DEVELOPMENT OF INTEGRATED BIOASSAY AND CHEMICAL METHODS TO CHARACTERIZE HEAVY-DUTY DIESEL EXHAUST

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ABSTRACT

The emissions from heavy-duty diesel-powered vehicles contains both particle and vapor-phase associated compounds. With respect to particulate matter (PM), diesel-powered vehicles are a major source in California. PM has especially been studied for potential adverse health effects to humans. Almost all diesel PM emitted is smaller than 10 μm (PM10) aerodynamic diameter and typically submicron in size. These particles can penetrate the deepest portions of the human lung, and in turn can lead to adverse health effects such as respiratory problems, mortality, and association with cancer. Diesel PM is considered a probable human carcinogen, and a complex mixture of toxic compounds can be adsorbed to these particles. Although many toxicological studies have focused on diesel PM, few studies have focused on vapor-phase toxic compounds, especially those emitted from the combustion of fuels available in California. These vapor phase compounds are more volatile than those typically found in the particulate phase. We investigated the particle- and vapor-phase emissions from a heavy-duty diesel engine using a California Pre-October 1993 fuel (Fuel 1) and a newer fuel (Fuel 2) that was available from the MTA storage tanks during the Main Study sampling period in March 1995. Emissions were collected under the controlled conditions of an engine dynamometer and dilution tunnel (a facility where engine exhaust is diluted and measured). The particle- and vapor-phase emissions were chemically analyzed using gas chromatography/mass spectral (GC/MS) analyses and by bioasssay using a *Salmonella* microsuspension assay. For the particulate-phase, the mass of PAHs per mass of particulate matter (μg/g), or concentration of PAH were determined for a series of 15 PAHs, and from this value, emissions (μg PAH/hp-hr) were determined. With the exception of phenanthrene, the concentration (μg PAH/mg PM) of most of the PAHs in the particle-phase was higher for Fuel 2 emissions. When the emission values for the PM samples were calculated, emissions of pyrene and benzo[a]pyrene were approximately 30% higher from the use of the Fuel 2 than from use of Fuel 1. Overall, for both fuel types, the highest relative emissions were for phenanthrene and pyrene. For the vapor-phase, numerous PAHs and PAH-containing substituent groups were detected. Overall, the level of vapor-phase PAHs were elevated in the emissions from Fuel 1 compared to those in the emissions from Fuel 2. Different types of substituted PAHs were found at elevated levels in

emissions from both fuels. For example, the emissions from Fuel 1 contained higher concentrations of alkylated PAHs, while the emissions from Fuel 2 contained higher levels of C_1-, C_2-, and C_3-tetrahydronaphthalenes. The bioassay analyses were conducted using the *Salmonella* microsuspension assay for both the PM and vapor phases. For PM collected during the hot cycles, the mutagenic activity per mass of PM was similar for both fuel types. However, since there were greater emissions of PM for Fuel 1 than for Fuel 2, the total emissions of mutagenic compounds were higher for Fuel 1. The total mutagenic activity emitted per horsepower-hour was approximately 13% and 9% higher (with and without metabolic activation), respectively, for the emissions from Fuel 1 than for emissions from Fuel 2. With respect to the bioassay results for the vapor-phase, there were measureable levels of mutagenic activity in the emissions from combustion of the two fuels. A mutagenic profile, or mutagram, was developed for the vapor-phase emissions and was based on chemical fractionation of the vapor-phase extracts. The profiles for emissions from each fuel were very similar. However, the total mutagenic emissions from Fuel 1 were greater than from Fuel 2. In conclusion, the methodology for measurement of vapor-phase emissions from the combustion of diesel fuel was successful in collecting and measuring PAHs and mutagenic activity from a heavy-duty diesel engine. The methodology for measuring differences in emissions from the use of different fuels was also established. There was a consistent finding that the PAHs and mutagenic activity were higher in the emissions from Fuel 1 than in those from Fuel 2. The integrated chemical and bioassay approaches helped to evaluate emissions from these fuels and to better assess public exposure to the compounds in diesel exhaust.

I. EXECUTIVE SUMMARY

A. Background

The emissions from heavy-duty diesel engines are known to contain high concentrations of particulate matter (PM) and particle-associated mutagenic compounds. PM from the emissions of diesel-powered vehicles are a known source of PM10 (particulate matter with aerodynamic diameter of less than 10 microns) in California. The adverse human health effects reported resulting from exposure to PM10 include increases in respiratory infections, decreased lung functionality, and morbidity. These health effects and exposure to PM10 are currently being evaluated by a number of research groups and governmental agencies. To lower diesel emissions of PM and oxides of nitrogen, the use of new formulations of diesel fuels was implemented in California in October, 1993. Since the particulate exhaust from diesel-powered vehicles is a major source from all vehicles, and control strategies such as the use of new fuels are being implemented for PM, the emissions of toxic compounds needs to be investigated to help better evaluate potential human exposure. Further, although diesel exhaust is suspected to be a cancer causing agent in humans, there have been relatively few studies on vapor-phase mutagens present in diesel exhaust. The vapor-phase of diesel exhaust has been responsible for inducing tumors in laboratory animals. In previous work, we developed methods for the collection, extraction, fractionation, and concentration of vapor-phase mutagens from the undiluted exhaust of a medium heavy-duty diesel truck and integrated these methods with chemical analysis and a sensitive mutagenicity bioassay. In the current research effort, we further optimized these methods and used them to characterize particle- and vapor-phase mutagens present in heavy-duty diesel exhaust using a Pre-October 1993 fuel (Fuel 1) and a stock MTA fuel (Fuel 2) that was available during the Main Study sampling period in March 1995. The emissions were collected under the controlled conditions of an engine dynamometer and dilution tunnel (a facility where engine exhaust is diluted and measured).

B. Methods

The particulate emissions from Fuel 1 and Fuel 2 were analyzed using integrated chemical and bioassay techniques. To collect PM and vapor-phase emission samples for chemical and bioassay analyses, a sampling unit was designed that consisted of two identical and parallel sampling trains.

Each sample train was composed of a Teflon filter, polyurethane foam (PUF), XAD-4 (XAD) adsorbent, and rotometers, in series, to directly trap PM and vapor-phase mutagens in heavy-duty diesel truck exhaust acquired under the controlled operating conditions of a chassis or engine dynamometer and dilution tunnel. The particle-phase mutagens from the exhaust were extracted from the filters for both chemical and bioassay analyses. The solvent extracts from the filters were chemically analyzed using an isotope dilution method (a method that incorporates internal deuterated PAHs during processing) and gas chromatography-mass spectrometry (GC/MS). The extracts were also directly analyzed by a modified Ames *Salmonella* mutagenicity assay (microsuspension assay) that measures damage to DNA. The extracts from the particle emissions from Fuel 1 and Fuel 2 were further fractionated using solid-phase separation techniques to compare chemical and mutagenic profiles in the particulate emissions from the two fuels. The vapor-phase mutagens from the exhaust were trapped on the PUF and XAD adsorbents, which were extracted using a supercritical fluid extraction (SFE) procedure. Supercritical fluids are gases that have many characteristics of a liquid, including the ability to extract compounds from various collection media. Supercritical fluids are used at specific temperatures and pressures and behave as liquids, but they do not generate liquid solvent waste. The SFE extracts of PUF and XAD were analyzed by a gas chromatograph-mass spectrometer (GC/MS). Further, the SFE extracts were also directly analyzed by a modified Ames *Salmonella* mutagenicity assay and the results were compared to those obtained for the particulate-associated mutagens.

C. Results and Conclusions

The regulated pollutant emissions for the two fuels were measured. On average, PM emissions for Fuel 1 (0.319 ± 0.017 g/hphr) were approximately 13% greater than Fuel 2 (0.277 ± 0.007 g/hphr). The regulated gaseous pollutants (NO_X and CO_2) were virtually identical and CO_2 was slightly higher in the Fuel 2 emissions.

Particulate matter obtained from the emissions from both fuels contained measurable amounts of polycyclic aromatic hydrocarbons (PAHs) that included both semi-volatile compounds (typically composed of three or four fused benzene rings) and essentially non-volatile PAHs (typically

composed of four or more fused benzene rings). The levels of PAHs are reported in two forms: 1) the mass of PAH per mass of PM (μg of PAH per mg of PM, and 2) the total emissions of PAH (μg PAH per hp-hr).

The mass of PAHs per mass of PM (μg/g), or concentration of PAH, were determined for a series of 15 PAHs. All concentrations were based on triplicate analyses and duplicate pooled samples. The concentrations for each PAH were highly precise. With the exception of phenanthrene, the concentration of most of the PAHs were higher for Fuel 2 than for Fuel 1 particles. When the sum of PAHs was calculated for each fuel type, the total concentrations of PAHs measured was approximately 16% higher for Fuel 2 than for Fuel 1 particles. Although concentrations of PAHs are valuable for purposes of comparison, the measurement for evaluating human exposure is based on specific emissions (μg/hp-hr).

Emissions are presented as μg of PAH per hp-hr. The emissions for two PAHs, pyrene and benzo[a]pyrene, are approximately 30% higher from use of Fuel 2 than from Fuel 1. Overall, for both fuel types, the PAHs with the highest relative emissions were phenanthrene and pyrene. When all PAH emissions were added together for each fuel type, the total emissions (dominated by phenanthrene and pyrene) were similar.

The bioassay results are reported in two ways: 1) the number of revertants per mass of PM (an index of the mutagenic activity) and 2) total revertant emissions (revertants per hp-hr). Comparing the hot cycles, the mutagenic activity per mass of PM was similar for both fuel types. However, since there were greater emissions of PM for Fuel 1 than Fuel 2, the total emissions of mutagenic compounds was higher for Fuel 1. The total mutagenic activity emitted per hp-hr was approximately 13% and 9% higher (with and without metabolic activation, respectively) for the emissions from Fuel 1 than emissions from Fuel 2.

The complex mixture of the extracts from PM were further analyzed by first fractionating the sample using solid phase extraction (SPE), and then chemically analyzing each fraction.

There were 5 fractions collected using a variety of organic solvents, starting from a relatively polar solvent (methanol), and gradually increasing to a more non-polar solvent (hexane). The first fraction (methanol) contained the PAHs and substituted PAHs. The second fraction contained more substituted PAHs. The remainder of the fractions were dominated by saturated hydrocarbons. These fractions were highly complex mixtures of compounds and require further fractionation to obtain a more detailed chemical identification. Bioassay analyses of the fractions were conducted and almost all the mutagenic activity was present in the first fraction (methanol) for the particles from both fuel types.

In general, the PUF samples contained considerable amounts of PAHs that are chemically composed of two or three fused benzene rings. Examples of these types of compounds include naphthalene, acenaphthylene, fluorene, and phenanthrene. There were lower amounts of similar compounds, including anthracene, fluoranthene, and pyrene. PAHs containing four or more fused benzene rings were detected in the filter extracts, but were not detected in the PUF samples, indicating that the sampling flowrate and sampling time minimized the loss of PAHs (4 rings or greater) from the filter to the vapor-phase.

The XAD samples contained higher concentrations of naphthalene than the PUF samples, but had much lower levels of PAHs containing more than two fused benzene rings such as acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene, and pyrene. The XAD samples did not contain PAHs with four or more fused benzene rings.

The PUF and XAD results were used to calculate the total vapor-phase PAH emissions from Fuel 1 and Fuel 2. The engine was run starting from the first run of the day (cold-start) to the remaining runs for the day (hot-start). For the hot-start cycles, there were higher emissions of most of the semi-volatile PAHs in the emissions from using Fuel 1 than Fuel 2. The exhaust from Fuel 1 contained higher levels of certain alkylated naphthalenes such as 2,6-dimethylnaphthalene, 2,7-dimethylnaphthalene, 1,3-dimethylnaphthalene, 1,2-dimethylnaphthalene, and 1,5-dimethylnaphthalene. However, the emissions from Fuel 2 contained higher levels of

C_1-, C_2-, and C_3-tetrahydronaphthalenes. For the cold-start exhaust samples, there were greater emissions of PAHs and substituted PAHs from using Fuel 1 than from using Fuel 2.

To help identify compounds in the complex mixture of the extracts, it was necessary to fractionate the whole extract into chemically separable and identifiable classes. The PUF extract was fractionated by solid-phase extraction (SPE) into five (5) distinct fractions using a series of organic solvents. The SPE fractions were analyzed by both bioassay and GC/MS, to facilitate identification of individual chemical components and potential mutagens. Each fraction was very chemically complex. For the emissions from both fuel types, most of the aromatic compounds that were detectable by GC/MS were found in Fraction #1 (methanol) and in Fraction #2 (methanol: acetonitrile). Partially hydrogenated naphthalenes were found mainly in Fraction #2, while PAHs and substituted PAHs were found in both Fractions #1 and #2, respectively. The major components in Fractions #3, #4, and #5 appear to be saturated hydrocarbons. These fractions require further fractionation to obtain a more detailed chemical identification.

The SPE fractions from the PUF samples were also tested for mutagenic activity to develop mutagrams of the extracts. There were elevated levels of mutagenicity in Fraction #1 from the exhaust from both Fuel 1 and Fuel 2. In Fraction #1, there was overt toxicity, or killing, of cells at the highest doses of the fraction derived from the Fuel 2 exhaust extract. Elevated levels of mutagenicity were also observed in Fraction #2. There was also overt toxicity to cells in Fraction #2 from the PUF sample extract of the exhaust from Fuel 1. There were no indications of mutagenicity for any sample in either Fraction #3 or #4. However, significant mutagenic activity was observed in Fraction #5 for both exhaust samples from Fuel 1 and Fuel 2. The mutagrams of the vapor-phase exhaust from using each fuel type were similar in profile with Fraction #1, #2 and #5 having the highest mutagenic activity.

Based on the results for PM, there were differences in the emissions from Fuel 1 and Fuel 2, especially for specific PAHs such as benzo[a]pyrene. There were also differences in the emissions of mutagenic compounds for the two fuel types and most of the mutagenic activity was present in

of mutagenic compounds for the two fuel types and most of the mutagenic activity was present in the fraction where PAHs, substituted PAHs, and other unidentified compounds were present. A more detailed analyses of the most active fraction, including subfractionation, would be important to further compare the emissions from these and other types of fuels. For the vapor-phase, there was a consistent finding that the PAHs and mutagenic activity were higher in the emissions from using Fuel 1 than from Fuel 2.

II. INTRODUCTION

A. Statement of Problem

The measurement of human exposure, both indoor and outdoor, to airborne particles that are 10 μm or less (PM10) is important because particles of this size can enter and deposit on the trachea, bronchi and alveoli (Schlesinger, 1985). These particles have been associated with increased respiratory infections, decreased lung functionality and morbidity (Further, 1986; Pope *et al.*, 1992; Dockery *et al.*, 1992). In California, PM10 has also been associated epidemiologically with cancer (Abbey *et al.*, 1991). Consistent with the potential carcinogenicity of PM10, these particles are known to contain numerous carcinogenic and mutagenic compounds (Daisey, 1987; Sheldon *et al.*, 1992; Atkinson et. al., 1988). Although there are numerous outdoor sources of PM10 in urban areas, a major emission is the PM generated from heavy-duty diesel vehicles. These particles are also a component of road dust in urban environments. The particles and their associated mutagenic activity that are present outdoors are thought to infiltrate indoors (Kado *et al.*, 1994). Numerous toxic compounds are associated with these particles, but these compounds are difficult to characterize by emission source.

The toxic compounds associated with PM from emission sources are difficult to routinely characterize chemically due to the complex mixture of the compounds present. Further, changes in fuel composition can alter the toxic emissions from these sources. For example, for heavy-duty diesel engines in California, diesel fuel produced after October 1993 (Reformulated fuels) had lower levels of sulfur and aromatic compounds. By changing to a Reformulated diesel fuel, the emissions of toxic compounds are thought to decrease, especially those compounds associated with PM. However, few experiments have been reported that are specifically designed to compare differences in toxic compound emissions associated with PM from Pre-October 1993 and newer diesel fuels. Further, the vapor-phase toxic compounds have not been typically investigated for these fuel types.

Unlike mutagens associated with PM, vapor-phase mutagens are not routinely monitored due to a lack of methodology that integrates vapor trapping and extraction with bioassay and chemical analysis. Vapor-phase mutagens consist of both volatile and semi-volatile compounds and are potentially a major class of toxic substances present in the ambient air and in vehicular emissions. These compounds are diverse and include halogenated hydrocarbons, aldehydes, and polycyclic aromatic hydrocarbons (PAH), many of which are carcinogenic to animals or suspected to be carcinogenic to humans. It is possible that there are unidentified vapor-phase mutagens present in ambient air, as well as in stationary and mobile source emissions.

Working with vapor-phase mutagens has required a different approach to trapping, extracting, and analysis compared to methods used for PM. Based on our previous studies of vapor-phase mutagens (Hsieh *et al.* 1990, 1993; Kado *et al.* 1992, 1996) supported by the California Air Resources Board (CARB), we developed methods to trap diesel emission samples, to extract these samples using supercritical fluid extraction (SFE) technology, and to integrate these methods with a sensitive mutagenicity assay and chemical analysis. The current study further tests these integrated methods.

The PM collected in these samples were mutagenic when tested in the bioassay in tester strains TA98 and TA100 (with metabolic activation, or +S9). Higher mutagenic activity was observed in tester strain TA98 (+S9). Since these tester strains can detect different classes of compounds, the differences in response indicates that the mutagenic compounds present in the particulate phase are different than those that are present in the vapor-phase. Both phases are therefore important to study in parallel. Vapor-phase mutagens have also been tested in the bioassay and by gas chromatography-mass spectrometry (GC/MS) analytical techniques. Bioassay used in conjunction with chemical analyses, termed "bioassay-directed chemical analysis", has been a powerful method used to identify important mutagenic compounds associated with diesel particles. Our previous studies indicate that heavy-duty diesel engine exhaust may be a major source of vapor-phase mutagens.

