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A Method for the Determination of Polycyclic Aromatic Hydrocarbons in Animal Tissue

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A number of methods of varying complexity have been used for the isolation and analysis of polycyclic aromatic hydrocarbons (PAH) in air (LUNDE and BJORSETH 1977, LAO et al. 1973), water (ANDELMAN and SUESS 1970, SCHWARZ and WASIK 1976), soil (SHABAD et al. 1971, HITES et al. 1977) and biological samples (TOMINGAS et al. 1976, GRIMMER and HILDEBRANDT 1972). These methods range from very simple ones, involving extraction and spectrophotometric analysis, to highly complex computerized gas chomatrographic-mass spectrometric (GC-MS) analysis (LAO et al. 1973, SCHWARZ and WASIK 1976). For analysis of biological samples, a large number of investigators rely upon lengthy manipulative and time-consuming procedures. In addition, without adequate purification the isolated extract may contain sufficient impurities to warrant additional steps. The need for a simple nonradiolabeled method is illustrated by continued use of these methods in biological studies (HENRY and KAUFMAN 1973, CRESIA et al. 1976). The present paper describes a method that is rapid, precise and avoids a number of the manipulative steps of other methods.

MATERIALS AND METHODS

All organic solvents and chemicals were analytical reagent grade and were used without any further purification. Fritted glass thimbles were used with the Soxhlet extractors.

Male Fisher-344 rats, 8 to 10 weeks of age were used in this study. For the deposition of pyrene in the lungs of animals by intratracheal instillation, animals were anesthetized with halothane and placed on special boards designed to hold their mouths open at the correct angle for instillation. After positioning of the animal, pyrene (1 mg/kg), in a sterile gelatin-saline suspension, was instilled directly into the lung. The animals were kept on the board for a few minutes to avoid regurgitation of the suspensions. The animals were sacrificed by CO_2 asphyxiation shortly after instillation of pyrene and the lungs were removed, blotted free of liquid and weighed. The lungs were transferred to tubes containing acetone and homogenized using a Tissumizer® (Tekmer Co., Cincinnati, OH). The homogenate was transferred to Soxhlet thimbles (85 x 25 mm) and extracted for 2 hours using acetone. Following extraction, the acetone extract was evaporated to dryness at 35°C using a rotary evaporator. The residue was taken up in one to two ml of acetone and pyrene was quantitated spectrofluorometrically at an emission wavelength of 387 nm and excitation wavelength of 323 nm. These wavelengths gave a maximum fluorescence reading of the pyrene standard which was used for instillation.

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An additional modified procedure was used for the quantitation of pyrene following instillation. This procedure is as follows:

Lung

↓ Homogenize with 15 ml of hexane and shake for 30 minutes

Lung homogenate

↓ Partition 5 ml of hexane (from homogenate) with 5 ml of 25% dimethylsulfoxide-H_2O (V/V)

Hexane

↓ Wash hexane two times with 5 ml of distilled H_2O

Hexane

↓ Record fluorescence
Ex λ 323 nm
Em λ 387 nm

Calculate Pyrene (tissue) by comparing to pyrene standard carried through the extraction procedure.

Distribution of Polycyclic Aromatic Hydrocarbon in 25% DMSO-H_2O V/V Hexane Phases

Six to eight micrograms of anthracene, fluoranthene pyrene and benzo(a)pyrene were added to hexane. An equal volume of 25% DMSO-H_2O was added and the distributuion coefficients were determined following mixing and separation of the two layers. Quantity of material was spectrophotometrically determined at the following wavelengths (nm): Anthracene, 355; fluoranthene, 358; pyrene, 344; and benzo(a)pyrene, 295.

RESULTS

A Soxhlet procedure was initially used to extract and quantitate pyrene and it was found that the amount of pyrene detected by this procedure was 25-50% of the amount instilled. Further investigation revealed that the low amount was primarily due to interfering substances present in the initial extract. A simple procedure for removing the substances was developed. When the acetone extract from the soxhlet extraction was evaporated to dryness, and partitioned between hexane and 25% (DMSO)-H_2O (v/v) the amount of pyrene fluorescence in the hexane extract increased with an overall recovery of approximately 92%. Thus the initial low fluorescence was apparently due to interferences that quenched pyrene fluorescence.

