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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH REDUCTIVE ELECTROCHEMICAL DETECTION OF MUTAGENIC NITRO-SUBSTITUTED POLYNUCLEAR AROMATIC HYDROCARBONS IN DIESEL EXHAUSTS

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SUMMARY

A method is described for the measurement of nitro-substituted polynuclear aromatic hydrocarbons (nitro-PAHs) which employs high-performance liquid chromatography with reductive electrochemical detection. A series of reference nitro-PAHs has been separated with a reversed-phase column and quantified over a linear range of 10^3 with a sensitivity of 10–100 pg per compound. Extracts of several samples of diesel-exhaust particulates contained numerous compounds which were reduced at an electrode potential of -0.6 V (*vs.* Ag–AgCl). By comparing retention times and hydrodynamic voltammograms of these peaks with those of reference nitro-PAHs it was possible to confirm the presence of 1-nitropyrene at levels of between 5 and 44 ng/mg in 5 of 6 diesel extracts and to differentiate peaks representing nitro-PAHs from those representing other reducible species (*e.g.*, aldehydes, ketones and quinones).

INTRODUCTION

Recent investigations of emissions from diesel engines have focussed on mutagenic and carcinogenic nitro-substituted polynuclear aromatic hydrocarbons (nitro-PAHs)[1–6]. Of the nitro-PAHs which have been tentatively identified in extracts of diesel-exhaust particulates, several are potent mutagens in Ames' *Salmonella* bioassay[7], a short-term test designed to detect chemicals which may be potential carcinogens. These compounds include 1-nitropyrene (1-NP)[1,4,5], 2,7-dinitrofluorene (2,7-DNF)[1], and 2- and 3-nitro-9-fluorene (2- and 3-NFO)[1]. Other mutagenic nitro-PAHs in diesel exhausts, including 2-nitrofluorene (2-NF)[1,2] and 4-nitrobiphenyl (4-NB)[1], are known to be carcinogenic in laboratory mammals[8]. Numerous other nitro-PAHs whose presence has been indicated in diesel exhausts[1,4] have not been tested

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for mutagenicity or carcinogenicity because of the unavailability of reference compounds.

The quantification of nitro-PAHs in diesel exhausts has been hampered by the small amounts of individual compounds present (pg–ng per mg of extract) and by the extreme complexity of the extracts which contain a myriad of potentially interfering aromatic species including polynuclear aromatic hydrocarbons (PAHs), nitrogen- and sulfur-containing heterocycles and oxidation products of PAHs[5,9]. Thus, methods are required which are not only extremely sensitive but which also provide great selectivity or resolution of individual nitro-PAHs from interfering compounds. Scheutzle *et al.*[4] used tandem mass spectrometry to quantify the prototypical and abundant nitro-PAH 1-NP in extracts of particulates from five diesel engines. Unfortunately, such instrumentation is generally unavailable to most researchers.

We report here the application of high-performance liquid chromatography (HPLC) with reductive electrochemical detection (RED) to measure nitro-PAHs at pg–ng levels and to quantify 1-NP in extracts of diesel exhaust particulates. The use of RED has been proposed by Kissinger *et al.*[10] for several classes of reducible organic compounds, including nitroarenes, which can be conveniently separated by reversed-phase HPLC. This application also illustrates the use of hydrodynamic voltammetry, as proposed by Kissinger *et al.*[10], to provide additional information concerning the identities of compounds whose retention characteristics match those of reference nitro-PAHs.

EXPERIMENTAL

Chemicals and standards

All solvents were distilled in glass (Burdick and Jackson or J. T. Baker). High purity monochloroacetic acid (99.7%) and sodium acetate (99.3%), used for the buffer solution, were obtained from J. T. Baker. Water was purified with a Millipore Q System. Analytical grade 1-nitronaphthalene (1-NN), 2-nitronaphthalene (2-NN), 2-nitrobiphenyl (2-NB), 3-nitrobiphenyl (3-NB), 4-nitrobiphenyl (4-NB), 2-nitrofluorene (2-NF), 9-nitroanthracene (9-NA), 3-nitro-9-fluorenone (3-NFO), 2,7-dinitrofluorene (2,7-DNF), 2-acetamido-3-nitro-9-fluorenone (2-ANFO), 4-nitrofluoranthene (4-NFA), 2,7-dinitro-9-fluorenone (2,7-DNFO), 7-nitrofluorene-1-carboxylic acid (7-NFCA), 1,4-naphthoquinone, anthraquinone, 2-naphthaldehyde, 9-anthraldehyde and 1-pyrenecarboxaldehyde were obtained from Aldrich. 1-Nitropyrene (1-NP) was obtained from Pfaltz and Bauer, 1,3-Dinitropyrene (1,3-DNP) and 1,3,6-trinitropyrene (1,3,6-TNP) were generously supplied by R. Mermelstein of Xerox Corp., Rochester, NY, U.S.A. Pyrene-3,4-dicarboxylic acid anhydride was synthesized as described elsewhere[11]. All reference standards were used without purification.