In the present study, particle samples from a heavy-duty diesel engine were collected and the extracts were analyzed by bioassay and chemical techniques. Vapor-phase samples were also collected concurrently and were tested using these same techniques.

B. Goals and Objectives

The overall objective of this project was test and integrate methodology to chemically characterize the particulate- and vapor-phase emissions from a heavy-duty diesel engine exhaust using a Pre-October 1993 fuel (Fuel 1) and a newer fuel (Fuel 2) that was available from the MTA storage tanks during the Main Study. The newer fuel had lower sulfur and polycyclic aromatic hydrocarbon content. We also evaluated the genotoxic activity of the PM and its relationship to fuel type, vapor-phase compounds, and criteria pollutant emissions. We developed and compared particle-associated mutagenic activity profiles, or mutagrams. We also validated integrated methods for the collection, quantitative transfer, assay for mutagenicity, and chemical identification of vapor-phase compounds present in the diesel engine emissions. This mutagenicity-directed chemical analysis procedure was also evaluated for assessing the significance of vapor-phase mutagens as a class of airborne toxic substances. The current work reported herein had the following specific goals:

1. Prepare a testing plan and validate sampling media for dynamometer testing. Design and test a new sampling unit to maximize the amount of diesel PM and vapor-phase sample collected from an engine dynamometer dilution tunnel, while maintaining the advantages of low volume sampling. Validate the sampling unit at the dynamometer facility. Validate our procedures for the collection, extraction, concentration, and quantification of vapor-phase mutagens using diesel exhaust as a model complex mixture.

2. Collect diesel PM and vapor-phase samples from the heavy-duty diesel engine emissions from Fuel 1 and Fuel 2. Prepare PM and vapor-phase samples for biological and chemical analyses. Extract vapor-phase compounds using SFE extraction procedures established for PAH and substituted PAH compounds.

3. Test all sample extracts using a sensitive mutagenicity assay and characterize. Determine specific mutagenic activity of PM and vapor-phase samples using bioassay. Develop mutagenicity profiles or mutagrams for each sample collected and compare results for each fuel type.

4. Develop bioassay-derived emission values for each fuel type.

5. Chemically fractionate the particulate and SFE extracts using SPE. Test the extracts for mutagenicity. Chemically characterize compounds with the highest peaks in the mutagrams by GC/MS.

6. Determine PAH emission rates from both fuels.

7. Submit draft final and final reports.

C. Background

Short-term tests for mutagenicity can be used to screen for the mutagenic components of complex environmental mixtures such as diesel exhaust. Mutagenic activity, as determined by short-term bioassays in cells in culture or in whole animal studies, indicates that there has been damage to DNA, the principal genetic material in all living organisms. Mutagenic activity, as determined in *Salmonella typhimurium* as the indicator organism, indicates that DNA damage has occurred in these cells. The mechanism that leads to cancer in humans and animals is thought to be the result of a complex and multi-staged process that involves mutation of DNA in specific genes (Sugimura et al, 1992). For example, two types of "target genes" or "cancer genes" thought to be important in the development of cancer are the proto-oncogenes and the tumor suppressor genes. Many of the proto-oncogenes, for example, are activated to cancer-causing oncogenes by simple mutations (Anderson *et al.*, 1992). The same types of mutations are detected in *S. typhimurium.* Most of the chemicals that are known to be carcinogenic to humans also damage DNA.

The *Salmonella*/microsome mutagenicity assay, or the Ames test (Ames *et al.*, 1975), is the most widely used and validated of all assays for mutagenicity. The test has been very useful in

directing chemical identification of mutagenic compounds present in environmental mixtures (Lewtas, 1988). Previously, Kado *et al.* (1983, 1986) developed a simple modification of the Ames test, known as the microsuspension assay, for the detection of mutagenic activity of urine from cigarette smokers and non-smokers and from extracts of airborne PM. This assay is 10 to 20 times more sensitive than the standard Ames test for a given amount of mutagen tested. This assay was adjusted for use with vapor-phase compounds in our previous work supported by CARB (Hsieh *et al.*, 1990; Kado *et al.*, 1992; Hsieh *et al.*, 1993; Kado *et al.*, 1996).

The exhaust from diesel engines has been determined by the International Agency for Research on Cancer to be a probable human carcinogen (IARC, 1989). In a number of animal studies, whole diesel exhaust was carcinogenic (IARC, 1989). Bagley *et al.* (1987) investigated the effect of ceramic particle traps on the chemical mutagens present in diesel exhaust. In their study, the mutagenic activity in both the soluble organic fraction of PM and volatile organic compounds of diesel exhaust was measured. The samples were collected in a dilution tunnel from an 8-cylinder medium-duty diesel engine. The PM was trapped on a filter, while the volatile organic compounds were collected on XAD-2 resin using high volume sampling. A number of driving modes were evaluated in using the standard Ames test to measure mutagenicity. Based on the number of revertants normalized to kilowatt-hours, the PM extract accounted for two-thirds and the volatile organic compound fraction contained about one-third of the total mutagenic activity. A number of compounds were tentatively identified in both phases which included PAHs such as fluorenone, anthracene/phenanthrene, ketones, and methyl anthracene/phenanthrene.

Much of the experimental work regarding diesel exhaust has been on PM. However, few studies have investigated the potential carcinogenicity of the vapor-phase. For example, Iwai *et al.* (1986) conducted chronic inhalation studies in rats who were exposed to unfiltered and filtered diesel exhaust. The primary cause of death among rats exposed only to the vapor-phase compounds present in diesel exhaust was malignant lymphoma, and the incidence was statistically significant compared to controls (Iwai, *et al.* , 1986).

In a major animal exposure study, Mauderly *et al.* (1994) exposed rats to emissions from a light-duty diesel engine or carbon black. The carbon black particles were similar to the soot particles in the diesel engine exhaust, but they contained markedly lower amounts of adsorbed organic compounds. The investigators found that prolonged exposure to diesel engine exhaust and carbon black particles at the concentration of particles tested, produced nearly identical carcinogenic and noncarcinogenic effects in the strain of rats used. The investigators concluded that the organic fraction may not be responsible for the observed effects and that mechanisms by which inhaled diesel soot and carbon black cause lung neoplasms in rats remain undefined.

Rasmussen et al (1990) tested the effect of varying fuel properties on the emission of mutagenic compounds present in the exhaust particles from a heavy duty diesel engine. Particles were collected using transient speed and load conditions while using nine fuels varying in aromatic content, sulfur content and boiling point. Mutagenic activity of the extracts of the PM was determined using the microsuspension assay or the Ames *Salmonella* /microsome test with strain TA98, with and without S9 metabolic activation. Increasing mutagenic activity relative to fuel consumed (mutants/lb fuel) or to engine work output (mutants/hp-hr) was correlated with increasing fuel aromatics ($p < 0.05$), but not with fuel sulfur content. Mutagenic activity with S9 was generally lower than without, but the correlations were not changed. However, the experiments were not designed to compare emissions from different fuel types.

Much of the information concerning diesel exhaust mutagenicity has been associated with extracts of PM (Slaga *et al.*, 1980). A number of potent mutagens have been isolated and identified using the microsuspension assay in conjunction with chemical analysis (Scheutzle *et al.*, 1982; Lewtas, 1988). Many of these compounds are suspected human carcinogens. Diesel PM was collected on various filter media, and the mutagenic compounds were extracted from the PM with organic solvents (Montreuil *et al.*, 1992). The extracted mutagens were concentrated and exchanged into a solvent that was compatible with the mutagenicity assay. Ambient airborne PM and its associated mutagenic compounds have been examined by a number of investigators (Talcott

and Wei, 1977; Pitts *et al.*, 1977; Moller and Alfheim, 1980; Chrisp and Fisher, 1980; Alfheim *et al.*, 1983; Chrisp *et al.*, 1978). The microsuspension method has also been used for determining mutagenic activity in model chamber studies (Arey *et al.*, 1992) and has been reviewed by the World Health Organization for methods for indoor air (IARC, 1993).

Mutagenicity-directed chemical analysis has been instrumental in identifying many important mutagenic compounds present in complex environmental mixtures (Lewtas, 1988). The analysis involves fractionating a complex mixture and determining the mutagenic activity of the various fractions. The mutagenic fractions are further fractionated and the bioassay is used to characterize the mutagens in the subfractions. In this manner, pure mutagenic compounds can be chemically characterized and identified. Using this technique, 1-nitropyrene and other nitro-PAH have been determined to be potent mutagens and carcinogens (Schuetzle, 1982; IARC, 1984). The mutagenic properties of extracts of PM from diesel engine emissions have been extensively studied and much of the mutagenic activity has been attributed to nitro-PAH (NRC, 1981; Schuetzle and Lewtas, 1986). In addition to PM, unfiltered diesel exhaust also contains many toxic gases or vapors that can cause acute respiratory problems (Slaga *et al.*, 1980).

Supercritical fluid chromatography has also been used in the bioassay-directed fractionation of coal tar (SRM 1597), a complex mixture of PAH standards (Parrish *et al.*, 1992). Coupling of this chromatographic technique with the microsuspension assay resulted in the quantitative recovery of coal tar mutagenic activity in tester strain TA98. Briefly, the coal tar was fractionated by supercritical fluid chromatography into six fractions. Three of the fractions contained over 97% of the mutagenicity, consisting of high molecular weight PAHs.

The mutagenic activity of volatile compounds from diesel engine emissions have been studied to a limited extent and with conflicting results. Scheutzle and Lewtas (1986) reported that compared to extracts of PM, 3% of the total direct-acting and about 5% of the total indirect-acting mutagenicity was due to vapor-phase compounds. Egeback (1982) however found that gaseous

emissions had greater direct-acting mutagenic activity than PM. The discrepancy between the results of these two studies may be attributed to differences in the efficiency of sample collection, preparation, and testing, although differences in fuels, engines, and test conditions cannot be ruled out.

Dorie *et al.* (1987) reported on the collection of particulate and gaseous exhaust samples from a stainless steel dilution tunnel (15.2 m^3/min with a dilution ratio of 15:1), with and without the use of ceramic particulate traps. The organic solvent extracts from exhaust particles and from volatile organic compounds were tested for mutagenic activity using tester strain TA98 (with and without metabolic activation). The solvent extracts with and without metabolic activation appeared to have similar activity, based on revertants/m^3. However, they reported that the mutagenic activity of the volatile organic compound extract was considerably less than that from the organic solvent extracts from particles.

Matsushita *et al.* (1986) studied the mutagenicity of PM and particle-free phases of diesel exhaust using a flow-through exposure chamber system to treat *Salmonella* bacteria to the gaseous and vapor compounds of the exhaust. The exhaust from a 2369 cubic centimeter diesel engine was diluted 10 times in a dilution tunnel. Mutagenic activity was detected under these test conditions without the addition of S9 microsomal enzymes. Bacterial tester strain responses in order of highest response, were: TA100, TA104. The authors reported little or no increase in mutagenic activity when S9 microsomal enzymes were incorporated into their test.

Westerholm *et al.* (1991) studied the chemical composition and mutagenicity of exhaust from a heavy-duty diesel vehicle (14.2 L engine) tested during transient driving conditions. The authors used a cryo-trap, XAD-2, and polyurethane foam (PUF) to collect the diluted volatile organic compounds from the exhaust. Mutagenic activity was detected on the XAD-2 and PUF samples. The contribution of mutagenic compounds in the semi-volatile phase was 20% in tester strain TA100 (±S9), 10% in TA98 (-S9), and 37% in tester strain TA98 (+S9). A number of 3-ring PAH

(substituted and unsubstituted) were tentatively identified in these samples. Phenanthrene and methyl phenanthrene, for example, were the highest emitted PAHs in the particulate phase. These PAHs were also present in high concentration in the semi-volatile phase.

A common procedure for trapping trace organics in the air involves passing an air sample through some trapping medium such as an adsorbent or filter. Methods which have been used for trapping vapor-phase compounds from air include collection of the whole sample into canisters or bags, condensation into cryogenic traps, absorption into solvent impingers, and adsorption onto solid matrices. A solid matrix was chosen for the present study due to its ease of use and the availability of a number of commercial adsorbents. Examples of solid matrices include XAD-2 and XAD-4 adsorbents. These adsorbents are macroreticular cross-linked polystyrene, divinylbenzene copolymers with high specific surface areas (290-750 m^2/g), small mean pore diameters (50-90 Å) (Nunez and Gonzales, 1984), and large mesh sizes (20-50). Volatile organic compounds that have been collected on the XAD adsorbents include PAH, mutagens from combustion sources, volatiles from diesel exhaust, chlorinated benzenes, polychlorinated biphenyls, alcohols, alkanes, carbonyls, carboxylic acids and esters, nitrogen and sulfur compounds, phenols, phthalates, and pesticides (Chuang *et al.*, 1987; Bennett *et al.*, 1979; Junk and Richard, 1984; Woodrow and Seiber, 1978; Wehner et al, 1984; Wong et al, 1988). Moller and Alfheim (1983) used XAD-2 resin and found that of the total mutagenic activity present in both the particulate and vapor phase, 40-90% of the activity was found in the vapor phase of combusted wood chips, oil, and coal.

Polyurethane foam (white polyether and charcoal gray polyester forms), or PUF, is another adsorbent that has been extensively used in air sampling. Because of its low resistance to air flow, PUF is useful for sampling at high flow rates. PUF has a large sorption surface, is easy to handle and store, and is relatively inexpensive. However, PUF requires thorough pre-cleaning with organic solvents before use. Westerholm *et al.* (1991) used PUF and XAD-2 to collect diesel exhaust emissions and found that the amounts of PAH emitted in the semi-volatile phase was approximately three times higher than that emitted in the particulate phase. In another study, De

Raat *et al.* (1987) used PUF to trap volatile organic compounds from air, and found mutagenicity in the water and various alcohol extracts of the PUF. Umlauf and Kaupp (1993) developed a sampler that used a filter to trap PM, followed by XAD-2 resin to trap semi-volatile organic compounds. Unlike our sampling system for diesel emissions which uses both PUF and XAD-4 adsorbents, the authors relied on a standard Soxhlet cartridge containing XAD-2 alone to trap semivolatile compounds in ambient air.

Recently, Peltonen and Kuljukka (1995) reviewed methods of air sampling for PAHs on PM trapped on filters and for vapor-phase compounds trapped by back-up adsorbents. Active sampling (using sampling pumps to draw air sample through adsorbent) was found to be the most common sampling method, with samples traditionally extracted by Soxhlet, ultrasonication, or supercritical fluid extraction (SFE). Analyses are typically carried out by HPLC equipped with a fluorescence detector or by GC/MS. Techniques requiring solvent extraction are not suitable for highly volatile mutagens because of considerable losses during sample extraction and concentration. An alternative approach for the extraction and concentration of volatile mutagens is the use of SFE.

When a fluid is above its critical pressure and temperature, it possesses gas-like mass transfer properties but behaves like a solvent. Carbon dioxide is commonly used as a supercritical extraction fluid above its critical conditions of 72 atm and 31.1°C. In previous work, we successfully integrated SFE with a *Salmonella*/microsuspension mutagenicity assay and chemical analysis for model semi-volatile organics (Hsieh *et al.*, 1990; Wong *et al.*, 1991; Kado *et al.*, 1992; Hsieh *et al.*, 1993; Kado *et al.*, 1996). SFE has great potential for extracting organics from complex matrices (Hawthorne, 1990) and has many advantages over liquid extraction. First, the solvating properties can be changed by varying the temperature and pressure. Second, supercritical fluids are gases at ambient temperatures. Therefore, a concentration step (as required for liquid extraction) is unnecessary because the fluid is vented from the sample upon reaching ambient pressure in the collection vessel. Third, many supercritical fluids have relatively low critical temperatures (for example, 31°C for CO_2) and are considered inert or have low chemical reactivity.

Fourth, for compounds that are rather difficult to extract, the polarity of the supercritical fluid can be influenced by using organic modifiers such as methanol to effect a more efficient extraction.

SFE is a reliable and efficient technique that can expedite the separation and analysis of complex chemical mixtures (Taylor, 1992). This technique has been used for the extraction of organic pollutants from a variety of adsorbents, including Tenax-GC, XAD-2, PUF, and Spherocarb (Hawthorne and Miller, 1986; Hawthorne and Miller, 1987; Schantz and Chester, 1986; Wright *et al.*, 1987). Kanagasabapathy *et al.* (1995) developed a method for trapping indoor airborne PAHs on XAD-2. They optimized SFE conditions by first spiking PAHs onto XAD-2 and using chemical modifiers, such as chlorobenzene, to improve extraction efficiency. Hawthorne and Miller (1986) used SFE to extract SRM 1650 (National Institute of Standards and Technology reference sample for diesel exhaust PM) and reported the quantitative recovery of PAHs with extraction times as short as 30 min. The same investigators later found that 30 to 60 min of SFE resulted in better recoveries of PAH compared to either a 4 hr sonication of the sample matrix with solvent or an 8 hr Soxhlet extraction (Hawthorne and Miller, 1987). This was due to the fact that supercritical fluids have the solvating power of a normal liquid, but with better mass transfer properties. By varying the extraction pressure, class-specific extractions of alkanes and PAH from diesel exhaust PM were accomplished. Since changes in pressure can influence the solvating power of a supercritical fluid, pressure gradients are commonly used to extract compounds of increasing molecular weight. The SFE-extracted sample can then be easily dissolved in a solvent suitable for bioassay or chemical analysis. SFE can also be directly interfaced with gas chromatography, high performance liquid chromatography, or SFC to provide powerful on-line analytical capabilities (Hawthorne and Miller, 1987; Hawthorne *et al.*, 1988; Engelhardt and Gross, 1988; McNally and Wheeler, 1988; Raynor *et al.*, 1988; Vannoort *et al.*, 1990).

