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DETECTION OF NITRO-POLYCYCLIC AROMATIC HYDROCARBONS IN LIQUID CHROMATOGRAPHY BY ZINC REDUCTION AND PEROXYOXALATE CHEMILUMINESCENCE

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SUMMARY

Detection limits in the range 0.25–8.5 pg have been obtained for nitro-polycyclic aromatic hydrocarbons (nitro-PAHs) in high-performance liquid chromatography (HPLC). The nitro-PAHs are reduced on-line to the corresponding amino-PAHs and detected by peroxyoxalate chemiluminescence.

Quantitative reduction is achieved on a short (3.5 × 0.32 cm) column packed with a 1:1 mixture of glass beads (*ca.* 40 μm) and zinc particles (40–80 μm). The mobile phase consists of 70–80% acetonitrile, the balance being a 50-m*M* tris(hydroxymethyl)aminomethane hydrochloride (pH 6.5) buffer, which is necessary both for the reduction and for catalysis of the chemiluminescent reaction.

The amino-PAHs are excited by energy transfer from the decomposition products of the reaction between hydrogen peroxide and bis(2,4,6-trichlorophenyl)oxalate. These reagents are introduced by post-column mixing, and the emission is detected by means of a conventional fluorescence detector with its light source turned off. We reported the high sensitivity and selectivity of peroxyoxalate chemiluminescence toward amino-PAHs in earlier work. The method yields a linear response over at least three orders of magnitude, and in most cases the detection limits are better than those obtained by fluorescence detection with the same fluorometer.

The reduction column may be placed either before or after the analytical column, so that the analytes are eluted either as the nitro-PAHs or the corresponding amino-PAHs. This feature provides a second characteristic retention time and is useful in identifying the detected compounds.

The technique has been applied to the selective detection of nitro-PAHs in carbon black. The carbon black samples were Soxhlet extracted with toluene, evaporated to dryness, and the extracts redissolved in methylene chloride for direct injection into the HPLC column. The principal compounds found in the carbon black samples were poly-substituted nitro-PAHs and unsubstituted PAHs. This method provides a sensitive and selective method for the analysis of complex samples for these environmentally important mutagens.

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INTRODUCTION

Interest in nitro-substituted polycyclic aromatic hydrocarbons (nitro-PAHs) has grown recently, since it was demonstrated that these compounds are potent, direct mutagens in Ames assays[1,2]. Pitts *et al.*[3] first showed that PAHs react with oxides of nitrogen to form nitro-PAHs. More recently, nitro-PAHs have been found in environmental samples by gas chromatography (GC) combined with mass spectrometry (MS) and by tandem mass spectrometry (MS–MS) methods[4–7]. Particulates from diesel exhaust have the largest concentrations, while those from ambient air and gasoline engines contain significantly less[8,9]. Particulate extracts from these sources are complex samples requiring fractionation to enrich the nitro-PAHs before analytical chromatography can be performed. These samples typically contain alkanes and aromatic compounds having various functional groups such as hydroxy, ketone, quinone, aldehyde, carboxylic acid and nitro groups. Fractionation of these samples has been performed by thin-layer chromatography (TLC)[10], high-performance liquid chromatography (HPLC)[4,7] and conventional adsorption column chromatography[11]. Paputa-Peck *et al.*[7] have shown that at least 60 specific nitro-PAHs are present in diesel exhaust particulates. Normal-phase HPLC was used to isolate the nitro-PAH fractions, and the analytical chromatography was carried out with fused-silica capillary GC and detection by MS and a nitrogen–phosphorus (thermionic) detector. MS–MS methods, such as mass-analyzed, ion-kinetic energy spectrometry (MIKES) and triple-stage quadrupole MS (TSQ), have been used to identify nitro-PAHs in similarly prepared extracts[4]. On the average, light-duty diesel engines produce 0.5 g of particulates per mile[11], of which 10–40% (w/w) are extractable with organic solvents. It appears that combustion processes and photochemical reactions in the lower atmosphere between oxides of nitrogen and PAHs are the major sources of environmental nitro-PAHs.