Collection and extraction of diesel-exhaust particulates

Two types of particulate samples were used in this study. Four of the six samples were obtained from Dr. Thomas Baines of the U.S. Environmental Protection Agency in Ann Arbor, MI, U.S.A. These samples consisted of pleated glass-fiber filters (Dustfoe Type, 13 m^2 collection area per filter, Mine Safety Appliance, Pittsburgh, PA, U.S.A.) containing diesel particulates collected at ambient temperature

from a dilution tunnel where diesel truck engines were tested. Sample No. 1 was collected from a 1979 International Harvester engine, Sample No. 2 from a 1979 Caterpiller engine and Samples No. 3 and 4 from a 1980 Mack engine. All engines were of the 4-stroke, 6-cylinder type with turbochargers and aftercoolers typical of long distance trucks and were designed to meet California emission standards. An electric dynamometer provided the mechanical loading of the engines. The protocol used in the generation and collection of the samples has been described elsewhere[3]. Single sections of each multi-pleated filter were removed from the filter frame for extraction.

The remaining two samples were obtained from Mr. Frank Robben of the Lawrence Berkeley Laboratory of the University of California, Berkeley, CA, U.S.A. These samples consisted of 20.3 × 25.4-cm PTFE-coated glass-fiber filters (Pallflex Products Corp., Putnam, CT, U.S.A.) containing diesel particulates from a medium speed diesel engine manufactured by the Engine and Compressor Division of Transamerica Delaval, Oakland, CA, U.S.A. Designated a DSR-46, this 4-stroke, 6-cylinder engine, with a shaft power of 2700 kW, is a turbocharged and intercooled model of the type used commercially for generating power. Samples were collected by drawing a portion of the diluted exhaust through an in-line filter holder at temperatures of $< 50°C$. Sample No. 5 was obtained when the engine was operated on diesel fuel No. 2. Sample No. 6 was obtained when 5 of the 6 cylinders were operated on diesel fuel No. 2 and the sixth cylinder was operated on a solvent refined coal middle distillate fuel (SRC-II).

Each filter was placed on a Sohxlet extraction apparatus and extracted for 24 h with 300 ml of dichloromethane using a cycling time of about 45 min. Extracts were filtered through PTFE membrane filters of 0.45 μm pore size (Millipore Type FH) and were reduced in volume to a few ml by rotary evaporation. Concentrated extracts were transferred to tared vials with dichloromethane rinsings, dried under nitrogen and weighed. Extracts were stored at $-4°C$ prior to analysis and manipulations were performed under shaded lighting to reduce the possibility of photooxidation.

Preparation of samples

Approximately 20 mg of each extract of diesel-exhaust particulates were dissolved in a minimum volume of dichloromethane (≈ 200 μl) and applied to a small silica gel cartridge (Sep-Pak, Waters Assoc.) with minimal rinsings of dichloromethane. The cartridge was eluted in serial order with 3 ml of hexane, 6 ml of dichloromethane and 3 ml of methanol. Each eluate was collected separately. Eluates were dried under nitrogen at 40°C, weighed in tared vials, dissolved in 200 μl of dichloromethane–*n*-propanol (1:1), and diluted to the desired concentration (0.5–2 mg/ml) with the HPLC mobile phase.

High-performance liquid chromatography

Samples were purged of dissolved oxygen with nitrogen (presaturated with the mobile phase) for about 10 min and aliquots were introduced into the 20-μl loop of a Rheodyne injection valve. The HPLC system consisted of a Beckman/Altex 100A pump, a 25 cm × 4 mm I.D. Beckman Ultrasphere ODS column (5-μm spherical particles) with a short precolumn containing C_{18} Corasil (Waters) both maintained at 50°C in a column oven, and a Bioanalytical Systems electrochemical detector com-

