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Partition and ion-exchange thin-layer chromatography of water-soluble fluorescent compounds*

It is essential for quantitative studies of energy transfer that the energy acceptors be free from impurities that either emit fluorescence or absorb strongly in the spectral regions of interest. In preparation for a study of excitation energy transfer between molecules in solution, twelve fluorescent water-soluble compounds have been tested in fourteen thin-layer chromatography systems to find satisfactory methods for analysis and for preparative separations.

Thin-layer chromatography (TLC) is a useful technique for the separation of impurities that would prove troublesome in energy transfer studies, since fluorescent compounds and compounds that absorb ultraviolet light strongly are easily seen on developed chromatograms. Reports in the literature[1] generally indicate silica gel as the adsorbent of choice for the analyses of such fluorescent compounds as anthranilic acid, vitamins B_2 and B_6, fluorescein, and rhodamine B, although an ion-exchange resin has been used for separation of B vitamins[2]. Air-dried silica gel layers are also reported to give good results on occasion[3], suggesting that separation may sometimes depend on partition as well as on adsorption, and that layers of unmodified and ion-exchange cellulose might be useful. Also WOLLENWEBER[4,5] and GÄNSHIRT *et al.*[6] have reported the use of cellulose layers for the chromatography of synthetic food colors. In addition, adaptation of successful thin-layer systems to column chromatography should be relatively much easier with cellulose than with air-dried silica gel with its poorly defined water content.

The results to be discussed below indicate that partition and ion-exchange TLC on cellulose is useful for the analysis of a variety of fluorescent water-soluble compounds. Adaptation to column chromatography has not yet been attempted.

Methods

The layers were either spread in the laboratory (with Bio-Sil A silicic acid, Bio-Sil CM cellulose for TLC, or Serva DEAE cellulose for TLC) or purchased precoated (Eastman Chromagram Sheet, Brinkmann silica gel HF, or Brinkmann MN 300 cellulose). Layers prepared in the laboratory were made by mixing the silica gel or cellulose powder with water in a blender, then spreading the slurry on glass plates with an adjustable-thickness spreader (Research Specialties Co.).

The compounds to be studied were dissolved in water or alcohol (except for

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TABLE I

R_F VALUES* ON SILICA GEL LAYERS

Layer and solvent key:

I = Activated silica gel (Eastman), *n*-propanol–formic acid (80:20).
II = Activated silica gel HF (Brinkmann precoated), chloroform–methanol–acetic acid (75:20:5).
III = Activated silica gel, *n*-propanol–H_2O–28% NH_3 (65:30:5).
IV = Activated silica gel, benzene–methanol (95:5).
V = Air-dried silica gel, benzene–methanol (95:5).
VI = Air-dried silica gel, 0.28% NH_3 in H_2O.
VII = Air-dried silica gel, first development with acetone, secondly with acetone–ethanol (95:5).

Compound, concentration in spotting solvent	*Layer and solvent*						
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*
9-Aminoacridine hydrochloride, 4 g/l methanol	94 (0)	—	—	0 (29, 62)	0 (21, 56)	2 (23)	7 (90)
Acriflavine neutral, 1 g/l water	78 (0)	—	—	0, 2, 5	0	2, 30	U
Anthranilic acid, 10^{-2} *M* in methanol	U	84	—	46	31	92	—
4,5-Dihydroxy-2,7-naphthalenedisulfonic acid, 10^{-2} *M* in water	28 (0)	U	—	U	0	U	—
Fluorescein, 1 g/l water	U	83 (55, 32, 0)	—	19 (0, 27, 33, 56)	12 (0, 17, 20)	79 (0,95)	94 (62, 71, 0)
3-Hydroxyanthranilic acid, 10^{-2} *M* in methanol	U	77	—	15 (3,0)	10 (20, 0)	U	—
Pyridoxal hydrochloride, 10^{-2} *M* in water	74	—	77	—	U	46	51
Pyridoxamine dihydrochloride, 10^{-2} *M* in water	48 (54)	—	61	—	0	13	0 (5)
Pyridoxine hydrochloride, 10^{-2} *M* in water	72	—	75	—	—	45	33
Quinine bisulfate, 10^{-2} *M* in water	60	55	—	0	0	U	5
Rhodamine B, 4 g/l ethanol	U	—	—	2 (7, 11, 15, 26, 80)	3 (8, 14, 17, 24, 27, 68)	U	—
Riboflavin, $2 \cdot 10^{-3}$ *M*, pyridine–acetic acid–water (10:1:40)	—	—	—	1 (13)	0 (7, 13)	U	—

* Values in parentheses are R_F-values of contaminants (assuming that the major spot is the named compound). U indicates that the TLC system was unsatisfactory for the compound. A dash (—) indicates that the compound was not tested with the TLC system.

riboflavin, which was dissolved in a 10:1:40 mixture of pyridine, glacial acetic acid, and water) and spotted onto the layers with micropipets. A stream of room-temperature air was directed across the layer to speed evaporation of the spotting solvents. All developments were ascending. Those involving organic solvents were done in a jar or tank lined with solvent-saturated filter paper, or in the Eastman Chromagram developing chamber. No filter paper was used with aqueous solvents. During development the jar, tank, or chamber was placed inside a hood with an amber Plexiglas window to protect the compounds from photodegradation. After development the plates were left inside this hood until the solvent had evaporated.

