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Two-dimensional Dual-band Thin-layer Chromatographic Separation of Polynuclear Hydrocarbons

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A two-dimensional dual-band thin-layer chromatographic procedure for the separation of a mixture containing many polynuclear hydrocarbons is described. A chromatoplate composed of two adsorbent layers, aluminum oxide G and acetylated cellulose, is used in this chromatography. A sample containing various polynuclear hydrocarbons is first developed on an aluminum oxide G layer with *n*-hexane - ether (19 : 1, v/v) and then on an acetylated cellulose layer with methanol-ether-water (4 : 4 : 1, v/v). A mixture of 15 polynuclear hydrocarbons was completely separated into its components by this treatment. This method was then applied to the separation of a vacuum-sublimation extract of particulates obtained from Tokyo air. The extract was separated into 69 spots, and it was proved that several hydrocarbons, including benzo(*a*)pyrene, has been isolated in a pure state.

The separation and determination of polynuclear hydrocarbons including benzo(*a*)pyrene, a potent carcinogen, in urban air pollutants is of importance in relation to the rapid increase in lung-cancer mortality in many countries in the world within the last 30 years. Thin-layer chromatography has recently attracted much attention in connection with the separation of the hydrocarbons, and several methods have been reported.[1-12] However, these methods do not seem to give a satisfactory separation for a sample containing many polynuclear hydrocarbons, such as an extract from air pollutants. For example, thin-layer chromatography using aluminum oxide G as the adsorbent and *n*-hexane-ether (19 : 1,v/v) as the developing solvent gives a good separation of a mixture of phenanthrene, pyrene, perylene, and dibenzo(*a,h*)anthracene, but it gives only a poor separation for a mixture of benzo(*a*)pyrene, benzo(*b*)fluoranthene, perylene, and benzo(*e*)pyrene. On the other hand, chromatography using a partially-acetylated cellulose as the adsorbent and methanol-ether-water (4 : 4 : 1,v/v) as the developing solvent gives a good separation for the latter mixture, but a poor separation for the former.

This paper is concerned with a new method for the two-dimensional thin-layer chromatographic separation of a complex mixture of polynuclear hydrocarbons. The thin-layer chromatoplate in the present method is composed of two adsorbent layers, aluminum oxide G and acetylated cellulose. The former layer is used in the first development, and the latter, in the second development.

Experimental

Hydrocarbons. The polynuclear hydrocarbons tested were anthracene, phenanthrene, pyrene, benzo(*a*)anthracene, chrysene, benzo(*e*)pyrene, benzo(*b*)-fluoranthene, benzo(*a*)pyrene, perylene, anthanthrene, benzo(*ghi*)perylene, dibenzo(*a,h*)anthracene, coronene, dibenzo(*a,i*)pyrene, and benzo(*k*)fluoranthene. The benzo(*b*)fluoranthene was a kind gift to us from Dr. B. T. Commins (MRC Air Pollution Research Unit, St. Bartholomew's Hospital Medical College, London). The benzo(*e*)pyrene and benzo(*k*)fluoranthene were kindly presented by Professor M. Kuratsune (Kyushu University, Japan). The other hydrocarbons were obtained from the Koch-Light Co., Ltd., London.

Adsorbents. Aluminium oxide G acc. to Stahl (E. Merck) and 26% acetylated cellulose were used

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[1] E. Sawicki, T. W. Stanley, W. C. Elbert and J. D. Pfaff, *Anal. Chem.*, **36**, 497 (1964).

[2] G. M. Badger, J. K. Donnelly and T. M. Spotswood, *J. Chromatog.*, **10**, 397 (1963).

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[6] H. Matsushita, Y. Suzuki and H. Sakabe, This Bulletin, **36**, 1371 (1963).

[7] H. Kessler and E. Müller, *J. Chromatog.*, **24**, 469 (1966).

[8] R. G. Harvey and M. Halonen, *ibid.*, **25**, 294 (1966).

[9] R. H. White and J. W. Howard, *ibid.*, **29**, 108 (1967).

[10] M. Frank-Neumann and P. Jössang, *ibid.*, **14**, 280 (1964).

[11] E. Sawicki, T. W. Stanley, W. C. Elbert, J. Meeker and S. McPherson, *Atomospheric Environ.*, **1**, 131 (1967).