In 1980, nitro-PAHs were first implicated as the causative mutagens in certain electro-photographic toners[12]. The nitro-PAHs were shown to originate in the carbon black contained in the toners used to make photocopies. This result was confirmed, and isomers of dinitropyrenes were isolated and identified, using toluene extraction, HPLC and direct insertion probe MS[13]. It was believed that an oxidation step with nitric acid was responsible for the significant amounts of nitro-PAHs found. This method of oxidation has been employed to alter the surface properties of carbon blacks, resulting in desirable properties such as adhesion and surface charge[14].

The strong electron withdrawing effect of a nitro group renders the nitro-PAHs non-fluorescent. Maccrehan *et al.*[15] have shown that nitro-PAHs can be rapidly reduced to the corresponding aromatic amines, on-line and at a pH compatible with reversed-phase HPLC according to the reaction

$$\text{Ar–NO}_2 + 6\,\text{H}^+ + 3\,\text{Zn}^0 \rightarrow \text{Ar–NH}_2 + 2\,\text{H}_2\text{O} + 3\,\text{Zn}^{2+} \quad (1)$$

where Ar is any aromatic moiety. From this reaction, it can be seen that reduction is favored by low pH conditions. Once produced, the amino-PAHs can be detected in HPLC by fluorescence.

In previous work we found that amino-PAHs can be detected with extremely high sensitivity and selectivity by using peroxyoxalate chemiluminescence in con-

junction with HPLC[16,17]. Detection limits for amino-PAHs in this method were typically in the sub-picogram range and were one to two orders of magnitude lower than could be obtained by optimizing fluorescence detection. Because of the high degree of selectivity, the amino-PAHs could be detected in complex matrices such as synthetic fuels with minimal sample preparation. The high sensitivity to amino-PAHs is apparently due to a combination of low oxidation potential and high fluorescence quantum yield[17]. Because of the good results we obtained for amino-PAHs, we have investigated the use of on-line reduction of nitro-PAHs to amino-PAHs in combination with peroxyoxalate chemiluminescence as a means of detecting these important mutagens in complex matrices, such as carbon black.

EXPERIMENTAL

Chemicals

All nitro-PAHs were obtained from either Aldrich or Foxboro/Analabs. Pyrene was obtained from Aldrich. The chemiluminescence reagent bis(2,4,6-trichlorophenyl)oxalate (TCPO) was prepared as described in the literature[18], further recrystallized in spectrograde ethyl acetate, and then washed with spectrograde hexanes. Zinc dust (40–80 μm) was obtained from J. T. Baker (cat. No. 4280). Glass beads were purchased from Alltech Assoc. HPLC-grade acetonitrile was purchased from Fisher Scientific. Reagent grade hydrochloric and nitric acids were also obtained from Fisher Scientific.

Samples

Carbon black samples were provided by IBM Corp. (Information Products Division, Boulder, CO, U.S.A.). The two samples, CB-1 and CB-2, differ in that CB-2 was prepared by a process known to result in a significant reduction in nitro-PAHs. Extracts were prepared by Soxhlet extracting 5-g quantities in 300 ml of spectrograde toluene (Fisher Scientific) for 24 h. The toluene extracts were then evaporated on a rotary evaporator until dry. The residue was redissolved in 0.5 ml of methylene chloride and stored in amber borosilicate glass vials with PTFE-lined septa. Nitropyrenes were prepared from pyrene and nitric acid. A 0.5-g sample of pyrene was added to 25 ml of nitric acid with gentle stirring. After 2 min, the reddish product was isolated on a fritted-glass filter and washed with excess water to remove all nitric acid. The solid mixture of nitropyrenes was dried at room temperature and stored in amber glass vials.

