

# A SUPERIOR THIN-LAYER CHROMATOGRAPHIC PROCEDURE FOR THE SEPARATION OF AZA ARENES AND ITS APPLICATION TO AIR POLLUTION

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## INTRODUCTION

A number of systems are available for the separation of aza heterocyclic compounds from each other and from polynuclear hydrocarbons and other types of aromatic compounds<sup>1</sup>. Of these systems column, paper, and thin-layer chromatography have been used most often.

At the present stage of development, column chromatography provides the most information, since a dozen aza heterocyclic compounds can be determined and the absorption spectra of many unknown basic compounds can be obtained<sup>2</sup>. The disadvantages of column chromatography are that it requires a large amount of organic particulates ( $\sim 100$  mg), several days for collection of an air sample, and several days for separation, spectral examination, and calculations. In addition the method requires a recording spectrophotometer.

The paper chromatographic methods are valuable in that the techniques involved are simple and are readily performed by untrained personnel. The disadvantages are the length of time needed for separation and the difficulty of separating some of the aza heterocyclic compounds. In recent work<sup>3</sup> with Whatman No. 1 paper, some compounds were not separated satisfactorily; these compounds are listed with their  $R_F$  values: phenanthridine, 0.48; benzo(*h*)quinoline, 0.48, from benzo(*f*)quinoline, 0.45; and 12-methylbenz(*a*)acridine, 0.10, from 7-methylbenz(*c*)acridine, 0.06. In some of our recent paper-chromatographic work<sup>4</sup>, alkyl derivatives were readily separated from their parent compounds, but some groups of compounds were not separated; these are listed with their  $R_F$  values: acridine, 0.78; benzo(*f*)quinoline, 0.78; and benzo(*h*)quinoline, 0.78, from phenanthridine, 0.75; acenaphtheno(1,2-*b*)pyridine, 0.62, and indeno(1,2,3-*i,j*)isoquinoline, 0.62, from benzo(*l,m,n*)phenanthridine, 0.60; benz(*a*)acridine, 0.44, from benz(*c*)acridine, 0.44; pyrenoline, 0.28, from 7,9-dimethylbenz(*c*)acridine, 0.22; and dibenz(*a,h*)acridine, 0.44 from 14-phenyldibenz(*a,j*)acridine, 0.44.

Thin-layer chromatography is useful for separating complex mixtures into various types of polycyclic compounds preliminary to their spectral characterization. The procedure offers many advantages: only small samples are required; the method involves less technique than does paper chromatography; analysis is fast – a few

hours; excitation and emission spectra can be obtained by use of an automatic fluorimetric scanner for thin-layer and paper chromatograms; and a chromatogram can be examined directly for an individual compound or for a family of compounds<sup>5</sup>. In addition, quenchofluorimetric techniques can be applied to the thin-layer plate to eliminate interferences and improve sensitivity. Disadvantages include the decreased availability of a spectrophotofluorimeter as compared to a spectrophotometer, and elution problems that may occur if the compound is strongly bound to the adsorbent. In previous work<sup>1</sup> with alumina as the adsorbent and pentane-ether (19:1, v/v) as the solvent system, the following groups of aza heterocyclic compounds were not satisfactorily separated: 8,10-dimethylbenz(*a*)acridine, 0.54, 7-methylbenz(*c*)acridine, 0.53, and benz(*c*)acridine, 0.53, from benzo(*h*)quinoline, 0.51; and phenanthridine, 0.16, and benzo(*f*)quinoline, 0.14, from benz(*a*)acridine, 0.12. In addition the  $R_F$  values of five other aza heterocyclic compounds have a range of  $0.19 \pm 0.03$  and the values of six others are in the range of  $0.125 \pm 0.015$ .

In this paper we discuss a new superior system of separation by which many of these compounds can be separated. With this improved separation, spectrophotometric and spectrophotofluorimetric characterization and assay of aza heterocyclic compounds is simplified.

#### EXPERIMENTAL\*

##### *Equipment*

The fluorescent colors were examined in a Chromato-Vue Cabinet (Kensington Scientific Corp., Berkeley 10, Calif.) under a 3660 Å light source.

Absorption spectral characterization and determinations were made with a Cary recording spectrometer, Model 11, equipped with 3-ml cells of 1-cm path length. An Aminco-Bowman spectrophotofluorimeter was used with the following settings: sensitivity 50, slit arrangement No. 2, and phototube RCA type 1P 21. An Aminco thin-film scanner was used in fluorimetric examination and scanning of the thin-layer chromatograms<sup>5</sup>.

A Technicon time-flow fraction collector was used in the column chromatographic separations of the basic subfractions of the organic airborne particulate fractions preliminary to the analysis of the polynuclear aza heterocyclic compounds.

