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COMPARISON OF METHODS FOR SEPARATING POLYCYCLIC AROMATIC HYDROCARBONS BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A comparison is made of different stationary phases (silica gel, cellulose, acetylated cellulose, and 3-aminopropyl-, ethyl-, octyl-, octadecyl-, and diphenylsilanized silica gel), mobile phases, and development techniques (conventional, continuous, and multiple) for the separation of environmentally important polycyclic aromatic hydrocarbons by high-performance thin-layer chromatography. Reversed-phase separations using octadecylsilanized silica gel and methanol–water or methanol–water-based ternary solvent systems were found to give the best separations. A new development technique, multiple development with application of the mobile phase further up the plate at each successive development, provided superior resolution of the polycyclic aromatic hydrocarbons compared with conventional multiple development.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants. They enter the environment primarily as by-products of incomplete combustion of carbonaceous materials. As several PAHs are know or suspect indirect mutagens and carcinogens in animals and man, their presence and distribution in the environment is of some concern.

Polycyclic aromatic hydrocarbons are usually determined by gas or liquid chromatography after suitable sample cleanup[1]. These methods provide an accurate determination of individual PAHs but involve fairly lengthy and costly analytical procedures. To complement these methods, thin-layer chromatography (TLC) is useful for the rapid screening of environmental samples[1–5]. Improvements in materials and practices of thin-layer chromatography, generally known as high-performance thin-layer chromatography (HPTLC), have improved the sensitivity, speed, and separating power of this technique while preserving its traditional advantages of methodological simplicity, static sample detection, and high sample throughput[6–9]. Although numerous TLC methods have been described for the determination of individual PAHs and mixtures of PAHs from environmental extracts, there is a shortage

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of similar information for HPTLC. This is of some importance, since different stationary phase materials and development techniques are currently employed in HPTLC. In this paper a broad spectrum of HPTLC materials and techniques are compared for the separation of environmentally important PAHs.

EXPERIMENTAL

Anthracene (Ant), benzo[*a*]anthracene (B*a*A), benzo[*a*]pyrene (B*a*P), benzo [*e*]pyrene (B*e*P), benzo[*g,h,i*]perylene (BPer), chrysene (Chr), corenene (Cor), dibenzo[*a,h*]anthracene (DB*ah*Ant, or DBAnt), fluoranthene (Flt), fluorene (Flu), perylene (Per), phenanthrene (Phen), pyrene (Pyr), and triphenylene (Tri) were obtained from Aldrich (Milwaukee, WI, U.S.A.). Benzo[*b*]fluoranthene (B*b*Flt), benzo[*j*]fluoranthene (B*j*Flt), benzo[*k*]fluoranthene (B*k*Flt), and indeno[1,2,3-*c,d*]pyrene (In*cd*Pyr or InPyr) were obtained from the Community Bureau of Reference (Brussels, Belgium) and dibenzo[*a,i*]pyrene (DB*ai*Pyr or DBPyr) from Sigma (St. Louis, MO, U.S.A.). All solvents were HPLC grade (Burdick & Jackson, Muskegon, MI, U.S.A.). Cellulose and 3-aminopropylsilanized silica HPTLC plates were obtained from E. Merck (Darmstadt, F.R.G.). Silica gel and ethyl-, octyl-, octadecyl-, and diphenylsilanized silica plates were obtained from Whatman (Clifton, NJ, U.S.A.). Acetylated cellulose HPTLC plates were prepared by modification of the precoated cellulose HPTLC plates with benzene–acetic anhydride and 4-dimethylaminopiridine (Aldrich) as catalyst. The details are given below.

Each 10 × 10 cm cellulose HPTLC plate was precleaned by development in acetone and acetone–methanol (1:1) prior to drying in an oven at 110°C. The dried plate was carefully immersed in a 15-cm Petri dish containing 75 ml of benzene and 25 ml of acetic anhydride containing 4-dimethylaminopyridine (1 mg ml^{-1}). The Petri dish was placed in an evacuated vacuum desiccator (water aspirator) for 1 h. The plate was soaked in a series of acetone baths until no further yellow coloration was observed. The plate was then oven dried and predeveloped with acetone successively, until a stable densitometric baseline was obtained.

