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# THE TRACE FLUOROMETRIC DETERMINATION OF POLYNUCLEAR AROMATIC HYDROCARBONS IN NATURAL WATER

by

RICHARD EDWARD KEEGAN

B. S., College of the Holy Cross, 1965

A THESIS

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

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Graduate School

Department of Chemistry

May, 1971

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Richard E. Keegan

# DEDICATION

This thesis is dedicated to my wife, Linda, whose patience and understanding provided the necessary encouragement for the completion of this investigation; and to my children, Kevin and Kelly.

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ABSTRACT

Polynuclear aromatic hydrocarbons (PNA) have received considerable attention in the last decade because of their carcinogenic properties. A variety of analytical methods have been developed for their determination in many substances (air particulates, cigarette smoke, soil, commercial solvents, soot, etc.). PNA's are discharged into the environment as an incomplete combustion product of coal tar pitch fumes as well as being indigenous to many soils. Their presence in air particulate matter has been firmly established and it is reasonable to assume that some are transported into the natural water system.

A procedure has been developed for the determination of PNA present in natural water in the parts per trillion (nanogram/liter) concentration range. The PNA are removed from the water by either continuous or batch extraction with n-pentane. Removal of interfering basic compounds is accomplished by extraction of the pentane solution with 72% $H_2SO_4$. Separation of the individual PNA is carried out on a Baker-flex cellulose TLC sheet developed with 50% aqueous DMF. The developed chromatogram is analyzed fluorometrically using a Farrand MK-1 Spectrofluorometer equipped with a TLC scanner. Qualitative analysis is based primarily upon $R_B$ values (migration distance of a compound relative to that of a benzo(a)pyrene standard) and fluorescence spectra obtained directly from the TLC sheet. Quantitative determination is performed by scanning across the unknown spot at fixed excitation and emission wavelengths. The area of the resulting fluorescence peak is then compared with that of a standard PNA separated on the same TLC sheet.

Water samples from three New Hampshire rivers have been analyzed using this method. Two of these rivers were found to contain traces of

PNA ranging from 80 to 1000 parts per trillion. Four PNA have been identified as dibenz(a,h)anthracene, benzo(a)pyrene, benzo(b)fluorene, and fluoranthene. Several other compounds have been tentatively characterized by their $R_B$ values and their fluorescence spectra.

Analysis of the data suggests that the PNA found in this study are naturally occurring and not likely due to air particulate pollution. Samples taken from a relatively unpolluted river showed the greatest number and largest quantities of PNA present.

INTRODUCTION

The presence of small amounts of polynuclear aromatic hydrocarbons (PNA) in many areas of the environment has focused attention on the development of improved analytical measurements for their determination. Much of this interest can be attributed to the fact that a substantial number of PNA have been shown to be highly carcinogenic. Carcinogenic activity of several PNA was demonstrated by Cook and co-workers[1] in 1932. Since that time, hundreds of papers have appeared dealing with the carcinogenic properties of many PNA.

The major source of PNA in the environment is the incomplete combustion of coal and other fuels[2]. As such PNA compose a significant percentage of chimney soot[3,4] and coaltar pitch[5]. Trace amounts of PNA have been found in association with a wide variety of other substances throughout the environment. Although most of these sources, such as airborne particulate matter or cigarette smoke, can be traced directly or indirectly to incomplete combustion, there is sufficient evidence to demonstrate that trace amounts of PNA are synthesized by naturally occurring organisms.

Sawicki[6] reviewed the separation and analysis of PNA present in the human environment in 1964. This review lists 244 references and includes 36 other systems in which traces of PNA have been found. Because these compounds are usually found in very complex mixtures, preliminary separation is almost always required. Sawicki discusses each of the common separation methods, namely sublimation, liquid-liquid extraction, vacuum distillation, solid-liquid extraction, and column, paper, thin-layer, and gas chromatography. Methods of analysis reviewed in this work are based on absorption, fluorescence, and phosphorescence spectra.

Since 1964 a considerable number of papers have been published in which one or more of the above techniques are used for PNA analysis. For example, Sawicki and co-workers[7] have published more than 50 papers dealing with the determination of aromatic hydrocarbons present in air particulate samples. In addition to the methods discussed in Sawicki's review several new techniques have been focused on the problem. In 1968 Jentoft and Gouw[8] investigated the use of high resolution liquid-liquid chromatography to separate synthetic PNA mixtures. Gel permeation chromatography was applied to thermally produced aromatic residues of PNA by Edstrom and Petro[9] in 1968. Majer and co-workers[10] in 1970 used thin-layer chromatography and mass spectrometry to analyze air particulate samples for trace amounts of PNA. In spite of these newer techniques, most investigators still used column and thin-layer chromatography combined with either absorption or fluorescence spectroscopy. Sawicki[11] has reviewed fluorescence analysis as applied to air pollution research up to 1969.

## Analysis of PNA in Natural Water

Relatively little emphasis has been placed on the quantitative analysis of trace amounts of PNA in natural water. Working in Germany, Borneff and co-workers have conducted the most extensive investigations of the PNA content of natural water. Beginning in 1959[12] they have published a continuing series of articles dealing with carcinogenic substances, mainly PNA, present in water and soil. At the present time, they have published 26 articles in this series, the most recent appearing in 1969[13]. This group has looked at several factors associated with the presence of PNA in natural water and soils. They have studied the carcinogenic activity of individual PNA using mice[14]. They have determined the

solubility of several PNA in drinking water as being approximately 0.01 parts per trillion[15] and have investigated the solubilization of PNA by detergents[16]. Additional investigations[17,18] concerned the presence of traces of PNA in activated carbon used for the preparation of drinking water. They concluded that the amounts present were so small that they presented no health hazard. Analysis of German forest soil samples[19] confirmed the presence of 6 carcinogenic PNA at concentrations of about 0.3 mg/kg of dry soil.

One of Borneff's earlier articles[20] discusses the fluorescence analysis of 8 common PNA both in benzene solution and directly from dry paper chromatograms. The developed spots were cut from the chromatogram and analyzed in the solid sample chamber of a spectrofluorometer. Excellent spectral data is presented. Their initial work[21] with surface water analysis involved liquid-liquid extraction of large volumes of water (500 liters) with 18 liters of benzene with subsequent separation using paper, thin-layer, and column chromatography. Absorption and fluorescence spectroscopy were employed for the final analyses. The major disadvantages of their methods were the extremely long analysis time, usually requiring several days, and the necessity of using large volumes of water and extracting solvent.

Borneff and co-workers have refined their earlier methods in subsequent papers. In 1967 Kunte[22], working with Borneff, described a TLC-fluorescence method suited for the analysis of 10 selected PNA. The PNA mixture was separated two-dimensionally on a mixed adsorbent layer composed of silica gel, alumina, and acetylated cellulose. The separated PNA were located with a UV lamp and subsequently eluted with benzene. Spectrofluorometric analysis of the eluted compounds produced errors ranging from 4 to 17% depending upon the compound determined.

In their most recent paper in this series, Borneff and Kunte[13] have modified their scheme for analyzing 10 liters of ground or drinking water. This amount of water was mixed (rotated) at 2000 rpm for 15 minutes with 600 ml of benzene. The solutions were allowed to settle for a few hours (preferably overnight) at which time 520-575 ml of the benzene could be recovered. The extract was concentrated to 0.1 ml and the entire sample applied to a TLC plate coated with a silica gel, alumina, acetylated cellulose mixture. The plate was then developed two-dimensionally (twice in each direction). Qualitative and quantitative analyses were performed under a UV lamp by comparison of the fluorescent spots with those on a standard chromatogram. The authors feel that visual comparison of spot size provides sufficient reproducibility in most cases. A coefficient of variation for 10 samples calculated from their data was 12.1%. The authors found total PNA concentrations of up to 50 and 100 ng/l for ground and drinking water respectively. They also propose that drinking water supplies showing total PNA concentrations greater than 200 ng/l should be rejected on the basis that above this PNA concentration, prolonged contact with the general population would constitute a serious health hazard.

Lijinsky and Shubik[23] examined 100 liters of Chicago tap water for the presence of PNA in 1965. The water was extracted in 2-liter portions with a single 200 ml volume of benzene which was subsequently removed by distillation. The residue was chromatographed on paper and analyzed spectrophotometrically using both absorption and fluorescence. No trace of any PNA was found in the water.

In 1968 Jager and Kassowitzova[24] described the determination of benzo(a)pyrene in drinking water. Ten 1-liter volumes of water were each extracted with 100 ml of n-heptane, the extracts combined and concentrated

to 10 ml. Analysis of low temperature fluorescence was performed either directly on the concentrated extract or after TLC separation. The method allows determination at a concentration of 3 ng/l. Relative errors of up to 40% for the direct method and up to 20% for the analysis with TLC were reported.

Scholz and Altmann[25] also determined benzo(a)pyrene in ground water in 1968. The method is reported to be useful for concentrations of benzo(a)pyrene from 0.1 to 1000 ng/l. A 1.0 liter water sample was extracted by stirring with 3 separate 30 ml portions of cyclohexane. The extracts were combined and concentrated to 100-150 µl. The total sample was then applied in 1-2 cm bands to a TLC plate coated with silica gel which had been previously washed with a 10% aqueous polyethyleneglycol solution. After development the adsorbent containing the benzo(a)pyrene spot was removed from the plate and soxhlet extracted for 30 minutes with 10 ml of cyclohexane. The solvent was evaporated and the residue dissolved in 3 ml of dioxane for fluorescence analysis. The "deviation" of the method was reported as less than 15% but it is not clear how this figure was obtained. The necessity of blank determinations was stressed when working in the lower concentration range of the method. Blank determinations fluctuated throughout the year ranging from about 0.3 to 1.5 ng/l.

## Separation Techniques as Applied to PNA

In the analysis of complex mixtures of PNA isolated from various sources, liquid-liquid extraction has been used primarily as a tool for separation of the acidic, basic, and neutral fractions. Sawicki[6] reviewed this phase of the analysis in 1964, including the separation of benzene[26], petroleum ether[27], cyclohexane[28], and diethyl ether extracts. Liquid-liquid extraction has been used by several authors as the initial step of a

PNA determination. Wedgwood and Cooper[30] extracted industrial effluents and sewage with chloroform prior to column chromatographic analysis. Howard and co-workers[31] used dimethyl sulfoxide to extract vegetable oils in 1966.

## Liquid-Liquid Extraction

Liquid-liquid extraction has been used for natural water analysis as discussed above. In these investigations water has been extracted with cyclohexane[25], benzene[13,21,23], and n-heptane[24]. The solvent, n-pentane, used in the present work has not been used in any previous liquid-liquid extraction procedures for the determination of PNA. Stanley[32] and co-workers have determined the efficiency of soxhlet extracting benzo(a)-pyrene from air particulate residues using pentane. Their results showed a 54% recovery of benzo(a)pyrene from an enriched air particulate sample.

## Thin-Layer Chromatography

Since the advent of thin-layer chromatography about 20 years ago, TLC has gradually replaced paper chromatography as the principal method for the separation of individual PNA. In 1964 Sawicki[6] reviewed the existing TLC methods for PNA. Since that time many papers have appeared using various TLC systems for PNA analysis; a partial listing of some of these papers follows.

Various types of adsorbent materials have been used. Silica gel layers were used by Stromberg and Widmark[33] in 1970 and Hood and Winefordner[34] in 1968. Biernoth[35], and Sawicki and co-workers[36] used alumina layers for PNA analysis in 1968 and 1970 respectively. Acetylated cellulose is one of the more widely used adsorbents. Sawicki[37], Shaad and co-workers[38,39] and Toth[40] have separated PNA with this adsorbent. In 1967

Keefer[41] separated several PNA on magnesium hydroxide. Libickova and co-workers[42] separated 24 PNA on silica gel layers impregnated with various electron-acceptor compounds. Two-dimensional TLC has been used by several authors[43-45] for the PNA analysis of very complicated mixtures. In 1969 Matsushita and Suzuki[46] separated a mixture containing many PNA using two-dimensional TLC on a plate coated with 2 different adsorbents.

The photodecomposition of PNA separated on TLC plates has also been studied. In 1964 Inscoe[47] studied the photochemical changes of 15 common PNA on silica gel, alumina, cellulose, and acetylated cellulose layers. Photodecomposition of PNA on caffeine impregnated silica gel layers was studied in 1965 by Lam and Berg[43]. They concluded that use of caffeine impregnated silica gel markedly decreased photodecomposition.

## Fluorescence Spectroscopy as Applied to PNA

Fluorometric methods have been used extensively in the analysis of PNA. While fluorescence work requires more precautions than does absorption, significant increase in sensitivity can be achieved. PNA are one of the most highly fluorescent classes of compounds and most of their excitation and emission spectra are well known. Sections on PNA analysis are included by Udenfriend[48] and by White and Argauer[49]. Fluorescence spectra of several PNA in various solvents are also presented in a fluorescence handbook by Berlman[50].

In 1964 Sawicki[6] reviewed the fluorescence analysis of PNA present in the human environment. Among the 31 references cited in connection with fluorescence there are 10 which include compilation of fluorescence spectra in various solvents. The major source of reference spectra for the present work was a paper published in 1960 by Sawicki and co-workers[51]. The excita-

tion and emission spectra of 41 PNA in pentane solution and 17 PNA in concentrated sulfuric acid are presented graphically.

## Quantitative Thin-Layer Chromatography

Quantitative TLC is a relatively new and rapidly growing method. It has only been in the past few years that the instrumentation necessary for precise quantitative analysis has become commercially available. Shellard[52] has edited a book dealing with the different aspects of quantitative paper and thin-layer chromatography.

Two general methods are used for quantitation after separation on thin-layer chromatograms. These are the analysis of components subsequent to elution from the adsorbent and analysis *in situ* on the thin-layer. Refinement of elution methods has depended upon the development of reproducible techniques for removing components from the adsorbent layer. Advances in *in situ* TLC analysis have parallelled the introduction of more sophisticated scanning instrumentation.

### Elution Methods

*Introduction*. Several reviews[53-55] of elution methods have been published describing the various techniques involved. In general, the compound of interest is extracted from the adsorbent with a suitable solvent and the resulting solution is analyzed quantitatively with appropriate instrumentation. Several techniques have been developed for the removal of the adsorbent layer together with the located spot. The most common method is to scrape off the adsorbent with a spatula or razor blade[56] and transfer it to a flask or test tube. The adsorbent is then extracted with an appropriate solvent and the solution either filtered through a glass filter or centrifuged to remove the adsorbent particles. To minimize losses caused by

the scraping process, some authors[57-59] have used vacuum-type spot collectors in which the spot can be sucked on to a filter or into an extraction flask and then eluted. With the development of glass fiber sheets, as well as carrier sheets composed of aluminum and plastic, the scraping procedure has been eliminated since the spot may be cut out and placed directly in a flask for elution[60].

A variety of analytical methods have been employed for quantitation subsequent to elution from TLC plates. These include ultraviolet and visible spectrophotometry[61-63], low temperature[34,64] and room temperature[65-66] fluorescence, phosphorescence[67], gas chromatography[68,69], polarography[70], and mass spectrometry[71].

Application to PNA Analysis. TLC-elution techniques have been used for the determination of a number of PNA employing a variety of adsorbents. About half of the literature dealing with PNA is concerned with the determination of benzo(a)pyrene. In 1964 Sawicki and co-workers[72] determined benzo(a)pyrene in air particulate samples after separation using TLC on alumina, cellulose acetate, and cellulose. After isolation of benzo(a)-pyrene, the spot was eluted with methanol followed by ultraviolet and fluorescence analysis. Fluorescence studies of standard benzo(a)pyrene samples showed that only 50 ± 15% of the compound was being recovered. Sawicki[36] also published a tentative method for the determination of benzo(a)pyrene in air particulates; TLC on alumina followed by fluorescence analysis in concentrated sulfuric acid was used. Siburn and co-workers[73] used fluorescence analysis subsequent to separation on silica gel layers impregnated with caffeine. In 1968 Scholz and Altman[25] determined benzo(a)pyrene in water after separation on silica gel. Spots were scraped from the plate, soxhlet extracted with cyclohexane, and determined fluorometrically in dioxane solution. Two other papers have been published

concerning benzo(a)pyrene analysis in which other instrumental methods were used subsequent to elution from the adsorbent layer. Stanley and co-workers[74] separated benzo(a)pyrene on mixed alumina-silica gel layers and analyzed the eluted spots spectrophotometrically. In 1970, Stromberg and co-workers[33] determined benzo(a)pyrene in air samples after separation on alumina layers. The spots were eluted with benzene followed by both spectrophotometric and gas chromatographic analysis. They reported 95% recovery of benzo(a)pyrene using gas chromatography.

Several other papers have appeared in which other PNA have been quantitatively estimated or determined after elution from TLC plates. In 1966 Pavlu[75] determined benzo(a)pyrene, dibenz(a,h)anthracene, pyrene, and several smaller PNA after separation on silica gel developed several times with hexane-benzene (10:1). The spots were eluted with benzene and determined fluorometrically. The following year, Kohler and Eichoff[76] determined several PNA in atmospheric dust after 2-dimensional TLC on a mixed alumina-cellulose acetate layer. Elution of the PNA spots was followed by fluorescence analysis. Radioactive tracer studies showed approximately 70% recovery of the PNA with a relative standard deviation of 15%. Bender[77] also used 2-dimensional TLC on mixed alumina-cellulose acetate layers for the identification and estimation of dibenz(a,e)pyrene. The identified spots were eluted with hot methanol and analyzed fluorometrically in pentane solution. In 1968 Biernoth[35] reported the quantitative determination of 13 PNA after separation on alumina. The method required preliminary purification on a silica gel column. The alumina plate was repeatedly developed (4 times) with isooctane and the separated spots were removed from the layer with cold ethanol. The individual PNA were then determined spectrophotometrically.

## *In Situ* Methods

*Introduction*. Quantitative TLC employing *in situ* methods is a fast growing field. It has the distinct advantages of requiring less time and usually being more sensitive than elution methods. Since *in situ* scanning is performed without removing the compound from the adsorbent layer, errors associated with the elution step are eliminated. Disadvantages inherent in the direct scanning technique are the possibilities of air oxidation or photodecomposition of compounds present on the adsorbent layer. In spite of these disadvantages, *in situ* TLC scanning is a very useful analytical method for a variety of compounds.

At present there are four photometric methods for quantitative *in-situ* scanning:

(1) Fluorescence

(2) Fluorescence quenching

(3) Densitometry

(4) Reflectance

There is another photometric method which has received very little attention in the literature. This is the use of phosphorescence measurements directly on TLC plates. In 1965 Sawicki and Pfaff[78,79] described the use of phosphorescence for the characterization and estimation of several types of compounds including a few PNA. The inherent difficulties in making these measurements makes this approach unsuitable for most broad range analyses.

A comprehensive review of fluorometric TLC scanning has not appeared in the literature. Two limited reviews[80,81] were published in 1968 in which instrumentation and parameters were discussed and examples of specific applications cited. The theory of photometric evaluation of thin-layer chromatograms was discussed by Klaus[82] in 1964.

Direct *in situ* fluorometric evaluation of thin-layer chromatograms was initially investigated by Seiler and co-workers[83] in 1963 and by Klaus[82] in 1964. Progress in the field was slow until about 1968 when the first commercial scanning instrumentation became available. Since that time the number of research papers in the field has increased considerably. At the present time there are about ten companies[84] marketing fluorescence scanners or scanning attachments. Descriptions and applications of several of these instruments have appeared in the literature[81,85-88].

Very little meaningful quantitative data has been published dealing with fluorescence scanning of thin-layer chromatograms. The data summarized in Table I has been taken from several papers where a relative standard deviation was reported. This data shows that, in general, the relative standard deviation for measurements on a single plate is better than 10%. This error is also shown to increase significantly when two or more plates are compared.

In the fluorescence quenching method the compound of interest absorbs some of the ultraviolet excitation radiation and causes a decrease in the fluorescence inherent to the adsorbent material. In most cases an adsorbent containing a fluorescent indicator is used. The advantages and disadvantages of quenching methods, as well as the important parameters, have been discussed by several authors[85,87,89]. The technique is very versatile and has been used for the determination of a wide variety of compounds.

*In situ* visible and ultraviolet densitometry on TLC plates was reviewed in 1968 by Shellard[90] and by Seiler and Moellar[91] and Lefar and Lewis[85] since that time. A number of studies have appeared dealing with *in situ* densitometric determination of a variety of substances including three papers[95-97] in which the advantages and disadvantages of the method are discussed.

Table I.

Summary of Reported Fluorescence Scanning Errors

| Compound Type | Relative Standard Deviation (%) Same Plate | Different Plates | Reference |
|---|---|---|---|
| Pyrene | 4 | - | 87 |
| DANS-amino acids | 3.5-5 | 10-15 | 92 |
| DANS-amino acids | 1.9-5 | 7.5-9.1 | 93 |
| PNA | 10 | - | 39 |
| Alkaloids | 2 | - | 94 |

Spectrophotometric reflectance measurements on TLC plates have been reviewed by numerous authors[85,98-101]. The theoretical basis for this technique has also been reported by Goldman and Goodall[102] and by Lieu and co-workers[86]. This method has the advantage over desitometry that the nature of the adsorbent support has a smaller effect on the measurements.

<u>Application to PNA Analysis</u>. Fluorometric scanning of PNA separated on thin-layer chromatograms has been described in two articles. In 1965, Sawicki and co-workers[37] briefly discussed the fluorometric scanning of several PNA isolated from air particulate samples and separated on acetylated cellulose. An Aminco-Bowman spectrofluorometer equipped with an Aminco automatic scanning attachment was used. Very little quantitative data is presented and the authors stress the use of the technique as an estimation method. One calibration curve is presented for benzo(a)pyrene showing an assumed linearity between 0 and 50 ng. No experimental values are given, and it is doubtful whether the points shown represent a statistically linear relationship. It is stated, however, that the slope of the regression line becomes less steep above 60 ng up to 200 ng of benzo(a)pyrene. Considering that this work was performed while TLC scanning was still in its infancy, the lack of good quantitative data is excusable. It is odd, however, that these prolific authors have neglected this specific area subsequently.

A more extensive study was published by Toth[40] in 1970. Thirteen of the most common PNA occurring in soot, smoked foods and tar were investigated after separation on mixed layers of cellulose acetate-alumina (2:1). An Aminco-Bowman spectrofluorometer fitted with a suitable scanning attachment was used. This system measured the fluorescence transmitted through the plate and hence was sensitive to the thickness of the layer and

the glass plate. Because of this the signal produced by a standard spot (100 ng of benzo(a)pyrene) was used as a calibration factor in all quantitative determinations. In a series of 15 measurements this correction factor fluctuated between 0.96 and 1.31 with a mean of 1.08.

Calibration curves were presented by Toth[40] for 12 PNA using a double logarithmic system with amounts ranging from 10 to 1000 ng. All of the curves were linear up to 100 ng with most showing slight negative curvature above this point. These curves were used in conjunction with the correction factor discussed above for the quantitative determination of the individual PNA. There was no mention of the necessity of obtaining calibration curves for each chromatogram.

The reproducibility of the method was poorly defined as a ± 10% variation in the measurement and evaluation by chromatography[40]. A description of just what this ± 10% is and how it was determined was lacking. It was stated that the major portion of the error was concerned with the application of the initial spots.

Considering the analytical techniques which had been used for PNA analysis, a general scheme was proposed for the trace analysis of PNA in natural water. The proposed method consisted of liquid-liquid extraction, preliminary separation of basic compounds, TLC separation of individual PNA, and fluorescence analysis.

## PRELIMINARY INVESTIGATION OF THE METHOD

The analytical scheme as envisioned initially was composed of four major steps. (1) Continuous liquid-liquid extraction of 5 liters of water with n-pentane followed by concentration of the extract to several milliliters. (2) Column chromatography of the resultant concentrate on acidic alumina to remove interfering basic components. (3) Thin-layer chromatography (TLC) of the concentrated eluent to separate the individual PNA. (4) Quantitative fluorescence analysis of the separated compounds. This tentative procedure was carried out on a water sample obtained on July 19, 1966 from the Oyster River immediately above the Geological Survey Dam near the then Route 4 in Lee, New Hampshire.

Eight gallons of water were collected and approximately four gallons were passed through a Millipore filter with a 0.45 u pore size. This process was extremely slow and took approximately one week. 3800 ml of the filtered water was placed in a 4 liter continuous liquid-liquid extractor (Pope Scientific, Inc.) and extracted with a total of 1250 ml of n-pentane. The course of the extraction was followed by removing aliquots from the pentane flask and measuring their fluorescence and ultraviolet absorption. The extraction was considered to be essentially complete after 40 hours when successive aliquots showed no significant fluorescence increase. The extractant was removed from the flask and concentrated to approximately 5 ml in an Erlenmeyer flask by aspiration. The solution was placed on a 125 mm x 25 mm acidic alumina column and eluted with successive 100 ml volumes of 0, 10, 20, and 30% benzene in pentane followed by 300 ml of 40% benzene in pentane. One hundred ml fractions were collected and concentrated to 50 ml by aspiration. Fluorescence and absorption spectra were run on each fraction. Both fluorescence and absorption reached a maximum in

fraction #2 and gradually decreased thereafter until fraction #7 in which the intensities were quite low. All fractions were combined and concentrated to an oily residue which was dissolved in 5.00 ml of n-pentane.

At this point several TLC systems were investigated in hopes of achieving maximum separation of the individual PNA. A detailed account of this study is presented in the section concerning development of TLC methods. The system employing a cellulose sorbent developed with 50% aqueous dimethylformamide (DMF) gave the best separation of the PNA concentrate. Four spots were observed under these conditions and tentatively identified by means of their $R_B$ values as dibenz(a,h)anthracene, benzo(a)-pyrene, benzo(b)fluorene, and fluoranthene. E. Sawicki[6] has defined the $R_B$ value of a particular PNA as the migration distance of that compound relative to that of benzo(a)pyrene.

The $R_B$ values of the four spots from the PNA concentrate and those of known reference compounds as well as the values given by Sawicki[6] for this TLC system are listed in Table II. According to Sawicki, $R_B$ values may vary by as much as 15% depending on development conditions. On the basis of these results which indicated that the proposed method was capable of indicating the presence of some PNA in natural water, it was decided to investigate in more detail each phase of the procedure.

Table II.

Tentative Identification of Compounds in the Preliminary Sample

| | $R_B$ Values | | |
|---|---|---|---|
| | Sawicki | Sample | Reference |
| Dibenz(a,h)anthracene | 0.66 | 0.64 | 0.66 |
| Benzo(a)pyrene | 1.00 | 1.00 | 1.00 |
| Benzo(b)fluorene | 1.33 | 1.36 | 1.37 |
| Fluoranthene | 1.89 | 1.87 | 1.82 |

# DEVELOPMENT OF THE METHOD

## Solvents

In any quantitative procedure dealing with nanogram ($10^{-9}$ gram) amounts of material, only the highest purity solvents can be used. Because PNA are among the most intensely fluorescent classes of compounds, the presence of even trace amounts in a solvent can lead to large errors. The solvents must also be purified to remove any other compounds whose fluorescence or quenching properties might interfere with that of the PNA being analyzed. In order to insure high purity solvents, each new batch was checked fluorometrically.

### n-Pentane

All fluorescence measurements (except those made directly from TLC plates) were made in n-pentane solution. Pentane was also used as the extracting solvent in removing PNA from the natural water. E. Sawicki[6] has cited several advantages in using pentane as a solvent in the trace fluorometric determination of PNA. Pentane purifies easily, evaporates rapidly at a low temperature, has a low fluorescence blank, and readily dissolves most PNA. These compounds also have more fine structure and sharper bands in pentane than in other more polar solvents. The principal disadvantage of using pentane is that all containers including optical cells must be kept tightly stoppered to prevent loss of solvent because of its very low boiling point ($36^{\circ}$ C).

Four grades of n-pentane were initially investigated using UV absorption from 200-500 nm. Both Pesticide Analysis Grade (Fisher Scientific Co.) and IR Spectranalyzed Grade (Fisher Scientific Co.) were much purer than either Practical (Eastman Organic Chemicals) or Technical (Eastman

Organic Chemicals) n-pentane. The latter two showed considerable absorbance from 200 to 275 nm. Pesticide Analysis Grade and IR Spectranalyzed Grade pentane were then checked spectrofluorometrically over the range 325-700 nm. Using an excitation wavelength of 313 nm, there was no detectable fluorescence with the Pesticide Analysis Grade while the IR Spectranalyzed Grade exhibited weak fluorescence from 340 to 390 nm.

Attempts to purify practical and technical grade n-pentane using distillation and adsorption chromatography were undertaken. Three different column types were used for distillation. Technical n-pentane distilled through a 30 cm Vigreux column showed a considerable decrease in fluorescence but the middle fraction of the distillate was not of sufficient purity to be used. Distillations were also carried out using a 90 cm column having a 5 mm I.D. containing a Monel spiral packing (Todd Scientific Co.) as well as a 90 cm column having a 25 mm I.D. packed with 4 mm glass helices. Reflux ratios for these two columns were automatically controlled and varied from 1:2 to 50:1. The helices column was slightly more efficient than the spiral band column. The quality of the distillates varied for both types emphasizing the need for absolute cleanliness. All purified pentane was checked either fluorometrically or by UV absorption and all contaminated batches were rejected. By the use of multiple distillations it was possible to purify both technical and practical pentane so that the fluorescence blanks were quite small. However, the Pesticide Analysis Grade pentane was still superior in that it showed a lower fluorescence blank.

Silica gel chromatography was also used to purify technical pentane. Lijinsky and Raha[103] have shown that all PNA are retained on a silica gel column which should therefore be suitable for removal of these compounds from technical pentane. A 2 cm chromatographic column was packed to a height of 40 cm with 100 grams of silica gel (Davison Chemical Co., Grade

923, 100-200 mesh). Five liters of technical pentane was passed through the column at a rate of 8 ml/minute. Fluorescence measurements taken before and after passage through the silica gel showed that while the fluorescence had been reduced 93% the eluate was still not as pure as Pesticide Grade pentane.

Considering that Pesticide Grade pentane showed a lower fluorescence blank, and considering the time required in attempted purifications, Pesticide Grade pentane was used in all subsequent experiments as obtained from the manufacturer after checking its purity fluorometrically. After initiation of this work, two other grades of pentane, Matheson, Coleman, and Bell's Chromatoquality Grade and Harleco's Fluorometric Grade, were obtained for preliminary comparison. Both of these solvents showed excellent low fluorescence blanks comparable to that of the Pesticide Grade pentane. Considering that both the Chromatoquality and Fluorometric grades were more expensive and considering that Pesticide Grade pentane had been used in all of the previous work, these two solvents were not used in subsequent work.

### Ethanol

Ethanol was purified by distillation of 95% ethanol through a 30 cm Vigreux column. The middle fraction was kept in each case. All batches were checked fluorometrically to insure purity.

### Sulfuric Acid

Concentrated sulfuric acid was obtained from Fisher Scientific Co. and used without further purification.

### Benzene

Technical grade benzene was purified by distillation through a 30 cm Vigreux column, the middle fraction being collected. All batches were checked fluorometrically to ensure purity.

### Water

Distilled water was obtained from a 20 gal. Barnstead automatic still fed by deionized water. Each new batch was checked fluorometrically. When the distilled water was unsatisfactory or the still inoperative, deionized water was distilled according to a method suggested in Perrin's book on the purification of solvents[104]. The water was distilled from 0.25% solid NaOH and 0.05% $KMnO_4$ and the middle fraction collected. This water was sufficiently pure for use as a blank.

### Ethyl Ether

Spectrograde ethyl ether (Eastman Organic Chemicals) was the purest grade commercially available. Further purification was attempted because of the large amount of fluorescence observed upon concentrating the ether 25-fold. Following the method cited by Perrin[105], 100 grams of alumina were washed several times with purified pentane and heated in a muffle oven at 400$^{\circ}$C for 5 hours. The alumina was allowed to cool in a vacuum dessicator and packed dry in a 2.5 cm column to a height of 22.5 cm. 200 ml of Spectrograde ether was passed through the column. The first 15 ml of ether was discarded and the remainder collected. Fifty ml of the eluate was concentrated to dryness and the residue dissolved in 2.00 ml of pentane. Fluorescence analysis of this solution, compared with that of a similarly treated residue of pure Spectrograde ether, showed that the ether passed

through the alumina had almost 3 times more fluorescence than the pure Spectrograde ether, indicating the necessity for prior cleaning of the alumina with highly purified more polar solvents. Attempts to purify Spectrograde ether by prior cleaning of the alumina with benzene and methanol were also unsuccessful. The Spectrograde ether was used in several TLC-elution studies but spectra could not be obtained with sample sizes less than 300 ng because of the high background caused by concentration of the ether subsequent to elution from the adsorbent.

## Chemicals

All reference compounds were purchased from commercial supply houses. The highest quality available was chosen and the purity checked using TLC and fluorescence. In all cases no fluorescent impurities were detected and the compounds were used without further purification. A list of the reference compounds and their source of supply follows:

Benzo(a)pyrene - Eastman Chemical Company - 97%

Dibenz(a,h)anthracene - Eastman Chemical Company - 97%

Benz(a)anthracene - Eastman Chemical Company - 97%

Fluoranthene - Eastman Chemical Company - Practical

Benzo(b)fluorene - Aldrich Chemical Company - 97%

Triphenylene - Aldrich Chemical Company - 98%

Carbazole - Aldrich Chemical Company - 99+%

Anthracene - J. T. Baker Chemical Company - Photosensitizer Grade

Pyrene - J. T. Baker Chemical Company - Photosensitizer Grade

Fluorene - J. T. Baker Chemical Company - Photosensitizer Grade

Chrysene - J. T. Baker Chemical Company - Photosensitizer Grade

## Liquid-Liquid Extraction

PNA are present in natural water in extremely small concentrations on the order of $10^{-7}$ gram/liter. Some concentration is a prerequisite before these small amounts may be measured quantitatively using presently available methods. After consideration of such techniques as carbon adsorption, freeze concentration, and the use of specific complexing agents, liquid-liquid extraction was chosen as the preliminary step in the present method. Continuous extraction with pentane was used for the majority of this work and proved to be quite satisfactory in spite of being time consuming. The possibility of using batch instead of continuous extractions was investigated later and found to give excellent results if certain precautions were taken.