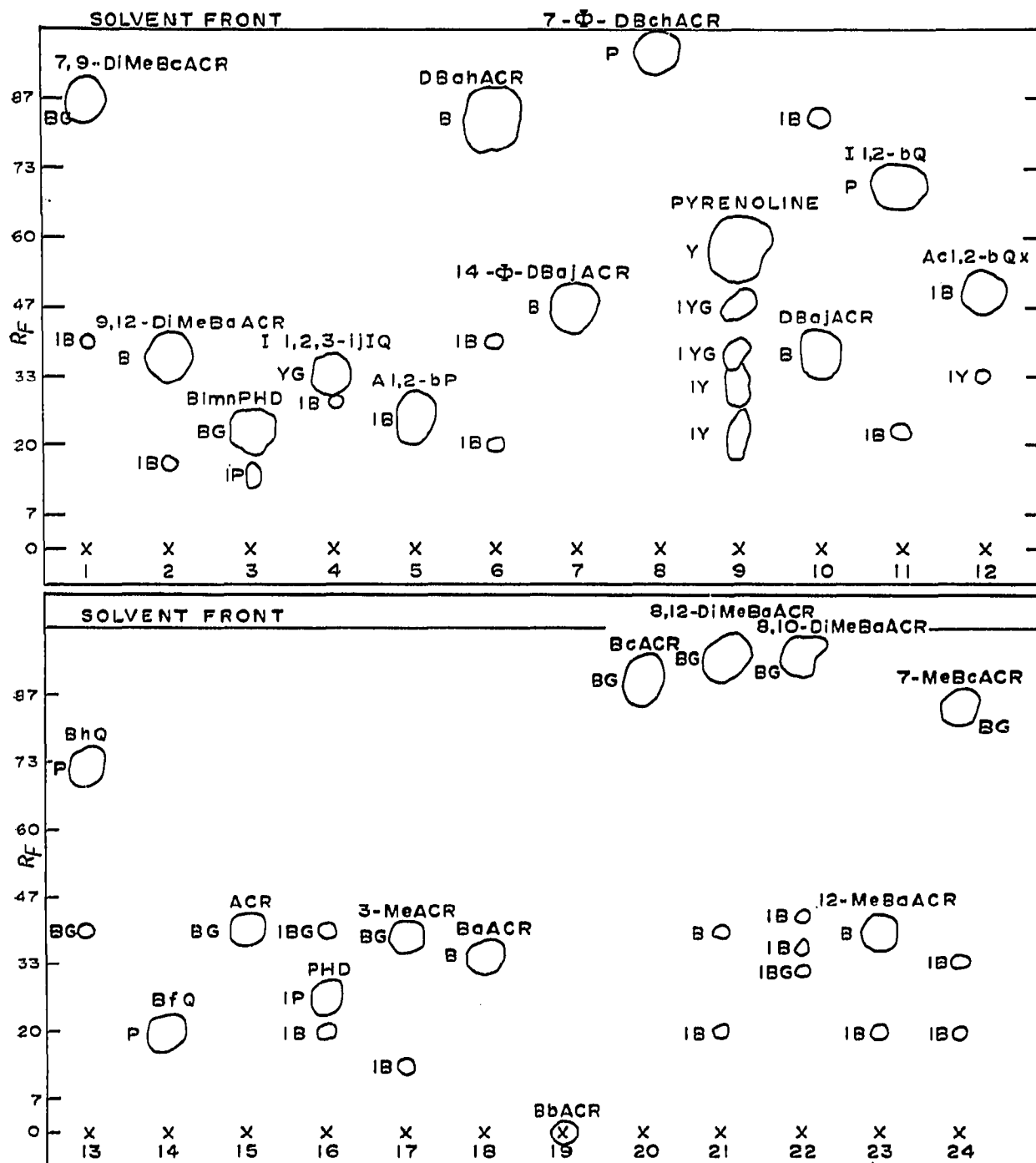
##### *Reagents*

Pentane was distilled before use. The aza heterocyclic compounds were obtained in the purest form from various sources and were used without further purification. The impurities present in these compounds were ascertained through thin-layer chromatography of 10 μg amounts of each compound on silica gel. By this method the amount and type of impurities can be ascertained, and the various aza compounds can be considerably purified (Figs. 1 and 2). In most cases the impurities were not a major problem in the analysis of these compounds.

The silica gel G plates (Analtech, Inc., Wilmington, Del.) and the silica gel G F254 plates (Brinkman Instruments, Inc., Westbury, N.Y.), both 20 by 20 cm, were

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\* Mention of commercial products does not constitute endorsement by the Public Health Service.