Several DMSO-H_2O mixtures were used for measuring pyrene in hexane (Figure 1). Maximum recovery of pyrene in the hexane layer was found when partitioned with 10 to 60% DMSO-H_2O. The separation

of DMSO-H_2O and hexane layers were sharper at approximately 25% DMSO-H_2O and thus, this DMSO concentration was used routinely. When higher DMSO-H_2O concentrations were used pyrene distributed more in the DMSO-H_2O layer. At approximately 90% DMSO-H_2O, the pyrene distributed equally between hexane and DMSO-H_2O.

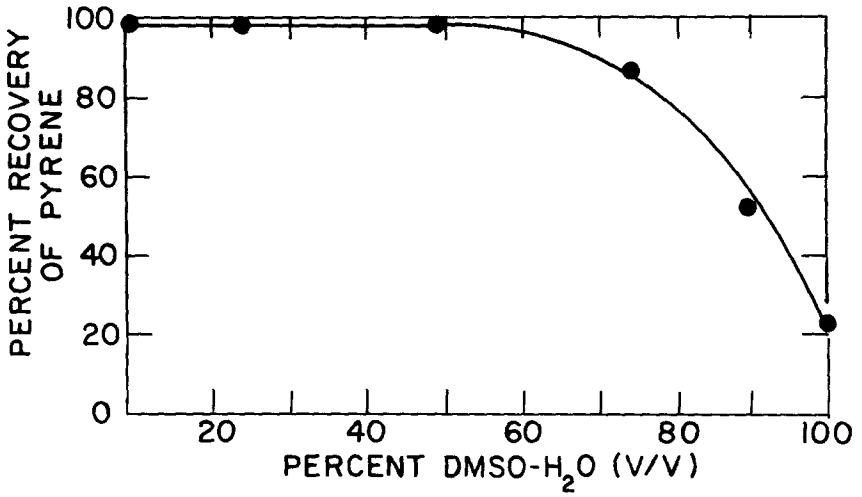

Figure 1. Recovery of pyrene in hexane following DMSO-H_2O partitioning; 8 µg of pyrene was added to 0.4 absorbance units of lung extract before partitioning.

In addition to showing the retention of pyrene in the hexane extract following the DMSO-H_2O partition, Figures 2 and 3 show that the maximum peak wavelengths of the spectrophotometric and spectrofluorometric spectra of the lung extract were essentially identical with that of a pyrene standard.

Although the DMSO-H_2O partitioning was efficient for removing interferences from lung extracts, the use of Soxhlet extractors did not lend itself to rapid analysis of large numbers of samples. Thus the modified procedure as outlined in the materials and methods was used for further analyses. Figure 4 shows the time that was necessary to extract pyrene from lung homogenates with this procedure. After 15 minutes of shaking the lung homogenates, 95% of the pyrene added to the tissue was found in the hexane extract. Table 1 shows the accuracy, precision and sensitivity of the method for pyrene.

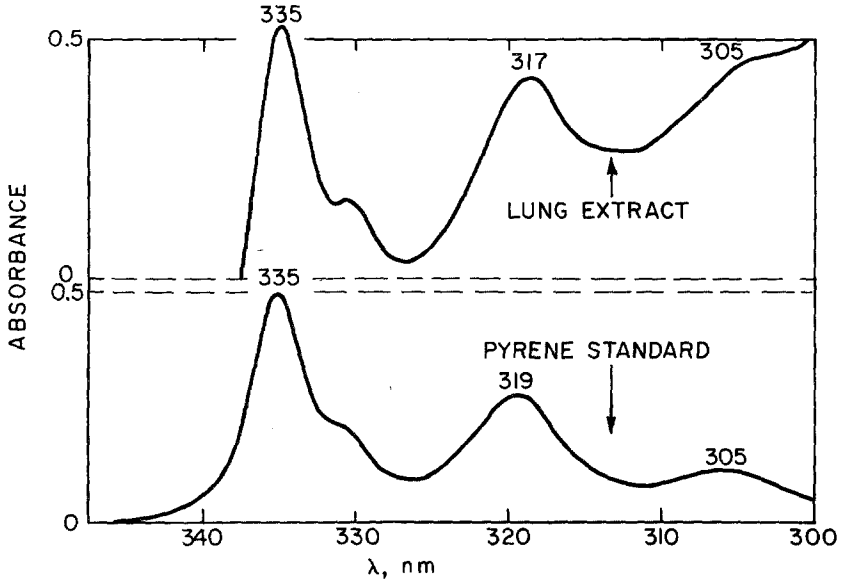

Figure 2. Absorbance spectra of a lung extract in hexane following intratracheal instillation of pyrene.