In both continuous and batch extractions, Pesticide Analysis Grade pentane was used exclusively as the extracting solvent. It is an excellent solvent for removing PNA from water for several reasons. (1) Most PNA are readily soluble in pentane. (2) It has a very low density (0.626 g/ml) and is quite immiscible with water. (3) It may be easily concentrated after the extraction because of its low boiling point ($36^{\circ}$ C.).

### Continuous Extractions

Three different continuous extractors were used. Extractor A was initially used as obtained from Pope Scientific Co. It was a 4 liter continuous extractor capable of being used for either lighter than water or heavier than water solvents. Because of the very low density of pentane, it was necessary to increase the height of the inner tube in order to produce a head of pentane sufficient to force the solvent from the bottom of the inner tube. The extractor had a round bottom which prohibited the use of a

magnetic stirring bar to increase the rate of extraction. The first six unknown extractions were performed using this extractor and large volumes of pentane solvent. A typical example would be the extraction of 3800 ml of water with 2100 of pentane.

The bottom of extractor A was broken and replaced with a flat bottom. At the same time the capacity was increased to 5 liters. The inner tube rested on the bottom of the extractor. A diagram of this extractor is shown in Figure 1. A heating mantle was used to heat the pentane in a 200 ml, 2-necked, round-bottom flask attached to the sidearm. The water level in the extractor was brought to about 3 cm from the sidearm. The initial extraction efficiencies on benzo(a)pyrene and benzo(b)fluorene were done with this extractor. In hope of increasing the rate of extraction, a new extractor was designed to accomodate the insertion of a magnetic stirring bar.

Extractor B was made from a 5 liter round-bottom flask and is shown in Figure 2. The bottom of the extractor was flattened and the inner tube held about 4 cm above this surface so that a 5 cm Teflon coated magnetic stirring bar was free to rotate. The total length of the inner tube was 50 cm. This extractor had a capacity of 5150 ml of water. In hopes of performing duplicate extractions, a new extractor which was similiar to B was purchased.

Extractor C is shown in Figure 3. No provision had been made to hold the inner tube suspended over the bottom of the extractor. It was necessary, therefore, to build an extension having a smaller internal diameter to hold the inner tube. The total length of the inner tube for this extractor was 70 cm. The capacity of the extractor was 5010 ml. A 5 cm stirring bar was also used with this extractor.

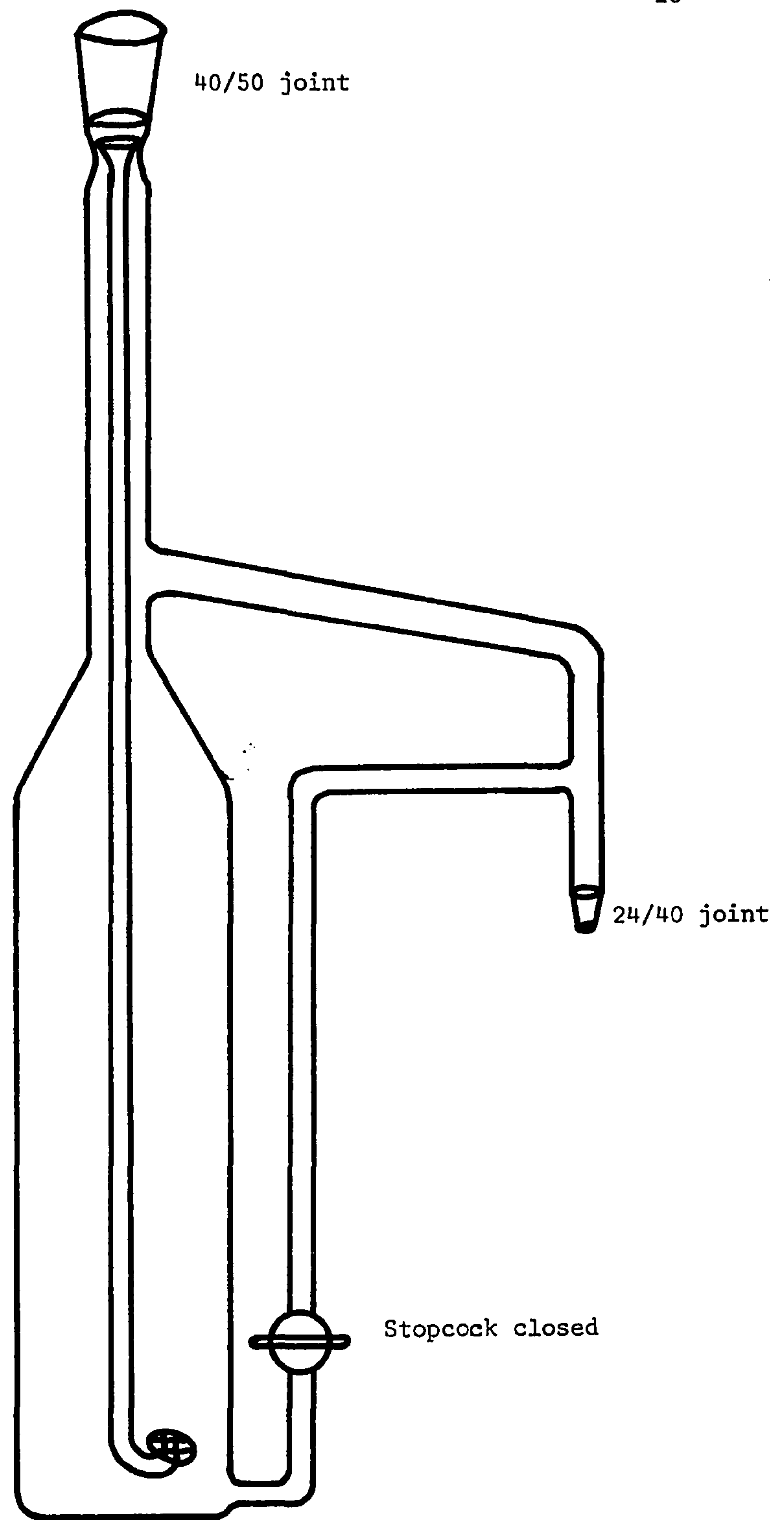



Figure 1. Extractor A

Scale: 1 inch = 10 cm

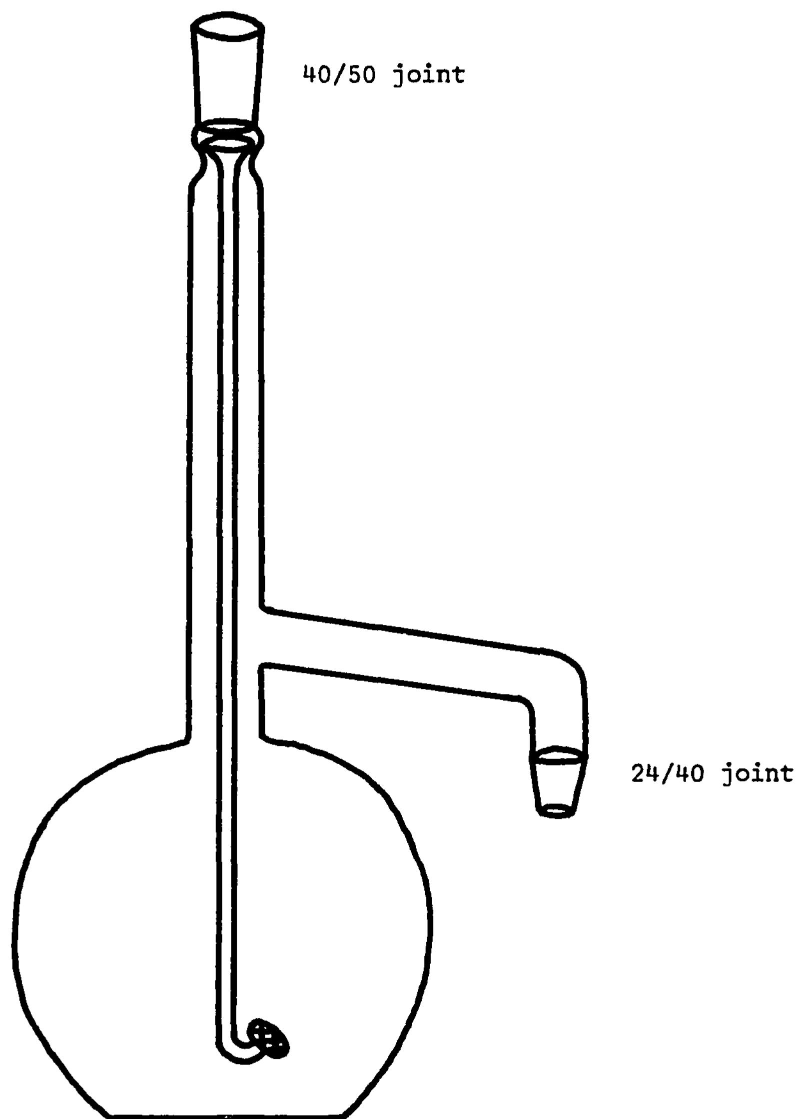



Figure 2. Extractor B

Scale: 1 inch = 10 cm

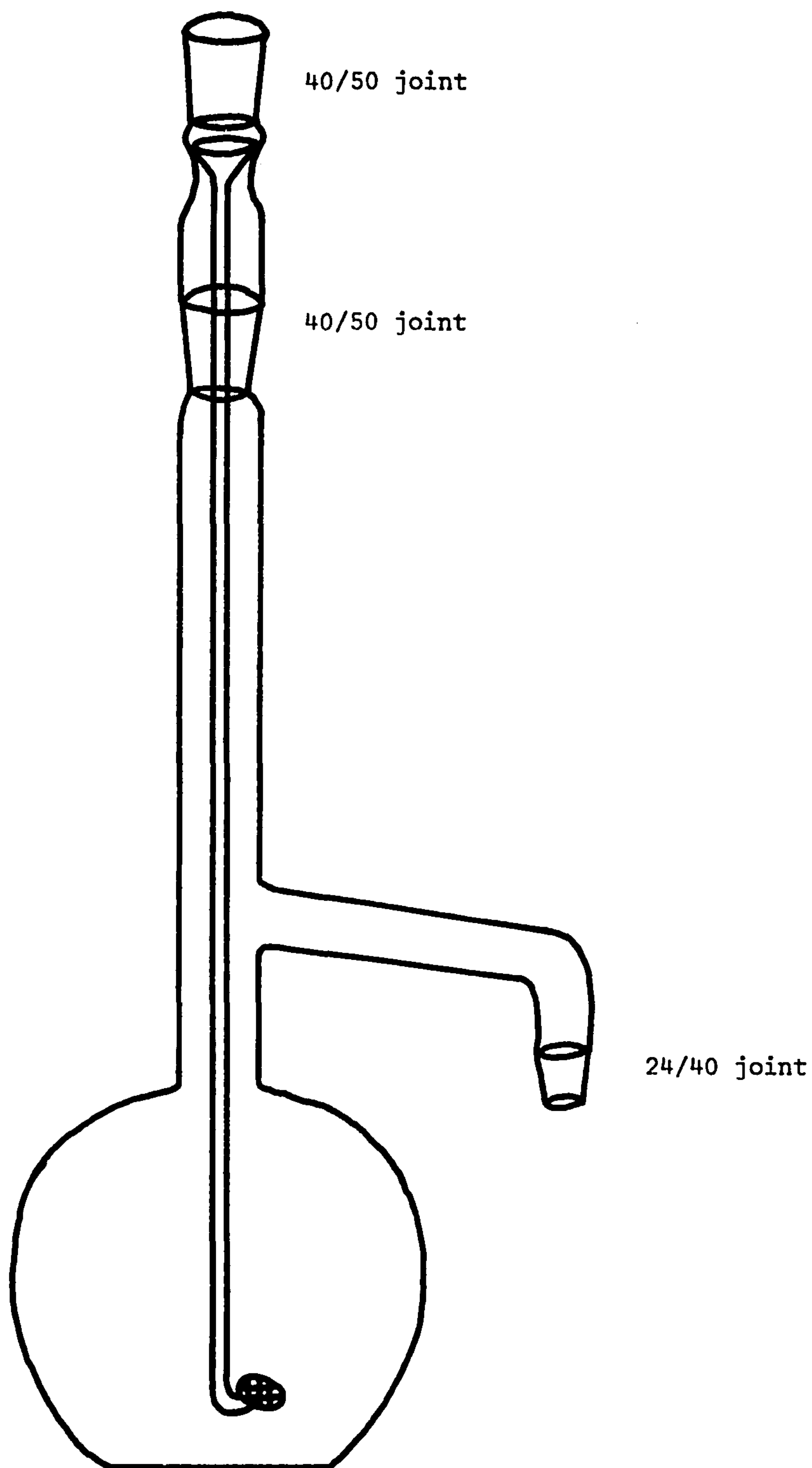



Figure 3. Extractor C

Scale: 1 inch = 10 cm

Extraction Time. The course of each extraction was followed fluorometrically using the Turner Fluorometer, Farrand Spectrofluorometer, or the spectrofluorometer built by B. Solomon[106]. Aliquots were periodically removed from the sidearm flask and the fluorescence measured. If necessary pure pentane was added to the flask prior to removing the aliquot so that a constant volume was being sampled. If this was not done, the small amount of pentane escaping through the condenser would reduce the total volume in the flask and cause an error in the observed fluorescence. The extraction was assumed to be complete when the fluorescence remained essentially constant for at least 4 hours. Figure 4 presents the course of typical extractions of benzo(a)pyrene and benzo(b)fluorene with extractors B and C. Fluorescence measurements were taken at 10, 18, 20, and 40 hours. The differences between the amounts extracted by the two extractors are due in part to variation in the amount of pentane in the extraction flask as well as to small variations in extraction efficiency. Benzo(b)-fluorene exhibited a greater fluorescence emission than benzo(a)pyrene. These curves also show that a large percentage of each compound was extracted within 24 hours.

A summary of times required for complete extraction with extractors A, B, and C is presented in Table III. These figures are slightly inflated because they were compiled from total extraction times which included the 4 hours allowed for the fluorescence to become constant. In some cases as much as 12 hours had elapsed between the final two aliquots. If measurements had been made every hour, the figures would have been considerably lower. The data in Table III clearly shows the effect of stirring on extraction time. The mean times for extractors B and C, in which stirring was used, are significantly lower than that for extractor A which was not stirred. The differences between extractors B and C can be attributed

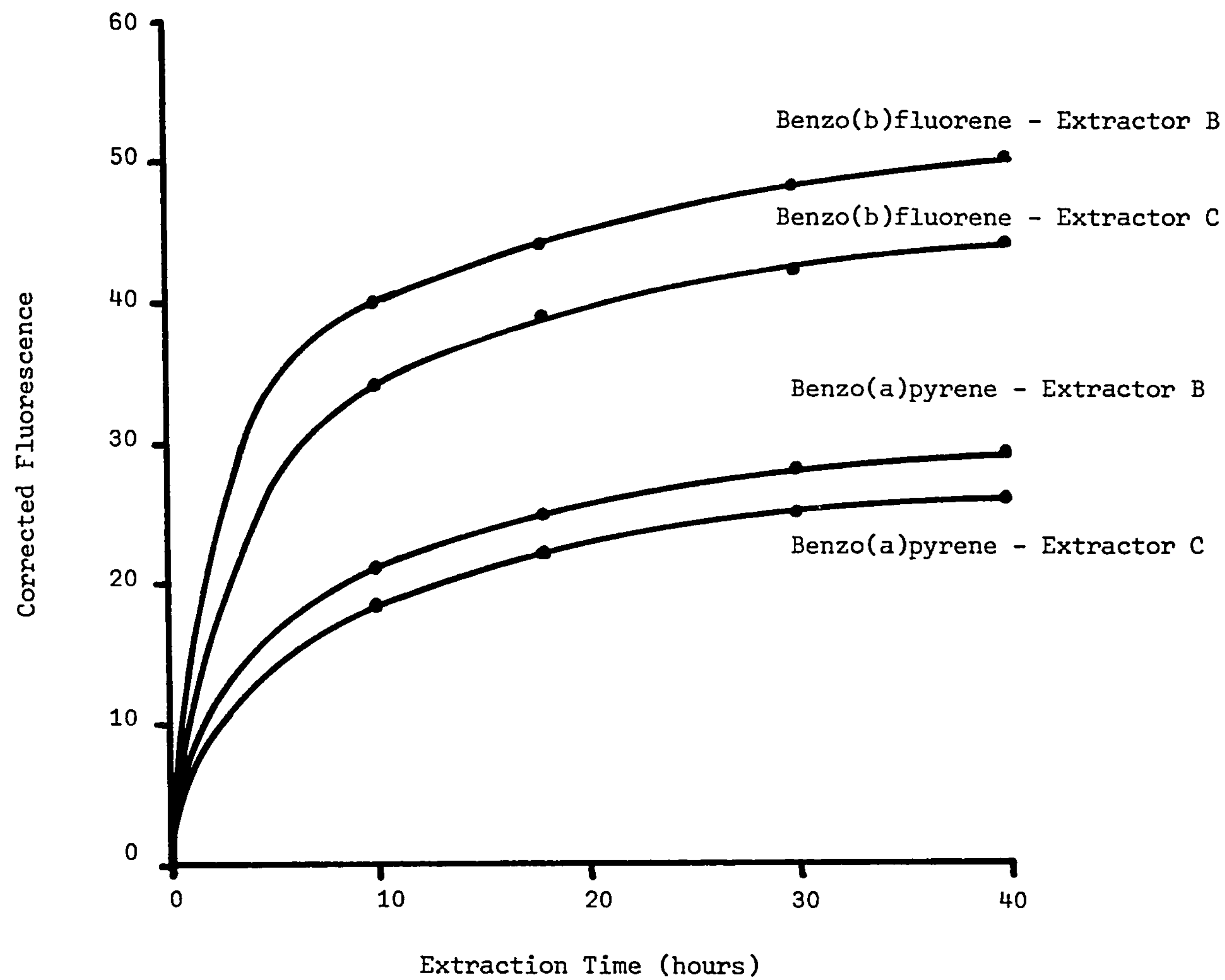



Figure 4. Variation of the amount of PNA extracted with time for typical continuous extractors.

Table III.

Summary of Time Required for Complete
Extractions of PNA with Extractors A, B, and C

| Extractor | No. of Extractions | Range (hours) | Mean (hours) |
|---|---|---|---|
| A | 22 | 29-101 | 54 |
| B | 20 | 16-75 | 42 |
| C | 15 | 16-68 | 37 |

somewhat to variations in stirring rates which were difficult to control precisely. By increasing the rate of stirring as much as possible, the last 16 extractions with B and C were completed in approximately 24 hours.

Extraction Efficiency. In order to determine the extraction efficiency of the extractors, synthetic samples of PNA dissolved in distilled water were prepared and extracted with pentane. Because of their extremely low solubility in pure water, PNA were introduced directly by means of a dilute ethanol solution into the extraction water. The percentage of ethanol in the water was always kept below 0.1%. Since the amount of each PNA dissolved in the water was known, percent recoveries were calculated by comparison of the fluorescence of the extract concentrated to an exact volume with that of a similar solution of known concentration. In all of these recovery studies, the four reference compounds were divided into two pairs such that the fluorescence spectrum of any one did not interfere with that of the other member of the pair. Accurate quantitative analysis could then be performed without prior separation of the individual compounds. For these studies benzo(a)pyrene was paired with benzo(b)fluorene and dibenz(a,h)anthracene was paired with fluoranthene.

The percent recovery data for extracting benzo(a)pyrene and benzo(b)-fluorene with extractor A is shown in Table IV. The concentrations of benzo(a)pyrene and benzo(b)fluorene in the water before extraction were 1.1 and 1.2 parts per billion (ppb) respectively. The first extraction gave unexplainably low results and these values have been statistically rejected on the basis of the later data. It can be seen from this data that greater than 95% of these two PNA may be extracted even without stirring the extraction solution.

Further recovery studies were conducted with all four reference

Table IV.

Efficiency of Extracting Benzo(a)pyrene and Benzo(b)fluorene with Extractor A

| | Percent Recoveries | |
|---|---|---|
| | Benzo(a)pyrene | Benzo(b)fluorene |
| | 65.5* | 81.5* |
| | 96.0 | 110 |
| | 90.0 | 106 |
| | 96.7 | 96.7 |
| | 98.4 | 100 |
| | 96.5 | 102 |
| Mean | 95.6% | 102.8% |
| Relative Standard Deviation | 3.0% | 4.7% |

* These values were omitted from the calculations of the means.

compounds at the part per trillion (ppt) level using extractors B and C and the results are shown in Tables V and VI. The concentration of each PNA was lowered to the ppt range to correspond with natural water levels. Approximately 250 ml of pentane was used for each extraction. All of the compounds were recovered using either extractor with better than 90% efficiency. The relative standard deviations were between 3 and 4% with the exception of dibenz(a,h)anthracene which was 1.1%. No reason can be found at the present time as to why this value is so much lower than the others.

When dealing with nanogram amounts of material, there is always the possibility of significant losses due to adsorption on glass surfaces. In light of this phenomenon, the recoveries obtained for all four reference compounds are quite good. The major drawback to continuous extraction is the length of time required. After development of the method was essentially complete, the feasibility of using batch extractions was studied in an effort to greatly decrease extraction time.

## Batch Extractions

Batch extractions were carried out in duplicate 4 liter separatory funnels. No grease of any kind was used on the ground glass joints. Since the joints were not allowed to set for any long period, it was sufficient to wet them thoroughly before use. Three liters of water was extracted each time. A convenient stand was made from wood and large cork rings to hold the two funnels in a vertical position. Synthetic samples were prepared from distilled water and the reference compounds; these were extracted with pentane. Recoveries of all compounds were calculated from fluorometric data.

Preliminary extractions with all four reference compounds indicated

Table V.

Efficiency of Extracting Benzo(a)pyrene and Benzo(b)fluorene with Extractors B and C

| | Benzo(a)pyrene | | Benzo(b)fluorene | |
|---|---|---|---|---|
| | Extractor B | Extractor C | Extractor B | Extractor C |
| Concentration | 412 ppt | 424 ppt | 375 ppt | 386 ppt |
| % Recoveries | 97.0<br>94.0<br>100 | 100<br>92.1<br>100 | 91.8<br>95.3<br>97.9 | 91.8<br>92.6<br>97.9 |
| Mean | 97.0% | 97.3% | 95.0% | 94.1% |
| Relative Standard Deviation | 3.1% | 3.8% | 3.2% | 3.4% |

Table VI.

Efficiency of Extracting Dibenz(a,h)anthracene and Fluoranthene with Extractors B and C

| | Dibenz(a,h)anthracene | | Fluoranthene | |
|---|---|---|---|---|
| | Extractor B | Extractor C | Extractor B | Extractor C |
| Concentration | 518 ppt | 533 ppt | 486 ppt | 499 ppt |
| % Recoveries | 95.2<br>95.1<br>93.0 | 89.4<br>90.4<br>92.0 | 97.0<br>104<br>98.2 | 93.4<br>100<br>97.3 |
| Mean | 94.4% | 90.6% | 99.7% | 96.9% |
| Relative Standard Deviation | 1.1% | 1.1% | 3.6% | 3.5% |

that greater than 90% of each compound could be recovered from water by shaking with 100 ml pentane for 5 minutes. The results of these extractions are shown in Table VII. However, when this procedure was applied to a real water sample from the Oyster River, a very stable emulsion was formed which prohibited quantitative separation of the two phases. Several methods were attempted to break up the emulsions.

Sodium chloride at concentrations of 0.33 and 3.3% was added to the river water before extraction to try to "salt out" the emulsions. Both attempts were unsuccessful. In the hope that emulsion formation might be caused by particulate matter present in the river water, 3000 ml of river water was filtered through a Hercules "fine" clarifying filter prior to extraction. This procedure was also unsuccessful in preventing the formation of an emulsion. At this point it was thought that the use of the suspending agents Superfloc and Aerofloc[107] (American Cyanimide Co.) might cut the emulsion. A combined solution of Superfloc and Aerofloc was obtained from the UNH Engineering Experiment Station and used in two ways: 2 ml of the Superfloc-Aerofloc solution was added to 3000 ml of river water before extraction, but no decrease in emulsion formation was noted. In the second procedure most of the water was drawn off after being shaken with pentane. Two ml of the Superfloc-Aerofloc solution was added to the remaining water and pentane. This procedure did reduce the emulsion moderately and so a recovery study was performed. Benzo(a)pyrene was added to 3000 ml of Oyster River water to give a concentration of 1 part per billion. The water was shaken with 100 ml of pentane for 5 minutes, allowed to settle for several minutes, and most of the water drawn off. Two ml of the Superfloc-Aerofloc solution was added to the emulsion and swirled lightly. The pentane was drawn off and made up to an exact volume for fluorescence measurement. The calculated percent recovery, however, was only 2%.

Table VII.

Percent Recoveries of Reference Compounds for Preliminary Batch Extractions

| | Concentration (ppt) | Percent Recoveries |
|---|---|---|
| Benzo(a)pyrene | 350 | 95.1, 97.0 |
| Benzo(b)fluorene | 350 | 98.3, 100 |
| Dibenz(a,h)anthracene | 440 | 92.8, 93.4 |
| Fluoranthene | 280 | 99.1, 101 |

Conditions: Compounds dissolved in distilled water and shaken thoroughly with 100 ml of pentane for 5 minutes.

Having learned that it was better to prevent the emulsion from forming than to try to break it up after formation, the funnel was swirled gently instead of being shaken vigorously. Preliminary tests were run using synthetic samples of benzo(a)pyrene dissolved in distilled water (350 ppt) to determine the time required for complete extraction with gentle swirling. Only 30% of the benzo(a)pyrene was recovered after 5 minutes of swirling whereas there was a 98% recovery after 10 minutes.

Based on these results a series of extractions were carried out with the four reference compounds. The compounds were dissolved in 3000 ml of distilled water and gently swirled with 100 ml of pentane for 10 minutes. The results of this work are shown in Table VIII. The mean percent recovery for each PNA was at least 97% while the relative standard deviation ranged from 1.76 to 2.36%. The recovery values are slightly higher and relative standard deviation slightly lower than those obtained using continuous extraction and constitute an improvement both in amount recovered and time required.

It would thus seem that batch extraction would be preferred to continuous extraction in most cases. The 10 minutes required for extraction permits the completion of the entire procedure in a single day. Continuous extraction, on the other hand, might still be necessary if emulsion formation cannot be prevented with some samples.

## Preliminary Separation

The pentane extract recovered from a natural water extraction consists of a wide variety of compounds ranging from long chain fatty acids to basic polynuclear heterocycles. Before TLC separation of the individual PNA and subsequent fluorescence analysis, it is desirable to remove as

Table VIII.

Percent Recoveries of Reference Compounds for Modified Batch Extractions

| | Concentration (ppt) | Percent Recovery | Mean | Relative Standard Deviation |
|---|---|---|---|---|
| Benzo(a)pyrene | 35 | 101,97.2,99.1,99.0 | 99.3 | 1.76 |
| | 350 | 102,100,97.3,98.6 | | |
| Benzo(b)fluorene | 30 | 97.4,96.7 | 98.8 | 2.36 |
| | 300 | 102,99.1 | | |
| Dibenz(a,h)anthracene | 445 | 98.8,95.6,99.0,96.1 | 97.0 | 1.83 |
| Fluoranthene | 280 | 100,97.0,97.6,95.8 | 97.5 | 2.08 |

Conditions: Compounds dissolved in distilled water and swirled gently with 100 ml of pentane for 10 minutes.

many interfering fluorescent substances as possible. This is particularly true of the basic heterocycles such as carbazole which not only are fluorescent but also have $R_B$ values comparable to those of PNA when separated on cellulose developed with 50% aqueous DMF. Several separation systems were investigated to find one which would remove most interfering basic compounds with a minimum loss of PNA.

During the preliminary stages of this work the first three unknown extracts were subjected to a column chromatographic procedure developed by Sawicki[108] as a means of removing interfering basic compounds and also possibly separating the PNA into several groups. The pentane extracts were concentrated to 5 ml and placed on a 5 x 125 mm acidic alumina column and eluted with successive 100 ml volumes of 0, 10, 20 and 30% benzene in pentane followed by a volume of 40% benzene in pentane sufficient to elute the remaining compounds. Absorption and fluorescence spectra were measured for the 100 ml fractions collected. While no detailed study of this system was made, the spectra indicated that, in addition to removing basic compounds, at least a partial separation of PNA into several groups was achieved with the use of approximately 700 ml of eluent. Since this method required the subsequent concentration of 700 ml of a benzene-pentane solution and since separation of the individual PNA could be achieved more easily using TLC, it was modified so that the eluent was pure 40% benzene in pentane. The column was reduced in size to 5 x 60 mm and 100 ml fractions checked with the Turner Fluorometer. This procedure was applied to the next four extracts with the result that most of the fluorescence was observed in the first 500 ml of eluent.

The recoveries of the four reference compounds using the above method were determined using approximately 1 mg of each compound. The percent recoveries were excellent and are listed below:

| | |
|---|---|
| Benzo(a)pyrene | 92.3% |
| Benzo(b)fluorene | 95.0% |
| Dibenz(a,h)anthracene | 91.4% |
| Fluoranthene | 91.9% |

In spite of this data, this approach was abandoned because of the time involved and the necessity of concentrating large volumes of solvent.

A variety of procedures were investigated in search of one which would remove most of the basic compounds with little loss of PNA. In these studies benzo(a)pyrene and carbazole were used as representative of the PNA and basic heterocycles respectively. Recovery values were calculated from fluorescence data and represent the percent of each compound remaining in the pentane layer. A description of these studies follows:

### Extraction of Carbazole with HCl

A 5 ppm solution of carbazole in 100 ml of pentane was extracted one time with 10 ml of 1 N HCl. The fluorescence spectra showed that less than 1% of the carbazole had been removed from the pentane.

### Treatment with Acidic Alumina

Both activated and deactivated acidic alumina were investigated as possible means of removing carbazole. Two procedures were employed using activated acidic alumina. The first attempt using activated alumina consisted of a batch contact of 10 grams of activated alumina with a 100 ml pentane solution containing 100 and 500 µg of benzo(a)pyrene and carbazole respectively in a 250 ml Erlenmeyer flask. The contents of the flask were shaken for 5 minutes, allowed to stand for 30 minutes, filtered through filter paper, and washed with 20 ml of pentane. The recoveries were 0.3% and 0.7% for benzo(a)pyrene and carbazole respectively. In the second

method 5 grams of alumina were placed in a fritted glass funnel which was attached to a 125 ml suction flask. A solution containing 100 and 500 μg of benzo(a)pyrene and carbazole respectively in 50 ml of pentane was filtered through the alumina and washed with three 5 ml portions of pentane. The recoveries for benzo(a)pyrene and carbazole were 0.6% and 0.7% respectively.

In view of the low benzo(a)pyrene recoveries obtained with activated alumina, it was decided to try deactivated alumina. Acidic alumina, activity IV[109], was prepared and 1 gram was placed on a fritted glass funnel. A solution containing 1.6 μg each of benzo(a)pyrene and carbazole in 25 ml of pentane was filtered through the alumina and washed with 75 ml of pentane. The recoveries were 92.5% and 90.8% for benzo(a)pyrene and carbazole respectively.

Since benzo(a)pyrene recoveries were too low using activated acidic alumina and carbazole recoveries were too high using deactivated acidic alumina, this approach was abandoned.

## Extraction with Perchloric Acid

Hartung and Jewell[110] have shown that partition of a benzene solution of neutral PNA with 72% perchloric acid concentrates the indoles, carbazoles, and phenazines in the acid phase. A solution containing 100 μg each of benzo(a)pyrene and carbazole in 50 ml of pentane was extracted with 10 ml of 72% perchloric acid. The recoveries for benzo(a)pyrene and carbazole were 46.9% and 15.4% respectively. No further work was attempted using perchloric acid.

## Extraction with Sulfuric Acid

Blom and Branken[111] have shown that successive extractions with 72

and 92% sulfuric acid of a benzene solution of a coal-tar fraction composed mainly of anthracene, carbazole, and phenanthrene separated the more basic carbazole from the other constituents. This method was applied to 75 ml of a pentane solution containing 0.98 and 5.70 mg of benzo(a)pyrene and carbazole respectively. The pentane layer was extracted once with 25 ml of 92% sulfuric acid. The recoveries in the pentane layer were 10.4% and 0.2% for benzo(a)pyrene and carbazole respectively. While this method removes essentially all of the carbazole it was unacceptable because of the loss of 90% of the benzo(a)pyrene. The feasibility of using a single extraction with 72% sulfuric acid was examined.

Three extractions were performed in which 75 ml of pentane solution containing 0.75 and 4.28 mg of benzo(a)pyrene and carbazole respectively were shaken with 25 ml of 72% sulfuric acid. The mean recoveries for benzo(a)pyrene and carbazole were 96.1% and 5.2% respectively. These results prompted a more detailed study using smaller amounts of benzo(a)-pyrene and carbazole.

Pentane solutions (75 ml) of benzo(a)pyrene and carbazole were extracted once with 25 ml of 72% sulfuric acid. Duplicate determinations of four solutions containing 1, 10, 100, and 1000 ppb benzo(a)pyrene and 5 times these amounts of carbazole respectively were extracted in random order. The recovery data for both compounds are shown in Table IX. The benzo(a)pyrene results agree quite favorably with those of the three preliminary extractions with 72% sulfuric acid. The mean benzo(a)pyrene recovery for the four concentrations was 98.1% with a relative standard deviation of 2.52%. The efficiency of removing carbazole from the pentane solutions is greater than 90% for all solutions except the lowest concentration of 5 ppb. Even at this low concentration, approximately 70% of the

Table IX

Summary of Recovery Data for Preliminary Separation with 72% Sulfuric Acid

| Benzo(a)pyrene | | | Carbazole | | |
|---|---|---|---|---|---|
| Concentration | % Recovery | Mean | Concentration | % Recovery | Mean |
| 1 ppb | 95.7, 91.0 | 93.4 | 5 ppb | 31.5, 30.9 | 31.2 |
| 10 | 101 , 97.3 | 99.2 | 50 | 4.8, 8.4 | 6.6 |
| 100 | 102 , 101 | 101.5 | 500 | 4.1, 12.4 | 8.3 |
| 1000 | 96.5, 100 | 98.3 | 5000 | 3.0, 3.0 | 3.0 |

Conditions: 75 ml of benzo(a)pyrene-carbazole (1:5) solution extracted one time with 25 ml of 72% sulfuric acid. % recovery data represent the % of each compound remaining in the pentane layer.

carbazole was removed. The 30% carbazole remaining in the pentane layer is tolerable in the over-all method because at this low concentration, the amount of carbazole spotted on the TLC plate would not constitute a serious interference. The sulfuric acid extraction was used in all subsequent analyses of unknown samples.

