel	C	Io–Ac–CCl_4 (3:1:1)	C-p-(a + L)-(b + V)-N
7	Silica Gel	C,B-P,B-S	Bz–Ac (7:3)	C-(a +b)-L-V-N
8	Silica Gel	C	Bz–Ac (7:1)	C-a, (b + L)-V-N
9	Silica Gel	C, EM	PE–Ac (7:3)	C-a-(b + L)-V-N
		B-P,B-S		C-a-b-L-V-N
10	Silica Gel	G-SG,G-SA	Io–Ac–E (3:1:1)	C-p-a-b-L-V-N
11	Silica Gel	G-SG	PE–ethyl acetate (13:1)	C-p-a-b-L-V,N
		G-SA		C-p-(a + b + L + V + N)
		M		C-(a + b + L + V + N)
12	Silica Gel	G-SG	PE + 1% *n*-Pr	C-a-b-L-V-N
		G-SA		C-a,b-(L + V + N)
		M		C-a-b-L-V,N
13	Silica Gel	G-SG	PE–Ac (7:3)	(C + a + b),L-V-N
		G-SA		C-a-b-L-V-N
		M		C-a,b-L-V-N

TABLE II

ABBREVIATIONS USED IN TABLES I, III AND IV

a = chlorophyll *a*
Ac = acetone
b = chlorophyll *b*
Bz = benzene
C = carotene
c = chlorophyll *c*
Ch = chloroform (water washed and dried over Drierite)
D = diadinoxanthin
E = diethyl ether
Io = isooctane
Ipr = isopropanol
L = lutein
Lx = loroxanthin
M = myxoxanthin
Mx = myoxanthophyll
N = neoxanthin
P = peridinin
p = pheophytin
PE = petroleum ether (20–40°)
Pr = *n*-propanol
S = siphonein
Sx = siphonaxanthin
V = violaxanthin
X = unidentified xanthophyll
Z = zeaxanthin
-a-b = well separated spots of chlorophylls *a* and *b*
a,b = partly separated spots (connecting) of chlorophylls *a* and *b*
(a + b) = mixed spots of chlorophylls *a* and *b*

front on Baker cellulose. The more weakly sorptive Baker and MN cellulose layers required less-polar wash liquids such as petroleum ether plus 0.5 to 1% *n*-propanol in order to achieve resolution of the pigments. Petroleum ether–benzene–chloroform–acetone–isopropanol (50:35:10:0.5:0.17) (ref. 12), which gives a separation of the pigments in leaf extract in the order C-L-V-a, (b + N) on cellulose paper, carries the pigments near the front without separation on Baker cellulose indicating that this is a weaker sorbent for the pigments than even paper.

On the cellulose layers, all of which were 100 μ in thickness, the optimum loading was about 0.5 to 2 μl of leaf extract. Chlorophylls separated in cellulose layers had double-tailing portions[2,9] which were eliminated if the initial zone was applied as a thin, uniform streak instead of a spot. With equal loading, the separations from a streak and in the central portions of the migrating spots were identical, however. All six major leaf pigments could be separated on Chromagram cellulose sheets by two-way development with solvent 1 (Table I) followed by solvent 2 or 3 (ref. 13), or by radial development with 1.5% *n*-propanol in petroleum ether[2].

Pigments in green leaf extract were not separated cleanly with any of the alumina layers. The green pigments and the xanthophylls remained at or near the origin unless very polar wash liquids (*e.g.*, 60% acetone, 40% petroleum ether) were employed, in which case the migrating green zones streaked badly. Isooctane–acetone–ether (3:1:1), and benzene plus 10% acetone had provided separations of the chlorophylls and carotenoids in the sequence C-(a + L)-(b + V)-N and (C + a + b)-L-V-N, respectively, with a minimum of chlorophyll alteration, when neutral Chromagram alumina was purchased and tested about three years ago[4]. With these same and similar wash liquids and our present supply of these layers (as well as the other brands of alumina), the chlorophylls and xanthophylls remained at or near the origin indicating that the activity of the layers supplied to us by the manufacturer apparently had increased.

Baker polyamide layers altered the chlorophylls in a manner similar to alumina so that they were either sorbed near the origin or streaked badly. Petroleum ether containing 1% *n*-propanol washed only carotene off the origin, while petroleum ether containing 10% *n*-propanol separated carotene and gave long streaking zones of the other pigments. Isooctane–acetone–ether (3:1:1) separated carotene and lutein but gave streaked, mixed zones of the other pigments.

Table I indicates five wash liquids that gave good separations of the pigments in leaf extract on silica gel layers. Isooctane–acetone–ether (3:1:1) provided complete separations of the six major pigments on Chromagram sheets, the two Baker sheets, and the two MN sheets. It is interesting that with this wash liquid, the sequence of lutein and the chlorophylls was different on Chromagram silica gel than on the other preformed layers and Silica Gel G columns and thin layers[3]. Petroleum ether plus 30% acetone also provided a complete separation of the six principal pigments on both Baker silica layers. In all cases the optimum loading was 0.5–2 μl of leaf extract even though some of the layers were 100 μ and some 200 μ. The maximum loading without loss of resolution was slightly higher on the thicker layers, being 10–15 μl rather than 5–10 μl. Slight chlorophyll alterations were indicated by traces of pheophytin and a faint green residue at the origin at higher loadings (5–15 μl), and a slightly low blue/red absorption maximum ratio for chlorophylls *a* and *b* separated in and eluted from these sheets.