Sample volumes of 200 nl were applied to the plates by using fixed-volume Pt–Ir dosimeters (Applied Analytical Industries, Wilmington, NC, U.S.A.) in conjunction with a Nanomat HPTLC Spotter (Camag, Muttenz, Switzerland). For conventional development a twin-trough development chamber (Camag) was used. For continuous and multiple development a short-bed continuous development chamber (Regis, Morton Grove, IL, U.S.A.) was used.

In situ sample detection was performed by means of a Shimadzu CS-910 scanning densitometer (Shimadzu, Columbia, MD, U.S.A.). All measurements were made under optimized conditions described previously[10–13]. Peak profiles were recorded on a Shimadzu U-135 strip chart recorder.

RESULTS AND DISCUSSION

The renaissance in TLC has brought with it many changes in the practice of the technique. The most important of these are new types of stationary phases, sample application and development protocols, and *in situ* detection techniques. Scanning techniques, principally the sequential wavelength scanning for optimized detection

and identification[10] and the calibration methods[11] for PAHs, are treated adequately elsewhere. This report deals with the selection of the stationary phase, mobile phase, and development technique for the separation of environmentally important PAHs. We will consider the three areas separately, although, of course, they are not independent variables.

Selection of the stationary phase

Several commercially available stationary phases are now widely used in HPTLC. The separation of PAH standards on silica gel and reversed-phase HPTLC plates has been recorded[10,14–17]. No studies have appeared on the comparison of different stationary phases for the separation of a wide range of PAHs. To provide such a comparison, a nine-component test mixture (BPer, Cor, DB*ah*Ant, Per, B*a*P, B*a*A, Ant, Flt, and Pyr) was separated on different HPTLC plates under conditions judged to be the best that could be arrived at empirically.

Several studies already exist on the separation of PAHs on silica gel and were used to help define the optimum separation conditions[1,4,14,15,18–20]. The optimum separation of the test mixture was obtained using hexane–methylene chloride (10:0.1) as the mobile phase (Fig. 1A). In this solvent system only four peaks are observed, and pyrene is the only PAH isolated from the mixture. Poor reproducibility of separations unless the solvents were perfectly dry, limited selectivity, and quenching of the fluorescence signal[14] made the choice of silica gel for the separation of the PAHs unacceptable.

The 3-aminopropylsilanized plates[21] exhibited little retention of the PAHs in the reversed-phase mode. In the normal-phase mode the plate behaves similarly to

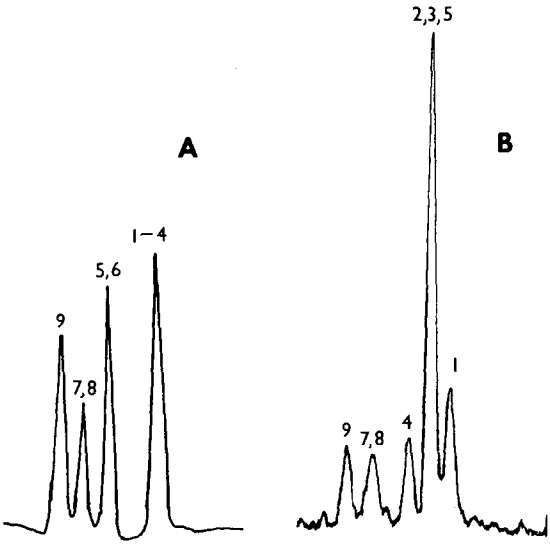

Fig. 1. Separation of PAH test mixture on silica gel (A) and 3-aminopropylsilanized silica (B) HPTLC plates. In both cases the mobile phase was hexane–methylene chloride (10:0.1). The silica gel plate was developed for three 5-min periods, and the aminopropylsilanized plate for one 5-min, one 8-min, one 10-min and one 13-min periods. Peaks: 1 = BPer; 2 = Cor; 3 = DB*ah*Ant; 4 = Per; 5 = B*a*P; 6 = B*a*A; 7 = Ant; 8 = Flt; 9 = Pyr.

silica gel but with some important selectivity differences (Fig. 1B). Note particularly the separation of BPer from Cor-DB*ah*Ant-B*a*P and the change in migration order of Per and B*a*P. In general, the mobility of the PAHs is high on these plates, which limits the possibility of obtaining adequate resolution.