## Thin-Layer Chromatography

### Materials

Adsorbents. The following adsorbents with the exception of cellulose were used to prepare and activate TLC plates according to instructions given by the manufacturers. Cellulose slurries were prepared by shaking the adsorbent and solvent with glass beads in an Erlenmeyer flask for 10 minutes. This was particularly necessary when using the fluorescent cellulose in order to produce a uniform fluorescent field. All slurries were spread using a 10 cm polyethylene bar spreader. Adsorbent thickness of 200, 600, or 1200 u could be selected.

Cellulose MN300 (Macherey, Nagel and Co.)
Cellulose $MN300_{254}$ (Macherey, Nagel and Co.)
Aluminum oxide $GF_{254}$ (E. Merck)
Silica gel $GF_{254}$ (E. Merck)
Silica gel $PF_{254}$ (E. Merck)

Microplates. Micro TLC plates were prepared from microscope slides in the usual manner. The adsorbent slurry was magnetically stirred in a small beaker and the slides were quickly dipped into the solution. The adsorbent was allowed to dry and then wiped from one side of the slide. These microplates were developed in sealed baby food jars.

Glass Plates. Glass plates were cut from double-thick window glass to standard sizes of 5 x 20, 10 x 20, and 20 x 20 cm. Prior to spreading, the plates were cleaned with hot chromic acid and washed thoroughly with distilled water. Prepared plates were desiccated in a dry box over calcium chloride until use.

Baker-flex Sheets. Baker-flex sheets (250 μ) of high purity cellulose were purchased from J. T. Baker Chemical Co. and used without further activation. 20 x 20 cm sheets were cut to appropriate sizes before use. Approximately 1 cm of adsorbent was scraped from each side edge to prevent "edge effects". The sheets were taped to a glass plate for use with sandwich-type developing chambers and the TLC scanner.

## Method of Development

Almost all TLC plates were developed using the sandwich technique[112]. The two exceptions to this were the use of microplates in preliminary studies and the use of a presaturated chamber in a comparison study to the sandwich technique. A sandwich chamber was prepared by first stripping about 1 cm of adsorbent from each side edge of the plate or sheet. If a sheet was being used, it was then taped securely to a glass plate. Then strips of white blotter paper were placed along the sides of the plate being careful that they did not touch the adsorbent layer. Finally, a clean glass plate was laid over the plate to be developed and fastened to it with spring clips. The blotter paper spacers created a low volume chamber between the two plates and also served to saturate the area ahead of the solvent front since the developing solvent migrated up the spacers more rapidly than up the adsorbent layer. The entire assembly was placed in a 3.5 x 21 cm paper chromatographic trough containing 15 to 20 ml of

solvent. Because of the extreme light sensitivity of most PNA, the entire apparatus was covered with a black cloth during development. Development was continued until the solvent front had travelled 15 cm. The average development time was approximately 4 hours.

## Sample Application

Samples were applied as spots 1.5 cm from the bottom of the plate and approximately 1.9 cm from each other. Ethanol was used as the solvent in which the PNA were dissolved because of the high volatility of pentane which made quantitative application impossible. Samples were applied in successive small doses to minimize the spot size. Four different types of spotting tools were studied:

(1) Capillary Tube. The preliminary qualitative TLC work was done using capillary tubes which were drawn out from heated melting point tubes. These tubes held approximately 10 to 20 µl of solvent.

(2) Lang-Levy Micropipets. These self-filling micropipets were obtained from Brinkmann Instruments, Inc. in 1, 2, 5, and 10 µl sizes. The manufacturer claims that they are reproducible to ± 5% of the stated volume. These were used for the majority of the TLC work where accurate quantitative results were not necessary.

(3) Plastic Micrometer Buret. A micrometer buret having a capacity of 0.2 ml was obtained from Brinkmann Instrument, Inc. The smallest scale division was 0.0002 ml and the stated accuracy was 0.5%. The buret is designed so that the

solvent comes in contact only with the interchangeable glass barrel and the Teflon plunger. This buret was used for all quantitative TLC work.

(4) Push-Button Adjustable Syringe. A Hamilton Co. syringe with a capacity of 0.2 ml and capable of delivering any volume between 1 and 20 µl was studied. The syringe was calibrated to 0.1 µl with an error of ± 1%. This syringe was found to be unsatisfactory for spotting even as little as 1 µl because of the large area of the spots. This was caused by the ejection of the entire sample at one time.

## Spot Visualization

Location of visible spots on developed chromatograms was accomplished under UV illumination. Both a short wave (UVS-11, Ultra-Violet Products, Inc.) and a long wave (UVL-22, Ultra-Violet Products, Inc.) lamp were used giving peak intensities at 254 and 365 nm respectively. The separated reference compounds were more distinct under 254 nm than 365 nm excitation light on cellulose layers. Illumination of the plates with UV light was kept as short as possible to minimize spot decomposition. The minimum amount of material necessary for visual observation varied among the four reference compounds. The observed fluorescence is dependent upon both the natural fluorescence quantum efficiency and each com-

pound's adsorptivity at 254 nm. Minimum observable amounts of benzo(a)-pyrene and fluoranthene were on the order of 10 ng while those for benzo(b)fluorene and dibenz(a,h)anthracene were about 30 ng.

## Initial Studies

Preliminary TLC work was done using the pentane concentrate from the first Oyster River water extraction. Samples were qualitatively spotted using a capillary tube. A number of chromatographic systems were investigated; these are listed in Table X. These possibilities were taken primarily from Sawicki's review article[6] on the determination of PNA in the human environment. The silica gel and alumina systems were developed on microplates while the cellulose plates were prepared on standard size glass. The different systems showed varying degrees of migration and separation. Under 254 nm light the fluorescence zones were easily observed. The separations observed with the cellulose systems were far superior to any of the others. Those systems in which hexane was the eluent produced the best separations for alumina and silica gel. The alumina $GF_{254}$, pentane-ether (19:1) system was unique in that the entire fluorescent band migrated with the solvent front. This is probably the reason why Sawicki[6] suggests that this TLC system is the best for separating PNA from complex mixtures. With cellulose TLC systems, the hydrocarbon mixture was separated into four distinct spots. Since cellulose gave the best separations, it was used exclusively in the remainder of the work.

## Separation of PNA on Cellulose

A study was made of the cellulose-DMF-water system to determine the variation in migration distances for the four hydrocarbon spots as

Table X.

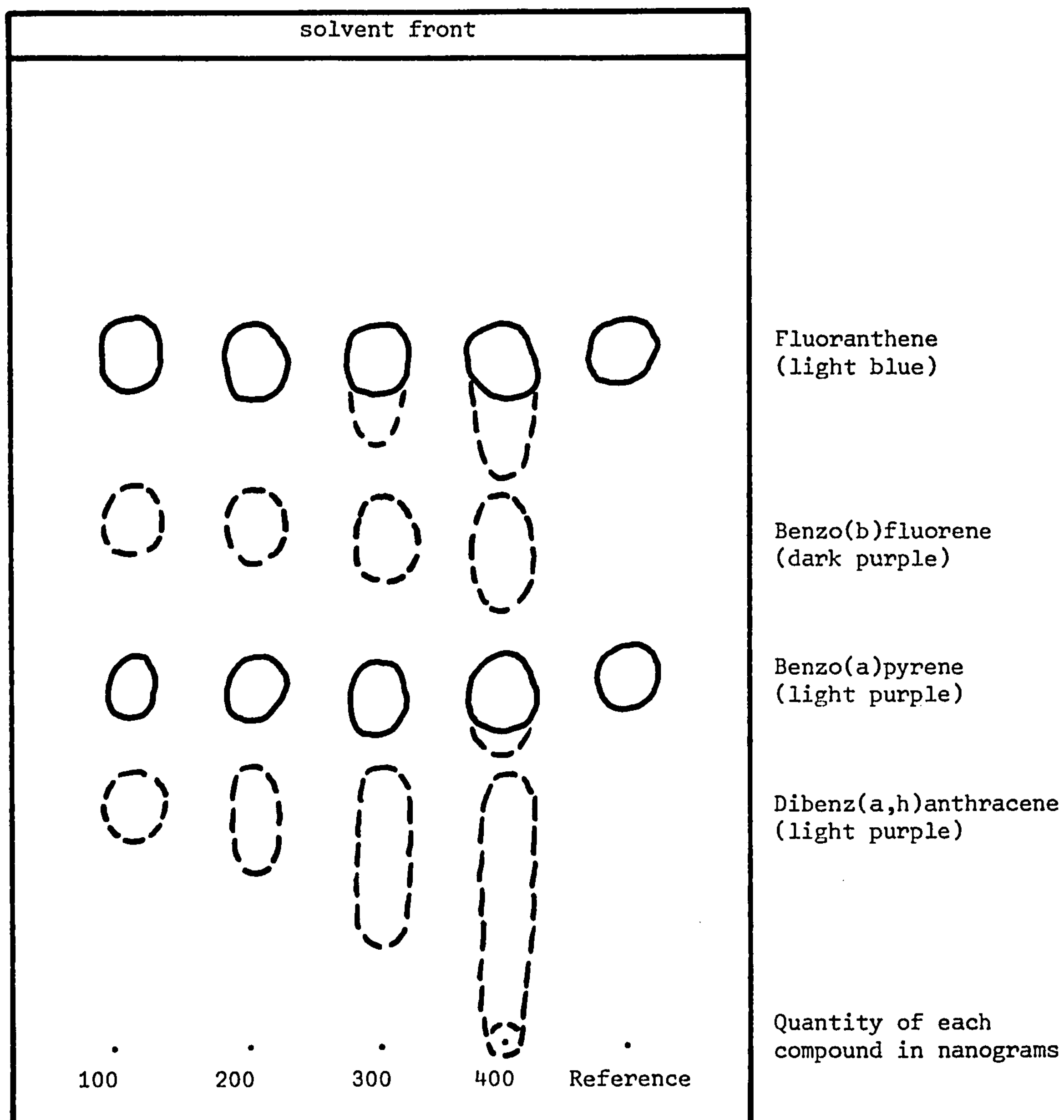
Summary of TLC Systems Investigated

| ADSORBENT | ELUENT | |
|---|---|---|
| Silica Gel $GF_{254}$ | Pentane-benzene | (1:1) |
| | Hexane-benzene | (1:1) |
| | Hexane | |
| | Carbon tetrachloride | |
| Silica Gel $PF_{254}$ | Pentane-benzene | (1:1) |
| | Hexane-benzene | (1:1) |
| | Hexane | |
| | Carbon tetrachloride | |
| Alumina $GF_{254}$ | Pentane-ether | (19:1) |
| | Hexane | |
| | Carbon tetrachloride | |
| Cellulose MN300 | Dimethylformamide-water | (3:1) |
| | Dimethylformamide-water | (1:3) |
| | Dimethylformamide-water | (3:2) |
| | Dimethylformamide-water | (1:1) |

the DMF-water ratio was changed. The four different DMF-water ratios studied are listed in Table X. It was found that the individual migration distances increased with decreasing water content and vice-versa with the best separation achieved using 50% aqueous DMF as eluent.

An attempt was then made to tentatively identify the four spots on the basis of $R_B$ values given by Sawicki[36]. Of those compounds for which Sawicki had determined $R_B$ values for the cellulose-50% aqueous DMF system, only anthracene was immediately available. It was chromatographed along with the hydrocarbon extract and the $R_B$ values of the four spots calculated from the known $R_B$ value of anthracene and the observed migration distances of the four spots and anthracene. The calculated values were then compared with Sawicki's list and the four most likely compounds purchased as reference compounds. These were dibenz(a,h)anthracene, benzo(a)pyrene, benzo(b)fluorene and fluoranthene. In subsequent TLC separations of these reference compounds, $R_B$ values, as shown in Table II, agreed consistently with those of the hydrocarbon extract if developing conditions were strictly controlled. The relative standard deviation of the $R_B$ values of the four reference compounds ranged from 2.4% for fluoranthene to 8.4% for dibenz(a,h)anthracene. This is consistent with Sawicki who claims that his values are good to ± 10%. This data was compiled from separations made both on Cellulose MN300 and on Baker-flex cellulose sheets. The separations achieved with both of these types of cellulose were identical.

The effect of concentration on the separation of the four reference compounds was studied and typical data are shown in Figure 5. Even though the separation is complete with 400 ng of each PNA, there is extensive tailing which would interfere severely with the analysis of other PNA present in a sample. For this reason, applied quantities were usually




Developer: 50% aqueous dimethylformamide
Time: 3 hours, 50 minutes
Reference: 50 ng each of BaP and Fluoranthene
_______ : Intense fluorescence
\- - - - : Weak fluorescence

Figure 5. Typical separation of reference compounds on cellulose as seen under 254 nm light.

kept below 200 ng so that reasonable spot areas could be maintained. For quantitative analysis using the TLC scanner, 50 ng was the maximum amount applied.

In conjunction with the concentration dependence of the separation, the effect of adsorbent layer thickness was also investigated. It was found that layers of 200, 250, and 600 μ produced essentially the same degree of separation with varying amounts of reference compounds. Cellulose MN300 and Baker-flex cellulose plates of 200 μ and 250 μ thickness respectively were used for the remainder of the work.

## Elution of PNA from Cellulose

Before the TLC scanning attachment was purchased, the removal of PNA from cellulose plates, prior to fluorometric quantitation, was investigated. The efficiency of removing benzo(a)pyrene and benzo(b)-fluorene from cellulose was measured using the method of T. W. Stanley and co-workers[32]. The TLC spot was scraped from the plate with a spatula and carefully transferred to a 25 ml fritted glass funnel attached to a 50 ml. suction flask which was immersed in a 50 ± 2° C water bath. The adsorbent was then washed with ethyl ether at a rate approximately equal to the rate of evaporation of the ether in the suction flask. A total of 100 ml of ether was used for each spot. The ether was evaporated to dryness and the residue dissolved in 1.00 ml of pentane for fluorometric analysis. The mean percent recoveries for triplicate 1.4 μg benzo(a)pyrene and benzo(b)-fluorene spots were 13 and 71% respectively. This same procedure was later tried with more realistic 250 ng benzo(a)pyrene spots and the mean recovery for three spots was 30.4% with a large relative standard deviation of 34.5%. Since Stanley was removing benzo(a)pyrene from an alumina layer and did not give any recovery values, it is impossible to compare this data with his

work. Obviously, this method was totally unacceptable for even semi-quantitative work.

A spot collector was obtained from Brinkmann Instruments, Inc. and used for further studies. The collector consisted of a bent, small-bore glass tube connected to a 25 ml round-bottom flask with a short tube having a side arm. A small fritted disc was clamped in place at the junction of the two tubes. When suction was applied to the side arm, the adsorbent layer containing the spot was drawn into the collector and deposited on the fritted disc. Ethyl ether was then drawn through the tube into the round-bottom flask, and the compound extracted from the adsorbent in the process. The amount of ether necessary for removal of 25 ng of benzo(a)-pyrene was determined. Volumes from 10 to 100 ml were studied, and it was found that 15 ml of ether removed as much benzo(a)pyrene as larger volumes. To further elucidate this recovery procedure, a complete statistical study was then made to determine the percent recoveries of 100, 250 and 500 ng quantities of benzo(a)pyrene and benzo(b)fluorene on Cellulose MN300 and Cellulose $MN300F_{254}$ plates. Duplicate determinations were made for each quantity on each type of adsorbent. Blank determinations were made by removing a section of adsorbent containing no PNA. Statistical analysis of the data by an analysis of variance showed that there was no significant difference in percent recovery between adsorbent types and among concentrations at the 95% confidence level. The 95% confidence intervals for benzo(a)pyrene and benzo(b)fluorene were 3.2 ± 0.9% and 8.5 ± 2.6% respectively. These exceptionally low recoveries were unacceptable and this method was abandoned with the acquisition of the Farrand TLC scanner.

## Fluorescence Analysis

### Instrumentation

All of the initial fluorescence work was done on a spectrofluorometer designed and built by B. Solomon[106]. Excitation was provided by a 1000 watt mercury arc lamp combined with a Bausch and Lomb High Intensity Grating Monochromator. The fluorescence emitted at a right angle to the excitation beam passed through a Jarrell-Ash Model 82-000 half-meter scanning monochromator and was detected by a 1P28 photomultiplier tube. The signal from the photomultiplier was amplified by a Leeds and Northrup Co. Microvolt Indicating Amplifier and recorded on a Houston Omnigraphic Corporation Model HR-96T x-y recorder. This instrument was dismantled with the acquisition of the Farrand MK-1 Spectrofluorometer which was used for the majority of the work.

The Farrand MK-1 Spectrofluorometer employed a 150 watt xenon arc lamp for fluorescence excitation. This lamp provided a continuous output throughout the ultraviolet and visible regions of the spectrum. Both the excitation and emission monochromators were of the modified Czerny-Turner type having a wavelength range from 200 to 700 nm. Wavelength accuracy was ± 2 nm. In all solution work slit widths were chosen to produce a band pass of 5 nm.

Radiation emerging from the excitation monochromator was focused on a 1 cm square fused quartz cell and the fluorescence was detected at a right angle by a 1P28 photomultiplier tube after passing through the emission monochromator. After amplification, the output was recorded on a Houston Omnigraphic Corporation Model HR-96T x-y recorder. The sensitivity of the x-axis was set at .1 volts/inch to produce a full scale scan of 300 nm; the y-axis sensitivity was 0.001 volt/inch.

The Farrand spectrofluorometer was fitted with a Farrand TLC Scanner which is described in a later section. This scanner was used in fluorescence measurements on TLC plates in the subsequent work.

A Turner Filter Fluorometer Model 110 was also used. It is a null-point instrument incorporating a general purpose ultraviolet lamp (G.E. #74T4/BL) with appropriate filters. All measurements were made using a #7-60 excitation filter which peaks at 360 nm and a #2A emission filter which passes wavelengths longer than 415 nm. Samples were contained in a cylindrical 1 cm quartz cell.

## Solution Fluorescence

All solution fluorescence measurements were made in Pesticide Analysis Grade n-pentane, the same solvent which was used in the extraction experiments. Each new batch of solvent was checked fluorometrically to assure the absence of contamination.

For quantitative fluorescence work, two different reference systems were employed. Comparison with a standard reference material is necessary to correct for variations in the light source or photomultiplier. Initially, the fluorescence emission at 450 nm from a 1 ppm solution of quinine sulfate in 0.1 $\underline{N}$ $H_2SO_4$ was used. The solution was excited with 350 nm light. Subsequent work was performed using a 1 cm solid pyrex reference cell obtained from Farrand Optical Co. The excitation monochromator was set at 350 nm, and the corresponding scatter peak at 350 nm was recorded.

In order that the intensities of fluorescence spectra obtained using the two reference systems could be compared, the relative emissions of the two references themselves were determined. This was accomplished by comparison of the peak heights at the maximum emission wavelengths des-

cribed above for the two systems. Under the standard conditions used, the quinine sulfate emission was equal to 94% of that of the pyrex cell.

Fluorescence excitation and emission spectra for the five reference compounds used in this study are shown in Figures 6-10. It can be seen, with the exception of fluoranthene, that all of these compounds exhibit excellent fine structure in both excitation and emission spectra. Even though the emission spectrum of fluoranthene was quite broad, its excitation spectrum does show pronounced fine structure. This property was very valuable in the characterization of unknown compounds and, in many cases with the proper choice of excitation and emission wavelengths, permitted the selective measurement of the fluorescence of a single component in a mixture.

Fluorescence spectra of these compounds dissolved in pentane were used for quantitative analysis of solutions. The analytical wavelengths used are shown in Table XI. More than one excitation wavelength is shown for three of the compounds; the choice of wavelength depended upon the composition of the mixture to be analyzed.

Calibration curves were not used for the quantitative determinations made during this study using the fluorescence of a pentane solution. These measurements were made during recovery studies of known amounts of specific compounds and the fluorescence of a solution to be analyzed was compared with that of a solution of known concentration. This technique is valid as long as PNA concentrations remain low enough to prohibit interference from concentration quenching. To insure that this condition was satisfied, concentrations were usually kept at the sub part-per-million level. If compliance with this requirement was questionable, several dilutions of the solution were made and their fluorescence spectra measured to check the linearity of the relationship between concentration and

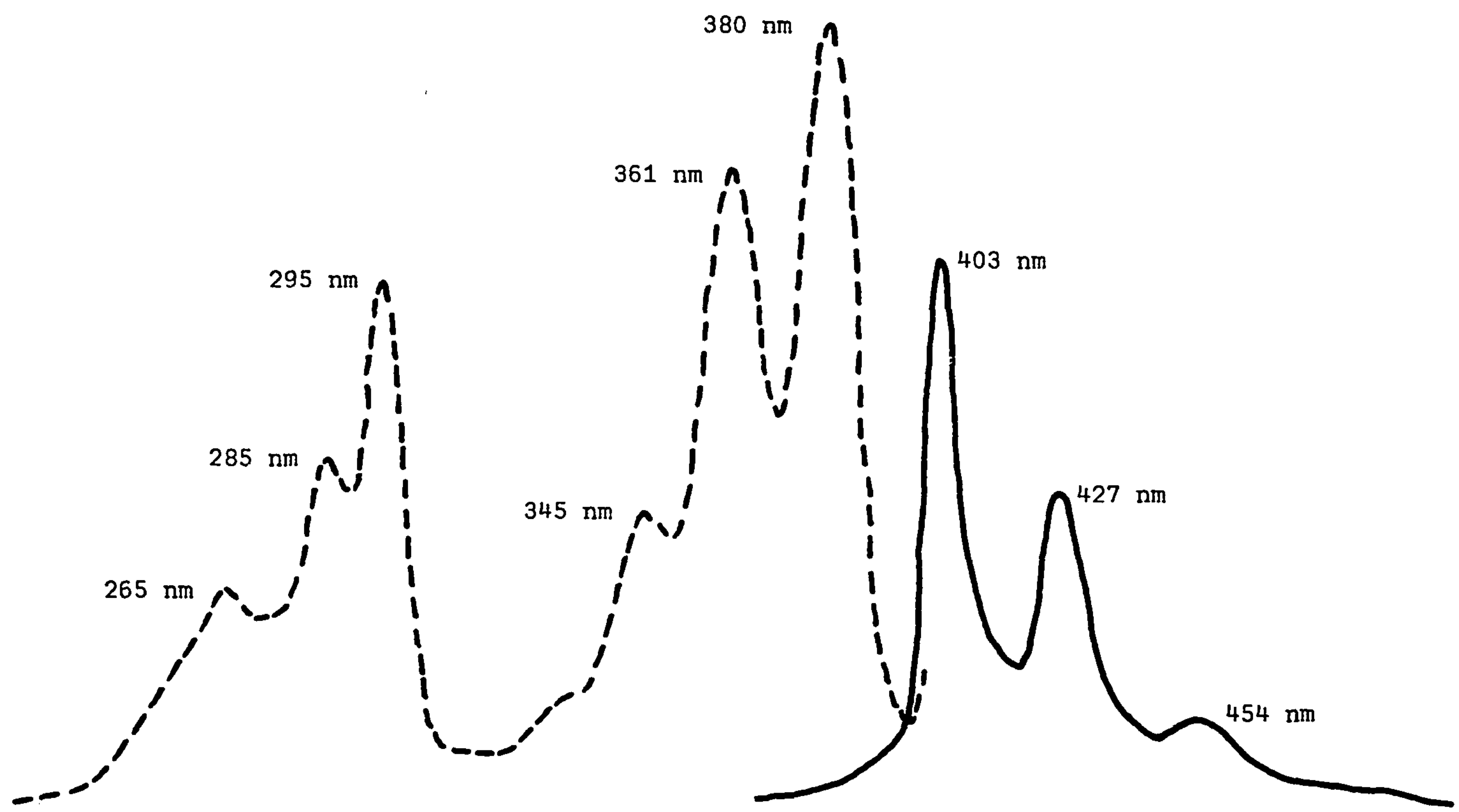



Figure 6. Fluorescence spectra of benzo(a)pyrene in pentane

- - - - Excitation spectrum at emission wavelength 403 nm

———— Emission spectrum at excitation wavelength 295 cm

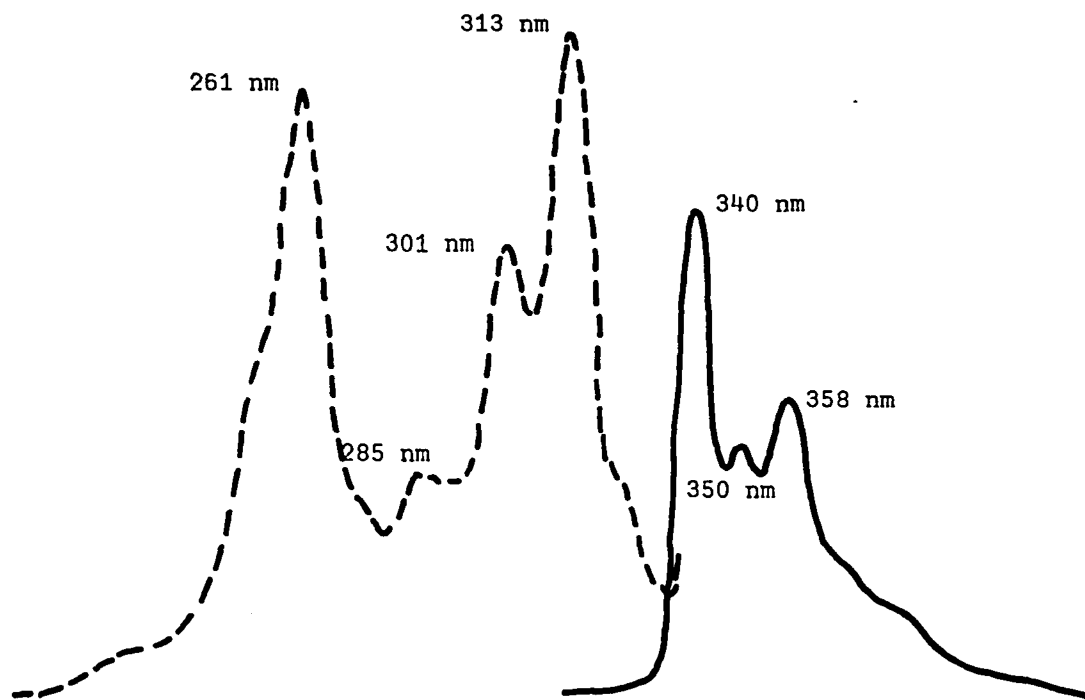



Figure 7. Fluorescence spectra of benzo(b)fluorene in pentane

- - - - Excitation spectrum at emission wavelength 340 nm

——— Emission spectrum at excitation wavelength 301 nm

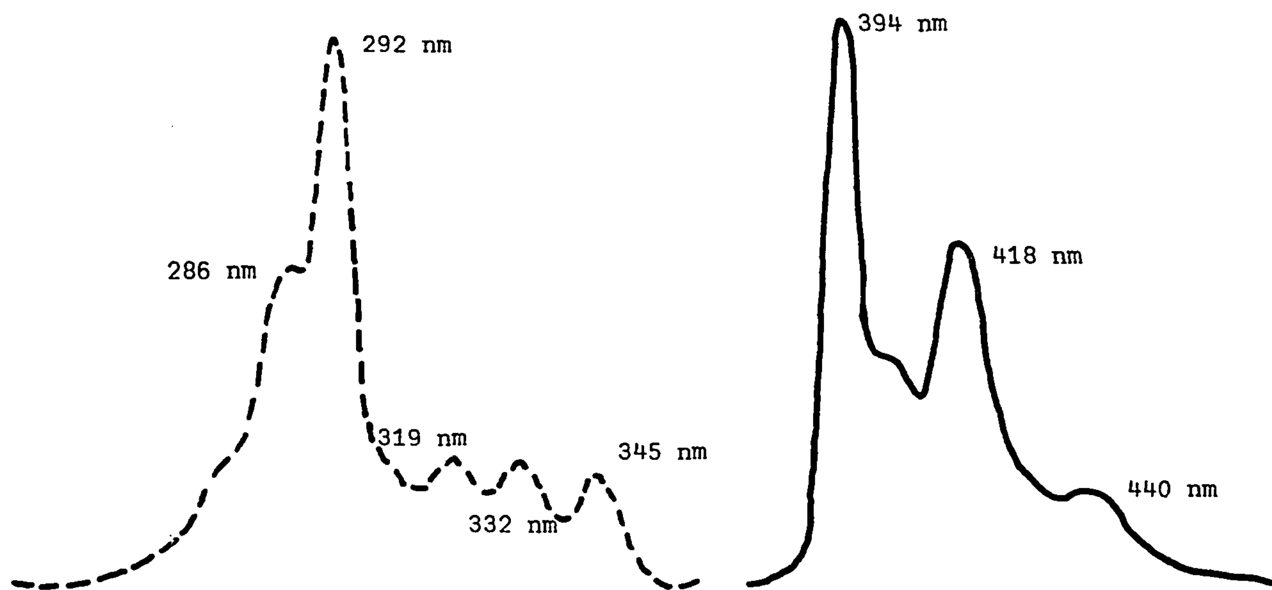



Figure 8. Fluorescence spectra of dibenz(a,h)anthracene in pentane.

- - - - Excitation spectrum at emission wavelength 394 nm

——— Emission spectrum at excitation wavelength 292 nm

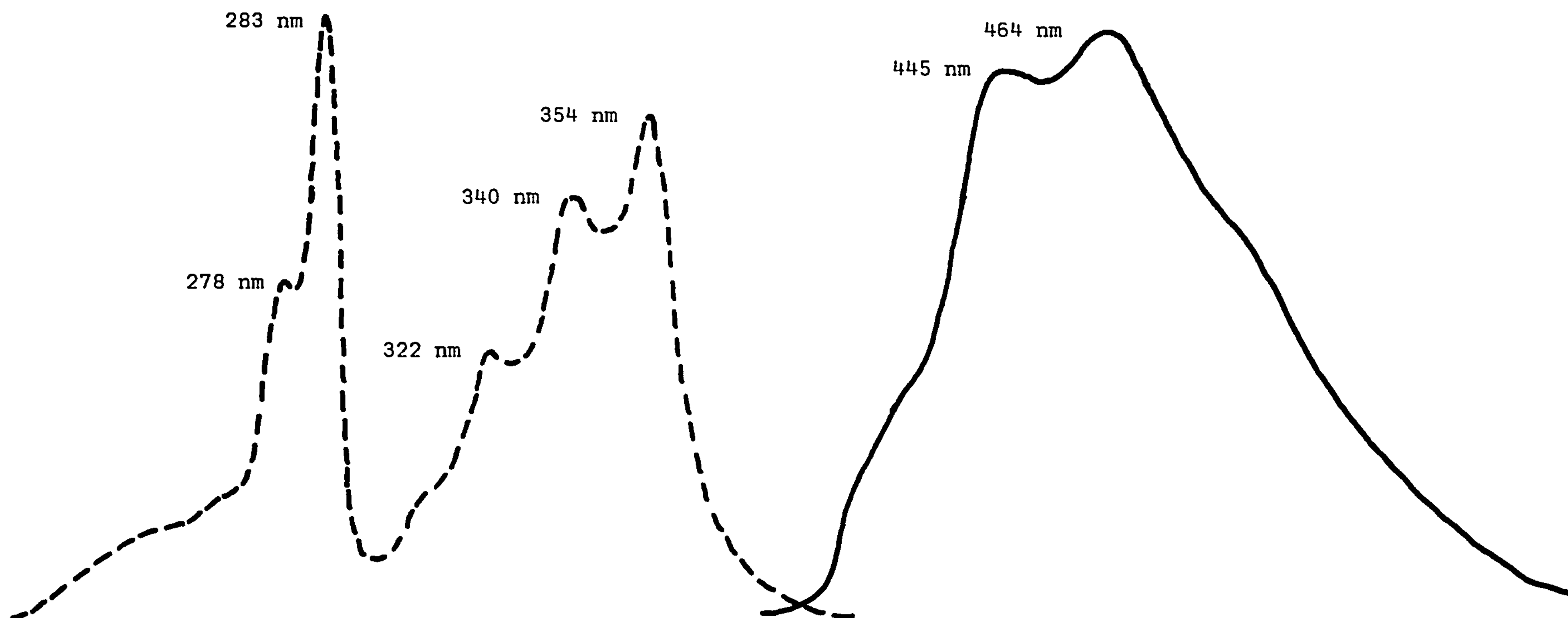



Figure 9. Fluorescence spectra of fluoranthene in pentane

- - - - Excitation spectrum at emission wavelength 464 nm

———— Emission spectrum at excitation wavelength 283 nm

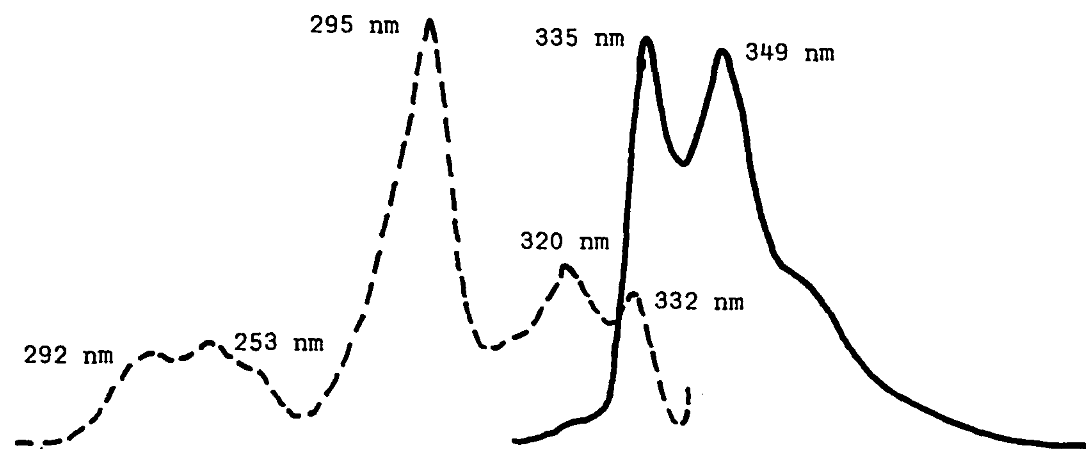



Figure 10. Fluorescence spectra of carbazole in pentane

- - - - Excitation spectrum at emission wavelength 349 nm

——— Emission spectrum at excitation wavelength 295 nm

Table XI.