HPLC apparatus

The chromatographic apparatus consisted of a Model 6000A solvent pump, Model U6K injector and a data module as a recorder, all manufactured by Waters Assoc. A DuPont Zorbax-ODS (25 cm × 4.6 mm) reversed-phase column was used for all separations. A Kratos Model FS 970 fluorescence detector and an Altex Model 151 UV detector were used for fluorescence and absorbance detection, respectively. All chromatography was carried out using a mobile phase of acetonitrile–Tris hydrochloride buffer (pH 6.5) (78:22, v/v).

Nitro-PAH reduction

To examine the ability of a variety of buffers to promote nitro-PAH reduction, a simple test was performed. A solution of 1-nitroanthracene (50 mg/l) in acetonitrile was mixed with the test buffer (4:1) in a clear 25-ml vial. Excess zinc dust (500 mg) was added and the solution was mixed by shaking for 1 min. The vial was then examined under a Model R52 UV lamp (Ultra-Violet Products) for fluorescence due to the formation of fluorescent aminoanthracene. Observations were made immediately after mixing, after 5 min, and after 10 min.

Nitro-PAHs were reduced on-line, in a short (3.5 cm × 3.2 mm) stainless-steel column, packed with fine-particle zinc and glass beads. Residence time in the reactor was approximately 7 sec. The reduction column was placed either before or after the analytical column, allowing the reduced nitro-PAHs to be retained as either amino-PAHs or nitro-PAHs. The on-line reduction was initially followed using pre-column reduction and UV absorbance detection. The percent reduction was determined by monitoring the appearance of the amino-PAH peak or the disappearance of the nitro-PAH peak.

The reduction column was packed by gravity with the assistance of a mechanical vibrator. Typically, after a day of use the column required repacking, as indicated by an increase in band broadening resulting from changes in the zinc located at the head of the column. The column was repacked by removing only the first centimeter of packing and refilling the column inlet.

Chemiluminescence detection

The HPLC apparatus with chemiluminescence detection and pre-column reduction is illustrated in Fig. 1. A Kratos URS 051 dual-reagent, post-column mixing device was used to pump the TCPO and H_2O_2 solutions. These reagents were combined in a low-dead-volume mixing cell and delivered to the Kratos FS 970 fluorescence detector with 25 cm of 0.25-mm I.D. stainless-steel tubing. The concentrations of TCPO and H_2O_2 were 13.4 m*M* and 1.15 *M*, and their respective flow-rates were 0.6 and 1.2 ml/min. The detector was operated with its light source off and without emission cut-off filter. Tris(hydroxymethyl)amino methane hydrochloride buffer (pH

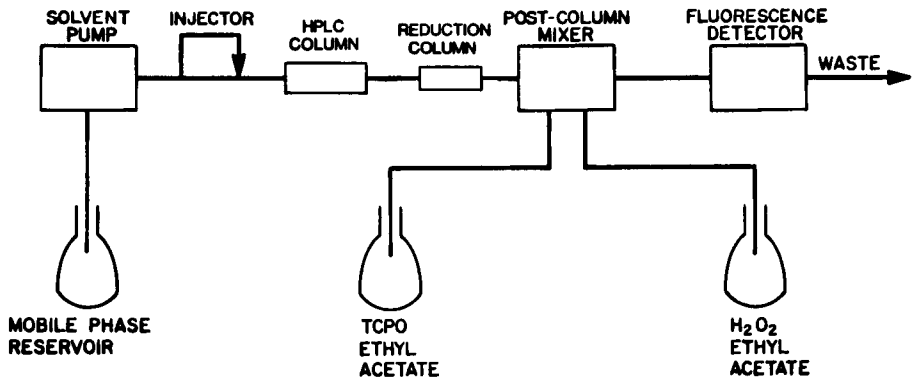


Fig. 1. Schematic diagram of the HPLC apparatus with post-column reduction and chemiluminescence detection.