While gas chromatography is the primary analytical method for volatile compounds, mass spectrometry is one of the most powerful methods for chemical identification. Mass spectral data libraries are readily available to aid in compound identification. A number of investigators have

identified previously unknown compounds present in atmospheric samples (Tong and Darasek, 1984; Yasuhara *et al.*, 1984; Arey *et al.*, 1992) and quantitation by gas chromatography-mass spectrometry (GC/MS) is possible with the use of 1) internal standards, 2) isotope dilution (adding standards before extraction), and 3) external standards (Tong and Karasek, 1984; Coleman *et al.*, 1983). Isotope dilution and the use of internal standards are the preferred methods of quantitation.

When a component in a mixture is present in low concentrations or there is some chemical interference, selected ion monitoring (SIM) can be used for analysis. SIM is highly selective and sensitive and several studies have reported success using this technique on vapor-phase pollutants (Yasuhara and Fuwa, 1978; Marano *et al.*, 1982; Jonsson and Berg, 1980). If a compound cannot be easily identified using GC/MS, further fractionation is necessary to facilitate isolation of the compound for mutagenicity-directed chemical analysis. Collecting adequate quantities of sample for subsequent subfractionation is an important part of the experimental design.

III. MATERIALS AND METHODS

A. Chemicals

HPLC grade methanol, acetone, hexane, and water were obtained from Fisher Scientific. For mutagenicity experiments, benzo[a]pyrene and dimethylsulfoxide (spectrophotometric grade) were from Aldrich Chemical Co. and were used without further purification. Dichloromethane (DCM, OmniSolve) was from EM Science. For solid phase extractions (SPE), acetonitrile (ChromPure) and hexane (UV, High Purity) were from Burdick and Jackson.

Deuterated standards included methylnaphthalene-d_{10}, fluorene-d_{10}, anthracene-d_{10}, fluoranthene-d_{10}, pyrene-d_{10}, benzo[b]fluoranthene-d_{12}, benzo[k]fluoranthene-d_{12}, benzo[e]pyrene-d_{12}, dibenz[a,h]anthracene-d_{14}, and benzo[g,h,i]perylene-d_{12} and were obtained from Cambridge Isotopes. Naphthalene-d_8, acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12} and perylene-d_{12} were from AccuStandard (New Haven, CT).

All other PAH standards, except for benzo[e]pyrene and perylene, were obtained from Restek (Bellefonte, PA). Benzo[e]pyrene, perylene and the substituted PAHs were obtained from Chem Service (West Chester, PA).

B. Adsorbents

A sampling train sorbent module was designed to quantitatively collect particulate matter (PM) and different classes of vapor-phase compounds representing a wide range of volatilities. Based on their physical and chemical characteristics, desorption techniques, and feasibility to integrate with the *Salmonella/* mutagenicity assay, Teflon filters, polyurethane foam (PUF) and XAD-4 (XAD) adsorbents were selected for trapping diesel exhaust emissions.

PM was collected on 70 mm diameter Teflon-coated glass fiber filters (T60A20; Pallflex; Putnam, CT). The filters were pre-cleaned by gentle shaking in methanol (3x) and DCM (3x) and dried in a Baker BioChemgard hood. Filters were pre-conditioned for at least 24 hrs in a humidity and temperature monitored room. The filters were weighed before and after diesel exhaust sample

collection using a Cahn microbalance (Cahn Scientific Instruments, Cerritos, CA) located in a humidity and temperature monitored room. The temperature in the room was 22°C (± 2°C) and the relative humidity was 50% (± 10%). The filters were stored in layers of glassine paper which were wrapped in aluminum foil.

Precut PUF plugs were obtained from Apico, Inc. (Baltimore, MD). Each PUF plug was nominally 27 mm in length and 48 mm in diameter. All PUF plugs were pre-cleaned by sequential sonication in methanol (3x) and DCM (3x) and dried in a Baker BioChemgard hood, equipped with HEPA filters. Purified XAD-4 resin was from Alltech (Philadelphia, PA). The XAD was further purified to remove potential background interferences by sequential 15 min sonications in methanol (3x) and dichloromethane (3x). The resin was dried for 5 days under vacuum at 50°C and then stored in solvent-cleaned amber glass jars.

C. Sampling Apparatus

We assembled and tested a larger sampling device than used for our previous vapor-phase sampling work (Hsieh et al., 1993; Kado et al., 1996). The sampling unit was designed to maximize the amount of sample collected from the dynamometer facility, while maintaining the advantages of low volume sampling. The sampling unit is illustrated in Figure 1 and consists of two sampling trains that are connected by a stainless steel "Y" whereby two parallel samples were collected during a single dynamometer run for bioassay and chemical analysis. Each diesel exhaust sampling train consisted of a two Teflon-coated glass fiber filters, a sorbent module containing three PUF plugs and one 40 mL bed of XAD, and a rotometer connected in series as shown in Figure 1. Teflon sorbent modules containing PUF and XAD were from Savillex Corp (Minnetonka, MN). The filters were placed into stainless steel filter holders that were used to collect the PM. Teflon tubing was used to connect both the filter holder assembly to the complete sampling train and to the vacuum pump. This sampling train was designed to efficiently trap PM, semi-volatile, and some volatile compounds.

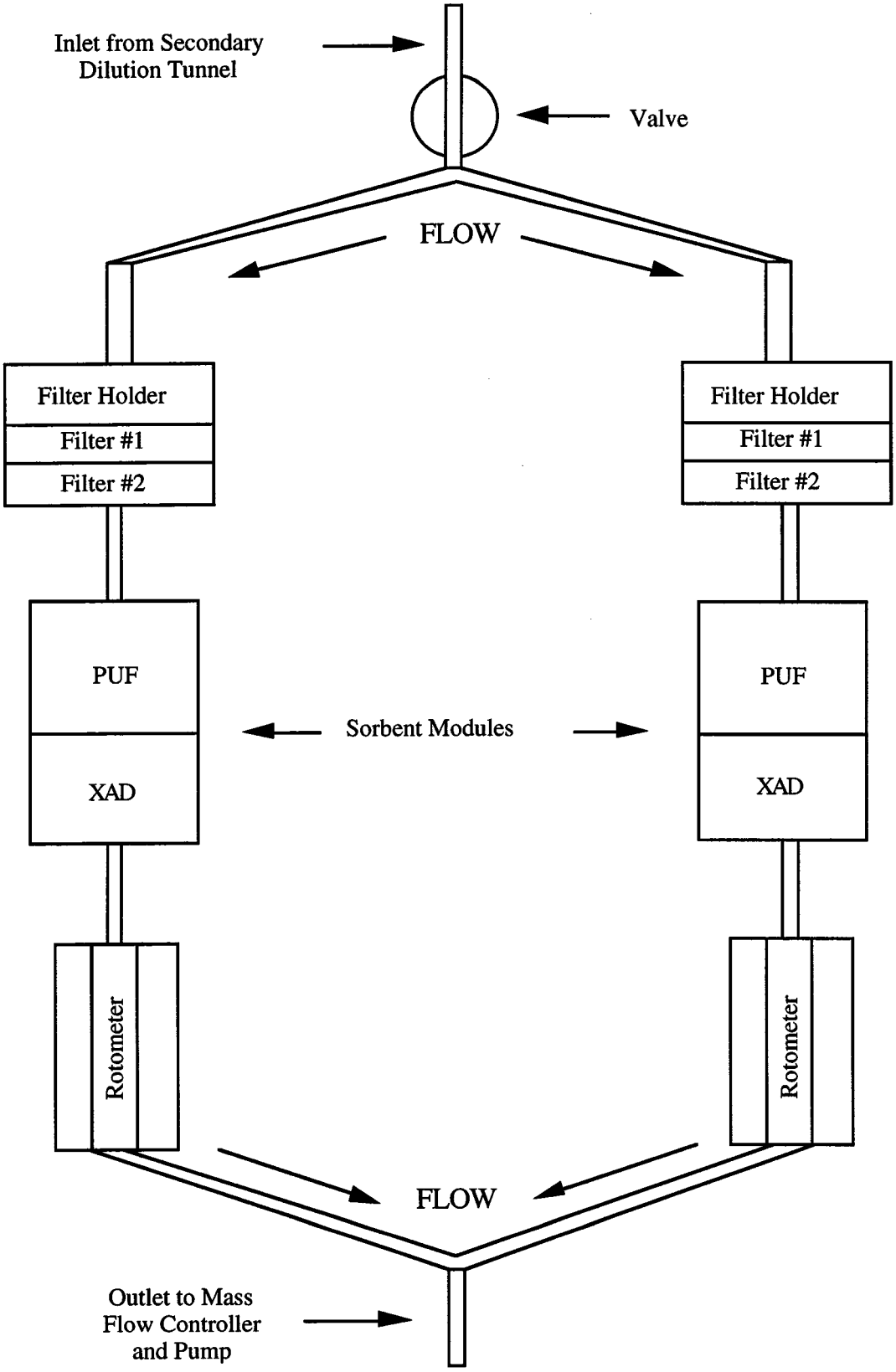


Figure 1. Particulate and Vapor-Phase Sampler.

D. Diesel Exhaust Sampling

In preparation for collecting diesel exhaust samples for the main study, two Pretest experiments were conducted at the Los Angeles County Metropolitan Transit District (LACMTA) dynamometer facility. Pretest #1 was conducted in August, 1994 and consisted of testing the sampling apparatus. A diagram of the diesel exhaust sampling set-up at the LACMTA facility is illustrated in Figure 2.

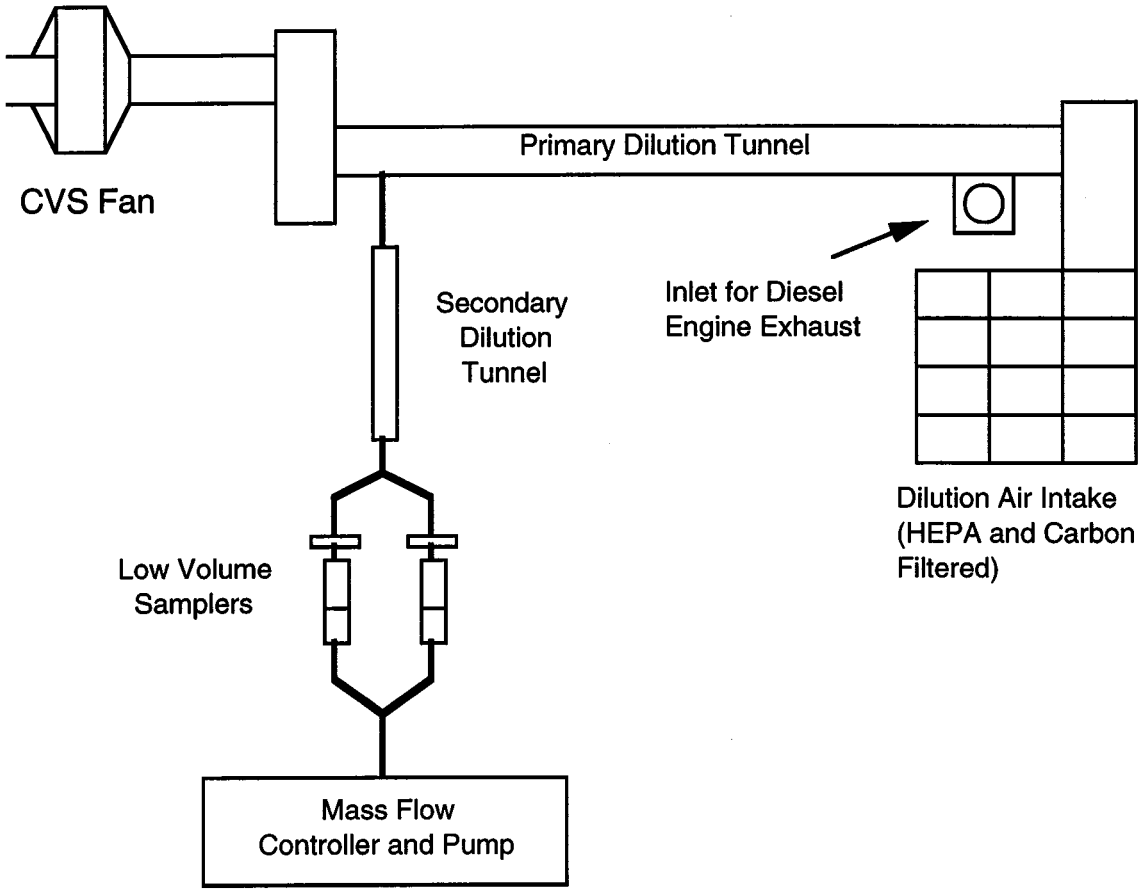


Figure 2. Dilution Tunnel for Heavy-Duty Diesel Exhaust and Sampler at the LACMTA Facility.

Pretest #2 was conducted on September 23, 1994, and consisted of collecting particle and vapor-phase samples from an actual test run of a vehicle on the chassis dynamometer. During this test, a diesel truck was being tested for standard emissions by Acurex Environmental, who

kindly allowed us to collect our samples from the same truck, subsequent to their sampling. Specifications for the diesel engine used in Pretest #2 are detailed in Table 1.

Table 1. Diesel Engine Specifications for Pretest #2.

Engine:	Cummins 6C78.3
Displacement:	8.3 liters
Horsepower:	210 @ 2200 r.p.m.
Transmission:	Allison MT643, 4-speed
Fuel:	Diesel No. 2

The Central Business District (CBD) test cycle was used, consisting of 14 consecutive 0 to 20 mph accelerations evenly spaced between idle speeds. The total cycle time was approximately 10 min and is shown in Fig. 3.

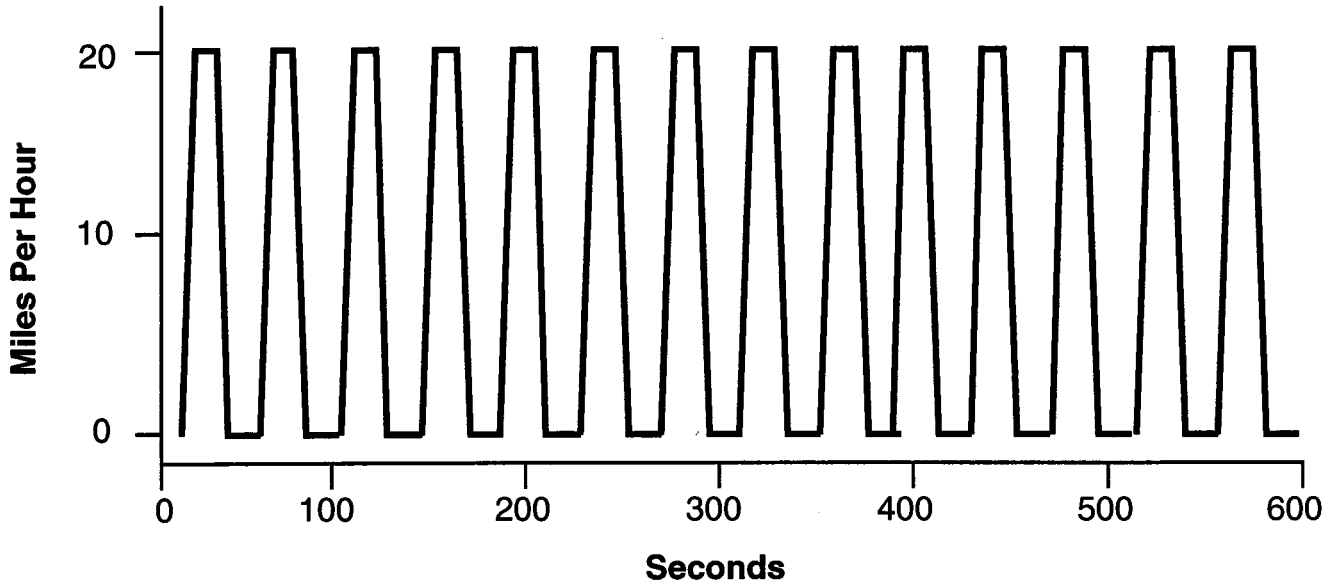


Figure 3. Central Business District (CBD) Cycle.

The Main Study was conducted at the LACMTA facility between February 27 and March 4, 1995. However, for the Main Study, we used an engine mounted at the facility, referred to as an engine dynamometer. A 1988 Detroit Diesel engine was tested with the specifications listed in Table 2.

Table 2. Diesel Engine Specifications for Main Study.

Engine:	1988 Detroit Diesel, Model 6V92 TA DDEC, Unit No. 06V158948
Displacement:	9.05 L
Horsepower:	277 Brake HP @ 2100 rpm
Fuel:	Diesel No. 2: Pre-October 1993 specification or stock MTA fuel

The test cycle used in the Main Study was a transient Federal Test Procedure as described in CFR40, Part 86, Subpart N. The total cycle time was approximately 20 min and is shown in Fig. 4.

