CONTRACT NO. A732-154
FINAL REPORT
AUGUST 1991

# Hydroxynitro-PAH and Other PAH Derivatives in California's Atmosphere and Their Contribution to Ambient Mutagenicity

State of California
AIR RESOURCES BOARD
Research Division

# HYDROXYNITRO-PAH AND OTHER PAH DERIVATIVES IN CALIFORNIA'S ATMOSPHERE AND THEIR CONTRIBUTION TO AMBIENT MUTAGENICITY

**Final Report**
**Contract No. A732-154**

Prepared for:

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California Air Resources Board
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**August 1991**

ABSTRACT

Extracts of collected ambient air particulate matter exhibit strong direct-acting mutagenicity in the Ames Salmonella typhimurium bacterial assay. Previous investigations have shown that the nitroarenes present in ambient air in the particle phase, the majority of which are formed in the atmosphere from the gas-phase OH radical and, to a lesser extent, $NO_3$ radical-initiated reactions of the gas-phase 4-ring PAH, contribute <10% of this direct-acting mutagenicity. During this experimental program aimed at identifying the chemical compounds, or class of chemical compounds, responsible for the majority of the ambient particle mutagenicity, we have conducted OH radical-initiated reactions of selected gas-phase PAH in the presence of oxides of nitrogen in a 6400 liter environmental chamber. Approximately 2000 liter samples of the chamber reaction contents were collected on polyurethane foam plugs, which were solvent-extracted, fractionated by high-performance liquid chromatography and subjected to mutagenicity testing using the Kado microsuspension modification of the Ames bioassay. Chemical analysis was conducted on the most mutagenic fractions. For naphthalene and fluorene, the most mutagenic fractions were those containing the nitronaphthalenes and nitrofluorenes, respectively, which accounted for the majority of the measured mutagenicities in these nitroarene fractions. For phenanthrene, the mutagenicity profile resembled that of collected ambient air particulate matter, with the majority of the mutagenicity being in a fraction more polar than the nitroarene-containing fraction. Chemical analysis showed the presence in this mutagenic fraction of two nitro-phenanthrene lactones. Through mutagenicity testing of available standard materials, it was determined that one isomer, 2-nitro-6H-dibenzo[b,d]pyran-6-one, is a highly potent, direct-acting mutagen. Combined bioassay-directed fractionation and chemical analysis of extracts of ambient air particulate matter collected in Riverside, CA, confirmed the presence of these two nitrophenanthrene lactones, and five nitro-methylphenanthrene lactones and two nitro-pyrene lactones were also tentatively identified. This class of polar chemical compound, the nitro-PAH lactones, may well contribute significantly to the observed mutagenicity of ambient air particulate matter. Further studies are needed to assess their contribution to ambient air mutagenicity and to determine the human health effects of these compounds.

ACKNOWLEDGMENTS

We express our appreciation to our Project Officer, Ralph Propper, for many helpful discussions and encouragement during this program. Dr. Barbara Zielinska is thanked for her contributions during the first year of this program involving the extraction, fractionation and attempted chemical analysis of collected ambient particles, and the synthesis and identification of several hydroxynitro-PAH. We thank Ms. Sara M. Aschmann for very able technical assistance during the environmental chamber experiments, Ms. Diane L. Skaggs for assistance in the fiscal management of this program, and Ms. Christy J. LaClaire, Ms. Lucille M. Sanchez and Ms. Barbara J. Crocker for typing and assembling this report.

This report was submitted in fulfillment of Contract No. A732-154, "Hydroxynitro-PAH and Other PAH Derivatives in California's Atmosphere and Their Contributions to Ambient Mutagenicity," by the Statewide Air Pollution Research Center, University of California, Riverside, under the sponsorship of the California Air Resources Board. Work on this program was completed as of April 19, 1991.

DISCLAIMER

The statements and conclusions in this report are those of the contractor and not necessarily those of the California Air Resources Board. The mention of commercial products, their source, or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products.

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# I. PROJECT SUMMARY AND CONCLUSIONS

It has been known for many years that extracts of collected ambient air particles are mutagenic in the absence of microsomal activation and are also carcinogenic (Leiter et al., 1942; Pitts et al., 1977, 1982; Holmberg and Ahlorg, 1983 and references therein). This direct-acting (i.e., in the absence of microsomal activation) mutagenicity is not due to polycyclic aromatic hydrocarbons (PAH), since they require microsomal activation for the expression of mutagenic activity. More recently, it has been shown that the nitro-PAH, most of which are formed from the gas-phase reactions of the parent PAH in the atmosphere, contribute ≤10% of the measured particle-phase mutagenicity. Two pieces of evidence suggest that the remaining mutagenicity of extracts of collected ambient air particles may be due to the products of the gas-phase atmospheric reactions of PAH: (a) ambient particle-phase mutagenicity data from seven sites in California correlated well with the ambient particle-phase concentrations of 2-nitropyrene, which is formed in the atmosphere from the OH radical-initiated reaction of pyrene in small (<1%) yield, and (b) the majority of the direct-acting mutagenicity of extracts of collected ambient air particulate matter resides in fractions which are significantly more polar than observed for direct combustion emissions (for example, diesel exhaust), indicating transformations in the atmosphere during transport from source to receptor.

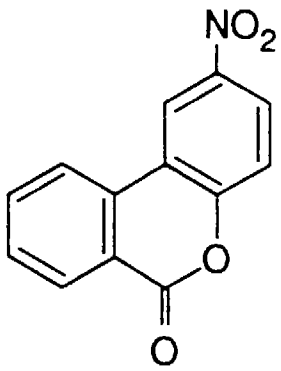
After initial efforts to identify the polar mutagenic compounds (or class of compounds) in ambient air particles by bioassay-directed fractionation of particle extracts were not successful, we redirected this experimental program to conduct bioassay-directed fractionation and chemical analysis of the products collected from the atmospherically-important reactions of selected gaseous PAH carried out in our 6400 liter all-Teflon environmental chamber. Specifically, hydroxyl (OH) radical-initiated reactions of the PAH naphthalene, fluorene and phenanthrene were carried out in the chamber, using the photolysis of methyl nitrite ($CH_3ONO$) to generate OH radicals at concentrations over an order of magnitude higher than present in the ambient atmosphere. Irradiations were carried out for ~10 mins, and ~2000 liters of chamber volume was collected on polyurethane foam plugs for solvent extraction and

fractionation by high performance liquid chromatography (HPLC). Mutagenicity testing of these HPLC fractions was carried out using the Kado microsuspension modification of the standard Ames Salmonella typhimurium microbial assay, and representative "mutagrams" [plots of mutagenic activity versus HPLC fraction number (fraction polarity)] are shown in Figure I-1 for naphthalene, fluorene and phenanthrene and for an extract of ambient air particulate matter collected in the Los Angeles air basin.

Chemical analyses were conducted on the most mutagenic fractions. For naphthalene and fluorene, the most mutagenic fractions (Fraction #4 in each case) were those containing the nitronaphthalenes and nitrofluorenes, respectively, which accounted for the majority of the measured mutagenicities in these nitroarene fractions. It is noteworthy that although 1- and 2-nitronaphthalene are only very weak mutagens in the standard Ames assay, both compounds showed significant activity when tested with the microsuspension modification. Since the nitronaphthalenes are the most abundant of the nitro-PAH in ambient air, this finding underscores the potential importance of volatile ambient mutagens and of gas-phase mutagens produced through atmospheric reactions.

As may be seen from Figure I-1, the mutagenicity profile of the phenanthrene reaction products resembled that of collected ambient air particulate matter, with the majority of the mutagenicity being in Fraction #6, a fraction more polar than the nitroarene-containing fraction. Chemical analysis showed the presence in this mutagenic fraction of two nitro-phenanthrene lactones. Through mutagenicity testing of available standard materials, it was determined that one isomer, 2-nitro-6H-dibenzo[b,d]pyran-6-one (I), is a highly potent, direct-acting mutagen.

(I)



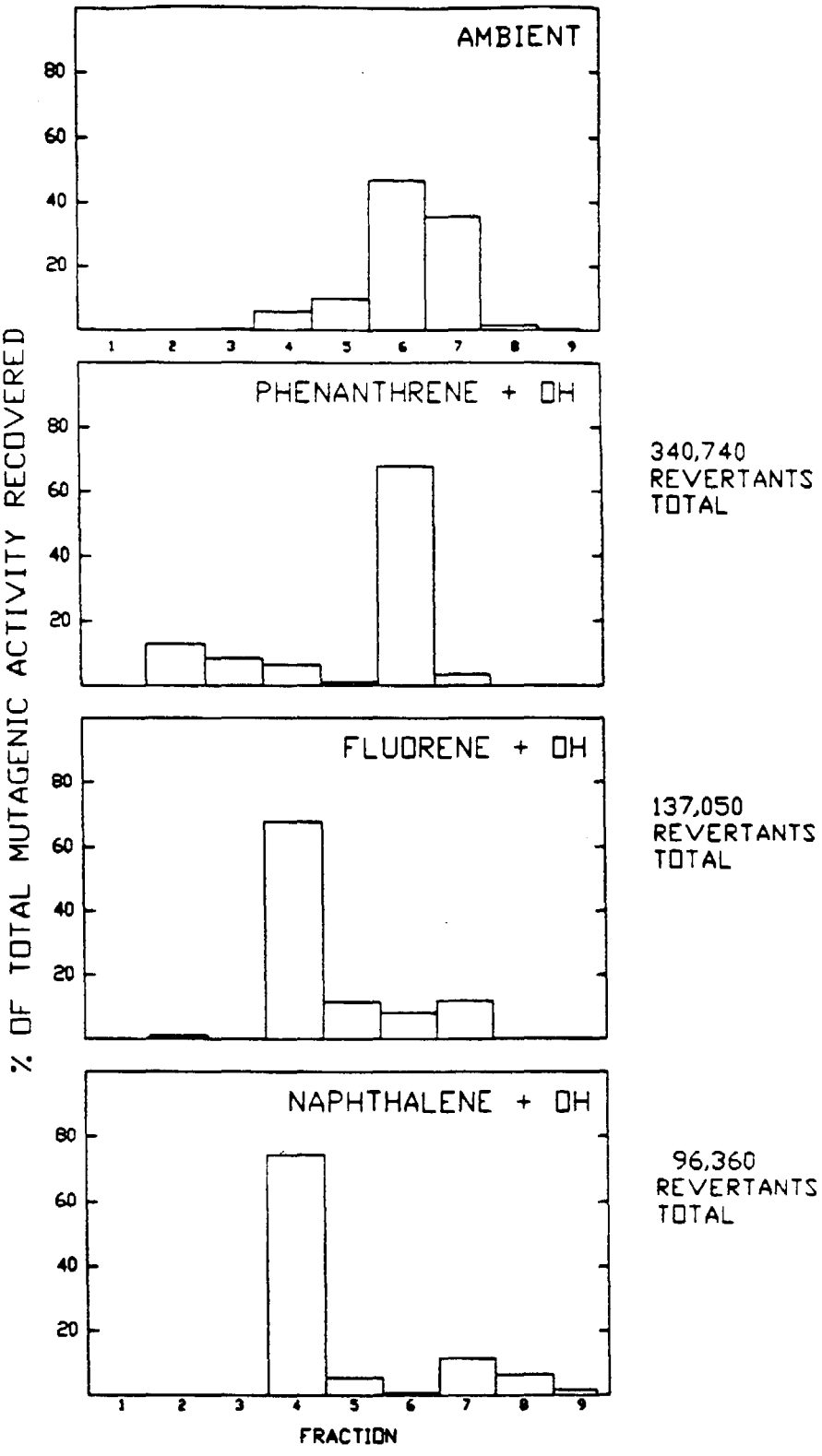



Figure I-1. HPLC mutagrams from the gas-phase OH radical - initiated reactions of phenanthrene, fluorene and naphthalene compared to the mutagram of Claremont, CA ambient air particle extracts. The plotted mutagenicity values have been normalized to the sum of the individual fractions (this sum is shown to the right of each chamber reaction data plot).

Analysis of the mutagenic subfractions from our earlier bioassay-directed fractionation of extracts of ambient air particulate matter collected in Riverside, CA, confirmed the presence of these two nitrophenanthrene lactones, and five nitro-methylphenanthrene lactones and two nitro-pyrene lactones were also tentatively identified. This class of polar chemical compound, the nitro-PAH lactones, may well contribute significantly to the observed mutagenicity of ambient air particulate matter. Further studies are needed to assess their contribution to ambient air mutagenicity and to determine the human health effects of these compounds.

## II. RECOMMENDATIONS

Bioassay-directed fractionation of the reaction products of PAH in environmental chamber studies has proven to be a very successful complement to bioassay-directed fractionation of ambient particle extracts. This approach has allowed the identification in ambient particulate extracts of a new class of mutagens, the nitro-PAH lactones. Additionally, assaying with the Kado modification of the Ames test has shown significant activity from the nitronaphthalenes in chamber reactions of naphthalene. In ambient atmospheres, volatile nitro-PAH will be formed from the gas phase reaction of the 2-ring PAH (and alkyl substituted 2-ring PAH) and will likely not deposit onto particles to any significant extent.

We make the following recommendations:

- The nitrophenanthrene lactones should be quantified in ambient samples and their contribution to ambient particulate mutagenicity determined.
- Hydroxyl radical-initiated reactions of methylphenanthrenes should be investigated in the environmental chamber with bioassay-directed fractionation and the contribution to ambient particulate mutagenicity from the nitro-methylphenanthrene lactones determined.
- Additional volatile PAH found in ambient atmospheres should be examined including fluoranthene and pyrene (whose atmospherically formed nitro-derivatives are generally the most abundant nitro-PAH in ambient particle extracts), methylnaphthalenes, biphenyl, benz[a]anthracene and dibenzothiophene.
- Bioassay-directed fractionation and chemical analysis of the semi-volatiles in ambient air should be conducted using the Kado modification of the Ames assay. The feasibility of using polyurethane foam plug samples for assessing the ambient mutagenicity of semi-volatiles should be examined.

# III. INTRODUCTION

## A. Background

A wide variety of organic chemicals are introduced into the atmosphere from anthropogenic sources, mainly as a result of fossil fuel combustion (Graedel et al., 1986). A class of organic compounds which has received much attention over the past 20 years is the polycyclic aromatic hydrocarbons (PAH). The PAH are formed in combustion systems at high temperatures (Bockhorn et al., 1982; Kittleson et al., 1985; Prado et al., 1985, Toqan et al., 1985; Frenklach et al., 1988; Frenklach, 1989), and hence are emitted from essentially all combustion sources. As discussed by Nikolaou et al. (1984), these combustion sources include emissions from automobiles, industrial processes, domestic and commercial heating systems, waste incineration facilities, tobacco smoking, agricultural burning, and several natural processes including forest fires and volcanic eruptions. Many of the PAH are animal carcinogens (NAS, 1983) and they are mutagenic in the presence of microsomal activation in the Ames Salmonella typhimurium bacterial assay (McCann et al., 1975).

In the atmosphere, the PAH are distributed between the gas and particle phases (see, for example, Arey et al., 1987; Atkinson et al., 1988; Bidleman, 1988; Coutant et al., 1988). Theoretical and ambient air data for several series of semi-volatile organic compounds (including PAH, alkanes, and organochlorine compounds) show that organic compounds with liquid-phase vapor pressures $>10^{-6}$ Torr exist in the atmosphere at least partially in the gas phase (Bidleman, 1988). Recent ambient atmospheric measurements of the PAH in California's atmosphere using high-volume samplers equipped with filters for the particulate matter and polyurethane foam solid adsorbent for the semi-volatiles show that the 2-4 ring PAH exist mainly in the gas phase (Arey et al., 1987, 1989a; Atkinson et al., 1988), although it must be recognized that the gas/particle distributions derived from adsorbent samplers using filter-solid adsorbent combinations are "operational" phase distributions and may be subject to a number of artifact problems (Bidleman, 1988).

It has been recognized for many years that extracts of collected ambient respirable particulate matter are carcinogenic (Leiter et al., 1942) and it has recently been shown that these extracts are strongly

mutagenic in the Ames assay without microsomal activation [i.e., they are direct-acting mutagens] (Pitts et al., 1977, 1982; Talcott and Wei, 1977; Tokiwa et al., 1977). This direct-acting mutagenicity cannot be due to the PAH, since the PAH require microsomal activation. To date, the chemical compounds responsible for the direct-acting mutagenicity of collected ambient particulate matter have not been determined to any appreciable extent. Nitroarenes, many of which are strong direct-acting mutagens and animal carcinogens (Tokiwa and Ohnishi, 1986), have been identified and quantified in ambient particulate matter collected at several locations throughout the world (see, for example, Gibson, 1983; Nielsen, 1983; Tokiwa et al., 1983; Nielsen et al., 1984; Pitts et al., 1985; Nielsen and Ramdahl, 1986; Ramdahl et al., 1986; Sweetman et al., 1986; Arey et al., 1987, 1988; Atkinson et al., 1988; Zielinska et al., 1988, 1989a). However, measurements of the nitroarenes present in ambient particulate matter and of the mutagenicity of extracts of the ambient particulate matter show that the nitroarenes contribute typically 1-10% of the measured mutagenicity (Atkinson et al., 1988; Arey et al., 1988; Strandell et al., 1987), with 2-nitrofluoranthene and 2-nitropyrene generally being the most abundant particle-phase nitroarenes and (together with 8-nitrofluoranthene in many cases) the major contributors to ambient particulate mutagenicity (Arey et al., 1988; Atkinson et al., 1988).

Laboratory studies carried out over the past eight years at the Statewide Air Pollution Research Center (SAPRC), University of California, Riverside, have shown that the 2-4 ring PAH present in the gas-phase undergo reactions in the atmosphere, primarily with the hydroxyl (OH) radical, to form nitroarenes, with the nitroarenes being distributed between the gas and particle phases and the nitrofluoranthenes and nitropyrenes being in the particle phase in the atmosphere (Sweetman et al., 1986; Arey et al., 1986, 1989a,b, 1990; Atkinson et al., 1987, 1990a,b; Atkinson and Aschmann, 1988). Indeed, the particle-phase nitroarenes observed in ambient air arise primarily from the atmospheric transformations of fluoranthene and pyrene (Arey et al., 1986, 1990; Zielinska et al., 1988, 1989a; Atkinson et al., 1990a), as shown in Figure III-1. In an analogous manner, the majority of the gas-phase 2-ring nitroarenes, 1- and 2-nitronaphthalene, methylnitronaphthalenes and 3-nitrobiphenyl, also appear to arise from the gas-phase reactions of the

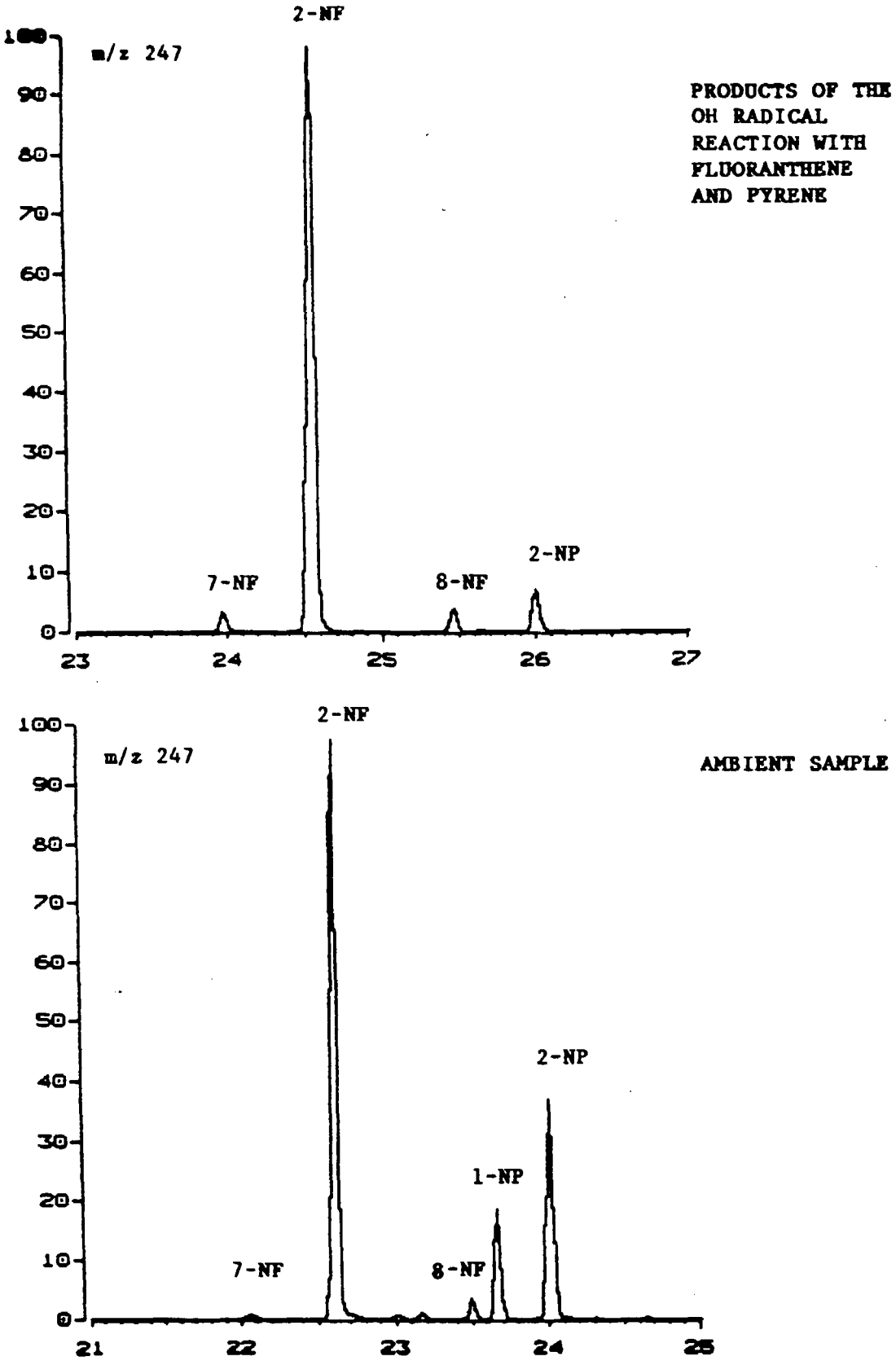



Figure III-1. GC/MS analyses of: a sample collected from a chamber exposure of fluoranthene and pyrene to OH radicals in the presence of $NO_x$ (top) and a daytime ambient air sample collected at Torrance, CA, during a high-$NO_x$ episode (bottom). Shown are the molecular ions m/z 247 for the nitrofluoranthenes (NF) and nitropyrenes (NP).

naphthalene, 1- and 2-methylnaphthalene and biphenyl precursors (Atkinson et al., 1987; Arey et al., 1987, 1989a, 1990; Zielinska et al., 1989b).

The formation yields of the nitroarenes from the OH radical-initiated reactions of the 2-4 ring PAH are ≤5% in all cases (Arey et al., 1989b; Atkinson et al., 1990a), with the other products presently being unaccounted for [hydroxy-PAH have been observed from the OH radical-initiated reactions of naphthalene and biphenyl with yields of ~10% and ~20%, respectively (Atkinson et al., 1987)]. Although the nitroarenes (mostly produced in situ in the atmosphere by reactions of the gas-phase 2-4 ring PAH) contribute <10% of ambient particle-phase direct-acting mutagenicity, the direct-acting mutagenicity of extracts of collected ambient air particulate matter at seven sites in California impacted by differing combustion emissions gave a reasonable (and the best) correlation with the 2-nitropyrene concentration (Atkinson et al., 1988) among the PAH and nitro-PAH measured. Since 2-nitropyrene is formed in the atmosphere from the OH radical-initiated reaction of pyrene, this observation suggests that the bulk of the direct-acting mutagenicity in ambient air particulate matter arises from the gas-phase atmospheric reactions of the 2-4 ring PAH (Atkinson et al., 1988). That the mutagenic profile of primary combustion-generated emissions undergoes change during transport from source to receptor is shown by the data given in Figure III-2 (Pitts et al., 1984). Figure III-2 shows that while a major portion of the direct-acting mutagenicity of diesel exhaust extract is present in a fraction containing the nitroarenes (fraction #7), this is not the most mutagenically potent fraction of the ambient particulate matter. Thus, as shown in Figure III-2, the mutagenicity of the ambient particulate matter extract occurred mainly in the more polar fraction #11. These findings have since been confirmed by ourselves (this report) and others (Siak et al., 1985; Schuetzle and Lewtas, 1986, Lewtas et al., 1990).

The observations that the PAH undergo atmospheric transformations to form nitroarenes and other reaction products, and that the nitroarenes contribute <10% of ambient particle-phase mutagenicity, but correlate with the ambient particle-phase mutagenicity, suggest that the majority of ambient mutagenicity, presently not accounted for, is due to PAH atmospheric reaction products. Moreover, the mutagenicity polarity profile of ambient air extracts show that the majority of the mutagenicity

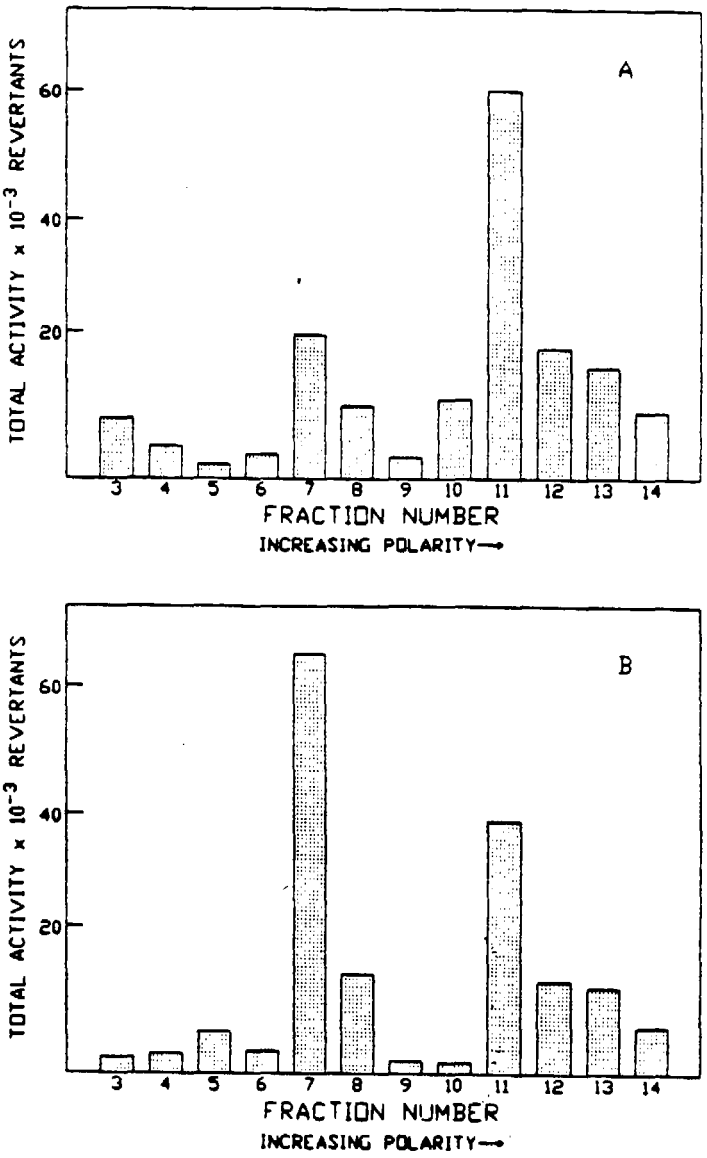
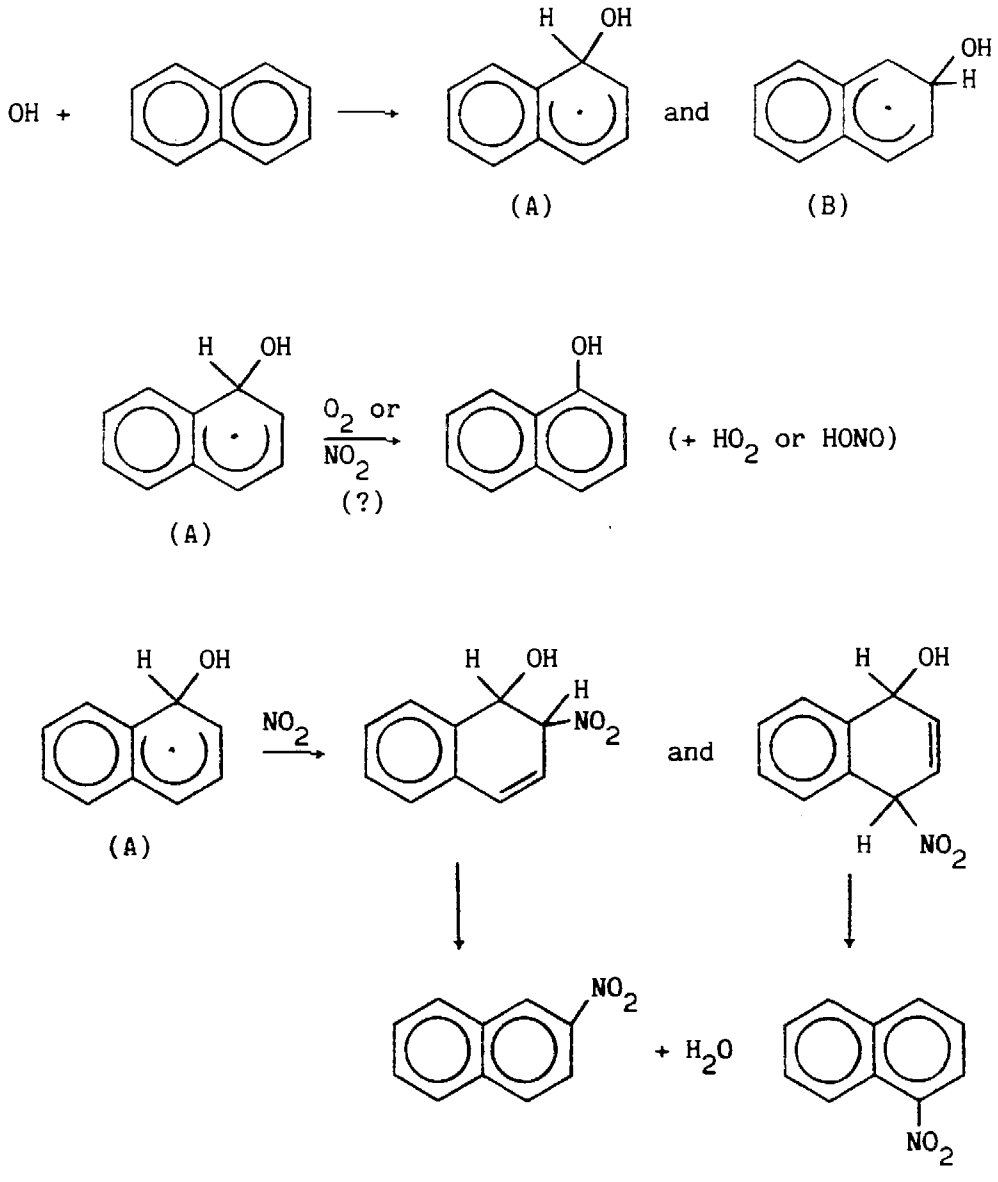
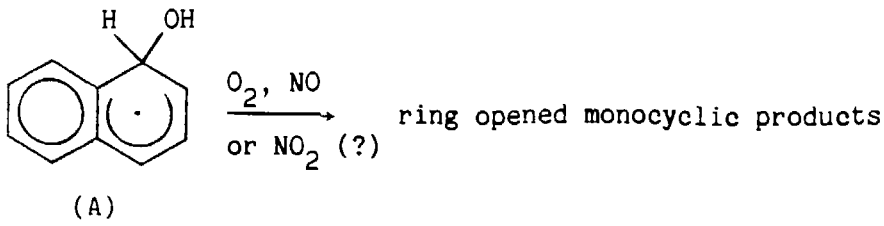



Figure III-2. Mutagenic activities (TA98, -S9) of HPLC fractions of $CH_2Cl_2$ extracts of (A) an ambient POM sample collected in El Monte, CA, and (B) a diesel exhaust particulate sample.

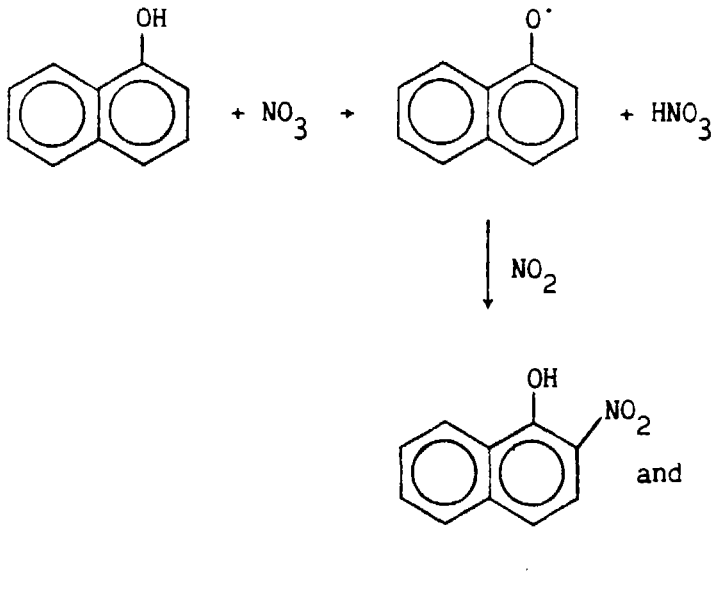
is due to the presence in ambient air particulate matter of chemical compounds more polar than the nitroarenes. Because of these considerations, the Air Resources Board contracted SAPRC to attempt to identify the chemical compounds responsible for the majority of the observed mutagenicity of ambient particulate matter. At the time that this program was initiated, we believed that a potential class of PAH reaction products responsible for the observed polar ambient mutagenicity was the hydroxynitro-PAH, expected to be formed from the hydroxy-PAH in high yield by reaction with the $NO_3$ radical. For example, for naphthalene:






(A)

followed by




The initial experimental approach in the program was to (a) carry out bioassay-directed fractionation of extracts of collected ambient air particulate samples with concurrent chemical analysis to identify the mutagenic chemicals responsible for the observed ambient mutagenicity, and (b) to identify the hydroxynitro-PAH present in ambient air and assess the contributions of these compounds to ambient mutagenicity. As described in Sections IV and V below, the hydroxynitro-PAH have low direct-acting mutagenicities, and the bioassay-directed fractionation and chemical analysis of ambient particulate matter was not successful in identifying polar mutagenic compounds. After consultation with the ARB staff, we thus changed the direction of the program, and investigated the atmospheric reactions of individual PAH in an environmental chamber, with bioassay-directed fractionation and chemical analysis of the collected reaction

products. As described in Sections VI through X, this approach was successful, and we have identified polar mutagenic compounds from the OH radical-initiated reactions of phenanthrene which we have also shown to be present in ambient air. In addition, through the use of the Kado microsuspension modification of the Ames assay in our bioassay-directed fractionations, we have found that 2-nitronaphthalene may be an important gas-phase mutagen in ambient air.

B. Organization of Report

As noted in the Background section above, a re-direction of effort was necessary during the conduct of this contract. Close consultation involving multiple discussions with the ARB staff allowed us to recognize that our bioassay-directed fractionation of ambient particle extracts had come to an impasse and the specific compounds we had targeted, the hydroxynitro-PAH, were unlikely to be the "missing mutagens". After changing our focus to "mutagenic PAH reaction products" through bioassay-directed fractionation of the atmospheric reaction products of those PAH most abundant in ambient atmospheres, we were able to return to our ambient samples and identify a significant new class of mutagens. This report is organized largely in a chronological fashion, since we strongly believe that reporting less than successful research efforts may be as important and informative to other scientific investigators as reporting successful endeavours.

We have established in the Background section above the rationale for our original approach. Our bioassay-directed fractionations of ambient air samples are detailed in Section IV below. We were able to isolate highly mutagenic subfractions of the ambient extracts, but our chemical analysis attempts using liquid chromatography/mass spectrometry proved futile.

Section V describes the synthesis, characterization and mutagenicity testing of the originally targeted hydroxynitro-PAH. The generally low mutagenic activity of this compound class (Section V-B) and new data regarding the expected yields of these compounds (Section V-C) are presented.

Section VI provides the rationale and describes the methods employed to re-direct this program to examine the mutagenic products formed from the atmospheric reactions of selected PAH. The three following sections

describe the results for each PAH examined: Section VII, naphthalene; Section VIII, fluorene and Section IX, phenanthrene. Having identified highly mutagenic nitrophenanthrene lactones formed from the simulated atmospheric reactions of phenanthrene, it was gratifying to return to our Riverside ambient particle extracts and be able to identify, as discussed in Section X, these specific compounds in mutagenic subfractions. Section XI provides a brief discussion of our findings.

## IV. BIOASSAY-DIRECTED FRACTIONATION OF AMBIENT AIR SAMPLES

As noted in Section III above, the initial approach in this program was to collect ambient air particulate samples and then to utilize bioassay-directed fractionation of the extracts of these samples with concurrent chemical analysis to identify mutagenic compounds present in the ambient air samples. The experimental procedures and results of this approach are described in the sections below.

### A. Particle Collections

A total of 18.1 g of ambient particulate matter was collected at the Riverside campus of the University of California (UCR). Since we had found in previous studies that collections of compounds such as the nitropyrenes and nitrofluoranthenes are quite low during the hottest part of the day when temperatures approach 40°C (as occurs frequently in Riverside), presumably due to "blow-off," we sampled from 1600-1000 hrs in order to maximize our collection efficiency without leaving the filters in place over extended periods. We sampled only on days with predicted high particulate loadings and collected samples ranging from 1-2 g per collection period.

Each sample was collected on four 16 in. x 20 in. precleaned (extracted in $CH_2Cl_2$ and methanol) Pallflex TX40HI20WW (high efficiency) Teflon-impregnated glass fiber (TIGF) filters using our ultra-high volume sampler (Figure IV-1). This sampler has an inlet with a 50% cut-point of 20 μm, allowing the collection of particles primarily in the respirable size range (respirable particles ≤10 μm). The total flow rate is equivalent to 16 times that of a normal high-volume (Hi-vol) sampler. Thus, the total flow rate was 640 SCFM with a face velocity equivalent to that of a normal Hi-vol sampler.

During the sampling periods, three Hi-vol samplers each fitted with a TIGF filter and a single polyurethane foam (PUF) plug and with flow rates of approximately 30 SCFM were also operated (see Figure IV-2). The PUF plugs were changed each night at approximately 2200 hrs. The single PUF plugs from each Hi-vol sampler were placed together in a glass jar and stored in a freezer. These PUF samples are expected to contain the semi-volatile compounds such as fluoranthene and pyrene and also the hydroxynitro-derivatives of the two-ring PAH; these compounds may be either

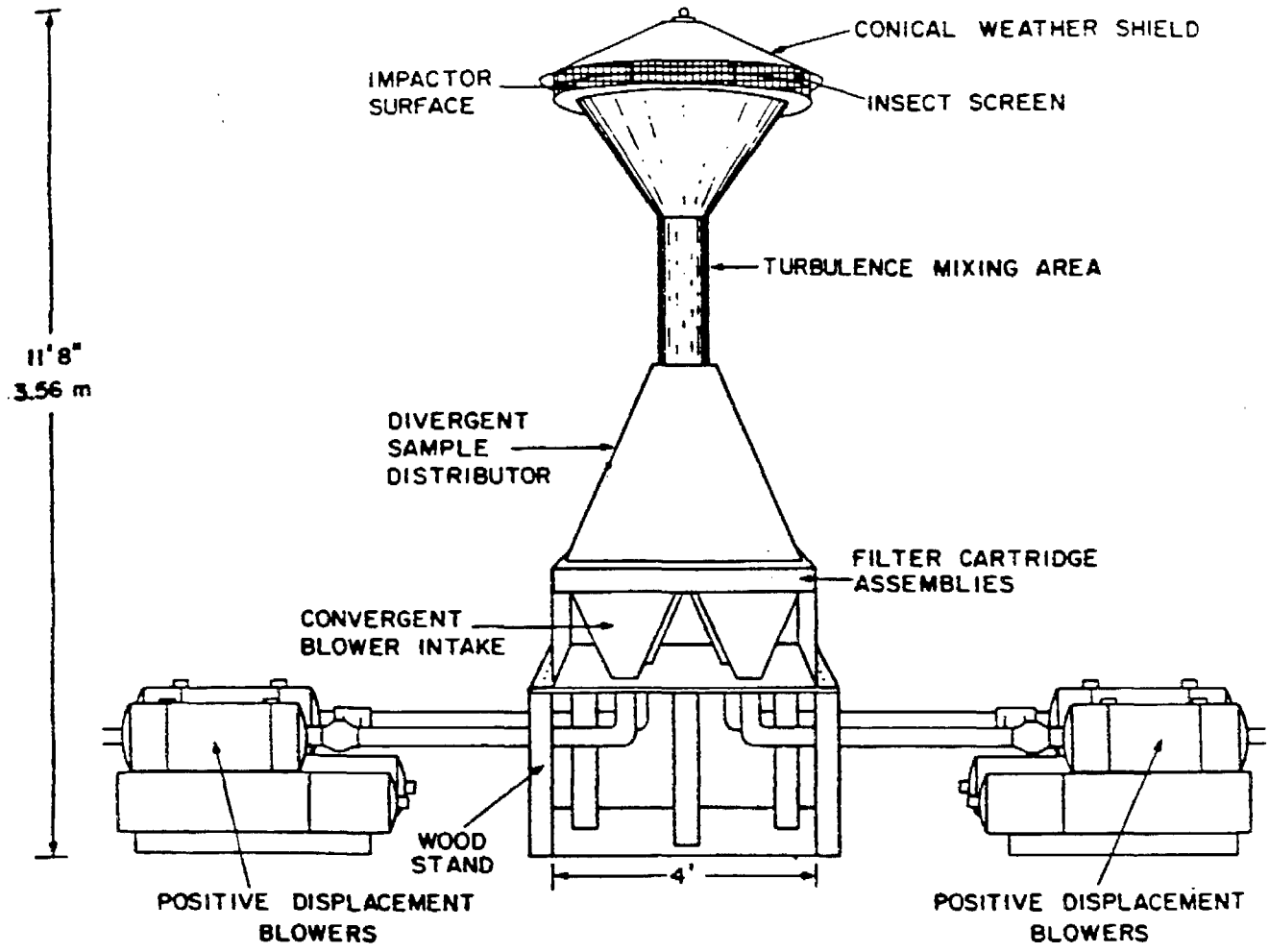



Figure IV-1. The SAPRC four-by-four high-volume mega-sampler.

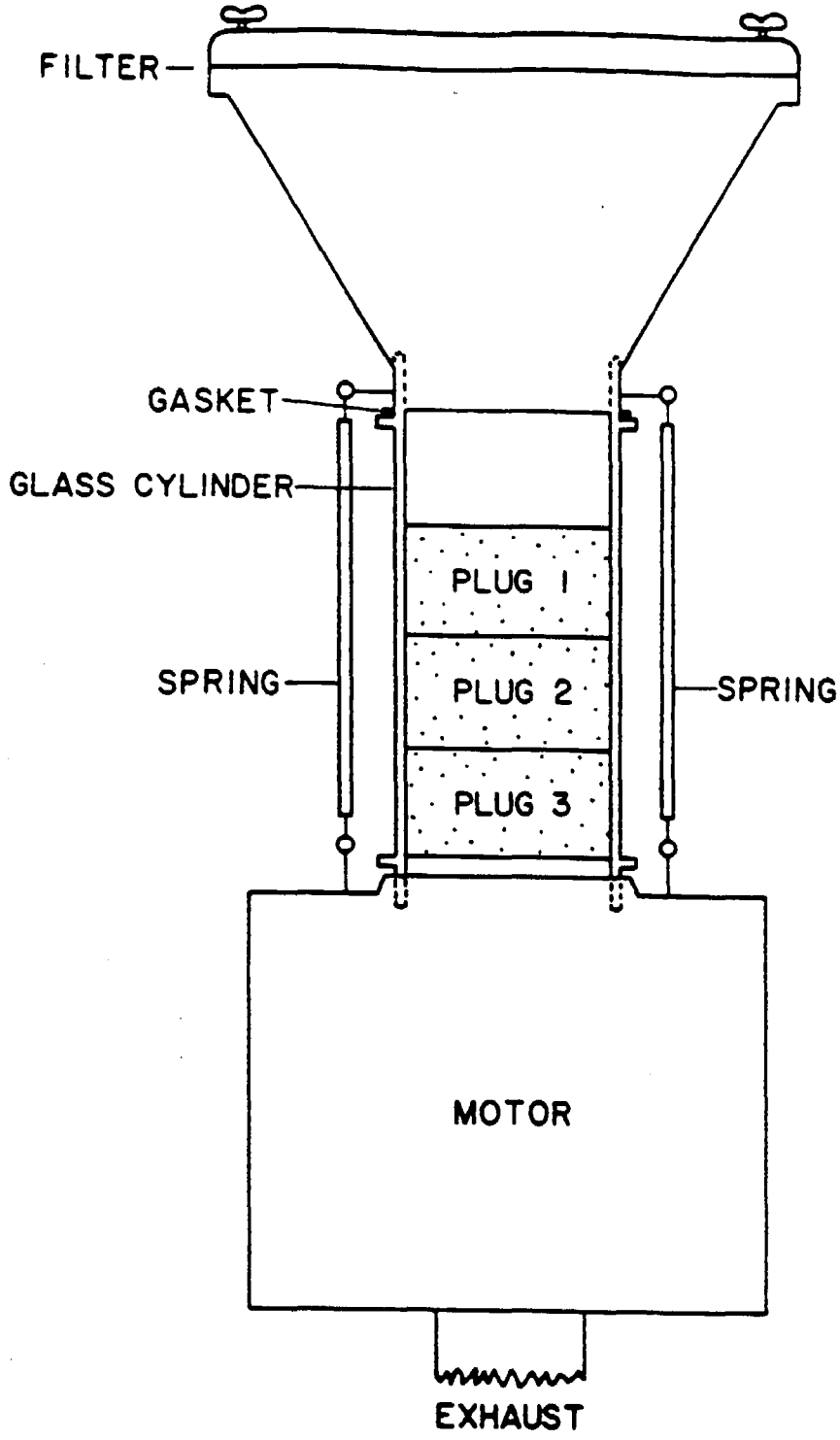



Figure IV-2. Schematic of modified Hi-vol sampler with PUF plugs underneath the filter to collect gas-phase species and compounds "blown off" the filter. Only a single PUF plug being used for current sample collection.

in the gas phase when sampled or be "blown-off" the particles during collection. Utilizing a single PUF plug in each Hi-vol sampler, with relatively short sampling times, was expected to maximize the quantity of semi-volatiles collected without resulting in an excessive number of PUF plugs requiring extraction.

B. Micro-Preincubation Mutagenicity Test

Optimization of Assay. The isolation and characterization of mutagenic compounds by serial bioassay-directed separations is best achieved through minimal sample consumption by the bioassay. We therefore used the modification of the Salmonella mutagenicity test of Kado and co-workers (Kado et al., 1983), which provides 10-30 times the sensitivity of the conventional Ames test and has been applied to extracts of ambient particulate matter (Kado et al., 1986). The test is a modified liquid preincubation assay in which the sample under test is incubated with ten times the usual number of bacteria in a small-volume liquid suspension (microsuspension) for 90 minutes prior to plating on the Ames test plate. In order to insure that the conditions reported by Kado et al. (1986) were optimal for our isolate of TA98, and to attempt to achieve further enhancement of sensitivity through the use of greater histidine concentrations, a control experiment was performed in which the bacterial cell density was varied together with the histidine concentration. The hypothesis was that greater cell densities may require greater histidine concentrations for full expression of mutagenicity. Additionally, we sought to determine the relationship between cell density and spontaneous reversion because of the possibility that increased "scatter" in the spontaneous reversion, which might occur with increased cell density, would counteract the increased sensitivity of the test.

The test consisted of a microsuspension assay of 2-nitrofluorene using four different histidine concentrations at each of four cell densities of TA98, without S9 activation. TA98 was chosen because of its sensitivity to ambient particulate mutagens, which exhibit activity in the absence of exogenous mammalian metabolism. The strain was cultured in L-broth at 37° C for 12 hours to approximately $5 \times 10^9$ cells $mL^{-1}$, the cells were harvested by centrifugation and resuspended in cold phosphate-buffered saline (PBS,0.02 M; pH 7.4) at a ten-fold higher density and placed in an

ice bath. From this suspension, three other cell densities were obtained by serial dilution in PBS at ice temperature. 2-Nitrofluorene (0.1 µg in 0.005 mL DMSO) was added to sterile glass culture tubes (13 mm x 100 mm) in an ice bath to which 0.1 mL of the TA98 suspension was added. Six replicates were tested at each of the 16 histidine/cell density regimes, as were six DMSO blanks. After addition of the bacteria, the tubes were incubated at 37° C for 90 minutes with shaking (180 rpm). At the end of the 90-minute incubation, the tubes were removed and placed on ice. Top agar (2.0 mL containing 100 nmol of biotin and varying amounts of histidine) was added to each tube, the contents were vortex-mixed, and then overlayed onto minimal glucose plates. The plates were incubated in the dark at 37°C for 63 hours, after which they were counted by means of an automatic colony counter.

The cell densities were determined by serial dilution and plating on histidine-supplemented minimal media. At each cell density, 0.1 mL of the bacteria were plated on histidine-free media for determination of pre-existing culture revertants (as opposed to those which arise on the plates during the 63-hour incubation). The proportion of spontaneous revertants which arise on the plates may be increased by increasing the histidine concentration which may, in turn, affect the sensitivity of the test.

The spontaneous reversion of TA98 as a function of cell density and histidine concentration is shown in Table IV-1. The number of spontaneous revertants increased with cell density of the bacterial suspension and with the histidine concentration, with the cell density being the more important factor over the range of values tested. The final number of unreverted Salmonella on each plate was determined by both the initial titer and the histidine concentration. In the conventional Ames test this "background lawn" is almost entirely due to growth on the plate, while at the higher two cell densities used here it is mostly due to the initial loading of bacteria. At the highest histidine concentration of the two highest cell densities, the heavy background lawn obscured the revertant colonies with the result that some colonies were not detected by the colony counter.

The number of revertants arising on the plates during their 63-hour incubation are shown in Table IV-2. These numbers were obtained by subtracting the number of pre-existing "culture" revertants from the number of spontaneous revertants. They can be seen to increase with the

Table IV-1. TA98 Spontaneous Reversion as a Function of Cell Density and Histidine Concentration (rev plate$^{-1}$)

| Cell Density ($10^9$ cells plate$^{-1}$) | Histidine (nmol plate$^{-1}$) | | | |
|---|---|---|---|---|
| | 100 | 150 | 200 | 250 |
| 1.37 | 43 | 52 | 62 | 68 |
| 2.73 | 77 | 79 | 86 | 95 |
| 4.10 | 109 | 108 | 112 | 105[a] |
| 5.46 | 127 | 131 | 141 | 141[a] |

[a]Revertant colonies partially obscured by heavy growth of background lawn.