Pyrene was used in these studies as a model of how an unaltered PAH may be analyzed in biological samples. To determine if the present method would be applicable to other PAH, the concentration of benzo(a)pyrene (BaP), anthracene, fluoranthene and pyrene was measured in the hexane layer following DMSO-H_2O partitioning (Table 2). Of the PAH investigated anthracene had the highest concentration in the hexane layer and BaP had the least, although all of PAH had very favorable distribution coefficients, i.e., recoveries greater than 85%. The difference in the distribution coefficients appears to be related to the molecular weight with the lower weight PAH being more soluble in hexane. With this procedure 85% of BaP would be retained in the hexane layer.

DISCUSSION

A method is described for the analysis of pyrene in lung tissue. The method is simple, specific, sensitive and may have applications in analysis of other unaltered PAH in lung and other tissues. The method is also rapid; a large number of analyses may be performed in a short time. Dimethylsulfoxide-H_2O was efficient for removing interferences from the hexane extracts, although the interferences encountered with hexane extracts were not as numerous

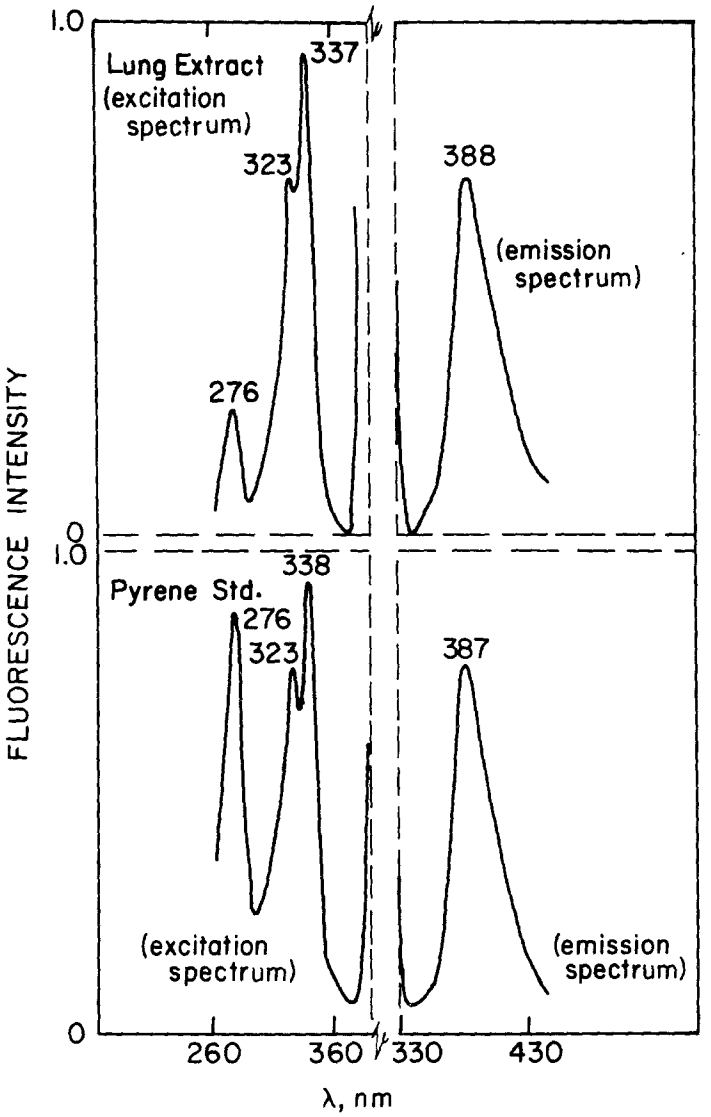

Figure 3. Spectrofluorometric excitation and emission spectra of a lung extract in hexane following intratracheal instillation of pyrene. Excitation was recorded at an emission wavelength of 387 nm. Emission was recorded at an excitation setting of 323 nm.