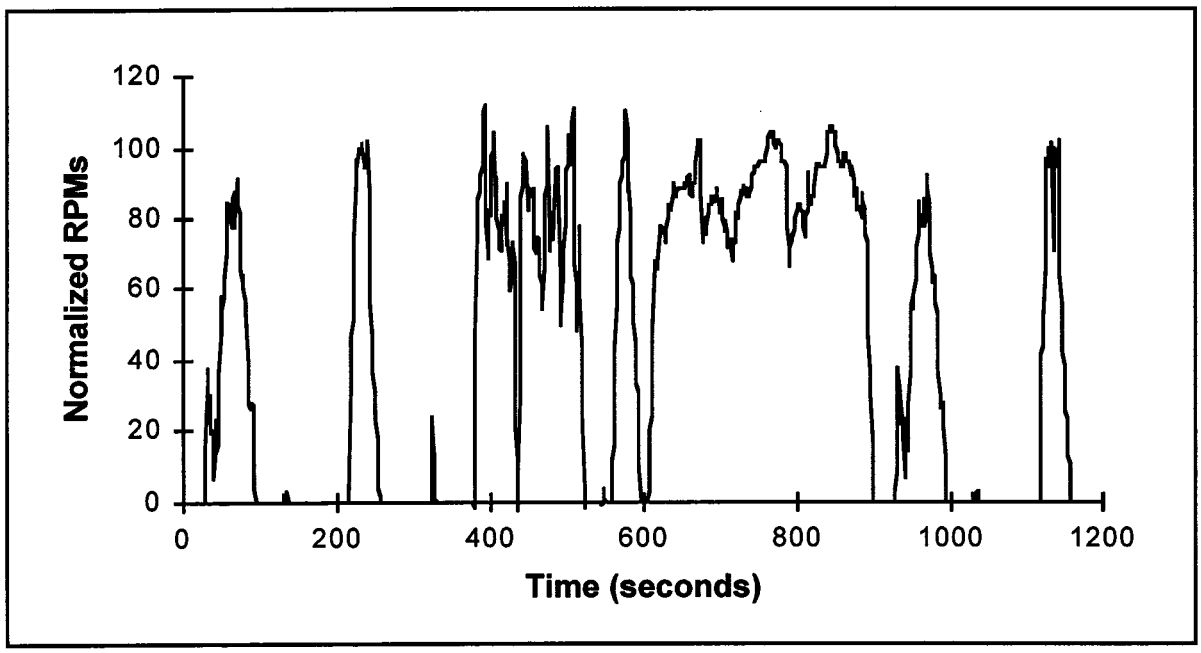


Figure 4. EPA Heavy-Duty Diesel Engine Transient Test Cycle (CFR40, Pt. 86, Subpt. N)

The following fuels were used in the Main Study: 1) a Pre-October 1993 specification fuel (Fuel 1) that was obtained from a petroleum company and 2) a newer fuel (Fuel 2) that was obtained from the stock supply at the LACMTA facility during the Main Study sampling period. The requested fuel specifications are summarized in Table 3.

Table 3. Requested Specifications for Fuel 1 and Fuel 2.

Fuel	Aromatic Content (%)	Sulfur Content (ppm)	Cetane Number
Fuel 1	30	500	43
Fuel 2	10	<500	50

Teflon sorbent modules containing the samples and requisite blanks were wrapped in aluminum foil throughout the sampling period to minimize potential photooxidation. Immediately after each sampling period, the sample and blank modules were stored at 4°C, until they were transported back to the laboratory, where they were stored at 4°C until further processing. Filter samples were stored at -20°C until processed. After the filters and adsorbents were extracted, the extracts were chemically analyzed by GC/MS.

E. Supercritical Fluid and Solvent Extraction

Supercritical fluid extraction (SFE) of the organic compounds present in the PUF and XAD samples was accomplished using liquid carbon dioxide (CO_2) as the extraction solvent at pressures and temperatures above its critical point of 72.9 atm and 31°C. SFE is a technique that is used for the extraction of compounds or complex mixtures from a wide variety of sample matrices. SFE fluids that are used above these critical points have the solvating properties of liquids. Since CO_2 is a gas at atmospheric pressure, this technique produces a concentrated extract without residual solvent. The concentrated extract can be directly analyzed by both GC/MS and the mutagenicity assay.

All supercritical fluid extractions were carried out using an ISCO Model 260D syringe pump (Lincoln, NE), an ISCO SFX2-10 extractor, and SFC/SFE grade CO_2 (Air Products,

Allentown, PA) under a helium headspace of 2000 psi. Prior to sample extractions, the PUF and XAD adsorbents were placed into a 10 mL stainless steel extraction cell. For chemical recovery studies, the adsorbents were spiked with either target or substituted PAHs in DCM. After spiking, the DCM was allowed to evaporate for a period of 10 min., after which approximately 1 mL of methanol was added as a modifier to the top of the adsorbent. For actual samples, approximately 1 mL of methanol was added as a modifier to the top of the adsorbent. Methanol was used as a modifier to increase the amount of organic compounds extracted from the adsorbents. The cell was then closed and placed into the extractor for a 5 min temperature equilibration period. A static extraction (SFE without CO_2 flow into the collection vial) was conducted for 10 min prior to conducting the dynamic extraction (SFE with CO_2 flow into the collection vial). The syringe pump was operated at 5000 psi, 75°C, 90 min for the PUF samples and at 7000 psi, 100°C, 60 min for XAD samples. These conditions were based on low levels of background mutagenicity and chemical contaminants and on chemical recovery studies (see Results). A length of stainless steel capillary tubing was used as a depressurizing flow restrictor to maintain supercritical conditions within the extraction cell. As the supercritical CO_2 exits the restrictor, it expands to a gas upon reaching ambient pressure. The effluent from the outlet of the capillary was directed into a graduated collection vial with a screw top fitted with a Teflon septum. The collection vial contained methanol and was placed into a dry ice-ethanol bath to trap the extracted organic compounds for direct mutagenicity and chemical analyses.

After establishing SFE conditions that were acceptable for both bioassay and chemical analysis, we proceeded to determine the chemical recovery of standard PAHs from the PUF and XAD-4 adsorbents using a standard mixture of 16 target PAHs. These PAH target compounds are presented in Table 4. The compounds listed in Table 4 were also used in the chemical analysis of the PM extracts. For the PUF and XAD, substituted PAHs were also quantitated and these compounds are presented in Table 5.

The diesel exhaust vapor-phase samples consisted of three PUF plugs followed by 40 mL of XAD-4 resin. The PUF and XAD were extracted separately by SFE. Each XAD sample was

spiked with a cocktail of deuterated internal PAH standards and methanol modifier was added. Following SFE, each extract was solvent exchanged with DCM. Two (2) µL of each extract was injected into the GC/MS without further sample preparation.

Table 4. PAH Target Compounds used for SFE Recovery Studies.

Compound	CAS No.	Molecular Wt.	Boiling Pt. (°C)
Naphthalene	91-20-3	128.17	218°
Acenaphthylene	208-96-8	152.20	280°
Acenaphthene	83-32-9	154.10	279°
Fluorene	86-73-7	166.22	298°
Phenanthrene	85-01-8	178.23	340°
Anthracene	120-12-7	178.23	340°
Fluoranthene	206-44-0	202.26	384°
Pyrene	129-00-0	202.26	404°
Benz[a]anthracene	56-55-0	228.28	438°
Chrysene	218-01-9	228.29	448°
Benzo[b]fluoranthene	205-99-2	252.32	---
Benzo[k]fluoranthene	207-08-9	252.32	480°
Benzo[a]pyrene	50-32-8	252.32	495°
Indeno[1,2,3-c,d]pyrene	193-39-5	276.30	---
Dibenz[a,h]anthracene	53-70-3	278.35	524°
Benzo[g,h,i]perylene	191-24-2	276.34	>500°

Table 5. Substituted PAH Target Compounds used for SFE Recovery Studies.

Compound	CAS No.	Molecular Wt.	Boiling Pt. (°C)
1-methylnaphthalene	90-12-0	142.08	243°
2-methylnaphthalene	91-57-6	142.08	242°
1-ethylnaphthalene	1127-76-0	156.09	259°
2-ethylnaphthalene	936-27-5	156.09	258°
1,2-dimethylnaphthalene	573-98-8	156.09	267°
1,3-dimethylnaphthalene	575-41-7	156.09	263°
1,4-dimethylnaphthalene	571-58-4	156.09	264°
1,5-dimethylnaphthalene	571-61-9	156.09	265°
1,8-dimethylnaphthalene	569-41-5	156.09	270°
2,3-dimethylnaphthalene	581-40-8	156.09	268°
2,6-dimethylnaphthalene	581-42-0	156.09	262°
2,7-dimethylnaphthalene	582-16-1	156.09	265°
1-methylfluorene	1730-37-6	180.25	-----
9,10-dihydrophenanthrene	776-35-2	180.25	169°
2-methylanthracene	613-12-7	192.26	-----
1-methylphenanthrene	832-69-9	192.26	358°
9,10-dimethylanthracene	781-43-1	206.29	-----
retene	483-65-8	234.32	390°

F. Chemical Analysis

1. Sample preparation

Prior to solvent extraction, each filter sample was spiked with a deuterated internal standard PAH mixture. Deuterated isotopes for most of the target PAHs were added to compensate for sample losses during sample preparation and changes in instrument response. Each sample was sonicated with DCM four separate times. After each sonication step, the sample was filtered and the four DCM extracts from each sample were combined and concentrated with nitrogen to 0.5 ml. The evaporation tube was rinsed with 0.5 ml of DCM and the rinsate was then combined with the DCM extract. The sample extracts were stored in amber vials until analyzed by GC/MS. A 200 µl aliquot of each sample extract was transferred to an autosampler vial for subsequent analysis by GC/MS.

Prior to SFE, each PUF sample was spiked with 100 µl of a 5 ng/µl deuterated PAH internal standard mixture. Deuterated isotopes for most of the target PAHs were added to compensate for sample losses during sample preparation. Following SFE, the final volume of each extract was adjusted to 2.5 ml. One ml of the SFE extract was removed, diluted with 25 mls of DCM, and then concentrated with nitrogen to 0.5 ml. One hundred (100) µl of a 5 ng/µl substituted PAH internal standard was added to each evaporation tube and the contents of each tube was transferred to a precleaned 2 ml amber vial, along with a 0.4 ml DCM wash of the evaporation tube. Samples from this concentrate were then injected into the GC/MS.

For the SFE XAD samples, 200 µl of the extract was removed and DCM and internal standards were added to make a final volume of 600 µl. Substituted PAHs and PAH internal standard final concentrations were adjusted to 0.5 ng/µl and 0.2 ng/µl, respectively. The samples were then injected and analyzed by GC/MS run in selective ion monitoring (SIM) mode.

2. Instrumental conditions and column selection

The PM and SFE extracts were analyzed using a Hewlett-Packard Model 5890 Series II Gas Chromatograph (GC) equipped with a Model 8290 autosampler interfaced to a Hewlett-Packard Model 5970A quadrupole mass selective detector (MSD). The GC was equipped with a split/splitless injector and an electronic pressure controller. The injector was run in the splitless mode and the electronic pressure controller was programmed for vacuum compensation and constant flow mode. For analysis of PM extracts, the GC was equipped with a 30 m x 0.25 mm i.d. DB-5 fused silica capillary column (0.25 µm film thickness; J&W Scientific). For analysis of SFE extracts, the GC was equipped with a 50 m x 0.20 mm i.d. HP-5 fused silica capillary column (0.5 µm film thickness; Hewlett-Packard). The MSD was run using both the selective ion monitoring (SIM) and electron impact (EI) modes.

A number of bonded methyl phenyl silicone columns were evaluated to achieve optimum separation of the numerous PAHs and substituted PAHs present in the PUF and XAD extracts. This type of column phase material generally results in good separation of moderately polar compounds. The HP-5 column had the best separation of compounds of the columns evaluated. The final instrument conditions are summarized in Table 6.

Table 6. GC/MS Conditions for Analysis of Particulate, PUF and XAD-4 Extracts.

Condition	Particulate Matter	PUF and XAD-4
Carrier gas / velocity	He; 35 cm/sec	He, 28 cm/sec
Temperature program	40°C hold for 4 min 10°C/min to 270°C 5 min hold @ 270°C 10°C/min to 305°C	40°C hold for 10 min 5°C/min to 270°C 5 min hold @ 270°C 10°C/min to 300°C 10 min hold @ 300°C

(cont'd)

Table 6 (cont'd). GC/MS Conditions for Analysis of Particulate, PUF and XAD-4 Extracts.

Condition	Particulate Matter	PUF and XAD-4
Injector temperature	295°C	295°C
Detector temperature	285°C	285°C
Solvent delay	6 min	6 min
Run time	38.5 min	80 min
Scan mode	SIM	SIM

The diesel particulate filter, PUF and XAD sample extracts and fractions were analyzed for PAHs by high resolution GC/MS. The isotope dilution method improves the quantitation by compensating for sample losses during the sample preparation step, since nearly each target PAH will have its own standard. The target and substituted PAHs that were quantitated are listed in Table 7. This table includes all of the C_1- and most of the C_2-substituted isomers of naphthalenes, as well as other mono-methylated vapor-phase PAHs. Other related compounds, such as retene, 9,10-dihydrophenanthrene, and 9,10-dimethylanthracene were also quantitated. Since deuterated isotopes were not commercially available for the substituted PAHs, an internal standard method of analysis was used.

Table 7. List of Analytes and Internal Standards for Vapor-Phase PAH Analysis by GC/MS.

Compound No.	Compound	Quantitation ion	Retention time (min)
1	naphthalene-d_8*	136	20.31
2	naphthalene	128	20.36
3	methylnaphthalene-d_{10}*	152	22.64
4	2-methylnaphthalene	142	22.36
5	1-methylnaphthalene	142	22.74
6	1- & 2-ethylnaphthalene	141	24.29
7	2,6- & 2,7-dimethylnaphthalene	141	24.41
8	1,3-dimethylnaphthalene	156	24.53
9	1,4-dimethylnaphthalene	156	24.88
10	2,3-dimethylnaphthalene	156	25.34
11	1,2-dimethylnaphthalene	156	25.42
12	1,5-dimethylnaphthalene	156	25.67
13	1,8-dimethylnaphthalene	156	26.14
14	acenaphthene-d_{10}*	160	25.64
15	acenaphthylene	153	26.40
16	acenaphthene	152	25.67
17	fluorene-d_{10}*	176	28.38
18	fluorene	166	28.50
19	anthracene-d_{10}*	188	32.95
20	9,10-dihydrophenanthrene	180	30.84
21	1-methylfluorene	180	31.10
22	2-methylanthracene	192	35.43
23	1-methylphenanthrene	192	35.78
24	9-methylphenanthrene	192	36.49
25	9,10-dimethylanthracene	206	39.87
26	retene	234	41.38
27	phenanthrene-d_{10}*	188	32.73
28	phenanthrene	178	32.83
29	anthracene	178	33.03
30	fluoranthene-d_{10}*	212	38.44
31	fluoranthene	202	38.52
32	pyrene-d_{10}*	212	39.65
33	pyrene	202	39.65
34	chrysene-d_{12}*	240	46.22
35	benz[a]anthracene	228	46.12
36	chrysene	228	46.34
37	benzo[b]fluoranthene-d_{12}*	264	52.93
38	benzo[b]fluoranthene	252	53.10
39	benzo[k]fluoranthene-d_{12}*	264	53.14
40	benzo[k]fluoranthene	252	53.28
41	benzo[e]pyrene-d_{12}*	264	55.14
42	perylene-d_{12}*	264	56.32
43	benzo[e]pyrene	252	55.34
44	benzo[a]pyrene	252	55.79
45	perylene	252	56.53

* internal standard
** not eluted from 50m HP-5 column

3. Isotope Dilution for PAH Analysis

For the development of an isotope dilution method for the trace analysis of PAHs in the diesel emissions, a standard reference material for diesel particulate matter (NIST 1650) was used. The NIST 1650 reference particulate matter was obtained from the heat exchangers of a dilution tube facility, following 200 engine hr of particle accumulation. Several direct injection four-cycle diesel engines were used to generate this particulate material, operating under a variety of conditions.

NIST reports both certified and non-certified concentrations of PAHs. The certified values represent the results from at least two different analytical procedures. The non-certified values are not based on two different analytical methods and are reported for informational purposes only. Chemical analyses were performed by GC/MS in both electron impact and negative ion chemical ionization modes. HPLC analysis was performed using wavelength programmed fluorescence detection. The target PAH analytes and the corresponding deuterated internal standards are listed in Table 8, along with the target and qualifier ions and retention times that were used to identify and quantitate the PAHs.

Table 8. List of PAHs, Internal Standards, Target and Qualifier Ions, and Retention Times for Chemical Analysis of the Particulate Extracts and SPE Fractions by GC/MS.

Compound[a]	Target and Qualifier Ions	Retention Time (min)
Naphthalene-d_8	136, 68	13.03
Naphthalene	128, 129, 127	13.56
Acenaphthene-d_{10}	162, 164, 160	17.19
Acenaphthylene	152, 153, 151	16.83
Acenaphthene	153, 154, 152	17.26
Fluorene-d_{10}	176, 174, 177	18.44
Fluorene	166, 165, 167	18.50
Phenanthrene-d_{10}	188, 94, 90	20.71
Phenanthrene	178, 179, 177	20.76
Anthracene-d_{10}	188, 187, 97	20.83
Anthracene	178, 177, 179	20.89
Fluoranthene-d_{10}	212, 106	23.53
Fluoranthene	202, 203	23.57
Pyrene-d_{10}	212, 106	24.05
Pyrene	202, 200	24.09
Chrysene-d_{12}	240, 120, 236	26.98
Benz[a]anthracene	228, 229, 227	26.97
Chrysene	228, 229, 227	27.04
Benzo[b]fluoranthene-d_{12}	264, 132	20.91
Benzo[b]fluoranthene	252, 253, 126	30.00
Benzo[k]fluoranthene-d_{12}	264, 132	30.02
Benzo[k]fluoranthene	252, 253, 126	30.09
Benzo[a]pyrene-d_{12}	264, 132	31.09
Benzo[e]pyrene	252, 132	31.09
Benzo[a]pyrene	252, 253, 126	31.18
Perylene-d_{12}	264, 265, 260	31.43
Perylene	252, 126	31.52
Dibenz[a,h]anthracene-d_{14}	292, 293	35.27
Indeno[1,2,3-c,d]pyrene	276, 275, 138	35.21
Dibenz[a,h]anthracene	278, 279, 139	35.36
Benzo[g,h,i]perylene-d_{12}	288, 144	35.83
Benzo[g,h,i]perylene	276, 275, 138	35.89

[a] Underlined compound indicates internal standard.