TABLE I

BUFFER TESTS FOR 1-NITROANTHRACENE REDUCTION

Buffer composition	*Time after mixing (min)**		
	0	*5*	*10*
Tris nitrate, pH 7, 100 m*M*	–	–	–
Tris nitrate, pH 5, 100 m*M*	–	–	+
Tris hydrochloride, pH 7, 100 m*M*	+	+	+
Tris nitrate, pH 7, 100 m*M*; 10 m*M* NaCl	+	+	+
Tris acetate, pH 7, 100 m*M*	–	+	+
Tris hydrochloride, pH 7, 10 m*M*	+	+	+
Distilled water, 100 m*M* NaCl	–	+	+

* + Indicates fluorescence detected; – denotes the absence of fluorescence.

6.5) was used with HPLC grade acetonitrile as the chromatographic solvent. This buffer base catalyzes the peroxyoxalate chemiluminescent reaction and allows the reduction of nitro-PAHs to occur at neutral pH values. The system was flushed each day after use with an acetonitrile–water mixture to avoid chloride corrosion problems.

Gas chromatography–mass spectrometry

Carbon black extracts were analyzed by fused-silica capillary GC–MS. The GC–MS system consisted of a Hewlett-Packard 5790 gas chromatograph, interfaced directly with a Hewlett-Packard 5982 mass spectrometer. Splitless injections were made with 2-μl sample volumes and a 45-sec valve time. The chromatographic column was a Hewlett-Packard ultra-performance fused-silica capillary column, coated with crosslinked methyl silicone (5% phenyl). The column was 25 m × 0.32 mm I.D. and had a film thickness of 0.52 μm. The gas chromatograph was programmed from 50° to 350°C, with a ramp rate of 4°C/min and an initial hold time of 1 min. The mass spectrometer scanned the mass range 50–450 a.m.u.

TABLE II

REDUCTION OF 1-NITROANTHRACENE WITH VARYING TRIS BUFFER CONCENTRATION

Aqueous Tris buffer concentration (mM)	*Reduction (%)*
0	0.0
5	6.0
10	8.1
20	13
25	23
30	32
35	80
40	>99.9

RESULTS AND DISCUSSION

Nitro-PAH reduction

In order to reduce nitro-PAHs at near neutral pH values, a buffer must be employed containing ions that will promote both the reduction and the chemiluminescence reaction. Since Tris buffer catalyzes the chemiluminescence reaction and does not promote microbial growth, it was selected as the buffer for reduction testing. The effects of several Tris buffers having different anions on nitroanthracene reduction in 75% acetonitrile are shown in Table I. These results indicate that nitroanthracene can be rapidly reduced if the pH is maintained at 7 or below and chloride ion is present. Therefore, Tris hydrochloride was selected as the buffer for HPLC with combined nitro-reduction and chemiluminescence detection.

The on-line reduction of nitroanthracene in HPLC was examined for a variety of Tris hydrochloride (pH 6.5) concentrations with a 78% acetonitrile mobile phase. The results of this experiment are summarized in Table II. Percent reduction values were computed by measuring the decrease in the area of the nitroanthracene peak. A peak due to aminoanthracene was observed in chromatograms where significant reduction occurred. As the concentration of buffer used (in the 78% acetonitrile mobile phase) increases from 0 to 40 m*M*, the percent reduction increases from 0 to 100%.

After about 8–10 h of operation the zinc column began to cause significant band broadening. This was caused by a decrease in particle size and the creation of voids at the head of the zinc column. At pH 6.5 zinc would be expected to react with protons liberated from the dissociation of water according to the reaction

$$2\,H^+ + Zn^0 \rightarrow H_2 + Zn^{2+} \qquad (2)$$

The length of time in which the band broadening remains negligible can be extended by packing the reduction column with a 1:1 mixture of 40-mm glass beads and zinc. This decreases the severity of particle clumping, observed in reduction columns that have been used for long periods of time. Apparently, the size of the voids produced at the head of the column remains small due to the dispersion of the zinc particles by the glass beads.