Fluorescence Wavelengths Used for Quantitative Analysis

| | Wavelengths (nm) | |
|---|---|---|
| | Excitation | Emission |
| Benzo(a)pyrene | 295 | 403 |
| | 361 | 403 |
| Benzo(b)fluorene | 261 | 340 |
| | 301 | 340 |
| Dibenz(a,h)anthracene | 292 | 394 |
| Fluoranthene | 283 | 464 |
| | 354 | 464 |
| Carbazole | 295 | 349 |

fluorescence. Typical data for such a dilution with one of the most fluorescent compounds, benzo(a)pyrene, are shown in Table XII; excellent linearity is exhibited.

Fluorescence spectra of standard solutions of five additional PNA were also measured. These compounds were suspected of being present in unknown water samples. Individual fluorescence excitation and emission spectra of these compounds are shown in Figures 11-15.

## Fluorometric TLC Scanning

### Description of the Scanner

A TLC scanner was purchased from Farrand Optical Co. and affixed atop the Farrand MK-1 Spectrofluorometer. The scanner is a 50 x 48 x 11 cm light-tight metal box containing a movable carriage which holds any size TLC plate up to 20 x 20 cm. The plate is held in place in the carriage, adsorbent layer facing downward, with magnets placed around its edge. The carriage is moved by a reversible, variable speed synchronous motor in one direction and by a manually controlled calibrated dial perpendicular to the first direction. Scanning rates may be selected from 10 pre-set values ranging from 0.4 to 12 inches per minute. Transmission of the light beam from the excitation monochromator to the TLC plate and the reflected fluorescence emission from the plate to the emission monochromator is accomplished by a series of lenses and mirrors built into a small adaptor which fits into the sample compartment of the spectrofluorometer. This unit is easily installed and makes it possible to change from TLC scanning to solution fluorescence and vice versa in a few minutes. The image of the light beam on the TLC plate is defined by interchangable adaptor slits of various sizes which fit on the adaptor. A slit with dimensions 1.5 x 13 mm was

Table XII.

Linearity of Benzo(a)pyrene Fluorescence with Dilution

| | Range | Fluorescence | Corrected Fluorescence* |
|---|---|---|---|
| Original Solution | 30 | 103 | 3090 |
| 10:1 Dilution | 3 | 102 | 306 |
| 100:1 Dilution | 0.3 | 104 | 31.2 |

*Corrected Fluorescence = Range x Fluorescence

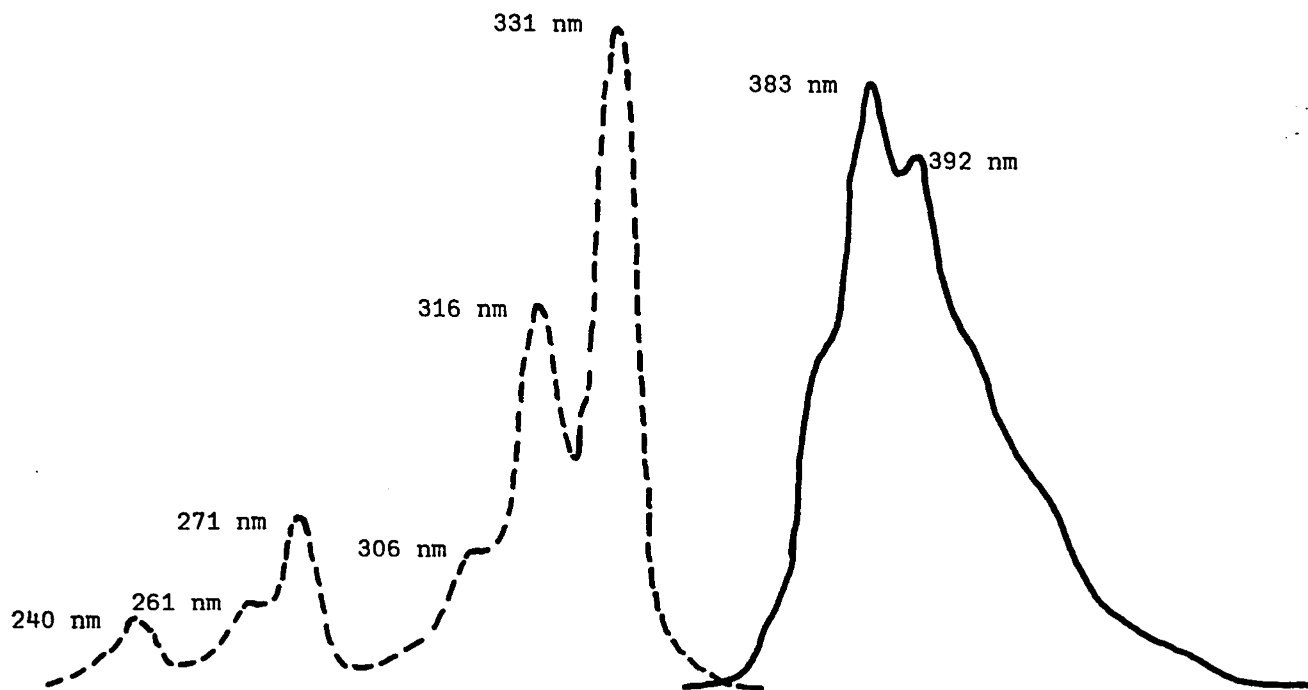



Figure 11. Fluorescence spectra of pyrene in pentane

- - - - Excitation spectrum at emission wavelength 383 nm

——— Emission spectrum at excitation wavelength 331 nm

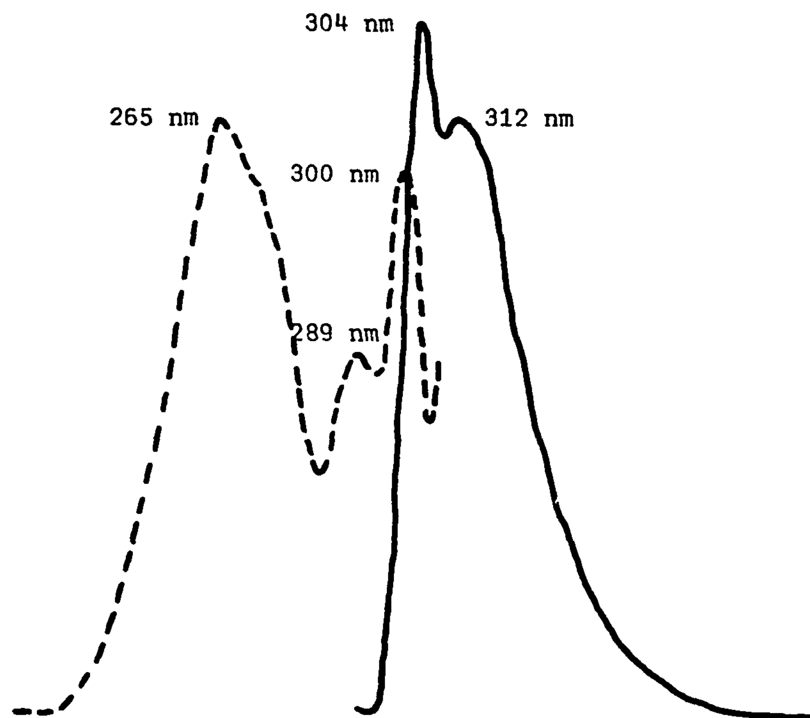



Figure 12. Fluorescence spectra of fluorene in pentane

- - - - Excitation spectrum at emission wavelength 312 nm

———— Emission spectrum at excitation wavelength 265 nm

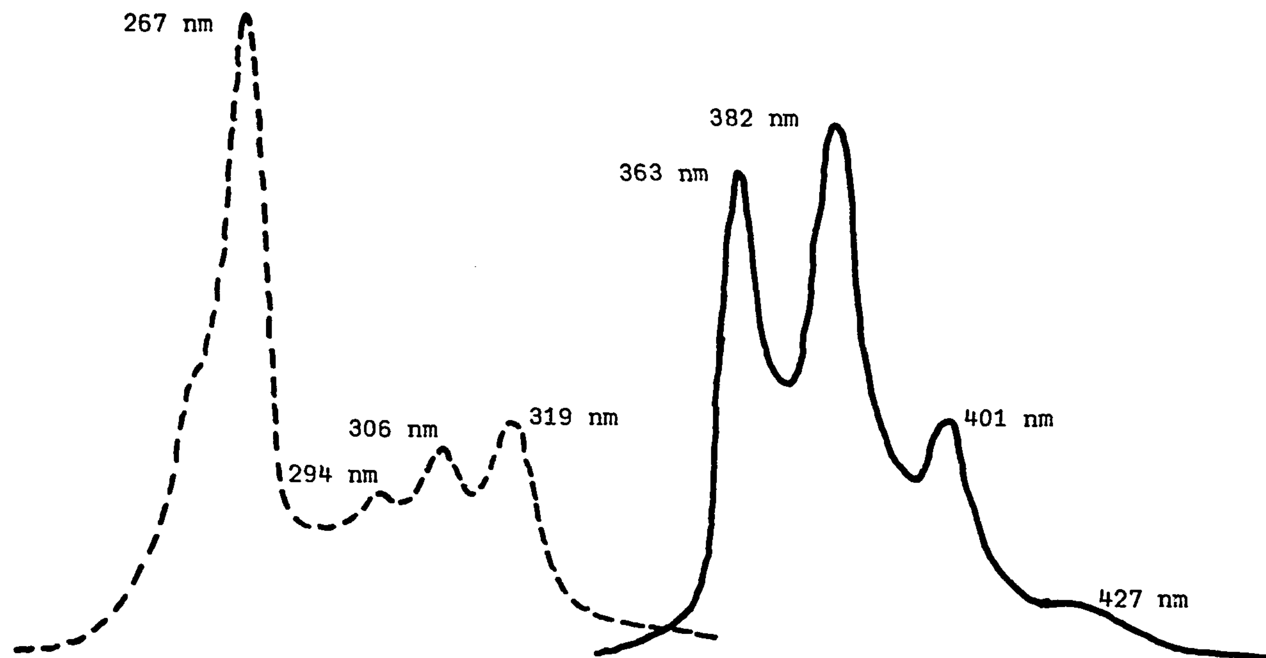



Figure 13. Fluorescence spectra of chrysene in pentane

- - - - Excitation spectrum at emission wavelength 382 nm

——— Emission spectrum at excitation wavelength 267 nm

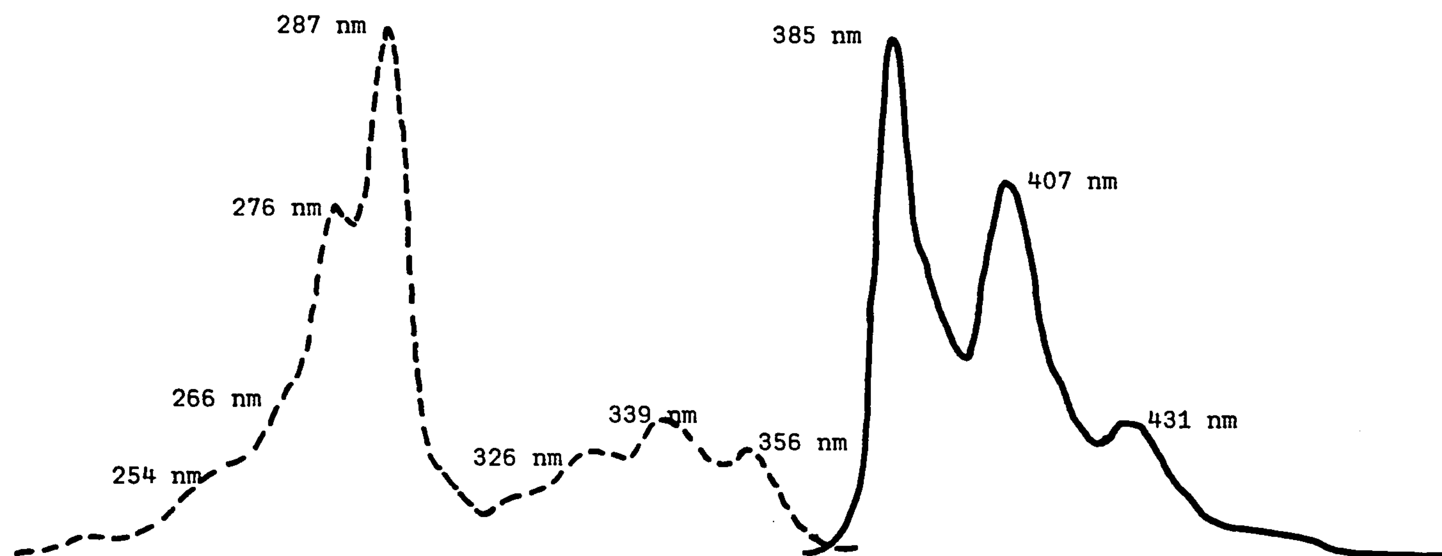



Figure 14. Fluorescence spectra of benz(a)anthracene in pentane

- - - - Excitation spectrum at emission wavelength 385 nm

——— Emission spectrum at excitation wavelength 287 nm

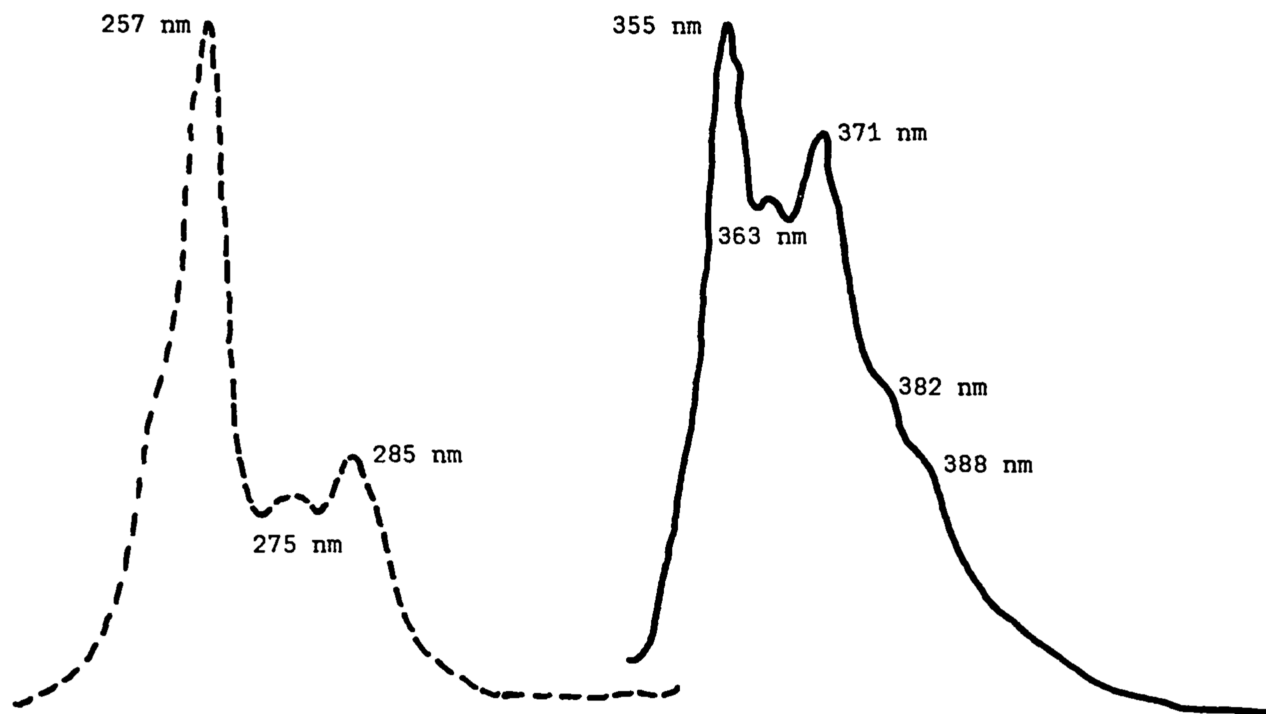



Figure 15. Fluorescence spectra of triphenylene in pentane

- - - - Excitation spectrum at emission wavelength 355 nm

——— Emission spectrum at excitation wavelength 257 nm

found to provide sufficient sensitivity with good resolution and was used throughout this work.

### Preparation of Baker-flex Sheets for Scanning

Because of their flexibility, Baker-flex sheets cannot be inserted directly into the scanner carriage. The edges of the sheet were therefore stripped of adsorbent and the sheet taped to a standard glass plate. These sheets had a tendency to buckle and extreme care was needed to insure that the sheet lay perfectly flat on the glass plate. Failure to observe this precaution resulted in variation in the distance from the adaptor slit to the adsorbent layer as the sheet was scanned, thereby producing significant errors.

One distinct advantage of Baker-flex sheets was the ability of the plastic support material to transmit light. When the excitation monochromator was set in the visible region of the spectrum, the image of the adaptor slit was visible through the adsorbent layer of the TLC sheet. This property greatly simplified positioning the light beam image on the sheet prior to scanning.

### Qualitative Analysis

Identification of PNA in unknown extracts was based primarily on $R_B$ values and fluorescence spectra obtained directly from the TLC adsorbent. Most of the commonly used instrumental techniques, such as ultraviolet absorptiometry and infrared analysis, are not sensitive enough to detect nanogram amounts of material on a TLC plate. Attempts to use a gas chromatographic-mass spectrometric system for qualitative identification of PNA are under investigation in this laboratory. This approach has been successful in the identification of fluoranthene in one unknown sample.

$R_B$ values are characteristic of a particular compound for a given TLC system. $R_B$ values were calculated from the migration distances of individual spots measured by scanning the developed chromatogram parallel to the direction of development. The output from the photomultiplier tube was applied to a strip chart recorder to obtain a permanent record of each scan. The speed of both the scanning carriage and the recorder were synchronized at the rate of one inch per minute so that migration distances could be read directly from the chart. In most separations, sufficient fluorescent material remained at the origin to be recorded as a peak from which migration distances could be measured. In those cases where the origin was nonfluorescent it was necessary to visually position the adaptor slit directly over the origin and begin the scan at a predetermined position on the strip chart.

Sawicki[36] states that the reproducibility of $R_B$ values of PNA separated with 50% aqueous dimethylformamide on cellulose is ± 10%. The mean $R_B$ values of the reference compounds as determined on fifteen chromatograms from the present work are shown in Table XIII. These values agree very well with those of Sawicki in Table II. The decrease in error with increasing migration distance can be attributed to the fact that the measurement error remains constant in spite of changes in migration distance.

The spectrofluorometer scanning system also permitted the measurement of fluorescence spectra directly from the TLC adsorbent. After location of the desired spot, the position of the scanner was adjusted to obtain the maximum fluorescence signal. Both excitation and emission spectra were then obtained by scanning with the appropriate monochromator.

For the four reference compounds studied, all fluorescence spectra measured directly from the cellulose layer showed no wavelength shifts from

Table XIII.

$R_B$ Values of Reference Compounds

| | $R_B$ | Relative Standard Deviation |
|---|---|---|
| Dibenz(a,h)anthracene | 0.66 | 8.4% |
| Benzo(a)pyrene | 1.00 | 5.1% |
| Benzo(b)fluorene | 1.40 | 4.1% |
| Fluoranthene | 1.90 | 2.9% |

those measured in pentane solution and shown in Figures 6-10. This is reasonable considering the non-polar nature of the cellulose adsorbent.

The smallest quantity necessary to produce a resolvable fluorescence emission spectrum was determined for the reference compounds as shown below:

| | |
|---|---|
| Dibenz(a,h)anthracene | 5 ng |
| Benzo(a)pyrene | 1 ng |
| Benzo(b)fluorene | 2 ng |
| Fluoranthene | 25 ng |

The fluoranthene value is high because of the lack of fine structure in the emission spectrum. The limiting factor in these measurements was the background caused by light scatter from the cellulose layer.

## Parameters Affecting Quantitative Scanning

Slits. Accurate reproducibility of all slit dimensions is absolutely necessary for quantitative TLC scanning. There are five variable slits in the Farrand-TLC Scanning system which was used. Four of these are the entrance and exit slits for both the excitation and emission monochromators and the fifth is the adaptor slit which defines the light beam image on the TLC plate. Fixed slits having 5, 10, and 20 nm band passes were available for each of the monochromator slits. All TLC scanning was done with a 10 nm slit at the entrance of the excitation monochromator and 5 nm slits at the three other positions. This combination gave the maximum sensitivity with sufficient resolution. Three fixed adaptor slits, having dimensions 0.5 x 7.5, 1.5 x 13, and 2 x 14 mm were supplied with the instrument. The 1.5 x 13 mm adaptor slit was used for all TLC scanning since it provided the best compromise between sensitivity and resolution.

Wavelengths. Any wavelength between 220 and 700 nm could be select-

ed with the excitation and emission monochromators with an accuracy of ± 2 nm. Qualitative scanning of developed plates to determine spot locations and subsequent $R_B$ values was done with fixed excitation and emission wavelengths. For the analysis of a particular PNA in both standard and unknown samples, excitation and emission wavelengths corresponding to maximum adsorption and fluorescence bands respectively were chosen. The same wavelength settings were used when comparing the intensity of a spot from an unknown sample with that of a known amount of reference compound.

The use of monochromators in place of filters decreased the sensitivity of the scanner somewhat but had several distinct advantages. By the appropriate choice of excitation and emission wavelengths, it was sometimes possible to resolve two closely adjoining spots having different excitation or emission spectra. Using this method it was possible to combine several scans and obtain a composite picture of an entire chromatogram. An example of this process is shown in Figure 16. A separated mixture of the four reference hydrocarbons was scanned four times at differing excitation and emission wavelengths corresponding to the maximum response of the individual PNA. The bottom trace is a composite of the entire separation combined from the four individual scans.

Monochromators were used in obtaining excitation and emission spectra of unknown spots. Either the excitation or emission monochromator was scanned automatically to obtain the corresponding fluorescence spectrum of a spot. If a scanner employing filters had been used, it would have been necessary to remove the sample from the adsorbent and dissolve it in an appropriate solvent in order to obtain fluorescence spectra.

<u>Distance from the adaptor slit to the adsorbent layer</u>. This parameter had to be held constant for all quantitative work. Variations in the

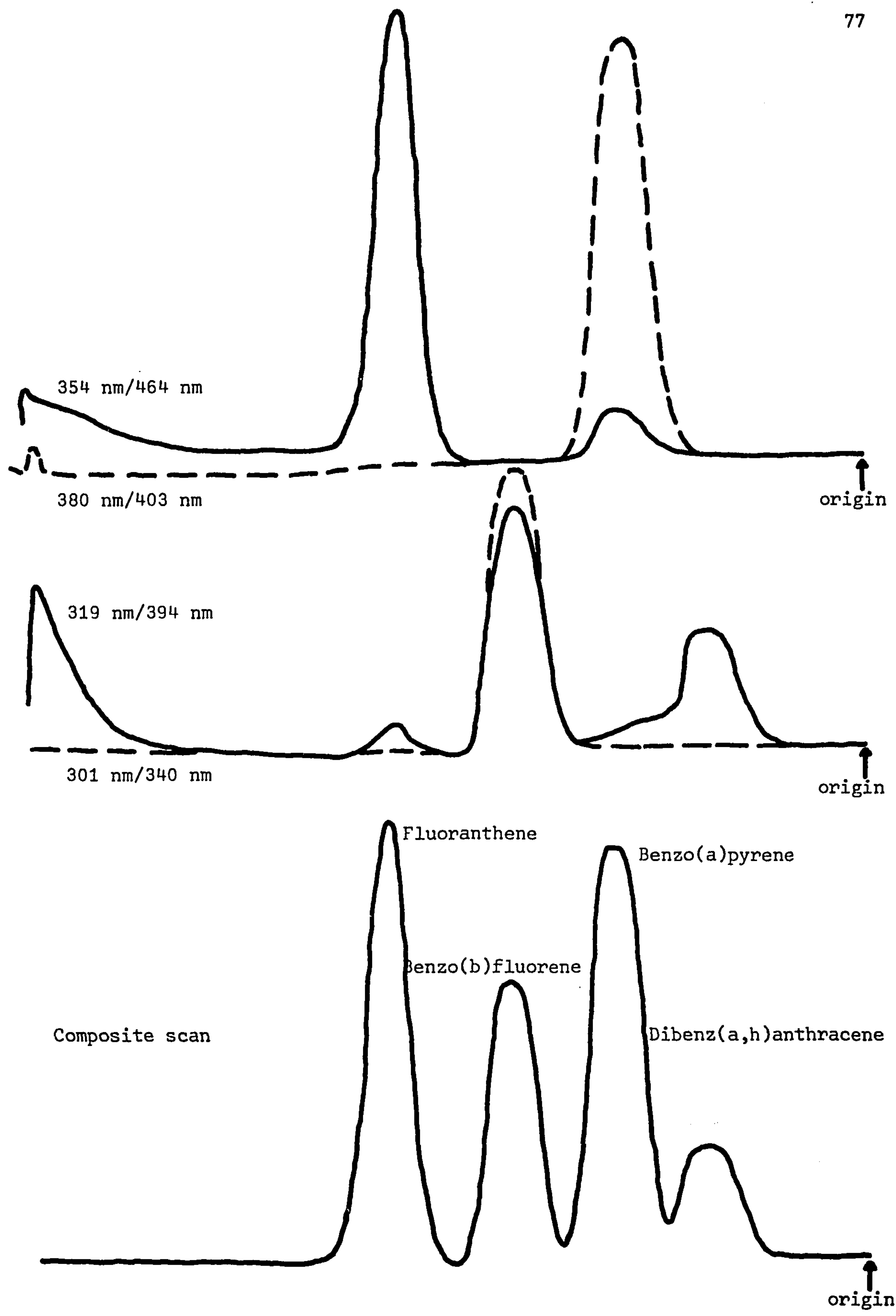



Figure 16. Formation of a composite TLC scan from individual TLC scans.

height of the layer above the adaptor slit would have caused changes in the distances the excitation light traveled before striking the surface and also in the distance the reflected fluorescence traveled before entering the emission monochromator. A decrease in fluorescence intensity would have resulted with increasing distance. A study was carried out using a 30 ng benzo(a)pyrene spot to determine the significance of this parameter. Three distances were obtained by resting the TLC plate first on the carriage itself and then increasing the distance successively by placing it on one and two of the magnets used to hold the plate in the carriage. These three distances obtained were 1.5, 5.0, and 8.5 mm respectively. The benzo(a)pyrene spot was scanned four times at each distance, and the relative fluorescence intensities were 100.0, 61.0, and 9.2 for the 1.5, 5.0, and 8.5 mm distances respectively. This clearly indicated a significant decrease in fluorescence with increasing distance. On the other hand separate studies, which are described below, showed that the photodecomposition of spots increased with decreasing distance between the adaptor slit and the adsorbent layer. Since the effect on the fluorescence intensity was greater than that on spot photodecomposition, 1.5 mm was chosen as the standard distance for all further work.

Lateral position of the scanner. In order to insure reproducibility when scanning a single spot, the same area of the spot must be scanned each time. It was soon realized that the fluorescence response was susceptible to very small changes in the lateral position of the scanner. Deviations as small as 1 mm caused significant changes in fluorescence intensity. It was necessary, therefore, to determine the lateral position giving maximum response for each spot whenever quantitation was necessary. This was accomplished easily in the following manner. The fluorescence response was observed on the microammeter of the spectrofluorometer as the

peak was scanned. The scanner was stopped approximately at the center of the spot, and the lateral position was quickly adjusted using the dial at the front of the scanner to obtain maximum response. The entire procedure was accomplished as quickly as possible to minimize spot decomposition.

Direction of scanning. Location and identification of separated compounds is uaually performed by scanning parallel to the direction of development. In this manner $R_B$ values of the various spots may be calculated. For quantitative analysis, however, scanning either parallel to or perpendicular to the direction of development may be employed. The advantages and disadvantages of each method have been studied previously[81,87,113]. Using single-beam instrumentation, perpendicular scanning is preferable when complete separation has been obtained because of the uniformity of the baseline. In those cases where separations are not complete, parallel scanning is more advantageous and usually permits the estimation of unresolved peaks. Double-beam scanning, on the other hand, always employs parallel scanning in which the samples are spotted in alternate rows so that the reference beam can continuously sample the blank layer.

The scanner used in this work was a single-beam instrument. After location of individual compounds, quantitative scans were always run perpendicular to the direction of development.

Photodecomposition of PNA spots. Most PNA are highly susceptible to photodecomposition. Inscoe[47] and Lam and Berg[43] studied photodecomposition of specific PNA on various thin-layer adsorbents. Because photodecomposition could cause a significant error in quantitative measurements, several investigations were conducted to determine the extent of photodecomposition with the Farrand scanning system.

The photodecomposition of a 30 ng benzo(a)pyrene spot was studied as a function of the distance from the adaptor slit to the adsorbent layer.

The three distances, 1.5, 5.0, and 8.5 mm, which were used to study the fluorescence intensity as a function of the distance from the adaptor slit to the adsorbent layer were used. The spot was scanned to a position directly over the spot, and the scanner was stopped for 2½ minutes. The spot was then rescanned, and the area obtained was compared with the previous scan. The percent photodecomposition values for 1.5, 5.0, and 8.5 mm were 27.4, 21.4, and 9.0% respectively. This demonstrates a definite increase in photodecomposition as the plate is brought nearer to the adaptor slit.

Another study of this type was performed on 5 and 50 ng benzo(a)-pyrene spots in which the spots were irradiated for one minute. The adaptor slit-plate distance was set at 1.5 mm and each spot irradiated three times. Average percent photodecomposition values were 8.0 and 3.9% for the 5 and 50 ng spots respectively. These results compare favorably with our earlier studies and also show the dependence of photodecomposition on the amount of sample per spot.

The photodecomposition of 5 and 50 ng benzo(a)pyrene spots was also studied as a function of the number of times each spot was scanned. Each spot was scanned eight times with the intensity being maximized each time as quickly as possible. The areas under the respective peaks were plotted versus scan number and are shown in Figure 17. This data shows about 1% photodecomposition per scan for both the 5 and 50 ng spots. Light source intensity was shown to be constant by measurement of a quinine sulfate standard before and after the experiment.

One interesting result of these studies was the observation that the actual shape of the recorded peak was changed when the scanner was stopped directly over the spot for several minutes. The outline of the adaptor slit was clearly visible when the spot was rescanned. This

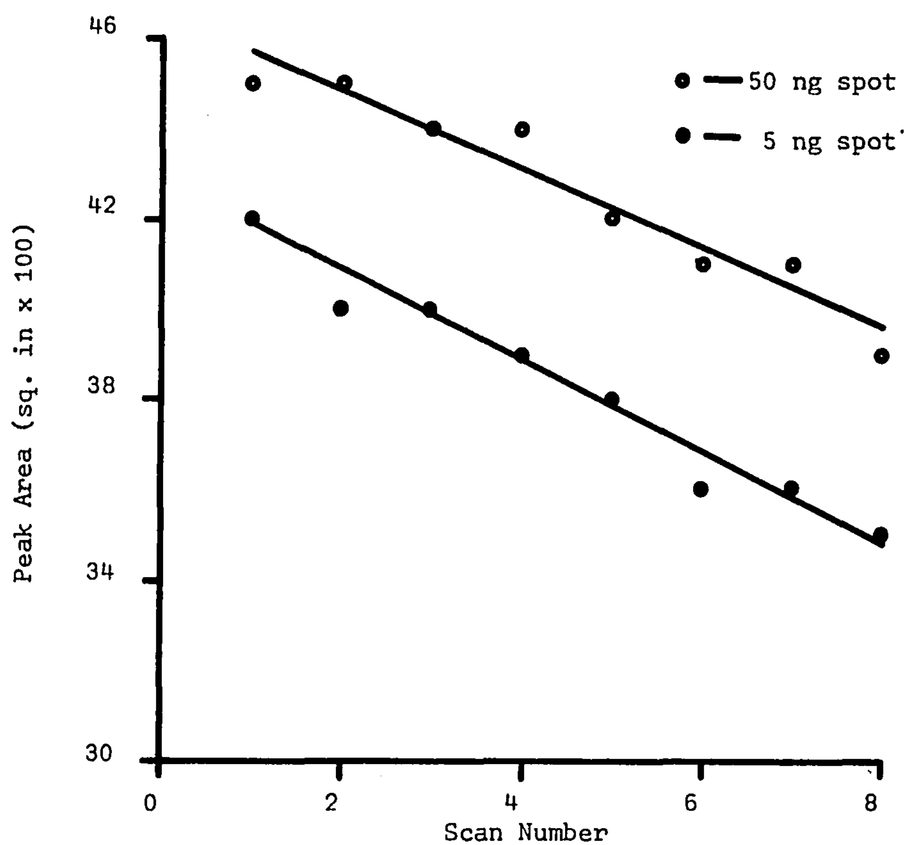


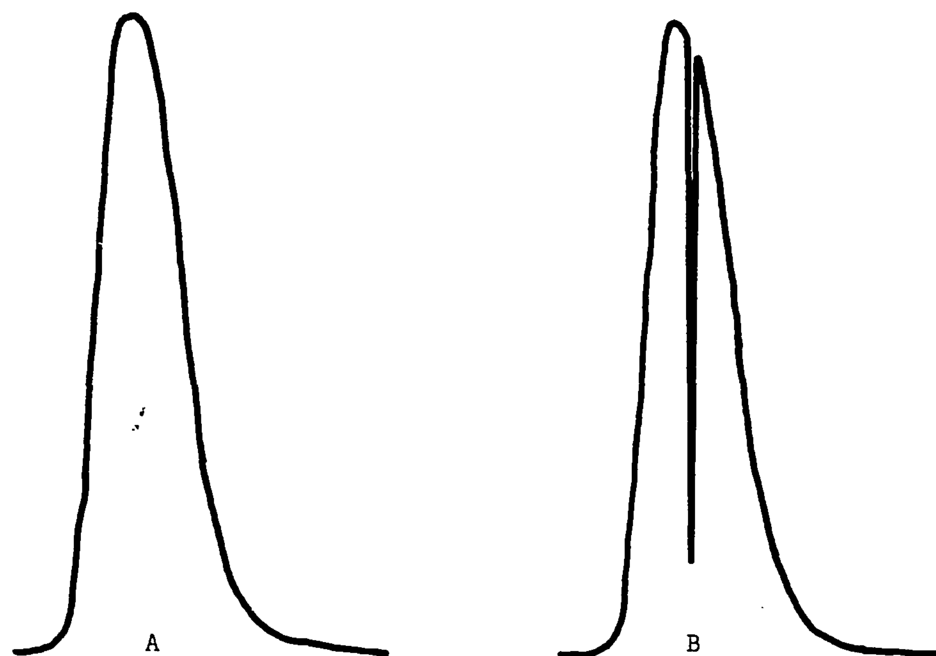

Figure 17. Photodecomposition of benzo(a)pyrene as a function of the number of scans.

phenomenon is shown in Figure 18 for a 50 ng benzo(a)pyrene spot which had been irradiated for about three minutes between scans.