Fibrous cellulose and acetylated cellulose TLC plates have been used for a number of years to resolve PAHs[1,22–29]. The separation properties of the microcrystalline HPTLC plates[30] for PAHs do not correlate with previous studies, in which fibrous cellulose TLC plates were used. Low selectivity and the uptake of organic solvent by the stationary phase, leading to poor reproducibility and a rising detector baseline (see Fig. 2A) limit their use for the separation of PAHs. Acetylation of the cellulose plates improves both the retention and the selectivity observed for the PAHs in the reversed-phase mode (Fig. 2B). However, the reaction conditions and extent of acetylation are difficult to reproduce exactly (*ca.* 30% of the plates prepared had to be rejected) and the plate cleaning process is tedious.

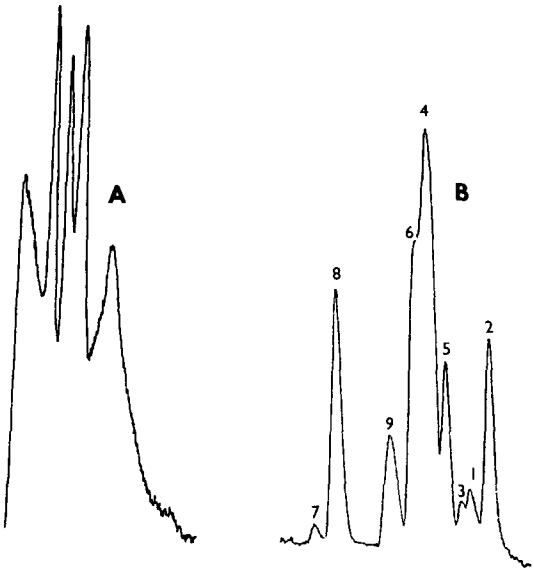

Fig. 2. Separation of PAH test mixture on cellulose (A) and acetylated cellulose (B) HPTLC plates. The cellulose plate was developed two 5-min periods in methanol–water (6:4). The acetylated cellulose plate was developed for one 10-min, one 15-min, one 20-min and one 25-min periods in methanol–water–diethyl ether (7:6:4). Peaks as in Fig. 1.

Reversed-phase plates with a chemically bonded ethyl-, octyl-, octadecyl- and diphenylsilanized silica layer are available from several sources. In Fig. 3 the separation characteristics of the ethyl-, octyl-, octadecyl- and diphenylsilanized silica plates, manufactured by Whatman, are compared for the separation of the PAH test mixture. Increasing the alkyl chain length of the bonded phase increases the retention of the PAHs and the selectivity of the separation process. The diphenylsilanized plates show poor selectivity and also lower efficiency than the alkylsilanized silica plates. The octadecylsilanized plates were selected for further optimization studies.

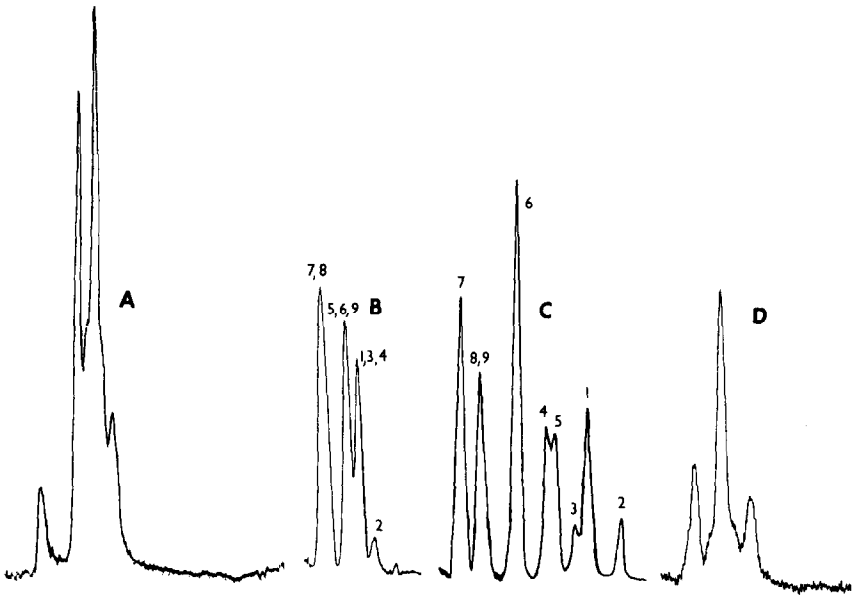

Fig. 3. Separation of PAH test mixture on (A) ethyl-, (B) octyl-, (C) octadecyl-, and (D) diphenylsilanized silica gel HPTLC plates. The ethylsilanized plate was developed for one 8-min and two 12-min periods in methanol–water–acetonitrile (10:6:1); the octylsilanized plate for one 5-min, one 10-min and two 15-min periods in methanol–water–acetonitrile (10:2:2); the octadecylsilanized plate for one 10-min, one 11-min, one 12-min, two 13-min and one 14-min periods in methanol–water–acetonitrile (3:1:1); and the diphenylsilanized plate for one 9-min and one 10-min periods in methanol–water–acetonitrile (10:3:1.5). Peaks as in Fig. 1.