The certified values, non-certified values and the values obtained from each method of analysis are listed in Table 9. NIST recommends that a minimum of 50 mg of the reference material should be used for chemical analysis. However, our filter samples from the Main Study had approximately 4-5 mg of particulate matter per filter. To more closely represent the mass collected from our filter samples, we analyzed 1 and 10 mg of the reference material. The results for these two analyses are listed in Table 9.

Table 9. Reported Concentration of PAHs in NIST 1650 Standard Reference Material.

	Amount Reported by Analytical Technique (µg / g particulate matter)				
Compound	Certified	Non-certified	GC/MS EI	GC/MS NICI	HPLC Fluorescence
Benz[a]anthracene	6.5		6		7.1
Benzo[a]pyrene	1.2		1.3	0.9	1.4
Benzo[g,h,i]perylene	2.4		2.3	2.6	2.4
Fluoranthene	51		48.5		49.8
Pyrene	48		49		45.5
Benzo[e]pyrene		9.6	9.6		
Benzo[k]fluoranthene		2.1			2.1
Chrysene		22			22
Indeno[1,2,3-c,d]pyrene		2.3	1.8	2.1	3.2
Perylene		0.13			0.13
Phenanthrene		71	79		63

EI = Electron Impact Mode
NICI = Negative Ion Chemical Ionization
HPLC = High Performance Liquid Chromatography
Certified = Average of results from two or more methods.
Non-certified = Results from one method.

The data shown in Table 10 indicates that the PAH values from the 10 mg extracts more closely match the NIST values than the 1 mg extracts. For the PAHs present in higher amounts (more than 20 µg / g), both the 1 and 10 mg extracts were comparable to the NIST values. For the 10 mg samples, benzo[g,h,i]perylene and indeno[1,2,3-c,d]pyrene were

significantly different from the certified and non-certified NIST values. The value obtained for indeno[1,2,3-c,d]pyrene for the 1 mg sample was comparable to that reported for NIST HPLC fluorescence. It should be noted that these differences may be due to the fact that the PAHs were at concentrations below the concentration of the lowest calibration standard. It is also possible that there was an inferring compound resulting from the different analytical procedure that was used by NIST. Also, the smaller sample size could have affected the representativeness of the sample.

Table 10. Comparison of NIST Reported PAH Concentrations with Current UC Davis (UCD) Experimental Results.

	GC/MS Analysis (µg / g particulate matter)					
Compound	Certified	Non-certified	10 mg Sample[a] (UCD)	% Recov.[b]	1 mg Sample[c] (UCD)	% Recov.[b]
Benz[a]anthracene	6.5		7	109	11	175
Benzo[a]pyrene	1.2		1	100	1	112
Benzo[g,h,i]perylene	2.4		5	225	8	332
Fluoranthene	51		46	90	42	83
Pyrene	48		44	92	44	92
Benzo[e]pyrene		9.6	9	90	7	69
Benzo[k]fluoranthene		2.1	2	100	ND	
Chrysene		22	22	100	21	95
Indeno[1,2,3-c,d]pyrene		2.3		165 (119)[d]	3	91
Perylene		0.13	ND		ND	
Phenanthrene		71	57	80 (90)[d]	57	80

[a] Value represents the average of three separate recovery experiments.
[b] Percent recovery calculated using NIST certified and non-certified values.
[c] Represents the value of two 1 mg samples that were pooled.
[d] Percent recovery calculated using NIST HPLC analysis values.

The values obtained for benzo[a]pyrene matched well with the NIST values even for the 1 mg sample which contained approximately 3 pg/µl, a level well below the lowest

calibration standard of 100 pg/µl. Seven of the PAH values from the 10 mg sample were within 10% of the NIST certified and non-certified values and eight of these values were within 20% of NIST certified values.

Because of the small amount of particulate matter that is typically collected on many filter samples, the PAHs are typically present at very low levels. This poses a problem for GC/MS analysis since the data from the NIST samples clearly shows better results when 10 mg of sample are extracted, compared with 1 mg. Since most diesel emission filter samples typically contain less than 10 mg of particulate matter, two separate filter sample extracts must be pooled for GC/MS analysis.

4. Solid Phase Extraction (SPE) Fractionation of SFE Extracts

To facilitate chemical identification, solid phase extraction (SPE) of the particulate and PUF extracts was performed. SPE is a chromatographic technique in which a liquid extract is fractionated into different chemical classes using a column containing a packed bed of sorbent material. Small volumes of solvent are typically used to elute the compound of interest or to fractionate the original extract. The particulate and PUF extracts from diesel exhaust samples collected using identical fuels were pooled and fractionated by SPE. Briefly, 0.5 ml was removed from each of three particulate extracts (HO5, HO6, and H07) and were pooled. Similarly, 0.5ml was removed from particulate extracts H12, H15, and H17 and pooled. The pooled samples were further concentrated with nitrogen. For the PUF extracts, 0.5 ml was removed from each of three extracts (HO4, HO6 and HO9) and pooled. Similarly, 0.5ml was removed from PUF extracts H12, H14, and H15 and pooled. The pooled samples were solvent exchanged into DCM and were further concentrated with nitrogen. From these pooled and concentrated samples, 0.5 ml was removed and fractionated by SPE using Waters C-18 SepPak cartridges (Milford, MA). Sample extracts C08, C13, and T19 were also fractionated by SPE and concentrated. The cartridges were eluted with 1.5 ml of methanol, followed by 1 ml of acetonitrile:methanol (50:50), 1 ml acetonitrile, 1 ml DCM and 2 ml of hexane. The fractions were then tested in the bioassay and were chemically analyzed by GC/MS.

G. Bioassay

Bioassay experiments were conducted to determine the specific mutagenic activity of the SFE extracts. For these samples, the specific mutagenic activity is reported as the number of revertants per microliter of sample extract collected by SFE. The specific mutagenic activity is determined from the slope obtained from the linear portion of the dose-response curve. A pilot experiment was conducted to determine the most sensitive tester strain. All vapor-phase extracts were then tested for mutagenicity using the microsuspension bioassay procedure (Kado et al, 1983) with tester strain TA100. For the PUF extracts, 5-, 10-, and 20-fold concentrates of each vapor-phase extract were tested. The concentrates were prepared by solvent exchanging the raw extract into dimethylsulfoxide (DMSO).

For the microsuspension procedure, bacteria were grown overnight in Oxoid Nutrient Broth No. 2 (Oxoid Ltd., Hants, England) to approximately 1 - 2 x 10^9 cells/ml and harvested by centrifugation (5,000 x g, 4°C, 10 min). Cells were resuspended in ice-cold phosphate-buffered saline (0.15M phosphate-buffered saline, pH 7.4) to a concentration of approximately 1 x 10^{10} cells/ml. The S9 (metabolic enzymes) and S9 mix (enzyme co-factors) were prepared and 300 μg S9/ml final concentration was used. The S9 from Aroclor 1254 pretreated male Sprague-Dawley rats was obtained from Molecular Toxicology, Inc. (Annapolis, MD.) and contained 39.2 mg protein/ml. For the microsuspension assay, the following ingredients were added, in order, to a 12 x 75 mm sterile glass culture tubes kept on ice: 0.1 mL S9 mix, 0.005 mL sample in dimethyl-sulfoxide, and 0.1 mL concentrated bacteria in PBS (1 x 10^{10} / mL phosphate-buffered saline). The mixture was incubated in the dark at 37°C with rapid shaking. After 90 min, the tubes were placed in an ice bath and taken out one at a time immediately before adding 2 ml molten top agar containing 90 nanomoles of histidine and biotin. The combined solutions were vortex-mixed and poured onto minimal glucose plates. Plates were incubated at 37°C in the dark for 48 hrs and counted using an automatic plate counter. Benzo[a]pyrene was used as a positive control. Strain markers were routinely determined for each experiment.

H. Quality Assurance and Control

The collection and storage of samples followed specifically defined procedures. All testing conditions for the Main Study were recorded on Field Data Sheets. For each sampling run, we recorded the date, time, type of sample (bioassay or chemical analysis), flow rate, temperature, and pressure of the sampling system. The parameters for each cycle were recorded. All samples were identified in a Master Log that was used throughout the main study. The Master Log contained sample ID's, sample type, and date of collection. Finally, all samples were tracked from collection to analyses using Chain of Custody forms. The Chain of Custody contained samples ID's, sample type, dates of transfer and acceptance, and the initials of personnel who transferred and accepted the samples.

For the sampling and chemical analysis, adsorbent and solvent blanks were analyzed along with the actual samples to determine possible background interferences. Field samples were stored on blue ice during transport to the laboratory where they were stored at 4°C before sample workup and extraction. All SFE extracts were chemically analyzed immediately following extraction or stored at -20°C until sample analysis and mutagenicity testing could be completed.

Prior to sample analysis, the MSD was manually tuned using perfluorotributylamine. The MSD was optimized for SIM analysis for PAHs by first injecting a reagent blank into the GC/MS to determine background contamination levels. If the background levels were acceptable, then a multi-point calibration curve was established by injecting 5 standards at 5 different concentrations. The actual samples were analyzed after the analysis of the calibration standards was completed. After every ten (10) samples, a calibration standard check sample was run to ensure that the instrument was still properly calibrated. If the target compounds were found at levels above the calibration curve, the samples were diluted and analyzed again. Duplicate analysis was performed for each sample.

All mutagenicity assays incorporated both positive and negative controls as well as filter and adsorbent blanks. All chemical and bioassay procedures were carried out in a room fitted with yellow fluorescent lights (G.E. F40Go) to minimize potential photooxidation of chemicals.

IV. RESULTS

A. Diesel Exhaust Sampling - Dynamometer Pretest Studies

1. Pretest #1

a. Collection of particulate matter and vapor-phase mutagens

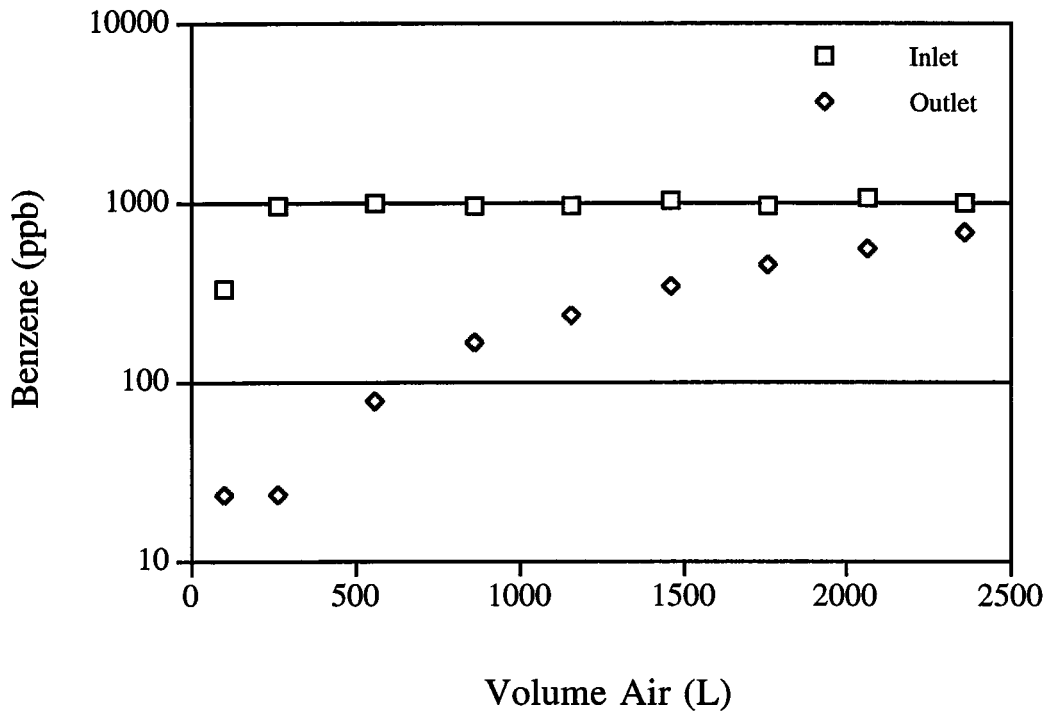
In preparation for collecting diesel exhaust samples for the main portion of the study, two Pretest experiments were conducted at the LACMTA facility, to acquire adequate sample mass for chemical and bioassay analyses, we assembled and tested a larger sampling device than that used for our previous vapor-phase sampling experiments (Hsieh et al., 1993; Kado et al., 1996).

The sampling unit was designed to maximize the amount of sample collected, while maintaining the advantage of low volume sampling to efficiently trap particulate matter (PM), semi-volatile, and volatile components. Low volume sampling is defined as sampling at less than approximately 40 LPM, with minimal loss of semivolatile compounds from the PM during sample collection. Prior to testing the sampling unit at the dynamometer facility, the sampling train was tested for leaks and its trapping efficiency was determined. The trapping efficiency experiments consisted of measuring the breakthrough of benzene, a highly volatile and model toxic compound.

Breakthrough refers to a compound that is no longer being efficiently collected by the trapping medium (in our case filter, PUF, and XAD-4). More specifically, breakthrough is measured as the volume of gas that is sampled before a specified percentage of the compound begins to pass through the sampler without being trapped. To determine breakthrough for our sampler, an experiment was conducted using a dilution manifold interfaced to the inlet of the sampler. Mass flow controllers regulated the flow rates of benzene and air entering the manifold. A manifold was used to mix zero-grade compressed air with a 770 ppm benzene compressed gas standard. The benzene was diluted to 1 ppm in the manifold and then directed into the sampler. The flow rate through the sampler was 20 LPM. A Photovac 10S70 gas chromatograph was used to measure the concentration of benzene at the sampler inlet and outlet. A comparison of the concentration of benzene at the inlet and the outlet of sampler versus the volume of air sampled is

illustrated in Figure 5. Approximately 50% of the benzene broke through after approximately 1700 L of the gas containing benzene flowed through the sampler. About 10% of the benzene broke through when approximately 750 L of gas was sampled. This 1700 L of gas is more volume than the volume of exhaust anticipated to flow through the sampler during a typical dynamometer cycle, or almost equivalent to a combination of two consecutive cycles.

Figure 5. Benzene Breakthrough for XAD.



Many of the toxic compounds to be trapped have considerably lower vapor pressures than benzene and therefore would have significantly higher breakthrough volumes. The breakthrough volume of benzene provides a lower limit volume of gas that can be sampled before the sorbent module should be changed. The sampling system, as designed, appeared to be efficient for collecting the semi-volatile compounds anticipated to be present in the vapor-phase of diesel engine exhaust.

Prior to conducting Pretest #1, the sampler was field tested at the LACMTA facility. The various connections of the sampler to the secondary dilution tunnel were established, the sampler was tested for leaks, and a background sample, or tunnel blank, was collected. For the tunnel blank sample, the exhaust pipe from the diesel engine was not attached, and only filtered air flowed through the tunnel. There were no leaks detected and no measurable amounts of PM were collected. This indicated that little or no ambient- or tunnel-associated PM would contaminate a sample under these conditions. Two Pretests were then conducted to collect samples to determine sampling conditions for the Main Study.

Pretest #1 was conducted in August, 1994 on a chassis dynamometer and consisted of collecting filter samples containing diesel exhaust PM from a heavy-duty diesel vehicle. The objective of this test was to acquire preliminary estimates of the particulate-associated mutagenicity. The run followed a Central Business District Cycle (CBD) previously described. Filter samples were collected from chassis dynamometer runs of a diesel bus (Test # 762 and #763). A summary of the particle mass collected as well as co-pollutants measured during the cycle is presented in Table 11 for the two cycles tested. Blank filters were also tested. The emissions of hydrocarbons, carbon monoxide, nitrogen oxides, and carbon dioxide were essentially equivalent between the two cycles measured. The amount of PM emitted was slightly higher in the first cycle by approximately 0.04 grams per mile.

Table 11. Summary of Pollutant Data for Pretest #1.
(Two CBD Cycles)

	Direct Emission Measurements of Regulated Pollutants			
Sample ID.	HC (ppm)	CO (ppm)	NOx (ppm)	CO2 (%)
762	9.82	35.56	46.27	0.37
763	9.78	34.54	46.22	0.37
	HC (gm)	CO (gm)	NOx (gm)	CO2 (gm)
762	4.58	33.00	62.01	5442.53
763	4.56	32.01	61.63	5422.60
	HC (gm/mile)	CO (gm/mile)	NOx (gm/mile)	CO2 (gm/mile)
762	2.18	15.67	29.45	2584.41
763	2.18	15.27	29.41	2587.85
	Particulate Emissions		Fuel Economy	
	gm	gm/mile	MPG	
762	1.72	0.82	3.89	
763	1.63	0.78	3.89	

b. Extraction and mutagenicity measurements

The filter samples from Pretest #1 were extracted with DCM by shaking and sonication. The extracts were then tested in the bioassay. For the bioassay, four doses were prepared from each sample extract, ranging in concentration from 1.25 to 50 μg particle equivalents/μl. Based on our previous work, diesel particulate mutagenic activity was approximately 10 revertants per μg equivalent of particles. For the bioassay, we needed to test approximately 50 μg per tube to produce 500 revertants. Particulate equivalent refers to the amount of material extracted equal to the amount of particulate indicated. The tester strain previously used

for the vapor-phase mutagenicity testing was TA100. However, tester strain TA98 has been the primary one reported in the literature (Schuetzle, 1983).