Influence of time on fluorescence intensity. Several authors[87,92,114] have discussed the effect of time on the fluorescence intensity of separated compounds. It has been shown that the intensity is dependent upon the moisture content of the layer due to incomplete drying. Jaenchen and Pataki[87] suggested standardization of the time between development and scanning, whereas Pataki and Wang[92] later found that thorough drying of the layer in a stream of cool air was sufficient to stabilize the fluorescence intensity.

Preliminary investigations showed that the fluorescence intensity of individual PNA spots varied significantly if the plate was not completely dry before scanning. For each spot the intensity decreased as the plate dried. Thorough drying of the plate was necessary for reproducible results. In order to insure complete drying of the adsorbent layer, the procedure of Pataki and Wang[92] was employed. The plate was removed from the eluent, and the Baker-flex sheet separated from the glass backing. The excess eluent was wiped from the edges and back of the sheet; the sheet was then dried in a slow stream of cool air for 30 minutes. The compressed air was passed through a tube containing glass wool and dry calcium chloride before striking the plate.

Benzo(a)pyrene and benzo(b)fluorene were used to check the scanning reproducibility after the 30 minutes drying time. Duplicate samples containing 50 ng each of benzo(a)pyrene and benzo(b)fluorene were separated on a Baker-flex sheet which was dried according to the above method. After drying, the four spots were scanned in random order for a total of 164 minutes. Each spot was scanned 12 times in this manner; the fluorescence being maximized before each scan. The results are shown in Figures 19 and 20. These data demonstrated that the fluorescence decrease was less than




A. Initial scan of spot.

B. Scan following $2\frac{1}{2}$ minute irradiation of spot.

Figure 18. Visual observation of photodecomposition of a benzo(a)pyrene spot.

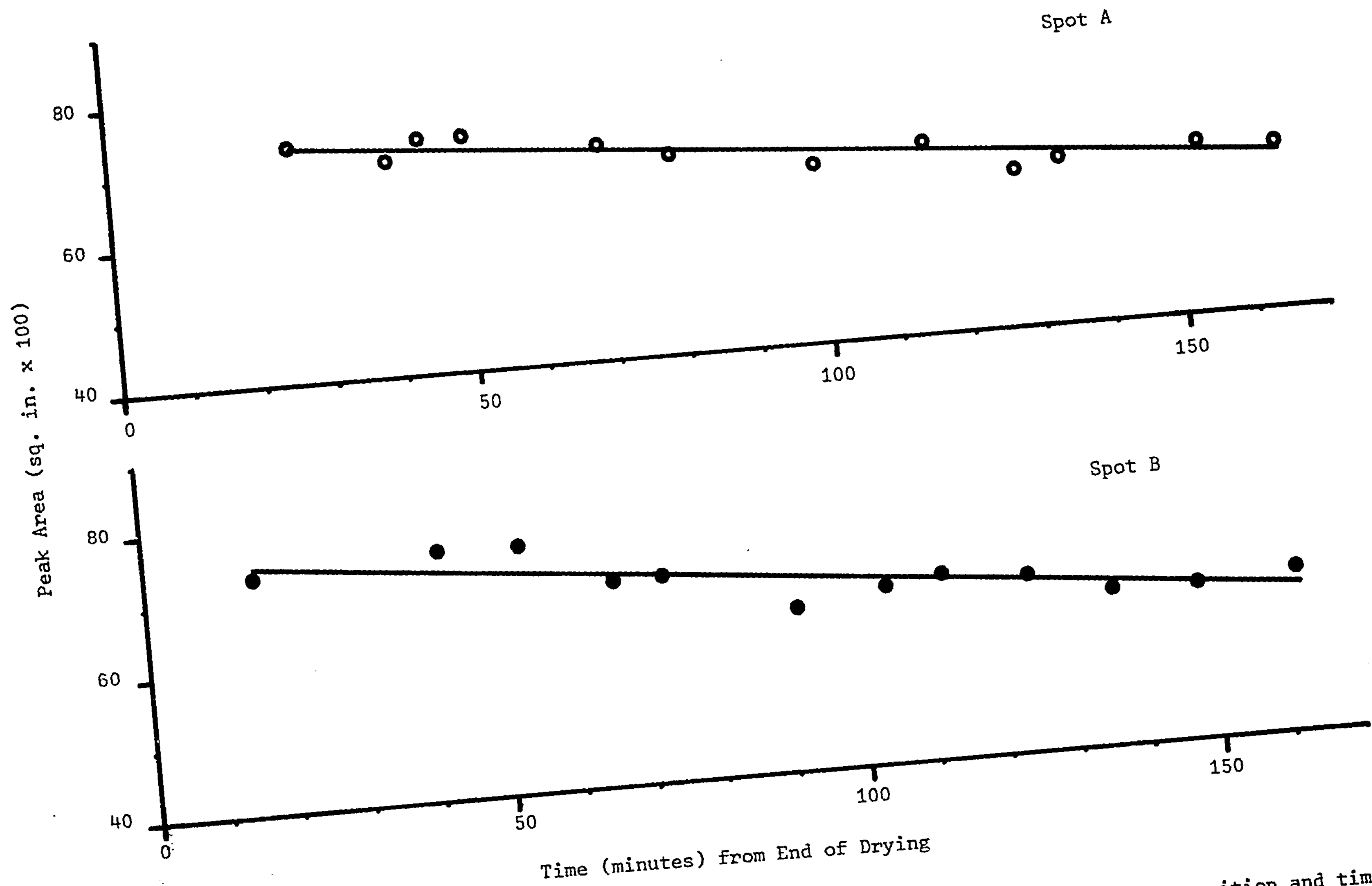



Figure 19. Variation of the fluorescence of a benzo(a)pyrene spot due to photodecomposition and time.

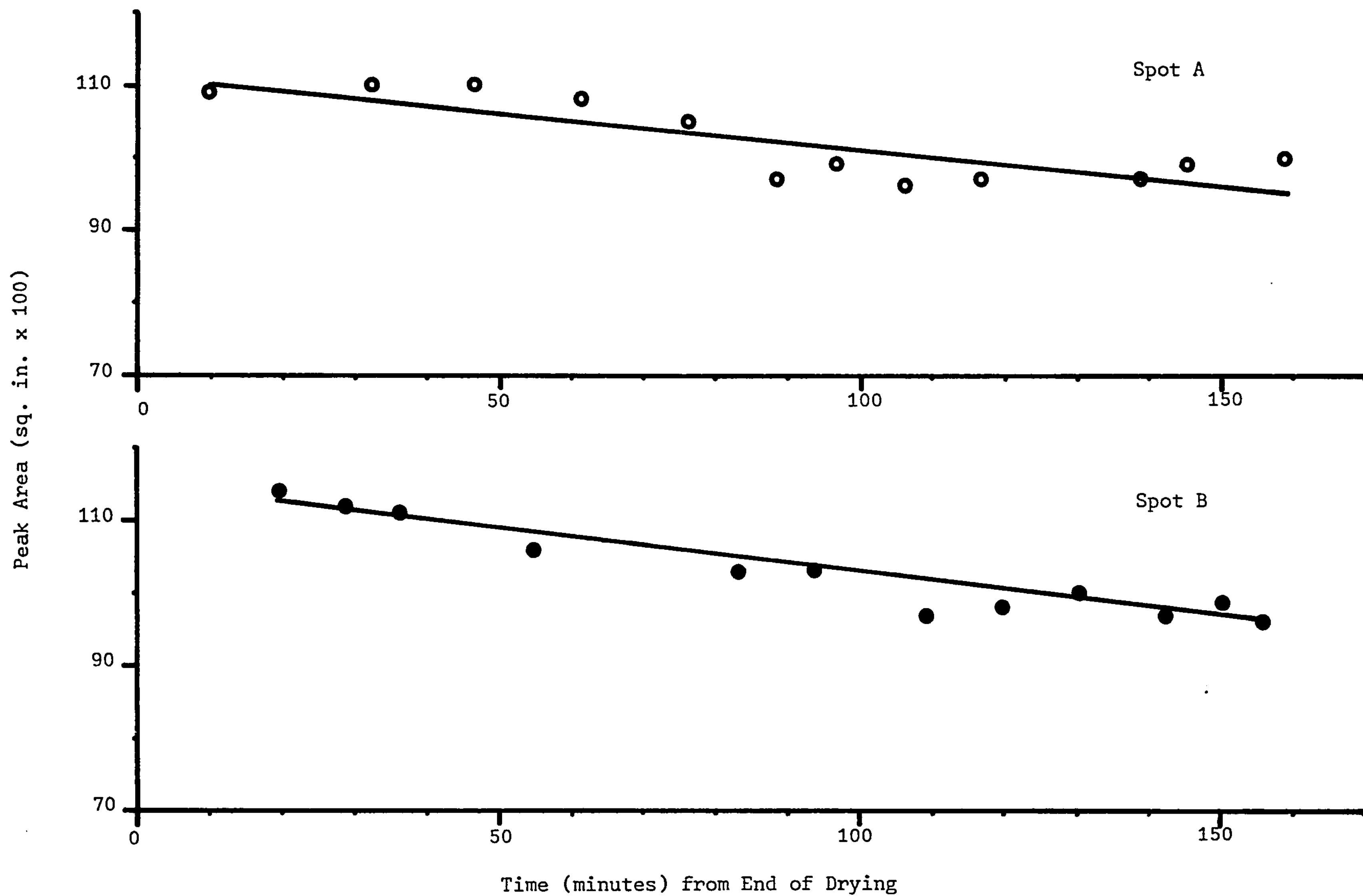



Figure 20. Variation of the fluorescence of a benzo(b)fluorene spot due to photodecomposition and time.

1.5% per scan for both compounds. Over the 2½ hour period the total fluorescence decreased 16.7% and 14.3% for the benzo(a)pyrene and benzo(b)fluorene spots respectively.

It has been shown above that photodecomposition was responsible for an approximate 1% decrease per scan. Since 12 scans were made on each spot in the 2½ hour period, the decreases due to time are 5% and 2% for benzo(a)-pyrene and benzo(b)fluorene respectively. As these values were within the limit of experimental error for the experiment, the conclusion that no significant decrease of fluorescence intensity with time if the adsorbent layer was thoroughly dried appeared justifiable.

Light source variation. The measured fluorescence of a spot is directly proportional to the light source intensity. For a single-beam system of the type used in this work, any changes in the intensity of excitation radiation produce corresponding fluorescence changes. While the xenon-arc lamp was quite stable when allowed to warm up for about 30 minutes, incorporation of a reference system proved more desirable for performing accurate quantitative analysis.

The possibility of using the recorded excitation scatter peak as a standard was investigated but was found unreliable as the resulting peak area was sufficiently susceptible to small changes of the adsorbent area being scanned to introduce significant error. Reproducible results were obtained only when the area illuminated by the adaptor slit was unchanged.

The use of a standard 100 ng quinine sulfate spot, applied to the plate after development, was also studied. Quinine sulfate, however, showed evidence of photodecomposition to about the same degree as the PNA studied earlier. It was decided, therefore, to use a 50 ng benzo(a)pyrene spot as a standard. The fluorescence emission was maximized before each scan, and correction was made to allow for the 1% photodecomposition

accompanying each scan. The standard spot was scanned at regular intervals during the quantitative analysis of known and unknown PNA.

## Calibration Curves

In order to accurately determine an unknown amount of a compound by _in situ_ fluorometry it was necessary to compare its fluorescence with that of known amounts of the same compound. This was usually done with the assistance of calibration curves in which the emitted fluorescence (area of the peak) was plotted as a function of the amount of compound applied to the layer. Measurement of peak areas was made with a planimeter. Although it was not absolutely necessary, it was advisable to work in a region where a straight line relationship between peak area and amount of sample is obtained.

A preliminary investigation was conducted to determine the linearity of the curves between 3 and 100 ng for each of the four reference compounds. Duplicate spots containing 3, 30, 60, and 100 ng of each PNA were separated in the usual manner. The separated spots were each scanned twice in random order and the results are shown in Figure 21. Only fluoranthene was linear over the entire range, while the other three reference compounds exhibit good linearity up to approximately 60 ng.

Further calibration curves were prepared for each of the reference compounds in the range of 5 to 50 ng. Typical curves for each of the reference PNA are shown in Figure 22. All of these curves were shown to be statistically linear by an analysis of variance. The range from 5 to 50 ng was therefore chosen as suitable for quantitative analysis of unknown amounts of each PNA.

Statistical analysis of the regression line equations obtained from four different plates showed that they were not identical at the 95% con-

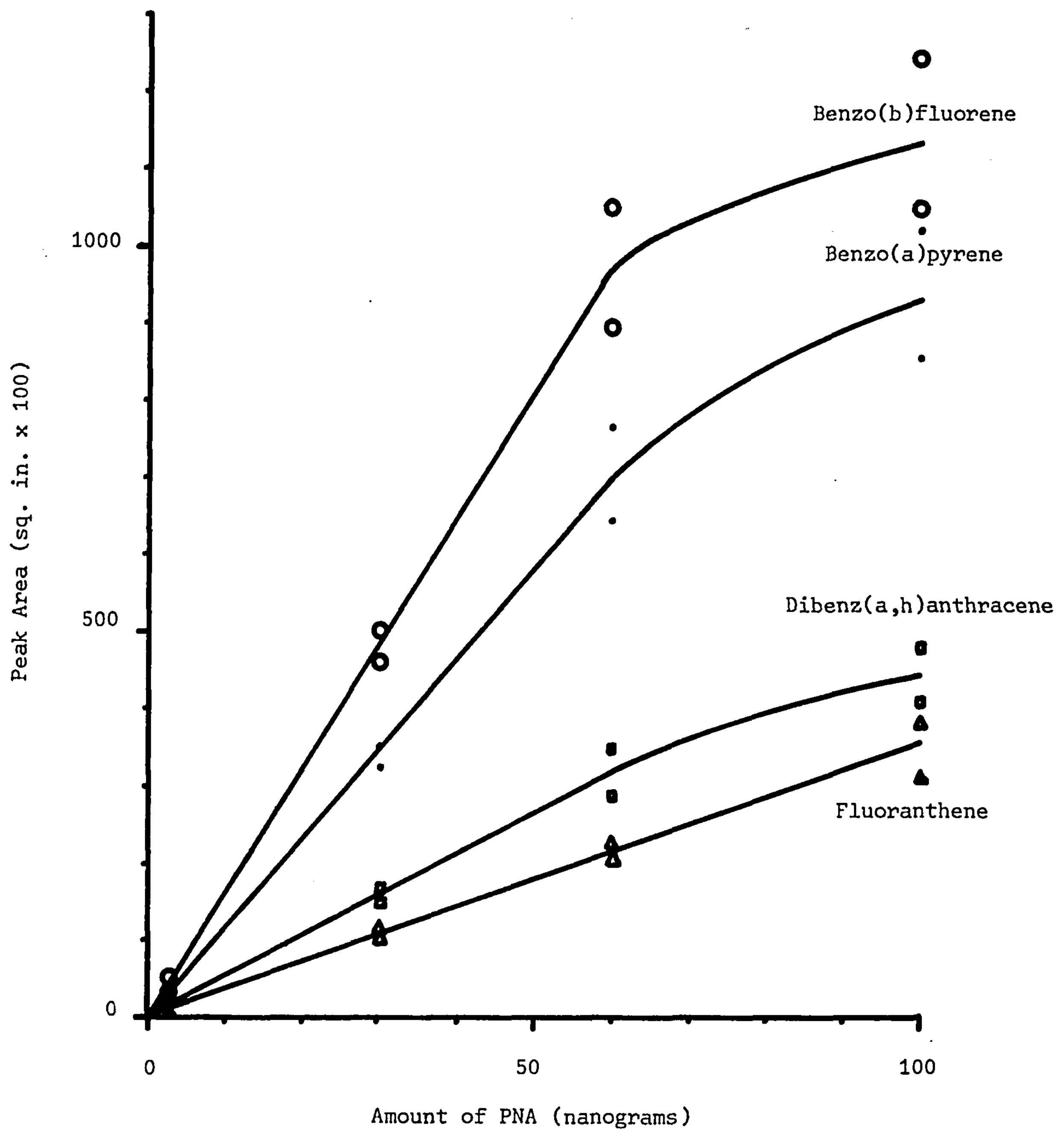



Figure 21. Preliminary calibration curves for the four reference compounds.

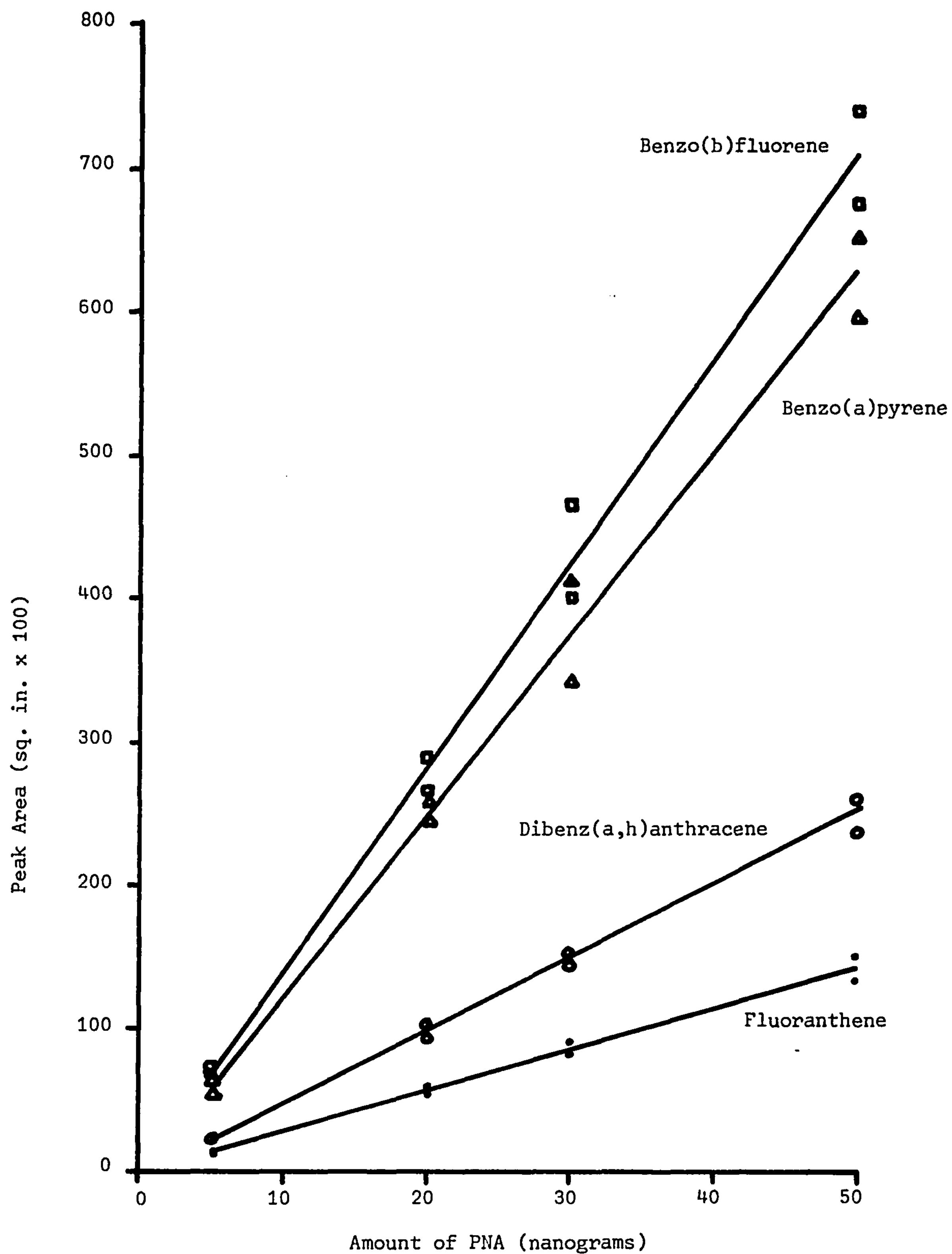



Figure 22. Calibration curves for four reference compounds.

fidence limit, in agreement with data presented in Table I. This meant that it was necessary to prepare a calibration curve on each plate. This was done in future unknown samples by spotting duplicate spots containing 5 and 50 ng of each of the reference PNA.

Figure 22 suggests that the measurement error increases with increasing amounts of PNA. This increase, however, is not associated wtih the measurement itself but with the manipulation of the data. A brief explanation follows. The current output from the photomultiplier was fed to a strip chart recorder for a permanent record. This current could be varied from 0.0001 to 100 microampers by means of a RANGE selector on the instrument. An appropriate RANGE was selected to bring the output signal on-scale on the recorder. It was not possible to determine an entire calibration on a single RANGE since the larger amounts of compound produced peaks which went off-scale using the lower RANGE setting. This meant that at least two RANGE settings were needed for each curve. In order to plot the data, it was then necessary to correct all of the values to a single RANGE. This is the step which introduces the increased error. For example, duplicate 50 ng benzo(a)pyrene spots produced peak areas of 59.5 and 65.0 (sq. in. x $10^2$) when recorded on a RANGE of 10. When these values are corrected to a RANGE of 1, they are 595 and 650 respectively, the difference in the two values being 10 times the difference in the original peak areas. It should also be noted that this is not simply a case of the error being directly proportional to the amount of sample. This error is associated solely with changes in the RANGE being used and subsequent data reduction to a common RANGE. The error could be eliminated by using only one RANGE for a single calibration curve but, except for fluoranthene, the slopes of the regression lines are too great to allow a ten-fold increase in the quantity of compound while still using a single

RANGE.

## Reproducibility

The factors involved in obtaining reproducible scans have been discussed above. Considering these parameters, two studies were made to determine the error associated with the scanning measurements.

The reproducibility of scanning a single 50 ng benzo(a)pyrene spot was determined in the following way. After thorough drying of the developed chromatogram, the 50 ng spot was scanned 10 times with the maximization procedure being repeated between each scan. The corresponding peak areas were measured; they ranged from 0.95 to 0.99 square inches with a mean of 0.969 square inches. The standard deviation was calculated to be 0.013 square inches. This resulted in a relative standard deviation of 1.33%.

The reproducibility of scanning six identical 50 ng benzo(a)-pyrene spots was also studied in a similar manner. After the plate was developed and dried, the spots were scanned four times each in random order. The replicate data is shown in Table XIV. Table XV contains the analysis of variance of this data. The relative standard deviation for a single spot was 2.41% and that for six spots was 10.0%. This is the error for the entire measurement including spot application, variation of light source, decomposition, and instrumental error. The large difference between the errors for a single spot and all of the spots shows that the major portion of the error is associated with spot application, which is in agreement with previously published results. Fairbairn and Relph[114] reported the spot application errors of seven experienced workers using conventional syringes or pipets. Relative standard deviations ranged from 3.3 to 11.9%. The use of an automatic spotting devise such as that described by Bridger and

Table XIV.

Scanning Data for Reproducibility of Six Benzo(a)pyrene Spots

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Replicates | 51 | 41 | 44 | 47 | 56 | 45 |
| | 53 | 42 | 47 | 47 | 54 | 45 |
| | 52 | 43 | 44 | 45 | 53 | 43 |
| | 52 | 41 | 44 | 47 | 55 | 43 |
| Mean | 52.0 | 41.8 | 44.8 | 46.5 | 54.5 | 44.0 |

Values are for peak areas expressed in square inches x 100

Table XV.

Analysis of Variance for Table XIV

| | Sum of Squares | Degrees of Freedom | Mean Square |
|---|---|---|---|
| Total | 54096.00 | 24 | |
| Correction Factor | 53581.50 | 1 | |
| Spots | 491.00 | 5 | |
| Error | 23.50 | 18 | 1.306 |

Standard Deviation for one spot = $\sqrt{1.306} = 1.14$

Relative Standard Deviation for one spot = $\frac{1.14}{47.25} \times 100 = 2.41\%$

Standard Deviation for all spots = $\sqrt{\frac{491.00 + 23.50}{23}} = 4.73$

Relative Standard Deviation for all spots = $\frac{4.73}{47.25} \times 100 = 10.0\%$

Relph[115] would significantly reduce the spot application error. Relative standard deviations using this instrument are approximately 2%.

Total Recovery of the Method

A series of experiments were carried out in order to determine the total recovery of the entire method for each of the reference compounds. In this way the amount of PNA lost because of incomplete extraction and surface adsorption could be calculated. Five analyses were carried out for each of the reference compounds in the following manner. Three liters of distilled water was spiked with 400 parts per trillion each of the reference compounds and consequently batch extracted with 100 ml of pentane. The extracts were extracted once with 72% $H_2SO_4$, and the pentane layer concentrated to dryness. The residue was dissolved in 0.10 ml of ethanol and two 10 ul spots applied to a TLC sheet along with appropriate standards. The developed chromatograms were analyzed with the TLC scanner, and the recoveries were calculated. The results are shown in Table XVI. The replicate data given are the mean values calculated from the duplicate spots applied from each extract.

The mean total recoveries agree quite well with the exception of fluoranthene which is significantly higher. Earlier recovery studies concerning only the extraction procedure did show slightly higher recoveries for fluoranthene, but the differences were not great enough to account for the much higher total recovery.

In order to determine the actual amount of PNA present in an unknown water sample, the amount determined by analysis must be corrected for losses which result during the experimental procedure. A correction factor was calculated for each compound on the basis of its mean total

Table XVI.

Summary of Total Recovery Studies

| | % Recoveries | | | | | Mean | Relative Standard Deviation |
|---|---|---|---|---|---|---|---|
| Dibenz(a,h)anthracene | 77.0 | 68.5 | 67.5 | 78.0 | 81.0 | 74.4 | 11.1% |
| Benzo(a)pyrene | 69.5 | 71.5 | 79.5 | 79.5 | 85.0 | 76.0 | 10.8% |
| Benzo(b)fluorene | 70.0 | 75.0 | 84.0 | 86.0 | 72.0 | 77.4 | 9.3% |
| Fluoranthene | 83.0 | 93.5 | 80.5 | 94.5 | 85.5 | 87.4 | 7.1% |

recovery.

$$\text{Correction Factor} = \frac{100}{\text{Percent Recovery}}$$

The correction factors for the four reference compounds are listed below:

| | |
|---|---|
| Dibenz(a,h)anthracene | 1.34 |
| Benzo(a)pyrene | 1.32 |
| Benzo(b)fluorene | 1.29 |
| Fluoranthene | 1.14 |

To apply the correction factor to real samples the amount of PNA determined analytically is multiplied by the correction factor to obtain the actual amount present in the water sample. The use of this correction factor assumes that the total recovery for real water samples is identical with the total recovery of PNA dissolved in distilled water. Because very little is known about the physical state of PNA present in natural water, the validity of this assumption is uncertain.

# ANALYSIS OF REAL SAMPLES

## Introduction

### Sampling Locations

The development method was used to analyze water from several New Hampshire rivers over a period of several years. Sampling locations on two of these rivers, the Oyster River and the Cocheco River, are shown in Figure 23. Sampling site A on the Oyster River was just above the U. S. Geological Survey Gaging Station in Lee. Three sites were chosen on the Cocheco River in Dover. Two of these were chosen above the city and one below the city in hopes of correlating PNA content with any possible pollution coming from the industries located in the city. Site B was located just upstream from the Washington Street bridge and the Dover sewage treatment plant below the city. Site C was located where County Farm Road crosses the river about 4 miles upstream from Dover. The final site D on the Cocheco River was just upstream from the Watson Road bridge approximately 2½ miles above the city. The final sampling site E was located on the Winnipesaukee River in Tilton, New Hampshire. Water samples were taken below the bridge where Rt. 106 crosses the river. This site was chosen because of its proximity to a large asphalt plant located about 500 yards upstream.

### Sampling Procedure

Water samples were collected in 2 gallon polyethylene containers which had been thoroughly cleaned. The containers were washed with detergent, rinsed, washed several times with concentrated $H_2SO_4$, and finally rinsed thoroughly with distilled water. At the sampling site, each con-

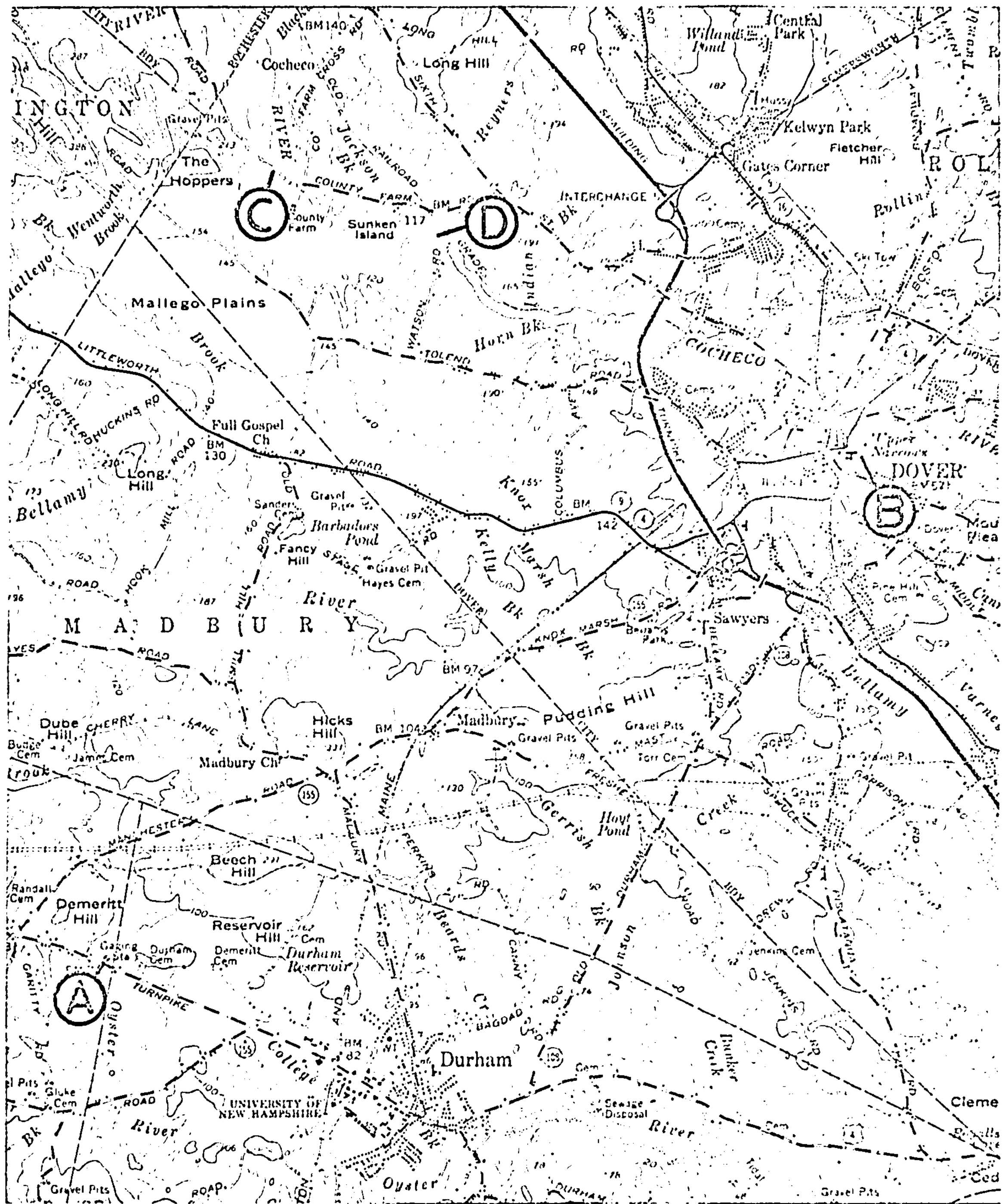



Figure 23. Location of Oyster River and Cocheco River sampling sites.

tainer was rinsed with river water before filling. Samples were taken just below the water surface being careful not to disturb the sediment on the river bottom. The water was transported quickly to the laboratory where the analysis was begun at once.

### Millipore Filtering

Several of the early samples were passed through a 0.45 μ Millipore filter to remove particulate matter. The filtering time was excessively long, often requiring up to two days to filter 5 liters of water. Further studies showed that there was a decrease in recovered PNA with increased time between sampling and extraction. The filtering was discontinued because of the large losses of PNA encountered during the time required for filtering.

### Samples Examined Without the TLC Scanner

Seven samples were analyzed from site A on the Oyster River during the initial development of the method, including the initial sample which has been described above. These samples were analyzed before the TLC scanner was obtained and quantitative methods had been developed. All of these extracts were also passed through an acidic alumina column with large amounts of 40% benzene in pentane.