[12] J. Janák and V. Kubecová, J. *Chromatog.*, **33**, 132 (1968).

as the adsorbents. The latter adsorbent was prepared by the acetylation of cellulose powder by the method of Wieland, Lüben, and Determann.[3] Camag's cellulose powder D for thin-layer chromatography was dried at 110°C for 30 min and then cooled in a desiccator containing concentrated sulfuric acid. The dried cellulose powder (30 g) was then acetylated in a mixed solution of benzene (675 m*l*), acetic anhydride (225 m*l*), and concentrated sulfuric acid (0.9 m*l*). Twenty-six percent acetylated cellulose was obtained by the reaction at 70°C for 9 hr. The cellulose was washed by methanol and ether and dried completely in an electric furnace at about 80°C.

Applicator and Coating. A chromatoplate with two adsorbent layers of aluminium oxide G and acetylated cellulose was prepared by the use of the applicator shown in Fig. 1. This is analogous to the applicator devised by Gilmore and Cortes.[13] The trough of the applicator (20 cm × 3.6 cm) was divided into two parts by a strip of stainless steel inserted into the grooves of the front and rear gates of the applicator. One part of the divided trough had the dimensions of 16 cm × 3.6 cm, and the other part, 4 cm × 3.6 cm. The strip-end in contact with the glass plate was shaped like a knife edge.

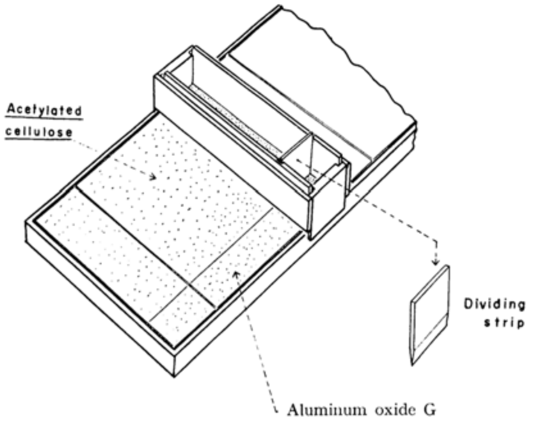

Fig. 1. Applicator for preparing dual band thin layer chromatoplate.

Slurries of the adsorbents were prepared by mixing them with a 10% methanol aqueous solution. The mixing ratio of the adsorbent to the methanol solution was 7 : 11 (g/m*l*) for aluminum oxide G and 10 : 45 (g/m*l*) for acetylated cellulose.

A slurry of aluminum oxide G was poured into the smaller part of the trough, and a slurry of acetylated cellulose, into the larger part of the trough. Then, they were immediately spread over glass plates (20 cm × 20 cm) in a thickness of 300μ. This gave a chromatoplate consisting of an aluminum oxide G layer (4 cm × 20 cm) and an acetylated cellulose layer (16 cm × 20 cm). The boundary line between the two adsorbent layers was very sharp. After having been left for about 3 hr for superficial drying at room temperature, the chromatoplate was activated by heating it at 110°C for 1 hr. The activated plate was then stored in a chamber kept at a relative humidity of about 20%. The humidity was easily controlled by putting a petri-dish containing a saturated potassium acetate aqueous solution on the bottom of the chamber.

Sample Application. A mixture of polynuclear hydrocarbons was applied to the lower left corner of the aluminium oxide G layer at a point 15 mm from the bottom of the layer and 10 mm from the layer boundary. The relative humidity of the atmosphere in the spotting box was kept at about 20% by putting a petri-dish containing a saturated potassium acetate aqueous solution into the box. The amounts applied to the layer were in the range of 0.03—0.4μg for each polynuclear hydrocarbon.

Development and Detection. The first development was carried out on the aluminum oxide G layer in a paper-lined chamber equilibrated with freshly-prepared *n*-hexane - ether (19 : 1, v/v) and also with a saturated potassium acetate aqueous solution. The development was continued until the front of *n*-hexane - ether (19 : 1, v/v) reached 150 mm. The developing time was about 35 min. The plate was then removed from the chamber, air-dried for about 5 min, rotated 90°, and inserted into the second chamber which had been equilibrated with freshly-prepared methanol-ether-water (4 : 4 : 1,v/v). The second development was continued until the solvent front reached 100 mm from the layer boundary. The developing time here was about 55 min.

