THIN-LAYER CHROMATOGRAPHY OF POLYCYCLIC AROMATIC HYDROCARBONS

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INTRODUCTION

The applications of thin-layer chromatography (TLC) to the separation of polycyclic aromatic hydrocarbons have been reviewed by several investigators[1,2]. Various absorbents including silica gel, alumina, cellulose, and cellulose acetate with different types of developing solvents have been evaluated for this purpose. In general, it can be stated that there is no universal or one method which is superior to another. The choice of a particular system or systems will depend upon the hydrocarbons to be separated and the chemical characteristics of the background material from which they are to be isolated. It is often found that where one system fails for a specific group of hydrocarbons, another may prove satisfactory. Consequently, because of the wide variety of polycyclic aromatic hydrocarbons it may be necessary to resort to several different systems to achieve the desired separations.

During the past few years we have been concerned with the development of sensitive, practical analytical methods for the determination of polycyclic aromatic hydrocarbons in foods[3,4]. In general, these procedures consisted of an initial extraction of the hydrocarbons, followed by a partition step between dimethyl sulfoxide and an aliphatic solvent. Column chromatography followed by paper and thin-layer separation is then used to reduce interfering background material and isolate the polycyclic compounds. Depending on the type of product under analysis and the specific hydrocarbons to be isolated, it may be necessary to employ all of these techniques to obtain satisfactory ultraviolet and spectrophotofluorometric spectra for characterization and estimation of the compounds present. If only benzo[*a*]pyrene is to be determined, the procedure may be shortened considerably by using thin-layer chromatography on cellulose acetate directly without the lengthy paper chromatographic method. In our work on the specific polycyclic hydrocarbons in smoked foods[3], we attempted to shorten the method by substituting thin-layer chromatography for paper chromatography. An examination of various thin-layer techniques indicated that good results were obtainable on cellulose plates of 500 μ thickness when 35% N,N-dimethylformamide (DMF) in ethyl ether was used as the immobile solvent and iso-octane as the mobile solvent. For example, excellent separations and recoveries were attained when mixtures of standard solutions of pyrene, 4-methylpyrene, benz[*a*]anthracene, benzo[*a*]pyrene, dibenz[*a*,*h*]anthracene, and benzo[*g*,*h*,*i*]perylene were applied to the plate. In the actual isolation of the extracted hydrocarbons from food, however, the

bands were close together or overlapping, and problems were encountered in quantitatively recovering the compounds from the plate. It was also noted that the higher condensed ring compounds (4-, 5- and 6-ring types) were localized near the starting line in the presence of small amounts of extraneous background material. In subsequent studies with refined vegetable oils[5], these difficulties were overcome by altering the concentration of DMF to 20%, thereby achieving good overall separation. This report describes the separation of polycyclic hydrocarbons by this technique of reverse phase thin-layer chromatography.

MATERIALS

Apparatus

Thin-layer chromatography apparatus. (1) Glass plates, 20 × 20 cm (25–10–11); (2) standard adjustable applicator, model S-II (25–09–00); (3) Plexiglass mounting board, standard size for plates up to 20 cm wide (04-10-00); (4) drying rack, with 10 shelves for 20 × 20 plates (25-09-15); (5) standard, rectangular developing tank, 22 cm deep × 8.5 cm wide × 20.5 cm long (25–10–22); (6) stainless steel desiccating storage cabinet, 30 cm wide × 25 cm deep (25-09-40) (Desaga/Brinkmann Instruments, Inc., Westbury, N.Y., or equivalent).

Dipping tank. Type 303, capacity 370 ml, inside dimensions 8 3/8 × 3/16 × 8 3/8″ equipped with cover (3106-H50) (A. H. Thomas Co., Phila., Pa., or equivalent).

Ultraviolet equipment. (1) Lamps: longwave, 3660 Å; shortwave, 2537 Å; and (2) Chromato-Vue Cabinet (Ultraviolet Products, Inc., San Gabriel, Calif., or equivalent).

Evaporation flasks. 250 ml capacity all-glass flasks (K-61725), with 24/40 S/T stopper (K-33175) having inlet and outlet tubes to permit passage of nitrogen across the surface of contained liquid to be evaporated. The inlet tube of the stopper used to convey the nitrogen is cut off 2 cm below the joint, and the outlet tube is constricted at the end and bent downward at a 45° angle to prevent flow-back of the condensate into the flask (Kontes Glass Co., Vineland, N.J., or equivalent).