Figs. 1 and 2. Thin-layer chromatogram on silica gel with pentane-ether (7:3, v/v) as developer. All compounds were present in 10  $\mu$ g amounts: (1) 7,9-dimethylbenz(c)acridine; (2) 9,12-dimethylbenz(a)acridine; (3) benzo(l,m,n)phenanthridine; (4) indeno(1,2,3-*i,j*)isoquinoline; (5) acenaphtho(1,2-*b*)pyridine; (6) dibenz(a,h)acridine; (7) 14-phenyldibenz(a,j)acridine; (8) 7-phenyldibenz(c,h)acridine; (9) pyrenoline; (10) dibenz(a,j)acridine; (11) 11H-indeno(1,2-*b*)quinoline; (12) acenaphtho(1,2-*b*)quinoxaline; (13) benzo(h)quinoline; (14) benzo(f)quinoline; (15) acridine; (16) phenanthridine; (17) 3-methylacridine; (18) benz(a)acridine; (19) benz(b)acridine; (20) benz(c)acridine; (21) 8,12-dimethylbenz(a)acridine; (22) 8,10-dimethylbenz(a)acridine; (23) 12-methylbenz(a)acridine; (24) 7-methylbenz(c)acridine. The fluorescence colors of the compounds and impurities are shown. For color abbreviations see footnote b to Table I.

coated with an appropriate adsorbent to a thickness of 250 m $\mu$ . The silica gel-cellulose (2:1) plates used for separation were made by the supplier's recommended procedure for silica gel plates (Brinkman Instruments, Inc.). The silica gel (Davison Silica Gel; mesh size 100-200) was used in column chromatography without activation.

#### *Developers*

Pentane-ether (7:3, v/v), main system. Pentane-nitrobenzene-triethylamine (80:20:0.01, v/v/v). Pentane-ether (9:1, v/v). Dimethylformamide-water (35:65, v/v).

#### *Fuming reagent*

Plates were sprayed with trifluoroacetic acid to bring out the fluorescent colors.

#### *Thin-layer chromatography procedure*

A solution (1-5  $\mu$ l) containing about 1  $\mu$ g each of several aza compounds was spotted with a pipet 1.5 cm from the base of a coated glass plate (20 by 20 cm). The spotted plates were placed in a glass jar containing 150 ml of the appropriate solvent system and were developed in an ascending manner. The shortest development time, 45 min, was obtained with silica gel and pentane-ether (7:3, v/v); the longest development time, 4 1/2 to 5 h, was obtained with silica gel-cellulose (2:1) and dimethylformamide-water (35:65, v/v). After the plates were developed, they were placed briefly under the ultraviolet light in the cabinet, and all fluorescent spots were marked. Some plates were sprayed with trifluoroacetic acid fumes, and the fluorescent spots marked.

With the silica gel F254 plate, the fluorescence colors were marked under long U.V. light, when the background was non-fluorescent. Under low U.V. light, dark non-fluorescent spots appeared against a green fluorescent background.

#### *Column chromatography procedure*

One gram of the untreated silica gel was added to a small volume of methylene chloride containing 20-50 mg of the basic fraction. The methylene chloride on the silica gel was evaporated so that the organic material was homogeneously dispersed. This silica gel was then added to a 0.5 by 15 in. column, which contained a lower layer of 9 g of untreated silica gel.

The column was eluted with successive 100 ml volumes of pentane solutions containing the following percentages: of ether 0, 8, 16, 24, 35, 45, 60 and 100; of acetone 20, and 40; the column was then eluted with a 100-ml volume of acetone. Fractions of approximately 20 ml were collected, placed in a vacuum oven, and evaporated to dryness.

The residue in each of the tubes was dissolved in a small volume of pentane and transferred quantitatively by repeated washings to a 3-ml spectral cell of 1-cm light path. The ultraviolet-visible absorption spectrum of each tube was then determined.

The various aza compounds were identified by the appropriate bands in their spectra<sup>1</sup>, and in some cases were estimated by the baseline technique<sup>2</sup>.

## RESULTS AND DISCUSSION

The satisfactory separation of a number of aza heterocyclic compounds that previously had not been separated was accomplished by using silica gel as the adsor-

bent and pentane-ether (7:3, v/v) as the developer. This system required about 45 min for the solvent front to travel 15 cm.

The separation of these compounds is dependent on the amount of steric protection of the aza nitrogen combined with the competitive attraction of the aza nitrogen atom for the silica gel and the eluent.

The adsorbent has a very strong attraction for the exposed aza nitrogen atom.