The spots separated on the chromatoplate were detected by means of their fluorescence under ultraviolet light. All the polynuclear hydrocarbons except chrysene and phenanthrene were detected under long-wave ultraviolet light (365 mμ). Chrysene and phenanthrene were detected under short-wave ultraviolet light (253 mμ). The irradiation time of the short-wave ultraviolet light should be made as short as possible in order to prevent the photochemical decomposition of polynuclear hydrocarbons.

Results

Separation of Polynuclear Hydrocarbons. The R_f values of the 15 hydrocarbons are listed in Table 1. The hydrocarbons which had overlapped each other on the aluminum oxide G layer upon the first development were adequately separated by the second development on the acetylated cellulose layer. This is clearly demonstrated in Fig. 2.

It must be noted that the first development on the aluminium oxide layer should be carried out in a chamber kept at a constant relative humidity of about 20%, because the R_f values of the hydrocarbons are highly affected by the humidity of the atmosphere in a developing chamber.[14,15] Development at a higher or at a lower humidity gave an unsatisfactory separation of the polynuclear hydrocarbons, as is shown in Table 1.

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[13] D. R. Gilmore and A. Cortes, *J. Chromatog.*, **21**, 148 (1966).

[14] H. Matsushita and Y. Suzuki, *Ind. Health*, **4**, 59 (1966).

[15] Y. Suzuki and H. Matsushita, *ibid.*, **5**, 65 (1967).

TABLE 1. R_f VALUES OF POLYNUCLEAR HYDROCARBONS ON TWO-DIMENSIONAL DUAL BAND THIN LAYER CHROMATOGRAM*

No.	Substance	Aluminum oxide G			Acetylated cellulose		
		Relative humidity			No. of repeated development**		
		20%	7%	49%	1	2	3
1	Anthracene	0.55	0.31	0.64	0.54	0.61	0.66
2	Phenanthrene	0.54			0.48	0.56	0.60
3	Pyrene	0.49	0.21	0.61	0.43	0.51	0.54
4	Benzo(*a*)anthracene	0.37	0.08	0.56	0.35	0.43	0.46
5	Chrysene	0.35			0.20	0.26	0.29
6	Benzo(*e*)pyrene	0.27			0.47	0.55	0.58
7	Benzo(*a*)pyrene	0.27	0.04	0.51	0.13	0.18	0.20
8	Perylene	0.26			0.39	0.47	0.50
9	Benzo(*k*)fluoranthene	0.25			0.28	0.34	0.37
10	Benzo(*b*)fluoranthene	0.25			0.19	0.25	0.28
11	Anthanthrene	0.18			0.19	0.24	0.27
12	Benzo(*ghi*)perylene	0.17	0.02	0.46	0.52	0.59	0.64
13	Dibenzo(*a,h*)anthracene	0.14			0.42	0.50	0.53
14	Coronene	0.10			0.58	0.66	0.71
15	Dibenzo(*a,i*)pyrene	0.07	0.01	0.36	0.28	0.35	0.38

* R_f values were mean of 3 determinations. Deviation of each R_f value from the mean value was within 0.03. Developing temperature was 18±2°C.

** Repeated development was carried out on acetylated cellulose layer with intermittent drying of 10 min in a room.

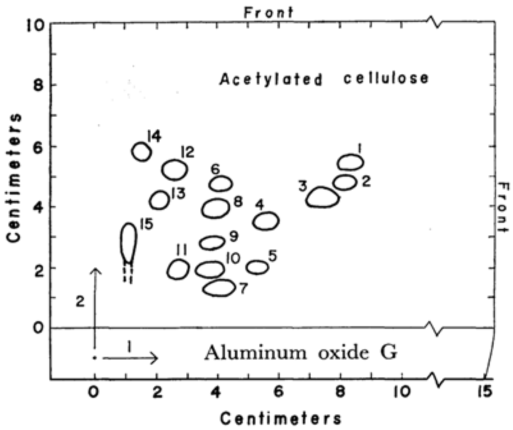

Fig. 2. Two-dimensional dual band thin layer chromatogram of polynuclear hydrocarbons.
The number of each spot corresponds to that of polynuclear hydrocarbon cited in Table 1.