The three silica gel layers stabilized in glass fiber were quite fragile relative to the film or aluminum backed layers. The ChromAR sheets were so limp that they could not be stood in wash liquid and so were supported by clipping to a glass rod at the top of the chamber. Gelman silica gel sheets were less sorptive than the silicic acid variety as indicated by the fact that the pigments were completely resolved by petroleum ether plus 1% *n*-propanol on the former and with petroleum ether plus 30% acetone on the latter. Isooctane–acetone–ether (3:1:1), a wash liquid with an effective polarity intermediate between the other two, resolved the pigments completely on both of these layers. Good separations were obtained on ChromAR layers with petroleum ether plus 1% *n*-propanol or 30% acetone. The separated pigments were in the usual sequence characteristic of Silica Gel G. When exposed to the vapors of concentrated HCl, carotenoids separated in these three media occasionally all turned blue, but the carotene and lutein zones slowly returned to yellow on standing. These glass fiber media readily soaked up the initial sample, and optimum loading was about 5–10 μl, higher than on film-backed layers.

The separations in Table I are all based on the mechanism of adsorption. Reversed-phase partition chromatography was performed by dipping a Chromagram silica gel sheet in a solution of 7% olive oil in petroleum ether (60–110°), air drying

TABLE III

SEPARATIONS OF SAPONIFIED LEAF EXTRACT

No.	*Adsorbent*	*Brands*	*Wash liquid*	*Order of separated spots of pigments from the solvent front*
1	Cellulose	C,EM	PE–Bz–Ch–Ac–Ipr (50:35:10:5:0.17)	C-L-V-N
2	Cellulose	C,B,MN	CCl_4 + 0.25% *n*-Pr	C-L-V-N
3	Cellulose	C,EM	Bz–PE (2:1)	C-L-V-N
4	Cellulose	C	PE + 1% *n*-Pr	C-L,V-N
		B,MN		C-L-V-N
5	Cellulose	C	PE–Ch (3:1)	C-L-V,N
6	Alumina	C,B	PE–Ac (1:1)	C-L-V-N
7	Alumina	C,B,EM-E	Bz–Ac (4:6)	C-L-V-N
8	Alumina	C	PE–Ac (7:3)	C-L,V,N
		B		C-L, (V + N)
9	Alimina	B, MN	Bz–Ac (7:3)	C-L,V,N
		C,EM-E		C-L-V-N
10	Polyamide	B,MN,EM	Io–Ac–E (3:1:1)	C-L,V-N
11	Silica Gel	C,B-N,B-S,MN-N, MN-S	Io–Ac–E (or CCl_4) (3:1:1)	C-L-V-N
		EM		C-(L + V + N)
12	Silica Gel	C,B-N,B-S,MN-N, MN-S,EM	PE–Ac (7:3)	C-L-V-N
13	Silica Gel	C,B-N,B-S	Bz–Ac (7:3)	C-L-V-N
14	Silica Gel	C,B-S	ethylene dichloride–ethyl acetate (4:1)	C-L-V-N
		B-N		C-L-V,N
15	Silica Gel	G-SG,G-SA	Io–Ac–E (3:1:1)	C-L-V-N
16	Silica Gel	G-SG	PE + 1% *n*-Pr	C-L, (V + N)
		M		C-(L + V + N)
17	Silica Gel	G-SA	PE–Ac (7:3)	C-L-V-N
		M		C-L-V-N

and then oven drying at 75° for 30 min. After cooling the layer and spotting the extract, development with oil-saturated methanol–acetone (20:1) gave a complete separation in the reverse order, N-V-L-b-a-C.

Saponified extract

Table III shows separations of saponified spinach or cocklebur extract on the four adsorbents. Saponification, which was carried out with alcoholic KOH, removes the two green chlorophylls as soluble salts but leaves the yellow carotenoids intact. In most cases, the four major carotenoids were well separated in the same sequence: carotenes (least sorbed), lutein, violaxanthin and neoxanthin. Occasionally, one or two minor yellow pigments were separated by these systems.

As noted above with leaf extract, the Chromagram and Merck cellulose layers sorbed the carotenoids more strongly than Baker and MN cellulose, but by controlling the polarity of the wash liquids, good separations were obtained on all the layers.

All brands of alumina acted quite similarly and provided good separations of the four leaf carotenoids with wash liquids of high enough polarity. The Baker and MN layers were slightly less sorptive than the Chromagram and Merck type E layers; the Merck type T layers were not usable with these wash liquids because the fronts stopped part way up the sheet. There was again indication that our current supply of Chromagram alumina was quite a bit more active than that received several years ago because a separation of the four carotenoids comparable to that achieved earlier[4]

TABLE IV

SEPARATION OF PIGMENTS FROM VARIOUS ORGANISMS

Chromagram cellulose sheets with PE–Bz–Ch–Ac–Ipr (50:35:10:5:0.17), Chromagram silica gel sheets with Io–Ac–E (3:1:1), or Chromagram alumina with PE plus 50% acetone as wash liquids are used.