The slope of the dose-response curve for each test sample in either tester strain was approximately equivalent for the two experimental runs. The slope of the dose-response curve is the specific mutagenic activity of a sample and is reported as the number of revertants per microgram of PM. The specific mutagenic activity of the diesel PM collected in these experiments was approximately 6 revertants/μg PM. This level is comparable to the specific activities we previously observed for a heavy-duty diesel truck. Tester strain TA98 was more sensitive than TA100 for these particle extracts. This is consistent with published reports and is a reflection on the compounds present in the diesel particle extract.

Chemical characterization of mutagenic compounds in the particle- and vapor-phase of heavy-duty diesel exhaust required an analytical method that was both sensitive and precise. A GC/MS method was set up to quantitate PAHs in the complex mixture of diesel exhaust. The following PAH standards were prepared and analyzed using gas chromatography/mass spectrometry (GC/MS): naphthalene, acenapthene, biphenyl, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-c,d]pyrene, dibenz[a,h]anthracene, and benzo[g,h,i]perylene. Since the objective of Pretest #1 was to acquire preliminary estimates of the particulate associated mutagenicity, no chemical analyses were performed on these filter extracts. A second pretest was initiated with the goal of collecting sample for chemical analyses.

2. Pretest #2

a. Collection of vapor-phase mutagens and particulate matter

Pretest #2 was conducted on September 23, 1994 using the chassis dynamometer to further test the sampling system and to collect adequate sample for chemical analyses. Diesel exhaust PM and vapor-phase compounds were collected and extracted as previously reported. The sample was acquired during sampling time kindly provided by Acurex Environmental, subsequent to their sample collection. The vehicle tested was a diesel hauler equipped with a Cummins 8.3 L engine (see Material & Methods for specifications). The dynamometer runs followed the Central Business District (CBD) driving cycle, as previously described. Four CBD cycles were run using our sampling system in-line. The standard gaseous pollutants measured during those runs are summarized in Table 9. Blank filters were also tested.

b. Extraction and chemical analyses

The filter samples were extracted with DCM by shaking and sonication. In preliminary chemical analysis, the sample extracts from the exhaust were found to contain a number of PAHs and characteristic long-chain hydrocarbons. Sixteen (16) PAHs both in the particle- and vapor-phase have been quantitated. The information gathered from these two Pretests allowed for the optimization of the sampling train system.

3. Summary

A sampling train consisting of a Teflon filter followed by sections of PUF and XAD was designed and assembled to maximize the amount of sample collected and to maintain the advantages of low volume sampling to efficiently trap PM, semi-volatile, and some volatile components. The sampling unit was leak tested and its trapping efficiency determined by measuring the breakthrough of benzene. The sampler was field tested at the LACMTA facility to evaluate sampler connections to the dilution tunnel system and to collect a background sample. There were no leaks detected and no measurable amounts of particulate matter were collected for the background, or tunnel blank sample.

Pretest #1 consisted of collecting filter samples containing diesel exhaust PM from a heavy-duty diesel vehicle on the chassis dynamometer, following a CBD driving cycle. Filter samples were collected to acquire preliminary estimates of the particulate associated mutagenicity. Blank filters were also tested. The specific mutagenic activity of the diesel PM collected in these experiments was approximately 6 revertants/μg equivalent of PM, which was comparable to the specific activities we previously observed for a heavy-duty diesel truck.

Pretest #2 was conducted using the chassis dynamometer to further test the sampling system. Diesel exhaust PM and vapor-phase compounds were collected from a heavy-duty diesel vehicle and also followed the CBD driving cycle. Standard gaseous pollutants, such as hydrocarbons (HC), carbon monoxide (CO), nitrogen oxides (NOx), and carbon dioxide (CO_2) were measured, as well as the emission rate of PM, as shown in Table 12. Blank filters were also tested. The compounds adsorbed onto PUF and XAD were extracted by SFE and preliminary GC/MS analyses and bioassay experiments were conducted.

B. Diesel Exhaust Sampling - Dynamometer Main Study

Prior to beginning the sampling for the Main Study, which focused on emissions from an engine dynamometer, we prepared a diesel sampling test protocol dedicated for the engine dynamometer. This protocol was circulated for comments, finalized, and submitted to LACMTA. During the week of February 27 to March 3, 1995, we used our sampling system to collect diesel exhaust PM and vapor-phase compounds from a heavy-duty diesel engine at the LACMTA facility.

Table 12. Summary of Pollutant Data for Four CBD Cycles for Pretest #2.

	Direct Emission Measurements of Regulated Pollutants			
Sample ID.	HC (ppm)	CO (ppm)	NOx (ppm)	CO2 (%)
1242	7.27	12.21	38.98	0.44
1243	7.32	11.76	38.68	0.44
1244	7.54	11.88	38.34	0.44
1245	7.75	10.97	38.43	0.43
	HC (gm)	CO (gm)	NOx (gm)	CO2 (gm)
1242	3.45	11.26	60.18	6416.69
1243	3.45	10.83	59.86	6397.10
1244	3.56	10.97	59.66	6309.07
1245	3.63	10.15	59.38	6244.28
	HC (gm/mile)	CO (gm/mile)	NOx (gm/mile)	CO2 (gm/mile)
1242	1.68	5.50	29.39	3134.01
1243	1.67	5.25	29.02	3101.44
1244	1.73	5.34	29.01	3067.34
1245	1.77	4.94	28.90	3038.83
	Particulate Emissions		Fuel Economy	
	gm	gm/mile	MPG	
1242	3.13	1.53	3.23	
1243	2.82	1.37	3.27	
1244	2.81	1.37	3.30	
1245	2.81	1.37	3.33	

The diesel sampling test cycles for the Main Study followed a transient Federal Test Procedure (FTP) for engine dynamometers, as previously described. Each run was transient and approximately 20 min in duration. As previously mentioned, a 1988 Detroit Diesel engine was

used for the Main Study and the following fuels were tested: 1) a Pre-October 1993 specification fuel (Fuel 1) that was obtained from a petroleum company and 2) a newer fuel (Fuel 2) that was obtained from the stock supply at the LACMTA facility during the Main Study sampling period.

1. Fuel analysis

Actual fuel samples were obtained before and after the completion of tests for each fuel type. The fuel samples were analyzed for sulfur, aromaticity, and polycyclic nuclear hydrocarbons (PNAs), by the ARB El Monte Laboratory. These results are presented in Table 13. The aromatic content of both fuels was similar. However, Fuel 1 had higher levels of PNAs and sulfur. The mass % of PNAs was greater in Fuel 1 than in Fuel 2. The amount of sulfur in Fuel 1 was almost triple that for Fuel 2.

Table 13. Chemical Analyses of Fuel 1 and Fuel 2.

Fuel	Condition*	Total Aromatics (vol%)	Total Aromatics (mass%)	PNA (mass%)	Sulfur (ppm)
Fuel 1	before	28.89	30.08	7.30	440.5
	after	28.90	30.15	7.39	427.1
Fuel 2	before	27.77	28.86	4.67	153.7
	after	27.83	28.93	4.65	156.1

* before = before start of first run of series of cycles.
after = after last run of all cycles for a specific fuel had been completed.

2. Collection of particulate matter and vapor-phase mutagens

Unfortunately, upon starting up for the test on Monday, February 27, 1995, a serious power outage occurred resulting in damage to the computer controlling the engine dynamometer. The problem was diagnosed, solved, and actual testing began on Thursday, March 2, 1995. The

MTA personnel generously agreed to stay through Saturday, March 4, 1995 to complete our testing. Fuel 2 was tested first for approximately 10 FTP cycles, followed by Fuel 1. The total number of individual samples collected is summarized in Table 14, according to fuel type and method of sample collection.

Table 14. Total Number of Diesel Particulate and Vapor-Phase Samples Collected from the LACMTA Engine Dynamometer Dilution Tunnel Facility.

	Number of Samples Collected			
Fuel Type	Primary Filter (70 mm dia.)	Secondary Filter (70 mm dia.)	PUF	XAD
Fuel 1	22	22	22	22
Fuel 2	24	24	24	24
Trip / Field Blanks	10	10	10	10

The regulated gaseous pollutants that were measured, such as hydrocarbons (HC), carbon monoxide (CO), nitrogen oxides (NOx), and carbon dioxide (CO_2) are shown in Table 15. The data for the mass of diesel PM collected for the respective fuels is summarized in Table 16. The mass per filter as well as mass per brake horsepower-hr is presented. The average ($\pm$ SD) particle mass emission from Fuel 1 hot cycles was 0.319 ± 0.017 g/hp-hr, while the average particle mass emissions from Fuel 2 was 0.277 ± 0.007 g/hp-hr. The average particle emission from the Fuel 1 was approximately 13% greater than from Fuel 2. The emissions from the cold start cycles are consistent with the difference in the hot start cycles. The particle emission from Fuel 1 (cold start) was 0.340 g/hp-hr and the emission from the Fuel 2 (cold start) was 0.300 g/hp-hr, an approximate 12% difference.

Table 15. Measurement of Regulated Pollutant Summaries for Main Study Samples.

Sample ID	Fuel Type	Cycle/ Type	Regulated Pollutant Emissions[a] (g/hp-hr)		
			Nitrogen Oxides (NO_x)	Carbon Monoxide (CO)	Carbon Dioxide (CO_2)
H12	Fuel 1	FTP/Hot	7.05	1.66	738.60
C13	Fuel 1	FTP/Cold/Single [b]	7.36	1.99	783.60
H14	Fuel 1	FTP/Hot	7.21	1.75	745.55
H15	Fuel 1	FTP/Hot	7.16	1.74	740.75
H16	Fuel 1	FTP/Hot	7.14	1.76	739.30
H17	Fuel 1	FTP/Hot/Single [c]	6.93	1.82	753.80
Average	Fuel 1	FTP/Hot only	7.14 ± 0.07	1.73 ± 0.05	741.05 ± 3.13
H05	Fuel 2	FTP/Hot	7.31	1.75	767.85
H06	Fuel 2	FTP/Hot	7.28	1.75	768.30
H07	Fuel 2	FTP/Hot	7.24	1.72	764.20
C08	Fuel 2	FTP/Cold/Single [b]	7.23	1.82	800.60
H09	Fuel 2	FTP/Hot	7.13	1.69	765.20
H10	Fuel 2	FTP/Hot	7.05	1.69	766.35
Average	Fuel 2	FTP/Hot only	7.20 ± 0.11	1.72 ± 0.03	766.38 ± 1.73

[a]All data are means of double FTP (Federal Test Procedure) runs based on our sampling system, except where noted. Hydrocarbon analyses in progress.

[b] Single cold start FTP.

[c] Single hot start FTP.

Table 16. Diesel Particle Emissions for Fuel 1 and Fuel 2.

Sample ID	Fuel Type	Cycle/Start Type [a]	Total PM Mass [b] (mg / filter)	Total Mass Emission (g / hp-hr)
H12	Fuel 1	FTP/Hot	5.536	0.345
C13	Fuel 1	FTP/Cold/Single [c]	6.200	0.401 [c]
H14	Fuel 1	FTP/Hot	6.065	0.384
H15	Fuel 1	FTP/Hot	6.059	0.382
H16	Fuel 1	FTP/Hot	5.984	0.377
H17	Fuel 1	FTP/Hot/Single [e]	6.298	0.397 [e]
Average	Fuel 1	FTP/Hot only	5.911 ± 0.253	0.372 ± 0.018
H11	No Diesel	Blank Run [d]	0.000	0.000
H05	Fuel 2	FTP/Hot	5.182	0.328
H06	Fuel 2	FTP/Hot	5.254	0.334
H07	Fuel 2	FTP/Hot	5.183	0.326
C08	Fuel 2	FTP/Cold/Single[c]	5.193	0.355 [c]
H09	Fuel 2	FTP/Hot	4.941	0.317
H10	Fuel 2	FTP/Hot	4.956	0.320
Average	Fuel 2	FTP/Hot only	5.103 ± 0.144	0.325 ± 0.007

[a] All data are means of double FTP (Federal Test Procedure) runs based on our sampling system.
[b] Total PM mass = primary filter mass + secondary filter mass.
[c] Single cold start FTP.
[d] Blank run = sample dilution tunnel without engine running.
[e] Single hot start FTP.

3. Extraction of Particulate Matter - Main Study

a. Extraction conditions

Prior to solvent extraction, all filter samples were reweighed at U.C. Davis and the weights were recorded and compared with those obtained after sampling at the LACMTA engine dynamometer-dilution tunnel facility. There were no significant changes in weight. For the bioassay, all filters were individually extracted three times with DCM and sonication. These extracts were then pooled, concentrated by nitrogen evaporation, and stored in the freezer at -20°C until tested in the bioassay.

For chemical analyses, sufficient amounts of particle extract were obtained by pooling the filters from two complete hot cycles. This resulted in having two sets of pooled filters for each fuel type. For the cold start samples, only one set of filters were extracted for each fuel type. Each pooled sample was chemically analyzed in triplicate by GC/MS. The filter particulate samples that were collected and extracted for bioassay and chemical analyses are summarized in Table 17 for both fuels that were used.

Table 17. Summary of Filter Samples Collected and Extracted from using Fuel 1 and Fuel 2.

Sample ID	Fuel Type	Cycle/Start Type [a]	Filters Extracted	
			Bioassay	Chem. Analysis
H12	Fuel 1	FTP/Hot	X	X
C13	Fuel 1	FTP/Cold/Single [b]	X	X
H14	Fuel 1	FTP/Hot	c	X
H15	Fuel 1	FTP/Hot	X	X
H16	Fuel 1	FTP/Hot	c	X
H17	Fuel 1	FTP/Hot/Single [d]	X	X
H04	Fuel 2	FTP/Hot	c	X
H05	Fuel 2	FTP/Hot	X	X
H06	Fuel 2	FTP/Hot	X	X
H07	Fuel 2	FTP/Hot	X	X
C08	Fuel 2	FTP/Cold/Single [b]	X	X
H09	Fuel 2	FTP/Hot	X	X
H10	Fuel 2	FTP/Hot	c	X
H11	No Diesel	Field Blank [e]	X	X
T18A	No Diesel	Trip blank	X	X
T19A	No Diesel	Trip blank	X	X
Solvent extraction blank			X	X

[a] All data are means of double FTP (Federal Test Procedure) runs based on our sampling system.
[b] Single cold start FTP.
[c] Sample has been archived.
[d] Single hot start FTP.
[e] Blank run = sample dilution tunnel without engine running.
X Sample has been extracted for bioassay or chemical analysis.

4. SFE - Main Study

a. SFE extraction conditions

For supercritical fluid sample extractions, we examined a matrix of different extraction pressures, temperatures and time points. We also tested the SFE extracts from blank adsorbents for background mutagenicity in the bioassay and chemically analyzed for background contaminants. Based on the information obtained from bioassay and chemical analyses, we selected the SFE extraction conditions for both PUF and XAD-4 as presented in Table 18.

Table 18. SFE Extraction Conditions for PUF and XAD-4 Samples.

Extraction Condition	PUF	XAD-4
Pressure	5000 psi	7000 psi
Temperature	75°C	100°C
Time	90 min	60 min
Modifier	10% methanol	10% methanol

b. SFE adsorbent spike recovery studies

Three separate chemical recovery studies were conducted for the target and substituted PAHs. The PAHs were spiked onto either PUF or XAD and the adsorbents were extracted by SFE. The PUF was spiked with 200 µL of a PAH standard mixture (5 ng/µL in DCM), containing the 16 target PAHs and 19 substituted PAHs. The XAD-4 was spiked with 150 µL of a PAH standard mixture (50 ng/µL in DCM). Following a 5 min equilibration period and a 10 min static extraction (CO_2 without flow) at either 5000 or 7000 psi, a dynamic extraction (CO_2 with flow) was conducted for either 60 or 90 min. for PUF and XAD, respectively. The resulting eluant was directed into a glass culture tube containing 1 mL of methanol placed in a dry ice-ethanol bath maintained at approximately -40°C.

Recovery data for the target PAH standards that were spiked onto PUF and XAD-4 from three separate experiments are summarized in Tables 19 and 20, respectively. Excellent recoveries of PAHs with 2 through 4 fused benzene rings were obtained from the PUF using SFE (for naphthalene with 2 fused benzene rings, F.W. = 128 through chrysene with 4 fused benzene rings, F.W. = 228). These compounds have boiling points up to 448°C. There was excellent reproducibility in the recovery values obtained from three separate spiking experiments. Compounds larger than chrysene, such as benzo[a]pyrene (5 fused benzene rings, F.W. = 252), had recoveries that were less than 30%, probably due to their higher boiling points and stronger adsorbing properties to the adsorbents. These compounds, however, are typically present in diesel exhaust PM captured on the filter and are not generally found in the PUF or XAD adsorbents. They were added as part of the spike recovery mixture to determine if these compounds could be extracted from PUF or XAD if present.

Excellent recoveries were obtained for 2- to 4-ring PAHs (naphthalene to anthracene) from the XAD using SFE. The recoveries decrease after anthracene. Essentially no 5-ring PAHs, such as benzo[a]pyrene), were recovered. The 3- to 5-ring PAHs were difficult to extract and recover under these extraction conditions. However, this does not pose any problems, since very few, if any, 5-ring PAHs are found on the XAD.

Table 19. SFE Recovery Results of Standard PAHs Extracted from PUF.