Table IV-2. "Plate" Revertants as a Function of Cell Density and Histidine Concentration (rev plate$^{-1}$)

| Cell Density ($10^9$ cells plate$^{-1}$) | Histidine (nmol plate$^{-1}$) | | | |
|---|---|---|---|---|
| | 100 | 150 | 200 | 250 |
| 1.37 | 11 | 15 | 25 | 31 |
| 2.73 | 9 | 11 | 18 | 27 |
| 4.10 | 14 | 13 | 17 | 10[a] |
| 5.46 | 11 | 15 | 25 | 25[a] |

[a]Revertant colonies partially obscured by heavy growth of background lawn.

histidine concentration, which was the limiting factor for growth on the plates, but were unrelated to the cell density used. By comparing the values in Tables IV-1 and IV-2, it can be seen that the spontaneous reversion was largely due to revertants contained in the overnight culture, which has implications for the error in the background response of the test (also for the conventional Ames test where an estimated 70% of the spontaneous revertants are contained in the initial inoculum). The day-to-day variation in spontaneous reversion is thus highly dependent on chance mutations, those arising during the initial growth of the culture and

amplified by exponential growth. Thus, the background response used for determining the significance of an induced response should not be taken from the historical value, but from the value determined for that particular culture. Also, the ratio of the induced response to the spontaneous response is not a good indicator of the test sensitivity, nor is it a good indicator of the significance of the induced response. Finally, because the error in the number of spontaneous revertants is due largely to pipetting error and inhomogeneity of the cell suspension, increasing the cell density does not result in a great increase in the error of measurement of the background response.

Table IV-3 shows the mutagenicity of 0.1 μg of 2-nitrofluorene, a standard mutagen for TA98, under the various conditions examined. The maximal response occurred at a histidine concentration of 200 nmol plate$^{-1}$ and a cell density of 1.37 x $10^9$ cells plate$^{-1}$. The mutagenicity of 15,000 rev μg$^{-1}$ is approximately 30 times the typical activity of 2-nitrofluorene as measured by our laboratory using the conventional Ames assay and occurred at approximately the cell density described by Kado et al. (1983) as being optimal for the microsuspension assay. The lower response with increasing cell density may be due to the lower average number of bacteria in the colonies of the background lawn (determined by the ratio of histidine concentration to cell density). Several divisions may be necessary for the expression of mutagenicity, whereas the estimated average number of divisions per colony is less than one at the 5.46 x $10^9$ cell loading and 100 nmol histidine regime.

Table IV-3. 2-Nitrofluorene Mutagenicity as a Function of Cell Density and Histidine Concentration (rev μg$^{-1}$)

| Cell Density ($10^9$ cells plate$^{-1}$) | Histidine (nmol plate$^{-1}$) | | | |
|---|---|---|---|---|
| | 160 | 150 | 200 | 250 |
| 1.37 | 11,000 | 13,000 | 15,000 | 14,000 |
| 2.73 | 6,900 | 7,600 | 9,700 | 11,000 |
| 4.10 | 5,500 | 7,300 | N.D.[a] | 8,100[b] |
| 5.46 | 3,200 | 4,200 | 4,600 | 6,000[b] |

[a]No data; contamination.
[b]Revertant colonies partially obscured by heavy growth of background lawn.

Mutagenicity Testing of Whole Ambient Extracts. In order to verify the applicability of the Salmonella micro-suspension preincubation mutagenicity test to ambient particulate extracts from southern California, tests were conducted on previously collected POM samples. An ambient particulate extract and the control mutagen, 2-nitrofluorene, were tested for mutagenicity by the Salmonella micro-suspension assay and simultaneously by the standard plate incorporation procedure. The enhancement of the micro-suspension test over the standard plate test was then calculated for both samples and compared.

The ambient particulate sample was collected on TIGF filters in Reseda, California, using two Hi-vol samplers with 10 μm cutoff inlets and operating at ~40 SCFM (Atkinson et al., 1988). The 12-hour sampling period was from 0700-1900 hours on May 28, 1987. The extract was obtained by Soxhlet extraction for 16 hours, using a benzene-methanol (80:20) azeotrope. The extract was tested at 0.625, 0.984, 1.55, 2.44, 3.84, 6.05, 9.53 and 15 μg plate$^{-1}$ in the micro-suspension test, and at 15, 30, 45, 60, 75, 90, 105 and 120 μg plate$^{-1}$ in the standard plate incorporation test. The standard mutagen, 2-nitrofluorene, was tested at 0.004, 0.00634, 0.01, 0.159, 0.0252, 0.0399, 0.0631 and 0.1 μg plate$^{-1}$ in the micro-suspension test and at 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 μg plate$^{-1}$ in standard mutagenicity test. The lower dose ranges for the micro-suspension test were chosen in anticipation of higher mutagenicities with this procedure. Each dose was tested in triplicate on TA98 without metabolic activation, and the mutagenicity (rev μg$^{-1}$) was determined by linear regression analysis of the dose-response data.

The results of this test are shown in Table IV-4. Although the control mutagen, 2-nitrofluorene, was 35 times as mutagenic in the micro-suspension preincubation test as in the standard test, the extract of the ambient particulate sample POM was only 2.4 times as active. Although this represents an increase in sensitivity, it is well short of the approximately 10-fold enhancement reported by Kado et al. (1986) for tri-solvent extracts of ambient particulate matter. One explanation for this small enhancement may be that the toxicity of the extract during the micro-preincubation step reduced the target cell density, essentially negating the advantage of this procedure over the standard test where the sample is less concentrated and the mutagenic response is less dependent on initial

cell loading. Further, the benzene-methanol azeotrope would be expected to extract polar compounds, many of which are likely to be toxic to the bacteria.

Table IV-4. TA98 Mutagenicity of the Extract of an Ambient Particulate Sample and 2-Nitrofluorene Using the Standard and Micro-suspension Procedures

| | Specific Activity (rev $\mu g^{-1}$) | | |
|---|---|---|---|
| | Standard Plate Incorporation Procedure | Micro-suspension Preincubation Procedure | Enhancement Factor |
| Ambient sample | 2.3 | 5.6 | 2.4 |
| 2-Nitrofluorene | 570 | 20,000 | 35 |

Therefore, a second micro-suspension test of the Reseda extract was performed, and the enhancement over the standard test was compared to that of a dichloromethane ($CH_2Cl_2$) extract of comparable mutagenicity. The protocol of the test was the same as above except that survival of the bacteria was measured after the preincubation step.

The second sample was obtained from particulate matter collected by a single Hi-vol sampler (23 SCFM) in Glendora, California on August 20, 1986 from 0800 to 2000 hours (Atkinson et al., 1988). The extract was obtained by a 16-hour Soxhlet extraction with $CH_2Cl_2$. In the micro-suspension test, the doses tested were 1.3, 2.1, 3.4, 5.5, 9.1, 15, 24 and 40 $\mu g\ plate^{-1}$, while in the standard plate incorporation assay they were 12.5, 25, 37.5, 50, 62.5, 75, 87.5 and 100 $\mu g\ plate^{-1}$.

The results of the mutagenicity tests and the enhancement of the micro-suspension procedure over the standard procedure are shown in Table IV-5. Although the mutagenicity of the particulate extracts in the micro-suspension test was not enhanced to the same degree as 2-nitrofluorene, enhancement of the $CH_2Cl_2$ extract was greater than that of the benzene-methanol extract. Table IV-6 compares the two extracts with

Table IV-5. Comparison of the TA98 Mutagenicity of a $CH_2Cl_2$ and a Benzene-Methanol Particulate Extract Using the Standard and Micro-suspension Procedures

| | Specific Activity (rev $\mu g^{-1}$) | | |
|---|---|---|---|
| | Standard Plate Incorporation Procedure | Micro-suspension Preincubation Procedure | Enhancement Factor |
| $CH_2Cl_2$ Extract | 1.8 | 10 | 5.6 |
| Benzene-Methanol Extract | 1.6 | 4.6 | 2.9 |
| 2-Nitrofluorene | 350 | 25,000 | 71 |

Table IV-6. Number of Cells Surviving the Micro-suspension Preincubation

| | Surviving Cells (Average of Three Determinations) |
|---|---|
| DMSO Blank | $8.1 \times 10^8$ |
| $CH_2Cl_2$ Extract | $7.5 \times 10^8$ |
| Benzene-Methanol Extract | $0.1 \times 10^8$ |

regard to the number of cells surviving the 90-minute preincubation in the micro-suspension test. Clearly, the sample extracted by the benzene-methanol azeotrope was more toxic, and this toxicity must surely have contributed to its lower mutagenicity enhancement in the micro-suspension assay.

Because of the interference of toxic compounds, the micro-suspension preincubation modification of the Salmonella test may not be as effective for crude environmental samples, such as ambient particulate matter, as it is for pure compounds. However, for the process of identification of mutagenic compounds by bioassay-directed fractionation, a six-fold increase

in sensitivity of the bioassay is still very useful in preserving sample material for chemical analysis. Although further tests are needed to demonstrate the effect of extraction solvent on the toxicity of extracts of ambient particulate samples in the Salmonella mutagenicity test, the above results, together with our previous experience with ambient particles collected in southern California, indicate that dichloromethane is the preferred solvent for the extraction of collected particulate matter for mutagenicity testing, and this solvent was used in all further work in this program.

C. Ambient Particle Extraction

The forty-four 16 in. x 20 in. filters on which the 18.1 g of particles were collected were extracted in two groups using an oversized Soxhlet apparatus. The filters were extracted for 16 hr in dichloromethane, and the extracts were concentrated by rotary evaporation and taken to dryness under a stream of nitrogen. Approximately 12% of the particle weight (2.12 g) was extractable. Preliminary Ames assays using both the standard protocol and the Kado modification were conducted, showing that the Kado modification gave a nine-fold increase in sensitivity over the standard assay.

D. Mutagenicity Testing of Fractionation Blanks

HPLC Blanks. Two solvent programs were used for the initial HPLC fractionation step. To assure that the column (a new semi-prep Ultra-Sphere Silica column) and the solvents contained no mutagens, both solvent programs were run and 9-min fractions (except where noted) were collected and concentrated for mutagenicity testing. The first solvent program employed was the "methanol program" which used n-hexane as one solvent (solvent A) and a mixture of dichloromethane/methanol (98:2) as the second bottle (solvent B). The program, at a flow rate of 3 mL $min^{-1}$, was as follows: 95% A and 5% B for 5 min, programmed to 65% A and 35% B over 25 min, held at this composition for 15 min, programmed over 10 min to 100% B, held at 100% B for 10 min, and then, returning to initial conditions, programmed over 5 min to 95% A and 5% B. Fraction #1 was the first 6 min of this program and fractions #2-#8 were each 9 min (27 mL).

The second solvent program, the "acetonitrile program," was as follows: 100% hexane for 10 min, programmed to 95% hexane and 5% dichloromethane over 5 min, followed by programming to 100% dichloromethane over the next 25 min, held at 100% dichloromethane for 10 min, programmed to 100% acetonitrile over 10 min and held at 100% acetonitrile for 10 min, and then, programming back to initial conditions, programmed to 100% dichloromethane over 5 min, held at 100% for 5 min, programmed to 100% hexane over 2 min. Nine fractions of 9 min, or 27 mL each, were collected for mutagenicity testing.

Other Blanks. In addition to the HPLC fraction blanks, a representative silica pre-column was eluted with solvents for blank testing. The three elution solvents were pentane, dichloromethane, and methanol. Additionally, four clean filters were extracted and tested, and blanks from the culture tubes and the rotary evaporator used for sample concentration were also carried out.

Results. The mutagenicities of these various solvent blanks were determined by the micro preincubation (microsuspension) modification of the Salmonella histidine reversion mutagenicity test. Each sample was transferred to sterile 13 mm x 100 mm disposable borosilicate culture tubes and evaporated to dryness under a stream of dry nitrogen. DMSO (Mallinckrodt SpectrAR grade, 5 µL) was added to each tube which was sonicated for 15 min and vortexed prior to the addition of the bacteria (100 µL, 1.1 x $10^9$ cells $mL^{-1}$). Each sample was tested in its entirety on a single plate with Salmonella strain TA98 without added metabolic activation. The standard 90-min preincubation was performed at 37°C with vigorous shaking (180 rpm) and was followed by the addition of 2.0 mL of soft agar to each tube. The contents of each tube were then mixed by vortexing and overlayed onto minimal glucose plates which were incubated at 37°C for 63 hr and scored by means of an automatic colony counter. Six DMSO blanks were tested for the determination of the background mutagenicity, and 2-nitrofluorene was tested in triplicate at eight doses in the range of 2-16 ng $plate^{-1}$ as a positive control.

The results of the test are shown in Table IV-7. The average background mutagenicity (56 rev ± 3.3 S.D.) was subtracted from each count; although negative counts may indicate a toxic effect, none of the negative counts was significant except that of sample #6, for which toxicity was

Table IV-7. Mutagenic Activities of Various Sample Blanks in the Microsuspension Test (TA98 without S9)

| Sample # | Description of Sample Blank | Net Revertants |
|---|---|---|
| 1 | Culture tube blank | -9 |
| 2 | Rotary evaporator blank | 152 |
| 3 | Pre-column pentane fraction | 53 |
| 4 | Pre-column $CH_2Cl_2$ fraction | 29 |
| 5 | Pre-column methanol fraction | 97 |
| 6 | Four blank filters | -56 |
| 7 | HPLC methanol program fraction #1 | 274 |
| 8 | #2 | 78 |
| 9 | #3 | 202 |
| 10 | #4 | 2053 |
| 11 | #5 | 130 |
| 12 | #6 | 90 |
| 13 | #7 | 213 |
| 14 | #8 | 695 |
| 15 | HPLC acetonitrile program fraction #1 | 10 |
| 16 | #2 | -7 |
| 17 | #3 | 0 |
| 18 | #4 | 23 |
| 19 | #5 | 15 |
| 20 | #6 | 1699 |
| 21 | #7 | 16 |
| 22 | #8 | 121 |
| 23 | #9 | 13 |

directly observed by the absence of the normal background lawn of unreverted bacteria on the test plate. The activity of the positive control, 2-nitrofluorene, was 16,000 rev $\mu g^{-1}$ (±710 S.D.).

E. Bioassay-Directed Fractionation of Ambient Particles

The mutagenic activity of the total particle extract is summarized in Table IV-8. The mutagen density of these Riverside particles (rev $m^{-3}$) was intermediate between those we measured for Oildale and Reseda, CA during our recent study of seven sites in California (Atkinson et al., 1988).

1. First Fractionation (~200 mg)

A preliminary fractionation was made on ~200 mg of the crude $CH_2Cl_2$ ambient particulate extract. Following an open-column silicic acid chromatography step, two different HPLC programs (detailed in Section D above) were tested. One HPLC program was that we have used previously for fractionation and subsequent analysis of PAH and nitroarenes in ambient extracts (Atkinson et al., 1988) and the second HPLC program was similar to that described in the literature for the analysis of hydroxynitroarenes in ambient extracts (Nishioka et al., 1988). For convenience, we will refer to the first HPLC program as the ACN-program (acetonitrile is the most polar solvent used in this program), and to the second HPLC program as the MeOH-program, (2% methanol in dichloromethane (v/v) is used as the polar solvent). The Kado modification of the Ames test was used to assay the resulting fractions.

The silicic acid open-column fractionation was performed prior to HPLC fractionation as described previously (Atkinson et al., 1988). Two grams of silicic acid (Mallinckrodt, 100 mesh), prewashed with methanol and reactivated at 400°C, was used to fractionate 190 mg of $CH_2Cl_2$ extract as follows. The extract was mixed with 0.5 g silicic acid, applied to the top of the column and eluted with 30 mL pentane, followed by 50 mL $CH_2Cl_2$ and then 50 mL methanol.

The mutagenicity assay results of the open-column fractionation are given in Table IV-9. Note that the crude $CH_2Cl_2$ extract was again tested and resulted in a somewhat higher value (as did the 2-nitrofluorene standard) for this test. As may be seen from Table IV-9, the recovery of mass from the silicic acid column was 98% and the mutagenicity recovery was >100%. The greater mutagenic activity from summing the open-column

Table IV-8. Mutagenicity of Riverside Ambient Particles

Collected August-October 1988 (~216,000 $m^3$ sampled)
Total Particle Weight: 18.1 g.
Extract Weight: 2.12 g. (Soxhlet extracted 16 hr with $CH_2Cl_2$)

TA98 Mutagenicity Ames Protocol
$CH_2Cl_2$ extract: 1.2 rev $\mu g^{-1}$ (~12 rev $m^{-3}$)
2-nitrofluorene standard mutagen: 420 rev $\mu g^{-1}$

TA98 Mutagenicity Kado Protocol
$CH_2Cl_2$ extract: 8.7 rev $\mu g^{-1}$ (~85 rev $m^{-3}$)
2-nitrofluorene standard mutagen: 20,000 rev $\mu g^{-1}$

Table IV-9. Mutagenicity of Silicic Acid Open-Column Fractions (First Fractionation: ~200 mg)

| TA98 Mutagenicity Kado Protocol | rev $\mu g^{-1}$ | Total Revertants |
|---|---|---|
| $CH_2Cl_2$ crude extract (190 mg fractionated) | 13 | $2.5 \times 10^6$ |
| 2-nitrofluorene standard mutagen | 28,000 | |
| Pentane fraction (4.1 mg) | 0 | 0 |
| $CH_2Cl_2$ fraction (31.1 mg) | 92 | $2.9 \times 10^6$ |
| Methanol fraction (151.5 mg) | 8.8 | $1.3 \times 10^6$ |
| Sum: | | $4.2 \times 10^6$ |

fractions than was present in the crude extract suggests that some toxic or antagonistic effect may have lowered the apparent activity of the crude $CH_2Cl_2$ extract.

As expected, the pentane fraction (containing aliphatic hydrocarbons) was inactive. The majority of the mutagenicity was contained in the $CH_2Cl_2$ fraction and only this fraction was used for further subfractionation by HPLC.

All of the hydroxynitronaphthalenes and hydroxynitrobiphenyls for which we have standards (syntheses of these hydroxynitroarenes are reported in Section V, below), as well as the ortho-substituted 2-hydroxy-1-nitrofluoranthene and 1-hydroxy-2-nitropyrene, are expected to have been in the $CH_2Cl_2$ fraction from the silicic acid column if they were present in the ambient particles. A check of the silicic acid column elution of the hydroxynitropyrenes suggests that the non-ortho substituted isomers may not be quantitatively eluted in the $CH_2Cl_2$ fraction.

The two HPLC programs discussed above were used to fractionate ten milligrams each from the 31.1 mg $CH_2Cl_2$ open-column fraction (see Table IV-9). Six HPLC fractions were collected from the ACN-program and fourteen fractions from the MeOH-program. Ten percent of each fraction was allocated for mutagenicity testing and was tested at two doses (80% and 20% of the sample). Figures IV-3 and IV-4 show graphically the HPLC programs utilized and the fractions collected. Tables IV-10 and IV-11 give the mutagenicity assay results.

As readily seen from the percent recoveries given in Tables IV-10 and IV-11, not all the activity placed on the HPLC column was recovered in the collected fractions. The recoveries calculated from the plates to which 20% of the sample was added (remembering that only 10% of the HPLC fractions were used for mutagenicity testing, with the remaining 90% being reserved for chemical analysis) were higher than those calculated from the plates on which 80% of the sample was tested, suggesting that some of the 80% fractions may have exceeded the linear region of the dose-response curve. Further, heavy toxicity (as evidenced by an abnormal background lawn) was seen in the "80% of sample" of the ACN-program Fraction #6 and in the "80% of sample" of the MeOH-program Fraction #11. Although no obvious toxicity was seen in the corresponding 20% of sample from these fractions, the net revertants at these doses may still have been low.

The activity profile of the HPLC fractions from the ACN-program was similar to those we obtained previously for ambient particulate extracts (Atkinson et al., 1988). Fraction #1, in which the PAH elute,

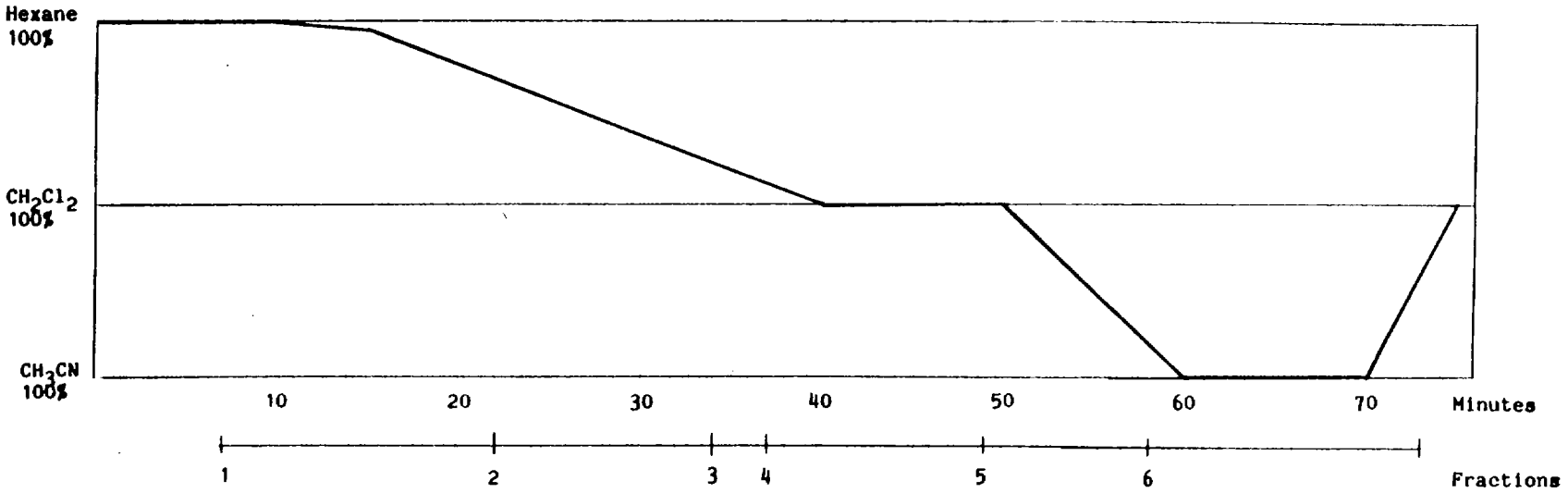



Figure IV-3. HPLC ACN-program used for sample fractionation on an Altex semi-preparative Ultrasphere Silica column (1 cm x 25 cm). The six fractions collected for mutagenicity assay and chemical analysis are given below the mobile phase program.

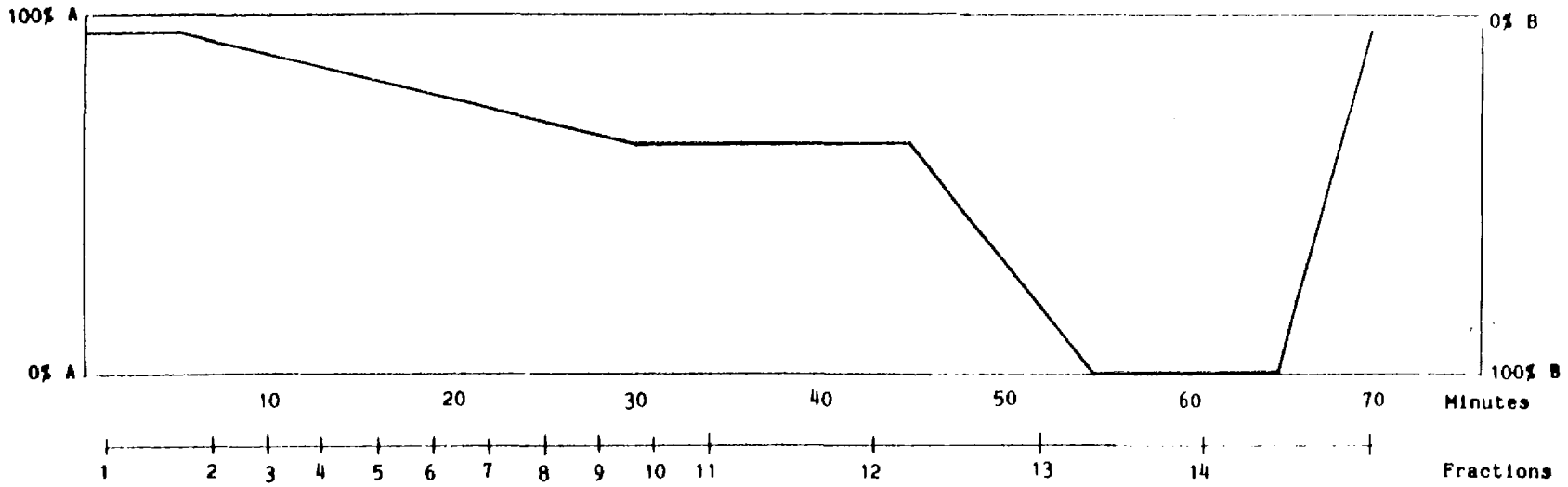



Figure IV-4. HPLC MeOH-program used for sample fractionation on an Altex semi-preparative Ultrasphere Silica column (1 cm x 25 cm). The fourteen fractions collected for mutagenicity assay and chemical analysis are given below the mobile phase program. Mobile phase A is hexane and mobile phase B is a mixture of $CH_2Cl_2$/methanol (98/2, v/v).

Table IV-10. Mutagenic Activities of HPLC Fractions (ACN-program) of $CH_2Cl_2$ Eluate from Silicic Acid Open-column Chromatography of an Ambient Particulate Extract

| 80% of Sample | | 20% of Sample | |
|---|---|---|---|
| Fraction # | Net Revertants | Fraction # | Net Revertants |
| 1 | 20 | 1 | 22 |
| 2 | 2050[a] | 2 | 794 |
| 3 | 974 | 3 | 106 |
| 4 | 4291[a] | 4 | 3736[a] |
| 5 | 1147[b] | 5 | 779 |
| 6 | 456[c] | 6 | 273 |
| SUM | 8938 | SUM | 5710 |
| %Recovery[d] | 12% | %Recovery | 31% |

[a]Above 1000 revertant colonies, the colony count is less accurate due to saturation of the colony counter response.
[b]Slight toxicity was observed.
[c]Heavy toxicity was observed.
[d]Based on the specific activity of the $CH_2Cl_2$ open-column fraction of 92 rev $\mu g^{-1}$.

Table IV-11. Mutagenic Activities of HPLC Fractions (MeOH-program) of $CH_2Cl_2$ Eluate from Silicic Acid Open-column Chromatography of an Ambient Particulate Extract

| 80% of Sample | | 20% of Sample | |
|---|---|---|---|
| Fraction # | Net Revertants | Fraction # | Net Revertants |
| 1 | 362 | 1 | 213 |
| 2 | 1727[a] | 2 | 459 |
| 3 | 441 | 3 | 52 |
| 4 | 1193[a] | 4 | 150 |
| 5 | 2050[a] | 5 | 289 |
| 6 | 1699[a] | 6 | 240 |
| 7 | 1155[a] | 7 | 241 |
| 8 | 1424[a] | 8 | 374 |
| 9 | 3362[a] | 9 | 2913[a] |
| 10 | 794[b] | 10 | 763 |
| 11 | 215[c] | 11 | 450 |
| 12 | 335 | 12 | 159 |
| 13 | 348 | 13 | 119 |
| 14 | 348 | 14 | 104 |
| SUM | 15,453 | SUM | 6,526 |
| % Recovery[d] | 21% | % Recovery[d] | 35% |

[a]Above 1000 revertants colonies, the colony count is less accurate due to saturation of the colony counter response.
[b]Moderate toxicity was observed.
[c]Heavy toxicity was observed.
[d]Based on the specific activity of the $CH_2Cl_2$ open-column fraction of 92 rev $\mu g^{-1}$.

showed no significant direct activity. Fraction #2, in which the mononitro-PAH elute, had ~14% of the total activity recovered from the HPLC column. This is consistent with our previous quantifications of nitrofluoranthenes and nitropyrenes and calculations (based on the specific activities of the individual isomers) that they accounted for ≤10% of the total ambient particulate mutagenicity. Dinitro-PAH most probably would elute in Fraction #3. As observed previously, the majority of the mutagenic activity resides in an HPLC fraction (Fraction #4) more polar than those containing the mono- and di-nitro-PAH.

After completing the ambient fractionation, the HPLC elution times of a number of standard compounds were determined using the MeOH-program. The elution times of the mononitro-PAH and several ortho-substituted hydroxynitro-PAH (including 1-hydroxy-2-nitropyrene) corresponded to the ambient Fraction #2. The elution times of 1-hydroxy-4-nitronaphthalene, 3-hydroxy-6-nitrobiphenyl, and three non-ortho-substituted hydroxynitro-pyrenes (1,3-, 1,6-, and 1,8-hydroxynitropyrenes) corresponded to the ambient Fraction #11. Thus, the MeOH-program separated the hydroxynitro-PAH over a number of fractions. As seen from Table IV-11, the most mutagenic fraction was Fraction #9.

Preliminary chemical analysis by GC/MS of Fraction #9 and several other fractions from the MeOH-program HPLC fractionation indicated that more material would be required for compound identification. 560 Mg of the crude $CH_2Cl_2$ extract was fractionated on silicic acid columns and further fractionated by HPLC. Only the MeOH-program was used since the separation achieved on this program was judged preferable. The 34-43 min HPLC eluant (corresponding to Fraction #11 in Table IV-11 and for which toxicity was observed in the mutagen assay) was subdivided into three 3 min fractions, resulting in a total of 16 fractions for mutagenicity assay and chemical analysis.

2. Second Fractionation (560 mg)

Silicic Acid Open-column Chromatography. As noted above, the $CH_2Cl_2$ fraction from the silicic acid open-column chromatography may not contain the non-ortho substituted isomers of the hydroxynitropyrenes quantitatively. Therefore, in addition to eluting the silicic acid with pentane, $CH_2Cl_2$ and methanol, a fourth solvent mixture of 5% methanol in $CH_2Cl_2$ was added prior to the 100% methanol elution. It was hoped that a

small fraction of intermediate polarity material could be eluted with the 5% methanol in $CH_2Cl_2$ with the large mass of very polar compounds still eluting in the 100% methanol fraction. As seen from Table IV-12, even 5% methanol in $CH_2Cl_2$ eluded a very large mass. Clearly, if in the future any attempt is made to quantify the hydroxynitropyrenes present in the ambient particles, deuterated internal standards will be required to correct for losses during analysis.

Table IV-12. Mass and Mutagenicity Balance of Silicic Acid Open-Column Fractions (Second Fractionation: 560 mg)

| TA98 Mutagenicity Kado Protocol | Mass (mg) | Specific Activity (rev $\mu g^{-1}$) | | Total Revertants |
|---|---|---|---|---|
| | | 1/16/89 | 1/23/89 | |
| $CH_2Cl_2$ crude extract | 560 | 8.1 | 21 | $1.2 \times 10^7$ |
| 2-Nitrofluorene standard | | 32,000 | 38,000 | |
| Eluting Solvent | | | | |
| Pentane | 17.3 | 0 | 0 | |
| $CH_2Cl_2$ | 135.8 | 17 | 60 | $8.1 \times 10^6$ |
| 5% Methanol in $CH_2Cl_2$ | 346.0 | 2.9 | 5.3 | $1.8 \times 10^6$ |
| Methanol | 21.3 | 0.4 | 1.0 | $2.1 \times 10^4$ |
| Totals | 520.4 | | | $9.9 \times 10^6$ |

The recovery of mass from the silicic acid open-column was good (93%). The specific activity (rev $\mu g^{-1}$) of the $CH_2Cl_2$ silicic acid open-column fraction as tested on January 16, 1989 was much lower than the value previously obtained for the corresponding fraction from our first

extract fractionation (92 rev $\mu g^{-1}$). The fractions were retested on January 23, 1989. The specific activity of the crude $CH_2Cl_2$ extract and the silicic acid column fractions are given in Table IV-12 for both tests. The January 23 test values are more similar to the activity we expected based on our first fractionation and this value has been used to determine the mutagenicity balance given in Table IV-12. A problem with the incubator humidity control may have caused these discrepancies. Previously, the sum of the mutagenicity of the silicic acid column fractions was greater than that of the crude extract applied to the column. The sum of the activities of the fractions given in Table IV-12 is ~80% of the mutagenicity estimated to have been applied to the columns.

As observed previously, the majority of the mutagenicity was in the $CH_2Cl_2$ eluant from the silicic acid column, and only this fraction was further fractionated by HPLC, as detailed below.

HPLC Fractionation (Level-One). Prior to this large scale (560 mg) fractionation, the HPLC column was cleaned with solvent rinses, and the MeOH program run twice with fourteen fractions collected each time. These "blank" fractions from the first collection showed low levels of mutagenic activity in some fractions and toxicity in others. The second MeOH program blanks showed acceptably low levels of activity in the Kado test and we, therefore, proceeded with the ambient sample fractionation.

The number of HPLC fractions collected was increased from 14 to 16 by subdividing the 34-43 min HPLC eluant into three fractions. This was done because of the high toxicity in the mutagenicity assay previously observed for the 34-43 min eluant. Figure IV-5 shows the solvent program used and the designated fractions collected. One hundred twenty milligrams of the 135 mg $CH_2Cl_2$ fraction from the silicic acid column was injected onto the semi-preparative HPLC column in five aliquots. The sixteen corresponding fractions from the five HPLC runs were combined and 0.2% of each fraction was taken for mutagenicity testing. The sixteen fractions were tested at doses of 1, 9 and 90% of the sample. Figure IV-6 shows the mutagenicity "chromatogram," that is, the net revertants in 0.2% of each HPLC fraction. Summing the activity in the sixteen HPLC fractions, ~31% of the activity applied to the HPLC column was recovered. This value agrees well with our previous fractionation, as does the profile of the activity.

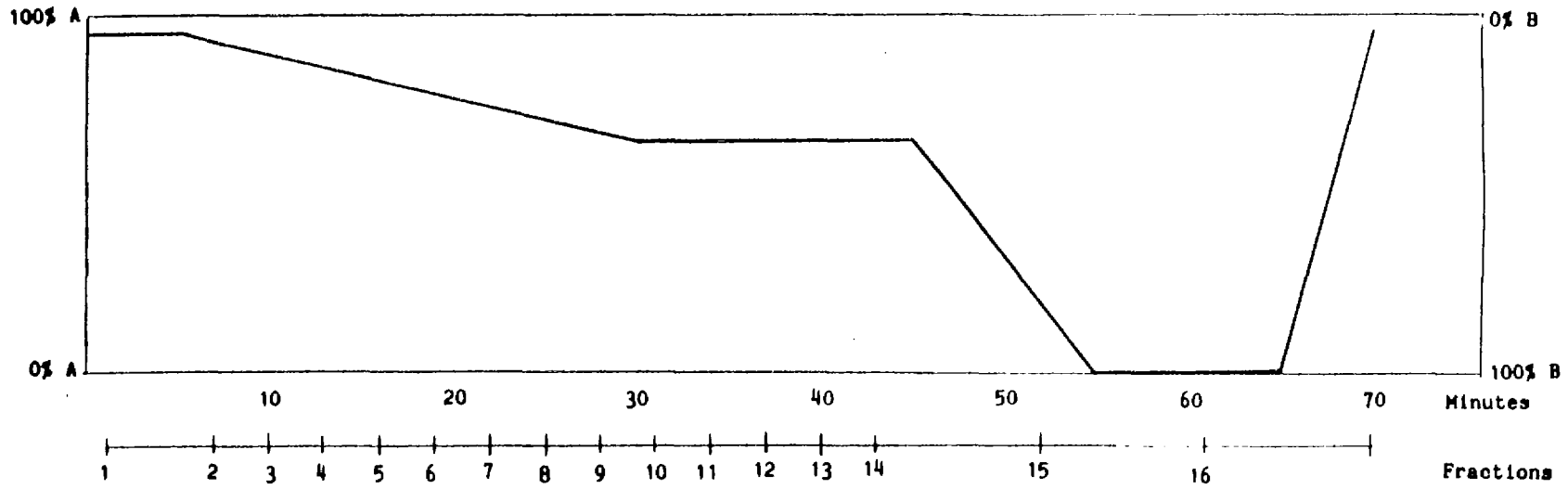



Figure IV-5. HPLC MeOH-program used for sample fractionation on an Altex semi-preparative Ultrasphere Silica column (1 cm x 25 cm). The sixteen fractions collected for mutagenicity assay and chemical analysis are given below the mobile phase program. Mobile phase A is hexane and mobile phase B is a mixture of $CH_2Cl_2$/methanol (98/2, v/v).

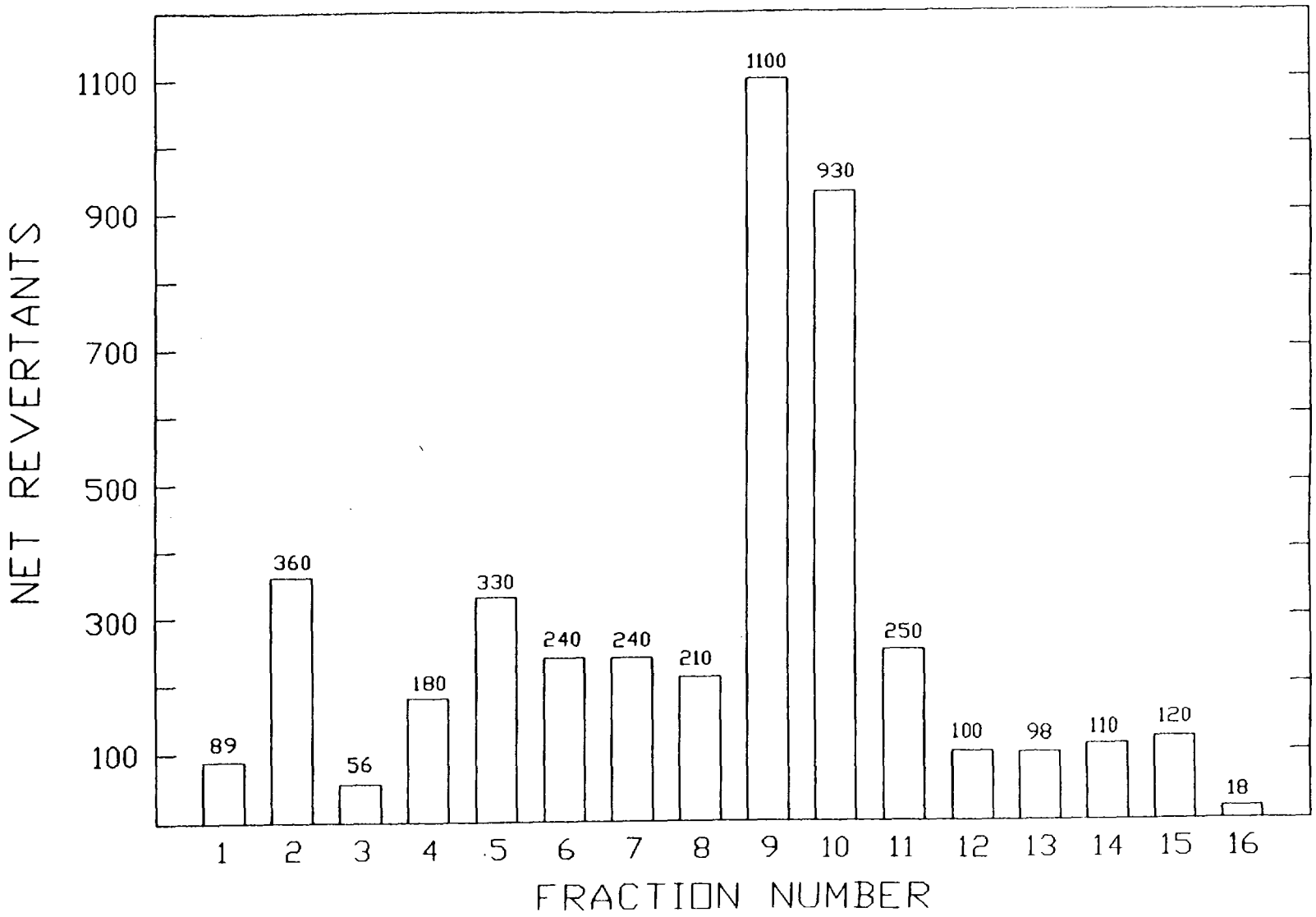



Figure IV-6. Calculated net revertants in 0.2% of each of the sixteen level one HPLC fractions collected as shown in Figure IV-5.

Fraction #9 was once again the most mutagenic fraction. Dividing the previous Fraction #10 (34-43 min HPLC eluant) into three, three-minute fractions (Fraction #10, 11 and 12 of Figure IV-6) apparently reduced the toxic effects and Fraction #10 (Figure IV-6) was nearly as potent as Fraction #9.

HPLC/MS. The instrumentation for liquid chromatography/mass spectrometry (LC/MS) in the Chemistry Department of the University of California was used for chemical analysis of the two most highly mutagenic level-one fractions, Fractions #9 and #10, of our ambient particulate extract. These level-one fractions were still quite complex and only oxygenated PAH-derivatives and azaarenes were tentatively identified in them. To our knowledge, however, no potent direct-acting mutagens which are oxygenated PAH-derivatives (that is, without any additional functional groups such as a nitro- group) have been reported.

HPLC Fractionation (Level-Two). Since GC/MS and HPLC/MS analysis of Fractions #9 and #10 showed many components at low levels, an attempt was made to further isolate the mutagen(s), by additional HPLC fractionation (Level-Two Fractionation). Two different reverse-phase HPLC columns were tried for the level two fractionation, a semi-preparative ODS column and a semi-preparative Vydac column.

The solvent program and fractions collected for the Vydac column level-two fractionation of the level-one Fractions #9 and #10 were: at 2 mL $min^{-1}$ flow; 50% $CH_3CN$, 50% $H_2O$ for 15 min; programmed to 100% $CH_3CN$ over 5 min; held at 100% $CH_3CN$ for 20 min, programmed over 5 min back to 50% $CH_3CN$, 50% $H_2O$ and held at this composition for an additional 5 min. Four fractions were collected from: 1-15 min, 15-21 min, 21-35 min and 35-50 min. Three percent of each fraction was submitted to mutagen assay and tested at doses of 1, 9 and 90% of the sample. The mutagenic activity of the four level-two fractions are shown in Figure IV-7 and the total revertants per fraction and the mutagenicity balance are given in Table IV-13. The recovery of the activity placed on the Vydac column was good, being 64% for the level-one Fraction #9 (HPLC I, Fraction #9) and 89% for the level-one Fraction #10 (HPLC I, Fraction #10).

The solvent program and fractions collected for the ODS column level- two fractionation were: at 2 mL $min^{-1}$; 80% $CH_3OH$, 20% $H_2O$ for 20 min; programmed to 100% $CH_3OH$ over 5 min; held at 100% $CH_3OH$ for 15 min;

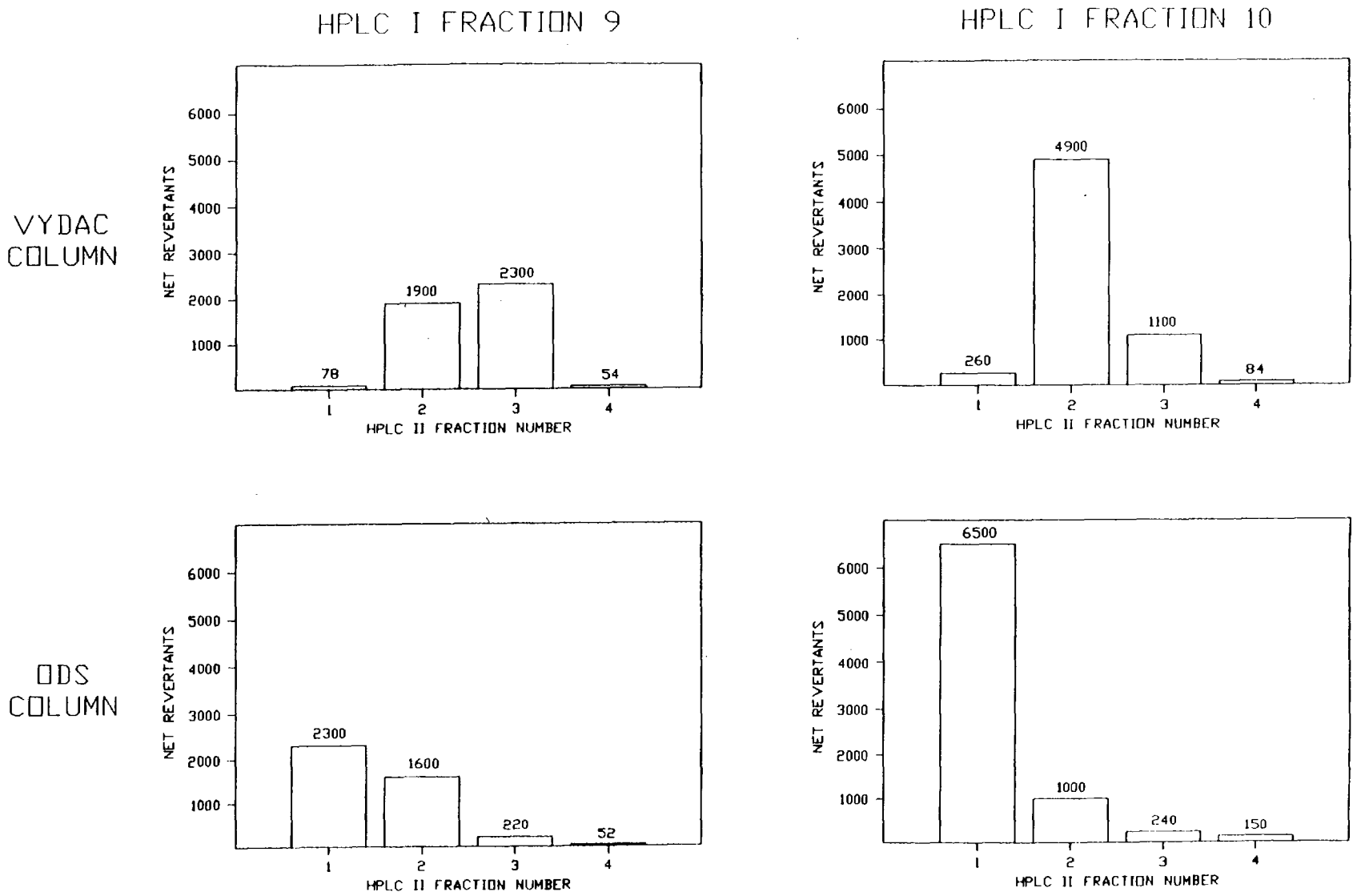



Figure IV-7. Net revertants for the level two fractionation of the level one Fractions #9 and #10 separated on a Vydac semi-preparative HPLC column (upper) and on an ODS semi-preparative HPLC column (lower).

Table IV-13. Mutagenicity of the Level-Two Fractionations of the Level-One Fractions #9 and #10

| Column | Fraction Number | Total Revertants Per Fraction | |
|---|---|---|---|
| | | HPLC I Fraction #9 | HPLC I Fraction #10 |
| Vydac | 1 | 78 | 260 |
| | 2 | 1900 | 4900 |
| | 3 | 2300 | 1100 |
| | 4 | 54 | 84 |
| | SUM | 4332 | 6344 |
| | CALCULATED | 6750 | 7125 |
| | RECOVERY | 64% | 89% |
| ODS | 1 | 2300 | 6500 |
| | 2 | 1600 | 1000 |
| | 3 | 220 | 240 |
| | 4 | 52 | 150 |
| | SUM | 4172 | 7890 |
| | CALCULATED | 6750 | 7125 |
| | RECOVERY | 62% | 111% |

programmed over 5 min back to 80% $CH_3OH$, 20% $H_2O$ and held isocratic for 5 min. Four fractions were collected from: 1-13 min, 13-20 min, 20-33 min and 33-50 min. Again, 3% of each fraction was assayed for mutagenic activity at 1, 9 and 90% of the sample. The activity of the fractions are shown in Figure IV-7 and the total revertants per fraction and the mutagenicity balance are given in Table IV-13. The recovery of the activity for the level-one Fraction #9 was similar for the two columns, while the activity recovered for level-one Fraction #10 was 100% (within the normal test variations) from the ODS column.

HPLC/MS of Level-Two Fractions. Prior to analyzing the ambient extract fractions, LC/MS analysis of a standard mixture was carried out to attempt to optimize the LC/MS system. As detailed below, the LC/MS system with plasma spray showed disappointingly low sensitivity. Figure IV-8 shows the separation by HPLC of a standard mixture containing 1-hydroxy-4-nitronaphthalene, two hydroxynitrobiphenyls, 3-hydroxyfluoranthene, 1-hydroxypyrene and two hydroxynitropyrenes (the 2 μL injected contained approximately 200 ng of each component). A Vydac (201TP5415/840919, 15 cm x 4.6 mm i.d.) reverse-phase HPLC column was used with a 1 mL $min^{-1}$ flow rate. The analysis was isocratic for 7 min (50% acetonitrile: 50% water), then programmed to 100% acetonitrile over 7 min. A similar column (201TP5415/870420) was taken to the UCR Chemistry Mass Spectrometry Facility for use in the LC/MS analysis employing the same solvent flow rate and program.

Since our standards would be expected to produce a high signal in the negative ion mode (the compounds being good electron capturing species), and additionally, in the positive ion mode, the background with the LC/MS solvent flowing was generally quite high, negative ion spectra were taken. Figure IV-9 shows the total ion current (TIC) from an injection of 10 μL of the standard solution (approximately 1 μg each component). Shown beneath the TIC are the mass chromatograms for m/z 188, 214, 217 and 262, corresponding to $[M-H]^-$ for 1-hydroxy-4-nitronaphthalene, the hydroxynitrobiphenyls, the hydroxyfluoranthene and hydroxypyrene, and the hydroxynitropyrenes, respectively. The resolution of the isomer pairs (i.e., the hydroxynitrobiphenyls, hydroxyfluoranthene and hydroxypyrene, and the hydroxynitropyrenes) seen in Figure IV-8 is not apparent in the LC/MS analysis. Figures IV-10 and IV-11 show the full spectra of the LC/MS peaks. The spectra all exhibit high $[M-H]^-$ ions and relatively little fragmentation. When methanol was substituted for acetonitrile as the solvent entering the mass spectrometer, only $[M]^-$ ions were observed for each of the standard compounds. It was not considered worthwhile to pursue LC/MS analysis of the level-two fractionation samples and the remaining sample was stored for future analysis (see Section X).