Selection of the development technique

Common methods of linear development in HPTLC include conventional, continuous, multiple, and continuous multiple development[31,32]. Two-dimensional development was not considered, as it makes scanning densitometry all but impossible and reduces the sample capacity of the plate. A comparison of conventional, continuous, and multiple development for the reversed-phase separation of a PAH mixture is shown in Fig. 4. In each case the total time for development was 41 min. Clearly, conventional development provides the poorest separation, continuous development gives a slight improvement, but the best results are obtained by multiple development. In the multiple development technique, each time the mobile phase traverses the spot a reconcentration of the sample takes place, counteracting the natural spot broadening mechanism that occurs whenever the spot migrates with the mobile phase. The principal advantage of the multiple development technique is that it leads to an increase in the separation efficiency. The multiple development technique was used in all subsequent studies.

Selection of the mobile phase

Fifteen water-miscible organic solvents were selected to encompass a wide range of solvent properties. For methanol–water mixtures at high water concentra-

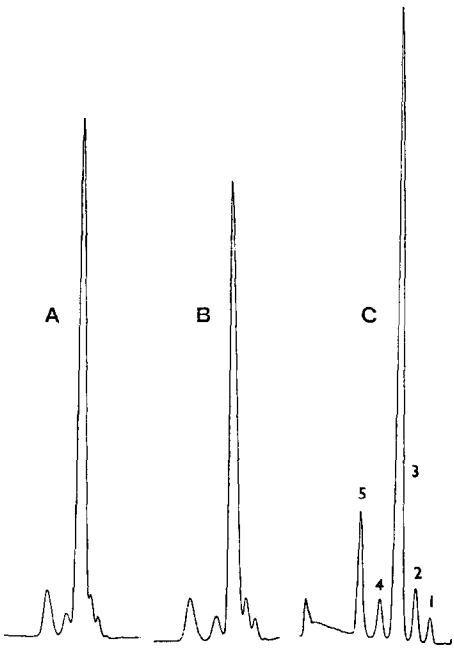

Fig. 4. Comparison of (A) conventional development, (B) continuous development, and (C) multiple development (four 5-min and three 7-min periods) in methanol–water (4:1). In each case the development time was 41 min. Peaks: 1 = Cor; 2 = BPer; 3 = Per and B*a*P and B*x*Flt; 4 = B*a*A; 5 = Flt.

tions little selectivity was observed. As the methanol concentration is increased, the mobility of the PAHs and the selectivity of the separation system increase. At high methanol concentrations the plate-cutting technique (see later) must be used to avoid pushing the sample into the solvent front. Higher-molecular-weight alcohols are much too viscous and provide low mobile-phase velocities without any additional gain in selectivity. Acetonitrile shows properties similar to methanol, but is a slightly stronger solvent as far as the PAHs are concerned. Acetone–water mixtures cause streaking of the sample and dioxane–water mixtures destroy the plate layer. Thus, neither solvent system can be considered useful. Solvents such as dimethylformamide and dimethyl sulfoxide show some useful selectivity but produce low mobile-phase velocities and are too difficult to evaporate from the plate surface between developments in the multiple development technique. Nitromethane was difficult to evaluate, as it is a very efficient fluorescence quencher. Methanol, acetonitrile, and tetrahydrofuran were judged the most selective solvents for the reversed-phase separation of PAHs.