TABLE I

$R_F$  VALUES AND FLUORESCENCE COLORS OF SOME AZA HETEROCYCLIC COMPOUNDS ON SILICA GEL

Compound	Pentane-ether (7:3, v/v)	
	$R_F^a$	Color <sup>b</sup> dry plate
7-Phenyldibenz(c,h)acridine	0.96	P
8,10-Dimethylbenz(a)acridine	0.94	BG
8,12-Dimethylbenz(a)acridine	0.93	BG
Benz(c)acridine	0.90	BG
Dibenz(a,c)phenazine	0.88	Y
11,12-Dimethyldibenz(a,c)phenazine	0.88	Pk
7,9-Dimethylbenz(c)acridine	0.87	BG
Benzo(a)naphtho(2,1-c)phenazine	0.86	Y
Tribenzo(a,c,h)phenazine	0.86	Pk
Tribenzo(a,c,i)phenazine	0.86	IO
7,10-Dimethylbenz(c)acridine	0.87	BG
7-Methylbenz(c)acridine	0.85	BG
Tribenzo(a,c,h)naphtho(2,1-j)phenazine	0.84	Y
Dibenz(a,h)acridine	0.83	B
Benzo(h)quinoline	0.73	BG
11H-Indeno(1,2-b)quinoline	0.69	P
Pyrenoline	0.58	Y
Phenazine	0.55	B
Acenaphtho(1,2-b)quinoxaline	0.50	IB
14-Phenyldibenz(a,j)acridine	0.46	B
Acridine	0.38	BG
3-Methylacridine	0.38	BG
Dibenz(a,j)acridine	0.38	B
9,12-Dimethylbenz(a)acridine	0.37	B
Benz(a)acridine	0.36	B
Indeno(1,2,3-i,j)isoquinoline	0.34	Y
3-Methylbenzo(f)quinoline	0.33	P
Phenanthridine	0.29	B
Acenaphtho(1,2-b)pyridine	0.25	IB
Benzo(l,m,n)phenanthridine	0.22	BG
Benzo(f)quinoline	0.21	B
Benz(b)acridine	0.00	—
9H-Pyrido(2,3-b)indole	0.00	—
9H-Pyrido(3,4-b)indole	0.00 <sup>c</sup>	B

<sup>a</sup> Average of 4 to 5 values.

<sup>b</sup> B = blue; Br = brown; G = green; l = light; P = purple; Pk = pink; R = red; Y = yellow; O = orange; V = violet. — = no fluorescence, so located by the quenching of a fluorescent plate.

<sup>c</sup> Other aza compounds that remained at the origin include adenine, adenosine, caffeine, guanylic acid, purine, and theophylline.

Therefore, the most sterically hindered molecule would be expected to move the greatest distance from the origin, and have the highest  $R_F$  value. This was found to be true, as shown in Table I, wherein 7-phenyldibenz(*c,h*)acridine has the highest  $R_F$  value of 0.96 and benz(*b*)acridine has the lowest  $R_F$  value, remaining at the origin.

The following groups of aza heterocyclic compounds that were not separable previously were satisfactorily separated by this system, as shown by their respective  $R_F$  values in Table I: 8,10-dimethylbenz(*a*)acridine, 0.94, 7-methylbenz(*c*)acridine, 0.85, and benz(*c*)acridine, 0.90, from benzo(*h*)quinoline, 0.73, and phenanthridine, 0.29, and benzo(*f*)quinoline, 0.21, from benz(*a*)acridine, 0.36. In addition, pyrenoline, 0.58, was separated from 7,9-dimethylbenz(*c*)acridine, 0.87, and dibenz(*a,h*)acridine, 0.83, was separated from 14-phenyldibenz(*a,j*)acridine, 0.46.

Comparison of the aromatic hydrocarbons with their aza derivatives clearly shows the effect of the attraction of silica gel for the aza nitrogen group. Where the aza nitrogen is sterically protected, the  $R_F$  value of the aza arene is approximately the same as that of the corresponding aromatic hydrocarbon; for example, dibenz(*a,c*)anthracene, 0.91 and dibenz(*a,c*)phenazine, 0.88; and dibenz(*a,h*)anthracene, 0.90 and dibenz(*a,h*)acridine, 0.83. Where the aza nitrogen is not sterically protected, the aza compound is strongly attracted to the silica gel and the  $R_F$  value is low, as shown in a comparison of the  $R_F$  values of the aza compounds with those of the analogous aromatic hydrocarbon compounds: for example, pyrenoline, 0.58, and benzo(*a*)pyrene, 0.90; acenaphtho(1,2-*b*)quinoxaline, 0.50, and benzo(*h*)fluoranthene, 0.90; acenaphtho(1,2-*b*)pyridine, 0.25, and fluoranthene, 0.92; benzo(*l,m,n*)phenanthridine, 0.22, and pyrene, 0.91. Another nitrogen in the molecule, as in the phenazines, weakens the attraction of the aza nitrogen for the silica gel. Thus, anthracene, phenazine, and acridine give  $R_F$  values of 0.93, 0.55 and 0.38.

The decreased attraction is probably due to the decreased basicity of the phenazine as compared to the analogous monoaza compound. For example, acridine,  $pK_a$  5.60, is more than 10,000 times stronger as a base than is phenazine,  $pK_a$  1.23. Thus, the electron densities at the phenazine nitrogens are decreased and the attraction of their nitrogens for the silica gel is considerably weakened, although some attraction is apparent. The remainder of the phenazines with their high  $R_F$  values definitely show this effect.