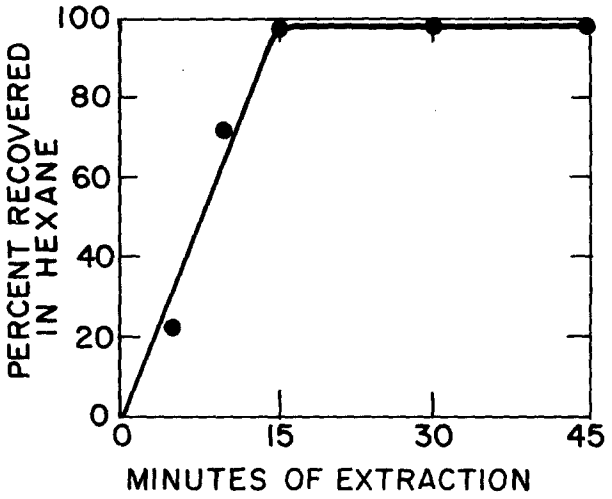

Figure 4. Kinetics of pyrene recovery in hexane from buffer and tissue homogenates.

TABLE 1

Precision, Sensitivity and Accurary of Spectrofluorometric Method for Pyrene

Precision			Accuracy		
range (µg/g)	mean	S.D.D.[a]	µg of pyrene added	recovery (%)	Sensitivity
1.5- 3.5	2.58	0.10	2- 5	85-95	0.01 µg/ml
10.5-17	14	0.42	4-16	90-95	

[a]Standard deviation of duplicates (S.D.D.) was calculated according to the following equation:

$$S.D.D. = \sqrt{\frac{\Sigma(d^2)}{2N}}$$

where d^2 equals the squared differences between the duplicate samples and N equals the number of duplicate samples analyzed. Thirty duplicate samples were analyzed for each range.

TABLE 2

Distribution of Polycyclic Aromatic Hydrocarbon in 25% DMSO-H_2O (v/v) Hexane Phases

PAH	Distribution coefficient[a]
Anthracene	52
Fluoranthene	16
Pyrene	18
Benzo(a)pyrene	7

[a]Distribution coefficient is expressed as the total quantity of material present in the upper phase (hexane) divided by the quantity of material in the lower phase (25% DMSO-H_2O (V/V)). Six to eight µg of PAH was used in each test. Quantity of material was spectrohotometrically determined at the following wavelengths (nm): Anthracene, 355; fluoranthene, 358; pyrene, 344; and benzo(a)pyrene, 295.

as those obtained with acetone. DUNN (1976) also found DMSO a useful reagent for removing interferences. However, in this latter study (DUNN 1976), DMSO was used to extract PAH and interfering substances from an organic extract followed by diluting the DMSO with water and re-extracting the PAH with an additional organic solvent. The interferences that are encountered in tissue samples are variable and thus do not always require an additional purification of the tissue extract, but because of these uncertainties, DMSO extraction was adopted for all samples. This strategy is consistent with that of DUNN (1976). The recoveries obtained with the present method are in good agreement with others (GRIMMER and HILDEBRANDT 1972, DUNN 1976, HENRY and KAUFMAN 1973).

The specific absorption and fluorescence spectra indicate that either could be used for quantitations, although fluorescence was on the order of 10^2 to 10^3 times more sensitive. When using the present method one person can analyze 120 samples in duplicate in one work week. The distribution coefficients of other PAH indicate that the method may also be useful for their analysis in tissues.

Although only data on lung tissues are shown in the present paper, other selected tissue, i.e., liver, kidney and stomach, were also used to determine the efficiency of the method for analyzing pyrene in these tissues. The amount of interferences that remained in hexane after DMSO-H_2O extraction were quite variable. The liver contained the highest and the most variable interferences and the

stomach the lowest. Thus it is recommended that internal standardization be used when analyzing tissues that contain considerable quantities of interfering materials.

In summary, the present method is simple, sensitive, rapid and useful for the determination of unaltered PAH in soft tissues of the body. The method should be of particular value in model systems designed to follow the retention, distribution and fate of PAH in tissues.

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