The reduction of four nitro-PAHs was examined using a reduction column

TABLE III

REDUCTION OF FOUR NITRO-PAHs OBTAINED WITH VARIOUS TRIS BUFFER CONCENTRATIONS

Compound	*Reduction (%)*				
	Aqueous Tris buffer concentration (mM)				
	0	*10*	*20*	*30*	*40*
9-Nitroanthracene	0.0	73	91	99	>99.9
6-Nitrochrysene	0.0	59	80	96	>99.9
3-Nitroperylene	0.0	82	>99.9	>99.9	>99.9
1-Nitropyrene	0.0	73	94	98	>99.9

packed with both glass beads and zinc. The pH of the buffer was again 6.5 and its volume fraction (before mixing) was 0.22 (78% acetonitrile). The results for this new reduction column were very similar to that achieved with the pure zinc column and are summarized in Table III. The reduction became quantitative for buffer concentrations $\geqslant 40$ m*M*. The major difference between the glass beads–zinc column and the column containing zinc only occurred at low buffer concentrations. The degree of reduction of 9-nitroanthracene was significantly higher with the glass beads–zinc column at lower buffer concentrations. For all remaining HPLC work, the mobile phase consisted of acetonitrile–50 m*M* Tris hydrochloride, pH 6.5 (78:22, v/v). This mobile phase contains sufficient buffer to reduce nitro-PAHs quantitatively and to enhance the chemiluminescent reaction by base catalysis. The acetonitrile concentration chosen previously was found to yield desirable retention behavior for a variety of nitro-PAHs.

HPLC–chemiluminescence detection

The HPLC of nitro-PAHs with chemiluminescence detection was performed with both pre-column and post-column reduction. Chromatograms of a mixture of six nitro-PAH standards are shown in Fig. 2. When the pre-column reduction was employed, the standards were eluted as amino-PAHs with short retention times identical to those obtained by direct injection of the corresponding amino-PAHs. In contrast, the chromatogram with post-column reduction shows the longer retention times characteristic of the nitro-PAHs. The photomultiplier tube potential was reduced slightly for the pre-column reduction chromatogram to compensate for the increased peak heights, due to the sharper peaks obtained. Detection limits for these six nitro-PAHs were obtained with combined post-column reduction and chemiluminescence detection. These values are shown in Table IV and are calculated for

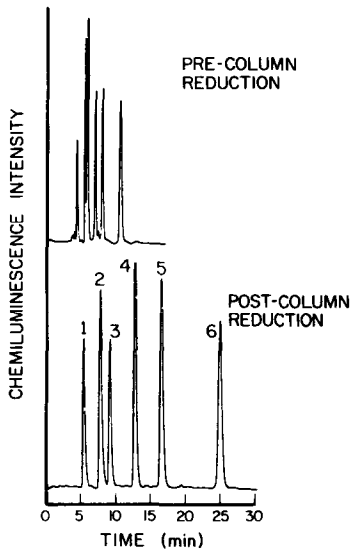


Fig. 2. HPLC chromatograms of a synthetic mixture of six nitro-PAHs with chemiluminescence detection. 1 = 1-Nitronaphthalene; 2 = 2-nitrofluorene; 3 = 9-nitroanthracene; 4 = 1-nitropyrene; 5 = 6-nitrochrysene; 6 = 3-nitroperylene; 50–500 pg each.