A few of the aza compounds that remained at the origin include adenine, adenosine, purine, theophylline and caffeine. These compounds contain NH and C=O groups, which are attracted more strongly to silica gel than is the aza nitrogen group. The non-fluorescent compounds were located by using the silica gel F254 plate and pentane-ether (7:3, v/v) as the developer. Neither the compounds nor the plate were fluorescent under long U.V. light. Under low U.V. light, all the compounds appeared at the origin as dark non-fluorescent spots against the green fluorescent background of the plate.

In an attempt to further the characterization of the aza arenes on a silica gel chromatogram, we investigated the fluorescence quenching effects of a non-volatile quencher in the developer<sup>6</sup>. Pentane-nitrobenzene-triethylamine (80:20:0.01, v/v/v) was used as the developer for the silica gel plate, and 3 h was required for the solvent front to travel 15 cm. The fluorescence of all the compounds, put on the plates in 0.1  $\mu$ g amounts, appeared to be quenched on both wet and dry plates. The compounds included benz(*c*)acridine, acridine, pyrenoline, phenanthridine, 9,12-dimethylbenz(*a*-

acridine, 14-phenyldibenz(*a,j*)acridine, indeno(1,2,3-*i,j*)isoquinoline, dibenz(*a,h*)-acridine, acenaphtho(1,2-*b*)pyridine, dibenz(*a,j*)acridine, benzo(*f*)quinoline, benzo(*h*)-quinoline, benz(*a*)acridine, and 7-phenyldibenz(*c,h*)acridine. After the plates were sprayed with trifluoroacetic acid fumes, the fluorescence colors were not intense (detection limits were about 0.05  $\mu\text{g}$ ); the compounds that remained quenched were pyrenoline, phenanthridine, indeno(1,2,3-*i,j*)isoquinoline, acenaphtho(1,2-*b*)pyridine, benzo(*h*)quinoline, and benzo(*f*)quinoline. Separation was similar to that obtained with the pentane-ether (7:3, v/v) developer; however, the separation of 7-phenyldibenz(*c,h*)acridine, benz(*c*)acridine, and dibenz(*a,h*)acridine was improved and benz(*a*)acridine and acridine remained at the origin.

The column chromatographic system discussed in this paper was used for the separation and determination of a standard mixture of five aza heterocyclic hydrocarbons. A mixture containing 1 mg each of 7-phenyldibenz(*c,h*)acridine, dibenz(*a,h*)acridine, benz(*a*)acridine, benzo(*h*)quinoline, and benzo(*f*)quinoline was chromatographed on the silica gel column. As indicated in Fig. 3, the largest molecule comes off the column first. This is because the aza nitrogen is most sterically hindered in this molecule. The silica gel adsorbent has a strong attraction for the aza nitrogen. The relative positions of the aza heterocyclic compounds can be affected by changes in the developer and in the moisture content of the adsorbent, by the availability of the aza