The most useful binary solvent system for the separation of PAHs was methanol–water in the composition range 20:3 to 10:3. Virtually identical results can be obtained by manipulating the solvent composition, within the above range, and the development time. Taking the analysis time into consideration, a convenient development sequence is 10 consecutive developments of 10 min with the solvent system methanol–water (10:3), as shown in Fig. 5. The principal problem with this solvent

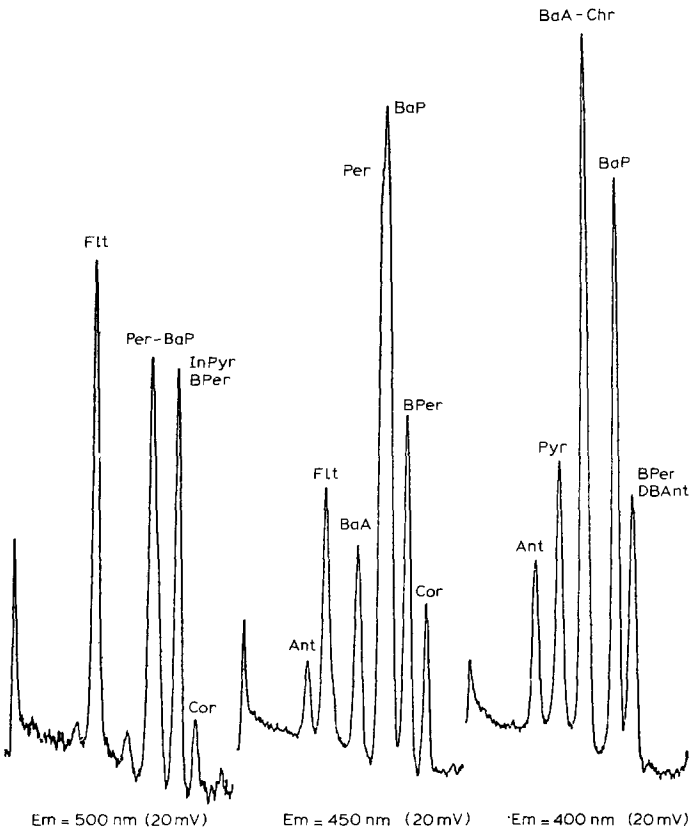

Fig. 5. Optimized separation of PAHs by reversed-phase chromatography on octadecylsilanized silica HPTLC plates with methanol–water (10:3) as the mobile phase. Ten 10-min multiple developments were used for the separation. The plate was scanned with excitation wavelength Ex = 266 nm. Em = Emission wavelength.

system is the poor resolution of the "benzo fraction", containing Per, B*a*P, B*e*P, and B*x*Flt (B*x*Flt is used throughout the text to represent the three benzofluoranthene isomers when they are unresolved), and the B*a*A–Chr overlap.

Ternary solvent systems containing methanol–water as the base solvent were investigated to see if additional selectivity could be found for the poorly resolved PAHs. Those solvents which demonstrated some selectivity were: acetone, dichloromethane, acetonitrile, dimethylformamide, benzene, and toluene. However, the influence of any of these additional solvents was small.

A characteristic of the multiple development technique is that it is necessary to increase the time for the later development steps in order to avoid pushing some of the sample components into the solvent front. Also, additional time is required to compensate for the longer time needed for the solvent to reach the first spot. Both of these processes lead to an increase in the analysis time without contributing to the separation. The aforementioned problem may be circumvented by applying the solvent further up the plate with each successive development. In practice, this can easily be implemented by cutting off a portion of the lower edge of the plate after each

development step. An example of the use of the plate-cutting technique is given in Fig. 6. Fig. 6A was obtained using five 10-min developments in methanol–acetonitrile–water (5:1:1). A 0.5-cm portion of the plate was removed before each successive development. The separation was continued (Fig. 6B) with three 13-min, two 15-min, and one 20-min developments for a total of eleven developments. Again, the plate was cut 0.5 cm after each successive development. The separation of poorly resolved spots increased between the fifth and eleventh developments, but the discernible information remains the same. Thus, the useful information in the chromatogram is available by the fifth development. Multiple development with plate cutting at each development step provides higher resolution of the PAHs than conventional multiple development (compare Fig. 6A with Fig. 5, for example). The improvement in sample resolution results primarily from an increase in the efficiency of the chromatographic system. For example, in Fig. 6B the slowest spot has migrated *ca.* 6 cm and the fastest *ca.* 11 cm. Yet, if the peak widths of the resolved components in Fig. 6A and B are compared, they are very similar, although the migration dis-