Pressure filter. 30 ml capacity, fine porosity filter (K-95500) modified to include a 24/40 S/T outer joint and an adapter equipped with a 24/40 S/T inner joint (K-18300) for connection to a tank of nitrogen (Kontes Glass Co., or equivalent).

Recording spectrophotometer and accessories. (1) Cary 11 (Applied Physics Corp., Monrovia, Calif., or equivalent). (2) Cells: (a) fused rectangular quartz cells, optical path length 10 ± 0.005 mm, 1.5 ml capacity (5-503 QS); (b) fused quartz cells, optical path length 50 ± 0.05 mm (2-228 Q), tolerance A (Optical Cell Co., Inc., Brentwood, Md., or equivalent).

Blender and accessories. Waring Blender, Model LB-1 (Waring Products Co., Winsted, Conn., or equivalent).

Reagents

All the reagents purified by distillation were distilled with an air-cooled reflux condenser (about 300 mm long) between the reservoir and the water-cooled condenser. The solvents were distilled in 2 l lots; the first 200 ml distillate was discarded and the next 1600 ml collected for use.

Iso-octane and benzene were purified to meet the specifications of the following test:

To the specified quantity of solvent in a 250 ml evaporation flask, 1 ml of purified *n*-hexadecane was added and the container was placed on the steam bath. The tube assembly was inserted; the inlet tube was connected to the nitrogen supply and the outlet tube to the solvent trap and vacuum line. Evaporation was discontinued when 1 ml of residue remained. (To the benzene residue 10 ml of purified iso-octane was added and the solution was re-evaporated. This procedure was repeated to insure the complete removal of benzene.) The 1 ml of hexadecane residue was dissolved in iso-octane and the volume was adjusted to 25 ml. The absorbance was determined in the 5 cm path length cells compared to iso-octane as reference. The absorbance of the solution of the solvent residue should not exceed 0.01 per cm path length between 280 and 400 mμ.

Iso-octane (2,2,4-trimethylpentane). Purified by distillation or by passage through a column of activated silica gel (Grade 12, Davison Chemical Co., Baltimore, Md., or equivalent) about 90 cm long and 5–8 cm diameter. 180 ml was used for the test described in the previous paragraph.

Benzene. ACS reagent grade. Purified by distillation and 160 ml was used for the test.

Methanol. ACS reagent grade. Purified as follows: 2 l of alcohol was refluxed with 10 g of KOH and 25 g of zinc dust for 3 h. Distillation was carried out with the air-cooled reflux condenser connected to a water-cooled condenser and the collection flask was provided with a drying tube to protect the distilled solvent from moisture.

50 ml of distilled methanol was placed in a 125 ml evaporation flask, 1 ml of *n*-hexadecane added, the tube assembly inserted, and evaporation to a 1 ml residue was carried out as previously described for benzene. 10 ml of purified iso-octane was added, the mixture re-evaporated, and then once again. The 1.0 ml of hexadecane was transferred into the 1 cm path length (total capacity 1.5 ml) and the ultraviolet spectrum recorded with iso-octane in the reference cell. The absorbance values should not exceed 0.03 per cm path length between 250 and 275 mμ, 0.015 between 275 and 300 mμ, 0.010 between 300 and 350 mμ, and 0.00 between 350 and 400 mμ.

n-Hexadecane. 99 % olefin-free. This is purified by percolation through a column of activated silica gel (Grade 12, Davison Chemical Co., or equivalent). 1 ml of *n*-hexadecane is transferred into the 1 cm path length cell (total capacity 1.5 ml) and the ultraviolet spectrum is recorded with iso-octane in the reference cell. The absorbance values should not exceed 0.02 per cm path length between 225 and 250 mμ, 0.010 between 250 and 275 mμ, and 0.00 between 275 and 400 mμ.

N,N-Dimethylformamide (DMF). Redistilled before use. (Matheson Co., Inc., East Rutherford, N. J., or equivalent.)

Ethyl ether. Analytical reagent.

Cellulose. Ultra Pure (MN300-HR, Brinkman Instruments, Inc.).

Polycyclic aromatic hydrocarbons. The compounds were obtained from various sources (see Acknowledgments). Purity of the compounds was checked by thin-layer chromatography before use.

Ethanol. USP grade. Redistilled before use.

Acetylated linters powder. 21 % acetylated (No. 124/21 ac, Schleicher and Schuell Co., Keene, N. H., or equivalent).