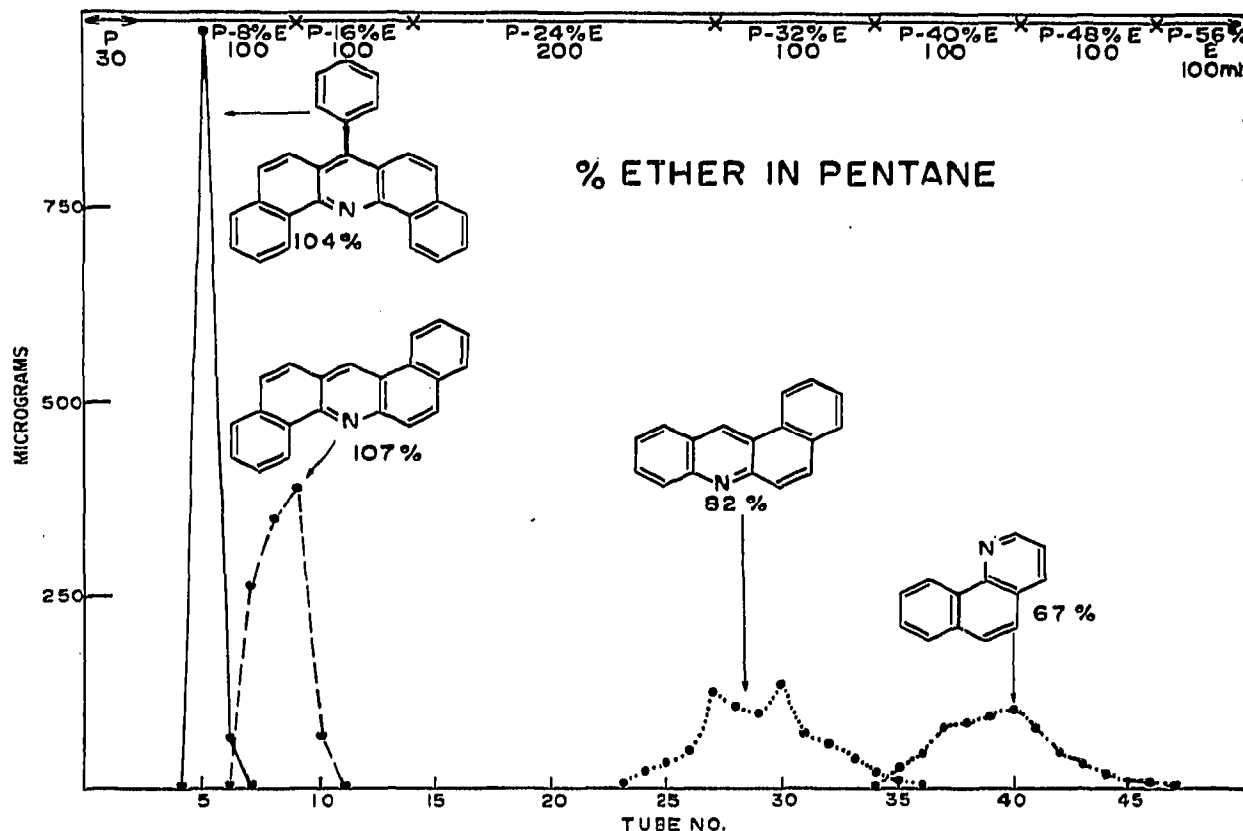


Fig. 3. Elution of 7-phenyldibenz(*c,h*)acridine, dibenz(*a,h*)acridine, benz(*a*)acridine, and benzo(*h*)quinoline on a silica gel column. All compounds put on in 1 mg amounts. The percent recovery calculated for each compound is shown near the appropriate structure. Benzo(*f*)quinoline was also put on but was not eluted.

nitrogen to the adsorbent and the solvent, and by the relative size of the molecules being separated. These factors explain why the benz(*a*)acridine ran before the benzo(*h*)quinoline on the column but the order was reversed on the thin-layer plate. Benz(*a*)acridine and benzo(*h*)quinoline appear to be the most sensitive to conditions in the system. The benz(*c*)acridine would be eluted between dibenz(*a,h*)acridine and benz(*a*)acridine, as shown by our separation of various basic fractions, discussed later. The benzo(*f*)quinoline was not eluted from the column even though acetone and methanol were passed through the column. The ultraviolet-visible absorption spectrum of each tube was then determined. By use of the appropriate bands in their spectra, the percent recovery of the aza compounds in the standard mixture was estimated by the baseline technique<sup>2</sup>. These values are also shown in Fig. 3.

#### APPLICATION

The separation of a basic fraction<sup>2</sup> of coal-tar pitch is shown in Fig. 4. The adsorbent was silica gel and the developer was pentane-ether (7:3, v/v). The standard