At this point in the program, the approach of bioassay-directed fractionation of collected ambient air particulate samples coupled with chemical identification did not appear to be fruitful in leading to the

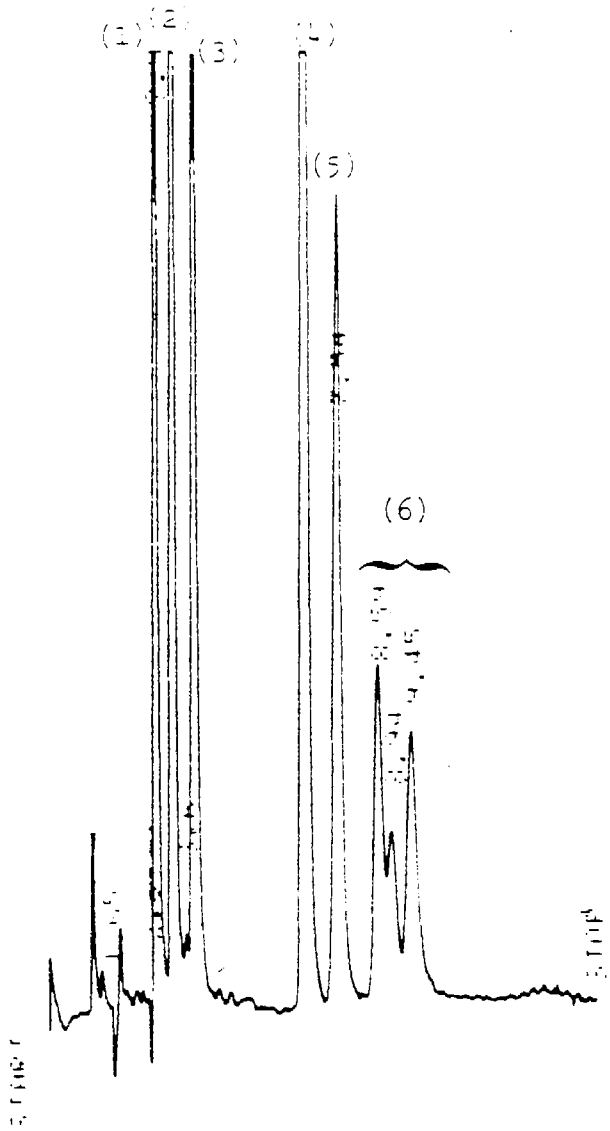



Figure IV-8. HPLC trace (UV 254 nm) of a standard mixture of hydroxy-nitroarenes: (1) 1-hydroxy-4-nitronaphthalene, (2) 3-hydroxy-6-nitrobiphenyl, (3) 2-hydroxy-5-nitrobiphenyl, (4) 3-hydroxyfluoranthene, (5) 1-hydroxypyrene, (6) hydroxy-nitropyrenes.

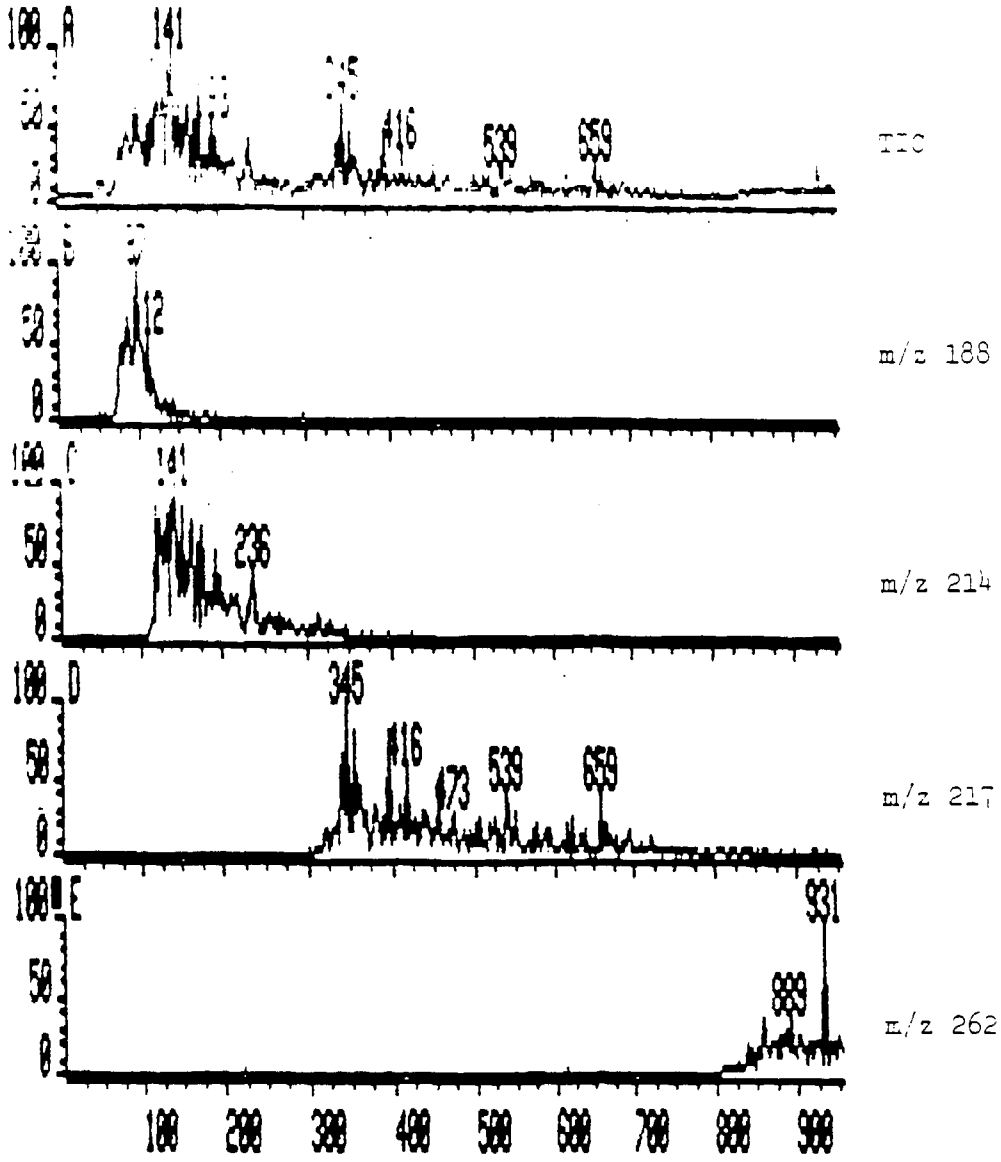



Figure IV-9. LC/MS traces from analysis of the standard mixture shown in Figure IV-8. A: Total ion chromatogram (TIC), B, C, D, and E: Mass chromatograms of m/z 188, 214, 217, and 262, respectively.

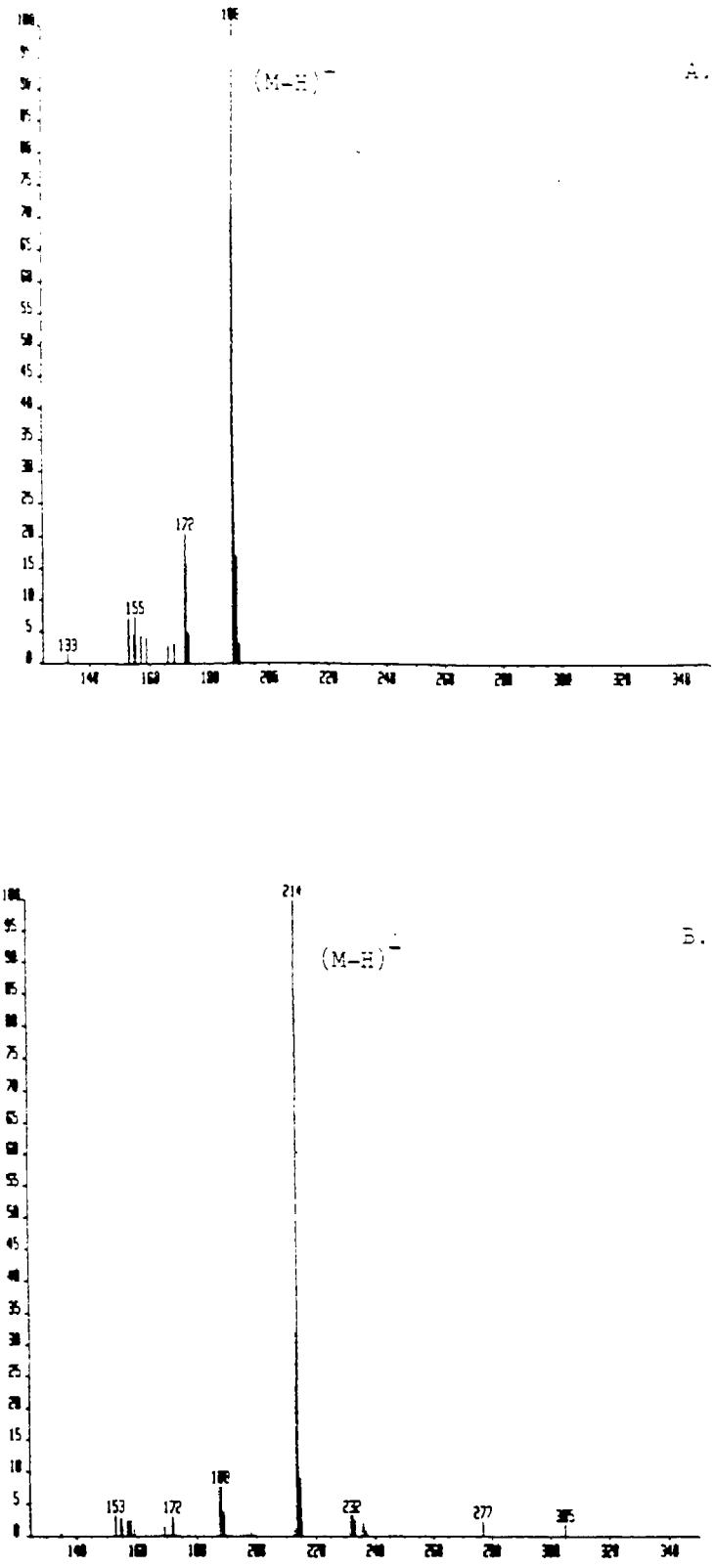



Figure IV-10. Negative ion mass spectra from LC/MS analysis of: (A) 1-hydroxy-4-nitronaphthalene, (B) hydroxynitrobiphenyls.

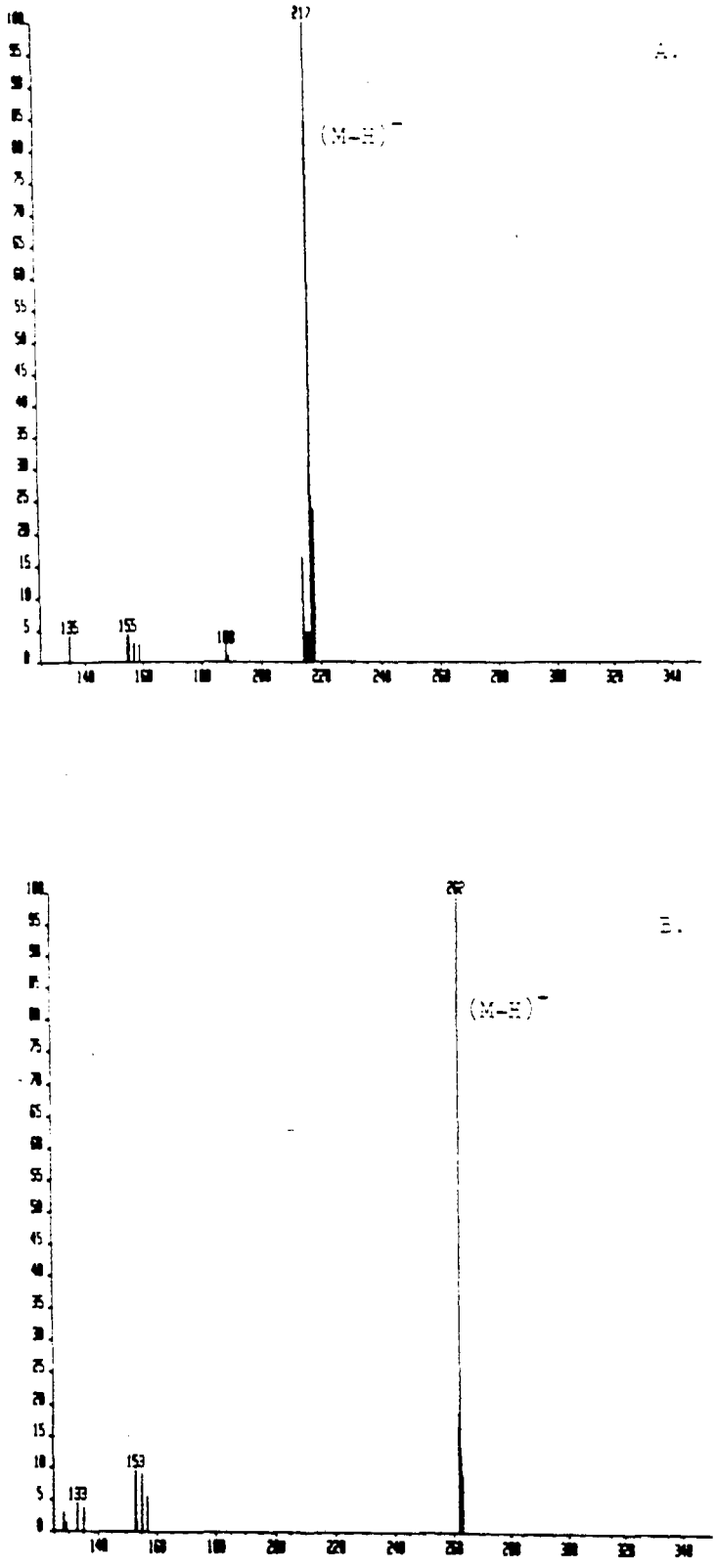



Figure IV-11. Negative ion mass spectra from LC/MS analysis of (A) hydroxyfluoranthene/hydroxypyrene, (B) hydroxynitropyrenes.

identification of new ambient mutagenic species. This conclusion, together with our determination that the hydroxynitro-PAH exhibit generally low mutagenic activities and appear in several HPLC fractions (rather than in one or two discrete fractions as is the case for the mutagenicity of extracts of ambient air particulate matter), led us to change the emphasis of the program.

## V. MUTAGENICITY TESTING AND CHEMICAL ANALYSIS OF HYDROXYNITRO-PAH

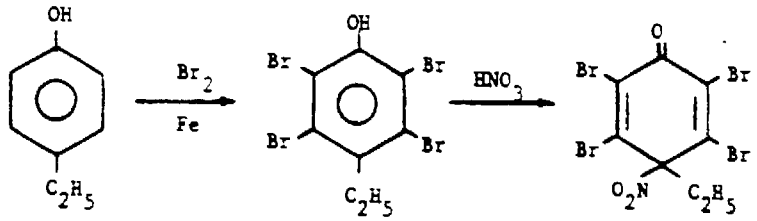
As noted in Section III, during the initial stages of this program, we anticipated that the hydroxynitro-PAH would be important products of the gas-phase reactions of the 2-4 ring PAH, and that they may be responsible for a significant portion of the observed direct-acting mutagenicity of the extracts of collected ambient air particulate matter. Accordingly, chemical characterization and mutagenicity testing of a series of hydroxynitro-PAH were undertaken. Since only a few hydroxynitro-PAH are commercially available, a number of hydroxynitro-PAH were synthesized. Specifically, 4-hydroxy-3-nitrobiphenyl was obtained from Lancaster Synthesis, Inc., 1-hydroxy-2-nitronaphthalene (95% stated purity) was obtained from Aldrich Chemical Company, and 2-hydroxy-1-nitronaphthalene was obtained from American Tokyo Kasei. 3-Hydroxy-2-nitrofluoranthene had previously been synthesized during our investigations of the products and mechanisms of the solution-phase reactions of fluoranthene with $N_2O_5$ (Zielinska et al., 1986). Eight other hydroxynitro-PAH were synthesized. The synthesis, $^1H$ NMR spectra, EI mass spectra, GC-MS analysis, and mutagenicity testing of these hydroxynitro-PAH are described in the sections below.

### A. Synthesis and Characterization of Hydroxynitro-PAH

Five hydroxynitrobiphenyls, two nitronaphthols, a hydroxynitrophenanthrene, a hydroxynitropyrene and a hydroxynitrofluoranthene were synthesized and fully characterized. For these syntheses, a method described by Lemaire et al. (1987) was used, which employed tetrabromonitrocyclohexadienone to induce selective nitration of phenols or naphthols:

This reaction was reported to produce nitrophenols and nitronaphthols with nitro-substitution in the ortho- and para-positions, with good overall yields (Lemaire et al., 1987).

Reagent Synthesis. 4-Nitro-4-ethyl-2,3,5,6-tetrabromo-2,5-cyclohexadiene-1-one was synthesized by bromination of 4-ethylphenol followed by nitration of the resulting 4-ethyl-2,3,5,6-tetrabromophenol:




680 mmoles (35 mL) of bromine was added dropwise over a period of 5 hrs to a suspension of 150 mmoles (18.3 g) of 4-ethylphenol and 0.5 g of iron dust in 225 mL of $CCl_4$. The mixture was allowed to react for 24 hrs at ambient temperature, then heated to boiling and filtered. Upon cooling, 4-ethyl-2,3,5,6-tetrabromophenol precipitated as pale yellow crystals with a 72% yield (47 g).

A solution containing 4.2 mL of 70% nitric acid in 20 mL of glacial acetic acid was then added dropwise (over a period of 10 min) to a solution of 8.76 g (20 mmoles) of 4-ethyl-2,3,5,6-tetrabromophenol in 60 mL of glacial acetic acid at 10°C. The suspension was allowed to react for 2 hrs at 5°C and precipitated by adding 60 mL of water. The crystals were filtered out, washed with water and hexane, and dried under vacuum. The 4-nitro-4-ethyl-2,3,5,6-tetrabromo-2,5-cyclohexadien-1-one yield was 8.6 g (89%).

General Nitration Procedure. 4-Nitro-4-ethyl-2,3,5,6-tetrabromo-2,5-cyclohexadien-1-one (2.42 g, 5 mmoles) was added to 5 mmoles of suitable substrate in 40 mL of diethyl ether. The mixture was allowed to react for 2 hrs at ambient temperature. For the hydroxynitrobiphenyls and the hydroxynitronapththols, the ether was evaporated under vacuum and 20 mL of a methanol/water (80/20) mixture was added. The 4-ethyl-2,3,5,6-tetrabromophenol was filtered out and saved. The solvent was then evaporated and the crude nitroproducts were purified by chromatography on a silica column with hexane/ethyl acetate elution. For the ortho-substituted hydroxynitrophenanthrene, hydroxynitrofluoranthene and hydroxynitropyrenes, the hydroxynitro-PAH precipitated from solution and were filtered

out and purified by chromatography. Since various amounts of brominated hydroxyaromatics were also formed, a second purification by open column chromatography or semi-preparative high performance liquid chromatography was necessary in some cases to obtain the hydroxynitro-PAH in pure form. Table V-1 shows the hydroxybiphenyls and naphthols nitrated by this procedure and the resulting hydroxynitro-PAH obtained from these reactions.

Hydroxynitroarene Characterization. In all, 12 hydroxynitro-PAH were characterized by $^{1}H$ NMR (Nicolet 300 pulsed Fourier transform NMR spectrometer) and by gas chromatography/mass spectrometry (GC/MS) [Hewlett-Packard 5890 GC with a 30 m DB-5 column interfaced to a HP 5970 mass selective detector]. Tables V-2, V-3 and V-4 show the $^{1}H$ NMR chemical shifts for the hydroxynitrobiphenyls, nitronaphthols, and other hydroxynitro-PAH, respectively.

Co-injection of the three nitronaphthols produced the total ion chromatogram (TIC) shown in Figure V-1. As apparent from this TIC, 1-nitro-2-naphthol and 2-nitro-1-naphthol were not resolved on the 30 m DB-5 column with the temperature program employed. Additionally, despite the use of cool on-column injection, 4-nitro-1-naphthol decomposed somewhat during the analysis (as evidenced by the broad peak preceding the 4-nitro-1-naphthol peak). Figure V-2 gives the electron impact (EI) mass spectra for the three nitronaphthols. 1-Nitro-2-naphthol and 2-nitro-1-naphthol were injected individually to obtain the spectra shown (note the differences in the fragmentation patterns of these co-eluting isomers).

Figure V-3 shows the TIC from the GC/MS analyses of a mixture of the six hydroxynitrobiphenyls. As seen from this TIC, all isomers were well resolved. Figures V-4 and V-5 give the EI mass spectra of these hydroxynitrobiphenyls.

## B. Mutagenicity Testing of Hydroxynitro-PAH

The mutagenicities of 10 hydroxynitro-PAH were determined in the standard Ames Salmonella plate-incorporation assay using TA98 without S9 metabolic activation. The compounds, three hydroxynitronaphthalenes, six hydroxynitrobiphenyls, and one hydroxynitrofluoranthene, were first tested in a preliminary test using the Kado microsuspension modification in order to estimate the activities without expending a great amount of each

Table V-1. Hydroxynitrobiphenyl and Nitronaphthol Synthesis: Structures of Substrates and Products

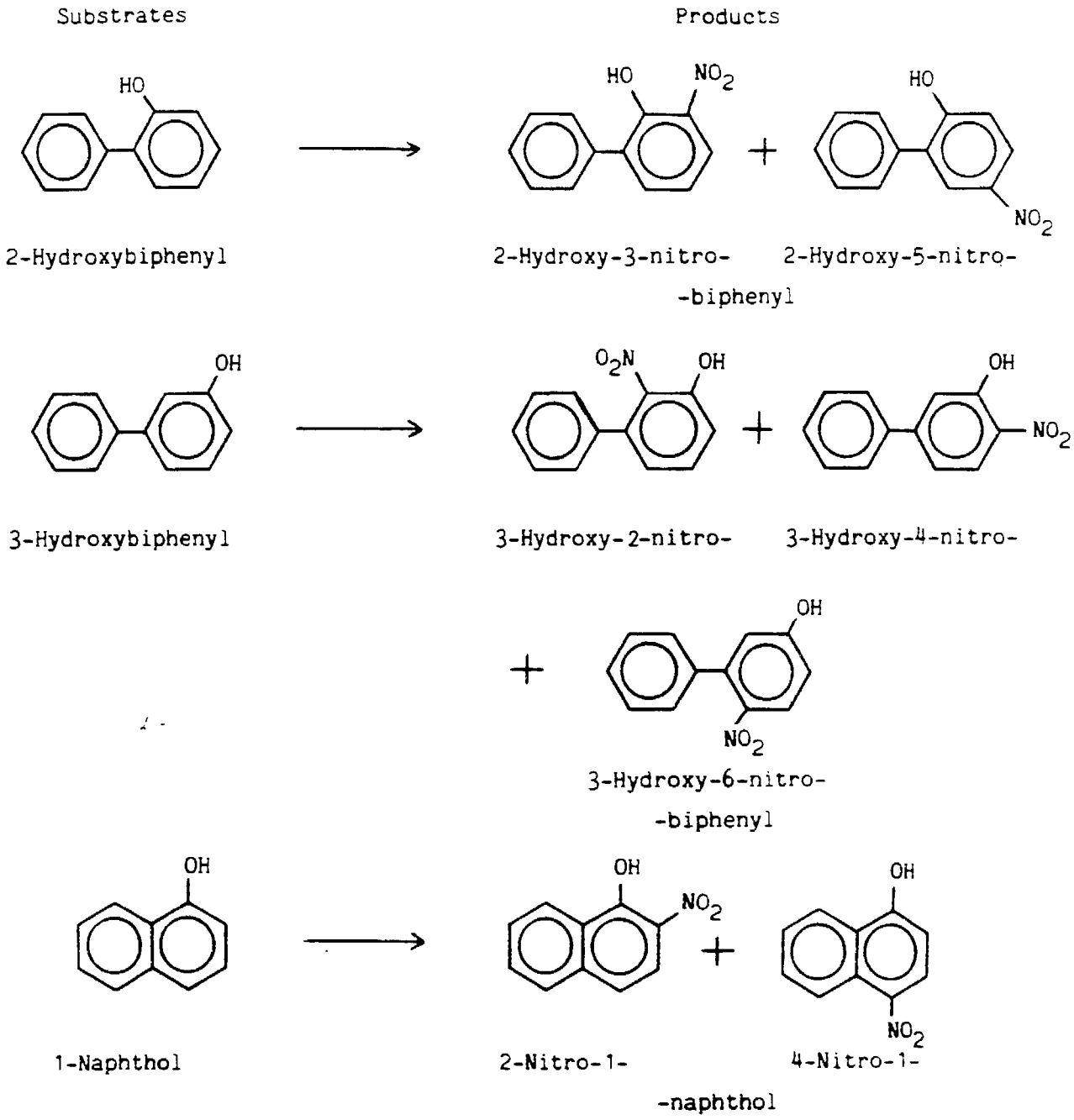
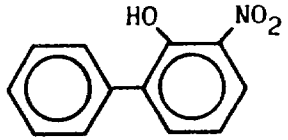
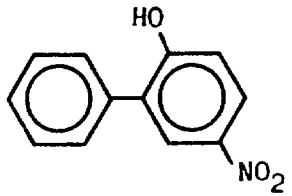
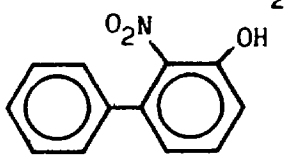
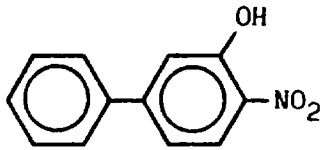
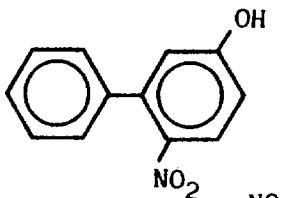
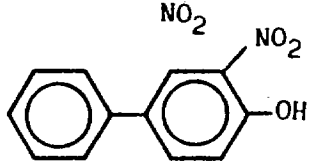
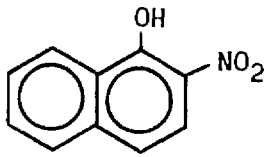
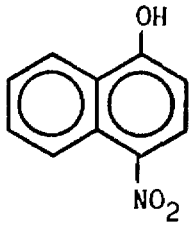
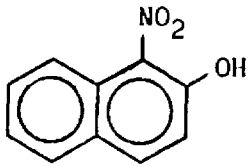


Table V-2. Chemical Shifts (in ppm)[a,b] and Coupling Constants (in Hz) of Hydroxynitrobiphenyls

| Compound | H-2 | H-3 | H-4 | H-5 | H-6 | H'[c] |
|---|---|---|---|---|---|---|
|  | -- | -- | 8.33(d)<br>$J_{4,5}$=8.9Hz | 7.3(t) | 7.86(d)<br>$J_{5,6}$=7.6Hz | 7.55-7.76(m) |
|  | -- | 7.2(d)<br>$J_{3,4}$=8.9Hz | 8.28(dd) | -- | 8.56(d)<br>$J_{4,6}$=3Hz | 7.5-7.8(m) |
|  | -- | -- | 7.07(d)<br>$J_{4,5}$=7.8Hz | ~7.55(m) | 7.18(d)<br>$J_{5,6}$=8.3Hz | 7.5-7.6(m) |
|  | 7.57(d)<br>$J_{2,6}$=~2Hz | -- | -- | 8.34(d)<br>$J_{5,6}$=8.8Hz | ~7.9(m) | 7.45-7.75(m) |
|  | 6.94(d)<br>$J_{2,4}$=2.7Hz | -- | 7.06(dd)<br>$J_{2,4}$=2.6Hz | 8.08(d)<br>$J_{4,5}$=9.1Hz | -- | 7.4-7.62(m) |
|  | 8.48(d)<br>$J_{2,6}$=2.2Hz | -- | -- | 7.42(d)<br>$J_{4,5}$=8.7Hz | 8.06(dd) | 7.4-7.8(m) |

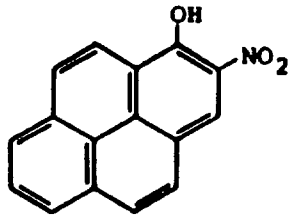
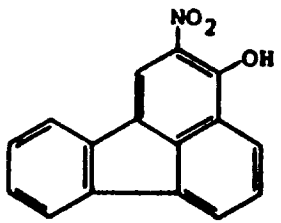
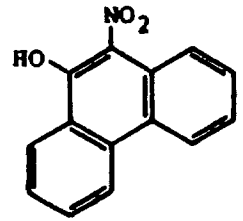
[a] $CD_3OD$ was used as a solvent.
[b] (d) = doublet, (dd) = doublet over doublet, (t) = triplet, (m) = multiplet.
[c] The five protons in the unsubstituted ring.

Table V-3. Chemical Shifts (in ppm)[a,b] and Coupling Constants (in Hz) of Nitronaphthols

| Compound | H-2 | H-3 | H-4 | H-5 | H-6 | H-7 | H-8 |
|---|---|---|---|---|---|---|---|
|  | -- | 8.71(d)<br>$J_{3,4}$=8.2Hz | 8.08(d) | 7.63(d)<br>$J_{5,6}$=9.3Hz | 7.93(t) | 7.84(t) | 8.23(d)<br>$J_{7,8}$=9.4Hz |
|  | 6.92(d)<br>$J_{2,3}$=8.6Hz | 8.59(m) | -- | 9.02(d)<br>$J_{5,6}$=8.8Hz | 7.88(t) | 7.70(t) | 8.59(m) |
|  | -- | 7.42(d)<br>$J_{3,4}$=9Hz | 8.15(d) | 8.05(m) | 7.80(t) | 7.63(t) | 8.05(m) |

[a] $CD_3OD$ was used as a solvent
[b] (d) = doublet, (t) = triplet, (m) = multiplet.

Table V-4. $^1$H NMR (δ in ppm, J in Hz) and Mass Spectral Data for the Hydroxynitro-PAH Synthesized

| Compound | $^1$H NMR | MS m/z (rel. int. %) |
|---|---|---|
|  1-Hydroxy-2-nitro-pyrene | 11.58 (s, 1H, OH)<br>8.75 (s, 1H, H-3)<br>8.58 (d, 1H, H-10, J 9.1)<br>8.2 - 8.02 (m, 4H, H-6,7,8,9)<br>7.87 (m, 2H, H-4,5) | 263 $[M]^+$ (100); 246 $[M-OH]^+$ (21);<br>217 $[M-NO_2]^+$ (24); 216 $[M-HNO_2]^+$ (45);<br>190(6); 189 $[M-NO_2-CO]^+$ (46); 188 $[M-HNCO_3]^+$ (90);<br>187 $[M-H_2NCO_3]^+$ (60); 186 (12) |
| 1-Hydroxy-2-nitro-pyrene-$d_8$ | — | 271 $[M]^+$ (100); 254 $[M-OH]^+$ (20);<br>253 $[M-OD]^+$ (3); 225 $[M-NO_2]^+$ (24);<br>224 $[M-HNO_2]^+$ (45); 223 $[M-DNO_2]^+$ (8);<br>198 (8); 197 $[M-NO_2-CO]^+$ (50);<br>196 $[M-HNCO_3]^+$ (93); 195 (31);<br>194 (58); 193 (17) |
|  3-Hydroxy-2-nitro-fluoranthene | 8.45 (s, 1H, H-1)<br>8.30 (d, 1H, H-4 or 6, J 8.2)<br>8.08 (d, 1H, H-6 or 4, J 7.1)<br>7.88 (m, 2H, H-7,10)<br>7.72 (t, 1H, H-5)<br>7.41 (m, 2H, H-8,9) | 263 $[M]^+$ (100); 233 $[M-NO]^+$ (14);<br>205 $[M-NO-CO]^+$ (6); 202 (36); 201 (11);<br>190 (12); 189 $[M-NO_2-CO]^+$ (28);<br>188 $[M-HNCO_3]^+$ (35);<br>187 $[M-H_2NCO_3]^+$ (41); 186 (7);<br>177 (6); 176 (12); 175 (8) |
|  9-Hydroxy-10-nitro-phenanthrene | 8.90 (d, 1H, H-4 or 1, J 8.7)<br>8.62 (m, 3H, H-1 or 4, H-5,8)<br>7.88 (t, 1H), 7.72 (m, 2H)<br>7.62 (t, 1H) | 239 $[M]^+$ (100); 223 $[M-O]^+$ (5);<br>222 $[M-OH]^+$ (9); 209 $[M-NO]^+$ (4);<br>206 $[M-HO_2]^+$ (6); 194 (6);<br>193 $[M-NO_2]^+$ (5); 181 $[M-NO-CO]^+$ (21);<br>178 (10); 177 (6); 168 (12); 167 (13);<br>166 (19); 165 $[M-NO_2-CO]^+$ (35);<br>164 $[M-HNCO_3]^+$ (36); 163 $[M-H_2NCO_3]^+$ (52);<br>162 (7) |

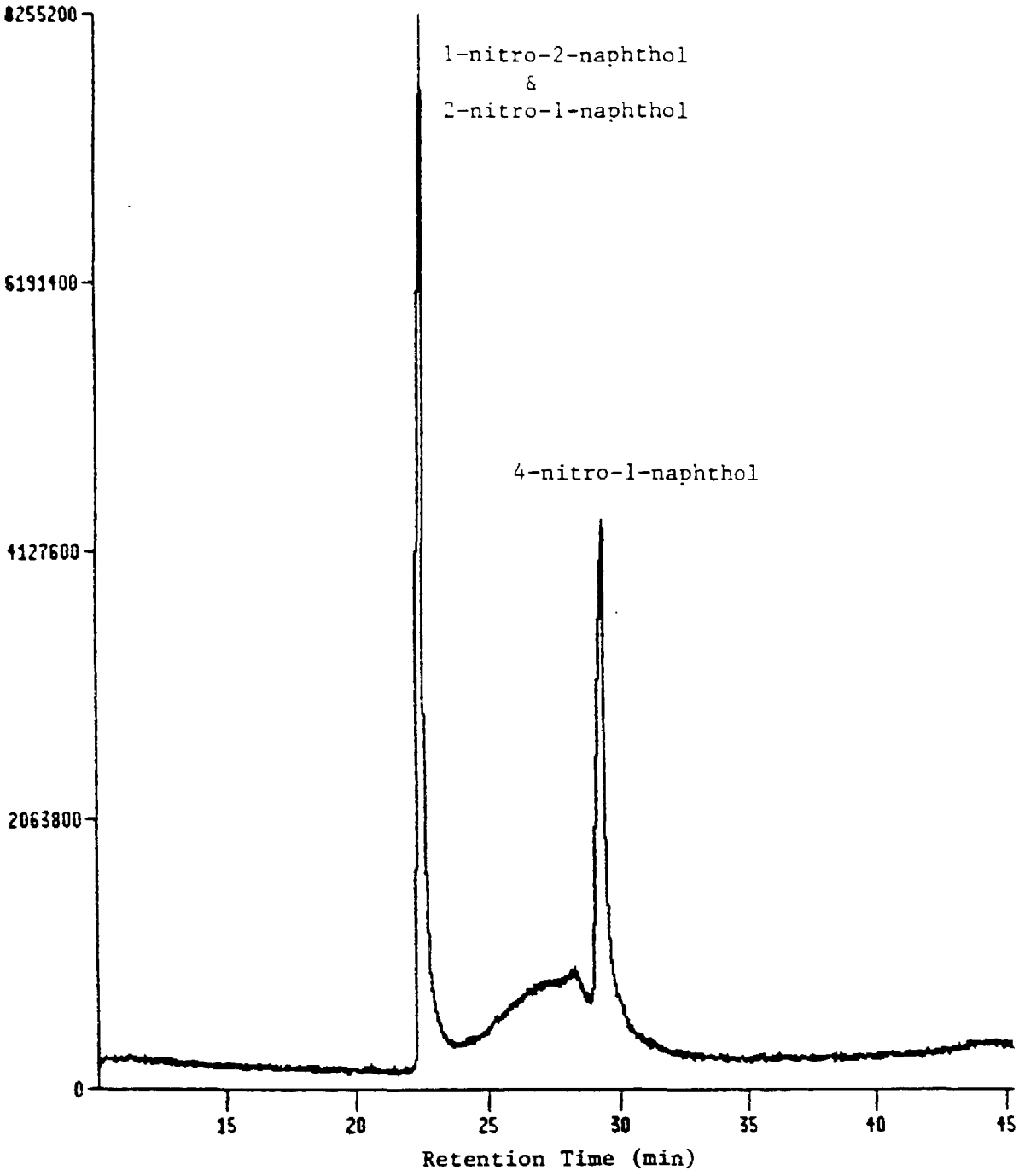



Figure V-1. TIC of the GC/MS analysis of three nitronaphthols. Column conditions: 30 m DB-5 column, cool on-column injection at 40°C then programmed at 6°C $min^{-1}$.

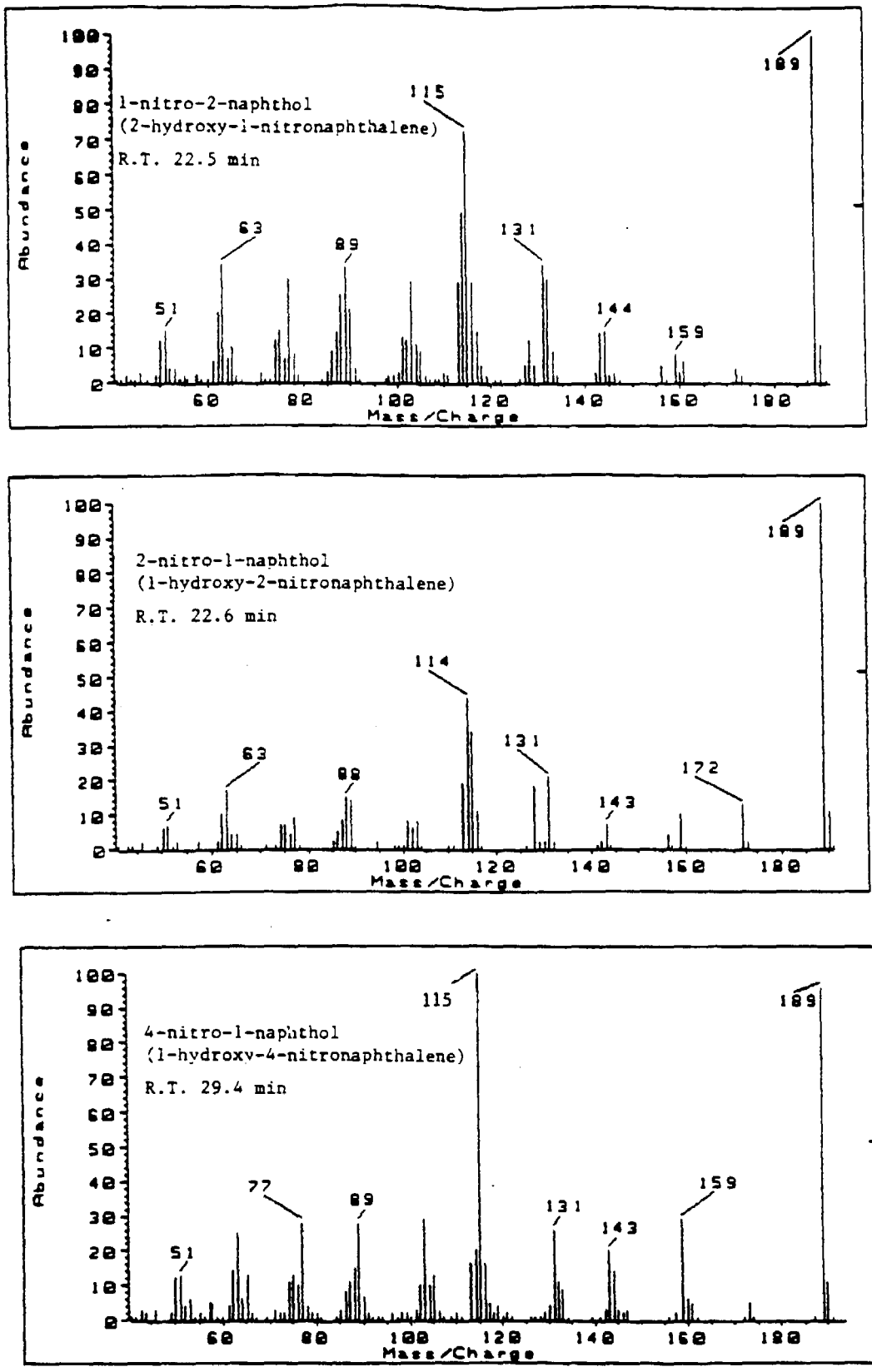



Figure V-2. Electron impact mass spectra of three nitronaphthols.

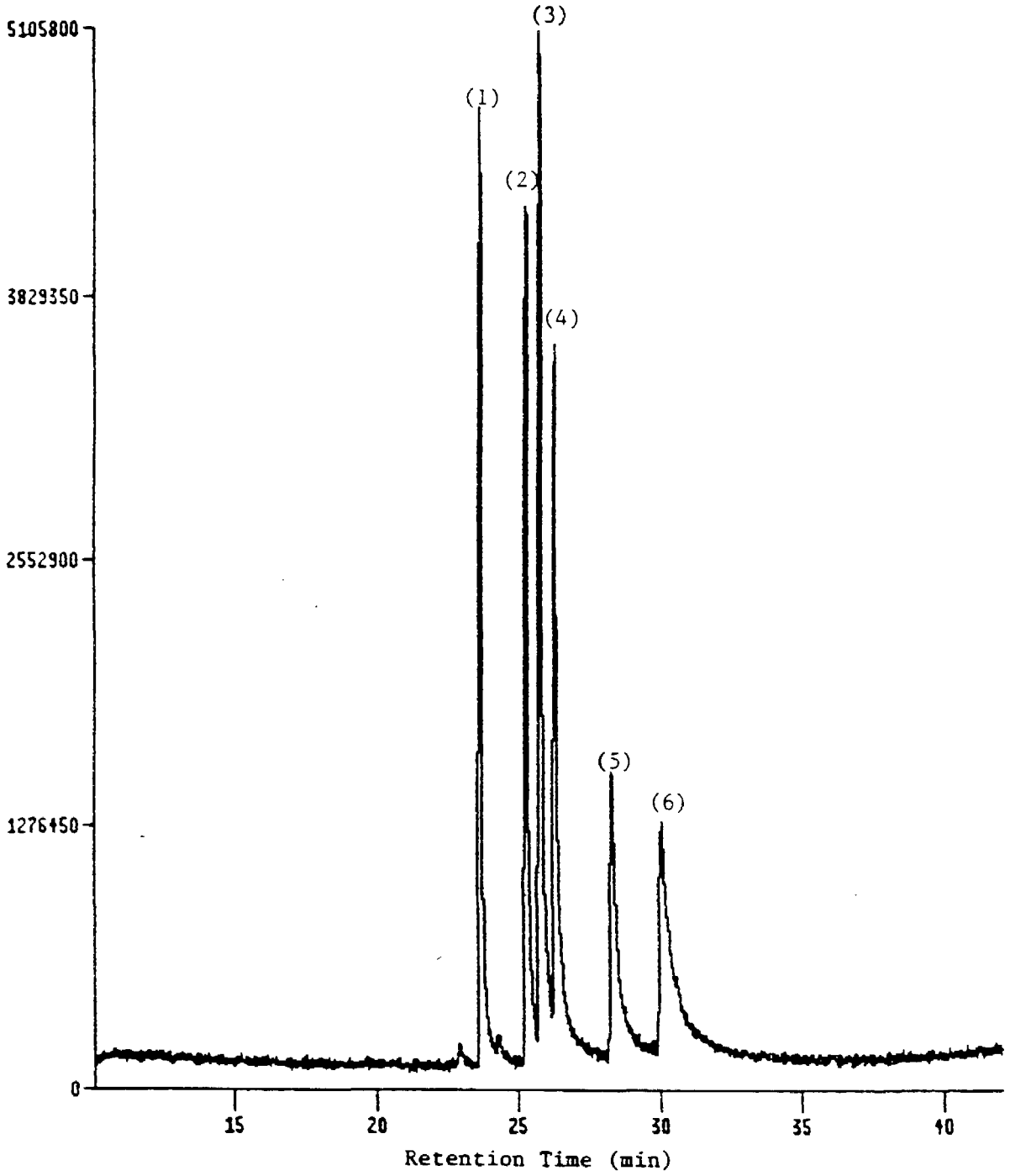



Figure V-3. TIC of the GC/MS analysis of a mixture of six hydroxynitrobiphenyls. The same column and temperature program as given if Figure V-1 were used. The peak identities are: (1) 3-hydroxy-2-nitrobiphenyl, (2) 2-hydroxy-3-nitrobiphenyl, (3) 4-hydroxy-3-nitrobiphenyl, (4) 3-hydroxy-4-nitrobiphenyl, (5) 2-hydroxy-5-nitrobiphenyl, (6) 3-hydroxy-6-nitrobiphenyl.

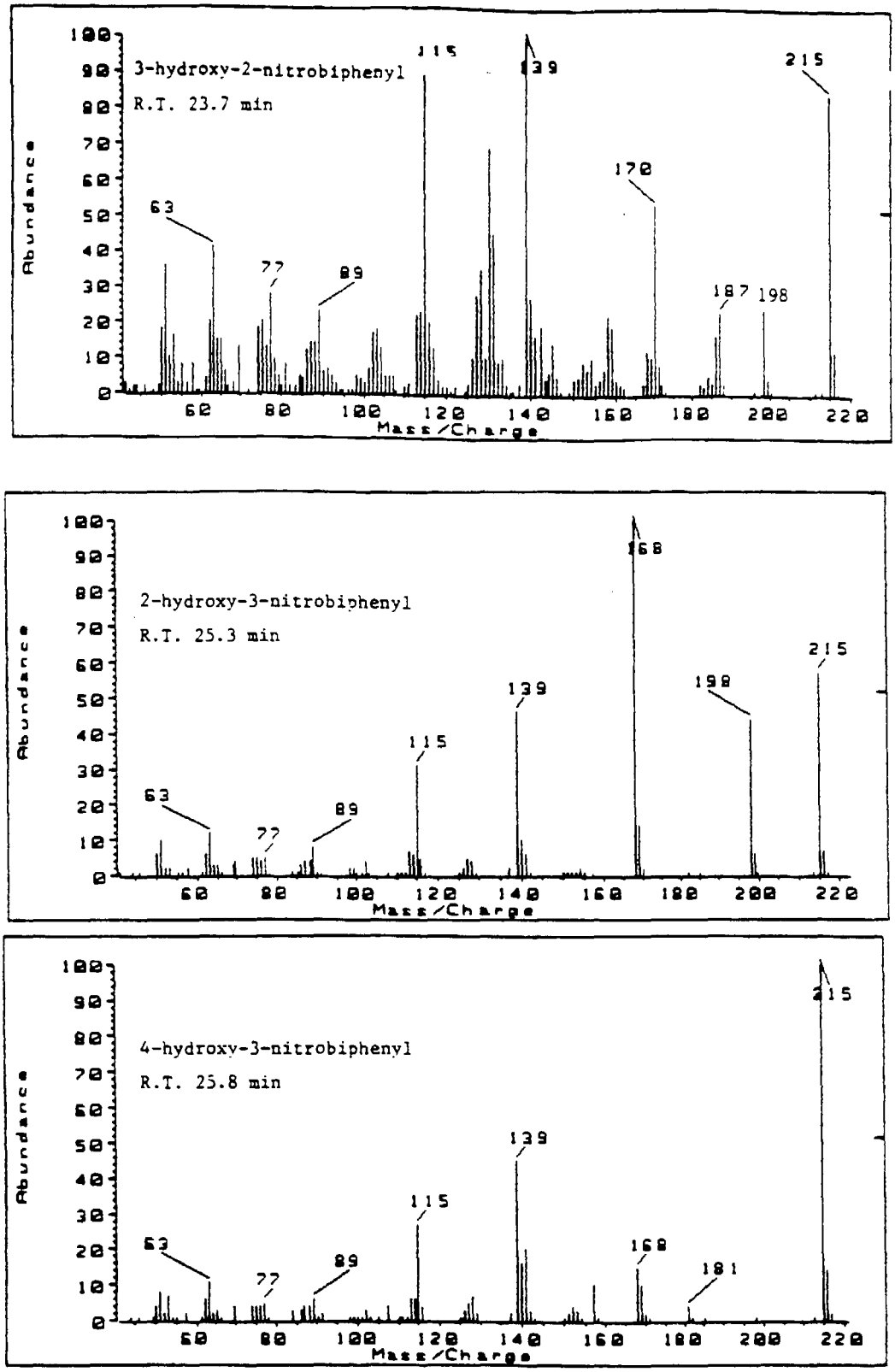



Figure V-4. Electron impact mass spectra of three hydroxynitrobiphenyls (peaks 1-3 in Figure V-3).

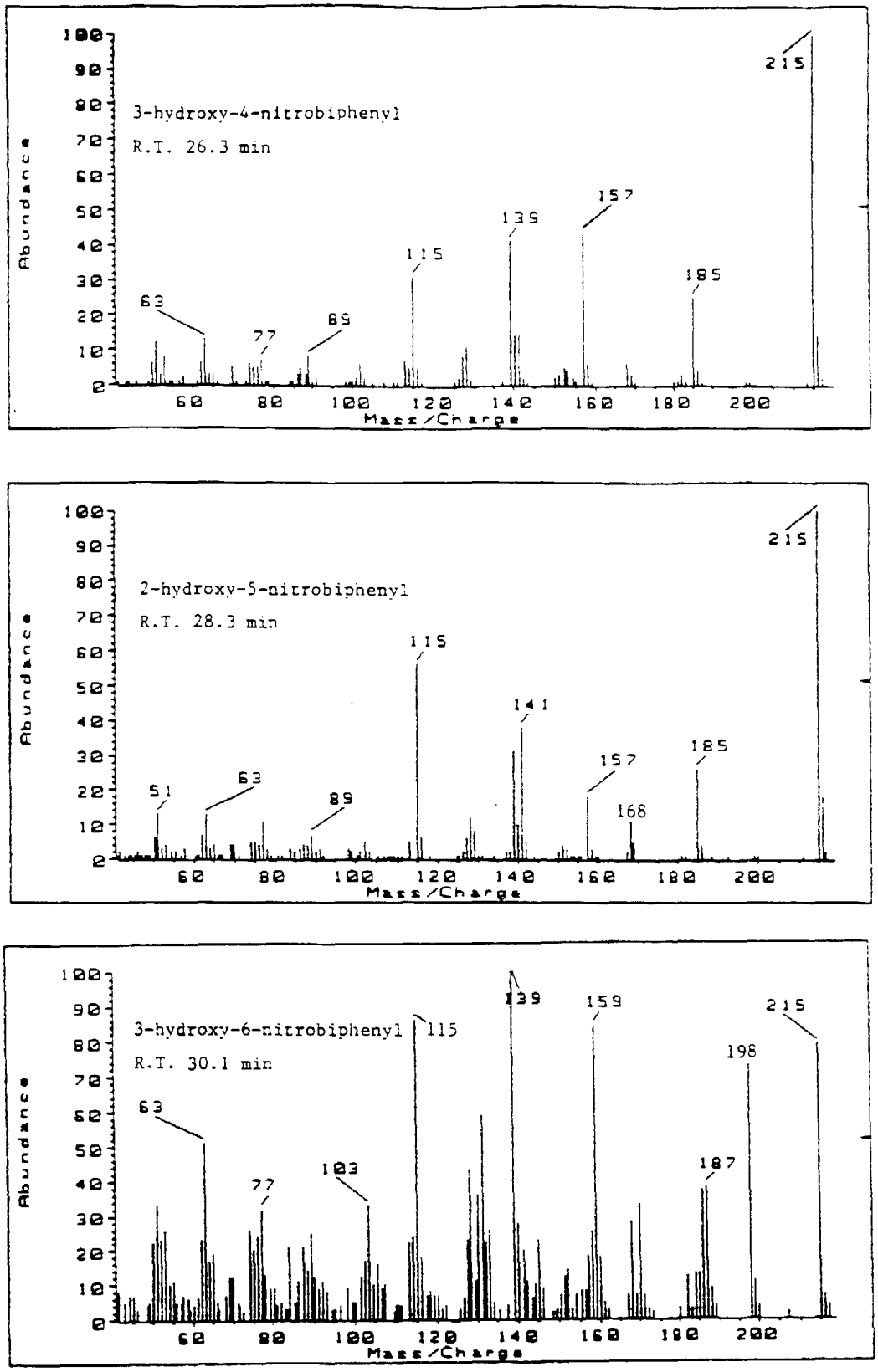



Figure V-5. Electron impact mass spectra of three hydroxynitrobiphenyls (peaks 4-6 in Figure V-3).

compound. The preliminary test (TA98; -S9) indicated that the activities of the compounds would be low and that toxicity could be expected at the higher doses.