prised of a LC-4A amperometric controller and TL-5 thin-layer flow cell with a glassy carbon working electrode. The electrode was operated at a potential of between −0.1 and −0.8 V *vs.* a Ag–AgCl reference electrode. All PTFE lines in the system were replaced with stainless steel to prevent permeation of oxygen into the mobile phase[10]. The mobile phase, 35% *n*-propanol in 0.05 *M* monochloroacetic acid–sodium acetate buffer at pH 3.8, was continuously heated to 50°C under nitrogen in a flask fitted with a reflux condenser to remove dissolved oxygen as recommended by the manufacturer of the detector (Bioanalytical Systems) for reductive work. The column was purged with the mobile phase at 0.1 ml/min overnight prior to analysis to remove dissolved oxygen. During analysis the flow-rate of the mobile phase was 1.0 ml/min. Some peak areas and retention times were determined manually while others were determined with a Varian Vista 401 data system.

The HPLC system required periodic maintenance to insure optimal performance. After a full day of analysis of diesel-exhaust samples, the system was washed with methanol to remove nonpolar residues. The glassy carbon electrode, when used in the reductive mode, is sensitive to passivation by heavy-metal contamination of the mobile phase. Using the high-purity salts listed, the background current was small and the working electrode required repolishing at roughly 1-week intervals. Initial work, performed with salts of reagent grade obtained from Aldrich, showed substantially higher background current and repolishing of the electrode was required more frequently.

RESULTS AND DISCUSSION

RED of reference nitro-PAHs

Fig. 1 shows a chromatogram of a mixture containing 10 ng each of 16 reference compounds. Although some components in the mixture were not completely resolved, this chromatogram illustrates that most nitro-PAHs containing between 2 and 4 rings, which are the most prevalent such species in diesel-exhaust extracts[1,4], are eluted within about 25 min of injection. Calibration curves of several reference compounds shown in Fig. 2 indicate that the linear range of the RED should typically be about 10^3 and that sensitivity should extend to 10–100 pg for most nitro-PAHs. The detector currents compiled in Fig. 2 were obtained by measuring peaks at −0.6 V, an electrode potential which, as will be shown, is at or above the "plateau" potential of many nitro-PAHs.

RED of diesel extracts

Diesel extracts had been prefractionated prior to HPLC by applying them to silica gel cartridges which were sequentially eluted with hexane, dichloromethane and methanol (see Experimental). Chromatograms of hexane fractions indicated the absence of species reducible at −0.6 V. However, chromatograms of both dichloromethane and methanol fractions contained numerous peaks at −0.6 V indicating the possible presence of nitro-PAHs. Compounds in dichloromethane fractions were eluted predominantly after oxygen (retention time = 6.4 min) in the reversed-phase HPLC system whereas compounds in methanol fractions eluted before oxygen. Because reference standards of nitro-PAHs and nitrosubstituted ketones of PAH eluted primarily after oxygen (Peak No. 2 in Fig. 1) priority was given to the investigation of

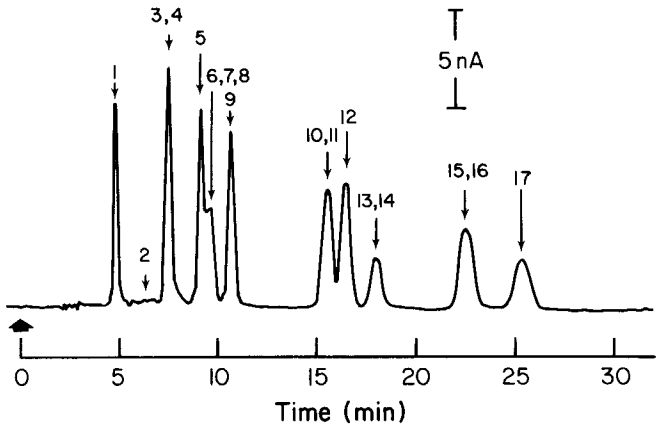

Fig. 1. Chromatogram of 16 reference nitro-PAHs. Chromatographic conditions: Column, 25 cm × 4 mm I.D. Ultrasphere ODS (5 μm spherical particles); Mobile phase, 35% *n*-propanol in 0.05 *M* monochloracetic acid–sodium acetate buffer at pH 3.8, 1.0 ml/min; electrode potential, −0.6 V *vs.* Ag–AgCl reference electrode. Identities of compounds present at 10 ng each: 1 = 2-ANFO; 2 = oxygen; 3 = 7-NFCA; 4 = 2,7-DNFO; 5 = 3-NFO; 6 = 2,7-DNF; 7 = 1-NN; 8 = 2-NB; 9 = 2-NN; 10 = 3-NB; 11 = 4-NB; 12 = 2-NF; 13 = 1,3,6-TNP; 14 = 9-NA; 15 = 1,3-DNP; 16 = 1-NP; 17 = 4-NFA.

dichloromethane fractions and the more polar species present in methanol fractions were not examined further in this study.