TABLE IV

CHEMILUMINESCENCE DETECTION LIMITS FOR NITRO-PAHs OBTAINED WITH HPLC AND POST-COLUMN REDUCTION

Compound	*Retention volume (ml)*	*Detection limit (pg)*
9-Nitroanthracene	8.6	0.40
6-Nitrochrysene	15.6	0.65
2-Nitrofluorene	7.4	8.5
1-Nitronaphthalene	5.2	3.6
3-Nitroperylene	23.3	0.60
1-Nitropyrene	12.1	0.25

peaks with heights equal to twice the peak-to-peak noise. The detection limits are significantly lower when pre-column reduction is used, since the amino-PAHs are eluted with shorter retention times and less band broadening. Detection limits for amino-PAHs in chromatography optimized for their separation were reported earlier[17]. Detection limits for amino-PAHs in chromatography optimized for their separation were reported earlier[17]. Detection limits ranged from 0.09 pg (90 fg) for 3-aminofluoranthene to 8.0 pg for 2-aminofluorene. Using pre-column reduction, the nitro-PAHs can be detected with similar sensitivities.

HPLC with chemiluminescence detection was applied to the detection of nitro-PAHs in carbon black extracts. Fig. 3 shows three chromatograms obtained for the CB-1 extract by pre-column reduction, post-column reduction and no reduction.

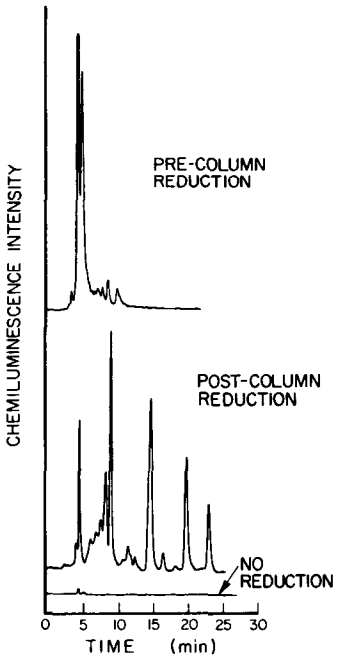
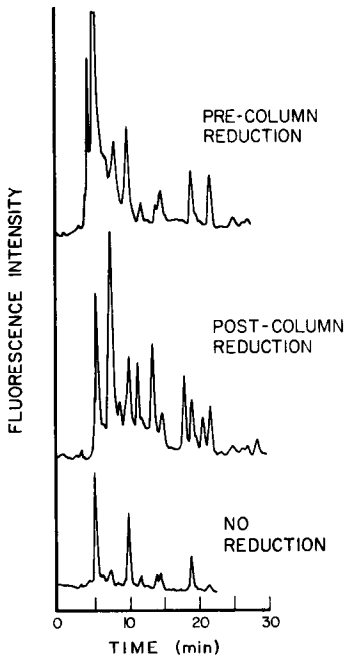


Fig. 3. HPLC chromatograms of the carbon black extract CB-1 with chemiluminescence detection.



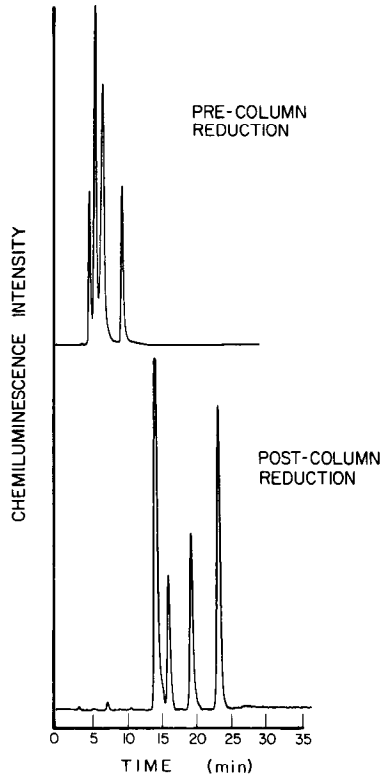
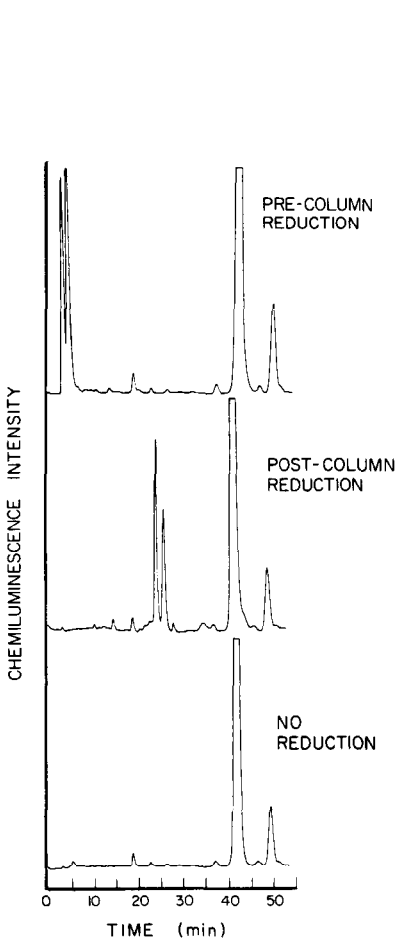