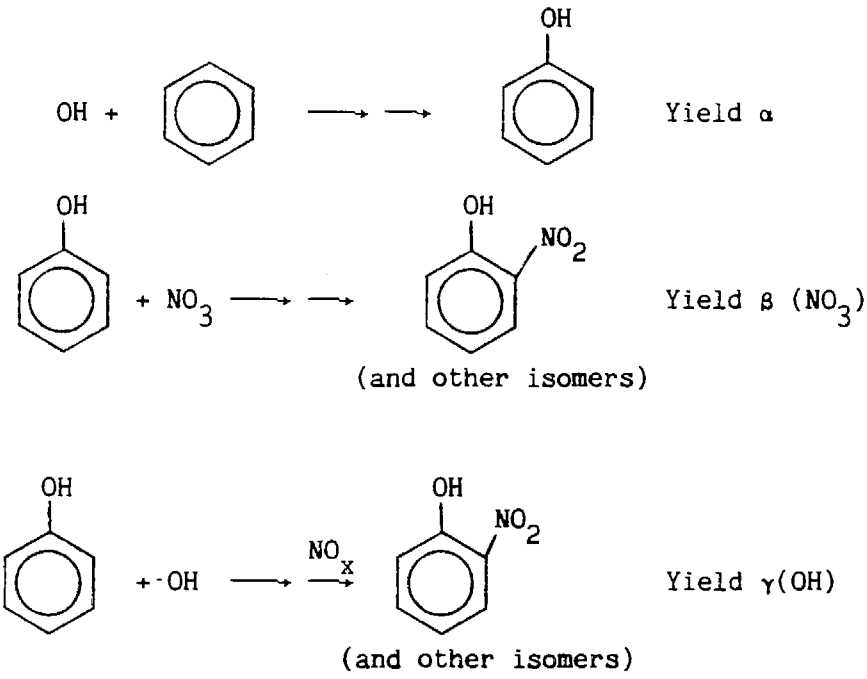
The following compounds were then tested in the standard Ames plate-incorporation assay: 1,2-hydroxynitronaphthalene, 1,4-hydroxynitronaphthalene, 2,1-hydroxynitronaphthalene, 2,3-hydroxynitrobiphenyl, 2,5-hydroxynitrobiphenyl, 3,2-hydroxynitrobiphenyl, 3,4-hydroxynitrobiphenyl, 3,6-hydroxynitrobiphenyl, 4,3-hydroxynitrobiphenyl, and 3,2-hydroxynitrofluoranthene. In anticipation of low activity, each compound was tested at eight doses up to 500 μg plate$^{-1}$, with the exception of 3,2-hydroxynitrobiphenyl and 3,4-hydroxynitrobiphenyl which were tested at eight does up to 400 μg plate$^{-1}$ because of limited amounts of the purified sample.

Of these compounds, only 2,1-hydroxynitronaphthalene was mutagenic toward TA98 (0.23 revertants μg$^{-1}$). Limiting concentrations were reached with the other hydroxynitroarenes: the hydroxynitrobiphenyls and the two other hydroxynitronaphthalenes were toxic at the higher doses, while 3,2-hydroxynitrofluoranthene, although nontoxic, was partially insoluble in the top agar at 190 μg plate$^{-1}$ and above. As observed by an abnormal background lawn of unreverted Salmonella, 2,5-hydroxynitrobiphenyl was the most toxic, with toxicity observed at the 130 μg dose. If they are present in ambient air, these hydroxynitronaphthalenes and the hydroxynitrobiphenyls may interfere with the Ames mutagenicity of collected organic matter by killing the test bacteria, resulting in an underestimation of the mutagenic activity.

Because of its low mutagenic activity, 2,1-hydroxynitronaphthalene would not be expected to be a major contributor to the TA98 mutagenicity of ambient POM. Low mutagenic activities of the hydroxynitronaphthalenes and hydroxynitrobiphenyls toward TA98 were not unexpected, because the two-ring nitroarenes tend to preferentially revert TA100, which detects base pair substitution mutations, as opposed to TA98, which detects frameshift mutations.

C. Formation of Hydroxynitro-PAH

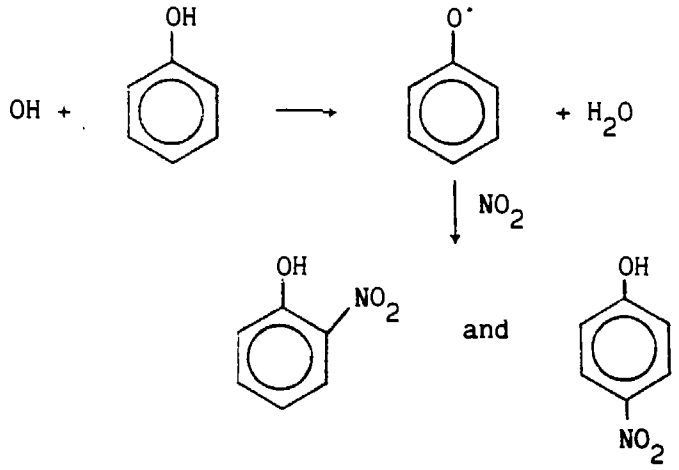
While at the beginning of this experimental program, we expected that the hydroxynitro-PAH would be formed in reasonably high yield from the atmospheric photooxidations of the 2-4 ring gas-phase PAH, experiments conducted under ARB funding (Contract A732-107) showed that this was not the case, at least for the simple monocyclic aromatic hydrocarbons benzene and toluene (Atkinson et al., 1990c). Thus, based upon the general reaction scheme (taking benzene as an example),




we have shown that the phenol and cresol yields from the OH radical-initiated reactions of benzene and toluene are ~0.25 for both aromatic hydrocarbons [the phenol yield from benzene is 0.236 ± 0.044 and the o-cresol and m- + p-cresol yields from toluene are 0.204 ± 0.027 and 0.048 ± 0.009, respectively (Atkinson et al., 1989)].

Furthermore, we have shown that the OH radical reaction of phenol, in the presence of $NO_x$, formed 2- and 4-nitrophenol and 1,2-dihydroxyphenol, with the 2-nitrophenol yield being 0.055 ± 0.015. In the case of o-cresol (the major cresol isomer formed from toluene), 2-methyl-6-nitrophenol and 2-methyl-4-nitrophenol were observed, with a 2-methyl-6-nitrophenol yield of 0.030 ± 0.014. The formation of these nitrophenol and nitrocresols is

consistent with the reaction sequence involving H atom abstraction from the -OH substituent group, followed by reaction of the resulting phenoxy-type radical with $NO_2$,




The measured yields are of a similar magnitude to the fractions of the reactions proceeding by H atom abstraction [~0.09 and ~0.06 for phenol and o-cresol, respectively (Atkinson, 1989)].

For the $NO_3$ radical reactions with phenol and o-cresol, we observed 2- and 4-nitrophenol and 2-methyl-6-nitrophenol and 2-methyl-4-nitrophenol, respectively, with the 2-nitrophenol yield from phenol being 0.16 ± 0.06 and the 2-methyl-6-nitrophenol yield from o-cresol being 0.05 ± 0.01. These data for the methylnitrophenols formed from o-cresol are in reasonable agreement with the product data of Grosjean (1985).

The 2-nitrophenol and 2-methyl-6-nitrophenol concentrations were measured during the OH radical-initiated reactions of benzene and toluene, respectively, under conditions where $O_3$ and hence $NO_3$ radicals were generated. The time-concentration profiles of the nitrophenols were in generally good agreement with the predictions based on the above product yield data. Clearly, while nitrophenols are formed from the OH and $NO_3$ radical reactions of phenol and the cresols, the formation yields are fairly low,

and large concentrations of hydroxynitro-aromatics relative to the parent aromatic hydrocarbon are not expected to be formed under atmospheric conditions. For example, the maximum yield of 2-nitrophenol from benzene (under conditions where the initially formed phenol is removed by reaction with the $NO_3$ radical) is ~3.5-4% of the benzene reacted, and the analogous maximum yield of 2-methyl-6-nitrophenol from toluene is ~1%.

Based on these low overall formation yields of hydroxynitroaromatics and the low mutagenic activities of this class of compounds, it appears that the hydroxynitro-PAH will not make a significant contribution to the direct-acting mutagenicity of extracts of collected ambient particulate matter. Accordingly, as described in Sections VI through IX, we changed the direction of this program, and focussed on the bioassay-directed fractionation of the products collected from environmental chamber reactions of individual PAH.

# VI. BIOASSAY-DIRECTED FRACTIONATION OF OH RADICAL-INITIATED REACTIONS OF PAH IN AN ENVIRONMENTAL CHAMBER

As noted in Section IV above, employing bioassay-directed fractionation and analysis on a Riverside ambient particulate sample, we did not succeed in the identification of previously unrecognized mutagenic species. The following provides the rationale for changing the emphasis of the program to the bioassay-directed fractionation of the reaction products formed by the OH radical-initiated reactions of abundant, gas-phase PAH. This change in program direction was made after full consultation with the ARB staff.

## A. Rationale

The majority of the nitro-PAH observed in ambient air, both gas-phase and particle-associated, are believed to be the products of the gas-phase atmospheric reactions of the parent PAH (Arey et al., 1986; 1987; 1989a,b; 1990; Zielinska et al., 1989a,b; Atkinson et al., 1990a). Thus, while some nitro-PAH such as 1-nitropyrene are emitted into the atmosphere in, for example, diesel exhaust, the more abundant 2-nitrofluoranthene is formed from the OH and the $NO_3$ radical-initiated reactions of gas-phase fluoranthene (Arey et al., 1986; Atkinson et al., 1990a,b). Although nitro-PAH make a significant contribution to the direct-acting mutagenicity of diesel exhaust extracts (Schuetzle, 1983), the nitrofluoranthenes and nitropyrenes, generally the most prevalent nitro-PAH and those found in highest concentrations in ambient particle extracts (Nielsen et al., 1984; Ramdahl et al., 1986; Nielsen and Ramdahl, 1986; Atkinson et al., 1988; Nishioka et al., 1988; Zielinska et al., 1989a; Arey et al., 1990), have been reported to contribute less than 10% of the direct-acting mutagenicity of ambient extracts toward Salmonella typhimurium strain TA98 (Arey et al., 1988).

In ambient air particle extracts, the major mutagenic fractions contain compounds more polar than the nitro-PAH (Nishioka et al., 1988; Lewtas et al., 1990; Section IV above). Two findings suggest that these polar mutagenic compounds may include PAH-derivatives, and that these derivatives are formed from the atmospheric reactions of PAH. Nishioka and coworkers (1988), using bioassay-directed fractionation of ambient

particle extracts, identified derivatives of PAH with tentative hydroxy- and nitro- substituents. Furthermore, in a study at seven sites in California (Atkinson et al., 1988; Arey et al., 1991), the direct-acting mutagenic activity of the extracts of particles collected at each site did not correlate with the PAH concentrations themselves, but rather correlated with 2-nitropyrene, a nitro-PAH formed in the atmosphere (Arey et al., 1986; Atkinson et al., 1990a). The nitro-PAH account for only a small fraction ($\lesssim$5%) of the atmospheric reaction products of the PAH with the hydroxyl radical, the species responsible for the relatively short lifetimes of the gas-phase PAH in the atmosphere (Arey et al., 1989a,b; 1990; Atkinson et al., 1990a), and the remaining PAH reaction products are expected to include polar compounds which may also be mutagenic.

We, therefore, began to apply the technique of bioassay-directed fractionation to the products of the atmospherically important gas-phase reactions of the PAH, in particular those reactions which are OH radical-initiated. The PAH were reacted in an environmental chamber, with the products being collected and subjected to HPLC fractionation with bioassay using the Kado modification (Kado et al., 1983; 1986) of the Ames assay with Salmonella typhimurium strain TA98. Mutagrams (bar graphs of activity versus HPLC fraction) from the reactions of those PAH known to be abundant in ambient air were compared to the mutagrams of ambient particle extracts to look for PAH which produced mutagenic products of the same polarity as the important, but unidentified, mutagens in ambient air. If the PAH are responsible for a significant portion of the polar direct-acting mutagenicity of ambient air particles through their atmospheric reactions, identification of the PAH reaction products could lead to a newly recognized mutagen or class of mutagens whose contribution to ambient mutagenicity could then be assessed.

## B. Methods

Due to problems with the reproducibility of HPLC fractionations made utilizing what we have referred to as the "MeOH program", we returned to the "ACN program" as detailed below. To allow comparisons of the chamber reaction product mutagrams with ambient particle samples, we extracted and fractionated additional ambient filter samples from collections made during the South Coast Air Quality Study.

Choice of PAH for Chamber Reactions. In a previous study of 7 sites within California, chosen to typify different dominant emission sources, gas-phase and particle-associated PAH, and nitro-PAH and ambient particle mutagenicity were measured (Atkinson et al., 1988; 1991; Arey et al., 1991). The abundances of some common PAH were as follows: naphthalene > fluorene ~ phenanthrene > fluoranthene ~ pyrene > benzo[e]pyrene, with the average naphthalene concentration reaching 3600 ng $m^{-3}$ at one site. On the basis of their high ambient concentrations, and since they are expected to be fully in the gas-phase (Bidleman, 1988; Coutant et al., 1988), naphthalene, fluorene and phenanthrene were chosen for bioassay-directed fractionation of their gas-phase OH radical-initiated reaction products. Significant daytime reaction of these PAH has been observed under ambient conditions (Arey et al., 1989a), and the dominant loss process by reaction with the OH radical has been calculated to lead to atmospheric lifetimes of ~9 hr; ~13 hr and ~6 hr for naphthalene, fluorene, and phenanthrene, respectively (Arey et al., 1989a,b).

Ambient Samples. Ambient particles were collected on the campus of Harvey Mudd College in Claremont, CA during the South Coast Air Quality Study (Lawson, 1990) in August, 1987. The particulate matter was collected on Pallflex T60A20 Teflon-impregrated glass fiber filters by a high-volume sampler equipped with a 10-micron size-selective inlet and operated at a measured flow rate of 38.2 SCFM. The three samples used for this study were obtained during daytime hours (0600-1800 PDT) on three successive days (8/27-29/87), using the same sampler. Each filter was Soxhlet extracted for 24 hr using $CH_2Cl_2$.

Environmental Chamber Reactions. As described in more detail elsewhere (Arey et al., 1986; Atkinson et al., 1987; Atkinson and Aschmann, 1988), reactions of PAH with the hydroxyl radical (OH) in the presence of $NO_x$ were conducted in a 6400 L all-Teflon environmental chamber equipped with two parallel banks of blacklamps (Sylvania F40/350BL). Hydroxyl radicals were generated by the photolysis of methyl nitrite in air at wavelengths >300 nm. The reaction products were sampled from the chamber by pulling ~2000 L of the chamber volume through polyurethane foam (PUF) plugs. The PUF plugs were Soxhlet extracted with $CH_2Cl_2$ for several hours, and the extracts fractionated as described below.

Naphthalene (ITC #1698; initial concentration 910 ppb) and fluorene (ITC #1611; initial concentration 90 ppb) were added to the chamber by flowing pure nitrogen through Pyrex tubes packed with the pure PAH. Phenanthrene (ITC #1649; initial concentration 160 ppb) was added to the chamber by spraying it in a methanol solution into the chamber and flushing briefly to remove most of the solvent. The naphthalene and phenanthrene reactions employed 2 parts-per-million (ppm) of methyl nitrite and 1 ppm NO with irradiation at 100% for 10 min. Fluorene was irradiated for 5 min at 100% light intensity and with 10 ppm each of methyl nitrite and NO. In general, the concentration of hydroxyl radicals increases proportionately with the methyl nitrite/NO concentration ratio and decays effectively instantaneously when the lights are turned off. Blank chamber runs in which $CH_3ONO$ and NO were photolyzed in the absence of the PAH were also sampled, extracted, fractionated and assayed for mutagenic activity.

HPLC Fractionation. The dichloromethane extracts of the ambient filters or PUF plugs from the chamber were filtered (0.45 μm Teflon filters, Acrodisc from Gelman Sciences) and fractionated by HPLC. The HPLC column was a preparative Regis Spherisorb S5W silica (5 micron) column, 25 cm x 10 mm. The HPLC instrumentation consisted of a Spectra-Physics Model 8100 gradient liquid chromatograph with a Model 8400 UV/Vis detector ($\lambda$ = 254 nm) and an ISCO fraction collector. The solvent program (at a flow rate of 3 mL $min^{-1}$) was: initially 100% hexane for 10 min, followed by a five min linear gradient to 95% hexane and 5% $CH_2Cl_2$. The solvent was programmed over the next 25 min to 100% $CH_2Cl_2$ where it was held for 10 min, then programmed to 100% acetonitrile over 10 min , held isocratic for 10 min and then programmed back to the initial conditions. In general, beginning after one minute, 9 nine-minute fractions were collected during the chromatogram for bioassay and chemical analysis.

Bioassay. Salmonella typhimurium, strain TA98, without S9 activation was used to assay the HPLC fractions from ambient air extracts and from the chamber PAH exposures. To enhance sensitivity, the Kado modification of the Ames assay (Kado et al., 1983; 1986) utilizing a 90 minute preincubation with ten times the usual number of bacteria (cultured in L-broth) in a small-volume liquid suspension was employed. The HPLC fractions were dissolved in DMSO (Mallinckrodt SpectrAR grade, 5 μL) by

sonication, combined with the bacteria (100 μL, 1.1 x $10^9$ cells $mL^{-1}$) and incubated for 90 min at 37°C with vigorous shaking (180 rpm). Following the addition of 2.0 mL of soft agar and mixing, each sample was overlayed onto minimal glucose plates which were incubated at 37°C for 63 hr and scored by means of an automatic colony counter. On each test day, 2-nitrofluorene was tested as a positive control. To obtain net revertants, all counts were corrected for the average background mutagenicity.

Chemical Analysis. A Hewlett-Packard 5890 GC interfaced to a 5970 mass selective detector was used for GC/MS analysis of aliquots from selected HPLC fractions. More complete characterizations of the reaction products of fluorene (Helmig and Arey, 1991 [Appendix A]; and Section VIII below) and phenanthrene (Section IX below) are provided in the sections that follow.

## C. Results and Discussion

The ambient sample mutagenicity data given in Table VI-1 is a tabulation of the revertants in each HPLC fraction from extracts of ambient particle samples collected on three consecutive days in August in Claremont, CA. Mutagrams graphically displaying these results are given in Figure VI-1. It is evident from Figure VI-1 that the mutagrams from all three days looked remarkably similar with the majority of the activity in HPLC fractions #6 and #7 (note that the nitro-PAH would be expected to elute in fraction #4 and possibly #5). Similar mutagrams, that is, with more activity in the later, more-polar, fractions have been observed for samples collected in El Monte, CA [assayed with the standard Ames test] (Pitts et al., 1984), and as noted above, these findings are consistent with our observations for Riverside particles (Section IV) as well as the work of other investigators (Nishioka et al., 1988; Lewtas et al., 1990).

The mutagrams of the products from the OH radical-initiated reactions of these three PAH are shown in Figure VI-2 and for comparison an averaged ambient mutagram is also shown. The ambient Claremont mutagram shown results from summing the activity for each fraction of the three samples and showing this as a percentage of the total activity recovered (sum of all individual fractions). It should be noted that, by rapidly sampling (~1000 liters $min^{-1}$) the chamber contents onto PUF plugs we sampled compounds produced in the gas-phase which under ambient conditions would

Table VI-1. Mutagenicity of HPLC Fractions of Ambient Particulate Organic Matter Collected in Claremont, CA, 8/27-29/87 (0600-1800 PDT) in the Kado Assay (TA98; -S9)

| HPLC Fraction # | Net Revertants per Fraction[a] 8/27/87 | 8/28/87 | 8/29/87 |
|---|---|---|---|
| 1 | 0 | 0 | 0 |
| 2 | 110 | 0 | 0 |
| 3 | 140 | 280 | 130 |
| 4 | 5,800 | 7,600 | 5,800 |
| 5 | 12,000 | 12,000 | 8,300 |
| 6 | 57,000 | 56,000 | 39,000 |
| 7 | 44,000 | 47,000 | 25,000 |
| 8 | 1,700 | 1,800 | 1,100 |
| 9 | 310 | 260 | 190 |
| Sum | 121,060 | 124,940 | 79,520 |
| Rev $m^{-3}$ | 160 | 160 | 100 |

[a]To obtain reliable data in the case of highly mutagenic fractions, each fraction was tested over a dose range spanning four orders of magnitude.

become particle-associated to an extent determined by their volatility. Reaction products eluting in fraction #4 were responsible for the majority of the mutagenic activity for both naphthalene and fluorene, while HPLC fraction #6 of the phenanthrene reaction was highly mutagenic. Clearly the product(s) responsible for the activity of fraction #6 from the phenanthrene reaction may be expected to contribute to the activity of fraction #6 of the ambient extracts. The activity observed in the chamber

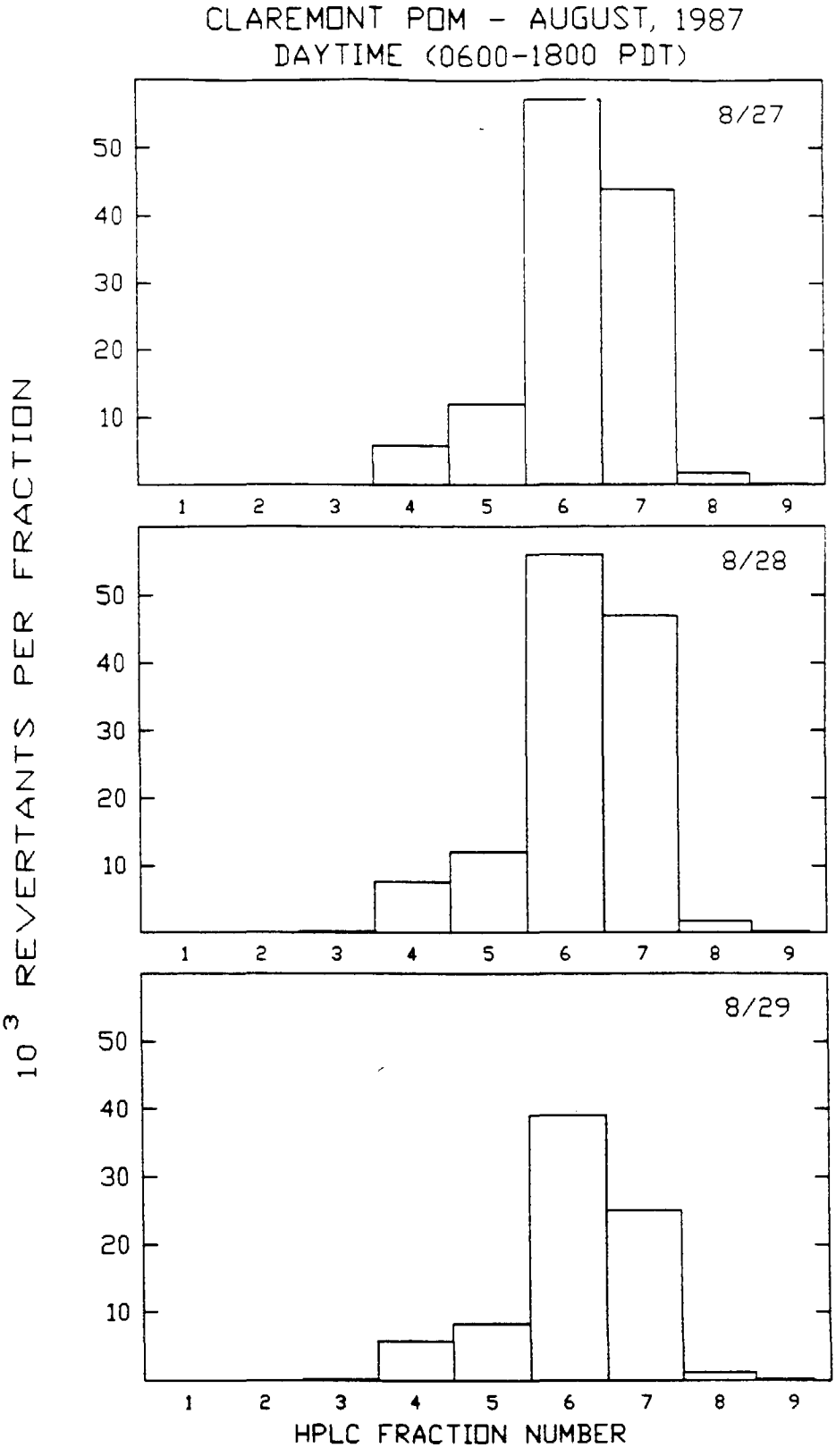



Figure VI-1. HPLC mutagrams of ambient particle extracts collected in Claremont, CA 8/27-29/91, during the daytime (0600-1800 PDT) hours.

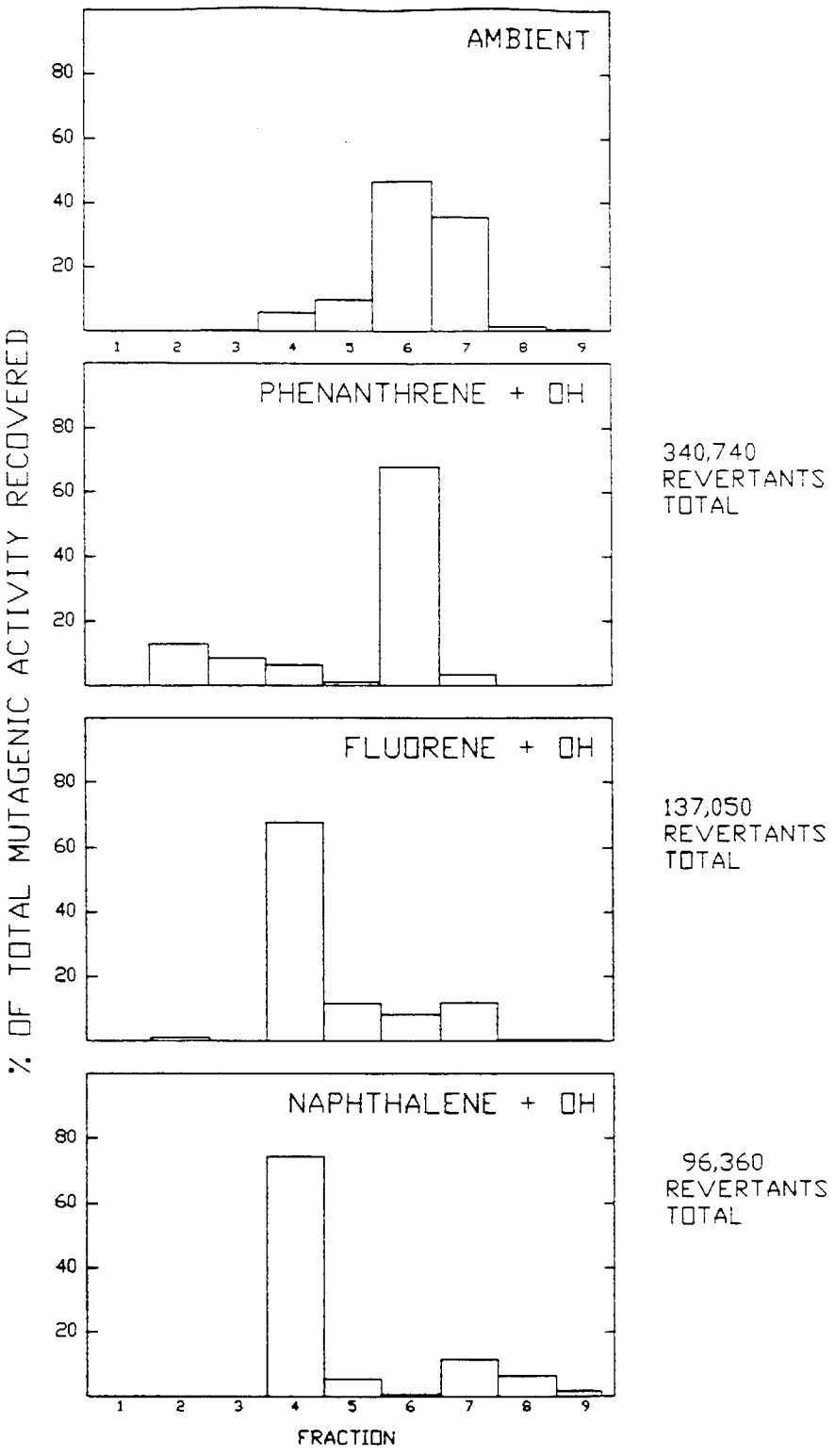



Figure VI-2. HPLC mutagrams from the gas-phase OH radical-initiated reactions of phenanthrene, fluorene and naphthalene compared to the mutagram of Claremont, CA ambient air particle extracts. The plotted mutagenicity values have been normalized to the sum of the individual fractions, shown to the right of the plots of the chamber reaction data.

blank runs was insignificant when compared with the activity of the PAH reaction products.

Both 1- and 2-nitronaphthalene and 1-hydroxy-2-nitronaphthalene were identified by GC/MS analysis of fraction #4 from the naphthalene reaction. We previously tested 1-hydroxy-2-nitronaphthalene in the standard Ames assay and in the Kado microsuspension assay and found it to be inactive (see Section V). Although 1- and 2-nitronaphthalene were reported to be only very weak mutagens toward TA98 in the standard Ames assay (see Table VI-2) we tested these compounds with the Kado modification and as shown in Table VI-2, both showed significant activity. By quantifying the nitronaphthalenes, it was estimated that ~90% of the activity of fraction #4 can be ascribed to the nitronaphthalenes, with the majority of the activity being from 2-nitronaphthalene.

The high activity of the nitronaphthalenes, in particular of the 2-isomer is consistent with the recent work of Kado et al. (1991a) and suggests that the standard Ames assay may underestimate the activity of volatile mutagens (Kado et al., 1991b). It is important to note that the ambient mutagenic activity, as shown in Figure VI-2, would include only a small fraction of the contribution from the nitronaphthalenes, since they are mainly in the gas-phase rather than on particles (Arey et al., 1987; 1989a). However, we have previously reported the ambient concentrations of the volatile nitro-PAH, including nitronaphthalenes, methylnitronaphthalenes and nitrobiphenyl, to be an order of magnitude greater than the particle-associated nitrofluoranthenes and nitropyrenes (Arey et al., 1987). Section VII below contains additional discussion of volatile nitro-PAH in ambient air.

A typical mutagram from the products of the gas-phase OH radical-initiated reaction of fluorene is shown in Figure VI-2. All four nitrofluorene isomers were identified by GC/MS analysis in HPLC fraction #4 of the fluorene chamber reaction. The activities of the nitrofluorene isomers in both the standard plate incorporation test and the Kado microsuspension modification of the Ames assay are listed in Table VI-2. A full discussion of the characterization of the nitrofluorene isomers is described in a manuscript now in press (Helmig and Arey, 1991), which is included as Appendix A of this report. Data on the nitrofluorene yields from the reaction for which the mutagram is shown in Figure VI-2, as well

Table VI-2. Mutagenicity of Nitronaphthalenes and Nitrofluorenes in the Standard Ames Assay and the Kado Modification

| Compound | Kado Microsuspension Assay (TA98; -S9) Revertants $\mu g^{-1}$ | Plate Incorporation Assay (TA98; -S9) Revertants $\mu g^{-1}$ |
|---|---|---|
| 1-nitronaphthalene | 280 | 0.3[a], 2.3[a] |
| 2-nitronaphthalene | 5,100 | 1.2[a], 5.2[a] |
| 1-nitrofluorene | 4,100 | 20 |
| 2-nitrofluorene | 20,000 | 440 |
| 3-nitrofluorene | 26,000 | 190 |
| 4-nitrofluorene | 160 | 1.9 |

[a]Data taken from Rosenkranz and Mermelstein (1983).

as for several additional fluorene reactions conducted, are given in Section VIII. Also discussed in Section VIII are the identifications of other products formed in these reactions. 3-Nitrofluorene was the isomer formed in highest yield and also the isomer which accounted for the majority of the mutagenic activity of fraction #4. Nearly 75% of the activity of fraction #4 shown in Figure VI-2 could be assigned to the nitrofluorenes quantified in this fraction.

GC/MS analysis of HPLC fraction #6 of the phenanthrene chamber reaction showed a lactone derivative of phenanthrene to be the major component of this fraction (Section IX). Two nitro-derivatives of this lactone were also found in fraction #6 and preliminary data suggest that these compounds are responsible for the mutagenicity of this fraction.

It is difficult to estimate the contribution of the mutagens in the phenanthrene reaction fraction #6 to the corresponding fraction in ambient air. The very high activity we observe for the chamber reaction is encouraging, but only quantification of the mutagen(s) in ambient particles will enable their true significance to be assessed.

The initial naphthalene concentration in the chamber reaction for which the product mutagram is shown in Figure VI-2 was nearly 1 ppm, and approximately 5 and 10 times the initial phenanthrene and fluorene concentrations, respectively. In ambient samples we have observed the ratio of naphthalene/phenanthrene to vary from ~20 to ~200. The activity of mutagen(s) in fraction #7 of the naphthalene reaction may be significant to ambient particle mutagenicity, depending on the ambient naphthalene concentrations and the amount of the mutagen that is ultimately present on the particles.

Fluoranthene and pyrene are among the additional PAH which should be studied in the future using the bioassay-directed fractionation described here. Although the ambient concentrations of fluoranthene and pyrene are lower than the PAH reported on here, as noted above, the nitro-derivatives produced from their OH radical-initiated reactions are generally the most abundant nitro-PAH observed in ambient particle extracts.

The utilization of an environmental chamber with a known PAH-starting material and, thus, the ability to produce sufficient product for isomer-specific identification is a promising complement to bioassay-directed fractionation of ambient particle extracts. Initial screening of abundant PAH for polar mutagenic reaction products has lead to the identification of nitro-phenanthrene lactones, which can now be quantified in ambient air and their contribution to ambient mutagenicity assessed. Furthermore, the importance of volatile mutagens which have been largely overlooked by the analysis of only particle extracts is illustrated by the activity of 2-nitronaphthalene formed from the OH radical-initiated reaction of naphthalene.

# VII. NAPHTHALENE

The average ambient concentrations of naphthalene measured at seven sites in California during our 1986-1987 ARB-sponsored study of PAH and their derivatives (Atkinson et al., 1988) are given in Table VII-1. Since naphthalene was always the most abundant PAH observed and is entirely in the gas phase under ambient conditions (Arey et al. 1987, 1989a; Atkinson et al. 1988; Bidleman, 1988), it was a natural choice for one PAH to "screen" for polar mutagenic reaction products.

## A. Methods

Two naphthalene chamber reactions (ITC-1697 and 1698) were conducted. The second of these naphthalene chamber experiments, ITC-1698, was used for the bioassay-directed fractionation and chemical analysis. As noted in Section VI, for this experiment the naphthalene mixing ratio in the chamber was 910 ppb and methyl nitrite and NO were added at mixing ratios of 2 and 1 ppm, respectively. A 10 min irradiation with 100% light intensity was conducted and a 2 min (~2000 L) PUF plug sample (with two plugs in series) was collected after the irradiation.

As described in Section VI, the PUF plug sample was extracted and fractionated by HPLC with nine fractions collected and assayed for mutagenicity in the Kado modification of the Ames assay. Each fraction was tested at a dose range of 0.05% to 45% of the sample to obtain reliable data in the case of highly mutagenic or cytotoxic fractions.

The nitronaphthalenes in HPLC fraction #4 were quantified by GC/MS analysis after spiking the extract with a known amount of 1-nitronaphthalene-$d_7$.

## B. Results

The results of the mutagenicity assay are summarized in Table VII-2. Naphthalene yields a significant amount of mutagenic activity, although it is important to note that the naphthalene concentration in the chamber was greater than that of the other PAH tested by ~10-fold. Approximately 75% of the activity was present in HPLC fraction #4. As noted in Section VI, the activity of fraction #4 was largely due to

Table VII-1. Average Values of Ambient Naphthalene, Nitronaphthalenes and 3-Nitrobiphenyl at Seven Sites in California

| Site | ng $m^{-3}$ | | | |
|---|---|---|---|---|
| | Naphthalene | 1-Nitronaphthalene | 2-Nitronaphthalene | 3-Nitrobiphenyl |
| Glendora | 3600 | 3.0 | 2.3 | 0.62 |
| Yuba City | 510 | 0.47 | 0.51 | 0.13 |
| Concord | 1500 | 0.36 | 0.29 | 0.07 |
| Mammoth Lakes | 780 | 0.09 | 0.08 | $\lesssim$0.03 |
| Oildale | 290 | 0.09 | 0.14 | 0.02 |
| Reseda | 810 | 0.59 | 0.48 | 0.10 |
| Pt. Arguello | 87 | 0.02 | <0.04 | ~0.002 |

Table VII-2. Mutagenicity of HPLC Fractions of the OH Radical-Initiated Reaction Products of Naphthalene in the Kado Assay (TA98;-S9)

| HPLC Fraction # | Net Revertants per Fraction |
|---|---|
| 1 | 0 |
| 2 | 0 |
| 3 | 0 |
| 4 | 72,000 |
| 5 | 5,200 |
| 6 | 730 |
| 7 | 11,000 |
| 8 | 6,200 |
| 9 | 1,500 |
| SUM | 96,630 |

2-nitronaphthalene. The results of the chemical analysis for the nitronaphthalenes and the calculated resulting mutagenicity are given in Table VII-3. The nitronaphthalenes account for 90% of the activity found in HPLC fraction #4 and 67% of the overall activity from the naphthalene chamber reaction. Thus, within the uncertainties due to the day-to-day reproducibility of the mutagenicity assay, the nitronaphthalenes account for all the activity of fraction #4.

Figure VII-1 shows the mass spectra of the three most abundant components of HPLC fraction #4. In addition to the nitronaphthalenes, a compound identified on the basis of its mass spectrum as 1-hydroxy-2-nitronaphthalene (compare with Figure V-2) was also present. As noted in Section V, we found this hydroxynitronaphthalene to be inactive in both the Kado assay and the standard Ames assay.

Table VII-3. Chemical Analysis of Nitronaphthalenes in Fraction #4 and Their Calculated Contribution to the Mutagenicity of the Naphthalene Chamber Reaction Products

| Observed Mutagenicity | Chemical Analysis | | Calculated Mutagenicity[a] |
|---|---|---|---|
| 72,000 rev. | 1-nitronaphthalene | 54 µg | 15,120 rev. |
| | 2-nitronaphthalene | 9.8µg | 49,980 rev. |
| | | SUM | 65,100 rev. |

[a]Using the activities measured for nitronaphthalene standards as given in Table VI-2.

As noted in Section VI, the significant activity of the nitronaphthalenes in the Kado modification of the Ames assay has important implications in terms of volatile ambient mutagenicity (Kado et al., 1991a,b). Moreover, we have previously reported that the concentrations of the volatile nitro-PAH such as the nitronaphthalenes, the methylnitronaphthalenes and 3-nitrobiphenyl (see Table VII-1) may be an order of magnitude greater than those of the particle-associated species such as the nitrofluoranthenes and nitropyrenes (Arey et al., 1987; Atkinson et al., 1988). These volatile nitro-PAH are largely the products of atmospheric reactions of the parent PAH (Arey et al., 1987, 1989a,b, 1990; Zielinska et al., 1989b) and we have estimated that in Southern California, the contribution of direct emissions to the ambient concentrations is small (Arey et al., 1990). Thus, the atmospheric photooxidations of naphthalene, 1- and 2-methylnaphthalene and biphenyl lead to volatile nitro-PAH which may result in gaseous mutagenicity levels which are comparable to particle-phase mutagenicities.

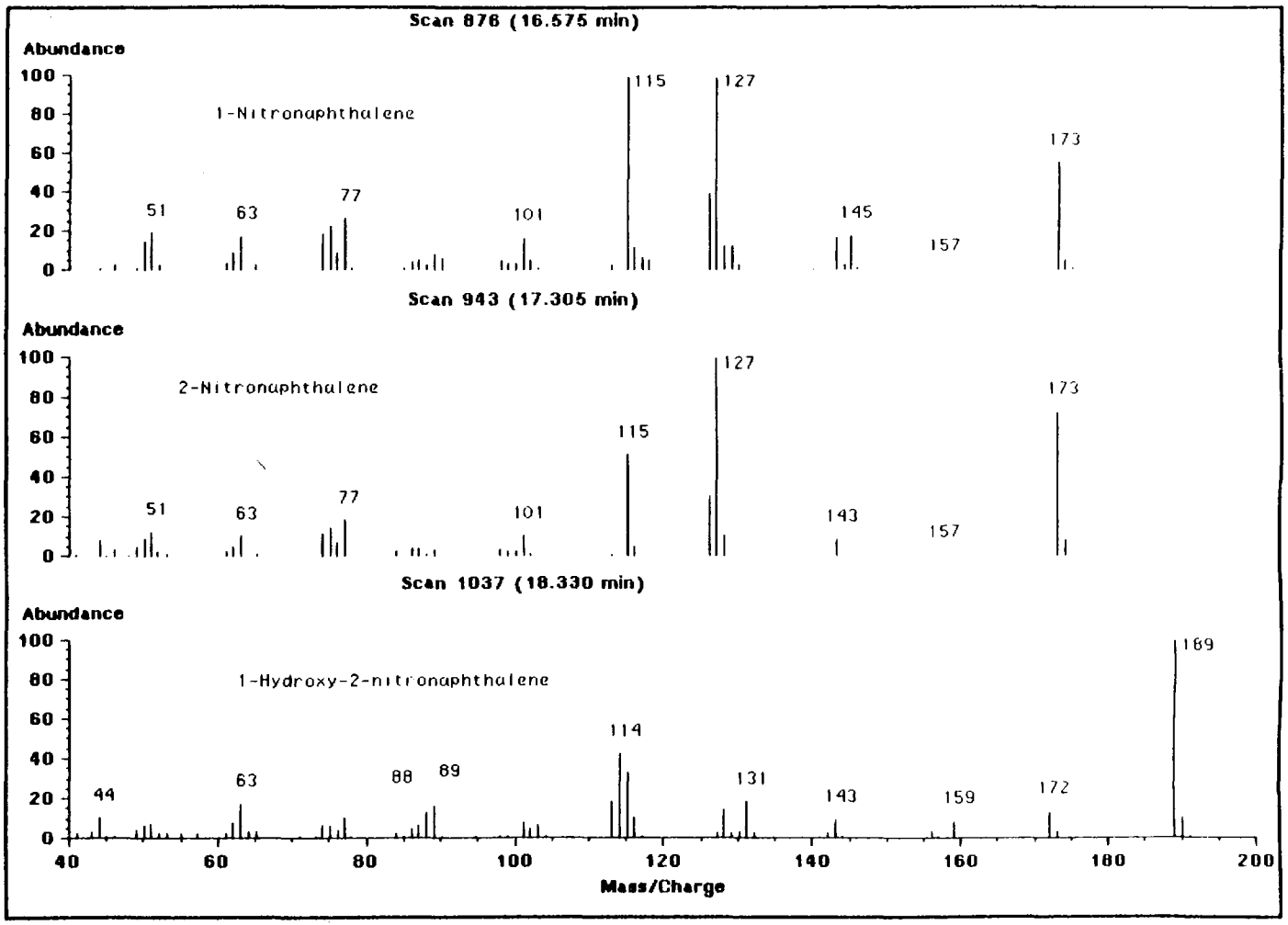



Figure VII-1. Mass spectra of the three most abundant components in HPLC fraction #4 of the naphthalene chamber reaction products. The compounds are (from top to bottom): at 16.6 min, 1-nitronaphthalene; at 17.3 min, 2-nitronaphthalene; at 18.3 min, 1-hydroxy-2-nitronaphthalene.

# VIII. FLUORENE

Fluorene is abundant in ambient air (see, for example, Atkinson et al., 1988) and it is a PAH whose OH radical-initiated reaction had not previously been examined. The major electrophilic nitration product of fluorene, 2-nitrofluorene, has been reported to be present in diesel exhaust and in ambient air (Xu et al., 1981; Beije and Möller, 1988 and references therein). Using bioassay-directed fractionation of ambient particle extracts with negative ion chemical ionization mass spectrometry, Nishioka and co-workers tentatively identified an hydroxynitrofluorenone in a polar mutagenic subfraction (Nishioka et al., 1988). These findings contributed to our decision to examine the reactions of fluorene in the environmental chamber.

When we found that the major nitro-derivative of fluorene produced in the OH radical-initiated reaction of fluorene was the 3-nitrofluorene isomer rather than the electrophilic nitration product (i.e., 2-nitrofluorene), a decision was made to examine a diesel particulate sample as well as ambient samples for the nitrofluorenes present.

## A. Methods

### 1. Chamber Analyses

The OH radical-initiated gas-phase reaction of fluorene in the presence of oxides of nitrogen was investigated under a variety of experimental conditions, as listed in Table VIII-1. Nitrofluorene formation yields were measured for a total of fourteen experiments. Mutagenicity testing of the products of selected chamber runs was conducted as described in Section VI.

The nitrofluorene isomers were synthesized, isolated and identified as described in Appendix A. As noted in Section VI, the four isomers were tested for their specific mutagenicity both in the Salmonella standard Ames plate-incorporation test and in the Kado microsuspension modification (see Table VI-2). The results from the two tests are consistent in that the 2- and 3-nitrofluorenes are more mutagenic than the 1- and 4-nitrofluorene isomers. Although the activity of the 2- and 3-nitrofluorenes are nearly identical in the Kado assay, 2-nitrofluorene has twice the activity of 3-nitrofluorene in the plate-incorporation assay.

Table VIII-1. Experimental Conditions and Product Yields for the Formation of Nitrofluorenes (NF) from the Gas-Phase OH Radical-Initiated Reaction of Fluorene

| | $10^{-13}$ x Initial Conc. (molecule $cm^{-3}$) | | | | Lights % for | Fluorene reacted in sampled volume[a] (molecule x $10^{-18}$) | Nitrofluorenes formed in sampled volume (molecule x $10^{-15}$) | | | | % Yield of reacted fluorene | | | | % Total Yield of nitro- |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ITC | Fluorene | $CH_3ONO$ | NO | $NO_2$ | t(min) | | 1-NF | 2-NF | 3-NF | 4-NF | 1-NF | 2-NF | 3-NF | 4-NF | fluorenes |
| 1611 | 0.215 | 24.7 | >24 | <0.1 | 100/5 | 1.2 | 3.1 | 1.7 | 7.4 | 1.1 | 0.26 | 0.14 | 0.62 | 0.09 | 1.1 |
| 1616 | 0.286 | 25.7 | 21.0 | 3.0 | 100/5 | 3.0 | 11 | 1.1 | 21 | 4.8 | 0.37 | 0.04 | 0.70 | 0.16 | 1.3 |
| 1617 | 0.328 | 5.2 | 22.9 | 1.1 | 20/10 | 0.46 | 9.7 | <0.3 | 13 | 1.6 | 2.1 | <0.07 | 2.8 | 0.35 | 5.3 |
| 1628 | 0.285 | ~25 | 22.9 | 1.6 | 100/10 | 3.4 | 13 | 1.4 | 31 | 6.8 | 0.38 | 0.04 | 0.91 | 0.20 | 1.5 |
| 1629 | 0.239 | 25.2 | 23.0 | 1.2 | 100/5 | 3.2 | 12 | 1.1 | 24 | 4.0 | 0.38 | 0.03 | 0.75 | 0.13 | 1.3 |
| 1630 | 0.280 | ~25 | 23.6 | 0.8 | 100/2 | 1.7 | 12 | 1.1 | 21 | 2.3 | 0.74 | 0.07 | 1.2 | 0.13 | 2.1 |
| 1631 | 0.273 | 26.0 | 23.1 | 1.4 | 100/5 | 2.0[b] | 11 | 2.0 | 22 | 4.0 | 0.56 | 0.10 | 1.1 | 0.20 | 2.0 |
| 1632 | 0.180 | 5.5 | ≥24 | <0.1 | 100/10 | 0.59 | 7.4 | 0.57 | 16 | 2.3 | 1.3 | 0.10 | 2.7 | 0.39 | 4.5 |
| 1620 | 0.136 | 23.3 | 2.2 | 15.1 | 100/6 | 0.98 | 7.7 | 1.1 | 16 | 4.0 | 0.79 | 0.12 | 1.7 | 0.41 | 3.0 |
| 1621 | 0.205 | 14.6 | 2.4 | 21.6 | 100/10 | 1.4 | 7.4 | 1.1 | 21 | 4.8 | 0.53 | 0.08 | 1.5 | 0.34 | 2.5 |
| 1622 | 0.219 | 24.0 | 2.4 | 21.6 | 100/6 | 1.5 | 10 | 1.7 | 24 | 6.3 | 0.68 | 0.11 | 1.6 | 0.42 | 2.8 |
| 1623 | 0.316 | 26.3 | 3.0 | 21.0 | 100/6 | 2.5 | 13 | 2.3 | 30 | 9.4 | 0.54 | 0.09 | 1.2 | 0.38 | 2.2 |
| 1624 | 0.221 | 28.2 | 2.4 | 21.6 | 100/6 | 1.4 | 13 | 2.3 | 28 | 8.0 | 0.89 | 0.16 | 1.9 | 0.55 | 3.5 |
| 1627 | 0.273 | 25.3 | 2.3 | 19.3 | 100/6 | 1.9 | 10 | 1.7 | 24 | 8.0 | 0.53 | 0.09 | 1.3 | 0.42 | 2.3 |

[a]Volume sampled = 2180 ± 270 liter in 2.0 min.
[b]No post-run fluorene concentration measured, reacted fluorene was calculated from observed fluorene decrease in other runs.

The activity of our stock solution of 2-nitrofluorene, which we routinely use as the positive control in our test procedure, was in excellent agreement with the newly prepared 2-nitrofluorene standard solution. A Kado test was also run on a mixture containing approximately equal concentrations of the four nitrofluorene isomers. This mixture gave a response of 8,900 revertants $\mu g^{-1}$, in excellent agreement with the calculated response (based on the concentration of each isomer) of 9,600 revertants $\mu g^{-1}$, indicating good additivity of the nitrofluorene activities.