Inspection of the developed chromatograms was done under ultraviolet light. Of these first seven samples, only the initial one showed any traces of PNA. This is probably due to the procedures used and lack of sensitivity in visually locating separated spots.

### Blank Determinations

Because the method is concerned with nanogram amounts of substances,

it is necessary to determine the contribution, if any, of contamination from sources other than the water sample. Lijinsky and Raha[103] and Sawicki[6] have discussed the presence of traces of PNA in commercial solvents and in particulate samples respectively.

Blank determinations were carried out periodically to determine any background contribution to the analytical method. Distilled water, whose purity had been checked fluorometrically, was either continuously or batch extracted, and the resulting extracts treated in the same way as unknown water samples. As in the analysis of unknown samples, the acid-washed extract was concentrated to dryness and the residue dissolved in 100 µl of ethanol. A 20 µl portion of this solution was applied to the TLC adsorbent so that 20% of the entire sample was analyzed.

Considering the smallest amount of each PNA producing a resolvable fluorescence spectrum, the minimum water sample concentrations which would be necessary to positively identify the four reference PNA by their fluorescence spectra are shown in Table XVII.

None of the blank determinations using either continuous or batch extraction showed the presence of an identifiable amount of any PNA. Trace amounts of benzo(a)pyrene and benzo(b)fluorene were evident in several blanks using the continuous extractors but dibenz(a,h)anthracene and fluoranthene were never seen. Any error contributed by background contamination, therefore, was negligible compared with the error of the total method.

## Continuous Extractions

Ten river water samples were analyzed using the continuous extractors. One extraction per sample was performed for the first six samples while duplicates were performed in the final four analyses. These analyses were carried out in the period April 21, 1968 to May 10, 1970 and are des-

Table XVII.

Identification Limits of Reference PNA in Blank Determinations

| | Continuous Extraction | Batch Extraction |
|---|---|---|
| Dibenz(a,h)anthracene | 5 ppt | 8 ppt |
| Benzo(a)pyrene | 1 ppt | 2 ppt |
| Benzo(b)fluorene | 2 ppt | 3 ppt |
| Fluoranthene | 25 ppt | 42 ppt |

Note: The detection limits for these compounds are approximately one-tenth of the identification limit values.

cribed below. Quantitative analysis was performed only on those spots which were positively identified by fluorescence spectra.

Sample No. 1

Water was collected from the Cocheco River at site C on April 21, 1968. The water temperature was 10° C and the pH was 6.6. Extractor A was used to extract 5000 ml of water for 43 hours. After extraction with 72% $H_2SO_4$, the extract was concentrated to dryness and dissolved in 1.00 ml of ethanol. A 20 ul portion of this solution as well as appropriate standards were applied to a cellulose sheet and developed for 3½ hours. Inspection of the chromatogram under short-wave UV light revealed three spots having $R_B$ values of 0.92, 1.38 and 1.79. Fluorometric TLC scans of this sample are shown in Figure 24. Although a spectrum could not be measured, the spot with $R_B$ = 0.60 was characterized as dibenz(a,h)anthracene on the basis of its $R_B$ value and maximum fluorescence excitation and emission wavelengths*. Fluorescence spectra of spots C, D, and G were obtained and are shown in Figure 25. Attempts to identify these spots by comparison of their spectra with those of standard PNA having known $R_B$ values within 10% of each spot were unsuccessful. A large number of other possibilities were not examined because $R_B$ values for the cellulose-50% aqueous DMF system have only been reported for a limited number of PNA.

Sample No. 2

Water was collected from the Oyster River at site A on August 19, 1968. The water temperature was 19° C and the pH was 6.7. Extractor B was

*The maximum fluorescence wavelengths referred to here and in subsequent samples are the excitation and emission wavelengths which produce the maximum fluorescence signal for a particular spot.

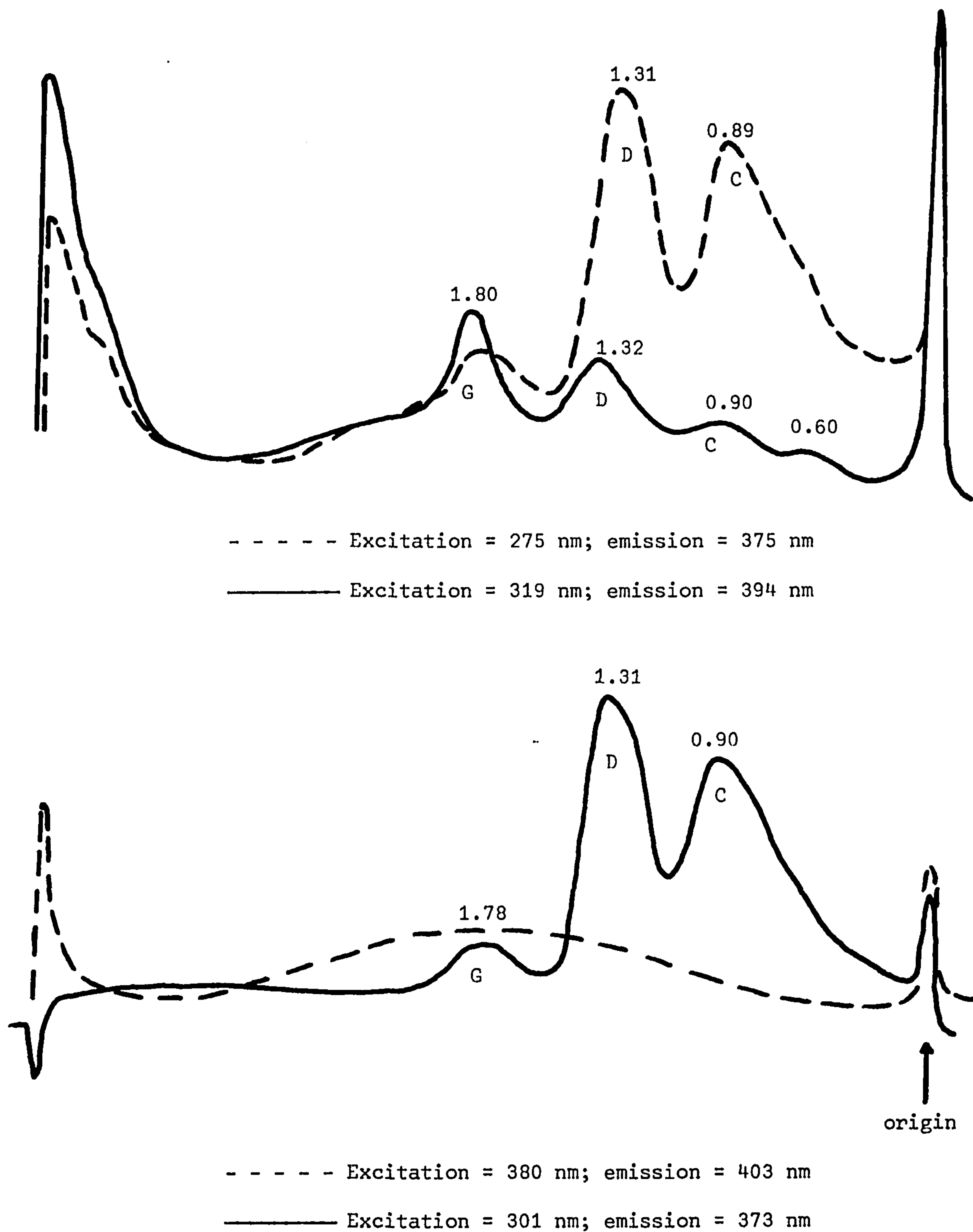



Figure 24. Fluorometric TLC scans of sample #1.

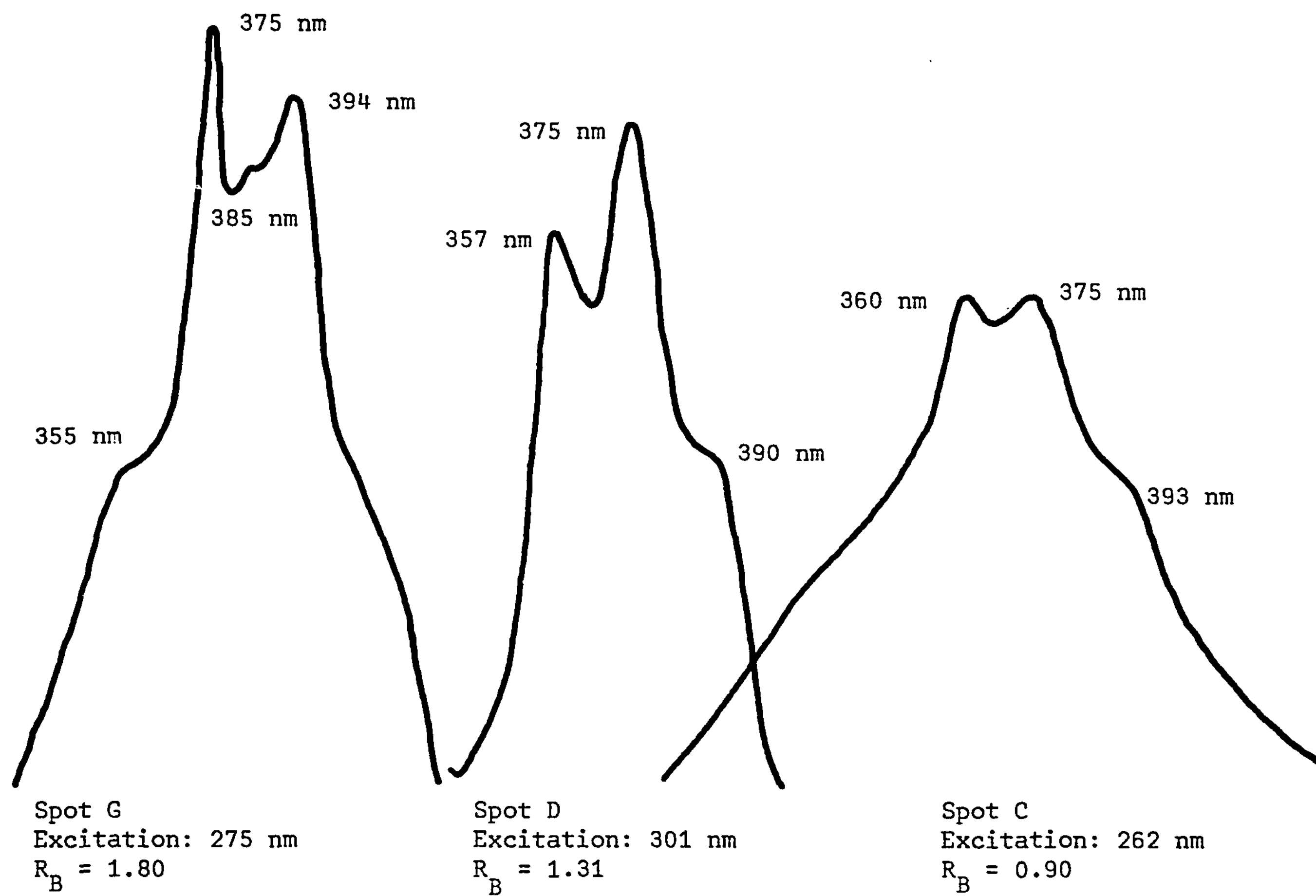



Figure 25. Fluorescence spectra of unidentified spots in sample #1.

used to extract 5150 ml for 75 hours. After extraction with 72% $H_2SO_4$, the extract was concentrated to dryness and dissolved in 1.00 ml of ethanol. A 20 ul portion of this solution as well as appropriate standards were applied to a cellulose sheet and developed for 4 hours. Inspection of the chromatogram under short-wave UV light revealed three spots having $R_B$ values of 0.99, 1.43, and 1.89. Fluorometric TLC scans of this sample are shown in Figure 26. Although a spectrum could not be measured, the spot with $R_B$ = 0.63 was characterized as dibenz(a,h)anthracene on the basis of its $R_B$ value and maximum fluorescence wavelengths. The spots with $R_B$ values of 1.00, 1.42, and 1.90 were identified as benzo(a)pyrene, benzo(b)fluorene, and fluoranthene respectively by their characteristic spectra. The following amounts of the identified PNA were determined from scans of the samples and standards.

| | |
|---|---|
| Benzo(a)pyrene | 6 ng |
| Benzo(b)fluorene | 8 ng |
| Fluoranthene | 29 ng |

Conversion of these values to concentration in the original water sample yields

| | |
|---|---|
| Benzo(a)pyrene | 78 ppt |
| Benzo(b)fluorene | 100 ppt |
| Fluoranthene | 320 ppt |

Sample No. 3

Water was collected from the Oyster River at site A on September 30, 1968. The water temperature was 18$^{o}$ C and the pH was 6.9. Extractor B was used to extract 5150 ml of water for 64 hours. After extraction with 72% $H_2SO_4$, the extract was concentrated to dryness and dissolved in 1.00 ml of ethanol. A 20 $\mu$l portion of this solution as well as appropriate stand-

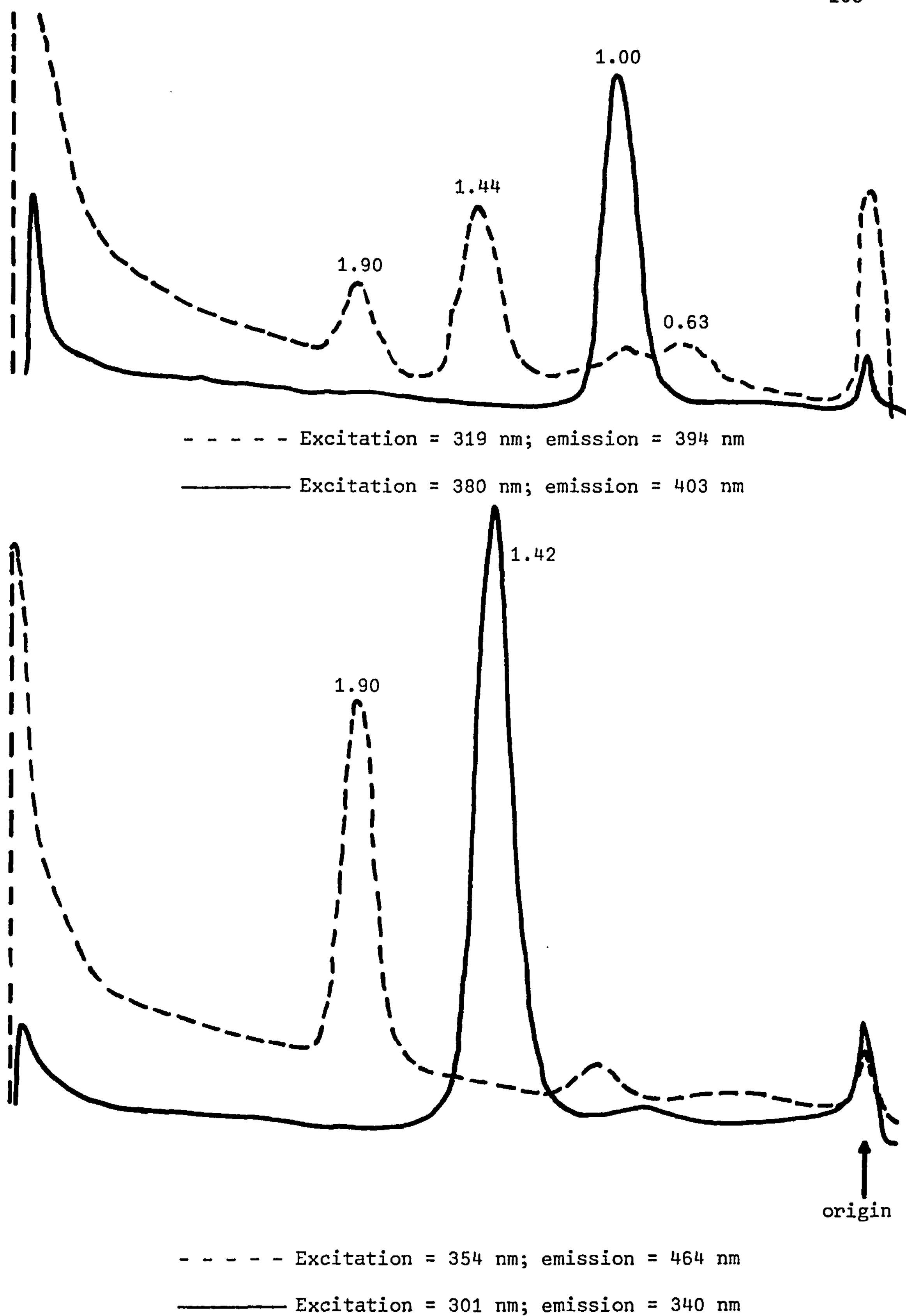



Figure 26. Fluorometric TLC scans of sample #2.

ards were applied to a cellulose sheet and developed for 4 hours. Inspection of the chromatogram under short-wave UV light revealed four spots having $R_B$ values of 0.62, 1.00, 1.42, and 1.89. Fluorometric TLC scans of this sample are shown in Figure 27. The spots at $R_B$ values 0.64, 1.00, 1.42, and 1.90 were identified as dibenz(a,h)anthracene, benzo(a)-pyrene, benzo(b)fluorene, and fluoranthene respectively by their characteristic spectra. The following amounts of each PNA were determined from scans of the samples and standards.

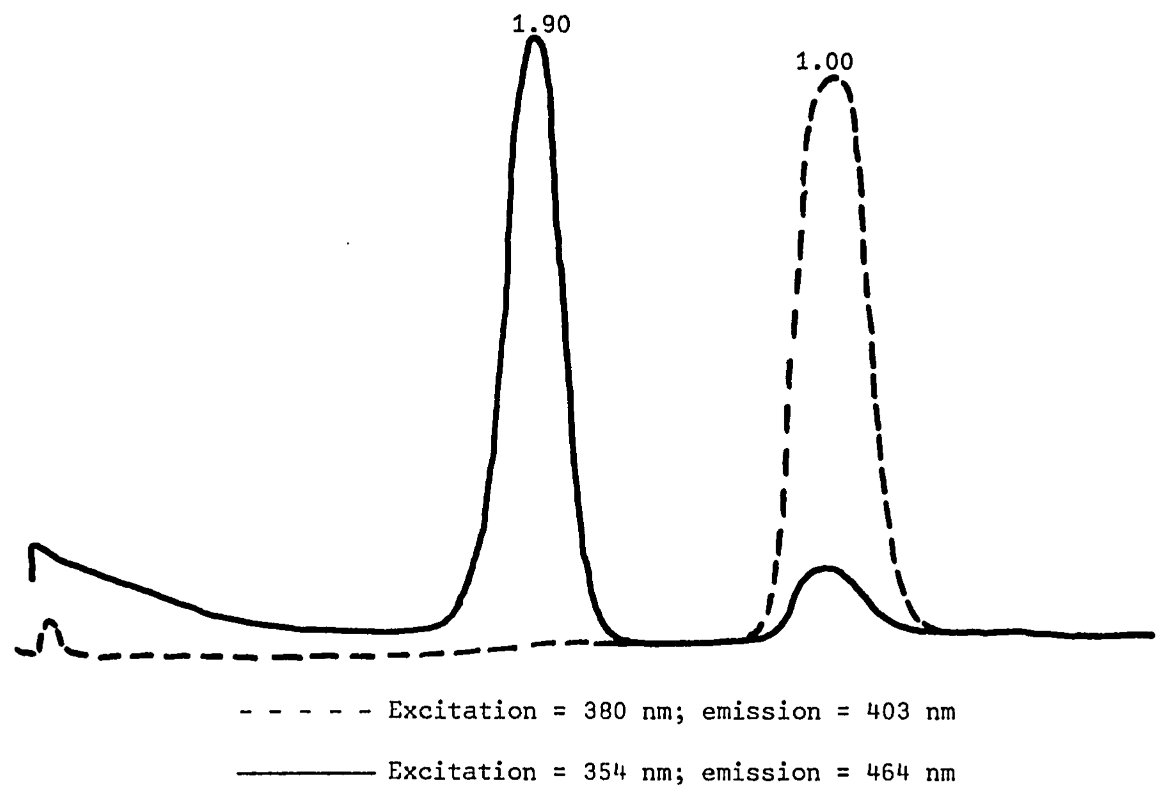
| | |
|---|---|
| Dibenz(a,h)anthracene | 7 ng |
| Benzo(a)pyrene | 10 ng |
| Benzo(b)fluorene | 44 ng |
| Fluoranthene | 50 ng |

The amount of fluoranthene was too great to fall on the linear portion of the calibration curve, therefore, 10 µl of the extract was chromatographed and the fluoranthene spot quantitatively scanned. It was found to contain 46 ng. Conversion of these values to concentration in the original water sample yields

| | |
|---|---|
| Dibenz(a,h)anthracene | 92 ppt |
| Benzo(a)pyrene | 125 ppt |
| Benzo(b)fluorene | 550 ppt |
| Fluoranthene | 1100 ppt |

Sample No. 4

Water was collected from the Oyster River at site A on November 7, 1968. The temperature of the water, which contained some particulate matter was 12° C and the pH was 6.6. Extractor B was used to extract 5150 ml of water for 54 hours. After extraction with 72% $H_2SO_4$, the extract was concentrated to dryness and dissolved in 1.00 ml of ethanol. A 20 µl portion



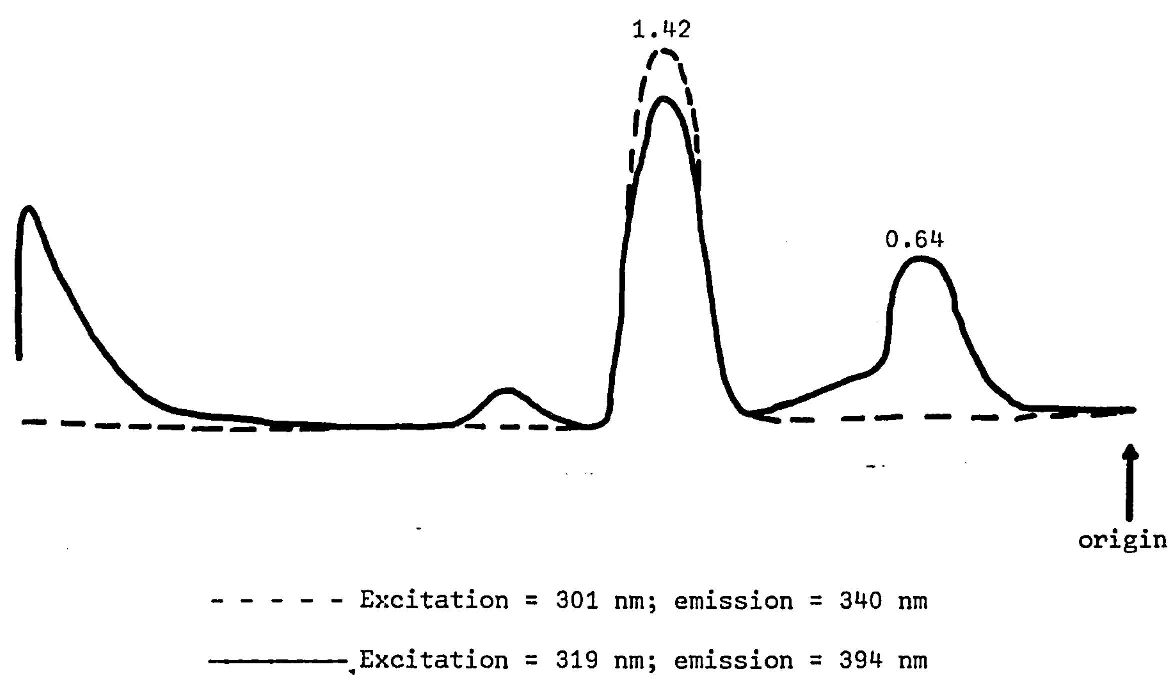




Figure 27. Fluorometric TLC scans of sample #3.

of this solution as well as appropriate standards were applied to a cellulose sheet and developed for 3½ hours. Inspection of the chromatogram under short-wave UV light revealed two spots having $R_B$ values of 1.86 and 2.09. Fluorometric TLC scans of the sample are shown in Figure 28. The spot with $R_B$ = 2.09 could not be located at any wavelength with the scanner. This spot showed a reddish fluorescence under UV light. The failure of the scanner to detect the spot is probably due to the insensitivity of the photomultiplier in the red wavelength region. Although spectra could not be measured, the spots with $R_B$ values of 0.68 and 1.41 were characterized as dibenz(a,h)anthracene and benzo(b)fluorene respectively on the basis of their $R_B$ values and maximum fluorescence wavelengths. The spot with $R_B$ = 1.86 was identified as fluoranthene by its characteristic spectrum. The fluoranthene spot was quantitatively scanned and found to contain 30 ng. Conversion of this value to concentration in the original water sample yielded a concentration of 330 ppt fluoranthene.

Sample No. 5

Water was collected from the Oyster River at site A on March 31, 1969. The water temperature was 1° C and the pH was 6.5. Extractor C was used to extract 5000 ml of water for 49 hours. After extraction with 72% $H_2SO_4$, the extract was concentrated to dryness and dissolved in 1.00 ml of ethanol. A 20 ul portion of this solution as well as appropriate standards were applied to a cellulose sheet and developed for 4 hours. Inspection of the chromatogram under short-wave UV light revealed one faint spot at $R_B$ = 1.40. Fluorometric TLC scans of the sample are shown in Figure 29. Insufficient material was present to obtain fluorescence spectra of spots B and F. The maximum fluorescence excitation and emission wavelengths were 325/385 and 335/391 for spots B and F respectively. The spot with $R_B$ = 1.37

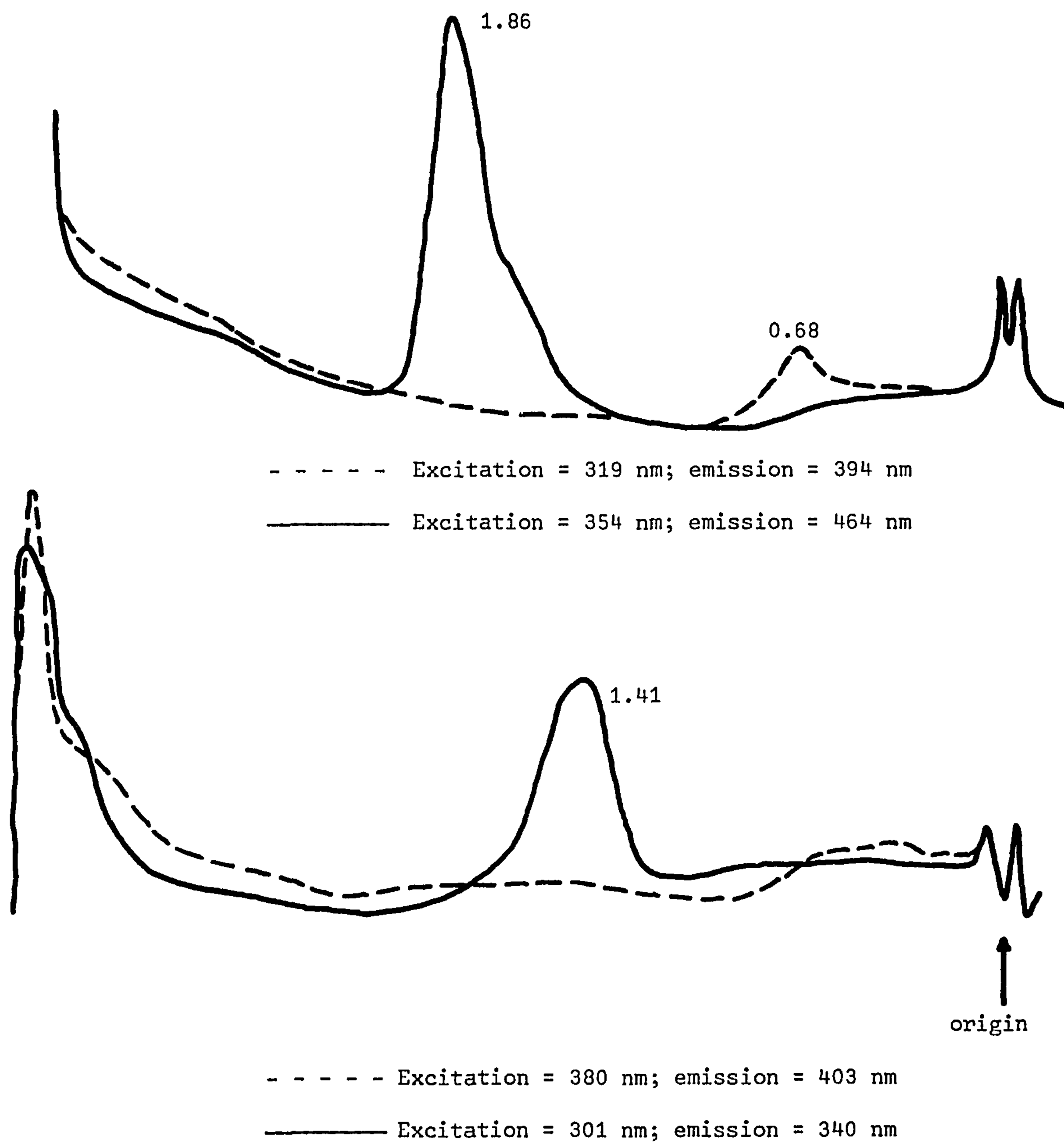



Figure 28. Fluorometric TLC scans of sample #4.

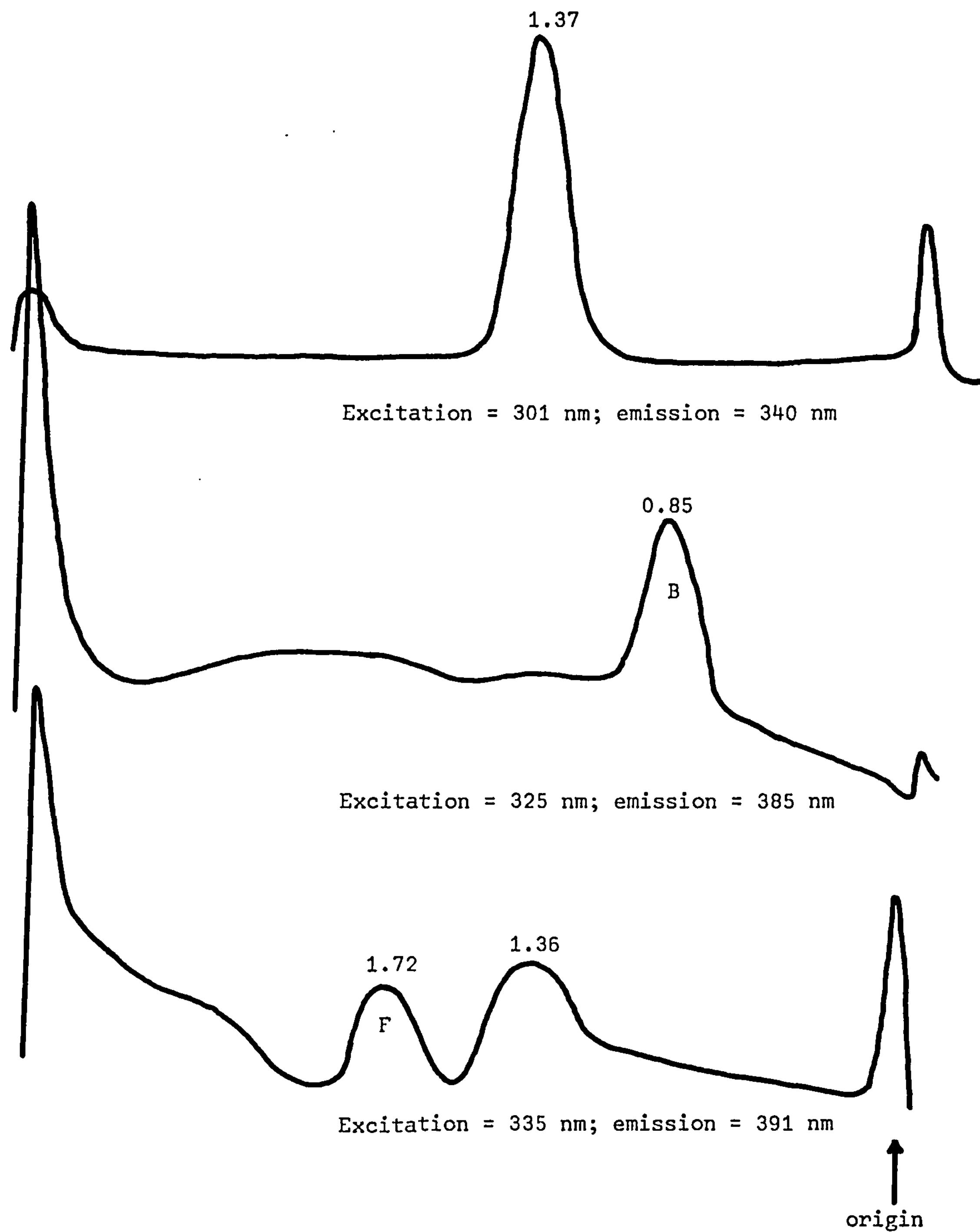



Figure 29. Fluorometric TLC scans of sample #5.

was identified as benzo(b)fluorene by its characteristic spectrum. The benzo(b)fluorene spot was quantitatively scanned and found to contain 12 ng. Conversion of this value to concentration in the original water sample yields a concentration of 155 ppt benzo(b)fluorene.