Fig. 4. HPLC chromatograms of the carbon black extract CB-1 with fluorescence detection.

Fig. 5. HPLC chromatogram of a nitrated pyrene sample with chemiluminescence detection.

The detector sensitivity was reduced ten-fold for the pre-column reduction, because the early-eluted peaks had significantly larger peak heights. The chromatogram obtained without reduction shows a negligible response towards the extract. At least ten major peaks were observed when post-column reduction was used. The qualitative value of using pre-column reduction is clearly demonstrated by the shifted retention volumes resulting from changing the position of the reduction column. The pre-column reduction chromatogram shows two major peaks with retention volumes less than aminonaphthalene. This retention behavior is characteristic of PAHs containing more than one amino group. The carbon black extract appears to contain nitro-PAHs with approximately two to four rings, most of these compounds containing more than one nitro-group. This result agrees with that of Rosenkranz *et al.*[13], who found a variety of di- and tri-substituted nitro-PAHs in carbon blacks subjected to a nitric acid oxidation. A set of three chromatograms obtained from the same sample with fluorescence detection is shown in Fig. 4. The excitation wavelength was set at 290 nm, which is nearly optimal for most amino-PAHs with the Kratos fluorescence detector. The main difference between fluorescence and chemiluminescence detection is the selectivity. The fluorescence chromatogram without reduction shows a variety of peaks that are due to normally fluorescent aromatic compounds. These extra peaks were observed in all chromatograms with fluorescence



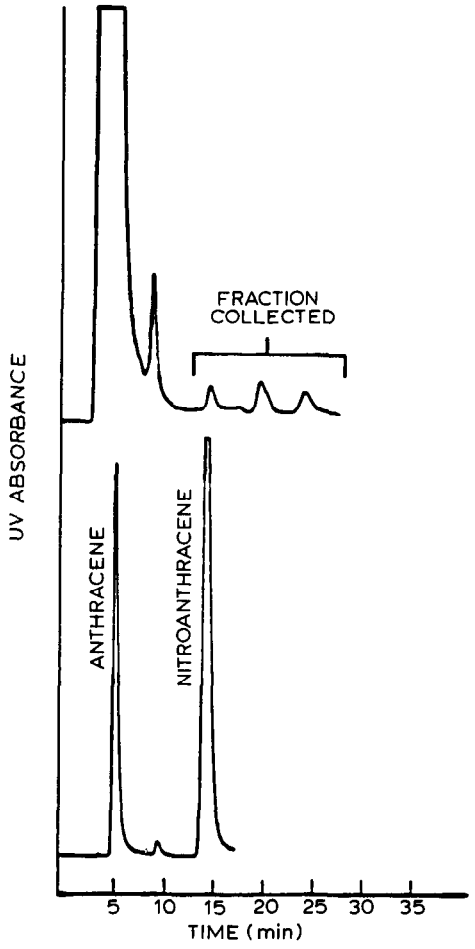


Fig. 6. HPLC chromatograms of the carbon black extract CB-2 with chemiluminescence detection.