Toluene. Redistilled before use.

Developing solvents. (1) Cellulose plates: mobile phase, iso-octane; immobile

phase, 20% DMF in ethyl ether (v/v). (2) Cellulose acetate plates: mobile phase, ethanol–toluene–water (17:4:4, v/v/v).

EXPERIMENTAL

Preparation of plates

The adsorbent is prepared by placing 20 g of cellulose and 100 ml of water in a Waring blender and homogenizing at high speed for 3 min. With a thin-layer applicator, the slurry is applied to the plates (20 × 20 cm) with a thickness of 0.5 mm or 500 μ. (Five plates can be prepared with this amount of slurry.) After coating they are allowed to completely air-dry. Before use, each of the prepared plates is washed in a chromatographic tank by allowing the mobile solvent, iso-octane, to migrate to the top of the plate. The plate is removed from the tank and the excess iso-octane allowed to evaporate. The plates are then stored in a desiccator until needed.

General development procedure

50 ml of the mobile phase, iso-octane, is poured into the development chamber and allowed to equilibrate for at least 30 min. The polycyclic hydrocarbons are prepared in benzene and spotted at levels of 0.2–0.5 μg along the starting line drawn 1.5 cm from the bottom of the pre-washed plates. In addition, 0.5 μg of benzo[*a*]pyrene is also spotted on the plate. Using a glass funnel the dipping tank is then filled to 1/8 in. of the rim with the immobile phase (20% v/v DMF in ethyl ether). The plate is inverted and carefully immersed in the dipping tank to within 0.5 cm of the starting line of the spots. The plate is then removed from the tank and the excess immobile phase allowed to drain for about 15 sec. The plate is inverted and placed (with the starting line facing downward) in the developing chamber containing the mobile phase, iso-octane. The chromatogram is allowed to develop in the dark until the solvent front has reached the top of the plate (about 1.25 h). Then the plate is removed from the chamber and immediately examined under both long and short wave ultraviolet light in the Chromato-Vue Cabinet. With the aid of a labeling template, the position of each fluorescent spot is determined and the R_F value calculated.

Quantitative estimation of hydrocarbons

The fluorescent spots on the adsorbent are outlined in the Chromato-Vue Cabinet. The plate is removed from the cabinet and the adsorbent around the spots is scraped off with a spatula and discarded. Each outlined spot of the adsorbent is then collected in a 125 ml beaker and the polycyclic hydrocarbon is eluted by extracting with 5 to 10 ml portions of hot methanol until fluorescence under ultraviolet light can no longer be seen in the last portion of solvent. (Three or four extractions are ordinarily enough to remove the polycyclic aromatic compounds from the adsorbent.) The flask is swirled repeatedly during the extraction operation and the individual extracts successively filtered through the 30 ml pressure filter under nitrogen pressure into a 50 ml glass-stoppered Erlenmeyer flask. The combined methanol eluate is concentrated to 0.5 ml or less on a steam bath under nitrogen. (Do *not* evaporate to dryness!) The solution is then transferred to a 1 cm cell, the volume adjusted to 1 ml with methanol and the ultraviolet spectra recorded using methanol in the reference cell. Any maxima observed are compared with those in the spectra of known polycyclic aromatic hydro-

carbons. Estimation of the quantities of the identified hydrocarbons is made by the baseline technique in conjunction with spectra of the hydrocarbon standard solutions recorded under the same instrumental conditions. Identification of the compound is also confirmed by applying the technique of spectrophotofluorometry. *Note*: If the spectra indicated the presence of hydrocarbon mixtures or extraneous background absorbance material, the solution is transferred quantitatively with small portions of benzene to a 50 ml glass-stoppered Erlenmeyer flask. The solvent is evaporated on the steam bath under nitrogen to 0.5 ml or less. 5 ml of benzene is added and the solution again concentrated to 0.5 ml or less. The concentrate is reserved for thin-layer chromatography on cellulose acetate plates[4].