Fluorene reaction products in the HPLC fractions from the OH radical-initiated fluorene chamber exposures were identified by GC/MS analysis. The measured mass spectra and retention indices (on DB-5 capillary columns) were compared with those obtained for standard compounds and data given in the literature. The linear programmed retention indices were calculated according to the relationship given by van den Dool and Kratz (1963), using as bracketing substances naphthalene (RI = 200), phenanthrene (RI = 300) and chrysene (RI = 400).

Nitrofluorene Quantification. GC/MS analyses were carried out on a Hewlett Packard 5890 GC with an Hewlett Packard 5970 mass selective detector (MSD). On-column injections were made onto a 30 cm x 0.32 mm uncoated precolumn at 50°C oven temperature. The GC columns used were a 30 m x 0.26 mm DB-1701 column (14 % cyanopropylphenyl, 0.25 µm film thickness, J&W Scientific) and a 60 m x 0.24 mm DB-5 column (5 % phenyl-silicone, film thickness 0.25 µm, J&W Scientific). After sample injection, the oven was programmed to 280°C at a rate of 6°C $min^{-1}$. The carrier gas was helium.

Quantification of the nitrofluorenes (NF) was performed by measuring the ratio of peak areas obtained from the single ion chromatograms of the molecular ions of the deuterated standard compound, 2-nitrofluorene-$d_9$, and the nondeuterated sample constituents. Relative response factors (RF) between 2-NF-$d_9$ and the nondeuterated nitrofluorene isomers were determined by comparing peak area ratios obtained from GC/flame ionization detection (FID) (assumed relative response = 1) with the peak areas for the molecular ion peaks from the GC/MS measurements. The mass of the NF analytes then were determined using the following general formula: the mass of analyte X ($m_x$) was determined from the peak

areas (A) of the analyte and the internal standard (IS) as follows: $m_x = A_x A_{IS}^{-1} m_{IS} RF^{-1}$.

2. Nitrofluorenes in Diesel Particles

200 Mg diesel particulate samples from the National Bureau of Standards standard reference material (SRM) 1650 were each spiked with 100 ng of 2-nitrofluorene-$d_9$ and Soxhlet extracted for 48 h with 200 mL of dichloromethane. The extract was concentrated to about 5 mL, filtered through 0.2 μm Teflon filters (Gelman Acrodisc CR PTFE), concentrated to about 100 μL and separated by HPLC on a preparative Regis Spherisorb S5W silica column as described in Section VI above. The fraction eluting between 31 and 36 min was collected, concentrated to 100 μL and further fractionated by reversed phase HPLC. The column used was a Beckman Ultrasphere ODS 5μ, 1 cm x 25 cm with a Beckman Gradient Liquid Chromatograph Model 334 system and a Beckman Model 164 UV Detector, $\lambda$ = 254 nm. The solvent program started with 50 % water and 50 % methanol. From 10 to 40 min a linear gradient to 100 % methanol was programmed, 100 % methanol was held for 20 min and then the eluent was programmed back to the initial composition over 5 min. The solvent flow was 3 mL $min^{-1}$. The fraction from 34 to 42 min was collected, concentrated to about 3 mL and extracted 5 times with 3 mL dichloromethane. The combined dichloromethane extracts were concentrated to 100 μL and analyzed by GC/MS as described above.

3. Nitrofluorenes in Ambient Air

Ambient air samples were collected during daylight hours (0600 to 1800 PST) at Long Beach, CA during the South Coast Air Quality Study in November and December 1987. The samples selected for analysis were those collected on the days following nights with high evening $NO_x$ and nitrous acid (HONO) concentrations (Winer et al., 1989). Monitored $NO_2$ concentrations throughout the sampling times and the evenings prior to sampling ranged between 30 and 250 ppb. Nitrous acid mixing ratios during the evenings prior to sampling reached maximum values between 8 and 15 ppb. High daytime photochemical activity from the early morning photolysis of nitrous acid was, therefore, expected.

Sampling was performed for gas-phase species (an operational definition of gas phase is employed here, meaning those compounds not present on the collected particles) using three polyurethane foam (PUF)

plugs positioned in series downstream from a Teflon-impregnated glass fiber filter (TIGF). The flow rate was ~27 SCFM, resulting in sampled volumes of 540 $m^3$ per 12-hr sampling period. After sampling, the PUF plugs were wrapped in aluminum foil, sealed in glass jars and stored in a freezer at -18°C. Analysis of a single PUF plug sample showed the nitrofluorene concentration to be quite low and, therefore, PUF plug samples from four sampling periods were composited for analysis, resulting in a combined sample volume of 2150 $m^3$. Before Soxhlet extraction with dichloromethane the PUF plugs were spiked with appropriate amounts of internal standard compounds (1-nitronaphthalene-$d_7$ [Aldrich Chemical Company] and 2-nitrofluorene-$d_9$). Extraction and sample workup were performed as described above for the diesel particulate analysis. 1- and 2-Nitronaphthalene and 3-nitrobiphenyl could be quantified in the normal phase HPLC fraction eluting from 28.5 to 31 min without any further reversed phase separation. Nitrofluorenes were quantified after a further reversed phase separation as described above.

Ambient particulate samples were collected at the same site and under the same ambient conditions as described for the gas-phase samples. The particulate samples were collected on sampling systems equipped with size selective inlets (50% cutoff, 10 μm). The respirable-sized particles were collected on 8 in. x 10 in. TIGF filters at a flow rate of about 40 SCFM (~1.1 $m^3$ $min^{-1}$). After sampling, the filters were folded, wrapped in aluminum foil and stored in a freezer at -18°C. The filters were spiked with appropriate amounts of the standard compounds and extracted with 200 mL of dichloromethane. The extract was then treated the same way as described above.

A blank sample was obtained by concentrating the same amount of solvent as used for the sample analysis. After running through the entire separation procedure, the GC/MS analysis of this sample showed no signals from the analytes of interest that were sufficiently intense to allow peak integration.

Quantification of the nitrofluorene isomers was performed as described above for the chamber samples using the molecular ions m/z = 211 for nitrofluorenes (m/z = 220 for 2-nitrofluorene-$d_9$). The masses used for additional nitroarene quantification were m/z = 173 for nitronaphthalenes (m/z = 180 for 1-nitronaphthalene-$d_7$) and m/z = 199 for nitro-

biphenyls. For each compound, an additional mass (or masses) of characteristic fragment ions were monitored to confirm the peak identifications. The RF values (applied with the general equation given above) for the nitronaphthalenes to 1-nitronaphthalene-$d_7$ were determined to be 1.18 for 1-nitronaphthalene and 1.75 for 2-nitronaphthalene, respectively. 3-Nitrobiphenyl was quantified with 1-nitronaphthalene-$d_7$ as an internal standard using an RF value of 3.23.

B. Results

1. Mutagenicity Assay of Chamber HPLC Fractions

Mutagenicity testing was conducted on four selected environmental chamber reactions of fluorene and the resulting mutagrams are plotted in Figure VIII-1 for chamber runs ITC-1611, 1625, 1643 and 1646. The highest activity was consistently present in fraction #4 which accounted for approximately 70-80% of the total recovered mutagenicity. The substances responsible for the mutagenic activity in fraction #4 were identified as nitrofluorene isomers. Other fractions with significant activities, i.e., in the 5 to 15 % range, were fractions #5, 6 and 7 (Figure VIII-1).

Runs ITC-1643 and ITC-1646 were conducted under conditions where sufficient NO to $NO_2$ conversion occurred to allow the formation of $O_3$ and hence of $NO_3$ radicals. It can be seen from Figure VIII-1 (where the activity in each fraction is shown normalized to the sum of the activity from all fractions) that the proportion of the total mutagenic activity recovered from the exposure which was found in HPLC fraction #4 was less for ITC-1643 and ITC-1646, and relatively more activity showed up in fractions #5 and #6. This indicates that under these experimental conditions the proportion of more polar components, presumed to be more highly substituted and/or oxidized reaction products, was somewhat greater. One candidate mutagen responsible for the activity in fraction #5 would be 3-nitrofluorenone (identified by GC/MS as noted below).

2. Chemical Analysis of Chamber HPLC Fractions

Qualitative Results. Each of the HPLC fractions (and in many cases smaller subfractions collected over shorter time intervals) were analyzed by GC/MS. The fluorene reaction products identified, the measured retention indices (RI) on the DB-5 column and the available literature RI data are listed in Table VIII-2. Separate injections were

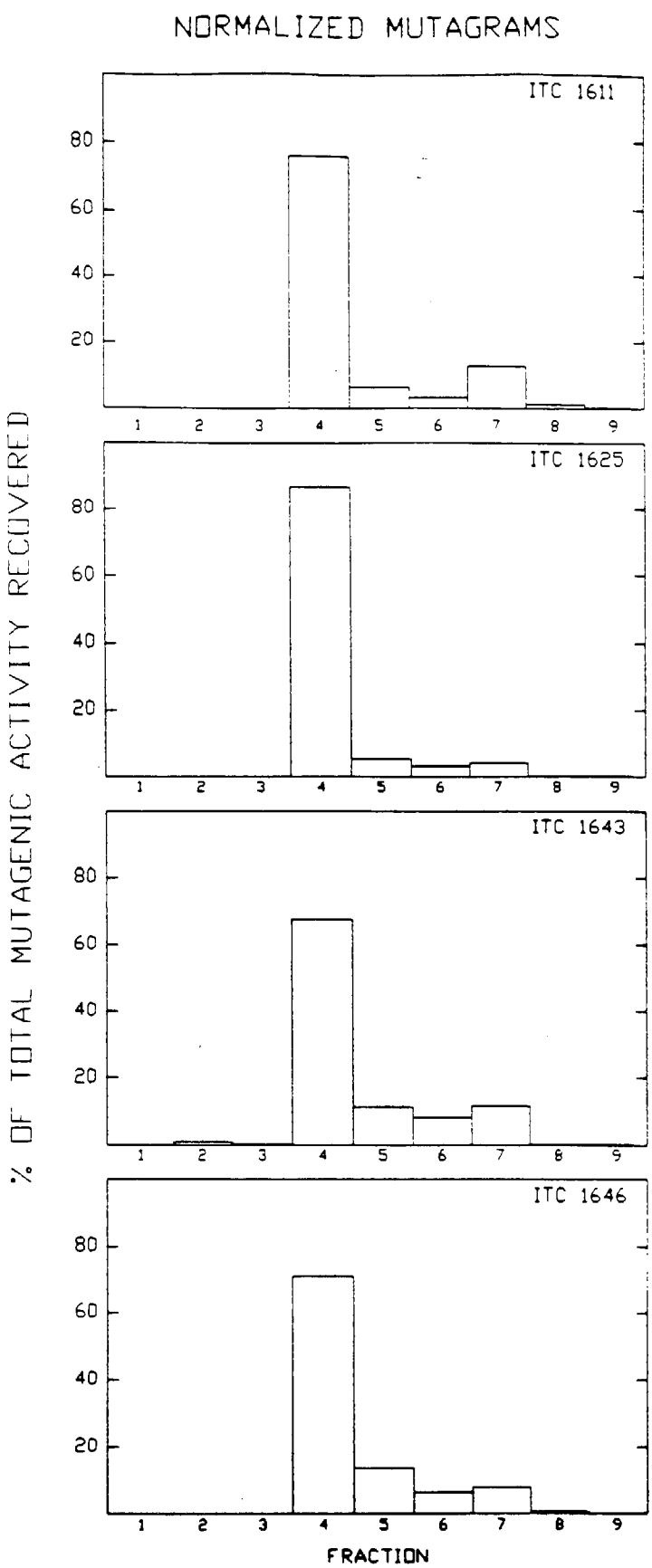



Figure VIII-1. Normalized mutagrams from the mutagen assay of HPLC fractions of the chamber exposures: ITC-1611, 1625, 1643 and 1646.

Table VIII-2. Identified Fluorene Reaction Products with Molecular Weight (MW), HPLC Fraction, and Retention Index (RI) Data on a DB-5 Capillary Column

| Compound | MW | HPLC Fraction | $RI_{sample}$[a] | $RI_{std}$[b] | $RI_{lit}$[c] |
|---|---|---|---|---|---|
| 9-hydroxyfluorene | 182 | 7 | 292.6 | 294.1[d] | |
| fluorenone | 180 | 5 | 293.5 | 293.1 | 294.8[e,f] |
| x-hydroxyfluorene | 182 | 5 | 316.6 | | |
| x-hydroxyfluorene | 182 | 5,6 | 318.9 | | |
| x-hydroxyfluorene[g] | 182 | 6 | 320.6 | | |
| 1-nitrofluorene | 211 | 5 | 337.4 | 337.4 | |
| 4-nitrofluorene | 211 | 5 | 337.4 | 337.4 | |
| 4-nitrofluorenone[h] | 225 | 5 | 342.5 | | |
| 3-nitrofluorene | 211 | 5 | 347.9 | 347.9 | |
| hydroxynitrofluorene | 227 | 5,6,7 | 349.1 | | |
| 2-nitrofluorene | 211 | 5 | 351.0 | 351.0 | |
| 3-nitrofluorenone | 225 | 5 | 353.8 | 354.3 | 355.1[e,i] |
| hydroxynitrofluorene | 227 | 7 | 354.4 | | |
| hydroxynitrofluorene | 227 | 5,6,7 | 356.1 | | |
| 1-nitrofluorenone[h] | 225 | 5 | 372.1 | | |

[a]Due to separate injections for the sample and standard solutions and poor peak shapes for the hydroxy and nitrohydroxy compounds, these RI values should be regarded as approximate.
[b]Retention index measured for an authentic standard analyzed on the same column and under identical conditions as the chamber reaction products.
[c]Values are rounded to one decimal unit.
[d]Approximate value due to broad peak shape.
[e]Retention index on SE-52 stationary phase.
[f]From Lee et al. (1979).
[g]May be 2-hydroxyfluorene ($RI_{std}$ = 322.5).
[h]Tentative identification.
[i]From White et al. (1983).

made for the HPLC fractions and the solutions containing the bracketing PAH standards used to calculate the RI values. For the more polar components, a relatively high concentration dependence of the RI values was observed and, therefore, the $RI_{sample}$ values given in Table VIII-2 should be regarded as approximate. The mass spectra of the reaction products are given in Appendix B.

As detailed by Helmig and Arey (1991) [Appendix A], the nitrofluorenes were synthesized by different methods and characterized by their mass spectra, $^1$H-NMR spectra and GC retention indices on three column phases. With this comprehensive data set, isomer-specific nitrofluorene analyses could then be achieved. 1- and 4-Nitrofluorene were not separated on the DB-5 column, and therefore, the identification of these two compounds was accomplished using a DB-1701 column, which separated all four isomers.

Isomer-specific identification of the hydroxyfluorenes, other than 9-hydroxyfluorene, was not possible as neither literature retention index data nor standard compounds were available. The identification of the nitrofluorenone isomers was based on the observation that 2-nitrofluorenone (RI = 366.80 on SE-52, White et al., 1983) could not be detected in the chamber samples and that the remaining nitrofluorenones had thus to be 1-, 3- and 4-nitrofluorenone. 3-Nitrofluorenone was identified as the the nitrofluorenone at RI = 353.8 based on reasonable agreement with the RI of an authentic standard of 3-nitrofluorenone as well as the reported literature value. The two remaining isomers had to be 1- and 4-nitrofluorenone and these were tentatively assigned by assuming that 1-nitrofluorenone, being the more polar isomer, should elute later than 4-nitrofluorenone.

As seen in Table VIII-2, at least three compounds of molecular weight 227 were identified as hydroxynitrofluorenes. Compounds of molecular weight 227 and RI values of 349.1 and 356.1 were observed from HPLC fractions #5, 6 and 7. The compounds observed in fractions #5, 6 and 7 may be different hydroxynitrofluorene isomers not resolved by the GC column. Alternatively, the HPLC program utilized may result in very broad elution of these hydroxynitrofluorenes.

As noted above, a tentative identification of an hydroxynitrofluorenone (molecular weight 241) was made by Nishioka and co-workers (Nishioka et al., 1988) in a polar mutagenic subfraction of an ambient particle extract. Interestingly, no compound of molecular weight 241 was observed in any of the HPLC fractions examined from the fluorene chamber reactions.

Quantitative Results. The fluorene reaction products quantified were the nitrofluorenes and fluorenone. All four possible nitrofluorenes were formed under the selected reaction conditions. Figure VIII-2 shows the mass chromatogram of a GC/MS analysis for the nitrofluorenes in the chamber sample from fluorene run ITC-1611. The product yields for the four nitrofluorenes are listed in Table VIII-1.

In order to establish that the observed isomer distribution was not affected by the instrumental sample work-up, 50% of the combined subfractions 4.1 to 4.6 of ITC-1625 was spiked with 2 μg of 2-nitrofluorene, analyzed as described and than reseparated by HPLC and analyzed again. The analytical results obtained are listed in Table VIII-3. The data shows no preferential loss for any NF isomer during the HPLC sample work-up, indicating that the NF distribution as measured is representative of that formed during the chamber reactions.

Under varying reaction conditions the distribution of isomers formed was relatively unchanged with 3-nitrofluorene being the main nitrofluorene isomer, followed by 1- and 4-nitrofluorene. The total nitrofluorene formation yield (NF formed/fluorene reacted) ranged from 1 to 5% with an average yield of 2.5 ± 1.2%. The experiments can be divided into two sets, with the initial NO and $NO_2$ concentrations being markedly different. Average production yields of these two sets, calculated from the experiments in which the light intensity was 100%, are summarized in Table VIII-4. Within the variability of the data, no significant effect of the $NO_2$ concentration can be discerned.

The overall nitrofluorene formation yield from the OH radical-initiated reaction of fluorene is within the range of nitro-PAH formation yields previously determined for the OH radical-initiated reactions of a series of two to four-ring PAH. Those PAH examined and their corresponding nitro-PAH formation yields are: naphthalene (0.6%), 1-methylnaphthalene (~0.4%), 2-methylnaphthalene (~0.2%), acenaphthylene

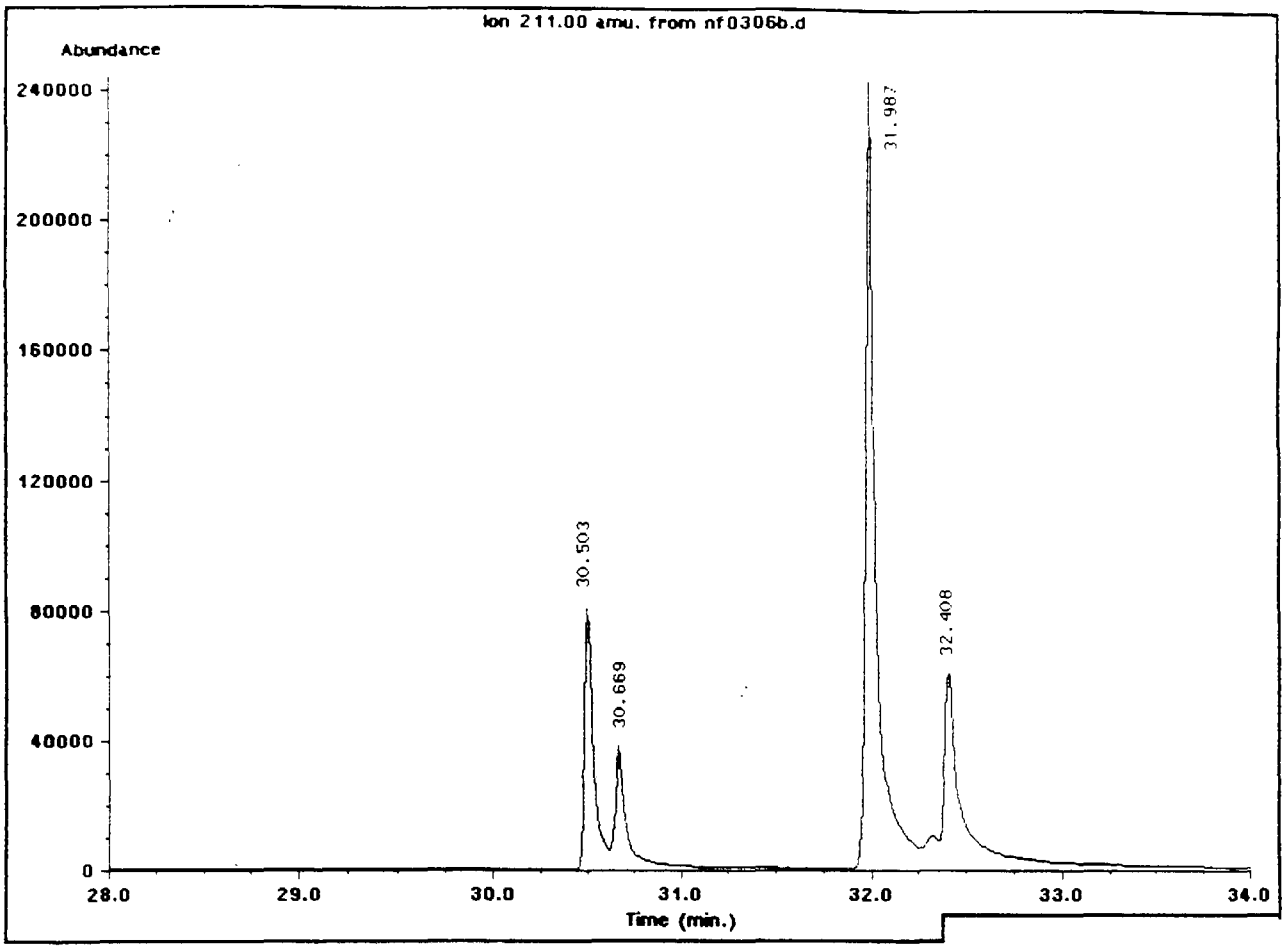



Figure VIII-2. Mass chromatogram from the selected ion monitoring GC/MS analysis of HPLC fraction #4 of ITC-1611 for the nitrofluorene molecular ion (m/z = 211). The elution sequence is 1-nitrofluorene (Rt = 30.50 min), 4-nitrofluorene (Rt = 30.67 min), 3-nitrofluorene (Rt = 31.99 min) and 2-nitrofluorene (Rt = 32.41 min).

Table VIII-3. Influence of HPLC Separation on Nitrofluorene Distribution

| Nitrofluorene | Relative Abundance of Nitrofluorenes[a] | |
|---|---|---|
| | 1st HPLC Separation | 2nd HPLC Separation |
| 1-NF | 0.151±0.003 | 0.151±0.001 |
| 2-NF | 0.296±0.004 | 0.304±0.004 |
| 3-NF | 0.448±0.007 | 0.446±0.003 |
| 4-NF | 0.105±0.007 | 0.102±0.002 |

[a]Average of three GC/FID determinations.

(2%), acenaphthene (~0.2%), biphenyl (5%), phenanthrene (≤0.1%), anthracene (~0.2%), fluoranthene (4.3%), pyrene (0.6%) and acephenanthrylene (~0.1%) [Arey et al., 1989b; Atkinson et al., 1990a].

The present observation that the nitrofluorene yields are, within the experimental errors, independent of the $NO_2$ concentration is consistent with our previous data for naphthalene and biphenyl (Atkinson et al., 1987) and is in accord with our data for a series of monocyclic aromatic hydrocarbons (Atkinson et al., 1989). These data suggest that despite the much higher $NO_x$ concentrations used in these experiments, the nitrofluorene formation yield of ~2-3% will be applicable to ambient atmospheric conditions.

The fluorenone yield was determined by GC/FID analysis in 9 chamber runs using external calibration with a fluorenone standard solution. The reaction yields determined were 9.2 ± 5.3 % of the fluorene reacted. This value should be regarded as a lower limit since sample loss occurring during the work-up procedure is not corrected for (as is the case for the nitrofluorenes where a deuterated internal standard is added prior to the sample work-up).

Table VIII-4. Comparison of Average Nitrofluorene Yields at Two Different $NO_2$ Concentrations

| $NO_2$ Concentration ($\times 10^{13}$ molecules $cm^{-3}$) | Average Time Lights On (min) | Nitrofluorene Yield (%)[a] | | | | |
|---|---|---|---|---|---|---|
| | | 1-NF | 2-NF | 3-NF | 4-NF | $\sum$ NF |
| 1.5 | 6.00 | 0.57 ± 0.35 | 0.07 ± 0.04 | 1.14 ± 0.72 | 0.19 ± 0.10 | 1.97 ± 1.18 |
| 15 | 6.66 | 0.66 ± 0.15 | 0.11 ± 0.03 | 1.53 ± 0.26 | 0.42 ± 0.07 | 2.72 ± 0.49 |

[a]Indicated errors are one standard deviation.

3. Nitrofluorenes in Diesel Particulates, Ambient Gas-Phase and Ambient Particulate Samples

Diesel. Due to the low total nitrofluorene concentrations observed, analysis of the diesel particulates was difficult. A second HPLC separation step was employed, but the signal to noise ratio in the GC/MS single ion chromatogram remained unsatisfactory, with the signals of the nitrofluorenes being close to, or below, the limit of detection. Only 2-NF could be positively identified and quantified at a concentration of about 0.25 $\mu g\ g^{-1}$. Although traces of 1-, 3- and 4-NF were detected, quantification of these isomers was not possible, and the concentrations of these isomers were estimated to be below 0.1 $\mu g\ g^{-1}$. In order to check these low concentration values, a second 200 mg diesel particulate sample was spiked with ~50 ng of 1-, 2-, 3- and 4-NF (equivalent to a concentration of ~0.25 $\mu g\ g^{-1}$) and run through the entire analysis procedure. All isomers were recovered in the final GC/MS analysis of this sample. The measured concentrations for this spiked sample ranged between 0.18 and 0.23 $\mu g\ g^{-1}$ for 1-, 3- and 4-NF, indicating reasonable recovery of the nitrofluorenes. Subtraction of the amount of 2-NF spiked into this sample from the measured 2-NF value of 0.55 $\mu g\ g^{-1}$ gives a 2-NF concentration in the SRM 1650 which is consistent with our first analysis. This experiment confirms that only 2-NF was present in the diesel soot sample at a significant level.

The 2-NF concentration we determined (0.25 $\mu g\ g^{-1}$) is in good agreement with the result of 0.27 $\mu g\ g^{-1}$ given for the same SRM 1650 sample by MacCrehan et al. (1988). The value of 15 $\mu g\ g^{-1}$ reported by Draper (1986) for this SRM diesel particulate appears too high and probably results from interferences in the GC with electron capture detection method employed in that work (Draper, 1986). In an extract of a different diesel particulate sample Campbell and Lee (1984) determined a 2-NF concentration equivalent to 4.1 $\mu g\ g^{-1}$ of the particles. Several other authors report the identification of 2-NF in diesel particulates, but give no quantitative data (Xu et al., 1981; Paputa-Peck et al., 1983; Hartung et al., 1984; Niles and Tan 1989). To our knowledge, nitrofluorene isomers other than 2-NF have not been identified by other workers in diesel particulates. For the SRM 1650 diesel sample, our results show

that concentrations of 1-, 3-, and 4-NF are at least a factor of 2-3 lower than the 2-NF concentration.

The finding that 2-nitrofluorene is the most abundant nitrofluorene isomer in the diesel SRM is consistent with our previous suggestion that the nitro-PAH in particles directly emitted to ambient atmospheres, such as diesel exhaust, will be dominated by the PAH electrophilic nitration products such as 2-nitrofluorene, 1-nitropyrene and 3-nitrofluoranthene. The presence of nitro-isomers distinct from these electrophilic nitration products in ambient particles can be attributed to OH and/or $NO_3$ radical-initiated atmospheric reactions of the parent PAH (Arey et al., 1987, 1989b).

Ambient Gas-Phase Samples. 1-NF and 3-NF were the only isomers that could be quantified in the PUF samples. The concentrations determined for 1-NF were 0.02 ng $m^{-3}$ in the single PUF and 0.09 ng $m^{-3}$ in the combined sample, while the 3-NF concentrations were 0.10 and 0.09 ng $m^{-3}$, respectively. The other isomers gave signals too weak for quantification and are estimated to be below the 0.02 ng $m^{-3}$ level. From the single and combined samples, the 1- and 2-nitronaphthalene concentrations were 2.1 ng $m^{-3}$ and 2.2-2.3 ng $m^{-3}$, respectively. 3-Nitrobiphenyl was measured at concentrations of 3.8 and 0.96 ng $m^{-3}$ in the single and combined samples, respectively.

Ambient Particulate Samples. As occurred with the diesel samples, the concentrations of nitrofluorenes in the ambient particles were low with the GC/MS signals again being close to the detection limit. Combining several filters into one sample did not improve the sensitivity markedly, since detection was mainly limited by the signal to noise ratio and not by the total amount of analyte present. Although weak signals for 1-, 3- and 4-NF were detected, an unequivocal quantification was not possible. The NF levels in these samples were estimated to be ~0.02 ng $m^{-3}$, the approximate limit of detection.

The nitrofluorene concentrations measured in the ambient air samples analyzed in this study fall into the lower range of data obtained from air measurements at other sites. The only NF isomer quantifications reported by other investigators have been for 2-NF, reported to be in the range of 0.02 to 0.7 ng $m^{-3}$ in an extensive dataset of samples collected in Beijing, China; Kawasaki, Japan; and Tokyo, Japan (Iida et al., 1985

cited in Beije and Möller, 1988). Concentrations measured in samples from Vienna, Austria were 0.01 and 0.07 ng $m^{-3}$ (Schneider et al., 1990). 2-NF concentrations up to 5.2 ng $m^{-3}$ reported in a set of aerosol samples from Berlin, FRG (Moriske et al., 1984) appear remarkably high when compared with our data and the other literature data. It is not clear whether the data reported in the literature is isomer-specific, i.e., whether 2-NF and 3-NF could be distinguished.

The concentrations determined for 1- and 2-nitronaphthalene and 3-nitrobiphenyl in these samples are well within the ambient concentration ranges previously reported (Arey et al., 1987; 1989a; Atkinson et al., 1988). The concentrations we found for the nitrofluorenes in the samples analyzed were at least one order of magnitude lower than the concentrations determined for the nitronaphthalenes and 3-nitrobiphenyl. Consistent with the present findings, the two-ring nitro-PAH have been reported to be mainly in the gas phase, that is, collected on PUF plugs rather than on filters (Arey et al., 1987). This is in contrast to the 4-ring nitrofluoranthenes and nitropyrenes which are particle-associated (Arey et al., 1987; Atkinson et al., 1988; Zielinska et al., 1989a). From the data reported here, it would appear that the nitrofluorenes are distributed between the gas- and particle-phases, being mainly in the gas phase at the ambient temperatures encountered in Southern California.

The ambient 3-nitrofluorene concentrations that we measured are consistent with ambient OH radical-initiated formation of this species. For example, based on the 3-NF and the 1- and 2-nitronaphthalenes (1-NN, 2-NN) being formed only from the OH radical-initiated reactions of fluorene and naphthalene, respectively, and assuming similar atmospheric loss processes, and hence lifetimes, for 3-NF and the nitronaphthalenes, then

$$\frac{[3\text{-NF}]}{[1\text{-} + 2\text{-NN}]} = \frac{k_F}{k_N} \frac{Y_{3\text{-NF}}}{Y_{NN}} \frac{[\text{fluorene}]}{[\text{naphthalene}]}$$

where $k_F$ and $k_N$ are the rate constants for the reaction of the OH radical with fluorene and naphthalene, respectively, $Y_{3\text{-NF}}$ and $Y_{NN}$ are the formation yields of 3-NF and 1- + 2-NN, respectively, and [fluorene] and [naphthalene] are the ambient concentrations of fluorene and naphthalene.

Using our present and previous (Arey et al., 1989a,b) kinetic and product data and typical ambient concentrations of naphthalene and fluorene in the Los Angeles area (Arey et al., 1989a) of 3000 and 60 ng $m^{-3}$, respectively, then it is calculated that [3-NF]/[1- + 2-NN] ~0.02. With our measured ambient concentration of 1- + 2-NN of 4.5 ng $m^{-3}$ (see above) this leads to a predicted ambient concentration of 3-NF of ~0.1 ng $m^{-3}$, in excellent agreement with the measured gas-phase value of 0.1 ng $m^{-3}$.

It should be noted that the nitronaphthalenes undergo photolysis in ambient air with a lifetime calculated to be ~2 hr, and the above calculation assumes that 3-NF also photolyzes with a similar lifetime. If 3-NF does not photolyze, and is stable in the atmosphere, the 3-NF concentration relative to 1- + 2-NN will be factor of ~3 higher than that calculated above (Arey et al., 1990), still in reasonable agreement. We have previously predicted with similar success the ambient air concentrations of the nitronaphthalenes, 3-nitrobiphenyl, 2-nitrofluoranthene and 2-nitropyrene (Arey et al., 1990) [note that the particle-associated PAH may well be stabilized against photolysis, and in this context see also Atkinson et al. (1990a) who observed a rapid gas-phase photolysis of 2-nitrofluoranthene and 2-nitropyrene]. Thus, in general, the observed ambient concentrations of the 2 to 4-ring nitro-PAH can be accounted for by formation from the OH radical-initiated reaction of the gas-phase PAH in the atmosphere during transport from source to receptor.

# IX. PHENANTHRENE

The most abundant three-ring PAH in ambient air is phenanthrene (see, for example, Atkinson et al., 1988), and it is expected to be mainly in the gas phase (Arey et al., 1987; Bidleman, 1988; Coutant et al., 1988). The dominant atmospheric loss process of phenanthrene is by gas-phase reaction with the OH radical, leading to a calculated lifetime of only ~6 hr (Arey et al., 1989a,b). We have previously estimated the nitrophenanthrene yield from the OH radical-initiated reaction of phenanthrene to be low, $\lesssim$0.1% (Arey et al., 1989b), but the major reaction products were unknown. Therefore, bioassay-directed fractionation and chemical analysis was next applied to the OH radical-initiated reaction of phenanthrene in our environmental chamber.

## A. Methods

Chamber Reactions. Due to its relatively low volatility, phenanthrene was added to the chamber in methanol solution (~30 mg in 100 mL solvent). The phenanthrene solution was sprayed into the chamber as a very fine mist using a glass atomizer and with the chamber mixing fan on, thereby spreading the phenanthrene over the chamber surfaces. Flushing pure air through the chamber for ~15 min removed most of the methanol. The volatilization of the phenanthrene from the surfaces established the gas-phase phenanthrene concentration in the chamber. In this way phenanthrene concentrations as high as 160 ppb were measured (higher, for example, than the fluorene concentrations achieved by filling the chamber with $N_2$ that had passed through a tube containing crystalline fluorene).

In all, twelve chamber reactions of phenanthrene were conducted: ITC-1649, 1650 and the series ITC-1712 through ITC-1721. The methyl nitrite and NO concentrations were 2 and 1 ppm, respectively, and the reactions proceeded for 10 min each with the lights at 100% intensity. Two minute polyurethane foam (PUF) plug samples (~2000 liters) were collected at the end of the reaction time.

Control exposures consisting of irradiated methyl nitrite and NO were conducted immediately prior to the phenanthrene exposures ITC-1649 and the series of exposures starting with ITC-1712. The chamber volume from each control exposure was sampled onto PUF plugs for testing in parallel with

the phenanthrene exposure products. Immediately prior to the HPLC separation of the phenanthrene products from ITC-1649, a blank HPLC run was collected as an additional control to be bioassayed in parallel.

The PUF plugs were extracted in dichloromethane and fractionated by HPLC as detailed in Section VI. The collected HPLC fractions were then subjected to bioassay and chemical analyses.

Mutagenicity Assay. The Kado microsuspension modification of the Ames assay on TA98 and without microsomal activation was used for mutagenicity testing of the HPLC fractions (see Section VI). Subfractions of the most mutagenic fraction were collected and assayed in a similar manner.

Chemical Analyses. Chemical analysis of the HPLC fractions was conducted by GC/MS as described previously for the fluorene reaction products (see Section VIII). For comparison with chamber reaction products, two nitrodibenzopyranones were purchased from Aldrich Chemical Company and dibenzopyranone was synthesized by the intramolecular substitution reaction of diphenic acid. 0.01 Mole diphenic acid (2.42 g) was added to 250 mL 0.4 N $H_2SO_4$ and 0.1 mole $H_2O_2$. The solution was refluxed for 6 hr and then neutralized with $Na_2CO_3$ to pH 7. The solution was filtered and extracted three times with 100 mL of ether. The combined ether fractions were dried over $MgSO_4$ and the solvent was evaporated. The crude product was recrystallized in ether. The purified product (44 mg) was analyzed by GC/MS, GC/FID and GC/ECD and by high resolution mass spectrometry. The chromatographic analyses showed no impurities. The result of the GC/MS analysis is shown in Figure IX-1. A high resolution mass measurement from a probe sample of the product gave a molecular ion mass of 196.0526, only 0.9 ppm off the calculated mass of $C_{13}H_8O_2$ (well within the ± 6 ppm regarded as an acceptable match). Further confirmation was achieved by the determination of the melting point. The measured melting point was in the range of 95.6 - 97.1 °C, which is slightly above the literature data for dibenzopyranone which range from 91.5 to 95 °C (Doering and Speers, 1950, Pan and Fletcher, 1960; Horner and Baston, 1973; Mehta and Pandey, 1975).

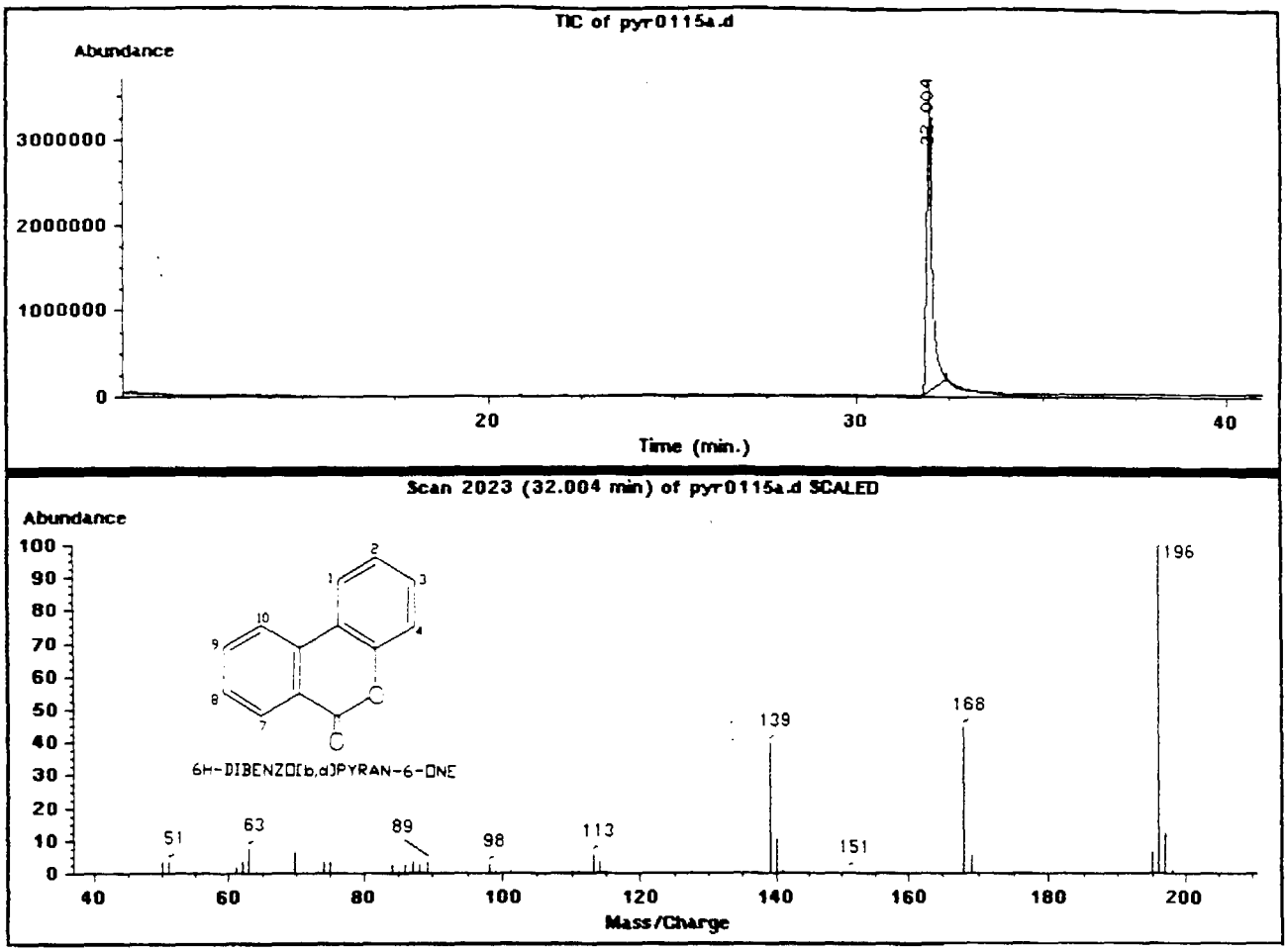



Figure IX-1. GC/MS analysis of a synthesized 6H-dibenzo[b,d]pyran-6-one standard. The upper trace shows the total ion chromatogram (TIC) and the mass spectrum of the peak at Rt = 32.00 min is shown below the TIC (structure shown on mass spectrum).

B. Results

Mutagenicity Assay of Chamber HPLC Fractions. The results of the mutagenicity test for ITC-1649 are given in Table IX-1. The total mutagenicity from the phenanthrene reaction was substantially greater than the total activity from either the naphthalene or the majority of the fluorene reactions (see Figure VI-2). The HPLC blank and the chamber control samples gave approximately 0.1% and 0.3% of the mutagenicity of the phenanthrene exposure sample, respectively, and were not significantly mutagenic except in the relatively nonmutagenic fractions #8 and #9 where they accounted for about half of the activity. The activity of an aliquot of the unfractionated extract of the phenanthrene reaction products was low relative to the sum of the individual fractions. Based on the unfractionated aliquot the sum of the sample activity would be 41,000 revertants in comparison with the calculated sum from the aliquot subjected to HPLC fractionation of 340,740 revertants (see Table IX-1), indicating possible suppression of mutagenicity in the whole extract due to toxicity. Toxicity was observed in fractions #2, 3, and 7, but not in the highly mutagenic fraction #6.

The interesting result of the phenanthrene chamber exposure is that the most mutagenic HPLC fraction was fraction #6, a more polar fraction than the most mutagenic fraction in the naphthalene (Section VII) and fluorene (Section VIII) exposures. Figure IX-2 shows the distribution of mutagenicity for the phenanthrene reaction products together with comparable mutagrams for a fluorene exposure (ITC-1643, total revertants 137,050) and a Riverside ambient particle extract (see Section IV). The HPLC fractions were composited differently for the chamber and ambient samples, but are displayed on the same axes. Figure VI-2 showed phenanthrene (ITC-1649) and fluorene (ITC-1611) mutagrams compared to ambient Claremont particle extracts. Both ambient mutagram - chamber reaction products mutagram comparisons make the same important feature apparent. The polarity distribution of the phenanthrene reaction products is a much better match with both the Riverside and Claremont ambient samples than is the polarity distribution for the fluorene reactions. The dominant mutagenic fraction of the phenanthrene exposure, fraction #6, overlaps with the dominant mutagenic fraction of the ambient sample shown in Figure IX-2 and corresponds to the most mutagenic fraction of the

Table IX-1. Mutagenicity of HPLC Fractions of Reaction Products From the OH Radical-Initiated Reaction of Phenanthrene (ITC-1649) in the Kado Assay (TA98; -S9)

| HPLC Fraction # | Total Calculated[a] Revertants per Fraction |
|---|---|
| 1 | 0 |
| 2 | 44,000 |
| 3 | 29,000 |
| 4 | 21,900 |
| 5 | 3,420 |
| 6 | 230,000 |
| 7 | 12,000 |
| 8 | 240 |
| 9 | 180 |
| SUM | 340,740 |

[a]The total has been calculated based on the fraction of the actual sample tested for activity.

ambient sample in Figure VI-2 (where the HPLC collection times of the ambient sample fractions were identical with those of the chamber reaction). Thus, the compounds in fraction #6 may be representative of the polar mutagens in ambient air. Indeed, given the abundance of phenanthrene, these compounds may be significant contributors to ambient particulate mutagenicity.

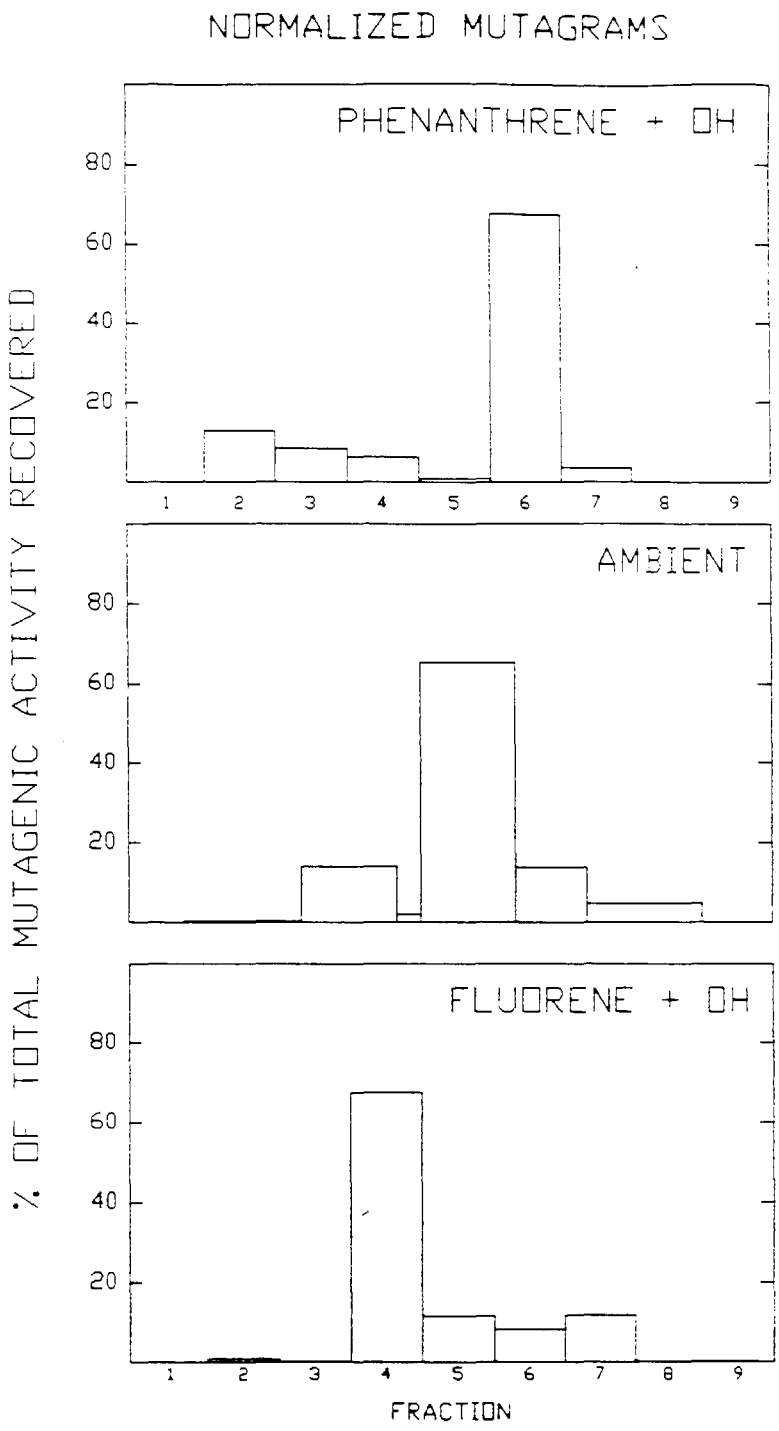



Figure IX-2. Comparison of the HPLC mutagrams from the gas-phase OH radical-initiated reactions of phenanthrene (ITC-1649) and fluorene (ITC-1643) with that of a Riverside ambient air particulate extract. The mutagenicity values have been normalized to the sum of the individual fractions.

Chemical Analysis of Chamber HPLC Fractions. The GC/MS chemical analysis of the HPLC fractions was focused initially on fraction #6 which had the highest mutagenic activity. Dibenzopyranone was tentatively identified in fraction #6 of ITC-1649. The extracts of two chamber phenanthrene exposures (ITC-1712 and 1713) were then pooled for HPLC separation as previously, except that nine one-minute "subfractions" were collected during the fraction #6 time window. The HPLC trace ($\lambda$ = 254 nm) showing the subfractions collected and a mutagram of their corresponding relative mutagenic activities are given in Figure IX-3. GC/MS analysis of subfractions 6.1 to 6.9 showed three assignable phenanthrene reaction products.

In subfraction 6.2 a large peak is evident from the HPLC trace. 6H-Dibenzo[b,d]pyran-6-one was identified in this subfraction by matching its mass spectrum and linear programmed retention index (RI = 326.27) with those of the synthesized standard (see Figure IX-1). Mutagenicity testing of dibenzopyranone in the standard Ames test has previously shown it to be a very weak direct-acting mutagen having an activity of 0.1 rev $\mu g^{-1}$. This is consistent with the very low proportion of the activity of fraction #6 that is found in subfraction 6.2.

Two nitro-substituted isomers of this parent lactone were found in the highly mutagenic subfractions 6.4 and 6.5. The earlier eluting of the two compounds was identified as 2-nitrodibenzopyranone by matching its GC retention time and mass spectrum with that of an authentic standard (mass spectrum shown in Figure IX-4). This isomer was distributed between subfractions 6.4 and 6.5 in approximately the same way as the mutagenic activity. Based on a comparison of the GC retention times, the second isomer found in the highly mutagenic subfraction 6.5 was determined not to be 3-nitrobenzopyranone (the only other available standard). The mass spectrum of the second phenanthrene reaction product (we have labelled it x-nitrodibenzopyranone) is given in Figure IX-5.