Fig. 7. Preparative LC chromatograms of a synthetic mixture and of the carbon black extract CB-2.

detection and not in any of the chromatograms with chemiluminescence detection.

Chromatograms of a nitrated pyrene mixture, with chemiluminescence detection, are shown in Fig. 5. The large decreases in pre-column reduction retention volumes due to multi-substituted nitro-PAHs were again observed. The retention behavior of compounds in this mixture was very similar to that of compounds present in the carbon black sample CB-1. In general, adding a second nitro group to a nitro-PAH results in an increase in retention volume with post-column reduction and a decrease in retention volume with pre-column reduction.

A second carbon black extract, CB-2, was prepared in the same manner as the CB-1 extract. This carbon black sample was prepared by a process known to result in a significant reduction in nitro-PAHs. The three chemiluminescence chromatograms of this extract are shown in Fig. 6. The majority of peaks observed were due to fluorescent aromatic compounds of unknown structure. The pre- and post-column

reduction chromatograms showed evidence of two nitro-PAHs in very low concentrations.

Gas chromatography–mass spectrometry

The carbon black extract CB-1 was examined by GC–MS. The prepared extract was injected directly without any other sample pre-treatment. The major compounds identified were alkyl- and oxygen-substituted PAHs.

The CB-1 extract was found previously to contain much larger amounts of nitro-PAHs by HPLC. It was obvious that without prior enrichment, GC–MS methods would not be able to detect any nitro-PAHs in the CB-2 extract. Normal-phase preparative-scale HPLC was employed in an attempt to enrich the sample with nitro-PAHs. Fig. 7 shows two chromatograms from the preparative separation. The bottom chromatogram is that of anthracene and nitroanthracene standards. These compounds were used to mark the retention volumes in which nitro-PAHs will be eluted. Nitroanthracene is eluted more than 20 ml after anthracene, and multi-substituted nitro-PAHs will be eluted after nitroanthracene. The top chromatogram is that of the CB-2 extract, with the collected fraction labeled. These fractions from several separate injections were collected, concentrated, and examined by GC–MS. The total ion current chromatogram of this fraction is shown in Fig. 8. The major compounds identified consisted of polar aromatic compounds and some phthalate esters, presumed to have been introduced during sample preparation. Attempts to detect nitro-PAHs in this fraction using selected-ion monitoring of the molecular ions of two- and three-ring compounds also failed. Very sensitive detection of nitro-PAHs using negative chemical ionization in combination with single ion monitoring has been reported[19], but this technique was not available to us.

It appears that the selectivity and sensitivity of HPLC with chemiluminescence detection is very useful in the analysis of complex mixtures of nitro-PAHs. Most complex samples containing nitro-PAHs are rich in both PAHs and polar PAH derivatives. These interfering compounds respond poorly to chemiluminescence detection, resulting in high selectivity for nitro-PAHs. For the nitro-PAHs, the sensitivity

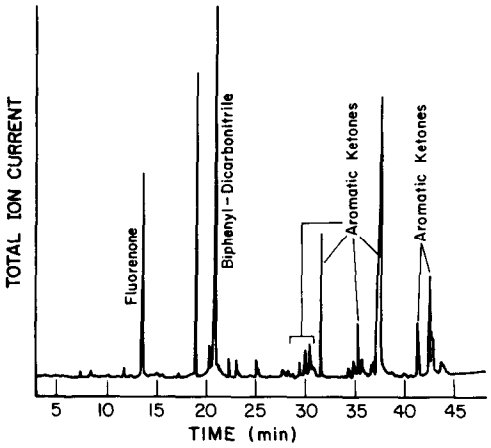


Fig. 8. GC total ion current chromatogram of the fractionated carbon black extract CB-2.

also appears to be very high compared to existing chromatographic detectors used in HPLC and GC. The principal drawback is the lower column efficiencies available for HPLC as compared to capillary GC.

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