Sample No. 6

Water was collected from the Oyster River at site A on July21, 1969. The water, containing some particulate matter, was 20° C and the pH was 6.8. Extractor B was used to extract 5000 ml of water for 62 hours. After extraction with 72% $H_2SO_4$, the extract was concentrated to dryness and dissolved in 1.00 ml of ethanol. A 20 µl portion of this solution as well as appropriate standards were applied to a cellulose sheet and developed for 3½ hours. Inspection of the chromatogram under short-wave UV light revealed two spots with $R_B$ values of 1.00 and 1.39. Fluorometric TLC scans of this sample are shown in Figure 30. Insufficient material was present to obtain a fluorescence spectrum of spot A which had maximum excitation and emission wavelengths of 355/465. Although a spectrum could not be measured, the spot with $R_B$ = 0.63 was characterized as dibenz(a,h)-anthracene on the basis of its $R_B$ value and maximum wavelengths. The spots with $R_B$ values of 1.01 and 1.40 were identified as benzo(a)pyrene and benzo(b)fluorene respectively by their characteristic spectra. The following amounts of the identified PNA were determined from scans of the samples and standards.

| | |
|---|---|
| Benzo(a)pyrene | 12 ng |
| Benzo(b)fluorene | 20 ng |

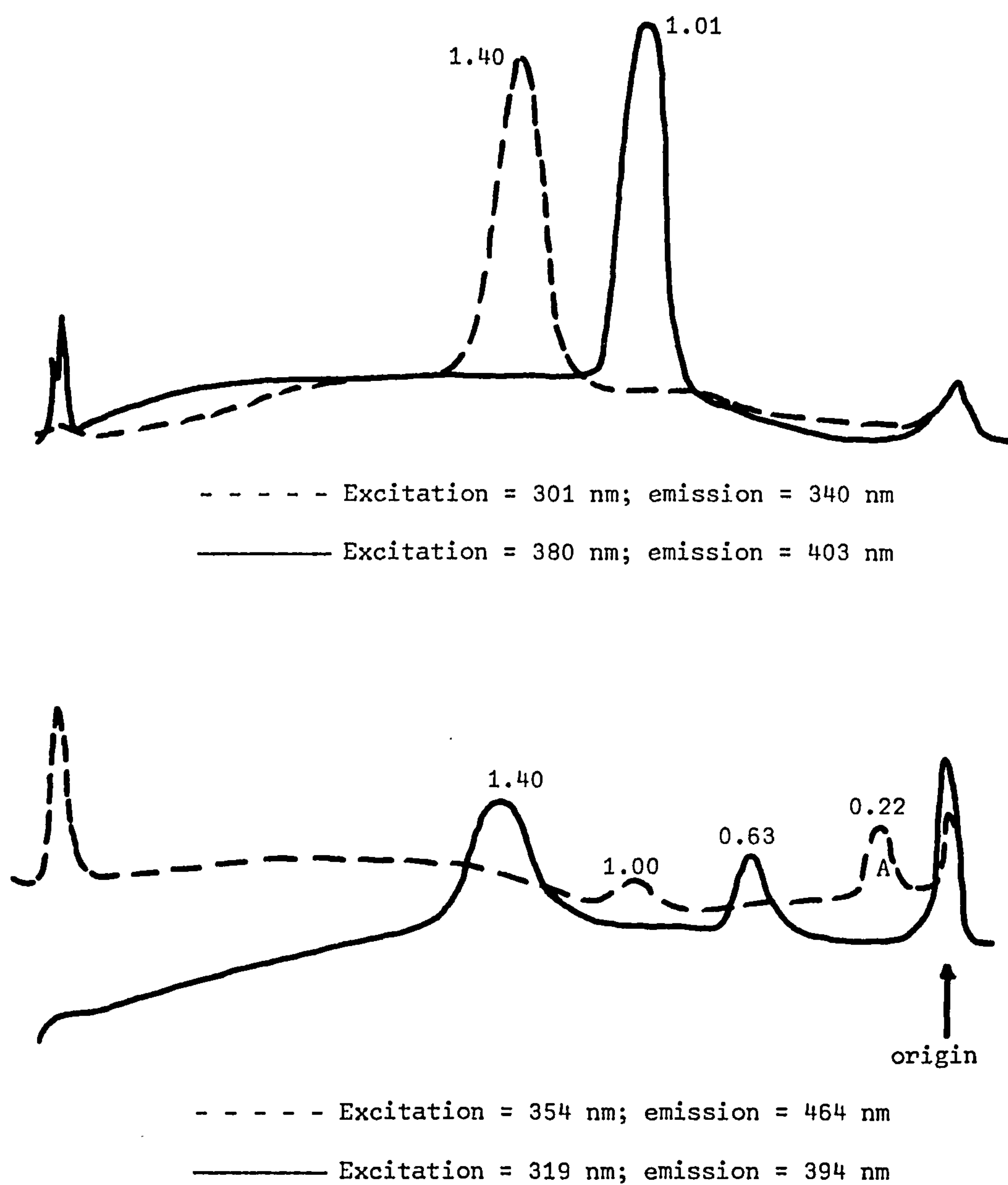



Figure 30. Fluorometric TLC scans of sample #6.

conversion of these values to concentration in the original water sample yields

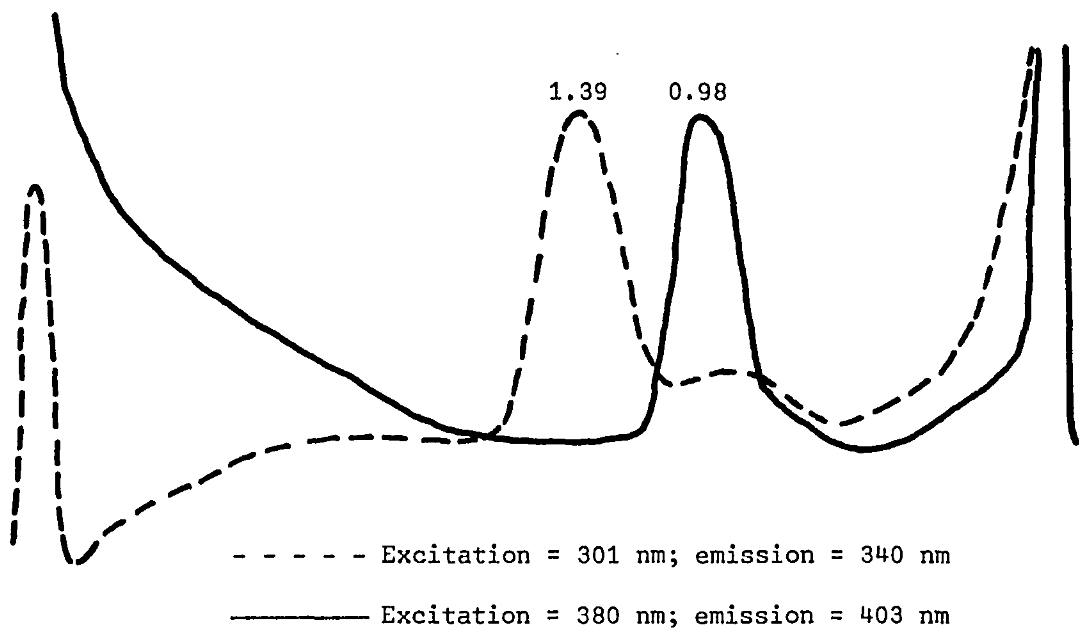
| | |
|---|---|
| Benzo(a)pyrene | 150 ppt |
| Benzo(b)fluorene | 240 ppt |

Sample No. 7

Water was collected from the Oyster River at site A on April 20, 1970. The water temperature was 8° C and the pH was 6.8. Duplicate analyses were performed on this sample using extractors B and C and are described below.

Sample No. 7-B. Extractor B was used to extract 5150 ml of water for 33 hours. After extraction with 72% $H_2SO_4$, the extract was concentrated to dryness and the residue dissolved in 100 µl of ethanol. A 10 µl portion of this solution as well as appropriate standards were applied to a cellulose sheet and developed for 4 hours. No spots could be seen under short-wave UV light. Fluorometric TLC scans of the sample are shown in Figure 31. Although fluorescence spectra of the four spots could not be obtained, they were characterized as dibenz(a,h)anthracene, benzo(a)pyrene, benzo(b)fluorene, and fluoranthene on the basis of their $R_B$ values and maximum fluorescence wavelengths.

Sample No. 7-C. Extractor C was used to extract 5000 ml of water for 33 hours. The extract was treated in the same manner as #7-B and separated on the same TLC sheet. Fluorometric TLC scans of the sample are shown in Figure 32. These scans show essentially the same amounts of three of the PNA identified in #7-B. Dibenz(a,h)anthracene is not observed in this extract. Considering possible errors in the method and the fact that only a trace of dibenz(a,h)anthracene was found in #7-B, the absence of this compound is not unreasonable.



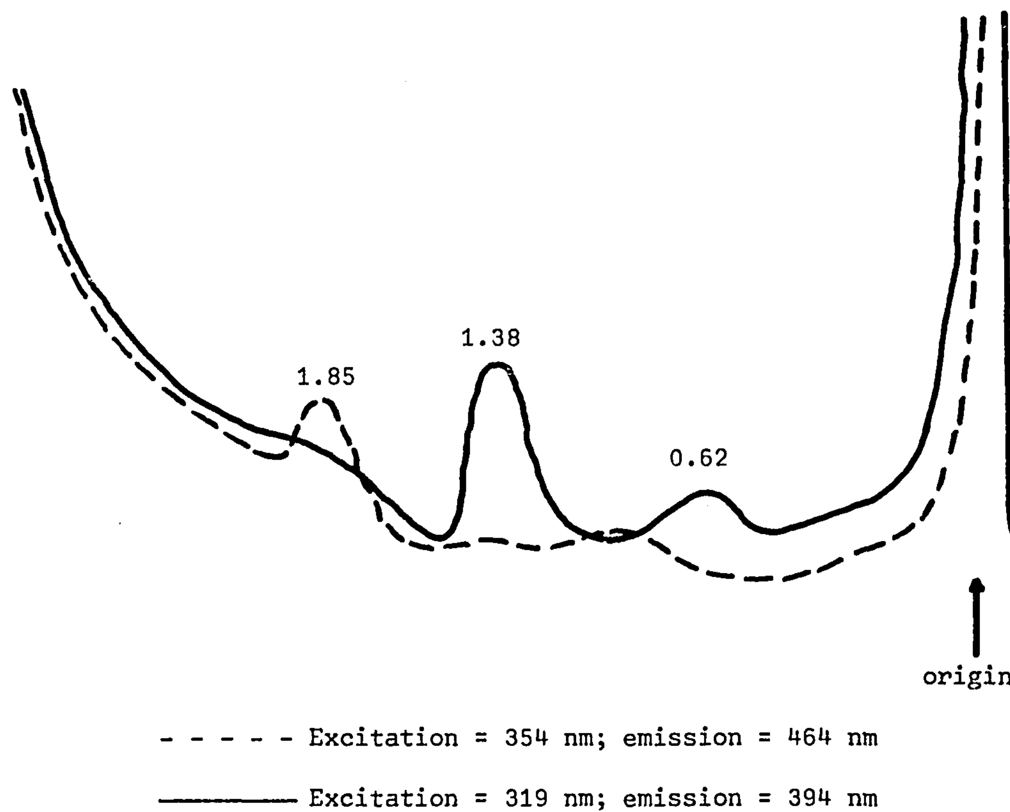
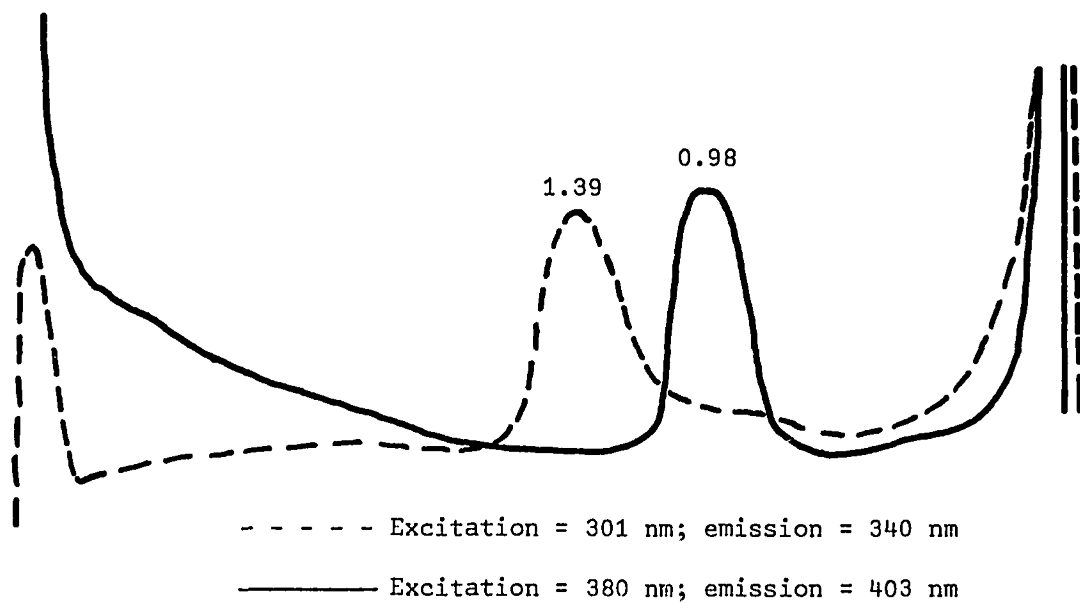




Figure 31. Fluorometric TLC scans of sample # 7-B.



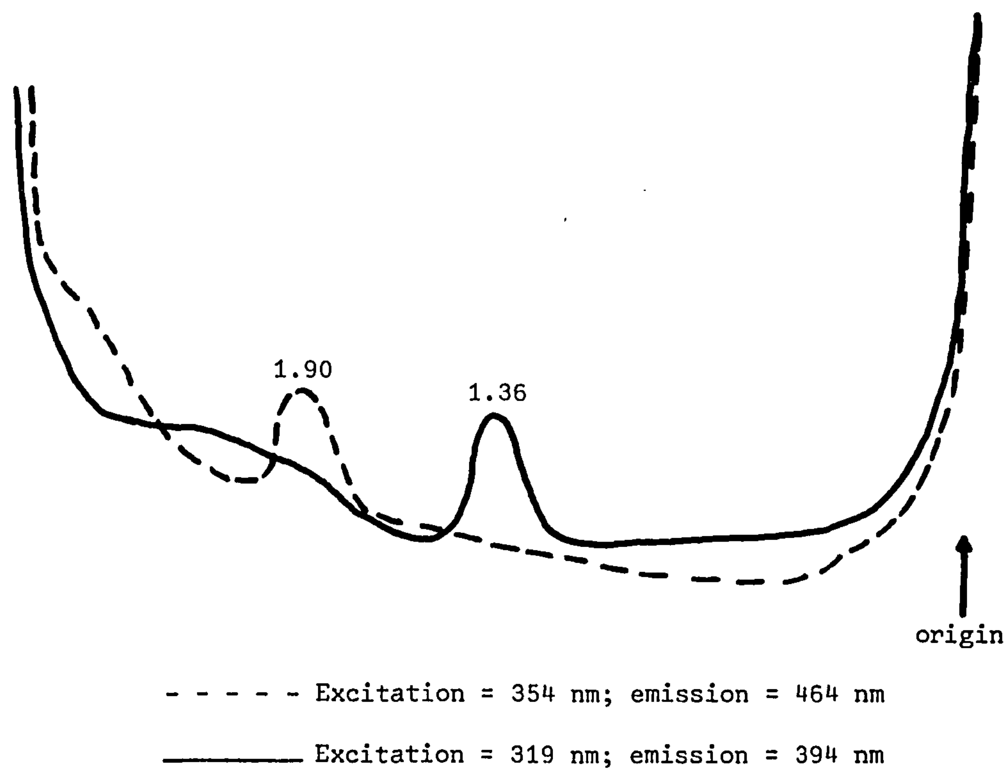




Figure 32. Fluorometric TLC scans of sample #7-C.

Sample No. 8

Water was collected from the Cocheco River at site B on April 27, 1970. The temperature of the water, which was slightly cloudy, was 11° C and the pH was 6.5. Duplicate analyses were performed on this sample using extractors B and C and are described below.

Sample No. 8-B. Extractor B was used to extract 5150 ml of water for 31 hours. After extraction with 72% $H_2SO_4$, the extract was concentrated to dryness and the solution as well as appropriate standards were applied to a cellulose sheet and developed for 4 hours. No spots could be seen under short-wave UV light. Fluorometric TLC scans are shown in Figure 33. The spot at $R_B$ = 0.98 was characterized as benzo(a)pyrene on the basis of its $R_B$ value and maximum fluorescence wavelengths.

Sample No. 8-C. Extractor C was used to extract 5000 ml of water for 31 hours. The extract was treated in the same manner as #8-B and separated on the same TLC sheet. TLC scans of the sample are shown in Figure 34. No spots were observed at any wavelengths including the trace amount of benzo(a)pyrene found in #8-B.

Sample No. 9

Water was collected from the Cocheco River at site D on April 29, 1970. The temperature of the water, which was slightly cloudy, was 15° C and the pH was 6.3. Duplicate analyses were performed on this sample using extractors B and C and are described below.

Sample No. 9-B. Extractor B was used to extract 5150 ml of water for 29 hours. After extraction with 72% $H_2SO_4$, the extract was concentrated to dryness and the residue dissolved in 100 µl of ethanol. A 10 µl portion of the solution as well as appropriate standards were applied to a cellulose

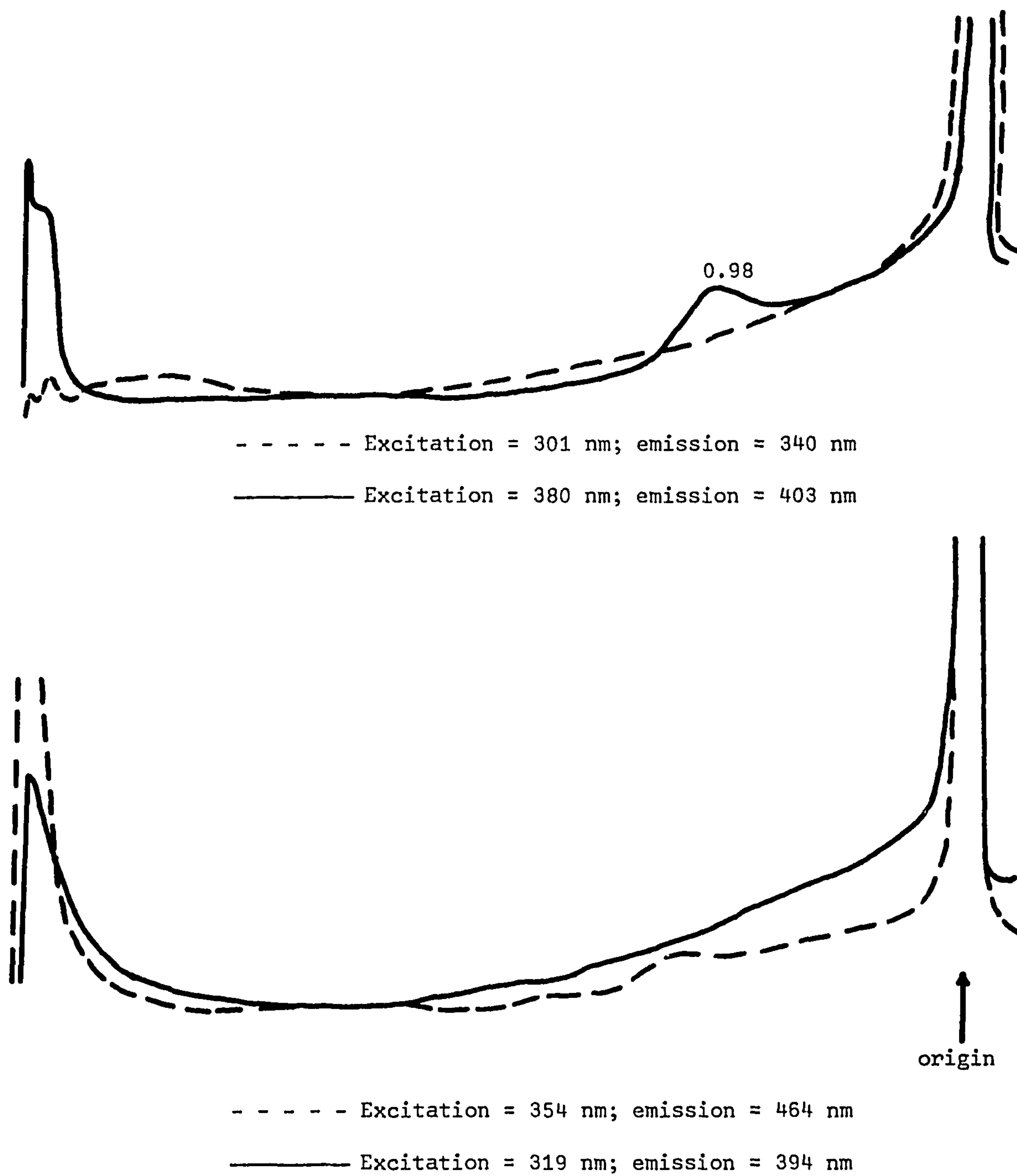
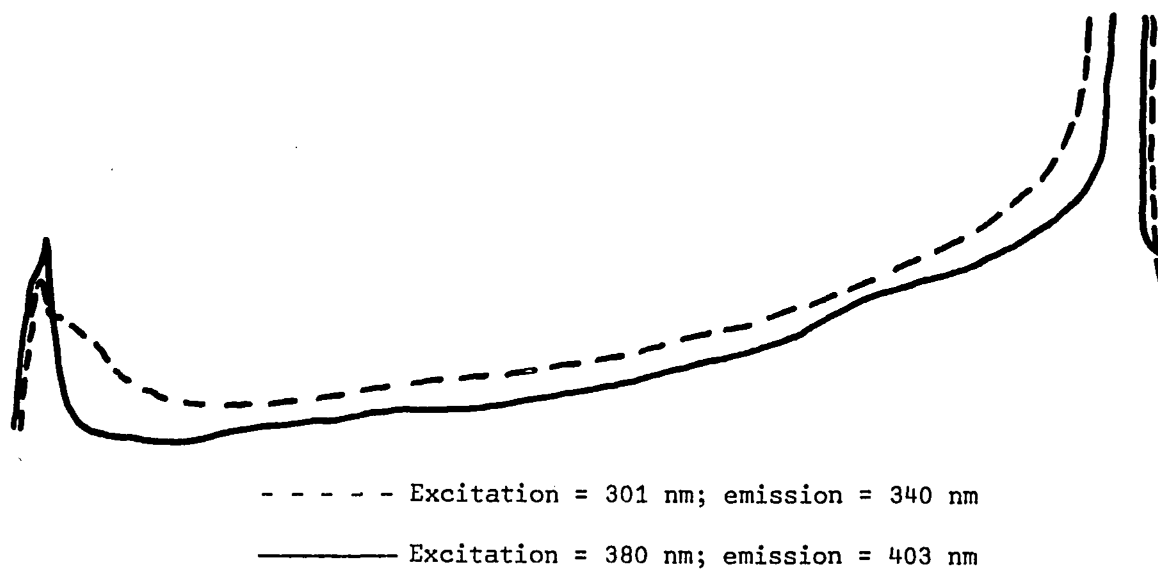



Figure 33. Fluorometric TLC scans of sample #8-B.



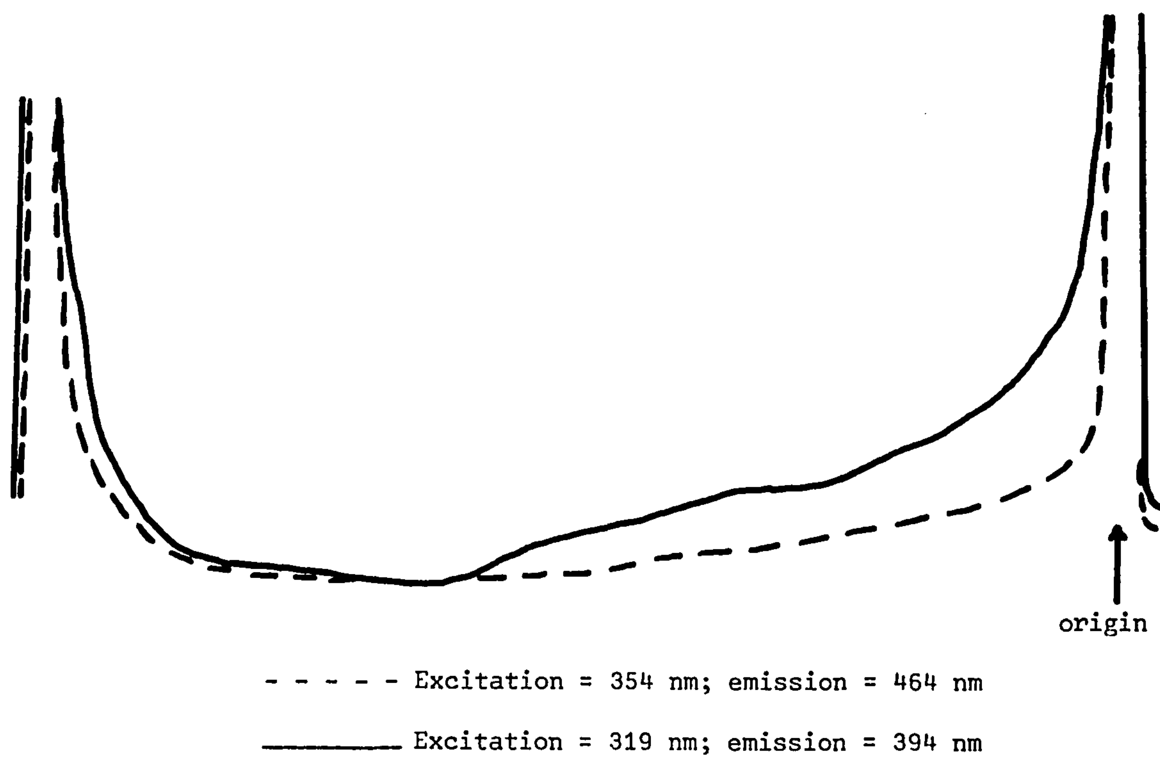




Figure 34. Fluorometric TLC scans of sample #8-C.

sheet and developed for 4 hours. No spots could be seen under short-wave UV light. Fluorometric TLC scans of the sample are shown in Figure 35. The spot at $R_B$ = 0.96 was characterized as benzo(a)pyrene on the basis of its $R_B$ value and maximum fluorescence wavelengths. Fluorescence excitation and emission spectra of spot E with $R_B$ = 1.63 were obtained and are shown in Figure 36. Attempts to identify this spot by comparison with standard PNA spectra were unsuccessful.

Sample No. 9-C. Extractor C was used to extract 5000 ml of water for 29 hours. The extract was treated in the same manner as #9-B and the fluorometric TLC scans are shown in Figure 37. The results were essentially the same as in #9-B.

Sample No. 10

Water was collected from the Winnipesaukee River at site E on May 10, 1970. The water temperature was 17$^{\circ}$ C and the pH was 7.1. Duplicate analyses were performed on this sample using extractors B and C and are described below.

Sample No. 10-B. Extractor B was used to extract 5150 ml of water for 35 hours. After extraction with 72% $H_2SO_4$, the deep yellow color of the pentane layer was transferred to the acid layer. The extract was concentrated to dryness and the residue dissolved in 100 ul of ethanol. A 10 $\mu$l portion of this solution as well as appropriate standards were applied to a cellulose sheet and developed for 4 hours. Fluorometric TLC scans of the sample are shown in Figure 38. No spots were observed at any wavelengths.

Sample No. 10-C. Extractor C was used to extract 5000 ml of water for 35 hours. The extract was treated in the same manner as #10-B and the TLC scans were essentially the same as those of #10-B shown in Figure 38. No spots were observed at any wavelength.

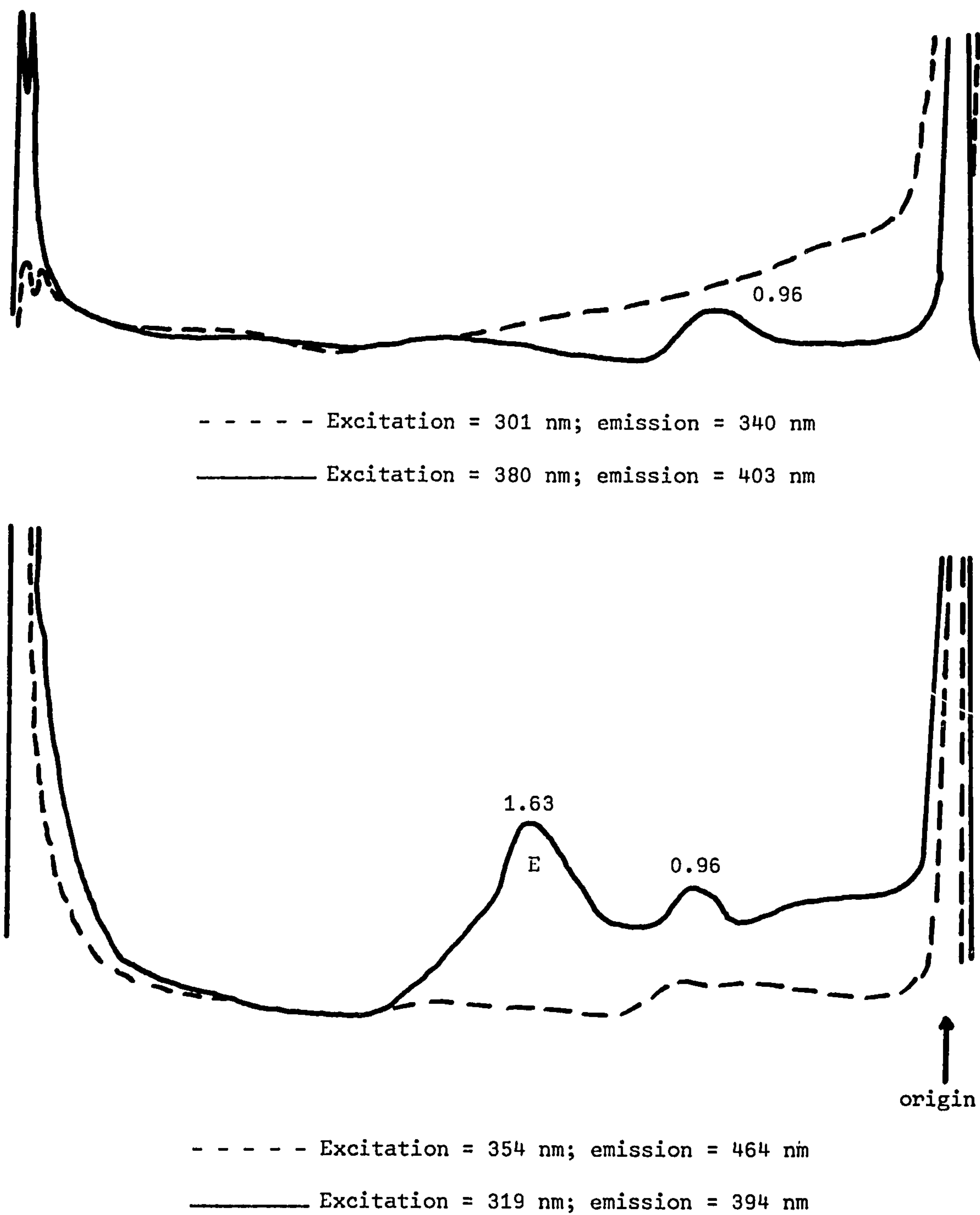



Figure 35. Fluorometric TLC scans of sample #9-B.

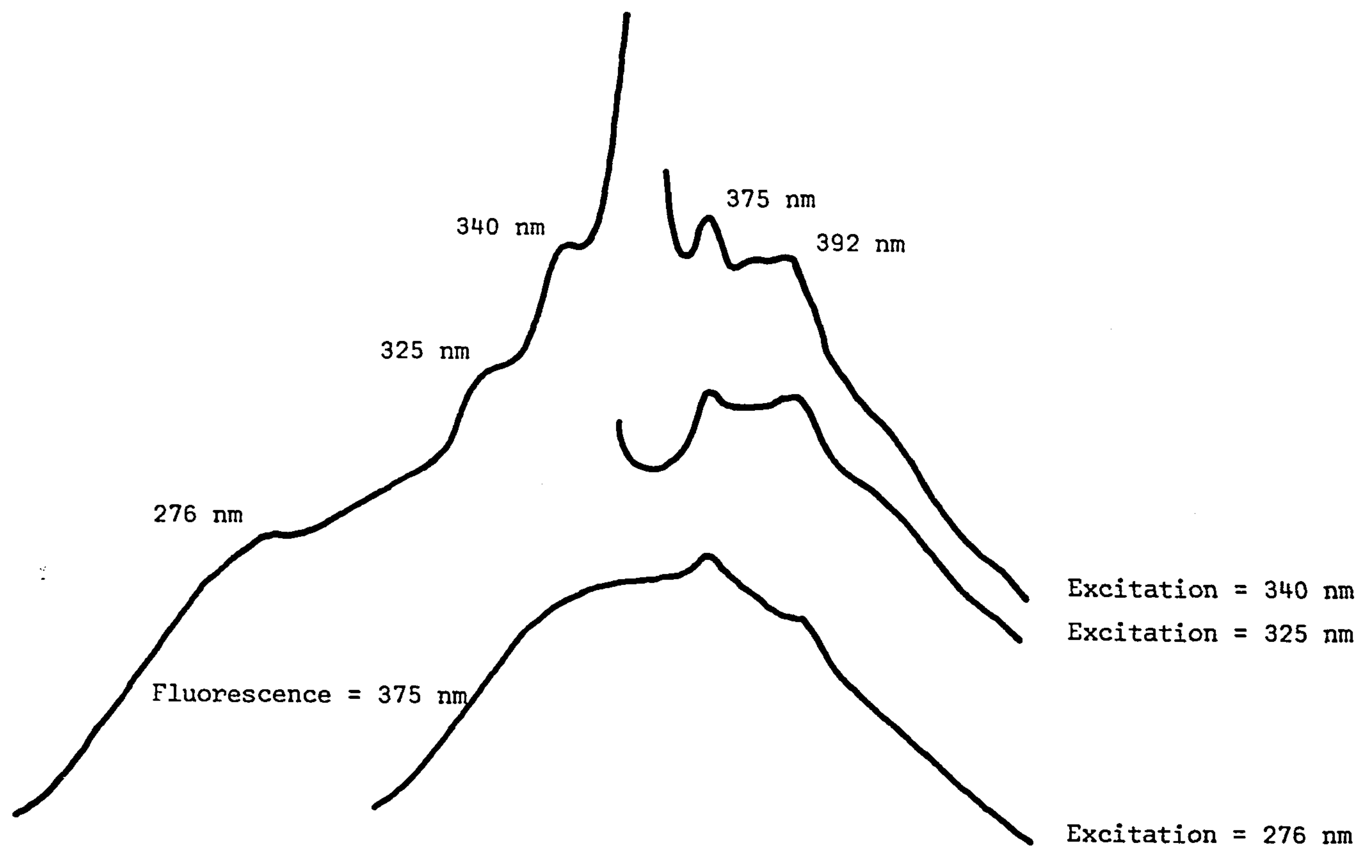



Figure 36. Fluorescence spectra of spot E in sample #9.