In the second development, spots of polynuclear hydrocarbons on the aluminum oxide G layer migrated rapidly with a rise in the solvent front, were deformed to a very small shape like a hairline on the layer boundary, and then were further separated into several spots on the acetylated cellulose layer. Since the spots separated on the acetylated cellulose layer took small, oval shapes, a high separability and detectability were obtained.

Separation of an Extract from Air Pollutants. The present method was used in the separation of an extract from air pollutants in Tokyo. Suspended particulates in Tokyo air were collected on a glass fibre filter (Gelman A, Gelman Instrument Co.) using a high-volume air sampler (Staplex Co.). The polynuclear hydrocarbons in

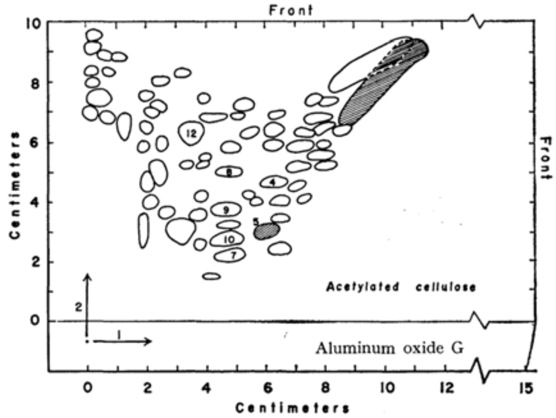

Fig. 3. Two-dimensional dual band thin layer chromatogram of vacuum sublimation extract from suspended particulates in Tokyo air.
Separation on acetylated cellulose layer was carried out by developing the plate three times to the 10 cm mark in methanol-ether-water with intermittent drying of 10 min in a room. The number indicated for several sports corresponds to that of polynuclear hydrocarbons cited in Table 1. The spot with oblique lines is that detected by short-wave ultraviolet light irradiation.

the particulates were extracted by vacuum sublimation, which had been proved to be more advantageous than solvent extraction for the extraction of polynuclear hydrocarbons.[16-18] The vacuum sublimation was carried out for about 30 min under conditions of 300°C and 0.007 mmHg for the particulates (185.5 mg) collected on a glass fibre filter. The sublimate which deposited on the narrow range of the inner surface of the sublimation tube (2 mm in inner diameter)was easily dissolved by 180 μl of benzene inserted into the tube by a microsyringe. Two microliters of the solution were used for the separation by the present thin-layer chromatography. Figure 3 shows the chromatogram for the sublimation extract of particulates in Tokyo air. Sixty-nine spots were detected on the chromatogram by means of their fluorescence under long- or short-wave ultraviolet light. A spot containing benzo(*a*)pyrene which had been obtained by the first development was further separated into 9 spots by the second development. The fluorescence and excitation spectra of the benzene extract of the spot corresponding to benzo(*a*)pyrene were entirely coincident with the spectra of the pure benzo(*a*)pyrene solution, as is shown in Fig. 4. Other polynuclear hydrocarbons, such as benzo(*b*)-fluoranthene, benzo(*k*)fluoranthene, perylene, benzo(*ghi*)perylene, chrysene, and benzo(*a*)anthracene, were also proved to be isolated in a pure state from the sublimation extract by spectrofluorometry.

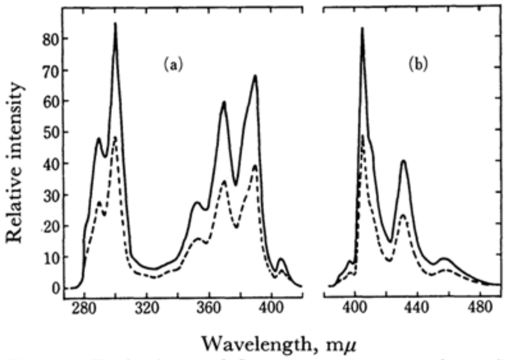

Fig. 4. Excitation and fluorescence spectra of spot 7 and benzo(*a*)pyrene solutions.
(a) Excitation spectra; (b) Fluorescence spectra.
—— Benzene extract of spot 7.
------ Benzo(a)pyrene benzene solution.