Compound	Percent Recovery from PUF (%)				
	Expt. #1	Expt. #2	Expt. #3	Avg.	S.D.
Naphthalene	104	101	107	104	3.36
Acenaphthylene	104	98.7	103	102	2.86
Acenaphthene	92.6	88.9	92.9	91.5	2.28
Fluorene	106	102	114	108	5.73
Phenanthrene	98.8	93.8	105	99.1	5.54
Anthracene	103	98.3	104	102	2.82
Fluoranthene	92.5	91.6	95.3	93.1	1.93
Pyrene	89.9	86.3	90.5	88.9	2.24
Benz[a]anthracene	96.9	80.8	83.5	87.1	8.59
Chrysene	82.2	60.1	63.8	68.7	11.8
Benzo[b]fluoranthene	39.1	16.4	22.9	26.1	11.7
Benzo[k]fluoranthene	43.7	18.8	26.8	29.7	12.7
Benzo[a]pyrene	19.3	8.6	12.5	13.5	5.38
Indeno[1,2,3-c,d]pyrene	1.56	0.79	1.25	1.20	0.39
Dibenz[a,h]anthracene	4.12	1.98	3.19	3.11	1.11
Benzo[g,h,i]perylene	0.54	0.35	0.47	0.45	0.10

Table 20. SFE Recovery of Standard PAHs Extracted from XAD-4.

Compound	Percent Recovery from XAD-4 (%)				
	Expt. #1	Expt. #2	Expt. #3	Avg.	S.D.
Naphthalene	103	104	90.7	99.2	7.41
Acenaphthylene	51.3	96.1	47.5	65.0	27.0
Acenaphthene	110	103	100	104	5.13
Fluorene	102	100	93.6	98.5	4.39
Phenanthrene	106	90.3	93.4	96.6	8.32
Anthracene	90.9	83.4	89.0	87.8	3.90
Fluoranthene	69.0	43.3	67.2	59.8	14.3
Pyrene	54.8	28.5	55.4	46.2	15.4
Benz[a]anthracene	17.9	3.83	15.5	12.4	7.53
Chrysene	16.3	3.01	16.1	11.8	7.62
Benzo[b]fluoranthene	3.86	0.00	0.00	1.29	2.23
Benzo[k]fluoranthene	3.44	0.00	0.00	1.15	1.99
Benzo[a]pyrene	0.95	0.00	0.00	3.17	5.48
indeno[1,2,3-c,d]pyrene	0.00	0.00	0.00	0.00	0.00
Dibenz[a,h]anthracene	0.00	0.00	0.00	0.00	0.00
Benzo[g,h,i]perylene	0.00	0.00	0.00	0.00	0.00

As presented in Table 21, excellent recoveries of all 18 substituted PAHs were obtained from the PUF, from 2-methylnaphthalene (F.W. = 142.08) through retene (3 benzene rings, F.W. = 234.32). These compounds have boiling points up to 394°C. The data in Table 21 also demonstrates the reproducibility in recovery values obtained from three separate experiments. Recoveries for the substituted PAHs from XAD are summarized in Table 21. Compounds from 2-methylnaphthalene through 2-methylanthracene (F.W. = 192.26) were quantitatively recovered from XAD in three separate SFE experiments with good reproducibility.

In summary, there is good recovery and reproducibility of standard PAHs of lower molecular weight extracted from PUF and XAD using SFE. For the PUF samples, we can efficiently and quantitatively extract PAHs ranging from naphthalene to chrysene for the target PAHs and to retene for the substituted PAHs.

For the XAD samples, we can quantitatively extract PAHs ranging from naphthalene to phenanthrene and anthracene. By using PUF and XAD in series for the sampling, we can effectively trap those PAHs containing 3 to 5 fused benzene rings onto the PUF, followed by the more volatile PAHs containing either 2 to 3 fused benzene rings or 2-ring substituted PAHs onto the XAD. These compounds can be quantitatively extracted from PUF and XAD using SFE.

After completing the SFE recovery experiments, we proceeded to extract the PUF and XAD samples obtained at the LACMTA facility during the March, 1995 diesel exhaust sampling trip using the SFE conditions previously outlined in Table 18.

Table 21. SFE Recovery Results of Substituted PAHs Extracted from PUF.

Compound	Percent Recovery from PUF (%)				
	Expt. #1	Expt. #2	Expt. #3	Avg.	S.D.
2-methylnaphthalene	85.6	107	102	98.3	11.3
azulene	67.1	88.9	93.7	83.3	14.2
1-methylnaphthalene	81.0	101	94.8	92.3	10.3
2-ethylnaphthalene	86.3	106	98.5	96.9*	9.80
1-ethylnaphthalene	87.4	106	92.1	95.1**	9.57
2,6 &2,7-dimethylnaphthalene	85.5	104	98.6	96.1	9.66
1,3-dimethylnaphthalene	82.8	103	99.0	95.0	10.8
1,4-dimethylnaphthalene	82.8	104	99.9	95.4	11.1
1,5-dimethylnaphthalene	85.6	107	99.3	97.1	10.7
1,2-dimethylnaphthalene	81.4	106	100	95.7	12.7
1,8-dimethylnaphthalene	87.3	109	103	99.9	11.4
9,10-dihydrophenanthrene	103	126	120	116	11.5
1-methylfluorene	103	119	117	113	8.52
2-methylanthracene	105	113	121	113	8.01
1-methylphenanthrene	104	107	114	108	5.35
9-methylphenanthrene	103	110	119	111	7.75
9,10-dimethylanthracene	98.0	108	115	107	8.74
retene	97.2	105	113	105	7.86

* 1- and 2-ethylnaphthalene are reported as a single value.
** 2,6- and 2,7-dimethylnaphthalene are reported as a single value.

Table 22. SFE Recovery Results of Substituted PAHs Extracted from XAD-4.

Compound	Percent Recovery from XAD-4 (%)				
	Expt. #1	Expt. #2	Expt. #3	Avg.	S.D.
2-methylnaphthalene	110.01	94.29	95.94	100.08	8.64
1-methylnaphthalene	102.42	89.06	89.80	93.76	7.51
2-ethylnaphthalene	98.71	83.08	84.04	88.61*	8.76
1-ethylnaphthalene	89.95	75.64	76.52	80.67**	7.96
2,6 &2-7-dimethylnaphthalene	100.76	86.89	86.66	91.44	8.07
1,3-dimethylnaphthalene	164.88	142.00	146.50	151.12	12.12
1,4-dimethylnaphthalene	75.38	62.20	95.03	77.54	16.52
1,5-dimethylnaphthalene	91.68	77.26	79.47	82.80	7.77
1,2-dimethylnaphthalene	125.13	103.11	107.49	111.91	11.65
1,8-dimethylnaphthalene	104.54	87.12	88.74	93.47	9.62
9,10-dihydrophenanthrene	145.14	118.67	119.20	127.67	15.13
1-methylfluorene	104.09	85.42	87.01	92.17	10.35
2-methylanthracene	75.70	70.67	73.74	73.37	2.53
1-methylphenanthrene	20.88	17.50	17.47	18.62	1.96
9-methylphenanthrene	63.10	57.96	59.63	60.23	2.62
9,10-dimethylanthracene	39.50	32.71	33.40	35.20	3.74
retene	52.14	47.58	48.15	49.29	2.48

* 1- and 2-ethylnaphthalene are reported as a single value.
** 2,6- and 2,7-dimethylnaphthalene are reported as a single value.

c. Samples for SFE extraction

A summary of the PUF and XAD-4 samples collected from Fuel 1 and Fuel 2 exhaust that have been extracted by SFE is presented in Table 23. There were numerous samples collected from the hot cycles for each fuel type. Since there was only one cold start each day, only a single cycle was collected for each fuel type. The SFE extracts of the PUF and XAD samples were tested separately in the bioassay.

Table 23. Summary of PUF and XAD-4 Samples Collected from Fuel 1 and Fuel 2 and Extracted by SFE.

Sample ID	Fuel Type	Cycle/Start Type [a]	Sample Trains Extracted by SFE			
			Bioassay		Chem. Analysis	
			PUF	XAD-4	PUF	XAD-4
H12	Fuel 1	FTP/Hot	X	X	X	X
C13	Fuel 1	FTP/Cold/Single [b]	X	X	X	X
H14	Fuel 1	FTP/Hot [c]	sample archived			
H15	Fuel 1	FTP/Hot	X	X	X	X
H16	Fuel 1	FTP/Hot [c]	field spike archived			
H17	Fuel 1	FTP/Hot/Single [d]	X	X	X	X
H04	Fuel 2	FTP/Hot [c]	sample archived		X	X
H05	Fuel 2	FTP/Hot	X	X	X	X
H06	Fuel 2	FTP/Hot	X	X	X	X
H07	Fuel 2	FTP/Hot	X	X	X	X
C08	Fuel 2	FTP/Cold/Single [b]	X	X	X	X
H09	Fuel 2	FTP/Hot	X	X	X	X
H10	Fuel 2	FTP/Hot [c]	sample archived			
H11	No Diesel	Field Blank [e]	X	X	X	X
T18A	No Diesel	Trip blank	X	X	X	X
T19A	No Diesel	Trip blank	X	X	X	c
CO_2 Extraction blank			X	X	X	X

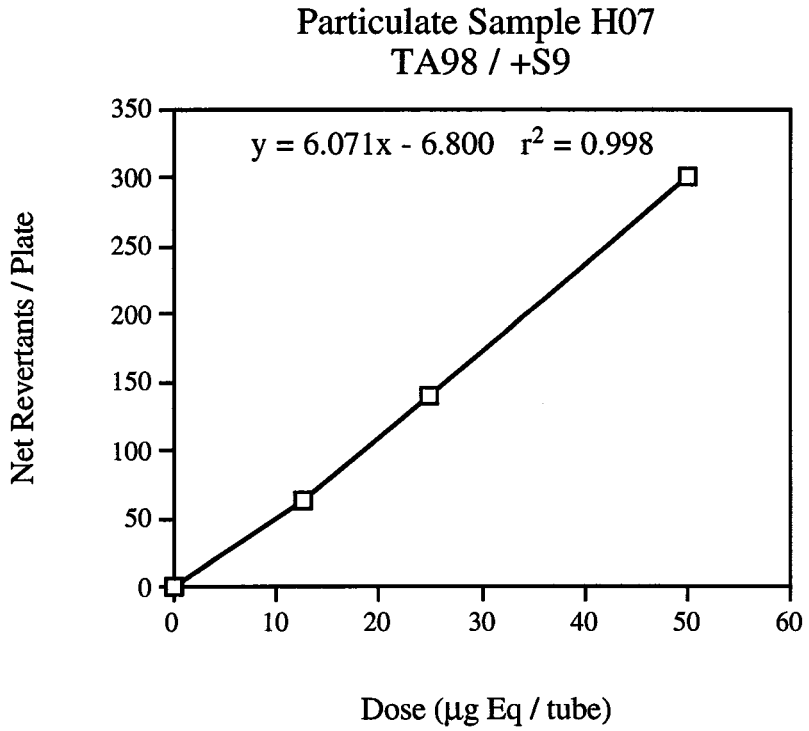
[a] All data are means of double FTP (Federal Test Procedure) runs based on our sampling system.
[b] Single cold start FTP.
[c] Sample has been archived. Chemical analyses completed using SFE extracts from bioassay train.
[d] Single hot start FTP.
[e] Blank run = sample dilution tunnel without engine running.
X Sample has been extracted for bioassay or chemical analysis.

5. Mutagenicity measurements

a. Filter samples

Mutagenicity experiments were conducted to determine the specific mutagenic activity of the diesel PM extracts. Specific mutagenic activity refers to the number of revertants per milligram of PM extracted. It is determined from the slope obtained from the linear portion of the dose-response curve. A pilot experiment was conducted to determine the most sensitive tester strain and TA98 was more sensitive to the mutagens present in the PM extracts (data not shown). All filter extracts were then tested for mutagenicity using the microsuspension bioassay procedure with tester strain TA98. For the Main Study, the specific mutagenic activity of the filter extracts was determined using the dose-response curves obtained from the bioassay. Representative dose-response curves for each fuel type for both cold and hot starts are illustrated in Figures 6 through 9.

As illustrated in these figures, all dose response curves are linear through the highest doses tested. Typically, mutagenic activity was higher when metabolic enzymes are added (+S9) to the test system. Further, mutagenic activity was slightly elevated during the cold start portion of the test. The specific mutagenic activities for all filter samples are summarized in Tables 24 and 25 for mutagenic activity with (+S9) or without (-S9) the addition of metabolic enzymes, respectively. The average of the hot cycles are also presented. The average (± SD) specific mutagenic activity with S9 for the particles collected from the emissions from Fuel 1 and Fuel 2 was 5.745 (± 0.710) and 5.605 (± 0.572), respectively. These values are essentially identical, with respect to the mutagenic activity per mass of particles collected. The average (± SD) specific mutagenic activity without S9 was 4.963 (± 1.55) and 5.140 (±0.137) for the emissions from Fuel 1 and Fuel 2, respectively. These values are lower compared to the mutagenic activity observed when metabolic enzymes are added. Although the specific mutagenic activities are similar per particle mass, the total emissions need to be calculated based on total particle emissions.



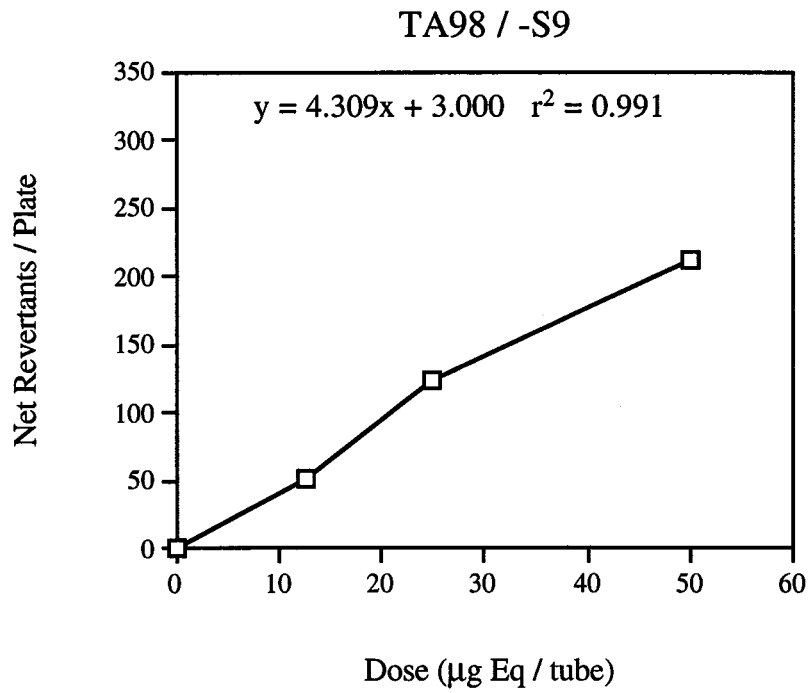
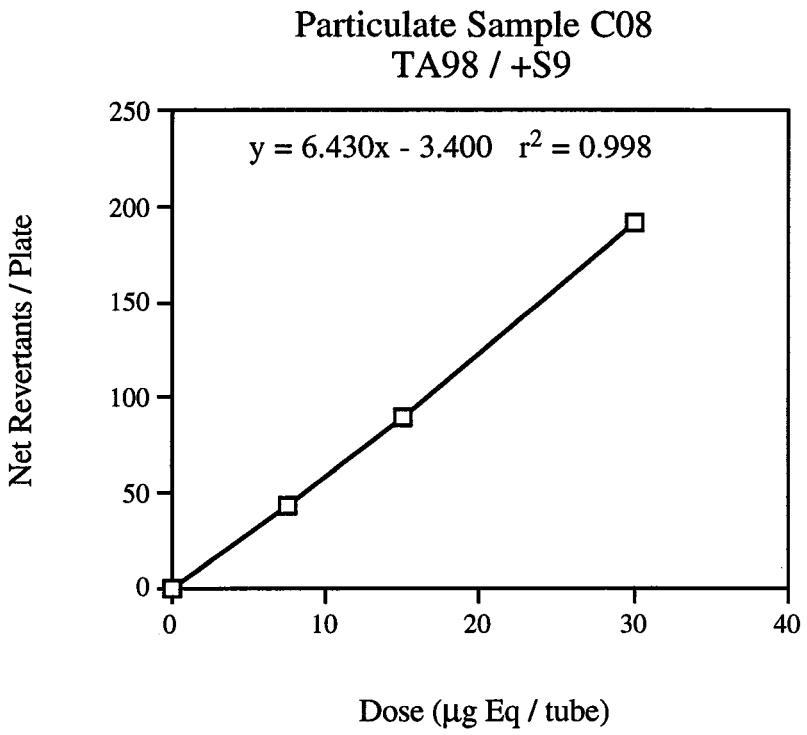


Figure 6. Dose-response relationship of a particulate matter extract (sample no. H07; Fuel 2/hot start) and mutagenic activity in Tester strain TA98 used with and without metabolic activation (±S9).