No.	*Adsorbent*	*Plant extract or saponified extract*	*Order of separated spots from the solvent front*	*Plant source*
1	Cellulose	sap. ext.	C-L-V-N	*Chlorella pyrenoidosa*
2	Silica gel	sap. ext.	C-L-V-N	
		plant ext.	C-p-a-L-b-V-N	
3	Alumina	sap. ext.	C-L-V-N	
4	Cellulose	plant ext.	C-L-V-(a + Lx)-(b + N)	*Chlorella vulgaris*,
5	Silica gel	sap. ext.	C-L-V-Lx-N	*Scenedesmus obliquus*,
		plant ext.	C-p-a-L-b,V-Lx-N	*Cladophora ovoidea*, or
6	Alumina	sap. ext.	C-L-V-(Lx + N)	*Cladophora trichatoma*
7	Silica gel	sap. ext.	C-L-V-N-S	*Codium fragile*, or
		plant ext.	C-p-a-b-S-L-V-N-Sx	*Codium setchellii*
8	Silica gel	sap. ext.	C-M-Z-Mx	*Phormidium luridum*
		plant ext.	C-M-p-a-Z-Mx	
9	Cellulose	sap. ext.	C-D-rearranged D-*cis*-D-N	*Euglena gracilis*
10	Silica gel	plant ext.	C-p-a-b,D-N	
11	Silica gel	plant ext.	C-p-a-D-P-c	Symbiotic alga of sea anemones (dinoflagellates)
12	Silica gel	sap. ext.	C-D-X-X (both X are orange)	

with petroleum ether plus 30% acetone now required about twice as much acetone. The loading limits were about the same on the Chromagram and doubly-thick Baker layers, about 0.25–20 µl of saponified extract solution.

Many wash liquids were tested with the three brands of polyamide layers and none gave a complete separation of the four leaf carotenoids. The best results were achieved with isooctane–acetone–ether (3:1:1), which gave separated zones of carotene and neoxanthin but connecting zones of lutein and violaxanthin.

Complete separations of the carotenoids were obtained on all of the siliceous layers with various wash liquids. If the carotenoids were eluted with ethanol at once, their spectra indicated no alteration. If the separated zones were dried in air or in a current of moist nitrogen for 30 min before elution, the epoxy carotenoids neoxanthin and violaxanthin were usually completely isomerized[3].

Algal organisms

Chromagram sheets were also tested with untreated and saponified extracts of various other plants. Typical results are shown in Table IV for species of green algae (both Chlorophytes and Siphonales), blue-green algae, *Euglena* and dinoflagellates. These pigment patterns confirm in general those obtained earlier in sugar columns[14,15] on paper[16,17] and on sucrose thin layers[18] for similar types of organisms. In all cases, the major carotene was found to be β with or without traces of α except for the Siphonales, which contained mostly α-carotene with minor amounts of β. The carotene isomers cannot be separated on any of the adsorbents tested above; they were studied on columns or thin layers of activated magnesium oxide[10]. These results, as well as those above for leaf extract, emphasize the great selectivity of these precoated thin-layer media for pigment separations. The extra yellow xanthophyll, loroxanthin, separated from extracts of many Chlorophytes, was first isolated and characterized by STRAIN and collaborators at the Argonne National Laboratory, Argonne, Ill.[10].

CONCLUSIONS

The above results indicate that all the commercially available brands of precoated thin layers are excellent media for separations of photosynthetic plant pigments. Cellulose sheets are somewhat superior to paper and the homemade cellulose layers we tested[2] as far as selectivity and sharpness of the zones. Alumina sheets are as selective as homemade layers of Alumina G[4] and provide good separations of carotenoid pigments without rearrangement. Silica gel sheets are as selective as homemade layers of Silica Gel G[3] and cause no more alteration of the chlorophylls and xanthophylls. Polyamide sheets did not provide complete resolution of the pigments in either untreated or saponified leaf extract; we did not test homemade polyamide thin layers.

In addition, these sheets are much more convenient to use. Not only is the chore of casting layers avoided, but the separated zones can be marked with a pencil on the layer, and the sheets can be covered with polyethylene (Saran wrap) and saved for future reference.

The good uniformity of these sheets was indicated by the fact that for any one band of a particular adsorbent developed with a certain wash liquid, there was virtually no change in the R_F values of the pigments (at a constant loading) from one

area to another on the same sheet or from one sheet to another in a given box. However, it was noted above that the activity of one brand of alumina layers was found to be greater than that received several years ago.

The observations reported also confirm the earlier conclusions[3,4,9,14] that leaves and many green algae contain six, and only six major pigments: carotene, lutein, violaxanthin, neoxanthin and chlorophylls *a* and *b*. Plants belonging to other major taxonomic groups contain other unique combinations of pigments.

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