To quantify the nitrodibenzopyranones in subfractions 6.4 and 6.5, a GC method with electron capture detection (GC/ECD) was developed. Although this method showed good sensitivity, it was found that the GC/ECD sensitivities for the two standard compounds available (2-nitrodibenzopyranone and 3-nitrodibenzopyranone) differed by a factor of 2.8 under the selected conditions, with 3-nitrodibenzopyranone having the higher

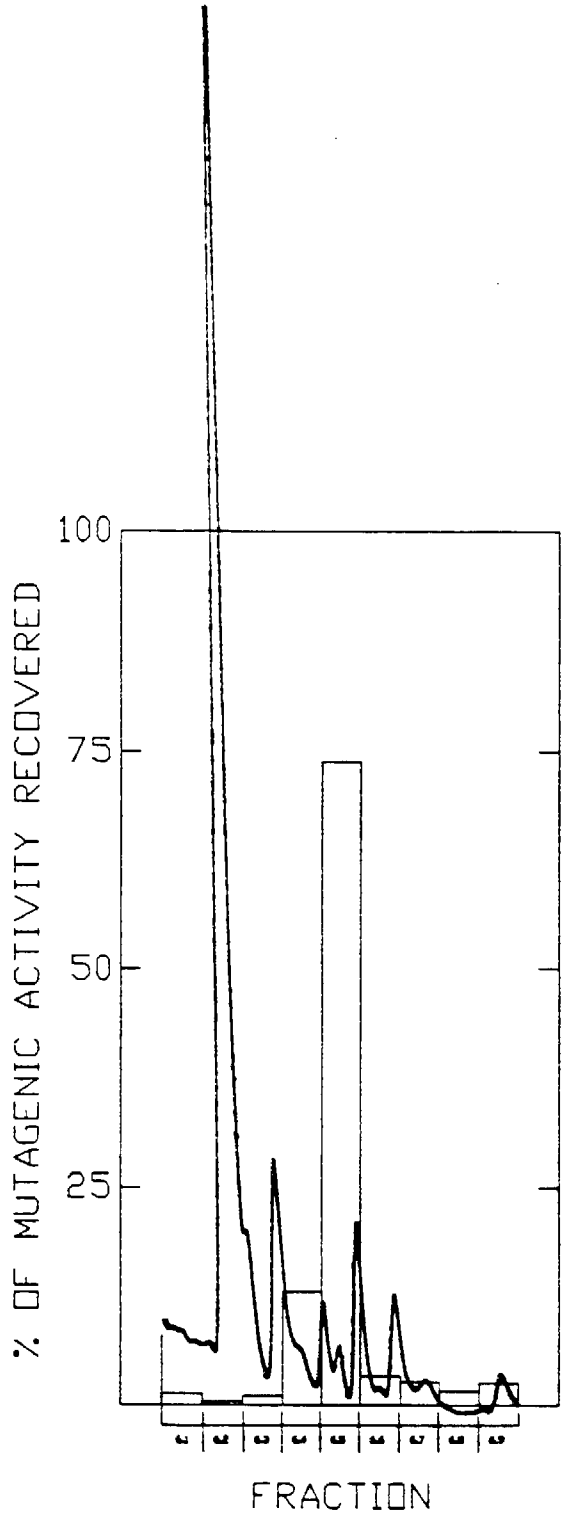



Figure IX-3. HPLC chromatogram ($\lambda$ = 254 nm) of fraction #6 of the reaction products from the OH radical-initiated reactions of phenanthrene in the environmental chamber (ITC-1712 and 1713) showing the subfractions collected (6.1 to 6.9) and the percent of mutagenic activity recovered in each subfraction.

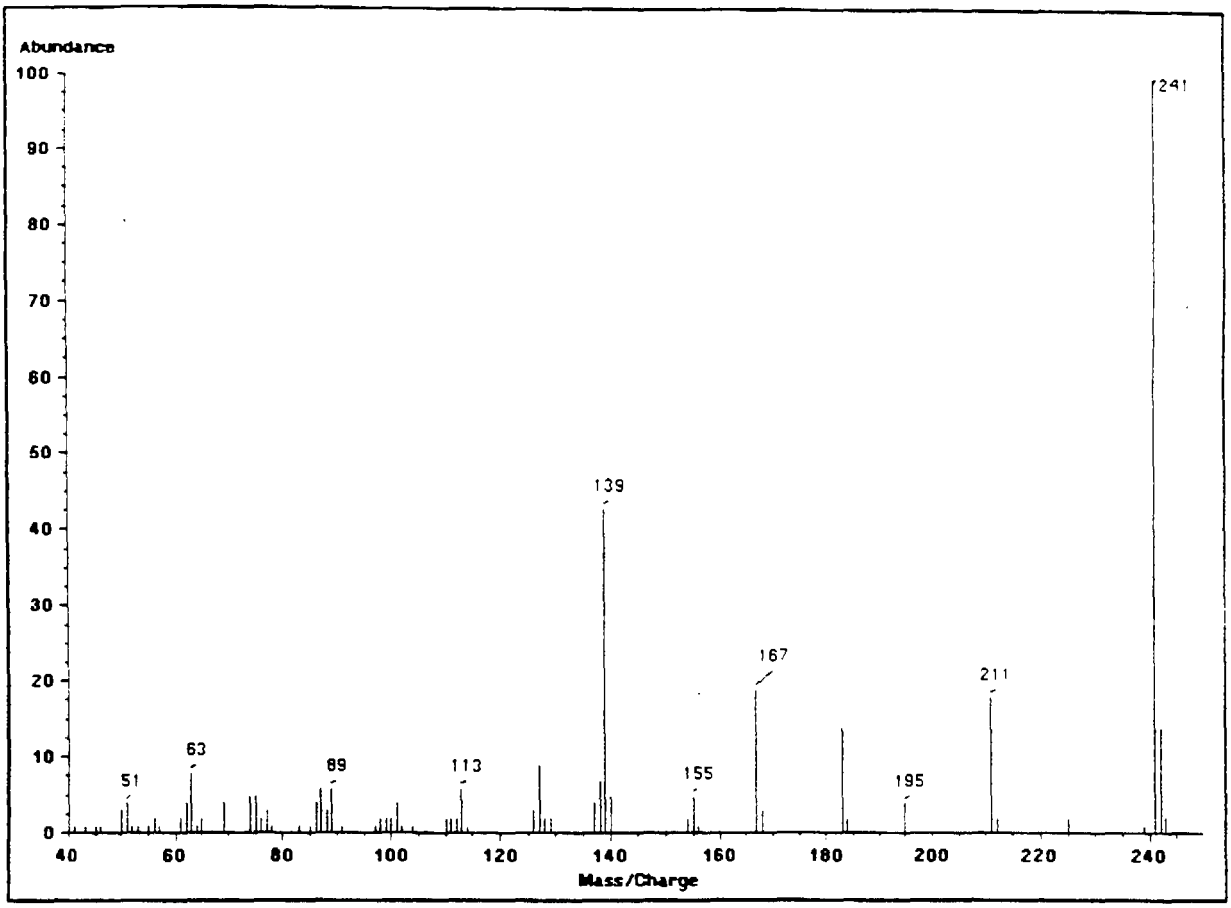



Figure IX-4. Mass spectrum of 2-nitro-6H-dibenzo[b,d]pyran-6-one.

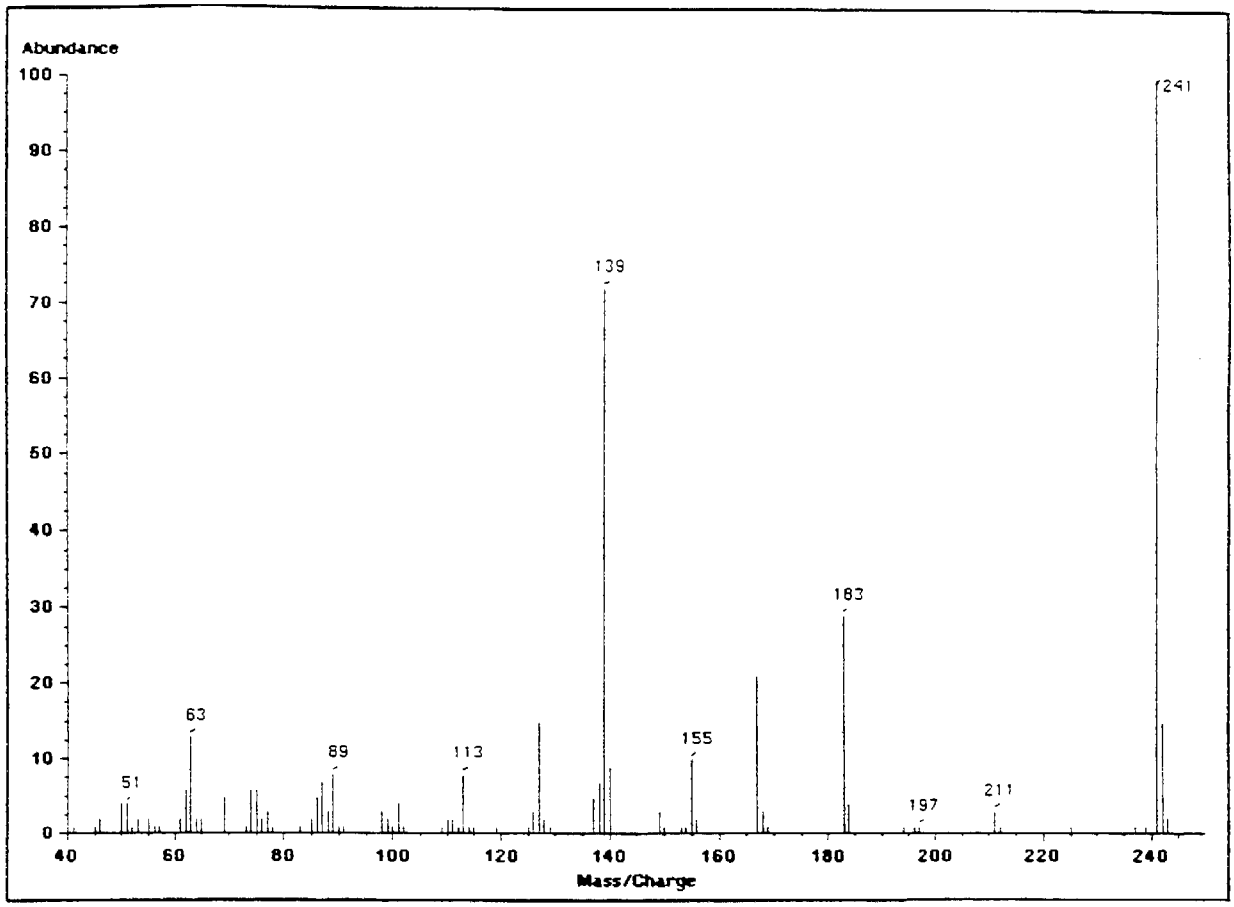



Figure IX-5. Mass spectrum of x-nitrodibenzopyranone.

sensitivity. It was thus not possible to accurately predict the ECD sensitivity of the second isomer found in the chamber reaction. Therefore an alternative GC/FID method was developed, which had sufficient sensitivity to quantify the nitrodibenzopyranones in the chamber extracts. The FID is expected to have almost equal sensitivities for all isomers. The sensitivity ratio of 1.16 determined for the two standards confirmed this assumption (the higher sensitivity was found here for 2-nitrodibenzopyranone). The 16 % difference in sensitivity may result more from the different GC peak shapes than from different FID sensitivities in themselves.

For quantification, the dichloromethane extracts of ITC-1714 and 1715 were combined and separated by our standard HPLC method with collection of 1.0 min subfractions of fraction #6. Half of each subfraction was allocated for mutagenicity testing and the remaining 50% of the highly mutagenic subfractions 6.4 and 6.5 was pooled and spiked with 3 µg of 3-nitrodibenzopyranone as an internal standard. The FID-chromatogram of this sample is shown in Figure IX-6. The amount of 2-nitrodibenzopyranone was calculated to be 1.0 µg and the amount of the unknown isomer to be 2.9 µg.

The extract of ITC-1716 was spiked prior to the HPLC separation with 2 µg 3-nitrodibenzopyranone. This isomer was recovered in HPLC subfraction 6.6. The quantification of the two other isomers was thus performed out of the combined HPLC subfractions 6.4 to 6.6. The amounts determined were 0.69 µg for the 2-nitrodibenzopyranone and 1.7 µg for the unknown isomer, respectively. Thus both analyses show the unknown nitrodibenzopyranone isomer to be formed in higher amounts. Using the value of ~3 mg phenanthrene reacted in the sampled volume of ITC-1716, we estimate the yield of the two isomers to be approximately 0.02 and 0.06%, respectively. This can be compared with the previously estimated nitrophenanthrene yield from the OH radical-initiated reaction of phenanthrene of $\lesssim$0.1% (Arey et al., 1989b).

Mutagenicity of Nitrodibenzopyranones. The two nitrodibenzopyranone isomers which are commercially available were tested for mutagenic activity using the Kado assay. Both compounds were mutagenic with the 2-nitrodibenzopyranone (observed from the phenanthrene chamber reactions) having the remarkably high activity of 240,000 revertants $\mu g^{-1}$. The

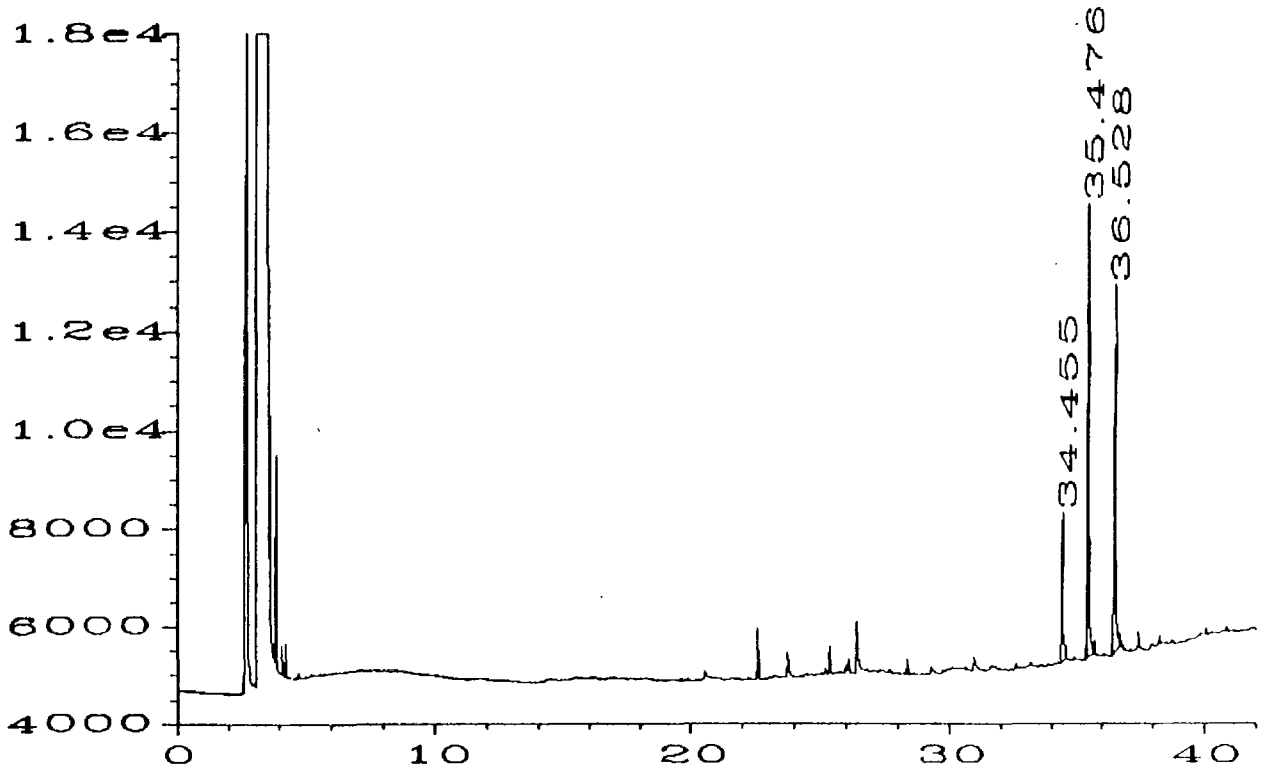



Figure IX-6. FID chromatogram of the combined subfractions 6.4 and 6.5 from ITC-1714 and ITC-1715 spiked with 3 µg 3-nitrodibenzopyranone. The peak assignments are: 34.455 min, 2-nitrodibenzopyranone, 35.476 min, 3-nitrodibenzopyranone (added spike) and 36.528 min, the unknown x-nitrodibenzopyranone isomer.

activity of the 3-nitrodibenzopyranone (not observed from the chamber exposures) was lower at 35,000 revertants $\mu g^{-1}$.

The distribution of mutagenic activity among the subfractions 6.1 - 6.9 of the combined extracts from ITC-1714 and ITC-1715 is shown in Figure IX-7 together with the HPLC chromatogram for fraction #6. As in the previous case (see Figure IX-3), most of the mutagenicity was distributed between subfractions 6.4 and 6.5, with the latter being the more mutagenic of the two. The combined activity of subfractions 6.4 and 6.5 was 330,000 revertants, 73% of which could be accounted for by 2-nitrodibenzopyranone, based on the activity of the standard and the GC-FID quantification. The remaining 21%, or 70,000 revertants, may well be due to the x-nitrodibenzopyranone present in subfraction 6.5, which would lead to an estimate for its mutagenic activity of 24,000 rev $\mu g^{-1}$. This value for the activity of the x-nitrodibenzopyranone is, of course, only a rough estimate, and a precise value will require identification of the isomer and measurement of its mutagenic activity. Thus, it is apparent that the highly mutagenic 2-nitrodibenzopyranone is the main contributor to the mutagenicity of the polar phenanthrene reaction products in fraction #6.

Since this class of mutagens, the nitro-PAH lactones, are both more polar than the nitro-PAH and yet can be analyzed successfully by GC/MS, we decided to return to our mutagenic ambient extracts from our bioassay-directed fractionation of Riverside particle extracts (see Section IV). The GC/MS analyses of these mutagenic fractions from the ambient Riverside extract are discussed in Section X.

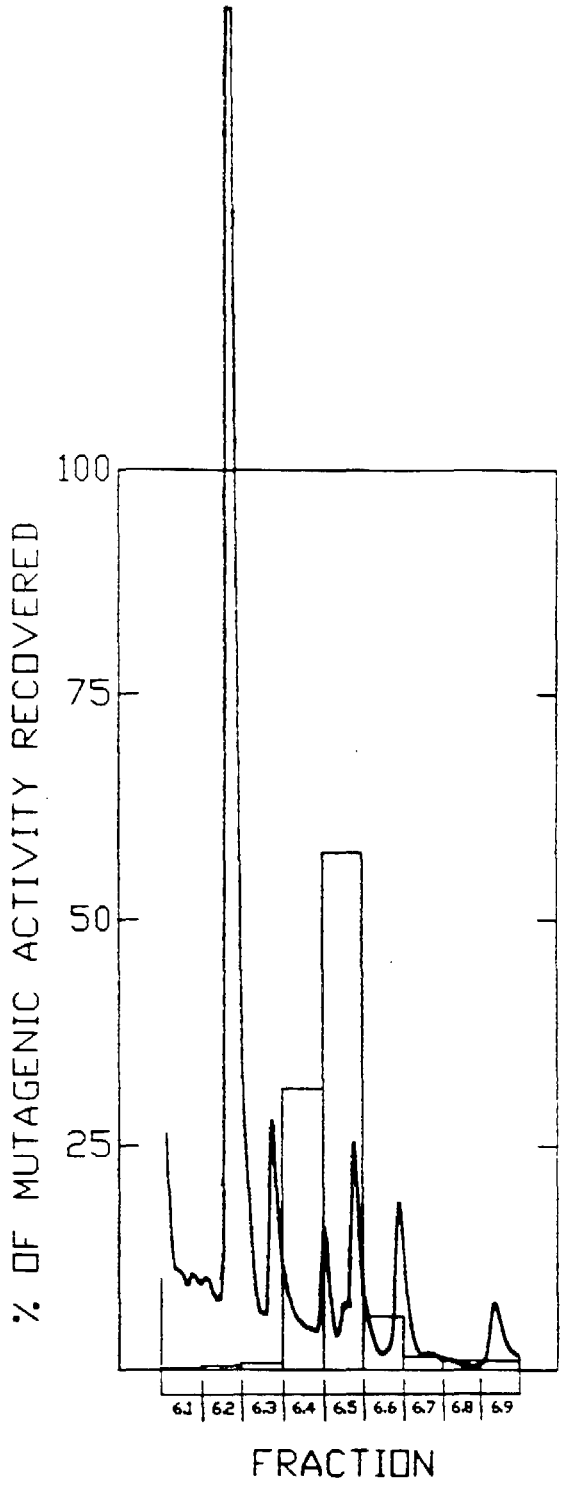



Figure IX-7. HPLC chromatogram ($\lambda$ = 254 nm) of fraction #6 of the reaction products from the OH radical-initiated reactions of phenanthrene in the environmental chamber (ITC-1714 and 1715) showing the subfractions collected (6.1 to 6.9) and the percent of mutagenic activity recovered in each subfraction.

## X. PRESENCE OF NITRO-PAH LACTONES IN AMBIENT PARTICLE EXTRACTS

As noted in Section IV, the weight of the dichloromethane extract obtained from 18 g of Riverside ambient particles was ~2 g. 560 Mg of this extract were fractionated by HPLC and resulted in the mutagram shown in Figure IV-6, in which fractions #9 and #10 were several-fold more mutagenic than any of the other fractions. After dividing each fraction in half, second HPLC separations were performed on fractions #9 and #10 using two different HPLC columns and solvent programs. The mutagrams from these level-two fractionations are shown in Figure IV-7. The percentages of the activity recovered from the level-two fractionations were quite good, varying from 62-100% (see Table IV-13). Thus, the significant polar ambient mutagens (more polar than the nitro-PAH) were present in these level-two fractions.

Each level-one fraction was separated into four level-two fractions. For each HPLC column, the same HPLC program was used to separate the level-one fractions #9 and #10 into four level-two fractions, leading to eight fractions from each reversed-phase HPLC column. We have analyzed by GC/MS all eight level-two fractions from the separations on the ODS reverse-phase HPLC column (Table X-1) and the three most mutagenic fractions from the separations of the Vydac HPLC column. It should be recognized that if a compound was distributed between the level-one fractions #9 and #10, it will be present in the level-two fractions of equal polarity, e.g. in #9-1 and #10-1 (see Table X-1), since as noted, the same HPLC program was used to separate both fractions #9 and #10. GC/MS analyses of these ambient extract subfractions were performed using a 5890 Series II GC attached to a 5971A mass selective detector and equipped with a 30 m DB-5 capillary column.

ODS Column Level-Two Fractions. 2-Nitrodibenzopyranone was identified in HPLC fractions #9-1 and #10-1, both significantly mutagenic fractions (see Table X-1). In fraction #10-1 a second isomer was observed whose retention time and mass spectrum match those of the x-nitrodibenzopyranone isomer we observed in our OH radical-initiated reactions of phenanthrene. Thus, both of the nitrodibenzopyranones identified from our environmental chamber reactions have been observed in ambient particle extracts! The mass spectra of these compounds from the ambient extract

Table X-1. Nitro-PAH Lactones Observed in the ODS Column HPLC Level-Two Fractions of the Most Mutagenic HPLC Level-One Fractions, I/9 and I/10, and the Corresponding Mutagenicity of the Level-Two Fractions

| HPLC I Fraction #9 | | | | HPLC I Fraction #10 | | | |
|---|---|---|---|---|---|---|---|
| HPLC II Fraction # | Total Revertants[a] | Nitro-PAH Lactones Identified or Tentatively Identified | Rt[b] | HPLC II Fraction # | Total Revertants[a] | Nitro-PAH Lactones Identified or Tentatively Identified | Rt |
| 1 | 2300 | 2-nitrodibenzopyranone | 29.0 | 1 | 6500 | 2-nitrodibenzopyranone | 29.1 |
| | | nitro-methylphenanthrene-lactone | 31.5 | | | x-nitrodibenzopyranone | 31.2 |
| | | | | | | nitro-methylphenanthrene-lactone | 33.4 |
| | | | | | | nitro-pyrene-lactone | 38.0 |
| 2 | 1600 | nitro-methylphenanthrene-lactone | 30.6 | 2 | 1000 | none identified[c] | |
| | | nitro-methylphenanthrene-lactone | 30.7 | | | | |
| | | nitro-methylphenanthrene-lactone | 31.4 | | | | |
| | | nitro-pyrene-lactone | 35.4 | | | | |
| 3 | 220 | none identified[c] | | 3 | 240 | none identified[c] | |
| 4 | 52 | none identified[c] | | 4 | 150 | none identified[c] | |

[a]Total Revertants per HPLC fraction (see Section IV and Table IV-13 for more details).
[b]GC Retention time. Under the conditions of the GC/MS analyses, a difference of ~0.1 min in Rt may occur for the same compound in two different injections.
[c]None identified refers to the fact that mass chromatograms for the nitro-PAH lactones for m/z = 241, 255 and 265 indicate that significant amounts of these compounds were not present.

analyses are given in Figure X-1, and these can be compared with those of the compounds shown in Figures IX-4 and IX-5.

A total of five compounds tentatively identified as nitro-methylphenanthrene lactones were observed in various HPLC level-two fractions. The mass spectra of these compounds are given in Figures X-2 and X-3. Significantly, each fraction in which these compounds were identified was substantially mutagenic. Two additional compounds, tentatively identified as nitro-pyrene lactones, were also observed in the mutagenic subfractions 9-2 and 10-1 (the mass spectra are given in Figure X-4). Subfraction 9-2 does not contain a significant amount of 2-nitrodibenzopyranone which suggests, not unexpectedly, that one or more of the nitro-methylphenanthrene lactone isomers is mutagenic and/or the nitro-pyrene-lactone also observed in this subfraction is mutagenic. It appears that this class of compounds, the nitro-PAH lactones, will contribute significantly to the mutagenicity of ambient particle extracts. Consistent with this assumption, analysis of the five least mutagenic fractions of the ODS column level-two fractions (9-3, 9-4, 10-2, 10-3 and 10-4) showed no detectable levels of these compounds.

Vydac Column Level-Two Fractions. The three most mutagenic subfractions from the level two separation on the Vydac HPLC column also contained 2-nitrodibenzopyranone, x-nitrodibenzopyranone and nitro-methylphenanthrene lactones (see Table IX-2). In the subfractions from the ODS column discussed above, the nitro-PAH lactones are relatively minor components in the total ion chromatograms (TIC). In the subfraction 9-2 from the Vydac column, however, 2-nitrodibenzopyranone is one of the most prominent components of the TIC. Consistent with the analyses from the ODS column subfractions, the x-nitrodibenzopyranone was observed only in a subfraction from HPLC level-one fraction #10, while 2-nitrodibenzopyranone was again found in two corresponding subfractions from each level-one fraction, in this case subfractions 9-2 and 10-2.

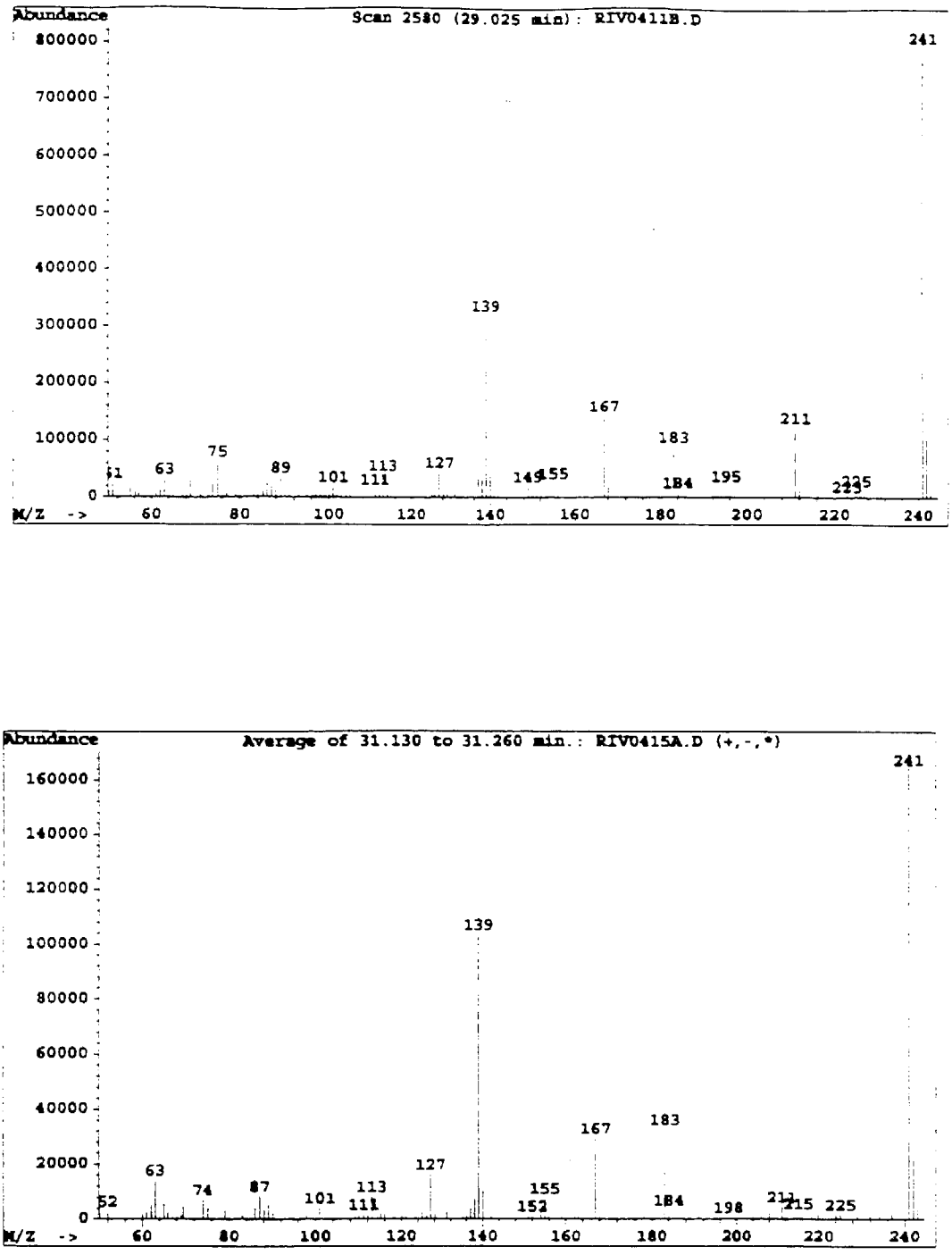



Figure X-1. Mass spectra of two nitrodibenzopyranone isomers observed in Riverside ambient particle extracts. The upper spectrum is of 2-nitrodibenzopyranone and the lower spectrum of x-nitrodibenzopyranone. The spectra are from subfractions 9-1 and 10-1, respectively, from the ODS HPLC column level-two fractionations.

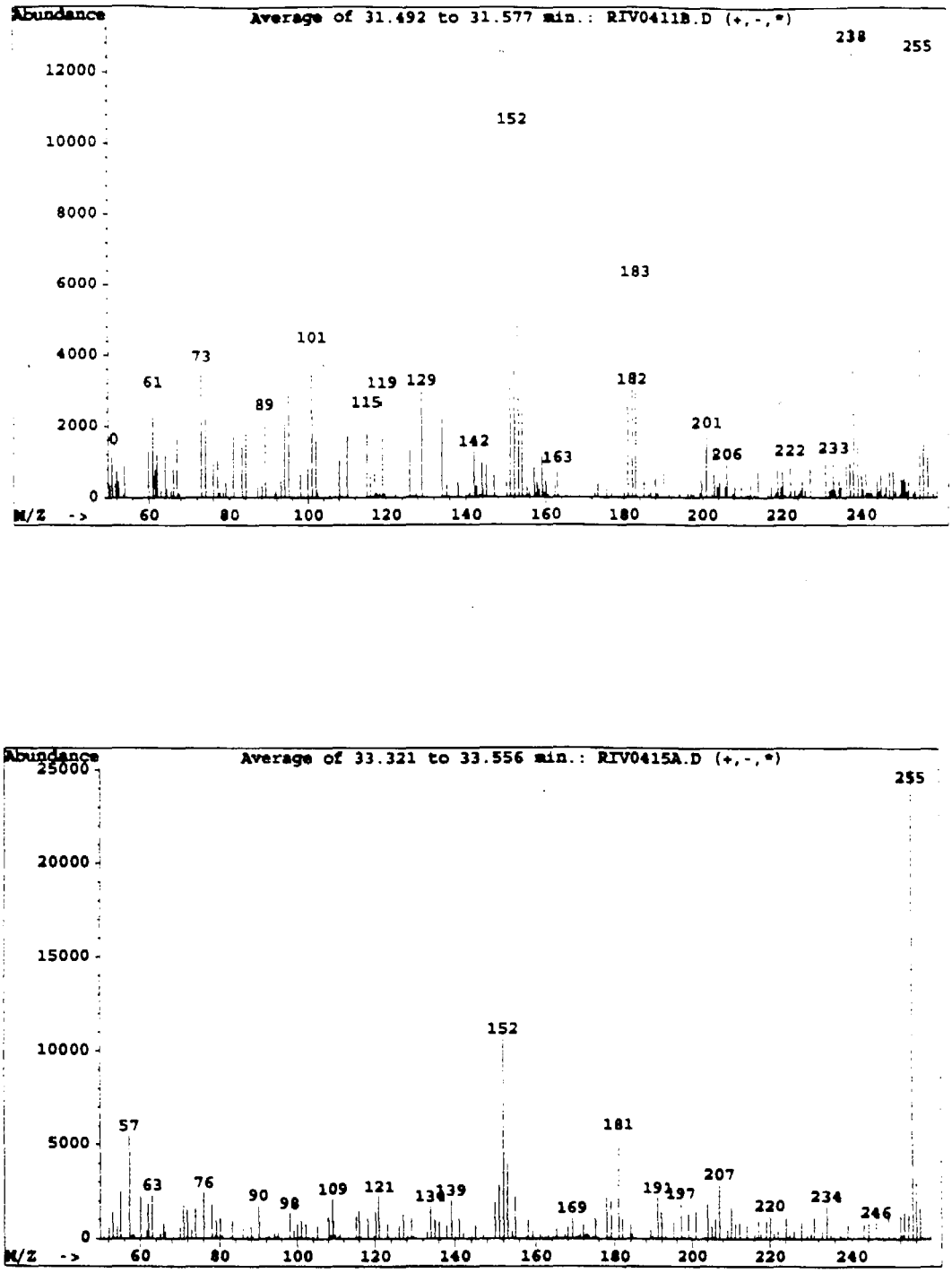



Figure X-2. Mass spectra of compounds from the Riverside ambient particle extracts which are tentatively identified as nitro-methyl-phenanthrene lactones. The spectra are from subfractions 9-1 and 10-1, upper and lower, respectively, from the ODS HPLC column level-two fractionations.

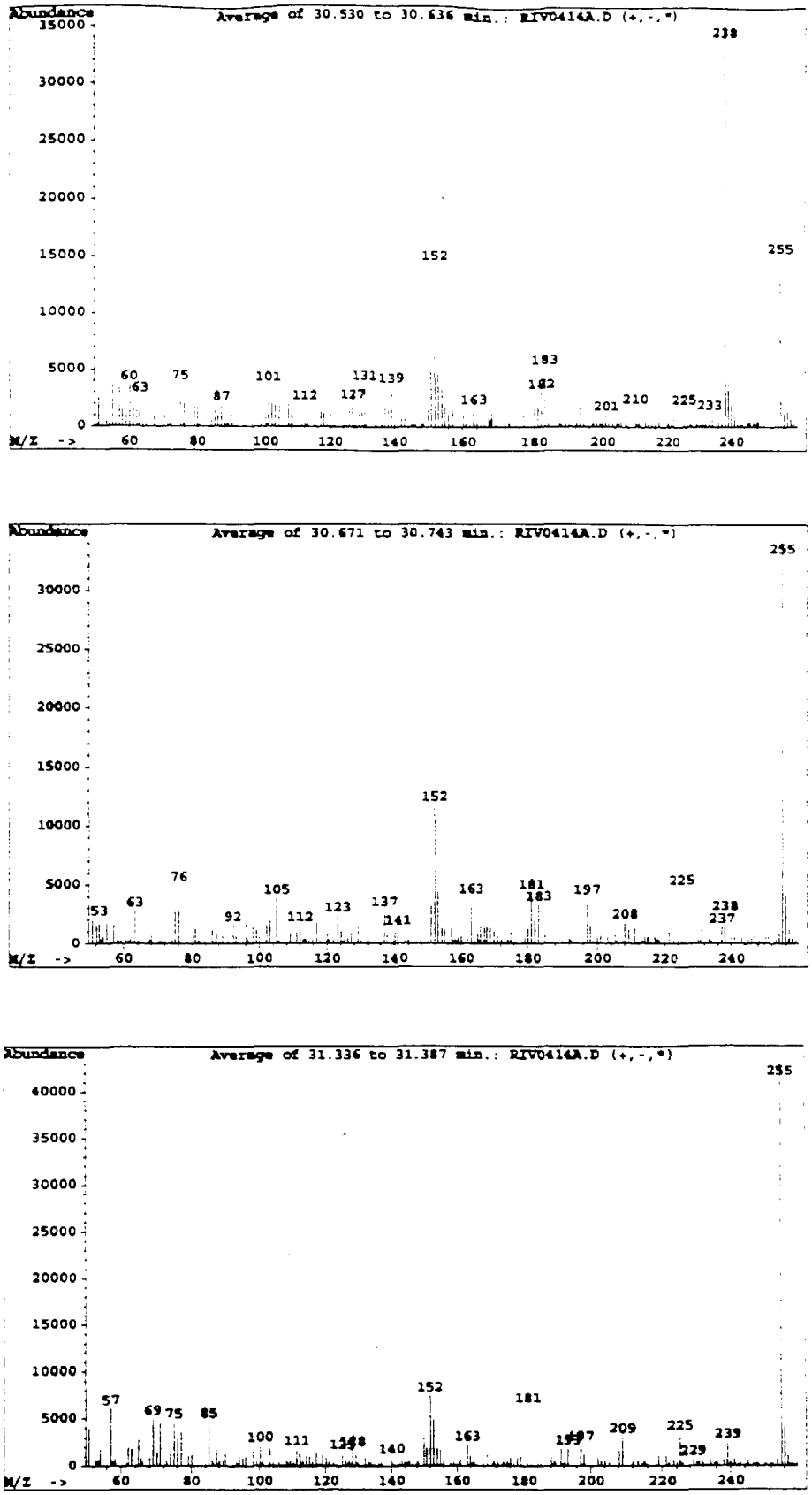



Figure X-3. Mass spectra of compounds from the Riverside ambient particle extracts which are tentatively identified as nitro-methyl-phenanthrene lactones. The spectra are from subfraction 9-2 from the ODS HPLC column level-two fractionations.

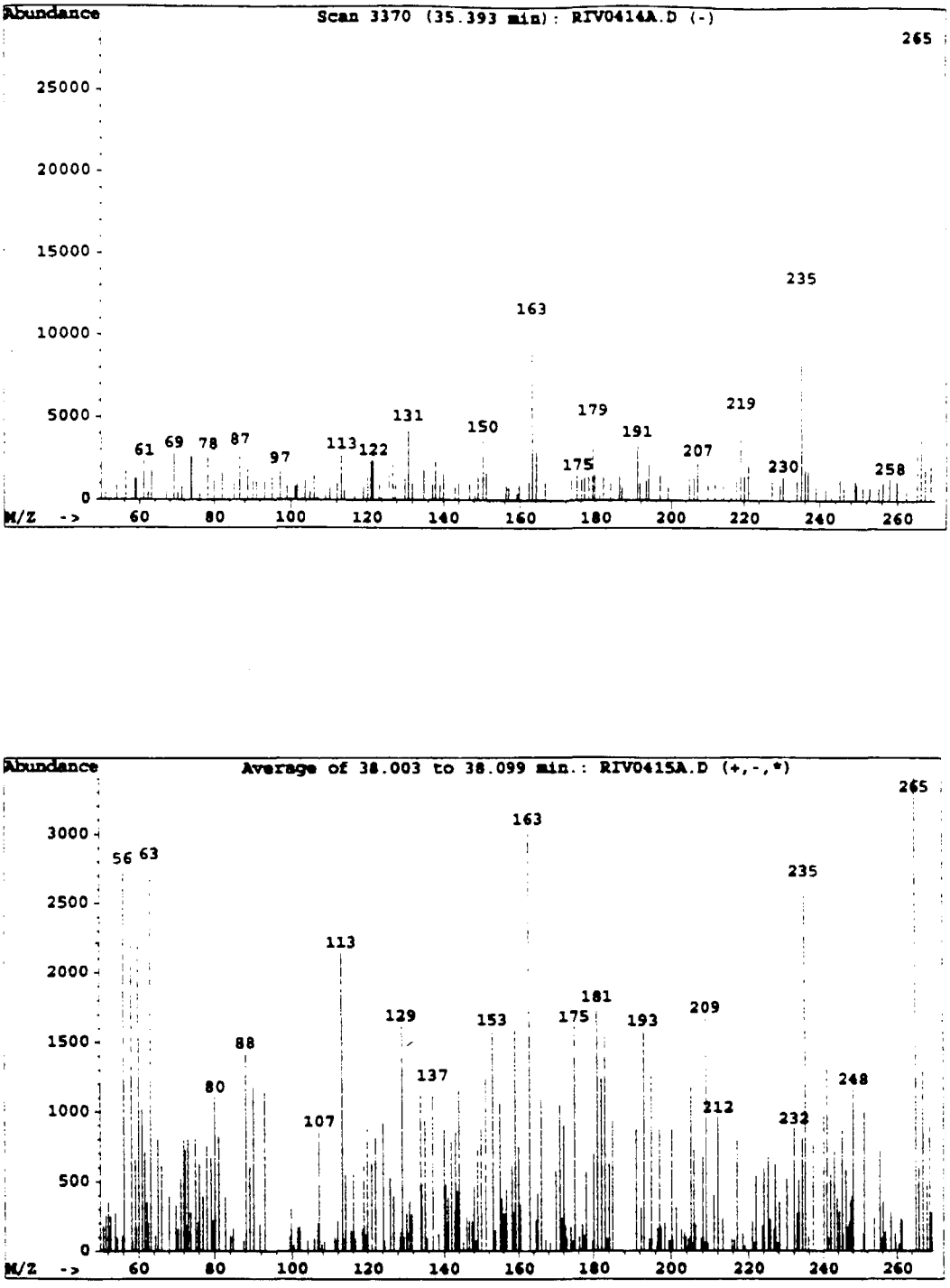



Figure X-4. Mass spectra of compounds from the Riverside ambient particle extracts which are tentatively identified as nitro-pyrene lactones. The spectra are from subfractions 9-2 and 10-1, upper and lower, respectively, from the ODS HPLC column level-two fractionations.

Table X-2. Nitro-PAH Lactones Observed in the Vydac Column HPLC Level-Two Fractions of the Most Mutagenic HPLC Level-One Fractions, I/9 and I/10, and the Corresponding Mutagenicity of the Level-Two Fractions

| HPLC I Fraction #9 | | | | HPLC I Fraction #10 | | | |
|---|---|---|---|---|---|---|---|
| HPLC II Fraction # | Total Revertants[a] | Nitro-PAH Lactones Identified or Tentatively Identified | Rt[b] | HPLC II Fraction # | Total Revertants[a] | Nitro-PAH Lactones Identified or Tentatively Identified | Rt |
| 1 | 78 | not analyzed | | 1 | 260 | not analyzed | |
| 2 | 1900 | 2-nitrodibenzopyranone | 29.0 | 2 | 4900 | 2-nitrodibenzopyranone | 28.9 |
| | | | | | | x-nitrodibenzopyranone | 31.0 |
| | | | | | | nitro-methylphenanthrene lactone | 33.3 |
| 3 | 2300 | nitro-methylphenanthrene lactone | 30.8 | 3 | 1100 | not analyzed | |
| 4 | 54 | not analyzed | | 4 | 84 | not analyzed | |

[a]Total Revertants per HPLC fraction (see Section IV and Table IV-13 for more details).
[b]GC Retention time. Under the conditions of the GC/MS analyses, a difference of ~0.1 min in Rt may occur for the same compound in two different injections.

# XI. DISCUSSION

The thousands of chemicals present in ambient air particulate extracts makes complete chemical characterization virtually impossible, and thus the concept of bioassay-directed fractionation with the goal of chemical identification of the biologically active compounds was developed. Bioassay-directed fractionation of extracts of collected ambient air particles results in mutagenicity profiles for these collected ambient particles which are different from those of combustion emissions (such as vehicle exhausts) in that the most mutagenic fractions in ambient air are more polar than those in, for example, diesel exhaust. This indicates that during transport in the atmosphere from source to receptor, polar mutagenic compounds are formed. Prior to this ARB contract, it had been demonstrated that the 4-ring nitroarenes (nitrofluoranthenes and nitropyrenes) accounted for up to 10% of the observed direct-acting mutagenicity of ambient particulate extracts. Furthermore, it was also recognized that the 2-4 ring PAH exist at least partially in the gas phase and undergo reaction with the OH radical to form, in the presence of $NO_x$, nitroarenes in small ($\lesssim$5%) yield. Despite the low nitroarene yields from these OH radical-initiated reactions, the nitroarene isomers formed from the OH radical reactions are precisely those observed in ambient air, thus showing the importance of atmospheric reactions. While the nitroarenes only account for a small fraction of the ambient particle-phase mutagenicity, we found that the direct-acting mutagenicity of ambient particles at seven sites throughout California correlated well with the 2-nitropyrene concentrations (2-nitropyrene is formed in the atmosphere from OH radical reaction with pyrene), implying that much of ambient particle-phase mutagenicity is due to atmospheric reaction products, possibly of gas-phase PAH.

These findings and conclusions strongly suggested that gas-phase reactions of the 2-4 ring PAH contribute in a significant manner to ambient mutagenicity, and that the unknown chemical(s) responsible for the majority of ambient mutagenicity may be 2-4 ring PAH reaction products. As detailed in Section IV above, we have shown that using the microsuspension modification of the Ames assay, ambient particle extracts can be fractionated with reasonable recovery of mutagenic activity after two

successive HPLC fractionation steps. However, employing LC/MS analysis of these HPLC fractions did not allow identification of the compounds responsible for the mutagenic activity.

The targeting for analysis of specific compounds and compound classes may be viewed as complementary to bioassay-directed fractionation. That is, quantifying a specific compound in an ambient extract and calculating its contribution to the ambient extract activity based on the known activity of the pure compound. Initially, we targeted hydroxynitro-PAH for analysis since we had observed this compound class as products from the atmospherically-important gas-phase reactions of aromatic compounds. As discussed in Section V, a number of hydroxynitronaphthalenes and hydroxynitrobiphenyls were synthesized and tested for mutagenic activity on TA98 (-S9) in the standard Ames plate-incorporation assay. Only 2,1-hydroxynitronaphthalene was mutagenic toward TA98 with an activity of only 0.23 revertants $\mu g^{-1}$.

It was decided that in order to target for analysis in ambient particle extracts mutagenic atmospheric transformation products of the PAH, bioassay-directed fractionation and chemical analysis of PAH reaction products collected from our environmental chamber was the approach to pursue. Thus, having a known PAH starting material and the ability to produce additional product as necessary to allow isomer-specific identification would greatly increase our chances for identification of mutagenic species. We chose to study the atmospheric reaction products of the most abundant PAH in ambient air, naphthalene, and two additional PAH present in high concentrations in ambient air, fluorene and phenanthrene. For each PAH reacted, a mutagram (mutagenicity versus HPLC fraction of increasing polarity) of the reaction products was produced with chemical analysis of the mutagenic HPLC fractions.

As discussed in Sections VI and VII, the major products in the most mutagenic fraction from the naphthalene reaction were 1- and 2-nitronaphthalene and 1,2-hydroxynitronaphthalene. While the hydroyxnitronaphthalene is inactive in the microsuspension assay, 1- and especially 2-nitronaphthalene were found to be active (280 and 5100 revertants $\mu g^{-1}$, respectively), and were calculated to account for ~70% of the overall activity from the naphthalene chamber reaction. These measured activities for the nitronaphthalenes are consistent with the recent work of Kado et

al. (1991a) and suggest that the standard Ames assay may underestimate the activity of volatile mutagens. Since the nitronaphthalenes are the most abundant of the nitro-PAH in ambient atmospheres, these findings have obvious implications for the importance of volatile ambient mutagens formed from atmospheric reactions.

As seen in the naphthalene reaction, the majority of the activity of the fluorene product mutagram was found in the HPLC fraction containing nitroderivatives. The major nitro-derivative of fluorene produced in the OH radical-initiated reaction was 3-nitrofluorene and not the electrophilic nitration product, 2-nitrofluorene. The average total nitrofluorene yield from the fluorene reaction was 2.5 ± 1.2%, consistent with the $\lesssim$5% nitro-PAH yields we have reported for the OH radical-initiated reactions of other PAH. Analysis of ambient samples collected with filters and PUF plugs showed that the nitrofluorenes are distributed between the gas- and particle-phases, and are mainly in the gas phase at the ambient temperatures encountered in Southern California. Thus, the nitro-PAH with molecular weight ≤211 appear to be mainly in the gas-phase, while the 4-ring nitro-PAH (m.w. 247) are mainly particle-associated.

Again using bioassay-directed fractionation and chemical analysis of the reaction products collected from our environmental chamber, we found that for phenanthrene the mutagenicity profile resembles that of ambient air particles, and that the mutagenic fraction(s) which are more polar than that in which the nitroarenes reside contain nitro-dibenzopyranones (nitro-phenanthrene lactones). These compounds are highly mutagenic, and account for a large fraction of the mutagenicity observed from the OH radical-initiated reactions of phenanthrene.

Furthermore, chemical analysis showed the presence of nitro-phenanthrene lactones (m/z 241), nitro-methylphenanthrene (or nitro-methylanthracene) lactones (m/z 255) and nitro-fluoranthene or nitro-pyrene lactones (m/z 265) in the ambient air particle extracts collected at Riverside and subjected to bioassay-directed fractionation. Of particular interest is that these nitro-PAH lactones were found in the most mutagenic fractions of the ambient particulate extracts. This indicates that the nitro-PAH lactones may to a large degree be responsible for the mutagenicity of the polar fractions of ambient air particle extracts and it is estimated that the activity of 2-nitro-6H-dibenzo[b,d]pyran-6-one

alone may account for up to 20% of the activity of the crude Riverside ambient particle extracts. Since the nitrophenanthrene lactones have molecular weights (m.w. 241) closer to that of the 4-ring nitro-PAH (m.w. 247) than to nitrofluorene (m.w. 211) and are more polar than the nitro-PAH, it is expected that these phenanthrene reaction products will be largely particle-associated in ambient atmospheres.

Further laboratory, ambient air and health effects studies of the nitro-PAH lactones need to be carried out to answer the questions as to the mechanism of formation of these compounds, their concentrations in ambient air, their contributions to ambient air mutagenicity, and their health effects on humans. Since this class of compounds also appears to be formed in low yield from the atmospheric reactions of the gas-phase PAH, further studies to more fully elucidate the full spectrum of products formed from the PAH in the atmosphere need to be carried out.