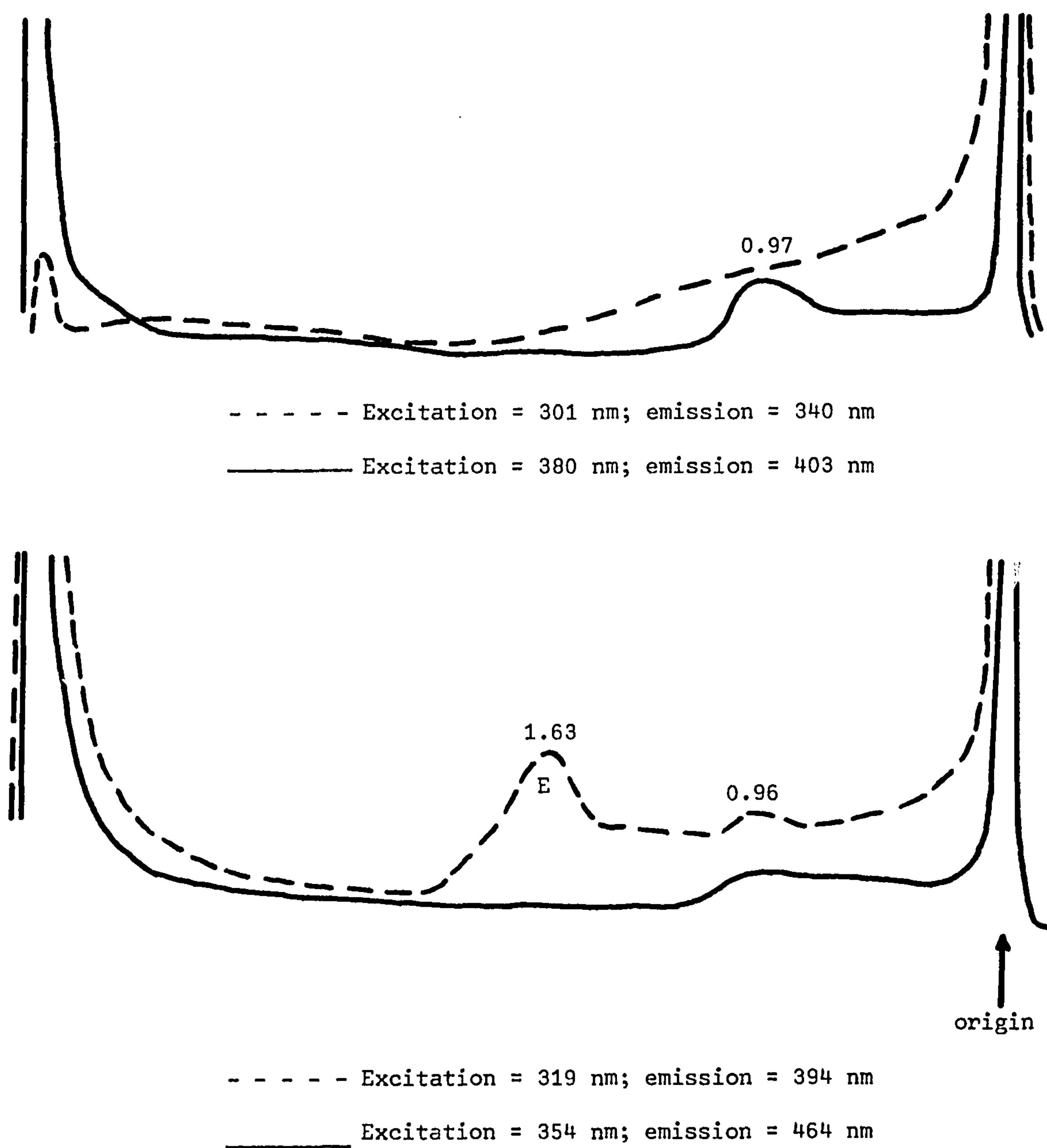



Figure 37. Fluorometric TLC scans of sample #9-C.

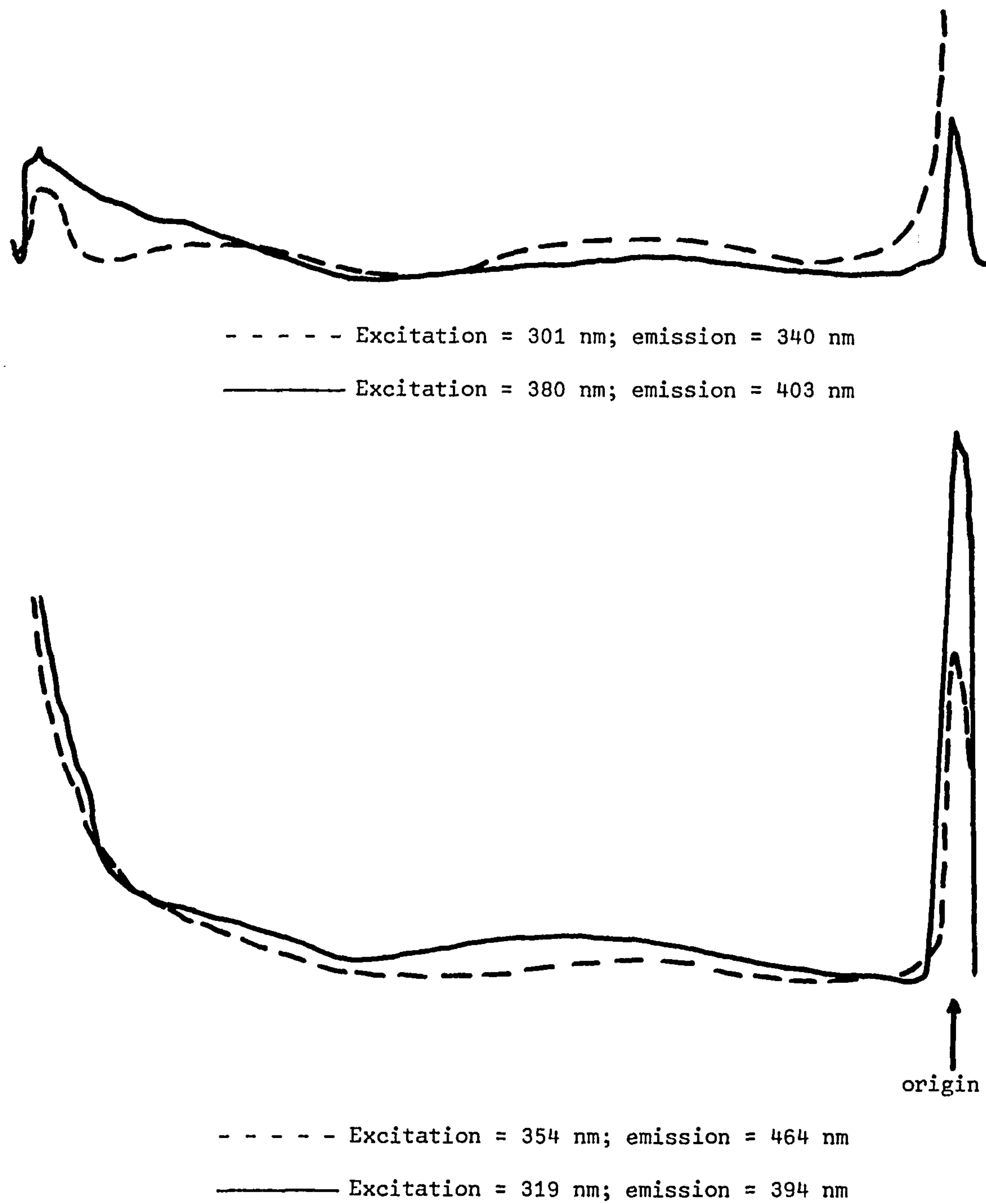



Figure 38. Fluorometric TLC scans of sample #10-B.

## Batch Extractions

Four river water samples were analyzed using the batch extractions from September 21, 1970 to October 1, 1970. Duplicate determinations were carried out for each sample using the technique of gently swirling the separatory funnel containing 3000 ml of water and 100 ml of pentane for 10 minutes. Each extract was washed with 72% $H_2SO_4$ and subsequently concentrated to dryness. Each residue was dissolved in 100 ul of ethanol and 20 ul applied to a cellulose sheet. No spots were visible in any of the chromatograms but fluorometric TLC scans did reveal some PNA. Sampling and fluorometric TLC scanning data are summarized below. No significant variation was apparent in any of the duplicate determinations. In the samples discussed below fluorometric TLC scans for only one member of each duplicate are presented.

### Sample No. B1

Water was collected from the Oyster River at site A on September 21, 1970. The water temperature was 14° C and the pH was 6.9. Fluorometric TLC scans of this sample are shown in Figure 39. The fluorescence spectrum of spot G was obtained and was identified with that obtained for spot G in Sample No. 1 and shown in Figure 25.

### Sample No. B2

Water was collected from the Cocheco River at site C on September 22, 1970. The water temperature was 19° C and the pH was 6.8. Fluorometric TLC scans of the sample are shown in Figure 40. The spots at $R_B$ values of 0.62 and 1.00 were characterized as dibenz(a,h)anthracene and benzo(a)pyrene respectively on the basis of their $R_B$ values and maximum

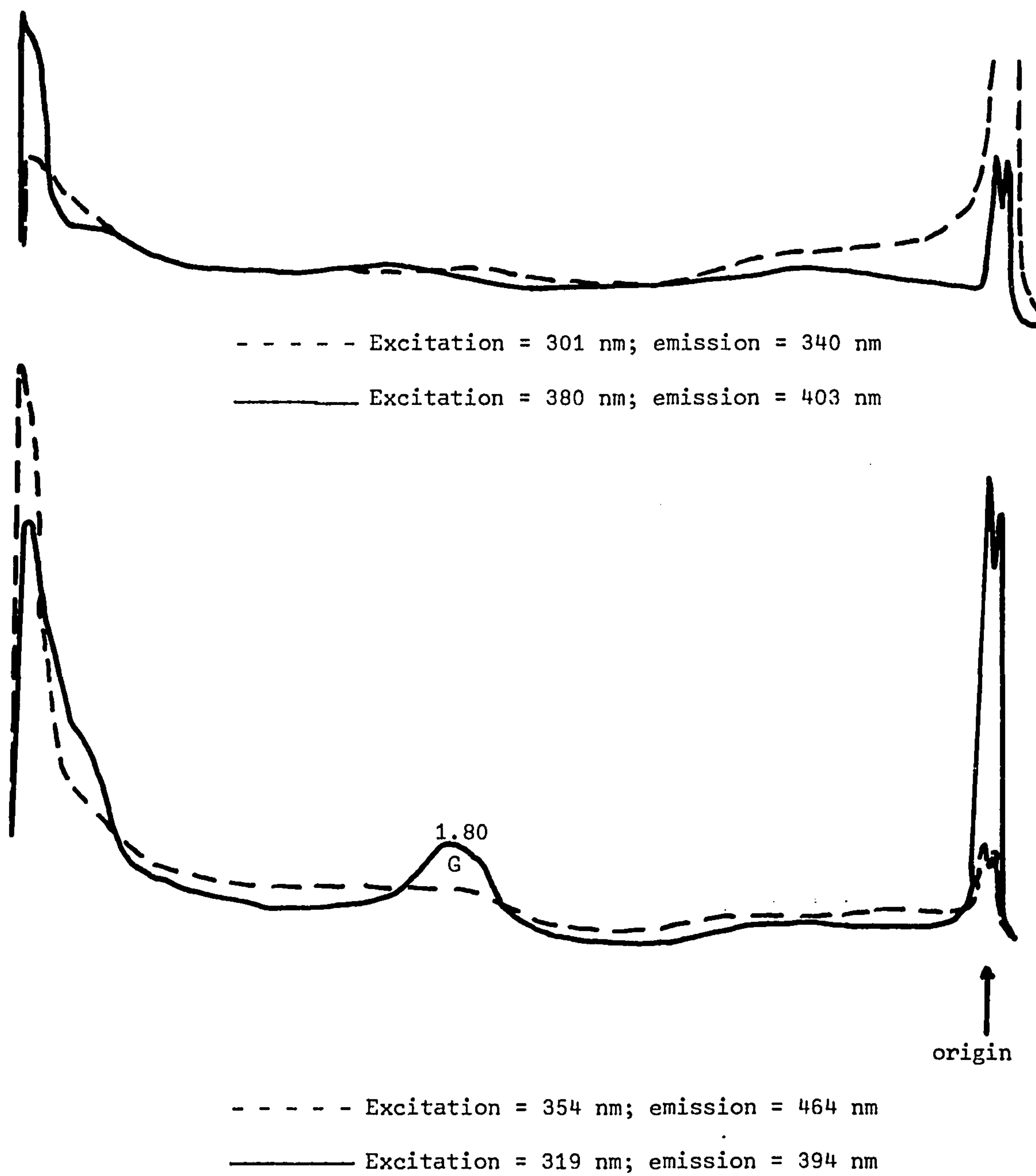



Figure 39. Fluorometric TLC scans of sample # B1.

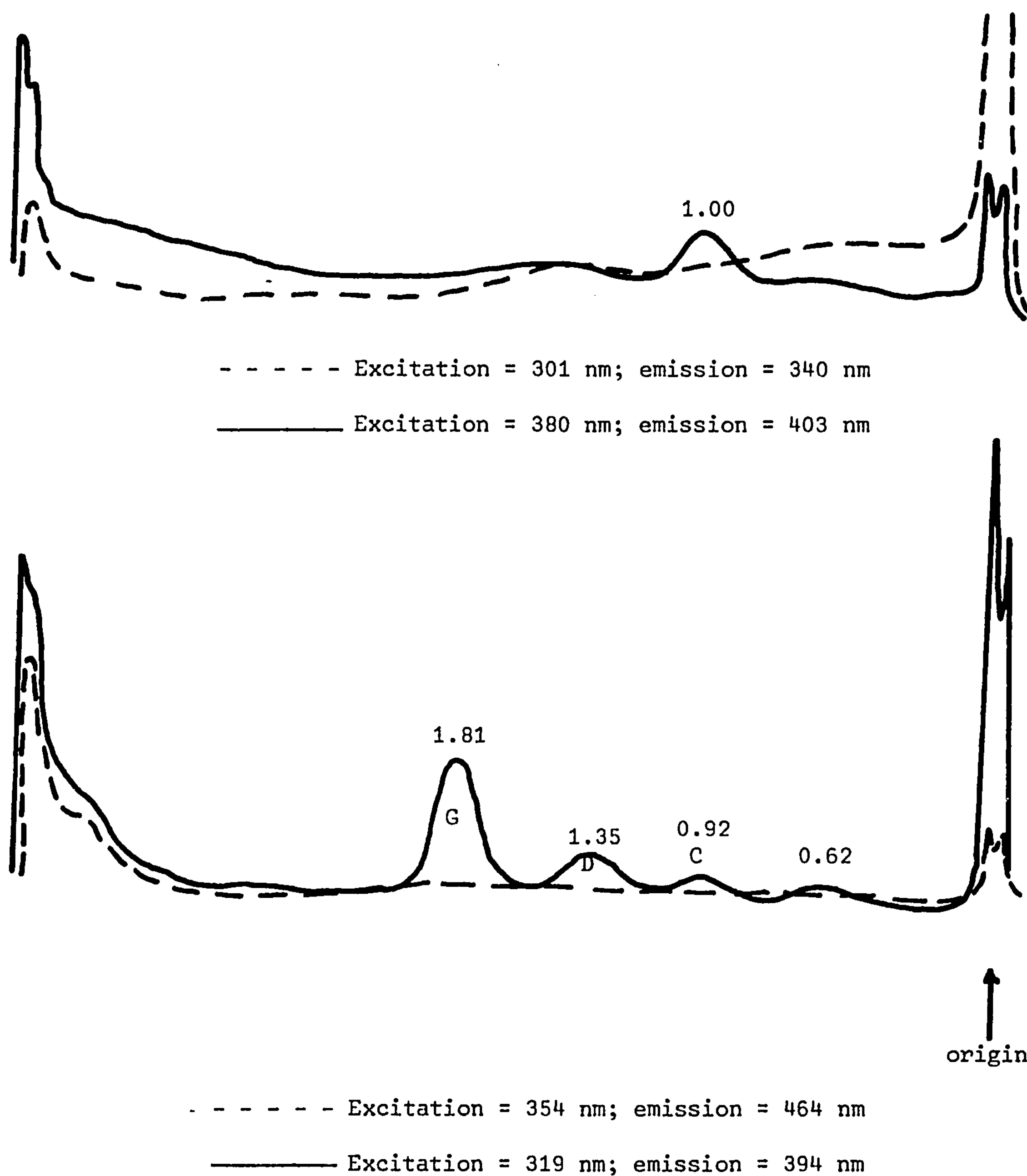



Figure 40. Fluorometric TLC scans of sample #B2.

fluorescence wavelengths. The spots at $R_B$ values of 0.92 and 1.35 showed maximum fluorescence wavelengths of 262/375 and 301/375 respectively. These $R_B$ values and maximum wavelengths are identical with those of spots C and D found in Sample No. 1. Spectra of these two spots are shown in Figure 25. The spot at $R_B$ = 1.81 was characterized by its fluorescence spectrum as being identical to that of spot G found in Sample No. 1. Its fluorescence spectrum is shown in Figure 25.

Sample No. B3

Water was collected from the Cocheco River at site D on September 29, 1970. The water temperature was 18° C and the pH was 6.6. Fluorometric TLC scans of the sample are shown in Figure 41. Fluorescence excitation and emission spectra for the spot at $R_B$ = 1.62 were found to be identical to those obtained for spot E found in Sample No. 9. The fluorescence spectra are shown in Figure 36.

Sample No. B4

Water was collected from the Winnipesaukee River at site E on October 1, 1970. The water temperature was 16° C and the pH was 6.8. As in the previous sample from this site the pentane extract was a deep yellow color. All of the color was removed by the acid extraction. TLC scans of this sample are shown in Figure 42. No spots were observed at any wavelengths.

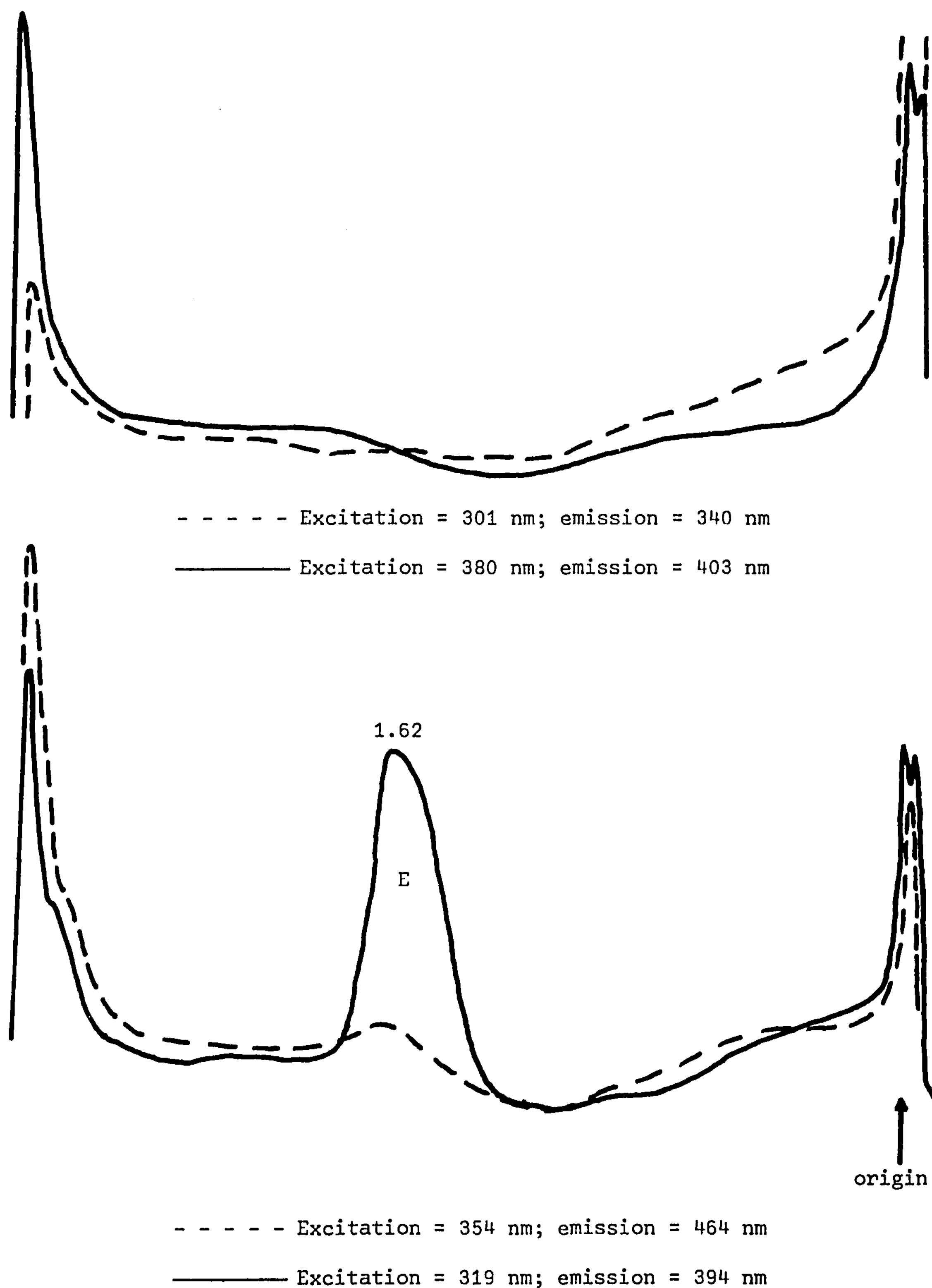



Figure 41. Fluorometric TLC scans of sample #B3.

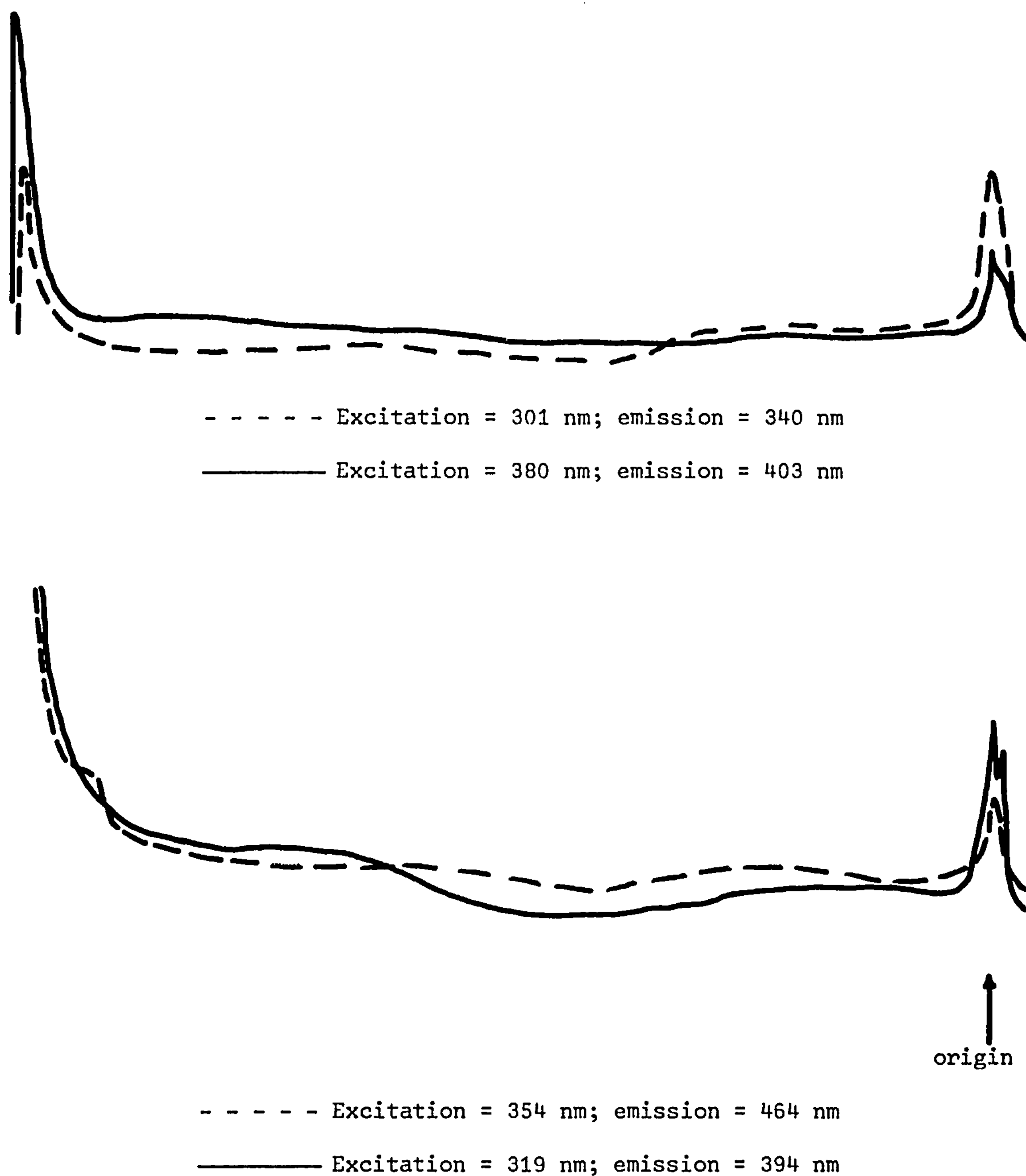



Figure 42. Fluorometric TLC scans of sample #B4.

# DISCUSSION

## Summary of the Method

The purpose of this research was the development of a fairly rapid method for the quantitative analysis of PNA in natural water. A summary of the method is presented below.

1. Extract 3 liters of water with 100 ml of pentane for 10 minutes. Swirl the separatory funnel gently to avoid emulsion formation.

2. Concentrate the pentane extract to approximately 75 ml and extract one time with 25 ml of 72% $H_2SO_4$.

3. Concentrate the pentane layer to dryness and dissolve the residue in 100 µl of ethanol.

4. Apply 20 µl of the ethanol solution, as well as standard solutions containing 5 and 50 ng of each reference compound to a cellulose plate. Develop the plate in the dark to a height of 15 cm (approximately 4 hours). Dry the chromatogram in a stream of cool, dry air for 30 minutes.

5. Scan the plate parallel to the direction of development to locate the separated PNA and determine $R_B$ values. Each separation should be scanned at a variety of fluorescence excitation and emission wavelengths. Record fluorescence spectra of located spots wherever possible.

6. Scan perpendicular to the direction of development those spots which have been qualitatively identified. Compare the measured peak area with those obtained for 5 and 50 ng standard spots of the same compound.

The method is extremely sensitive and most PNA may be determined in the parts per trillion range. Extension of this range to lower limits can be accomplished by combining the extracts from several 3 liter water samples. The time required for a complete analysis using batch extraction is about 8 hours. If continuous extractions are necessary, an additional

24 hours is required.

Accurate quantitative measurements may be obtained for those PNA which can be completely separated on a cellulose plate developed with 50% aqueous DMF. Even in cases where individual compounds are not totally separated, the fluorescence of a single PNA can sometimes be measured by appropriate choice of excitation and emission wavelengths. Complex mixtures of PNA, such as those associated with air particulate samples, would require preliminary separation into simpler groups prior to TLC analysis. Several different TLC systems would be necessary to separate each group of PNA into individual compounds. Although the TLC scanning technique should be adaptable to these TLC systems, basic studies of the scanning parameters would be necessary.

The relative standard deviation of the entire method as shown in total recovery studies is between 10% and 15% for the four reference PNA. Since a significant percentage of this error is associated with manual TLC spot application, incorporation of an automatic TLC spotting device would reduce the relative standard deviation to less than 10%.

## Analysis of Real Samples

The developed method has been applied to the analysis of 14 river water samples. Identification of unknown PNA was based upon comparison of their $R_B$ values and fluorescence spectra with those of known PNA. All identified spots were quantitatively determined by the fluorometric TLC scanning method described above. The data concerning these analyses are summarized in Table XVIII and Table XIX for continuous and batch extractions respectively. The values given in Table XVIII are the concentrations in the original water samples of the particular PNA expressed in parts per trillion. Four PNA have been identified and quantitatively determined.

Table XVIII.

Summary of Continuously Extracted Samples

| Sample | Identified Spots: DiBahA | BaP | BbF | Fluor | Unidentified Spots |
|---|---|---|---|---|---|
| 1 | * | - | - | - | C,D,G |
| 2 | * | 78 | 100 | 320 | - |
| 3 | 92 | 125 | 550 | 1000 | - |
| 4 | * | - | * | 330 | H |
| 5 | - | - | 155 | - | B,F |
| 6 | * | 150 | 240 | - | A |
| 7-B | * | * | * | * | - |
| 7-C | - | * | * | * | - |
| 8-B | - | * | - | - | - |
| 8-C | - | - | - | - | - |
| 9-B | - | * | - | - | E |
| 9-C | - | * | - | - | E |
| 10-B | - | - | - | - | - |
| 10-C | - | - | - | - | - |

Notes:

(1) * $R_B$ values and maximum fluorescence wavelengths are correct but not enough compound is present for positive identification using fluorescence spectrum.

(2) Concentrations of identified spots are expressed as parts per trillion (ng/l) present in the original water sample.

Table XIX.

Summary of Batch Extracted Samples

| | Identified Spots | | | | Unidentified Spots |
|---|---|---|---|---|---|
| Sample | DiBahA | BaP | BbF | Fluor | |
| B1 | - | - | - | - | G |
| B2 | * | * | - | - | C,D,G |
| B3 | - | - | - | - | E |
| B4 | - | - | - | - | - |

* $R_B$ values and maximum fluorescence wavelengths are correct but not enough compound is present for positive identification using fluorescence spectrum.

With the exception of fluoranthene in Sample No. 3, all concentrations were in the sub-part per billion range. Eight additional compounds, which were very likely PNA, were also detected in the water samples. A summary of the data concerning these unidentified spots is shown in Table XX. All spots were characterized either by fluorescence spectra or the fluorescence excitation and emission wavelengths which gave maximum response. Spot H could not be recorded spectrophotometrically because its maximum fluorescence emission occured in the red wavelength region where the photomultiplier was insensitive.

Because only nanogram amounts of PNA are present on the TLC adsorbent, the instrumental techniques which may be used for identification are limited. Infrared spectroscopy and ultraviolet absorptiometry require larger sample sizes in the ng and mg range. Mass spectrometry does possess the required sensitivity; its use in conjunction with TLC has been reported by Deverse and co-workers[116]. In this method, elution of the compound is not necessary and nanogram amounts of compound may be examined. This type of analysis would complement the use of fluorescence to facilitate unknown spot identification.

Of the three rivers sampled, only samples from the Oyster River showed sufficient amounts of the four reference PNA to obtain fluorescence spectra. The concentration values determined for these four PNA agree with the results of Borneff[117] who has found total PNA concentrations of 0-10 ppt in ground water and 500-2600 ppt in German river water.

Fewer PNA could be characterized in water samples taken from the Cocheco River downstream from the city of Dover than in those taken upstream from Dover. This indicates that there is no significant contribution to the PNA content from sources within the city. The most probable sources of PNA within the city would have been suspended air particulate

Table XX.

Summary of Data Concerning Unidentified Spots

| Spot | Unknowns | $R_B$ | Wavelengths (nm) Giving Maximum Fluorescence |
|---|---|---|---|
| A | 6 | 0.22 | 355/465 |
| B | 5 | 0.85 | 325/385 |
| C | 1,B2 | 0.90 | See Fig. 25 for spectrum |
| D | 1,B2 | 1.31 | See Fig. 25 for spectrum |
| E | 9,B3 | 1.63 | See Fig. 36 for spectrum |
| F | 5 | 1.72 | 335/391 |
| G | 1,B1,B2 | 1.80 | See Fig. 25 for spectrum |
| H | 4 | 2.09 | * |

* Spot H has reddish fluorescence under 254 nm light but cannot be seen with the TLC scanner.

matter or industrial or sewage effluents. The fact that more PNA are found in the water above the city would indicate that these sources are contributing very little to the PNA content of the water.

No PNA could be detected in the two samples from the Winnipesaukee River. This site had been chosen because of its nearness to a large asphalt plant. PNA are known to be products of incomplete combustion of asphalt-type materials and it was felt that water taken from this location would contain large amounts of PNA. That there was indeed a great deal of extractable material in the water was apparent from the deep yellow color of the extract. This material was shown to be basic however, by extraction with $H_2SO_4$.

When considering the possible sources of the PNA identified and characterized in this work, it is significant that the largest amounts of PNA were found in the relatively unpolluted Oyster River. The Oyster River is small in comparison with the other two rivers sampled, and is fed by several small streams, themselves originating in swampy land. The banks of the river above the sampling site are generally steep, often rising 100 feet or more in a short distance. The large PNA concentrations found in the Oyster River suggest that the trace amounts of PNA present are due to natural phenomenon and not to environmental pollution. Several authors[118-120] have shown unequivocally the biosynthesis of PNA in plants and soils and Borneff[121] and Blumer[122] have determined PNA in soil samples. Borneff[121] has also postulated that the runoff of rain carries some of these naturally occurring PNA into the ground water and streams to establish a natural level of PNA. It would seem that the amounts found in this study are associated with these naturally occurring amounts.

In summary, we have developed a procedure which enables one to analyze for trace amounts of a potentially hazardous series of compounds

present in natural water. This method is sufficiently rapid to be used for routine monitoring of PNA in natural water systems. It is also flexible enough to be adaptable to other PNA which have not yet been investigated.

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