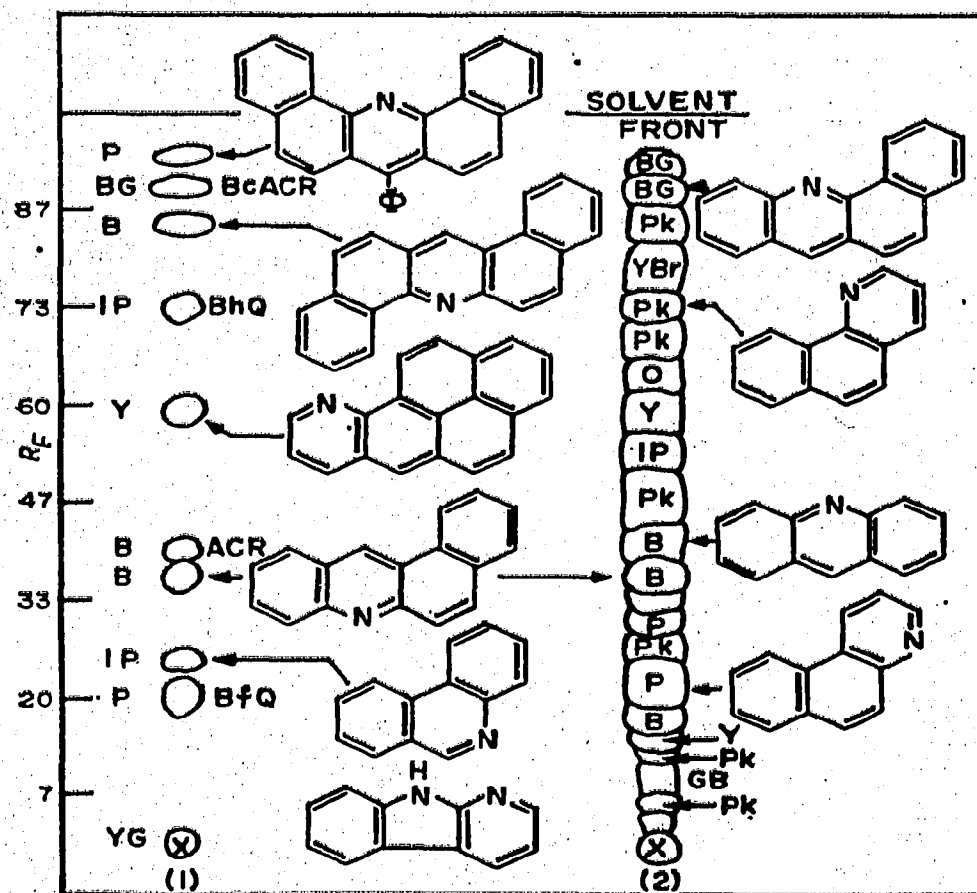


Fig. 4. Thin-layer chromatogram on silica gel with pentane-ether (7:3, v/v) as developer. Standard compounds on left present in 0.1  $\mu\text{g}$  amounts. Reading from top to bottom: (1) 7-phenyldibenz(*c,h*)acridine, benz(*c*)acridine, dibenz(*a,h*)acridine, benzo(*h*)quinoline, pyrenoline, acridine, benz(*a*)acridine, phenanthridine, benzo(*f*)quinoline, and 9H-pyrido(2,3-*b*)indole; (2) basic fraction equivalent to 200  $\mu\text{g}$  of coal-tar pitch. The fluorescence colors of the compounds are shown. For color abbreviations see footnote b to Table I.



compounds were put on the plate in  $0.1 \mu\text{g}$  amounts, and the amount of basic fraction put on was equivalent to  $200 \mu\text{g}$  of coal-tar pitch. By the relative  $R_F$  values and fluorescence colors, the following aza heterocyclic compounds were identified in the basic fraction: benz(*c*)acridine, benzo(*h*)quinoline, acridine, benz(*a*)acridine, and phenanthridine.

Two-dimensional thin-layer chromatography, especially with mixed adsorbents, followed by direct fluorimetric examination, has been found invaluable in characterizing a large variety of polynuclear air pollutants<sup>7</sup>. Two of the developers used successfully in the basic fraction separation were pentane-ether (9:1, v/v) and dimethylformamide-water (35:65, v/v)<sup>8</sup>. Used in conjunction, these solvent systems give even better separation. For our investigation the amount of basic fraction equivalent to 2 mg of coal-tar pitch was put on a silica gel-cellulose (2:1) plate. A standard mixture of  $0.2 \mu\text{g}$  each of benz(*c*)acridine, 11H-indeno(1,2-*b*)quinoline, acridine, dibenz(*a,j*)acridine, and benzo(*f*)quinoline was also put on the plate. The first dimension was developed by pentane-ether (9:1, v/v), which took about 3 h to travel 13 cm. The plate was allowed to dry, and the fluorescence colors of the standards were marked. The standard mixture was again put on the plate and was developed in the second