Spots were observed under ultraviolet light with a Chromato-vue cabinet. Records of the developed chromatograms were made by tracing, or more satisfactorily, by photographing the chromatograms with a Polaroid MP-3 camera placed above the viewing port of the Chromato-vue cabinet. Two Eastman Kodak 1A "Skylight" filters in series were attached to the camera to prevent scattered ultraviolet light from reaching the film. Both color and black-and-white photographs have proved useful.

Results and discussion

Results are summarized in Tables I and II. Three compounds, 9-aminoacridine hydrochloride, anthranilic acid, and quinine bisulfate, were chromatographed successfully with a variety of solvent and adsorbent systems.

Acriflavine neutral is described by the supplier as a mixture of 3,6-diamino-10-methylacridinium chloride and 3,6-diaminoacridine. With systems VIII and XI acriflavine neutral is separated into 5 or 6 components, with system VIII giving slightly better resolution. The sample we received appears to contain at least three major components, two of which emit green fluorescence and one blue.

Chromotropic acid (4,5-dihydroxy-2,7-naphthalenedisulfonic acid) could not be chromatographed successfully on silica gel. System IX resolved one major and four minor components. The major component migrated the farthest, indicating that this system will be useful for preparative separation.

Separation of fluorescein from several impurities was most successful on silica gel layers; several solvent systems gave useful results.

3-Hydroxyanthranilic acid tended to streak on both silica gel and cellulose layers. DEAE cellulose appeared to provide as successful a chromatogram as any layer. The two buffer systems tried with DEAE cellulose may well not be the best; no systematic search was made to determine optimal pH or ionic strength. Methanol proved to be a poor choice for a spotting solvent for 3-hydroxyanthranilic acid. This compound decomposed slowly in the solution, even in the dark at room temperature.

Pyridoxal also tended to streak in a number of systems, possibly because of decomposition in the solvents used, as suggested by BOLLIGER[3]. It was best separated from pyridoxamine and pyridoxine on silica gel, by sequential development (system VII). A useful separation was also obtained on a layer of the cation exchanger carboxymethyl cellulose with sodium acetate buffer at pH 5.0, ionic strength 0.1. Separation was less complete at pH 4.0 and nonexistent at pH 7.5.

Mixtures of pyridoxamine and pyridoxine were separated on both silica gel and cellulose layers; the latter adsorbent was slightly better for resolving an impurity contained in our sample of pyridoxamine.

TABLE II

R_F VALUES* ON UNMODIFIED AND ION-EXCHANGE CELLULOSE LAYERS

Layer and solvent key:

VIII = Cellulose, *n*-propanol–H_2O–28% NH_3 (65:30:5).
IX = Cellulose, isopropanol–H_2O–28% NH_3 (65:30:5).
X = Cellulose, 0.28% NH_3 in H_2O.
XI = Cellulose, isopropanol–H_2O–HCl (55:40:5).
XII = DEAE cellulose, sodium acetate buffer, pH 5.0, I = 0.1.
XIII = DEAE cellulose, phosphate buffer, pH 7.45, I = 0.1.
XIV = Carboxymethyl cellulose, sodium acetate buffer, pH 5.0, I = 0.1.

Compound, concentration in spotting solvent	*Layer and solvent*						
	VIII	*IX*	*X*	*XI*	*XII*	*XIII*	*XIV*
9-Aminoacridine hydrochloride, 4 g/l methanol	—	91	4 (10)	88 (32)	—	U	2 (10)
Acriflavine neutral, 1 g/l water	58, 65, 40, 76, 49, 0	26, 54, 36, 62, 0	0	U	—	U	U
Anthranilic acid, 10^{-2} *M* in methanol	—	78	96	85	59	60	56
4,5-Dihydroxy-2,7-naphthalenedisulfonic acid, 10^{-2} *M* in water	31 (10,0)	50 (19, 30, 14, 0)	U	96 (43)	—	U	U
Fluorescein, 1 g/l water	—	68	88 (98)	U	—	5 (0, 10, 23, 33)	—
3-Hydroxyanthranilic acid, 10^{-2} *M* in methanol	U	U	U	79 (46, 8)	51 (0, 19)	55 (0, 25)	44 (0)
Pyridoxal hydrochloride, 10^{-2} *M* in water	U	U	92	82	U	U	38
Pyridoxamine dihydrochloride, 10^{-2} *M* in water	U	62 (96)	87 (94)	60 (83)	U	U	28
Pyridoxine hydrochloride, 10^{-2} *M* in water	—	74	95	88	—	U	33
Quinine bisulfate, 10^{-2} *M* in water	97	97	23	90	—	68	—
Rhodamine B, 4 g/l ethanol	—	U	26 (37, 46, 50, 62, 65, 74)	U	—	U	—
Riboflavin, $2 \cdot 10^{-3}$ *M*, pyridine–acetic acid–water (10:1:40)	—	44 (10, 0, 68)	70 (95, 40)	54 (75, 31, 82)	—	—	—

* Values in parentheses are R_F values of contaminants (assuming that the major spot is the named compound). U indicates that the TLC system was unsatisfactory for the compound. A dash (—) indicates that the compound was not tested with the TLC system.

Rhodamine B was successfully chromatographed on both activated and air-dried silica gel, with a 95:5 mixture of benzene and methanol as the solvent, or on cellulose with 0.28 % NH_3 in H_2O; however, with the latter, the main spot was smeared.

Riboflavin was more successfully chromatographed on cellulose than on silica gel. Each of the three solvent systems tested gave good separation from the impurities (one of which may have been in the pyridine).

Lawrence Radiation Laboratory, Bio-Medical Research Division, University of California, Livermore, Calif., U.S.A. JAMES E. GILL

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