Discussion

The present chromatography has some advantages for the separation of polynuclear hydrocarbons over the hitherto-reported thin-layer chromatographies which use a single adsorbent layer.[1-12] The separation efficiency is high, since spots of the hydrocarbons are scattered all over the acetylated cellulose layer in the present method. The high separation efficiency comes from the combined use of the two adsorbent layers which are different in separation characteristics. Another advantage of the present method is the high sensitivity. For example, the minimum detectable value of benzo(*a*)pyrene is 0.001 μg or less in the present method, while the value is 0.007 μg in the one-dimensional chromatography using silica gel as the adsorbent layer and *n*-hexane - *o*-dichlorobenzene - pyridine (10 : 1 : 0.5,v/v) as the developing solvent.[6] This high sensitivity comes from the combined use of two different chromatographies; adsorption chromatography in the first and partition chromatography in the second development. The adsorption force of aluminium oxide G for the hydrocarbons is very much weakened by the second developing solvent, which contains water as one component, as may easily be understood from the facts that the R_f values of the hydrocarbons on the aluminum oxide layer are highly affected even by the moisture content in a developing chamber and increase remarkably with an increase in the moisture content.[14,15] For these reasons, the spots on the aluminum oxide G layer are rapidly transferred to the boundary between the two adsorbent layers as very small, hairline-like spots and then separated into components on the acetylated cellulose layer with the rise of the second developing solvent. The hairline-like spots cause very small oval spots on acetylated cellulose after the second development, resulting in the enhancement of the sensitivity in the detection of polynuclear hydrocarbons.

Furthermore, the fluorescence intensities of the hydrocarbons on the acetylated cellulose layer are not much reduced even after having been left for a prolonged period in a dark room, although the fluorescences on the aluminum oxide layer change in color and are reduced remarkably in intensity by the same treatment.

In the present method, the two adsorbents should be so selected as to equalize approximately the moving speeds of the first developing solvent on the two layers. If the moving speeds differ markedly, the spots of hydrocarbons on the aluminum oxide layer will be found on a heavily-curved line after the first development, as is illustrated schematically in Fig. 5. This often has an adverse effect on the separation. For this reason, we selected acetylated cellulose prepared from cellulose D (Camag) as the adsorbent in the second development. Acetylated cellulose prepared from cellulose D-O (Camag) and MN 300 acetylated cellulose (M. Nagel) were not suitable for the adsorbent in this method, because the moving speeds of the first developing solvent on these cellulose layers

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16) W. L. Ball, G. E. Moore, J. L. Monkmann and M. Katz, *Amer. Ind. Hyg. Assoc. J.*, **23**, 222 (1962).

17) H. Sakabe, *Proc. Roy. Soc. Med.*, **57**, 1005 (1964).

18) H. Arito, R. Soda and H. Matsushita, *Ind. Health*, **5**, 243 (1967).

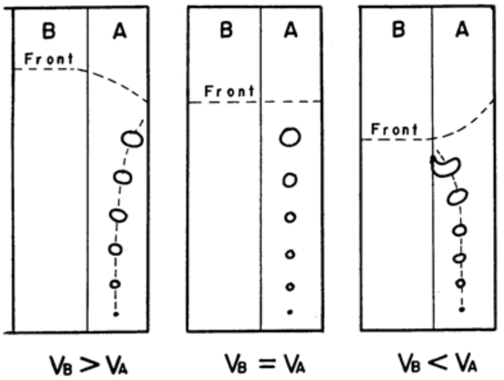

Fig. 5. Schematic representation of the 1st dimensional chromatograms.
A and B indicate aluminum oxide G and acetylated cellulose, respectively. V_A and V_B indicate the moving speed of developing solvent on A and B layers.

were considerably higher than that on the aluminum oxide G layer.

In an accurate determination of the benzo(*a*)pyrene in air pollutants, it should first be isolated completely from the other hydrocarbons. However, the benzo(*a*)pyrene fraction obtained by the column, paper or thin-layer chromatography, which is ordinarily used, is frequently contaminated by other hydrocarbons, some of which interfere with an accurate determination of the benzo(*a*)pyrene. The benzo(*a*)pyrene fraction obtained by the present chromatography was completely isolated from such hydrocarbons as benzo(*k*)fluoranthene, benzo(*e*)pyrene, benzo(*b*)fluoranthene, and perylene, and proved to be spectrofluorometrically pure. Thus, the present method may also make possible an accurate determination of the benzo(*a*)pyrene in air pollutants.