Chromatograms of dichloromethane fractions of extracts from various samples of diesel particulates were superficially similar, in that they all contained numer-

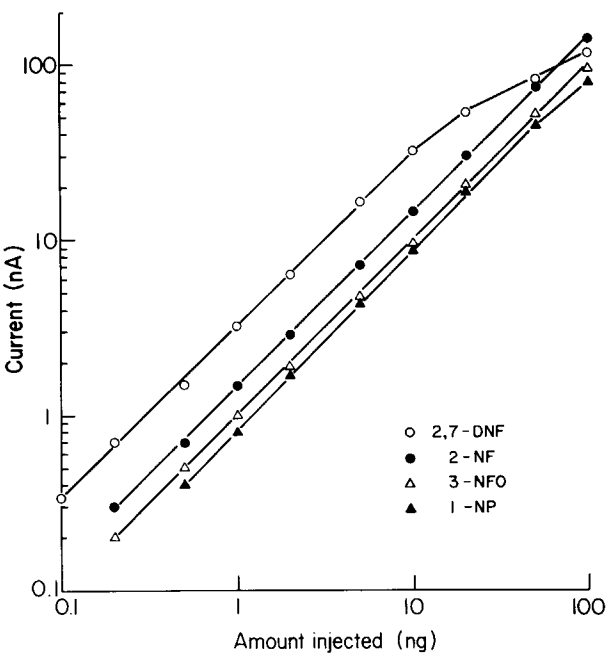

Fig. 2. Calibration curves of 4 nitro-PAHs (3-NFO, 2,7-DNF, 2-NF and 1-NP). Chromatographic conditions as in Fig. 1.

ous peaks in roughly the same range of retention times, but varied considerably in the actual retention times and areas of the peaks. Fig. 3 depicts chromatograms of the dichloromethane fraction from diesel extract No. 1 recorded at −0.6 V (top) and −0.3 V (bottom), respectively. This sample was selected for examination because 3 of the more abundant peaks in the chromatogram, labeled a, b and c, had retention times close to those of several reference nitro-PAHs which have been identified in diesel extracts. [Peak a possibly: 3-NFO, 2,7-DNF, 1-NN; peak b possibly: 2-NF; peak c possibly: 1-NP, 1,3-DNP.]

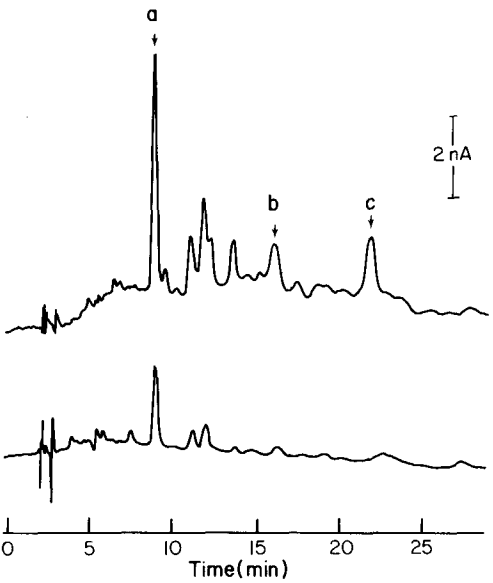

Fig. 3. Chromatograms of 10-μg portions of diesel extract No. 1. Chromatographic conditions given in Fig. 1. Top chromatogram, electrode potential = −0.6 V; bottom chromatogram, electrode potential = −0.3 V.

Hydrodynamic voltammograms

Additional information concerning the identities of peaks a, b and c was obtained by comparing the normalized hydrodynamic voltammograms[10] of these peaks with those of the suspected reference nitro-PAHs as shown in Fig. 4–6. In each case, the relative current ratio Φ, which is the ratio of the current at given potential to the diffusion-limited current, is plotted *vs.* applied potential. The only candidate nitro-PAH whose presence in the sample is entirely consistent with the volammetric data is 1-NP which produces a voltammogram that matches that of peak c within the range of experimental error (Fig. 6). The voltammograms of the other candidate nitro-PAHs are sufficiently different from those of the unknown peaks to preclude the confirmation of identity. However, the data do not eliminate the possibility that these compounds may have coeluted with additional nitro-PAHs or with other reducible species thereby confounding the interpretation of voltammograms.