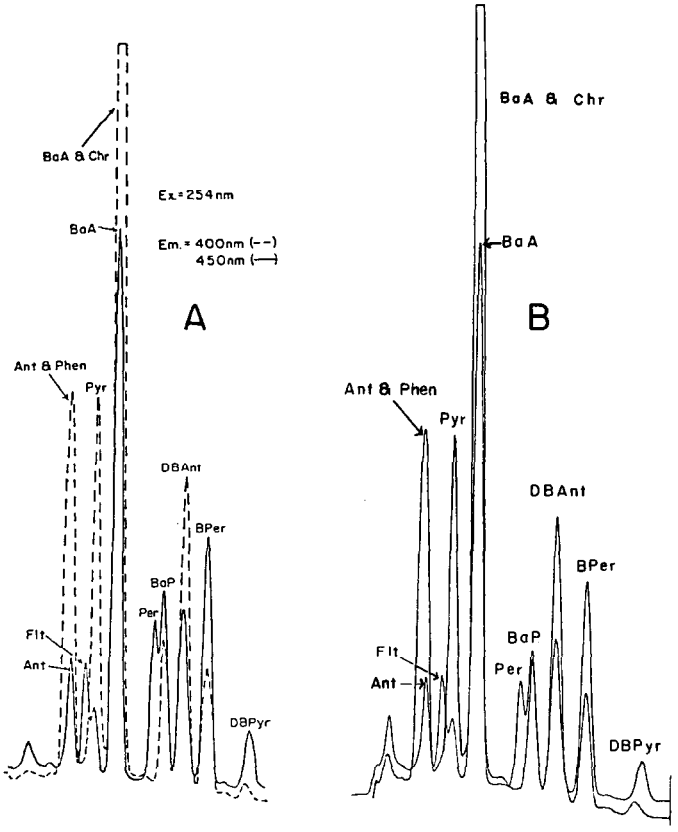

Fig. 6. Optimized separation of PAHs by reversed-phase HPTLC on octadecylsilinized silica plates by the plate-cutting technique. The mobile phase was methanol–acetonitrile–water (5:1:1). The plate was cut 0.5 cm from the lower edge before each successive development. The multiple development sequence was five 10-min, three 13-min, and two 15-min developments. The separation obtained at the end of the fifth (A) and eleventh (B) development is shown.

tances are different. Provided that the correct mobile-phase velocity, number of development steps, and time for each development are selected, a spot can be made to traverse virtually the whole length of the plate without experiencing significant spot broadening beyond that induced in the first few developments. This we attribute to a combination of the spot reconcentration mechanism of the multiple-development technique and the minimization of diffusion by maintaining the sample components in a high mobile-phase velocity zone. In terms of efficiency, an average value of *ca.* 2000–3000 theoretical plates can be obtained by using conventional development; for multiple development this becomes *ca.* 5000–10,000 theoretical plates, and for multiple development with change in position of the mobile phase entry point (plate-cutting technique) the efficiency increases to *ca.* 15,000–25,000 theoretical plates.

The chromatograms in Fig. 6 represent the best separation obtained. If In*cd*Pyr were included, it would overlap with BPer. This does not represent a problem, since these two compounds are easily distinguished by selective wavelength scanning[10]. Cor overlaps slightly with DB*ai*Pyr, but, again, wavelength discrimination is possible. Chr and B*a*A are inseparable in all systems investigated. B*a*A can be determined at 365 nm excitation and 400 nm emission in the presence of Chr. Chr cannot be determined in the presence of B*a*A, except by difference, using 266 nm excitation and 400 nm emission with subtraction of the contribution of B*a*A, calculated from its response at 365 nm excitation and 400 nm emission. The most severe limitation of the method is the lack of separation of B*e*P, Per, and B*x*Flt isomers. The fluorescence properties of these compounds are very similar, so that scanning at selective wavelengths can provide only qualitative information about their presence[10].

CONCLUSIONS

Octadecylsilanized silica gel and acetylated cellulose were found to be suitable stationary phases for the separation of PAHs. Experimental difficulties in reproducibly preparing partially acetylated cellulose plates prevents their more general use at present. Their selectivity is complementary to that of the octadecylsilanized plates, and further studies are called for. Using the octadecylsilanized silica plates, the best separations were obtained by multiple development methods with either a binary solvent system of methanol–water or a ternary solvent system containing methanol–water with either acetonitrile or dichloromethane. The ternary solvent systems are particularly useful with the plate-cutting technique. Cutting off a portion of the lower edge of the plate after each successive development step provides an increase in the separation efficiency and better utilization of the spot reconcentration mechanism. A theoretical model and a modified development chamber to replace cutting of the plate with a physical repositioning of the plate with respect to the mobile phase entry position are currently under development.

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therefore, does not necessarily reflect the view of the agency and no official endorsement should be inferred.

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