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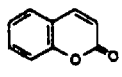
## GLOSSARY OF TERMS, ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| ACN | Acetonitrile |
| Alkyl-PAH | Alkylated polycyclic aromatic hydrocarbons |
| ARB | Air Resources Board |
| Azaarenes | PAH containing a nitrogen atom |
| $CCl_4$ | Carbon tetrachloride |
| °C | Degrees centigrade |
| $CD_3OD$ | Deuterated methanol |
| $CH_2Cl_2$ | Dichloromethane, methylene chloride |
| $CH_3CN$ | Acetonitrile |
| $CH_3OH$ | Methanol |
| $CH_3ONO$ | Methyl nitrite |
| cm | Centimeter |
| DMSO | Dimethyl sulfoxide |
| ECD | Electron capture detector |
| EI | Electron impact (mass spectrometry) |
| FID | Flame ionization detector |
| g | Gram |
| GC | Gas chromatography |
| GC/MS, GC/MSD | Combined gas chromatography/mass spectrometry |
| $^1H$ NMR | Proton NMR |
| $H_2O_2$ | Hydrogen peroxide |
| HONO | Nitrous acid |
| Hi-vol | High-volume sampler |
| HPLC | High performance liquid chromatography |
| hr | Hour |
| $H_2SO_4$ | Sulphuric acid |
| Hydroxynitroarene | PAH containing hydroxy (OH) and nitro ($NO_2$) groups |
| Hz | Hertz, cycle $sec^{-1}$ |
| i.d. | Inner diameter |
| ITC | Indoor Teflon chamber |
| Lactone | Cyclic ester; for example, coumarin  |

## GLOSSARY

(continued)

| | |
|---|---|
| L-broth or LB-broth | Growth medium for overnight culture of *Salmonella* strains |
| LC/MS | Liquid chromatography/mass spectrometry |
| Lifetime | The time required for the reactant concentration to fall to 1/e of its initial value |
| m | Meter |
| $m^3$ | Cubic meter |
| M | Molar |
| Megasampler | Ultra-high vol sampler (equal to 16 standard hi-vols) |
| MeOH | Methanol |
| mg | Milligram |
| $MgSO_4$ | Magnesium sulphate |
| min | Minute |
| $min^{-1}$ | Per minute |
| mL | Milliliter |
| mm | Millimeter |
| mol | Mole ($6.022 \times 10^{23}$ molecules) |
| MS | Mass spectrometry |
| MSD | Mass selective detector |
| Mutagen density | Atmospheric mutagenicity "concentration"; total activity divided by sampling volume (rev $m^{-3}$) |
| Mutagen loading | Specific mutagenicity of the particulate matter; total activity divided by particulate weight (rev $mg^{-1}$) |
| Mutagram | Plot of mutagenic activity v. HPLC fraction polarity |
| M.W. | Molecular weight |
| m/z | Mass to charge ratio |
| N | Normal |
| $N_2$ | Nitrogen |
| $Na_2CO_3$ | Sodium bicarbonate |
| NF | Nitrofluorene |
| ng | Nanogram ($10^{-9}$ gram) |
| nm | Nanometer |
| nmol | Nanomole ($10^{-9}$ mole) |
| NMR | Nuclear magnetic resonance |

GLOSSARY

(continued)

| | |
|---|---|
| Nitroarene | PAH containing nitro ($NO_2$) group(s) |
| $NO_3$ | Gaseous nitrate radical |
| $NO_x$ | Oxides of nitrogen (NO + $NO_2$) |
| $O_3$ | Ozone |
| ODS | C-18 polyfunctional silica-based reverse phase sorbent |
| OH | Hydroxyl radical |
| Open column chromatography | Liquid chromatography technique, used for compound separation or purification |
| PAH | Polycyclic aromatic hydrocarbons |
| PBS | Phosphate-buffered saline |
| PDT | Pacific daylight time |
| pH | $-\log_{10}[H]^+$; $[H]^+$ = hydrogen ion conc mol $l^{-1}$ |
| POM | Particulate organic matter, i.e., the organic extracts of the collected particles which are comprised of a spectrum of organic species, including PAH and PAH-derivatives. |
| ppb | Part per billion |
| ppm | Part per million |
| ppt | Part per trillion |
| PST | Pacific standard time |
| PUF | Polyurethane foam |
| rev | Revertants; net response above background in the Salmonella mutagenicity test |
| RF | Response factor |
| RI | Retention index |
| rpm | Revolutions per minute |
| RT | Retention time |
| S9 | Supernatant from a 9000 x g centrifugation of rat liver homogenate |
| SAPRC | Statewide Air Pollution Research Center |
| S.D. | Standard deviation |
| SCFM | Standard cubic feet per minute |
| Semi-prep column | Semi-preparative scale column used for compound separation or purification by HPLC |
| Specific activity | Specific mutagenicity of the particulate extract; slope of the Salmonella dose-response curve (rev $\mu g^{-1}$) |

GLOSSARY

(continued)

| | |
|---|---|
| TA98 | Ames Salmonella typhimurium strain, detects frameshift mutations. Most sensitive strain for detecting ambient particulate mutagens |
| TIC | Total ion chromatogram |
| TIGF | Teflon impregnated glass fiber (filters) |
| µg | Microgram ($10^{-6}$ gram) |
| µL | Microliter ($10^{-6}$ liter) |
| µm | Micrometer ($10^{-6}$ meter) |
| UV | Ultraviolet |
| λ | Wavelength (nm) |

APPENDIX A

Preprint of paper in press in
INTERNATIONAL JOURNAL OF ENVIRONMENTAL ANALYTICAL CHEMISTRY

"Analytical Chemistry of Airborne Nitrofluorenes"

# Analytical Chemistry of Airborne Nitrofluorenes

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## Abstract

Analytical techniques for the analysis of 1-, 2-, 3- and 4-nitrofluorene were developed to enable these species to be determined in the gas and particle phases of the atmosphere. The nitrofluorene isomers were synthesized by electrophilic and gas-phase OH radical-initiated nitration of fluorene and identified by their $^1$H-NMR and mass spectra. For quantitative analysis deuterated 2- and 4-nitrofluorene were also synthesized for use as internal standard compounds. The GC retention indices of all six compounds were determined on DB-5, DB-1701 and SB-Smectic columns.

**Key Words:**

1-, 2-, 3- and 4-nitrofluorene, NMR spectra, mass spectra, retention indices on DB-5, DB-1701 and SB-Smectic.

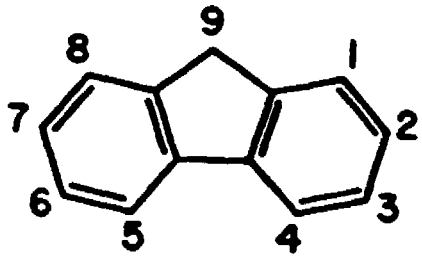
## Introduction

Although the mutagenic and carcinogenic properties of organic extracts of ambient particulate matter are well established, the individual compounds responsible for much of the overall activity are still unknown. Recent research has shown that airborne nitro- and

hydroxynitro-derivatives of polycyclic aromatic hydrocarbons (PAH) are responsible for a portion of the mutagenic activity.[1-3] These compounds can either be emitted during the combustion of organic materials or be formed in the atmosphere by transformations of the airborne PAHs. Due to the lower vapor pressures of these PAH-derivatives, they are partitioned more into the particle phase than the parent PAH.

Typical analyses of atmospheric nitro- and hydroxynitro-PAHs involve high volume sampling on inert filter (particle phase) and adsorbent (gas phase) materials, solvent extraction of the laden filters or adsorbents, fractionation of the extracts by liquid chromatography and identification and quantification by high performance liquid chromatography (HPLC) or gas chromatography (GC) using either nonspecific flame ionization detection (FID) or more specific detection techniques, e.g., mass spectrometric (MS) detection with different ionization techniques.[4,5] Thus, for correct and isomer-specific identifications, a knowledge of retention index data and mass spectra is essential.

Although, fluorene (I) is one of the major PAHs found in ambient air[6-11]




(I)

reports of the identification of its nitroderivatives in ambient samples are limited.[12] The only mononitrofluorene isomer reported has been 2-nitrofluorene, which is the main product of the electrophilic nitration

of fluorene[13] and the only commercially available nitrofluorene. This isomer, which has been identified in diesel exhaust particulate extracts[14-19] is well known for its mutagenic activity and is often used as a standard mutagen compound in mutagenicity assays.[12]

In contrast to electrophilic nitration of fluorene, gas-phase reactions of fluorene in the atmosphere are expected to lead primarily to nitro-isomers other than 2-nitrofluorene. The most important atmospheric degradation reaction of fluorene is expected to be initiated by OH radical attack, as has been shown for a series of structurally-related compounds.[20-23] The preferred sites of the OH radical addition are expected to be the 2- and 4-positions, which if followed by addition of $NO_2$ at the ortho position would lead, after elimination of $H_2O$, to 1- and 3-nitrofluorene.[24] To our knowledge these isomers have not been identified in ambient air, perhaps due to a lack of analytical procedures capable of distinguishing all four isomeric nitrofluorenes. Therefore, we have investigated the analytical properties of these compounds. All four nitrofluorene isomers were synthesized by appropriate procedures and spectroscopically and chromatographically characterized. For quantitative analysis purposes deuterated 2- and 4-nitrofluorene were also synthesized to be used for internal standard calibration.

## Synthesis and Isolation

Nitrofluorenes were synthesized for the spectroscopic studies according to a procedure by Radner[25] in which fluorene (Aldrich, 98%) in acetic acid anhydride is treated with concentrated $HNO_3$ at 0°C. The

synthesis mixture was extracted with dichloromethane and ice water and the organic layer was further separated by preparative HPLC on an Ultrasphere Si column (Beckman Semi-Prep 25 cm x 10 mm, using a Beckman Gradient Liquid Chromatograph Model 334 system with Beckman Model 164 UV Detector, $\lambda$ = 254 nm). All four nitrofluorenes were separated using a solvent program starting with 75% hexane and 25% dichloromethane. After all nitrofluorenes had eluted the solvent was programmed to 100% dichloromethane to elute more polar substances from the column. The flow rate was 3 ml $min^{-1}$. A chromatogram on which the fractions collected are indicated is shown in Figure 1. GC/MS analysis (Hewlett Packard 5890 GC with Hewlett Packard 5970 mass selective detector (MSD), GC column eluting directly into ion source, GC operating conditions given below under Gas Chromatography) showed that fractions D, E, G and H contained nitrofluorenes. The nitrofluorene present in fraction H was the main reaction product and was identified as 2-nitrofluorene by comparing its GC and HPLC retention time with that of a 2-nitrofluorene standard solution (Aldrich, 98%). The nitrofluorenes in fractions D and G were identified by $^1$H-NMR as described below. The yield of the nitrofluorene isomer at RT = 15.96 (Figure 1) was too low to produce sufficient material for NMR identification.

In a study of the gas-phase OH radical-initiated reaction of fluorene, samples were produced which contained all four nitrofluorene isomers.[26] Fluorene was introduced into a 6400 l indoor all-Teflon chamber overnight by flowing dry nitrogen through a tube filled with crystalline fluorene. The fluorene concentration prior to the irradiation was about 100 ppb. OH radicals were generated by irradiation of methyl nitrite as described previously.[22] Approximately 2000 l of the chamber

volume were sampled onto polyurethane foam plugs (PUF), which were then Soxhlet extracted with dichloromethane. The extracts were filtered (0.45 µm Acrodisc CR, Gelman Sciences), concentrated and separated by HPLC (preparative Spherisorb S5W silica column, 25 cm x 10 mm, Regis, using a Spectra Physics model 8100 gradient liquid chromatograph with Spectra Physics model 8400 UV/VIS detector and ISCO fraction collector; solvent program starting with 100% hexane, then a linear gradient to 95% hexane and 5% dichloromethane from 10 to 15 min, then programmed to 100% dichloromethane by 40 min, held at 100% dichloromethane for 10 min, then programmed to 100% acetonitrile over 10 min, held isocratic for 10 min then programmed back to the initial conditions; solvent flow 3 ml $min^{-1}$). The fraction ranging from 28 to 37 min was collected, concentrated to 100 µl and analyzed by GC/MS. After quantification of the nitrofluorenes in the HPLC fractions, these chamber samples were utilized to isolate enough of the fourth nitrofluorene isomer to allow $^1$H-NMR analysis.

The combined HPLC fractions of 13 chamber irradiation runs were further separated by reversed phase HPLC (Beckman Ultrasphere ODS 5µ column, 10 mm x 25 cm, Beckman HPLC as described above; solvent program starting with 50% water and 50% methanol, from 10 to 40 min programmed to 100% methanol, held at 100% methanol for 20 min and then programmed to the initial composition over 5 min, flow rate 3 ml $min^{-1}$). Under these conditions the desired isomer eluted last at RT = 40.1 min and was sufficiently separated from the other isomers.

Deuterated 2- and 4-nitrofluorene were obtained by treating deuterated fluorene (MSD Isotopes, 99.3%) with a solution of $N_2O_4$ in dichloromethane, adding a few drops of methanesulfonic acid as

catalyst.[25] The synthesis mixture was separated by open column liquid chromatography on silica gel (LPS-2, 37-53 μm, Whatman). The extracts containing the mononitrofluorenes were combined and 2-nitrofluorene-$d_9$ (93%) was separated from 4-nitrofluorene-$d_9$ (7%) by fractional recrystallization. A GC/MS analysis of the isolated 2-nitrofluorene-$d_9$ and a MS probe analysis showed no foreign substances. The total reaction yield of 2- and 4-nitrofluorene-$d_9$ was 70%.

## NMR Spectra

The identifications of the nitrofluorene isomers isolated from the fluorene + $HNO_3$ synthesis (HPLC fractions D and G in Figure 1) and the fourth isomer isolated from the chamber reactions were performed by taking the 300 MHz $^1$H-NMR spectra (Nicolet 300 MHz pulsed Fourier transform spectrometer, spectra recorded in deuterated acetone as solvent and internal standard). The spectrum of a fluorene standard solution was additionally taken to evaluate the downfield shifting effect of the $NO_2$ group in comparison to the parent non-nitrated compound. The aromatic section of the spectra as recorded for 2-nitrofluorene, HPLC fractions D and G, and the isolated chamber product are shown in Figures 2 to 5. A summary of the spectroscopic data obtained is given in Table 1.

The peak assignments were made by comparing chemical shift data of structurally related nitroarene compounds[27-34] and by performing suitable decoupling experiments: Irradiation of the fluorene sample at 7.57 ppm reduced the triplet at 7.30 ppm to a doublet signal, showing that the proton at 7.30 ppm is adjacent to H-1. Irradiation of the sample from

fraction G (Figure 4) at 7.85 ppm reduced the doublet at 8.21 ppm to a singlet signal and irradiation at 7.66 ppm caused the multiplet signal at 7.42 to 7.47 to be simplified which led to the assignments of H-1 and H-8 and the identification as 3-nitrofluorene. As even decoupling experiments did not allow assignment of the signals observed in the spectrum of fraction D (Figure 5), a 500 MHz spectrum (Varian VXR) of this sample was recorded which led to the identification of this sample as 4-nitrofluorene. With the higher resolution of this instrument the multiplet signal at 7.87 to 7.95 ppm split into three doublets at 7.89, 7.92 and 7.93 ppm. Because of its lower coupling constant (J = 6.41 Hz), the signal at 7.92 ppm was assigned to H-5. The remaining two protons (coupling constants 8.24 and 8.39 Hz) were distinguished by a nuclear Overhauser enhancement (NOE) experiment on H-9. The NOE difference spectrum showed enhancements of 5.4% for the doublet at 7.93 ppm and 4.6% for the split doublet at 7.68 ppm. The doublet at 7.93 ppm could thus be assigned to H-1 and the remaining signal at 7.89 ppm to H-3. This experiment also confirmed the assignment of H-8.

Since the nitrofluorenes in HPLC fractions D, G and H were identified as 4-nitrofluorene, 3-nitrofluorene and 2-nitrofluorene, respectively, the fourth nitrofluorene found in HPLC fraction E and isolated from the chamber irradiation runs had to be 1-nitrofluorene (Figure 2). The peak assignments of its NMR spectrum were performed by one NOE and two irradiation experiments. Irradiation of the overlapping multiplet and triplet signals at 7.68 to 7.73 ppm reduced both doublets at 8.18 and 8.32 ppm to singlets and simplified the multiplet at 7.43 to 7.46 ppm. Irradiation of the split doublet signal at 7.99 to 8.02 ppm only simplified the multiplet at 7.43 to 7.46 ppm showing that the former

signal can be assigned to H-5 and the overlapping signal at 7.68 to 7.73 ppm to H-8. Distinguishing between the two doublets at 8.18 and 8.32 ppm was attempted by an NOE experiment on H-5 using the 500 MHz instrument. The difference spectrum showed only a very small but not unequivocal enhancement for the doublet at 8.32 ppm. This signal was assigned to H-4 and the remaining doublet at 8.18 ppm to H-2. This assignment is consistent with the expected chemical shifts calculated on the basis of the observed data for the other nitrofluorene isomers, i.e., on the downfield shifts in comparison to fluorene which result from the $NO_2$ substitutions. However, an unequivocal assignment was not achieved and the reversed assignment cannot be excluded.

## Mass Spectra

The 70 eV electron impact (EI) mass spectra of the nitrofluorenes were recorded from the GC/MS analysis of a sample containing all 4 isomers and are shown in Figures 6 to 9. The intensities of the most abundant fragments are summarized in Table 2. The data were evaluated from the average of six scans around the peak maxima. It is apparent that for the isomers with the $NO_2$ group at the 1 or 4 position the loss of $HNO_2$ (- 47) is the dominant fragmentation, whereas for the nitrofluorenes with the nitro group on position 2 or 3 the $NO_2$ loss dominates, giving a base peak at m/z = 165 (-46). The loss of OH as seen from the abundances of the m/z = 194 peak, found for all four isomers with an intensity range of 18 to 55%, is strikingly high. Other related nitroarenes generally show lower abundances for this fragmentation process[27,35,36], with the exception of

nitro-PAHs having an $NO_2$ group in a bay position[35]. Consistent with bay region $NO_2$ groups giving an OH loss, the abundance of the m/z = 194 peak is highest for 4-nitrofluorene (followed by 1-nitrofluorene) which indicates that the adjacent aromatic bay H atom (H-5) in 4-nitrofluorene as well as the nonaromatic H-9 atom in the peri position in 1-nitrofluorene are favoring this elimination. Additionally, recent work[37] has shown that OH loss can be one of the most abundant fragmentations in methylnitronaphthalenes having an $NO_2$ group in an ortho or peri position to the methyl group and thus allowing the abstraction of a nonaromatic H atom.

## Gas Chromatography

Initial attempts to use a 50 m HP-5 column (crosslinked 5% phenylmethylsilicone, 0.2 mm i.d., 0.33 μm film thickness, Hewlett Packard) to separate the isomeric nitrofluorenes resulted in poor resolution of 1- and 4-nitrofluorene and of 3- and 2-nitrofluorene. Better resolution was achieved on a column with a DB-1701 liquid phase (14% cyanopropylphenyl [equivalent to OV-1701], 30 m x 0.26 mm, 0.25 μm film thickness, J&W Scientific, carrier gas He, 50°C on column injection, then programmed to 280°C at 6°C $min^{-1}$), which then was used routinely. A typical chromatogram showing the ion trace for the molecular ion at m/z = 211 for a standard mixture containing all four isomers is shown in Figure 10. Although 1- and 4-nitrofluorene still are closely eluting, the resolution is sufficient for quantitative analysis of all four isomers. The observed peak tailing is due to the polarity of the nitrofluorenes since nonpolar

substances showed a distinctly sharper peak shape under the same analysis conditions. Corresponding to their higher dipole moments, the strongest tailing is observed for 2- and 3-nitrofluorene.

For the determination of linear retention indices (RI) a solution containing approximately equal amounts of all four isomers and the deuterated compounds was spiked with a series of bracketing substances. Since two different retention index systems have been used for the analysis of nitro-PAHs, a total of four internal standards was added to allow calculation of the indices in both systems. The solution was spiked with phenanthrene (RI = 300) and chrysene (RI = 400) for determining the indices in the most commonly applied system using parent PAHs as bracketing standards.[38-44] Additionally, two nitrated bracketing substances (1-nitronaphthalene [RI = 200] and 9-nitrophenanthrene [RI = 300]), were added to calculate the retention indices in the system recently described by Robbat et al.[45,46] This index system is of special advantage when more selective and sensitive GC detection techniques [e.g. electron capture detectors (ECD), nitrogen phosphorous detectors (NPD), negative ion chemical ionization mass spectrometry (NICIMS), chemiluminescent detectors (CD)] with enhanced sensitivity for nitrogen containing compounds are applied, since these detectors respond poorly to the parent nonnitrated PAHs.

A total of three columns was tested: a 60 m x 0.24 mm DB-5 column (5% phenylsilicone, film thickness 0.25 µm, J&W Scientific), a second DB-1701 column having the same parameters as the one described above and a 25 m x 0.20 mm SB-Smectic (liquid crystalline polysiloxane, film thickness 0.15 µm, Lee Scientific). Helium was used as carrier gas in all cases. The measurements were performed on a HP 5880A GC using flame ionization

detection. On-column injections with the same oven program as described above were used for both the DB-5 and DB-1701 columns. Injections on the SB-Smectic column were made on a split injector (300°C) at 120°C oven temperature and the oven was then programmed to 280°C at a rate of 4°C $min^{-1}$. The linear retention indices were calculated from the relationship given by Van Den Dool and Kratz.[47]

The RI values obtained are listed in Table 3. Limited literature data are available which may be compared with our results for the DB-5 column. Retention index data for 2- and 3-nitrofluorene were reported on SE-30, SE-52 and SE-54 liquid phases which have similar chemical structures and retention characteristics as the DB-5 column. There is generally good agreement between our RI value for 2-nitrofluorene of 350.98 on the DB-5 column and the literature values of RI = 346.54 on SE-54,[16] 348.56 on SE-30[48] and data ranging from 352.12 to 353.06 on SE-52.[40,41,43] A discrepancy between our retention index for 3-nitrofluorene (RI = 347.86) and a reported value of RI = 328.71[41,43] on SE-52 seemed too high to be explainable by differences in the selectivity of the stationary phase or in GC parameters. Furthermore, we observed the same elution pattern on the HP-5 column, which has a similar stationary phase, with 3-nitrofluorene eluting just before 2-nitrofluorene. This discrepancy between our RI value for 3-nitrofluorene and that reported in the literature cited has been resolved, since the literature value was based on an apparent compound misidentification.[49] The elution sequence on these columns is related to the polarity of the isomers, with the nonresolved 1- and 4-nitrofluorene, which have the lowest dipole moments, eluting first, and the 2- and 3-nitrofluorenes eluting later due to the more exposed position of the nitro group.

The DB-1701 columns showed the same elution sequence as the DB-5 column, but as illustrated in Figure 10, 1- and 4-nitrofluorene were resolved.

A different elution sequence was found on the SB-Smectic column, with 4-nitrofluorene eluting prior to 1-nitrofluorene. The spread of the RI values reflects that the four isomers were more widely spaced than on the other columns tested. The separation on this phase is reported to be strongly dependent on molecule geometry (especially on the length-to-breadth ratio) and is thus very suitable for the separation of isomeric compounds.[37,50,51] Consistent with the importance of molecular geometry, 4-nitrofluorene, the broadest nitrofluorene, elutes first and 2-nitrofluorene, the longest isomer, elutes last.

In summary, the described chromatographic techniques enable isomer-specific determination of all four nitrofluorenes in ambient samples. The liquid chromatographic elution sequence on the Beckman Ultrasphere Si HPLC column was determined to be: 4-nitrofluorene, 1-nitrofluorene, 3-nitrofluorene, 2-nitrofluorene whereas the elution sequence on the reversed phase Ultrasphere ODS column was: 4-nitrofluorene, 2-nitrofluorene, 3-nitrofluorene, 1-nitrofluorene. GC analysis of all four mononitrofluorene isomers can be achieved on either a DB-1701 or SB-Smectic stationary phase and quantification can conveniently be performed by adding deuterated 2- or 4-nitrofluorene, since the perdeuterated isomers are sufficiently separated from the non-deuterated compounds.

## Acknowledgements

The financial support through the California Air Resources Board (Contract No. A732-154) and the U.S. Environmental Protection Agency through Assistance Agreement R814857-01 are gratefully acknowledged. We thank Dr. R. Lee, Prof. D.L. Rabenstein and their coworkers (Department of Chemistry, University of California Riverside) for recording the NMR spectra and for helpful comments on the data interpretation. Dr. R. Atkinson, Statewide Air Pollution Research Center, University of California is thanked for valuable discussions and for performing the chamber experiments. The results described in this article have not been subjected to the agencies' review and therefore no official endorsement should be inferred.

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Table 1. $^1$H-NMR data obtained from fluorene, 1-nitrofluorene (isolated from chamber samples), 2-nitrofluorene, 3-nitrofluorene (HPLC fraction G) and 4-nitrofluorene (HPLC fraction D).

| compound | chem. shift (ppm) | number of protons | coupling[a] | assignment | coupling constant (Hz) |
|---|---|---|---|---|---|
| fluorene | 3.90 | 2 | s | H-9 | |
| | 7.30 | 2 | t | H-2, H-7 | J(1,2) = 7.3 |
| | 7.37 | 2 | t | H-3, H-6 | J(3,4) = 7.4 |
| | 7.57 | 2 | d | H-1, H-8 | |
| | 7.86 | 2 | d | H-4, H-5 | |
| 1-nitro-fluorene | 4.42 | 2 | s | H-9 | |
| | 7.43 - 7.46 | 2 | m | H-6, H-7 | |
| | 7.68 - 7.73 | 1 | m | H-8 | |
| | 7.70 | 1 | t | H-3 | |
| | 7.99 - 8.02 | 1 | d | H-5 | |
| | 8.18 | 1 | d | H-2[b] | J(2,3)[b] = 8.0 |
| | 8.32 | 1 | d | H-4[b] | J(3,4)[b] = 7.6 |
| 2-nitro-fluorene | 4.12 | 2 | s | H-9 | |
| | 7.43 - 7.51 | 2 | m | H-6, H-7 | |
| | 7.67 - 7.71 | 1 | m | H-8 | |
| | 8.03 - 8.06 | 1 | m | H-5 | |
| | 8.12 | 1 | d | H-4 | J(3,4) = 8.4 |
| | 8.31 | 1 | d | H-3 | J(1,3) = 1.8 |
| | 8.46 | 1 | s | H-1 | |
| 3-nitro-fluorene | 4.11 | 2 | s | H-9 | |
| | 7.42 - 7.47 | 2 | m | H-6, H-7 | |
| | 7.66 | 1 | d | H-8 | |
| | 7.85 | 1 | d | H-1 | J(1,2) = 8.2 |
| | 8.12 | 1 | d | H-5 | J(5,6) = 7.0 |
| | 8.21 | 1 | d | H-2 | J(2,4) = 2.1 |
| | 8.70 | 1 | s | H-4 | |
| 4-nitro-fluorene | 4.09 | 2 | s | H-9 | |
| | 7.40 - 7.49 | 2 | m | H-6, H-7 | |
| | 7.54 | 1 | t | H-2 | |
| | 7.68 | 1 | d | H-8 | |
| | 7.89[c] | 1 | d | H-3 | J(2,3) = 8.24 |
| | 7.92[c] | 1 | d | H-5 | J(5,6) = 6.41 |
| | 7.93[c] | 1 | d | H-1 | J(1,2) = 8.39 |

[a] s = singlet, d = doublet, t = triplet, m = multiplet.
[b] These assignments may be reversed.
[c] Data obtained from 500 MHz acquisition.

Table 2. Relative abundances of major ions observed in the mass spectra of the four nitrofluorene isomers.

| fragment lost | relative abundance (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | - | OH | NO | $NO_2$ | $HNO_2$ | $H_2NO_2$ | $HCNO_2$ | $H_2C_2NO_2$ |
| m/z | 211 | 194 | 181 | 165 | 164 | 163 | 152 | 139 |
| 1-nitrofluorene | 66 | 36 | 7 | 82 | 100 | 54 | 15 | 15 |
| 2-nitrofluorene | 60 | 27 | 10 | 100 | 49 | 37 | 15 | 13 |
| 3-nitrofluorene | 54 | 18 | 4 | 100 | 41 | 31 | 10 | 10 |
| 4-nitrofluorene | 66 | 55 | 11 | 76 | 100 | 51 | 21 | 18 |

Table 3. Retention indices (RI) on DB-5, DB-1701 and SB-Smectic columns (mean values and standard deviations calculated from 6 replicates). PAH (bracketing standards phenanthrene and chrysene) and nitro-PAH (NPAH, bracketing standards 1-nitronaphthalene and 9-nitrophenanthrene) RI values.

| nitrofluorene | PAH retention index | NPAH retention index |
|---|---|---|
| | DB-5 | |
| 4-nitrofluorene-$d_9$ | 336.39 ± 0.05 | 269.56 ± 0.03 |
| 1-nitrofluorene[a] } 4-nitrofluorene[a] | 337.35 ± 0.11 | 270.55 ± 0.08 |
| 3-nitrofluorene | 347.86 ± 0.02 | 281.35 ± 0.05 |
| 2-nitrofluorene-$d_9$ | 350.07 ± 0.04 | 283.62 ± 0.04 |
| 2-nitrofluorene | 350.98 ± 0.04 | 284.56 ± 0.06 |
| | DB-1701 | |
| 1-nitrofluorene | 344.44 ± 0.05 | 268.50 ± 0.10 |
| 4-nitrofluorene-$d_9$ | 345.10 ± 0.03 | 269.19 ± 0.10 |
| 4-nitrofluorene | 345.83 ± 0.05 | 269.93 ± 0.07 |
| 3-nitrofluorene | 357.36 ± 0.03 | 281.97 ± 0.09 |
| 2-nitrofluorene-$d_9$ | 360.42 ± 0.08 | 285.17 ± 0.01 |
| 2-nitrofluorene | 361.09 ± 0.03 | 285.85 ± 0.06 |
| | SB-Smectic | |
| 4-nitrofluorene-$d_9$ | 331.97 ± 0.06 | 263.40 ± 0.06 |
| 4-nitrofluorene | 332.65 ± 0.04 | 264.27 ± 0.03 |
| 1-nitrofluorene | 341.82 ± 0.04 | 275.87 ± 0.02 |
| 3-nitrofluorene | 352.59 ± 0.03 | 289.49 ± 0.04 |
| 2-nitrofluorene-$d_9$ | 370.72 ± 0.09 | 312.36[b] |
| 2-nitrofluorene | 371.72 ± 0.09 | 313.65[b] |

[a]Peaks not resolved.
[b]Values determined with additionally 6-nitrochrysene added as bracketing standard. Only one single run was performed at a program rate of 2.5°C $min^{-1}$ as 6-nitrochrysene eluted 6 degrees above recommended maximum oven temperature.

## Figure Captions

Figure 1. HPLC chromatogram of 100 µl (fluorene + $HNO_3$) synthesis mixture with the collected fractions indicated.

Figure 2. $^1$H-NMR spectrum of 1-nitrofluorene (isolated from combined chamber samples).

Figure 3. $^1$H-NMR spectrum of 2-nitrofluorene (standard solution).

Figure 4. $^1$H-NMR spectrum or 3-nitrofluorene (HPLC fraction G).

Figure 5. $^1$H-NMR spectrum of 4-nitrofluorene (HPLC fraction D).

Figure 6. Mass spectrum of 1-nitrofluorene.

Figure 7. Mass spectrum of 2-nitrofluorene.

Figure 8. Mass spectrum of 3-nitrofluorene.

Figure 9. Mass spectrum of 4-nitrofluorene.

Figure 10. Portion of the GC/MS separation (ion trace at m/z = 211) of a standard solution containing all four nitrofluorenes (NF) on a 30 m DB-1701 column. The elution sequence is 1-nitrofluorene (Rt = 31.33 min), 4-nitrofluorene (Rt = 31.48 min), 3-nitrofluorene (Rt = 32.80) and 2-nitrofluorene (Rt = 33.23).