Quantitation of 1-NP

Five of the six diesel extracts investigated contained peaks whose retention

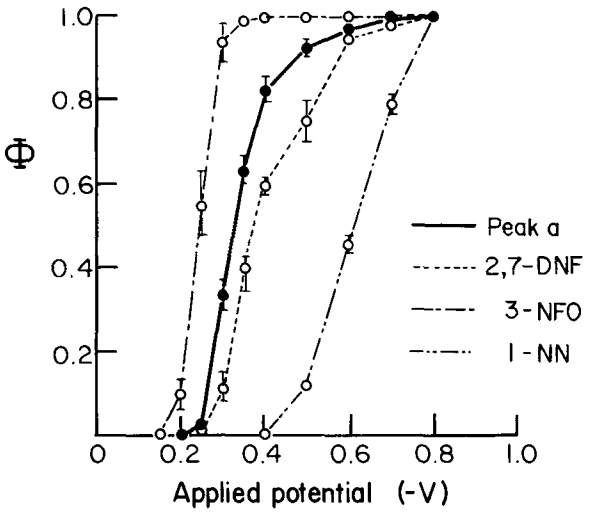

Fig. 4. Hydrodynamic voltammograms of peak a (Fig. 3) and 3 reference nitro-PAHs (2,7-DNF, 3-NFO and 1-NN) with similar retention times. (Φ is the relative current ratio; mean and range are plotted for 3 or more observations.)

times and hydrodynamic voltammograms matched those of 1-NP within the range of experimental error. To determine the concentrations of this compound whose presence is presumed in the extracts, the recovery of 1-NP was determined by spiking 10- to 20-mg portions of diesel extracts with between 0.3 and 0.5 μg of 1-NP, then applying them to silica gel cartridges and performing HPLC as described in Experimental. Based upon 6 trials the recovery was 95.0 $\pm$ 10.4% ($\bar{x} \pm$ S.D.) when corrected for equivalent control samples of the same extracts. The concentrations of 1-NP in the six diesel extracts after correcting for a recovery of 95% are given in

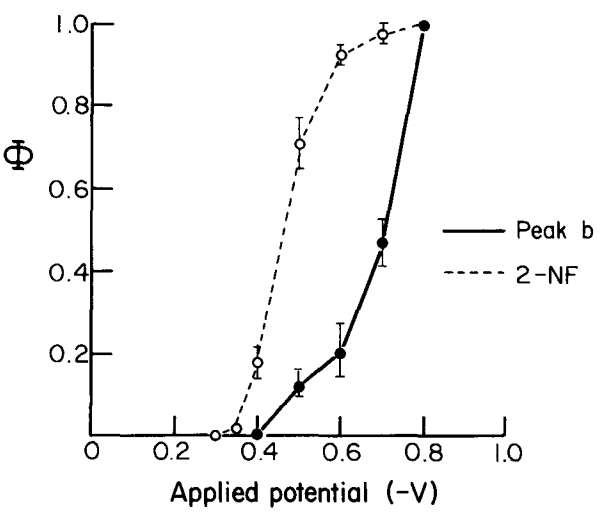

Fig. 5. Hydrodynamic voltammograms of peak b (Fig. 3) and reference 2-NF which has the same retention time. (Φ is the relative current ratio; mean and range are plotted for 3 or more observations.)

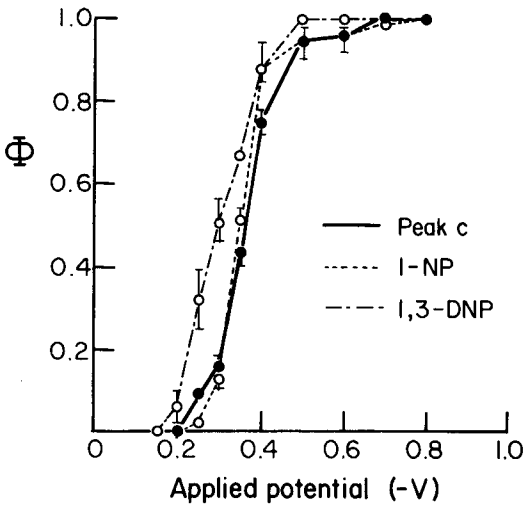

Fig. 6. Hydrodynamic voltammograms of peak c (Fig. 3) and reference 1-NP and 1,3-DNP which have similar retention times. (Φ is the relative current ratio; mean and range are plotted for 3 or more observations.)