RESULTS AND DISCUSSION

The R_F values for 29 polycyclic aromatic hydrocarbons on cellulose (immobile phase, 20% DMF in ethyl ether; mobile phase, iso-octane) and cellulose acetate (ethanol–toluene–water) are reported in Table I. These data indicate that each of the

TABLE I

R_F VALUES[a] OF POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic hydrocarbon	*Cellulose*[b]		*Acetylated cellulose*[c]	
	Range	*Av.*	*Range*	*Av.*
7,12-Dimethylbenz[*a*]anthracene	0.71–0.77	0.74	0.49–0.51	0.50
4-Methylpyrene	0.64–0.72	0.70	0.67–0.72	0.70
5,6-Dimethylchrysene	0.68–0.71	0.69	0.41–0.43	0.42
3-Methylcholanthrene	0.67–0.71	0.69	0.53–0.61	0.57
Anthracene	0.63–0.67	0.65	0.53–0.60	0.57
12-Methylbenz[*a*]anthracene	0.62–0.64	0.63	0.34–0.35	0.34
7,8-Dimethylbenz[*a*]anthracene	0.62–0.64	0.63	0.42–0.42	0.42
Benzo[*c*]phenanthrene	0.59–0.63	0.62	0.52–0.56	0.54
1,2-Dihydrobenz[*e*]aceanthrylene	0.59–0.63	0.62	0.38–0.39	0.39
Cholanthrene	0.59–0.60	0.60	0.49–0.50	0.50
5-Methylchrysene	0.58–0.59	0.59	0.39–0.39	0.39
Pyrene	0.57–0.59	0.58	0.59–0.64	0.62
Fluoranthene	0.56–0.57	0.57	0.58–0.62	0.60
7-Methylbenz[*a*]anthracene	0.56–0.56	0.56	0.44–0.45	0.45
4-Methylbenzo[*a*]pyrene	0.50–0.52	0.51	0.37–0.37	0.37
Benz[*a*]anthracene	0.44–0.47	0.45	0.42–0.42	0.42
Chrysene	0.43–0.44	0.44	0.35–0.36	0.35
Triphenylene	0.43–0.44	0.44	0.48–0.50	0.49
Benzo[*a*]pyrene	0.39–0.41	0.40	0.23–0.23	0.23
Benzo[*k*]fluoranthene	0.39–0.41	0.40	0.39–0.41	0.40
Benzo[*e*]pyrene	0.37–0.40	0.39	0.55–0.59	0.56
Anthanthrene	0.35–0.37	0.36	0.32–0.33	0.32
Perylene	0.32–0.33	0.33	0.45–0.49	0.47
Benzo[*g,h,i*]perylene	0.32–0.33	0.33	0.50–0.52	0.51
Dibenz[*a,h*]anthracene	0.30–0.30	0.30	0.47–0.49	0.48
Dibenzo[*a,i*]pyrene	0.28–0.28	0.28	0.24–0.24	0.24
Dibenzo[*a,i*]phenanthrene	0.27–0.28	0.27	0.55–0.59	0.57
Dibenzo[*a,e*]pyrene	0.25–0.27	0.26	0.38–0.38	0.38
Dibenz[*b,i*]anthracene	0.18–0.19	0.19	0.51–0.56	0.54

[a] Average of five determinations.
[b] Solvent system: immobile phase, 20% DMF in ethyl ether; mobile phase, iso-octane.
[c] Solvent system: ethanol–toluene–water, 17:4:4 (v/v/v).

systems has inherent advantages in the separation of various polycyclic hydrocarbons.

The cellulose reverse phase system is more effective in separating the compounds into groups according to their ring structure. The cellulose acetate multiphase technique is superior in the separation of the individual 4-, 5- and 6-ring compounds. For example, the hydrocarbon pairs, benzo[*a*]pyrene and benzo[*e*]pyrene and benz[*a*]-anthracene and chrysene, difficult to separate on cellulose, were readily separated with the other system. When the two systems are used in conjunction with one another, only four of the 29 compounds studied could not be adequately separated for subsequent quantitative analysis, *viz.*, pyrene and fluoranthene and perylene and benzo-[*g,h,i*]perylene. In our experience, other available methods also fail to give sufficient separation of the first hydrocarbon pair for quantitative estimations. However, both pairs of compounds can be identified and estimated from the ultraviolet absorbance spectrum. As discussed in a previous report[3], either the baseline or the variable reference technique can be utilized advantageously. For the quantitative determination of the polycyclic aromatic hydrocarbons it is estimated that a difference in R_F units of 0.03 is required for the reverse phase system and 0.05 units for the multiphase system.

SAWICKI *et al.*[1], in a study of the application of thin-layer to the analysis of atmospheric pollutants, compared the following adsorbents and developers: alumina with pentane–ether (19:1, v/v); cellulose with DMF–water (1:1, v/v); and cellulose acetate with ethanol–toluene–water (17:4:4, v/v/v).