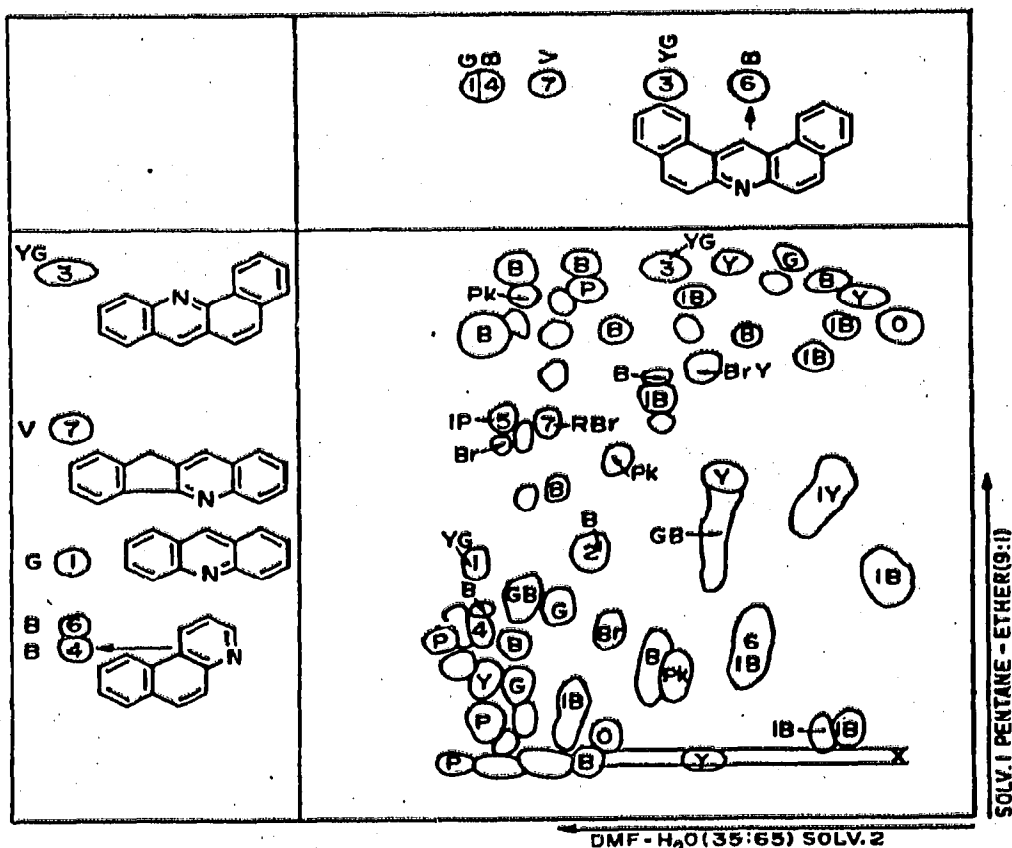


Fig. 5. Two dimensional thin-layer chromatographic separation on silica gel-cellulose (2:1) of basic fraction equivalent to 2 mg of coal-tar pitch and the characterization of seven spots. Standards put on in  $0.2 \mu\text{g}$  amounts: (1) acridine; (2) benz(*a*)acridine; (3) benz(*c*)acridine; (4) benzo(*f*)quinoline; (5) benzo(*h*)quinoline; (6) dibenz(*a,j*)acridine; (7) 11H-indeno(1,2-*b*)quinoline. The plate was sprayed with trifluoroacetic acid fumes and the fluorescence colors marked. For color abbreviations see footnote b to Table I.

dimension by dimethylformamide-water (35:65, v/v), which took about 4 1/2 h to travel 13 cm. The plate was allowed to dry, and the fluorescence colors were marked after it had been sprayed with trifluoroacetic acid fumes. Fig. 5 shows that benz(*c*)-acridine, acridine, benzo(*f*)quinoline, dibenz(*a,j*)acridine, and 11H-indeno(1,2-*b*)-quinoline can be identified in the basic fraction by use of the  $R_F$  values obtained in the two different solvent systems. Similarly, benzo(*h*)quinoline and benz(*a*)acridine were identified by developing these standards two-dimensionally on a similar plate with the basic fraction. All of these compounds have been identified previously by another system of separation and direct fluorimetric examination<sup>5</sup>. It must be emphasized that because this mixture was extremely complicated, each spot contains several compounds. For this reason fluorimetric characterization is necessary for any separation or assay method involving these compounds.

The column chromatographic procedure described earlier was applied to a complicated mixture, the basic fraction of a sample of coal-tar pitch. Twenty to fifty mg of the basic fraction was put on the column and the elution procedure followed. The absorption spectrum in pentane was then obtained for each tube. Spectral examination of a basic fraction of coal-tar pitch indicated the presence of 760  $\mu\text{g}$  of benz(*c*)acridine and 410  $\mu\text{g}$  of benz(*a*)acridine per gram of original sample. Previous work with a somewhat analogous sample<sup>2</sup> gave values of 600 and 1000  $\mu\text{g}$  of benz(*c*)acridine and benz(*a*)acridine, respectively, per gram of an airborne particulate sample polluted with coal-tar pitch. These values include the alkylated derivatives. Obviously the separation of alkyl derivatives needs to be studied. The difficulty is that alkyl derivatives are not available. Because of its differences from the alumina column chromatographic method, we believe that the silica gel method merits further development and study.

In conclusion, we recommend that the silica gel thin-layer chromatographic method be used for the improved separation of the aza arenes and that it be applied to air pollution analysis.

#### SUMMARY

A thin-layer chromatographic method for separation of polynuclear aza heterocyclic compounds with silica gel is presented which is superior to previously reported paper and thin-layer chromatographic methods. Many of the groups of compounds had run together in previous separation methods. This procedure has been applied to the separation of various basic fractions of interest in air pollution studies. Benz(*c*)acridine, benzo(*h*)quinoline, acridine, benz(*a*)acridine, and phenanthridine can be separated and identified in these samples with the help of two-dimensional thin-layer chromatography on silica gel-cellulose (2:1).

In addition, a column chromatographic separation of a basic fraction of coal-tar pitch, with silica gel as the adsorbent, was investigated, and the amounts of benz(*c*)acridine and benz(*a*)acridine were estimated. Evidence obtained from the absorption spectra indicates the presence of a large number of unknown and previously identified compounds in the fractions.

The various silica gel methods are recommended for use in air pollution studies.

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