Figure 1

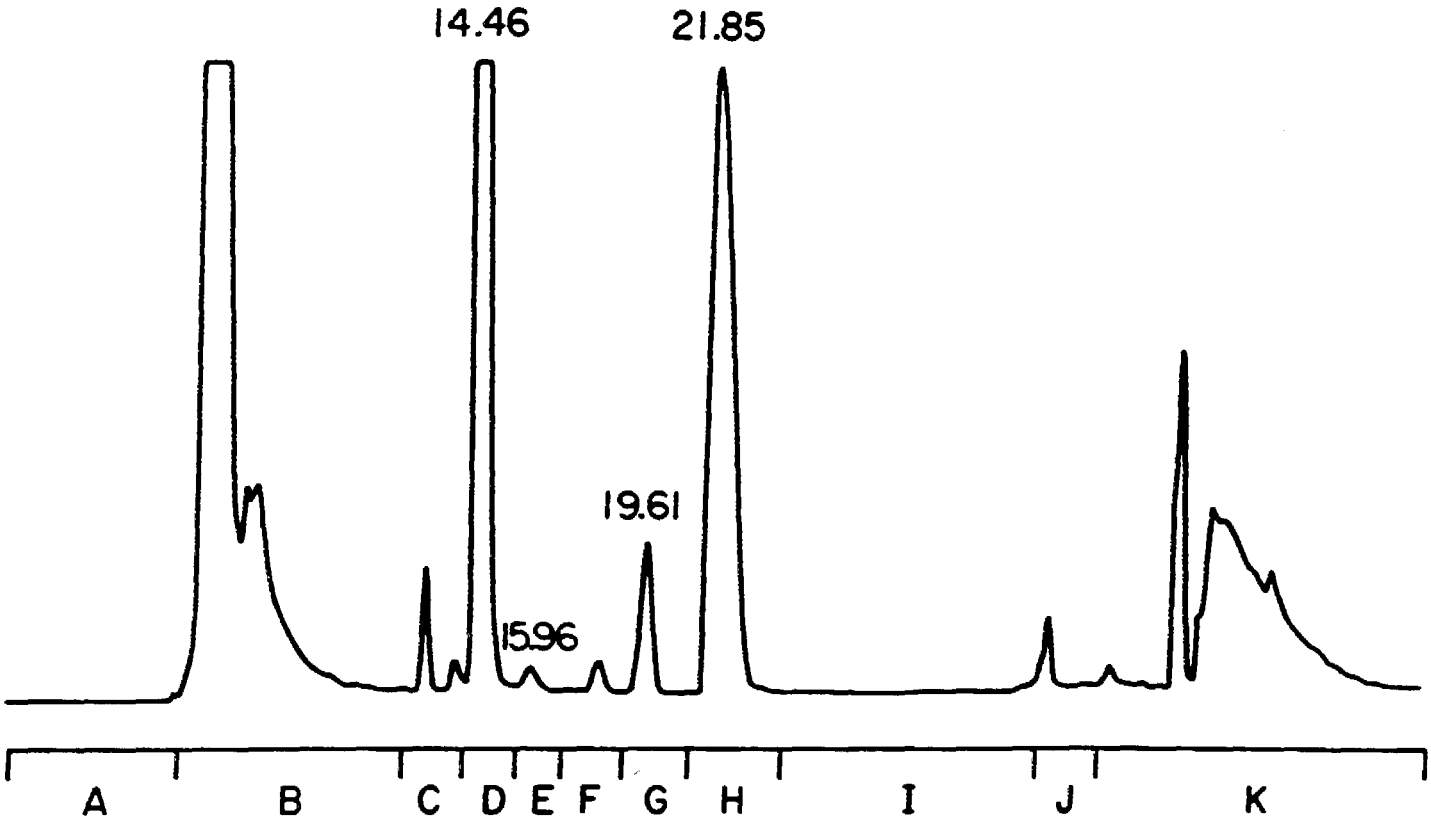


Figure 2

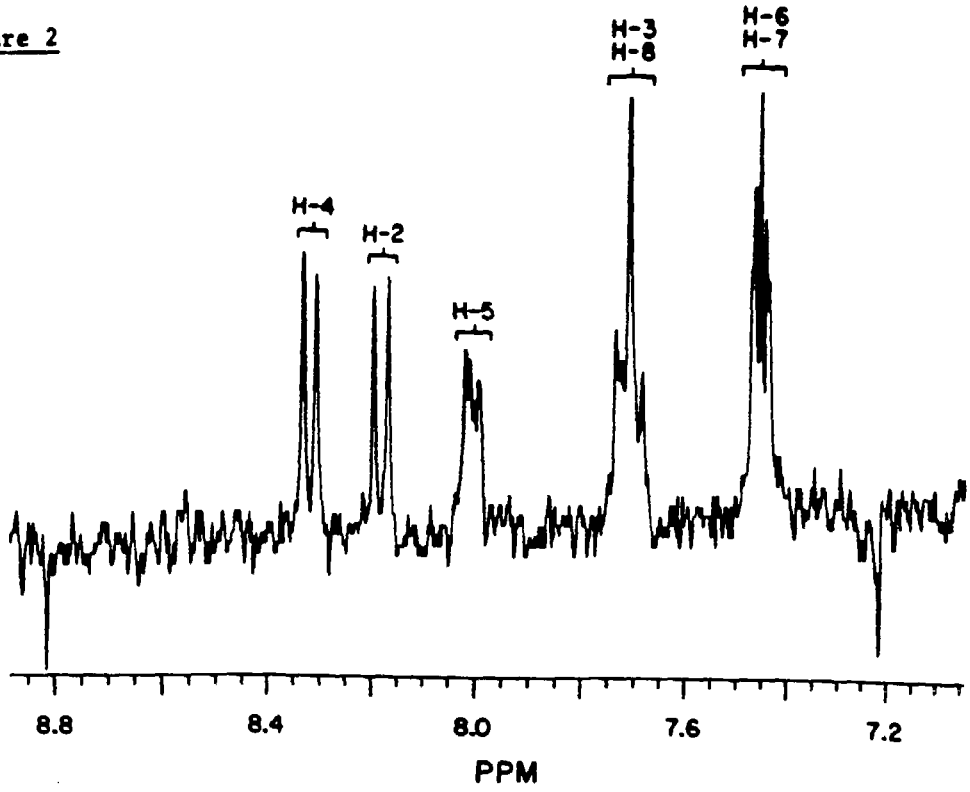



Figure 3

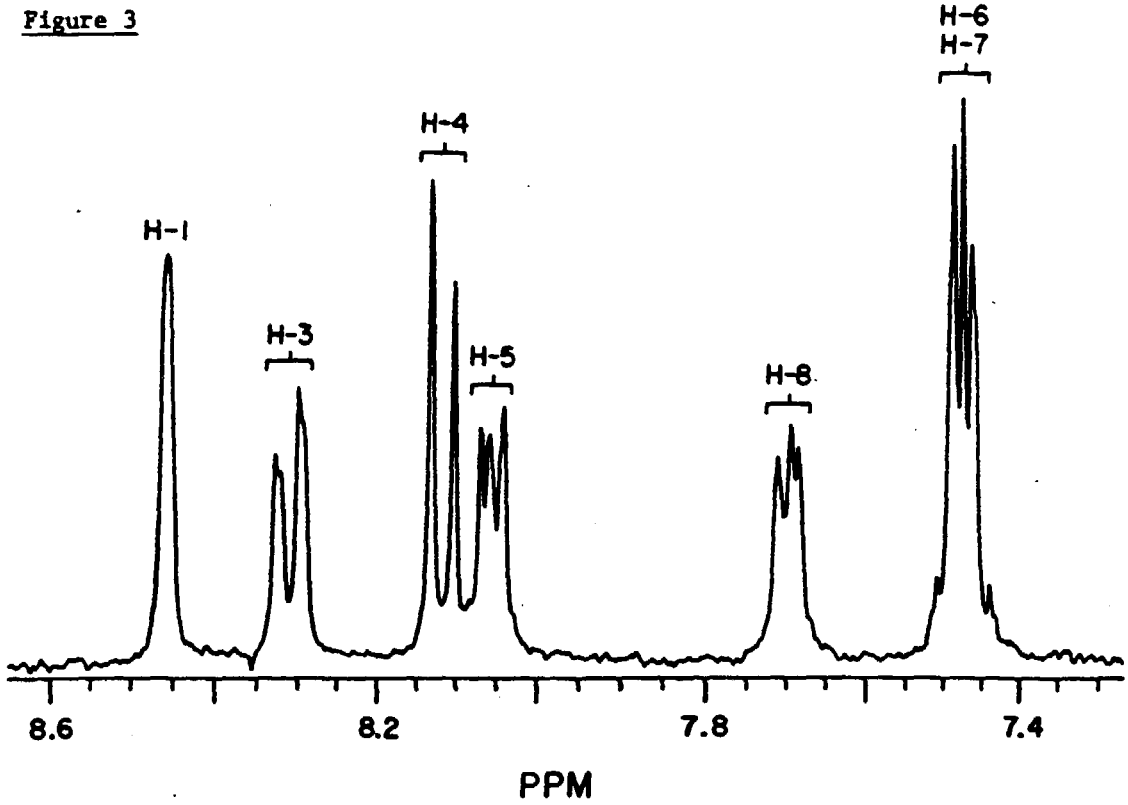


Figure 4

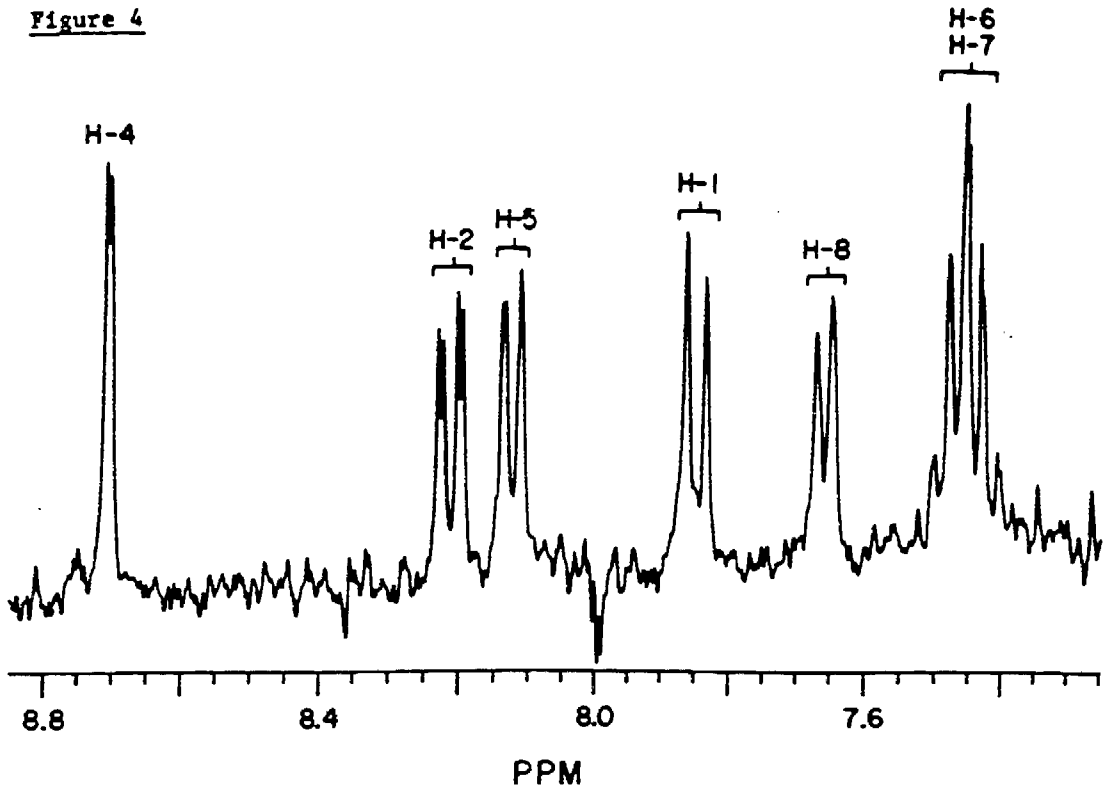



Figure 5

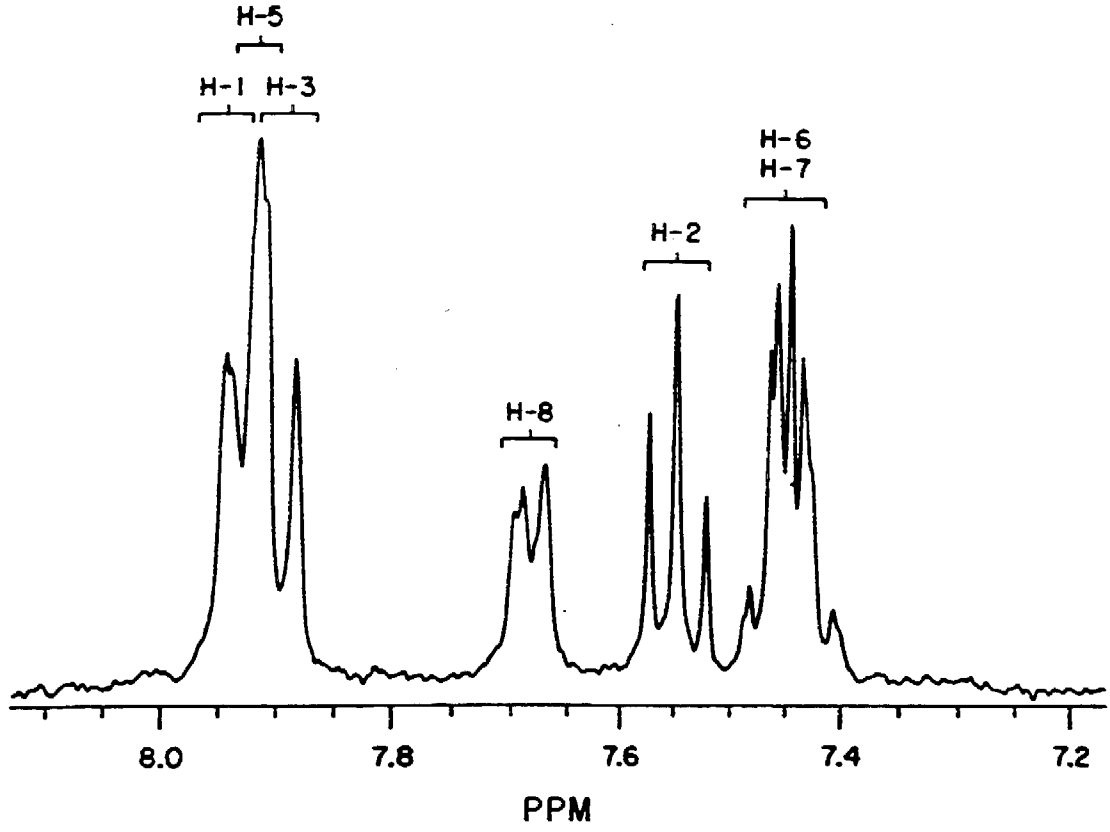


Figure 6

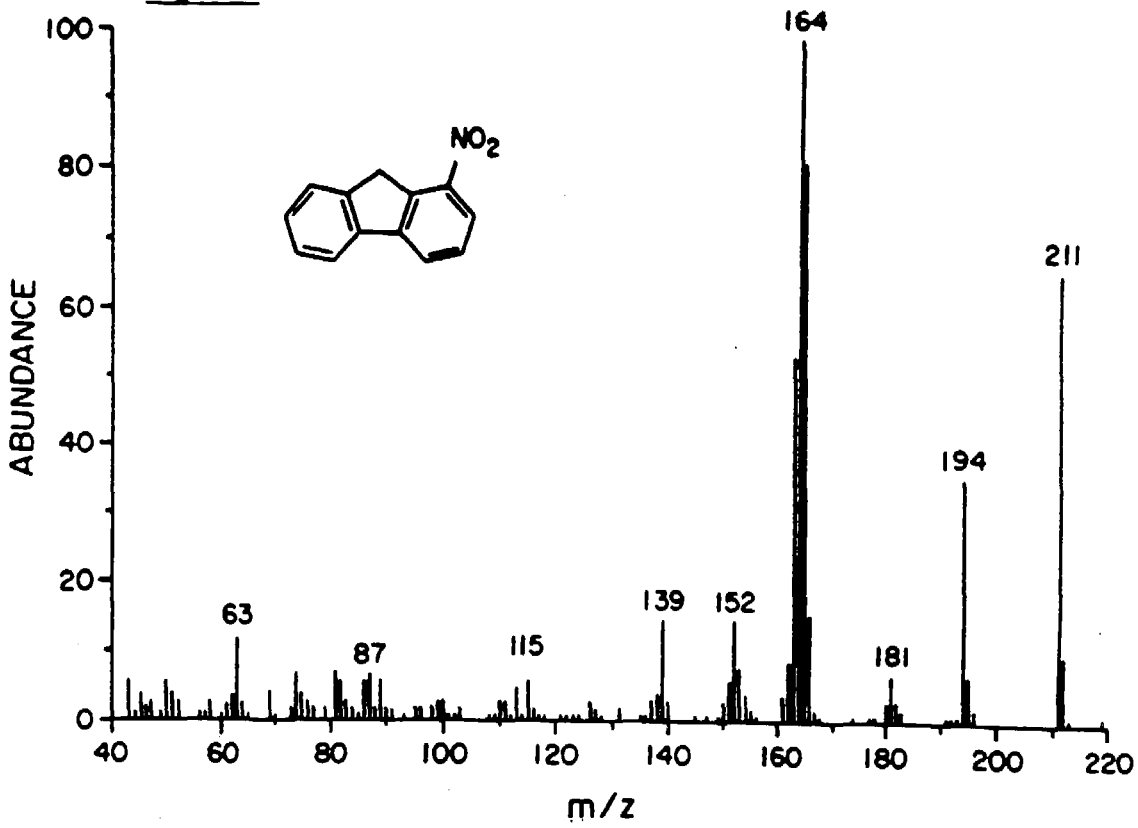



Figure 7

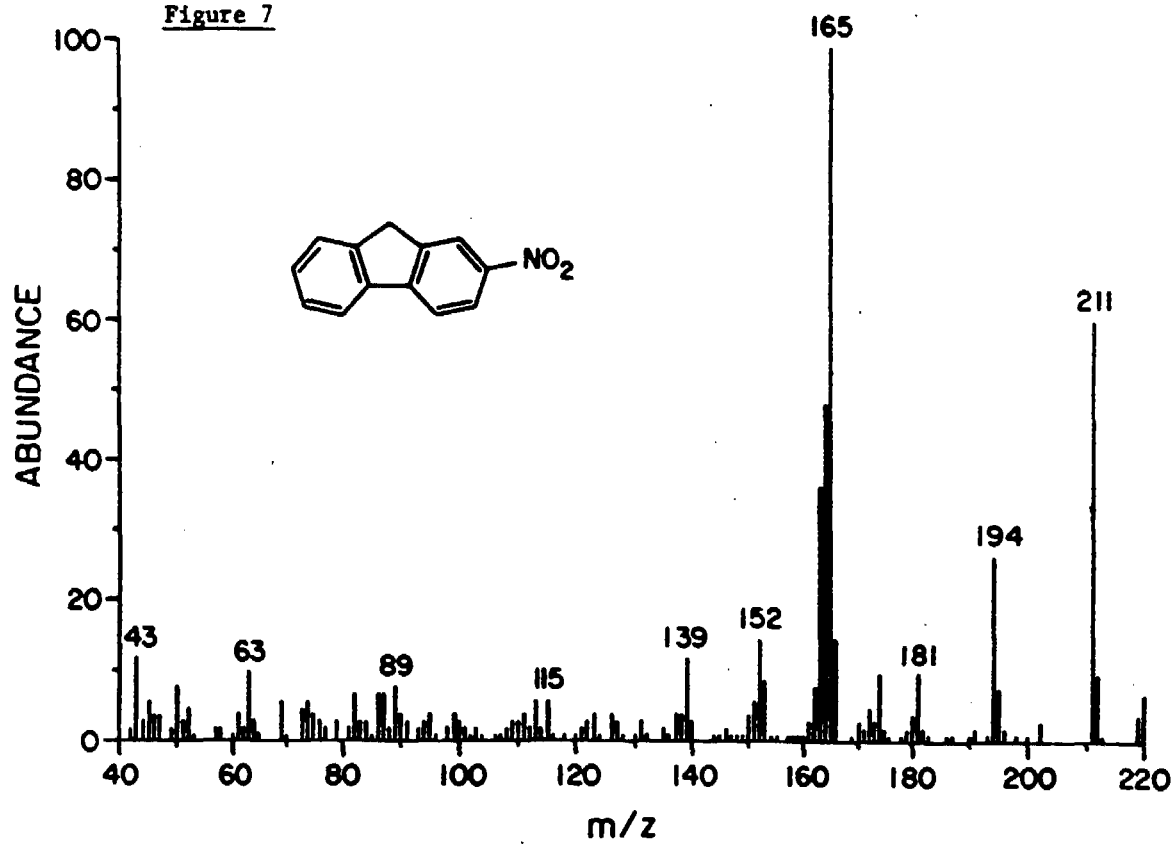


Figure 8

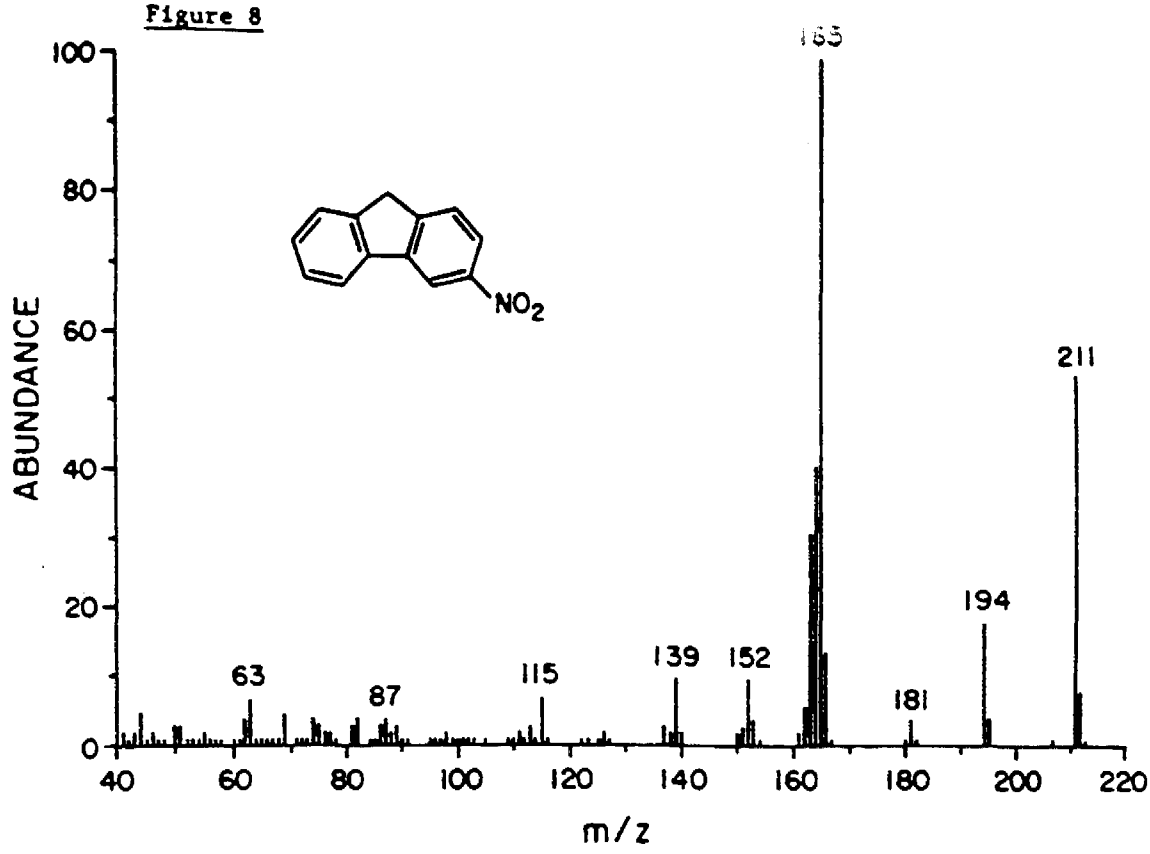



Figure 9

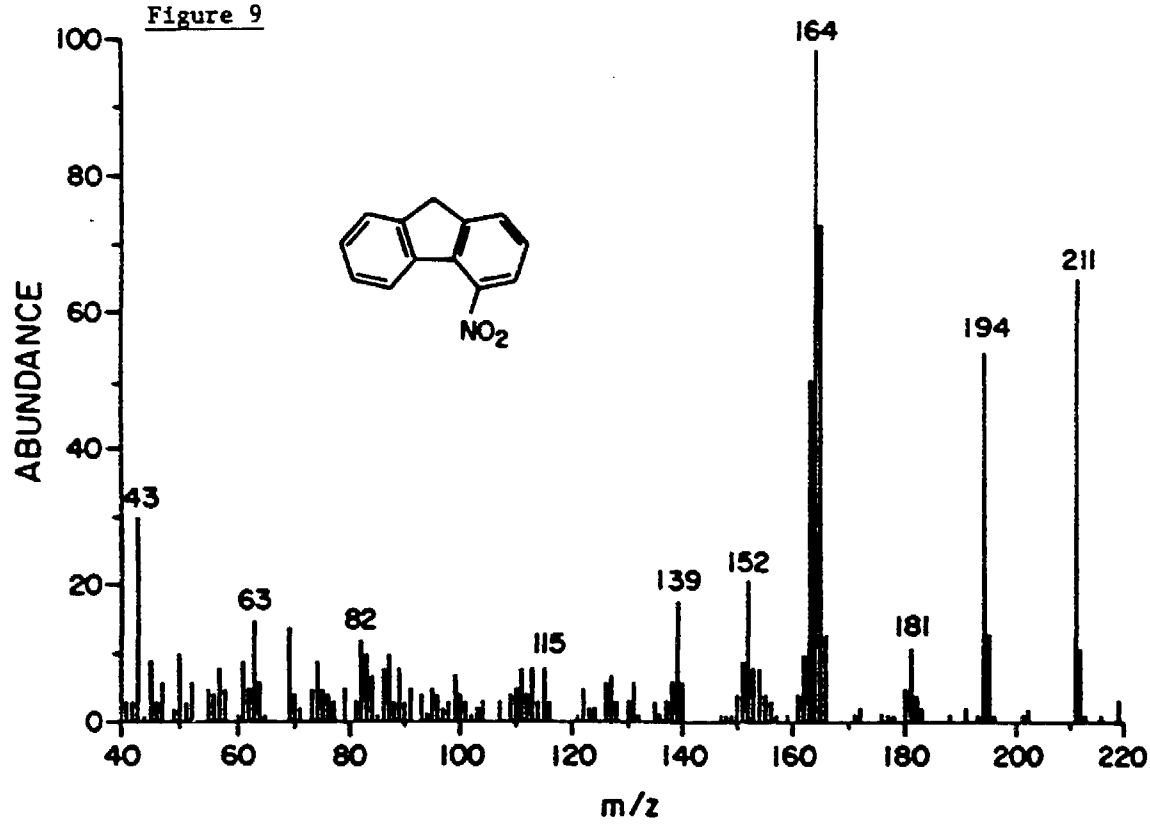
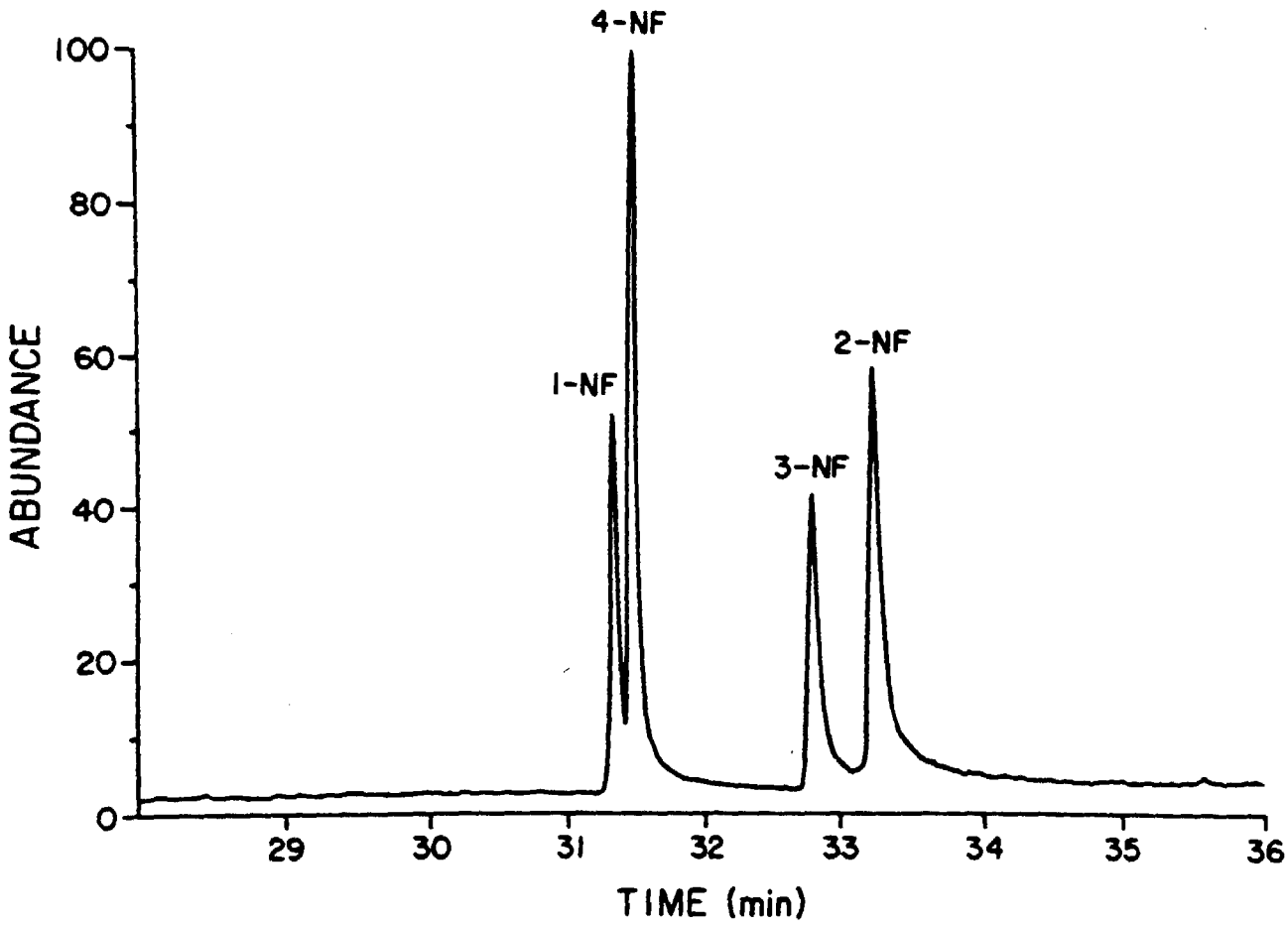
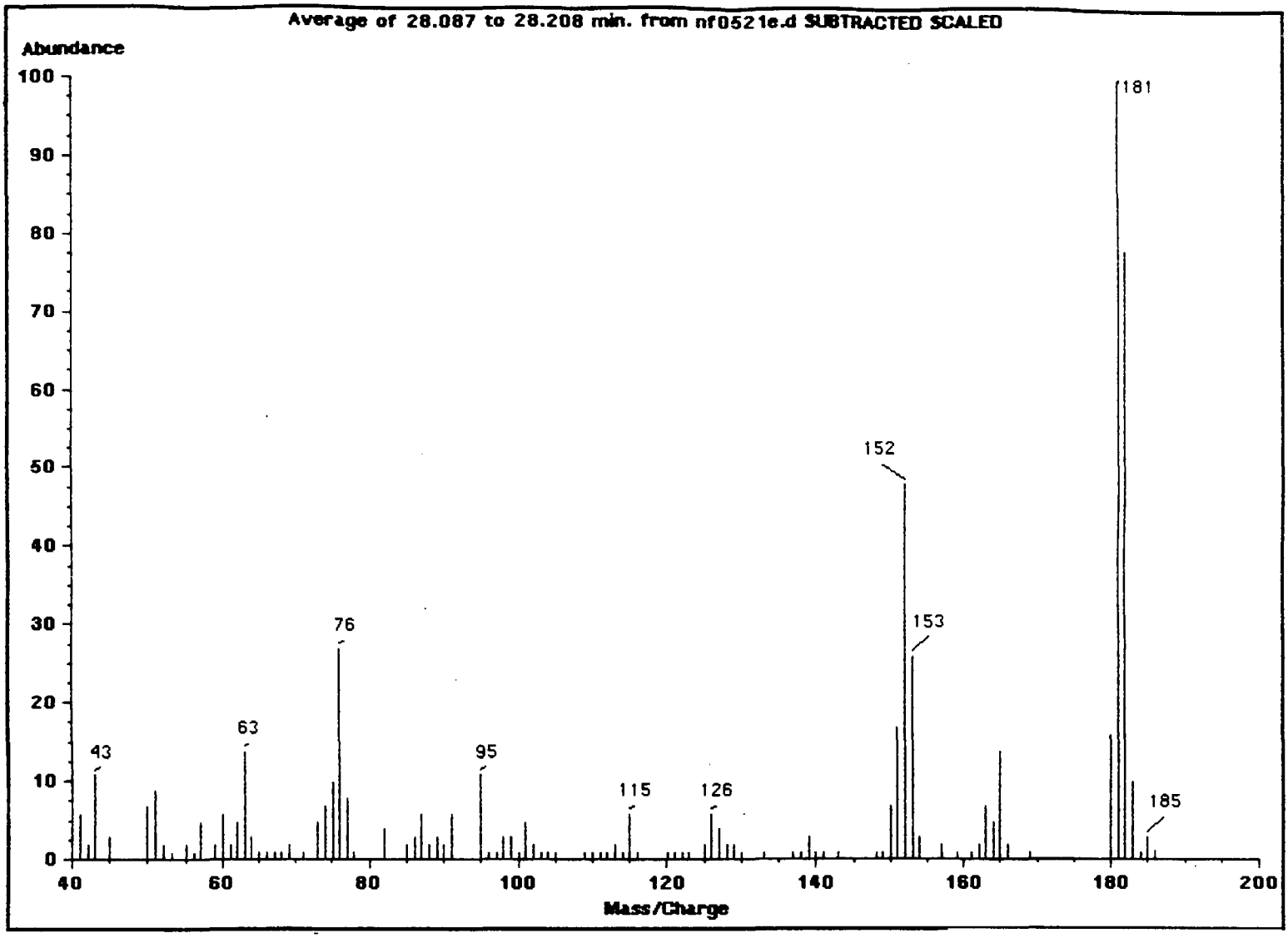


Figure 10

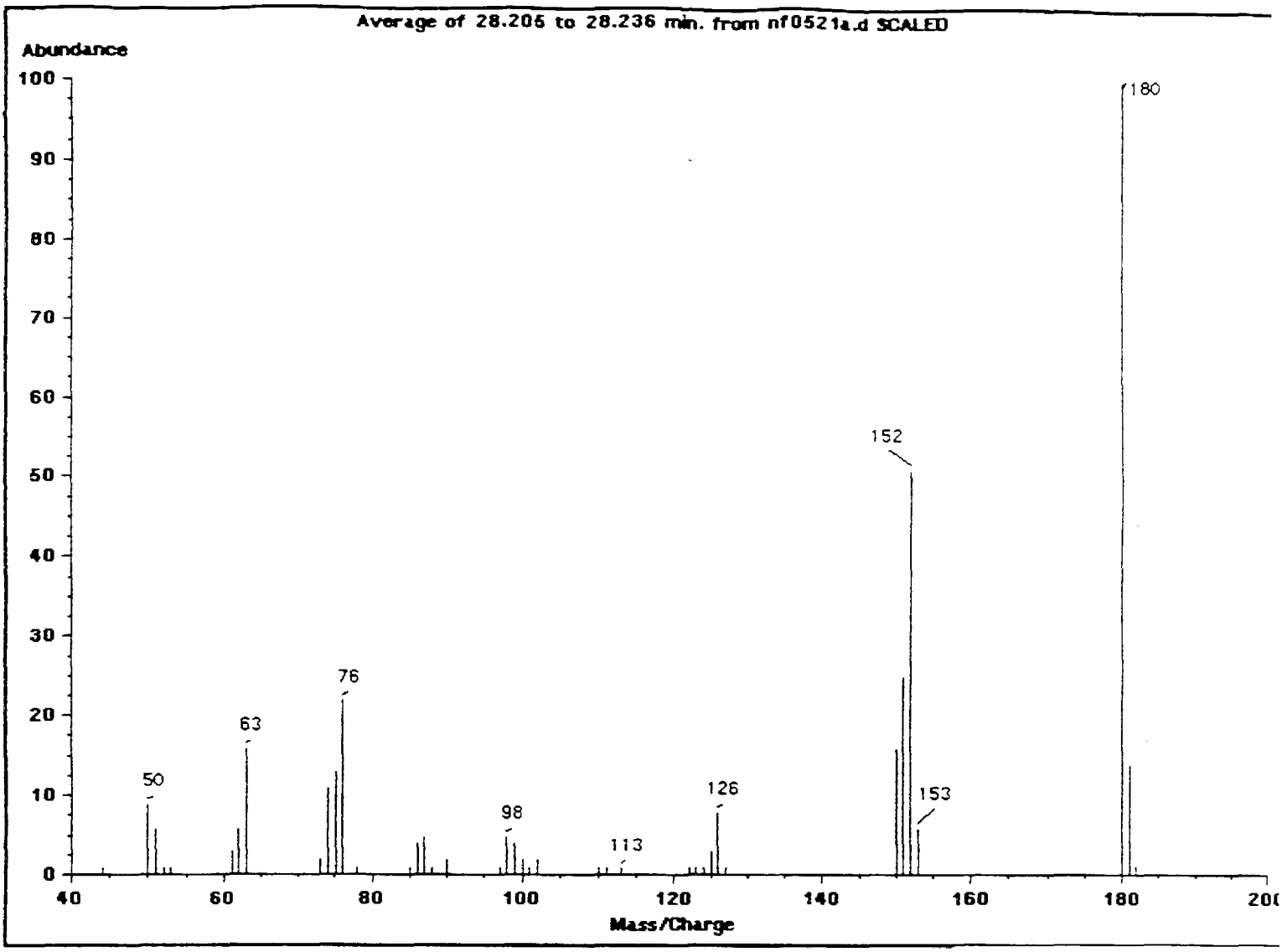

4-NF
1-NF
2-NF
3-NF
100
80
60
40
20
0
ABUNDANCE
29
30
31
32
33
34
35
36
TIME (min)

# APPENDIX B

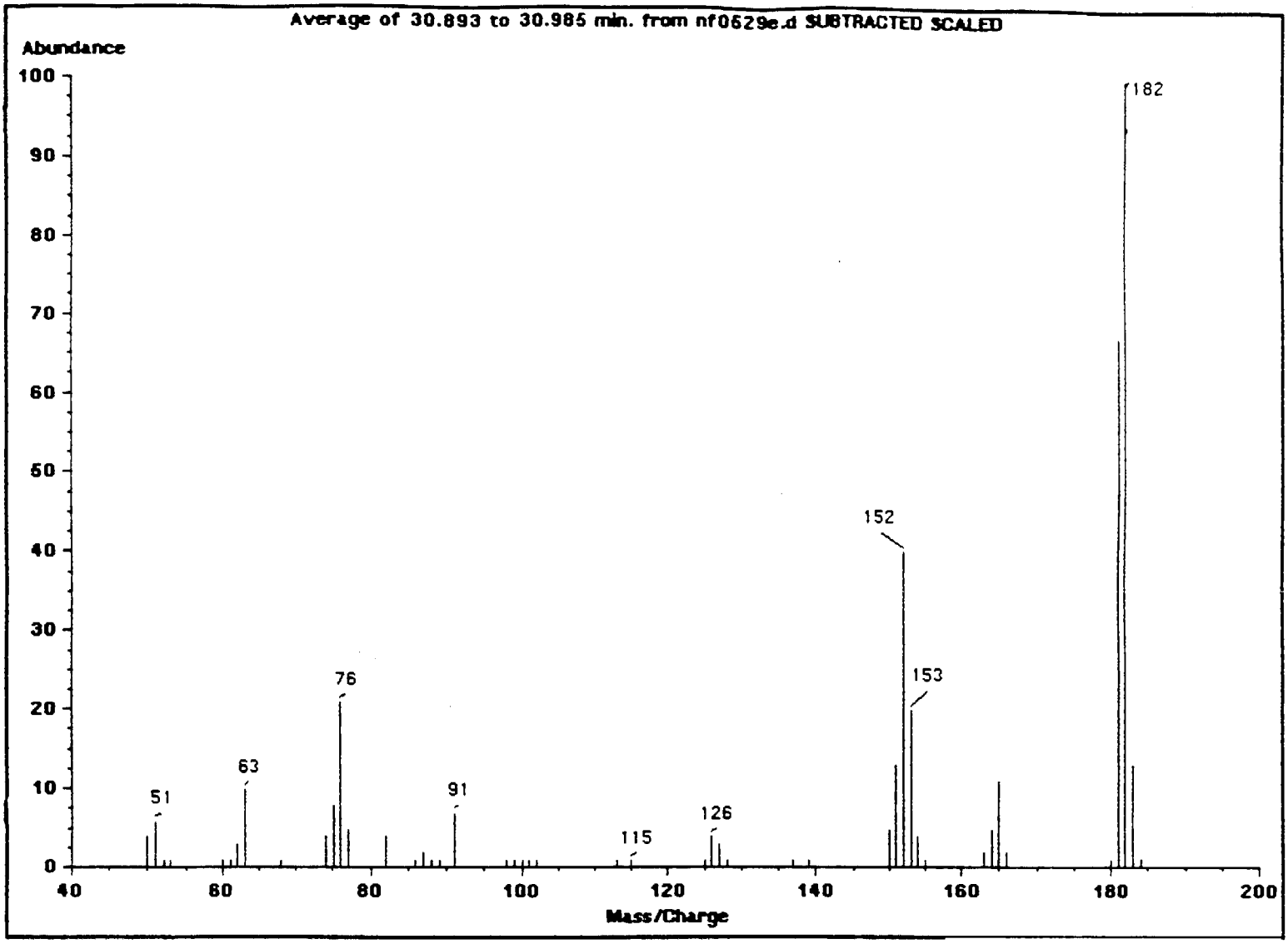
Electron Impact Mass Spectra of Compounds Identified in the OH Radical-Initiated Reactions of Gas-Phase Fluorene



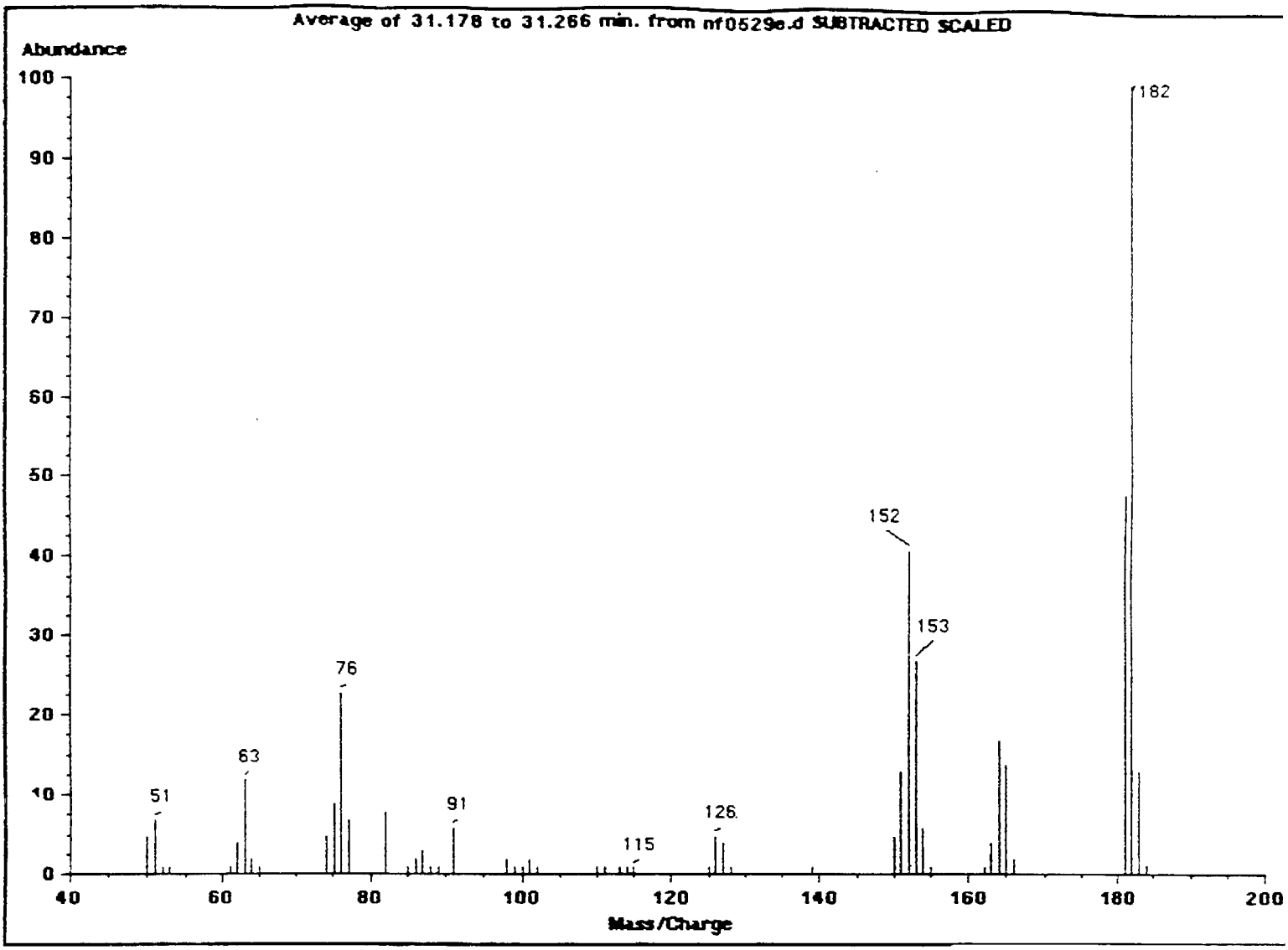

| | |
|---|---|
| compound identification | : 9-hydroxyfluorene |
| molecular weight | : 182 |
| measured retention index (n = 1) | : 292.59 |
| retention index of reference substance | : 294.06 |
| literature retention index | : 293.6 |
| compound found in HPLC fraction | : 7 |



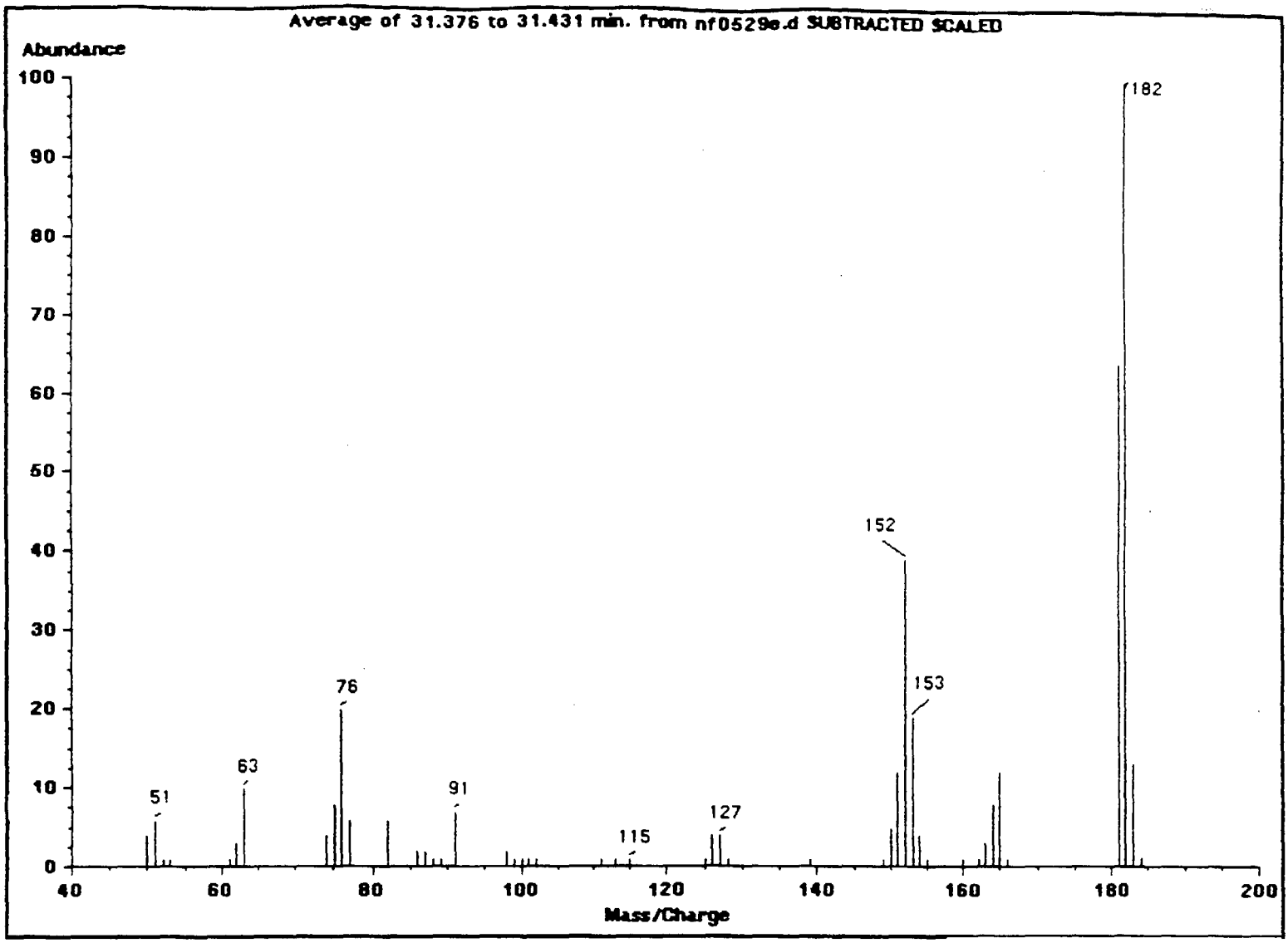

| | |
|---|---|
| compound identification | : fluorenone |
| molecular weight | : 180 |
| measured retention index (n = 1) | : 293.51 |
| retention index of reference substance | : 293.06 |
| literature retention index | : |
| compound found in HPLC fraction | : 5,6 |



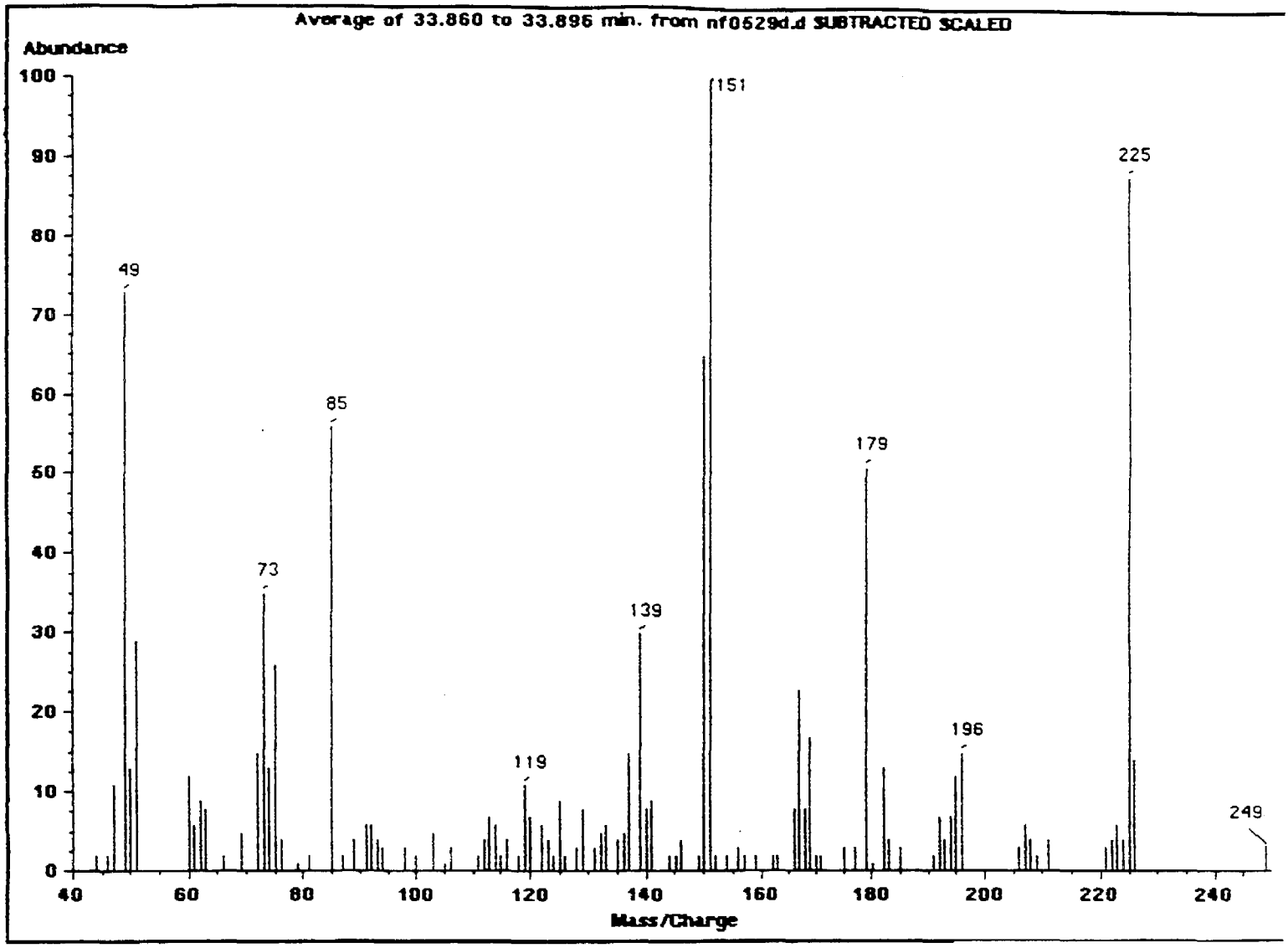

| | |
|---|---|
| compound identification | : hydroxyfluorene |
| molecular weight | : 182 |
| measured retention index (n = 3) | : 316.57 |
| retention index of reference substance: | |
| literature retention index | : |
| compound found in HPLC fraction | : 5 |



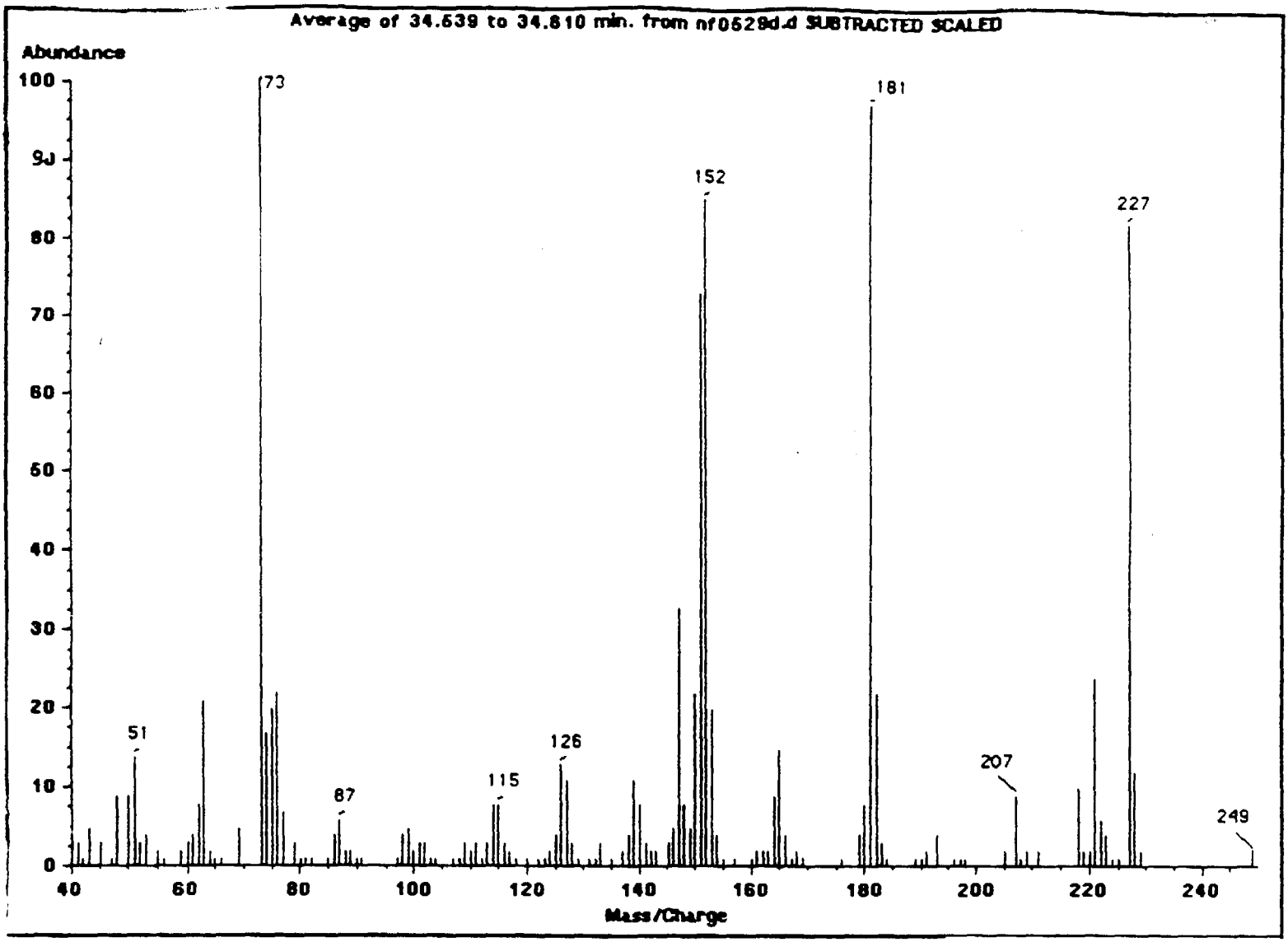

| | |
|---|---|
| compound identification | : hydroxyfluorene |
| molecular weight | : 182 |
| measured retention index (n = 4) | : 318.93 |
| retention index of reference substance: | |
| literature retention index | : |
| compound found in HPLC fraction | : 5,6 |



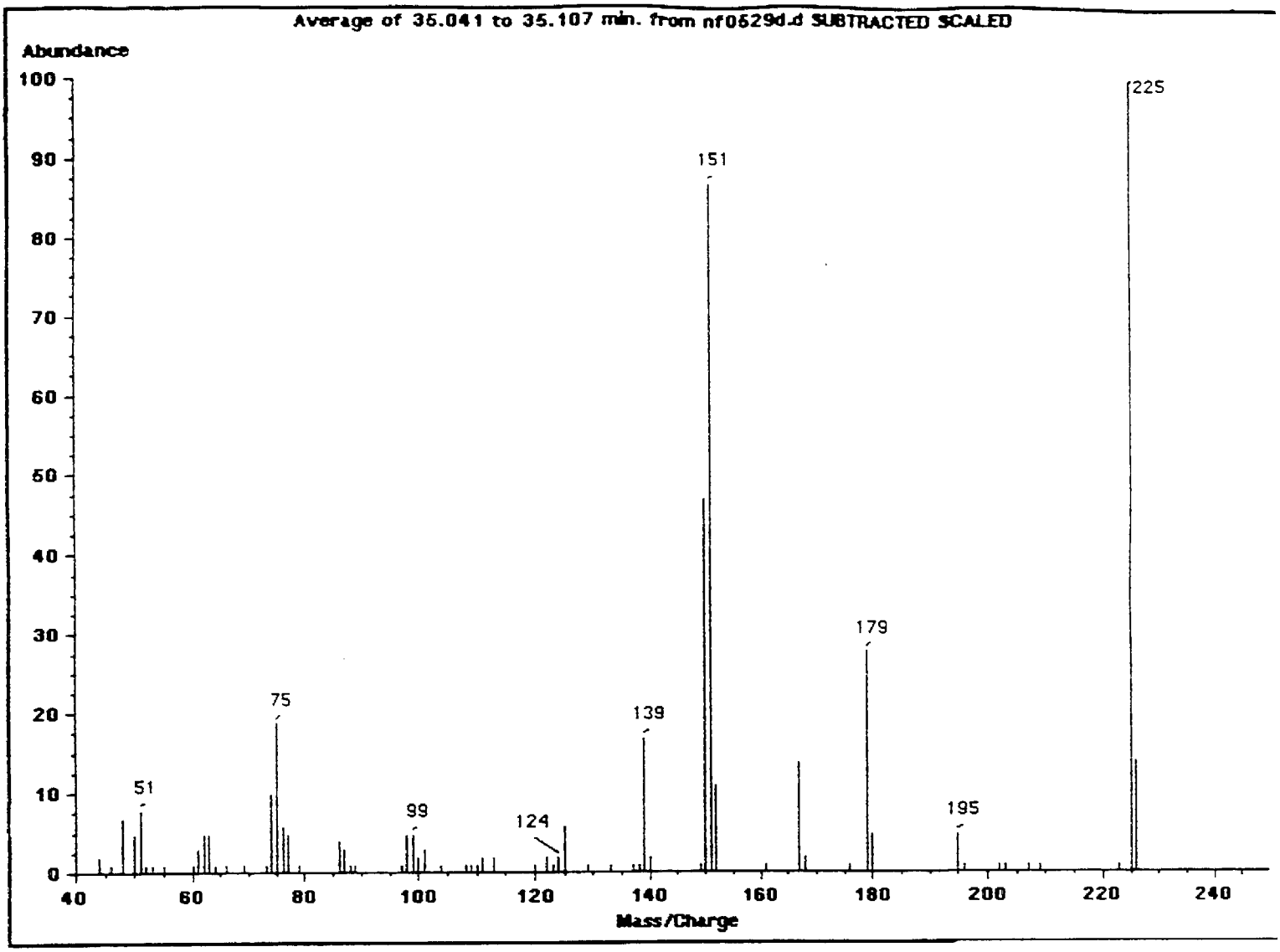

| | |
|---|---|
| compound identification | : hydroxyfluorene |
| molecular weight | : 182 |
| measured retention index (n = 2) | : 320.61 |
| retention index of reference substance: | |
| literature retention index | : 322.53 (2-hydroxyfluorene) |
| compound found in HPLC fraction | : 6 |



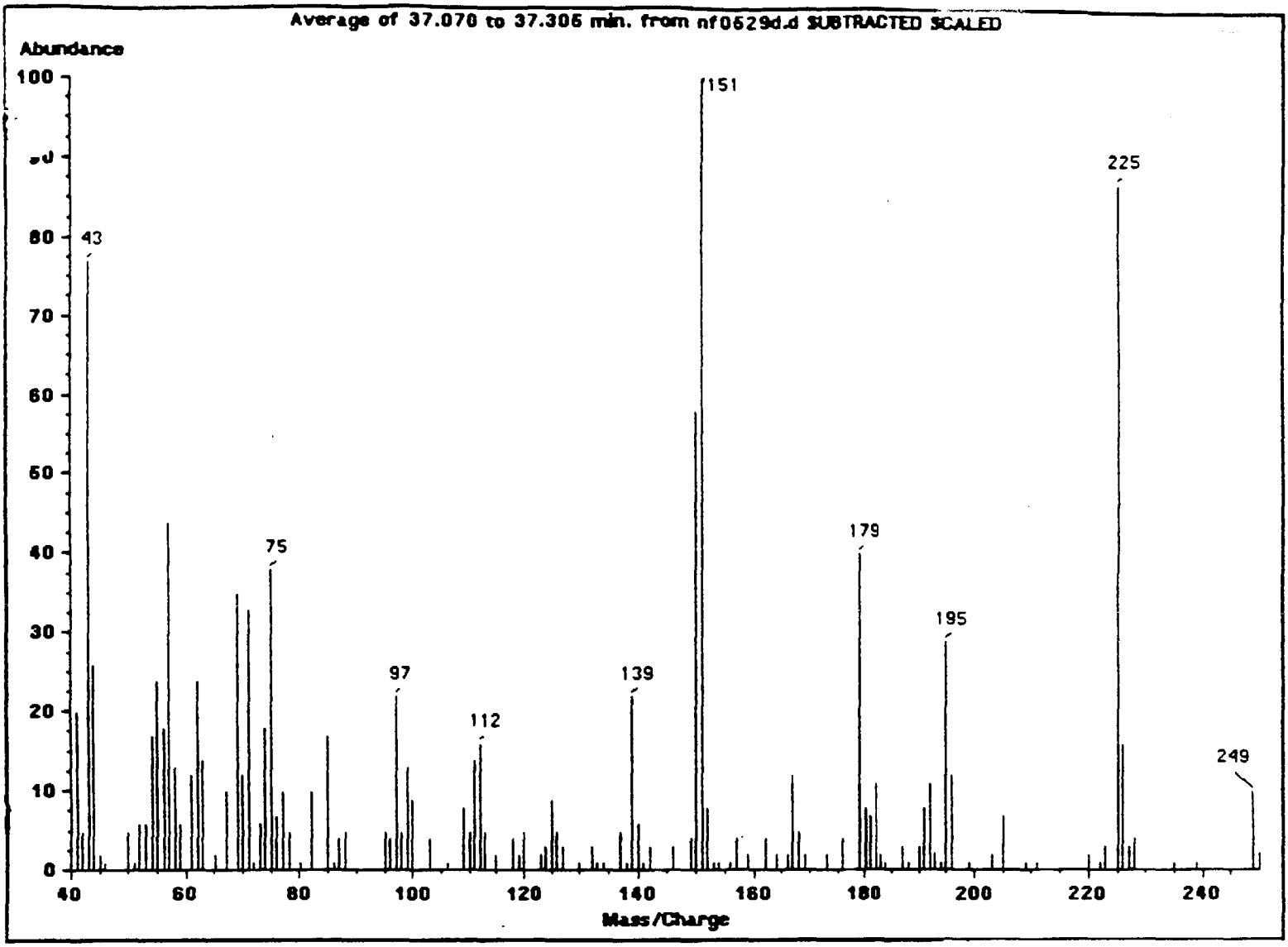

| | |
|---|---|
| compound identification | : 4-nitrofluorenone |
| molecular weight | : 225 |
| measured retention index (n = 1) | : 342.47 |
| retention index of reference substance: | |
| literature retention index | : |
| compound found in HPLC fraction | : 5 |



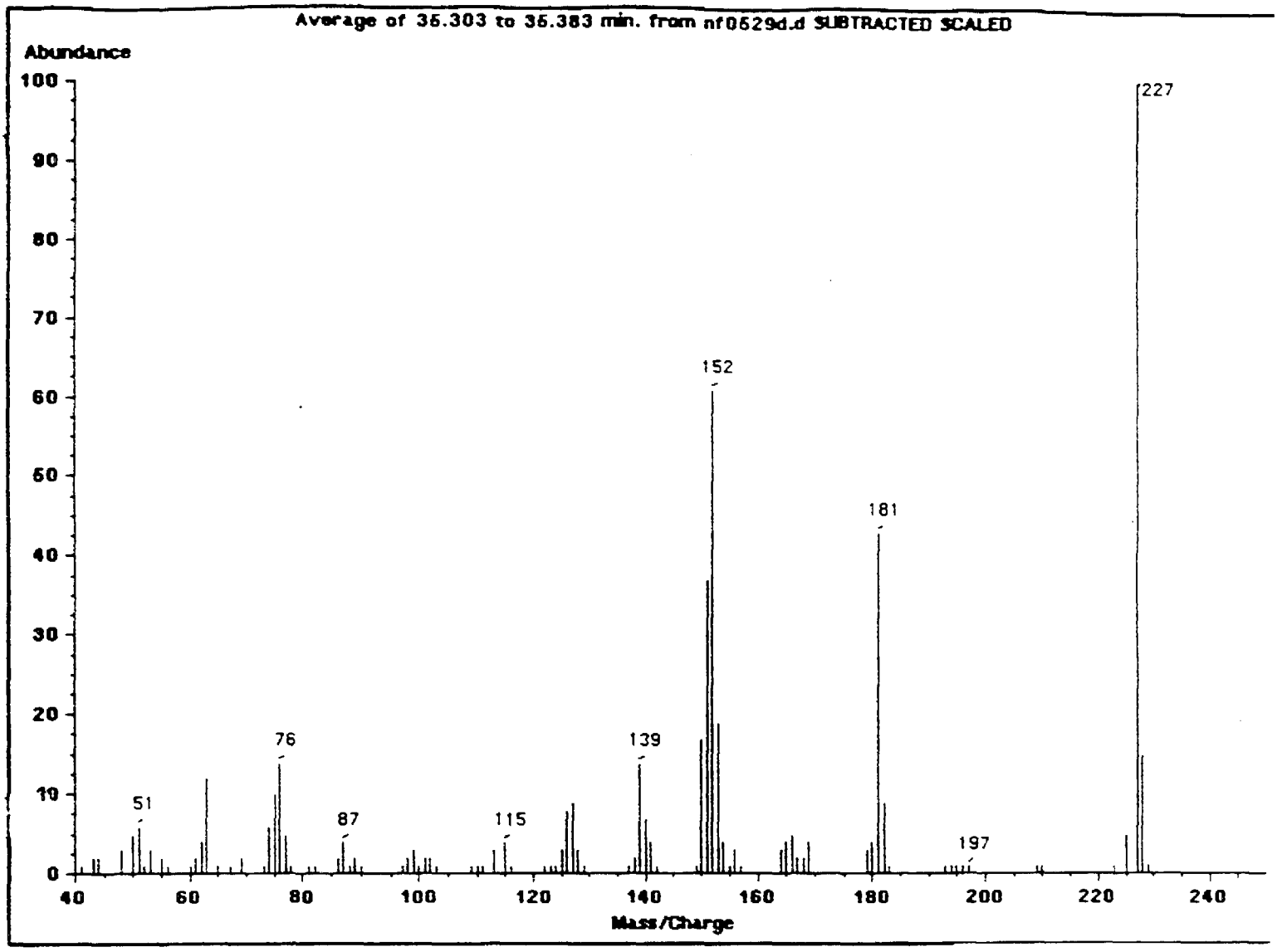

| | |
|---|---|
| compound identification | : hydroxynitrofluorene |
| molecular weight | : 227 |
| measured retention index (n = 5) | : 349.08 |
| retention index of reference substance: | |
| literature retention index | : |
| compound found in HPLC fraction | : 5,6,7 |




| | |
|---|---|
| compound identification | : 3-nitrofluorenone |
| molecular weight | : 225 |
| measured retention index (n = 4) | : 353.64 |
| retention index of reference substance | : 354.27 |
| literature retention index | : 355.07 |
| compound found in HPLC fraction | : 5 |




| | |
|---|---|
| compound identification | : 1-nitrofluorenone |
| molecular weight | : 225 |
| measured retention index (n = 2) | : 372.07 |
| retention index of reference substance: | |
| literature retention index | : |
| compound found in HPLC fraction | : 5 |




| | |
|---|---|
| compound identification | : hydroxynitrofluorene |
| molecular weight | : 227 |
| measured retention index (n = 6) | : 356.03 |
| retention index of reference substance: | |
| literature retention index | : |
| compound found in HPLC fraction | : 5,6,7 |