Table I. These concentrations, determined by both standard addition and external calibration, ranged from <2 to 44 ng/mg of extract. Three runs with extract No. 1 indicated values of 32, 39 and 36 ng/mg.

Scheutzle *et al.*[4] reported that 1-NP, at levels between 55 and 2285 ng/mg, was one of the more abundant nitro-PAHs in the diesel extracts they investigated. Based upon the relative sizes of peaks of presumed nitro-PAHs in our samples, these results confirm this observation; however, the concentrations of 1-NP we measured were substantially smaller than those reported by Scheutzle *et al.*[4].

Other reducible species

The utility of RED as a tool for measuring nitro-PAHs in engine exhausts and in ambient air rests in part upon its specificity. It is important, therefore, to know whether other classes of reducible compounds might interfere in the interpretation of

TABLE I

CONCENTRATIONS OF 1-NITROPYRENE IN DIESEL EXTRACTS

A recovery of 95% from the prefractionation procedure is assumed.

Sample No.	*Concentration of 1-nitropyrene (ng/mg)*
1 (3 trials)	32, 39, 36
2	44
3	<2
4	8
5	20
6	5

chromatograms. Since the technique is based upon reversed-phase HPLC, only aromatic species with 2 or more rings and little ionic character (at pH 3.8) should be sufficiently retained by the column to elute in the range of interest (*i.e.*, after oxygen in Fig. 1). Of these compounds, the most likely interfering species are oxidation products of PAHs including ketones and quinones, aldehydes and dicarboxylic acid anhydrides[5,9,11].

Compounds representative of these classes of potentially interfering species were tested in the system at an electrode potential of -0.6 V. Of these substances, all three aldehydes (2-naphthaldehyde, 9-anthraldehyde and 1-pyrene carboxaldehyde) and pyrene-3,4-dicarboxylic acid anhydride produced no response. Yet the two quinones, 1,4-naphthoquinone and anthraquinone, were detected with sensitivities comparable to those of the nitro-PAHs. The hydrodynamic voltammograms of these quinones, shown in Fig. 7, indicate that they are more easily reduced than the nitro-PAHs tested. If these reduction potentials are indicative of those of other ketones and quinones of PAH it may be possible to differentiate these compounds from nitro-PAHs by measuring Φ values of the peaks at different electrode potentials. By these criteria, none of the larger peaks shown in Fig. 3 would be attributed to ketones or quinones.

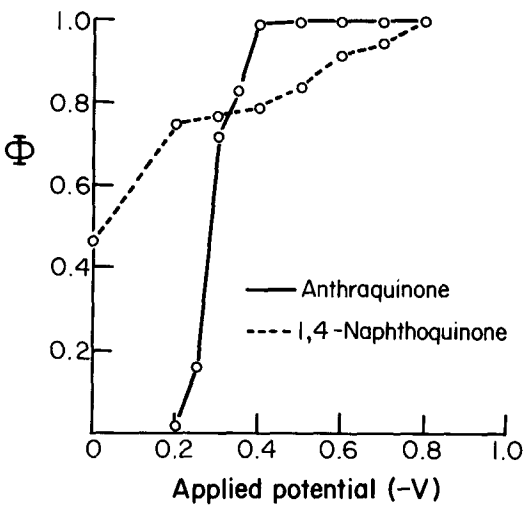

Fig. 7. Hydrodynamic voltammograms of anthraquinone and 1,4-naphthoquinone. (Φ is the relative current ratio.)

The reduction of ketones of PAH which also contain nitro groups is apparently initiated at the carbonyl oxygen (compare the voltammograms of 3-NFO, Fig. 4, and 2-NF, Fig. 5). Thus, it would probably not be possible to differentiate nitro-containing ketones from ketones and quinones which do not contain nitro groups solely on the basis of their Φ values.

CONCLUSIONS

The use of HPLC with RED offers the analyst a relatively straightforward approach to the measurement of nitro-PAHs at pg–ng levels in air samples. Our success in quantifying 1-NP in extracts of diesel-exhaust particulates illustrates the viability of the technique when applied to extremely complex mixtures. A particularly attractive feature of the method involves the use of hydrodynamic voltammetry to assist in the characterization of nitro-PAHs in samples. Preliminary results indicate that some reducible species which might interfere with the analysis (aldehydes and anhydrides) are not measured at the electrode potential used for nitro-PAHs (-0.6 V) while others (ketones and quinones) can probably be differentiated from nitro-PAHs by their hydrodynamic voltammograms.

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