The procedure using alumina was found to yield poor separations of individual polycyclic hydrocarbons but was more effective than the other systems in isolating the compounds from organic fractions of airborne and air pollution source particulates. Since this adsorbent effectively separates classes of aromatic compounds, it has been recommended as a preliminary separative technique. The best overall separations of polycyclic hydrocarbons were obtained on cellulose plates with aqueous DMF as the mobile phase but this system worked poorly for the so-called "benzpyrene fraction," *e.g.* benzo[*a*]pyrene, benzo[*e*]pyrene, benzo[*k*]fluoranthene, and perylene[1]. Other compound pairs which may not be completely resolved, as evidenced by the closeness of the reported R_B values, include dibenz[*a,h*]anthracene and benzo[*g,h,i*]perylene, chrysene and pyrene, benz[*a*]anthracene and triphenylene, and phenanthrene and anthracene. The aforementioned system and other types where high percentages of water were utilized in the developing solvent have been studied in our laboratory. One of the disadvantages noted was the long developing time, 2–4 h. With the reverse phase system, the chromatogram is developed in about 1.25–1.5 h when the solvent front is allowed to migrate a distance of 18.5 cm. The cellulose acetate system was found by SAWICKI *et al.* to be the most effective in the separation of the "benzpyrene" fraction, whereas most other paper and thin-layer chromatographic procedures fail in these respects. This procedure has also been used after paper chromatography in our studies on the determination of polycyclic hydrocarbons in smoked foods[3] with excellent results.

GUNTHER AND BUZZETTI[2], in their review of thin-layer procedures, report that better resolution of polycyclic compounds is obtained with Silica Gel G than alumina. According to these authors, the efficiency of extraction of the hydrocarbons from both of these adsorbents for quantitative work is often poor. It is also pointed out that the possibilities of accelerated oxidation (air, ultraviolet light) must be considered when sensitive polycyclic aromatic hydrocarbons are adsorbed on active surfaces, such as

alumina. For example, SAWICKI *et al.*[1] reported recoveries of 50–80 % for benzo[*a*]-pyrene after thin-layer chromatography on alumina. With the cellulose and cellulose acetate systems described here, benzo[*a*]pyrene recoveries ranged from 93 to 98 %, thus demonstrating that quantitative recoveries can be obtained in the application and transferral of the compound from one plate to the other. Similar recovery values have been obtained for benz[*a*]anthracene, benzo[*g,h,i*]perylene, and dibenz[*a,h*]-anthracene. In the actual isolation of trace quantities of polycyclic aromatic hydrocarbons from food products, such as refined vegetable oils[5], the extracts are best resolved when applied to the cellulose adsorbent in a narrow streak rather than a spot. With this technique, more definitive separations are afforded and the background material extracted from the oils tends to move more rapidly and completely with the solvent front.

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SUMMARY

R_F values have been obtained for thin-layer chromatography of 29 polycyclic aromatic hydrocarbons with the following systems: cellulose (immobile phase, dimethylformamide in ethyl ether; mobile phase, iso-octane) and cellulose acetate (ethanol–toluene–water, 17:4:4, v/v/v). The cellulose reverse phase system more effectively separated the compounds into groups according to their ring structure. The cellulose acetate multi-phase technique was superior in separating the individual 4-, 5- and 6-ring compounds. This technique is rapid and has been successfully used in the preliminary separation of these hydrocarbons from vegetable oils.

REFERENCES

1 E. SAWICKI, T. W. STANLEY, W. C. ELBERT AND J. D. PFAFF, *Anal. Chem.*, 36 (1964) 497.
2 F. A. GUNTHER AND F. BUZZETTI, *Residue Rev.*, 9 (1964) 90.
3 J. W. HOWARD, R. T. TEAGUE, Jr., R. H. WHITE AND B. E. FRY, Jr., *J. Assoc. Offic. Agr. Chemists*, 49 (1966) 595.
4 J. W. HOWARD, R. H. WHITE, B. E. FRY, Jr. AND E. W. TURICCHI, *J. Assoc. Offic. Agr. Chemists*, 49 (1966) 611.
5 J. W. HOWARD, E. W. TURICCHI, R. H. WHITE AND T. FAZIO, *J. Assoc. Offic. Agr. Chemists*, 49 (1966) 1236.