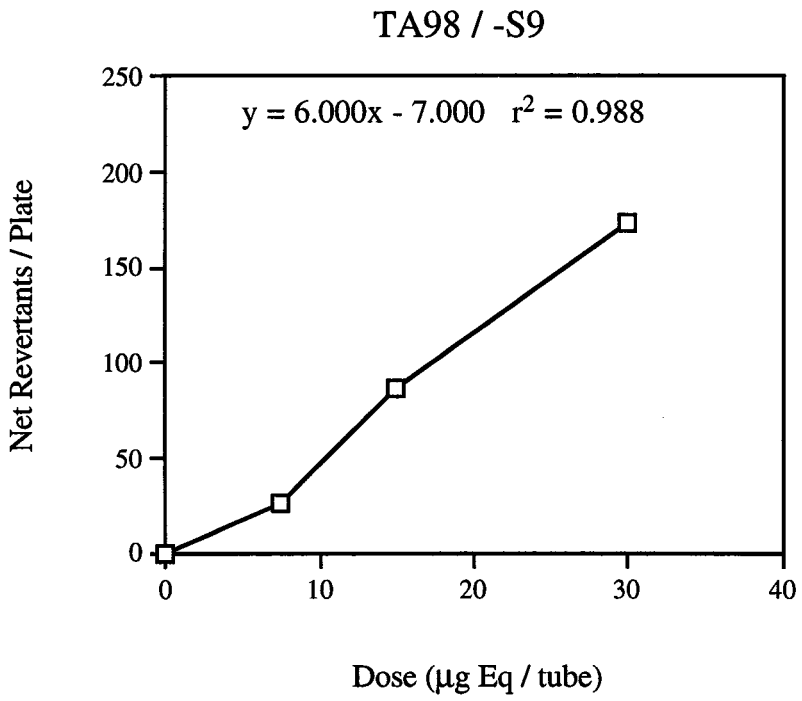
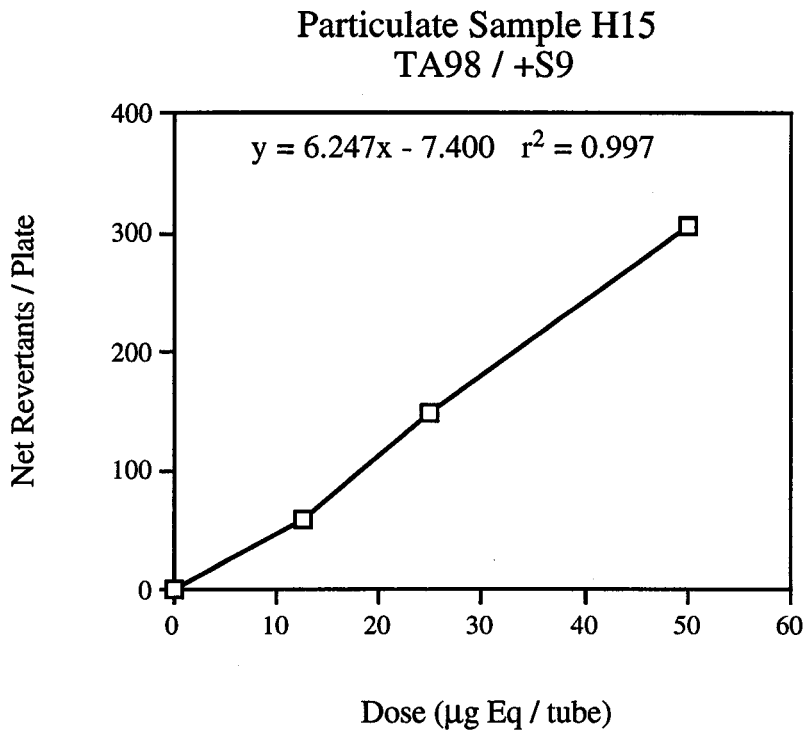


Figure 7. Dose-response relationship of a particulate matter extract (sample no. C08; Fuel 2/cold start) and mutagenic activity in Tester strain TA98 used with and without metabolic activation (±S9).



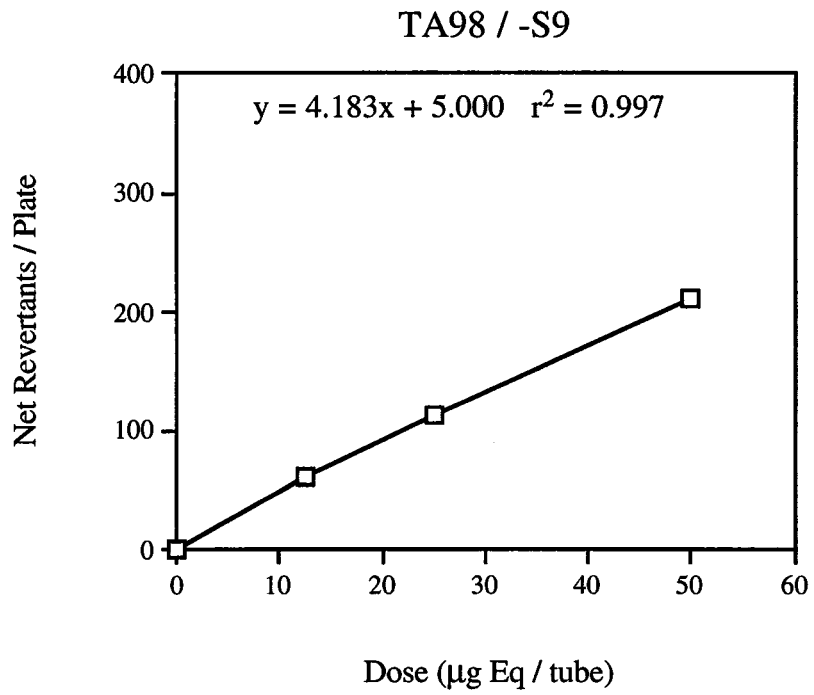
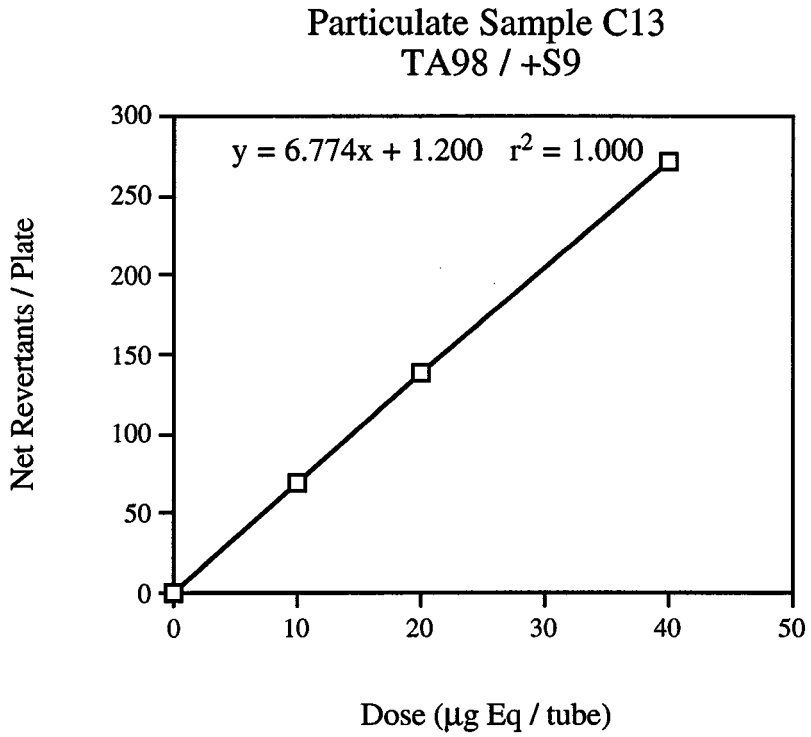


Figure 8. Dose-response relationship of a particulate matter extract (sample no. H15; Fuel 1/hot start) and mutagenic activity in Tester strain TA98 used with and without metabolic activation (±S9).



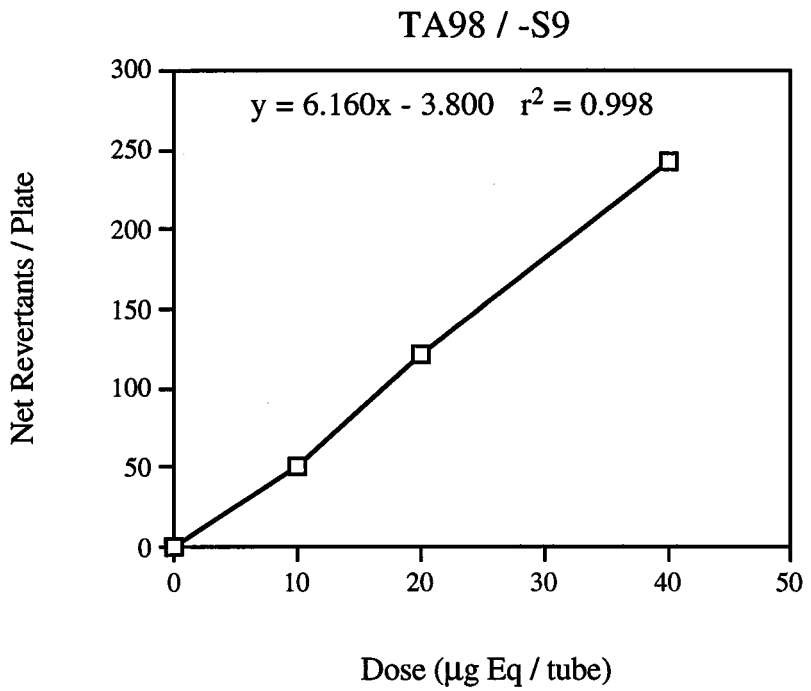


Figure 9. Dose-response relationship of a particulate matter extract (sample no. C13; Fuel 1/cold start) and mutagenic activity in Tester strain TA98 used with and without metabolic activation (±S9).

Total emissions were calculated by multiplying the total revertants per train (reported as total revertants per cycle) by a factor that accounts for dilution of the exhaust in the dilution tunnel. The emissions of mutagenic compounds were normalized to a unit of power called horsepower-hour (hp-hr) and are reported as the total number of revertants per hp-hr. The value obtained represents the total emissions from a single cycle.

Table 24. Specific and total emissions of mutagenic activity equivalents from the filter extracts. Emissions from Fuel 1 and Fuel 2. Filter extracts tested in the bioassay using Tester strain TA98 with S9 metabolic activation.

Sample ID	Fuel Type	Cycle/Start Type [a]	Specific Mutagenic Activity (TA98 revertants / μg) (+S9) [b]	Total Filter Revertants per Train	Total Revertant Emission (rev/hp-hr x 10^6)
H12	Fuel 1	FTP/Hot	5.243	29025	1.81
C13	Fuel 1	FTP/Cold/Single [c]	6.774	41999	2.71
H15	Fuel 1	FTP/Hot	6.247	37851	2.38
H17	Fuel 1	FTP/Hot/Single [e]	6.335	39898	2.51
Average	Fuel 1	FTP/Hot only	5.745 ± 0.710	33438 ± 6241	2.10 ± 0.40
H05	Fuel 2	FTP/Hot	4.905	25418	1.61
H06	Fuel 2	FTP/Hot	6.181	32475	2.06
H07	Fuel 2	FTP/Hot	6.071	31466	1.98
C08	Fuel 2	FTP/Cold/Single [c]	6.430	33391	2.28
H09	Fuel 2	FTP/Hot	5.262	26000	1.67
Average	Fuel 2	FTP/Hot only	5.605 ± 0.572	28840 ± 3865	1.83 ± 0.22
H11	No Diesel	Field Blank [d]	0.000	0	0.00
T18A	No Diesel	Trip Blank	0.000	0	0.00
T19A	No Diesel	Trip Blank	0.000	0	0.00
T20A	No Diesel	Trip Blank	0.000	0	0.00
Filter Extraction Blank			0.000	0	0.00

[a] All data are means of double FTP (Federal Test Procedure) runs based on our sampling system.
[b] Calculated from the linear portion of the dose-response curve for each sample extract
[c] Single cold start FTP.
[d] Blank run = sample dilution tunnel without engine running.
[e] Single hot start FTP.

Table 25. Specific and total emissions of mutagenic activity equivalents from the filter extracts. Emissions from Fuel 1 and Fuel 2. Filter extracts tested in the bioassay using Tester strain TA98 without S9 metabolic activation.

Sample ID	Fuel Type	Cycle/Start Type [a]	Specific Mutagenic Activity (TA98 revertants / µg) (-S9) [b]	Total Filter Revertants per Train	Total Revertant Emission (rev/hp-hr x 10^6)
H12	Fuel 1	FTP/Hot	3.867	21408	1.33
C13	Fuel 1	FTP/Cold/Single [c]	6.200	38192	2.48
H15	Fuel 1	FTP/Hot	6.059	25345	2.31
H17	Fuel 1	FTP/Hot/Single [e]	6.298	22289	2.50
Average	Fuel 1	FTP/Hot only	4.963 ± 1.550	23377 ± 2784	1.82 ± 0.69
H05	Fuel 2	FTP/Hot	5.182	25594	1.70
H06	Fuel 2	FTP/Hot	5.254	28555	1.75
H07	Fuel 2	FTP/Hot	5.183	22334	1.69
C08	Fuel 2	FTP/Cold/Single [c]	5.193	31158	1.84
H09	Fuel 2	FTP/Hot	4.941	24122	1.57
Average	Fuel 2	FTP/Hot only	5.140 ± 0.137	25151 ± 2632	1.68 ± 0.08
H11	No Diesel	Field Blank [d]	0.000	0	0.00
T18A	No Diesel	Trip Blank	0.000	0	0.00
T19A	No Diesel	Trip Blank	0.000	0	0.00
T20A	No Diesel	Trip Blank	0.000	0	0.00
Filter Extraction Blank			0.000	0	0.00

[a] All data are means of double FTP (Federal Test Procedure) runs based on our sampling system.
[b] Calculated from the linear portion of the dose-response curve for each sample extract
[c] Single cold start FTP.
[d] Blank run = sample dilution tunnel without engine running.
[e] Single hot start FTP.

The total emissions of mutagenic compounds are reported as revertants per hp-hr in Tables 24 and 25 for emissions calculated with and without S9, respectively. The emissions of mutagenic compounds was slightly higher for the particles emitted from Fuel 1 than from Fuel 2 for activity detected with or without S9. The average mutagenic emissions (±SD) for the particles emitted from Fuel 1 was 2.10 (±0.40) x 10^6 revertants/hp-hr and for Fuel 2 was 1.83 (± 0.22) x 10^6 revertants/hp-hr (+S9). The average emissions (±SD) for the activity determined without metabolic enzymes (-S9) was 1.82 (±0.69) x 10^6 revertants/hp-hr for the particles emitted from Fuel 1 and 1.68 (± 0.08) x 10^6 from Fuel 2.

b. PUF samples

For the Main Study, the specific mutagenic activity of the PUF extracts were determined using the dose-response curves obtained from the bioassay. Representative dose-response curves for each fuel type are illustrated in Figures 10 and 11.

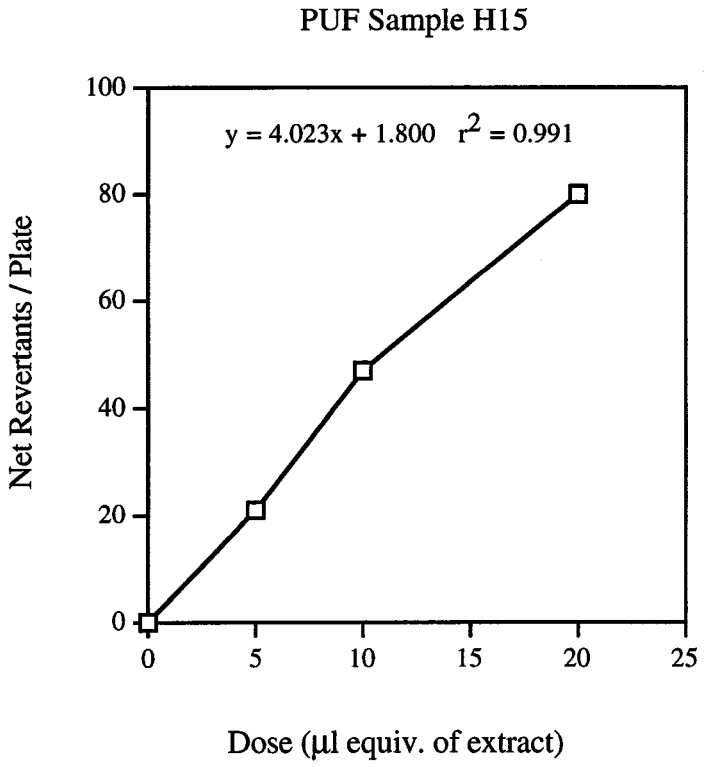


Figure 10. Dose-response relationship of a PUF extract (sample no. H15; Fuel 1 / hot start) and mutagenic activity in tester strain TA100 used with metabolic activation (+S9). Volume equivalent of extract represents ml of original SFE extract.

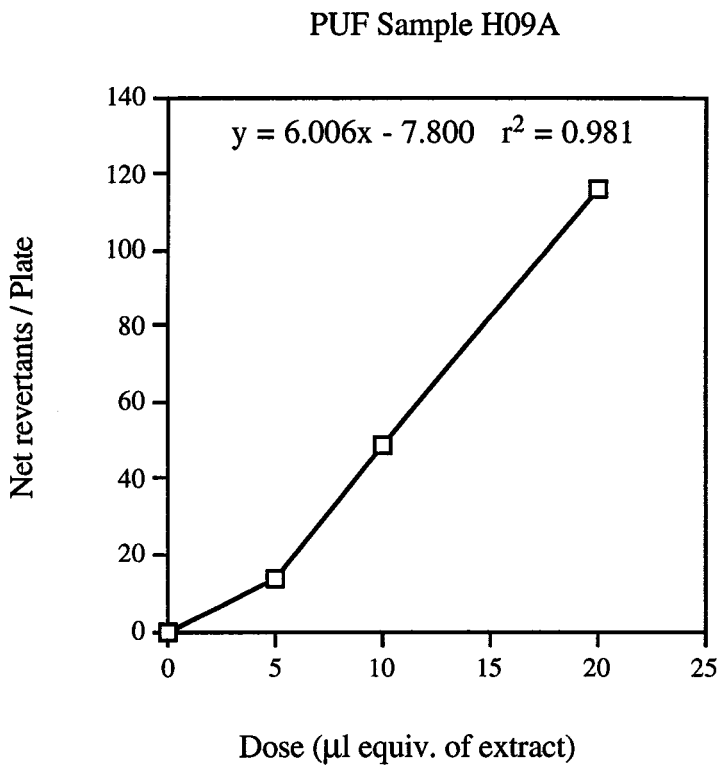


Figure 11. Dose-response relationship of a PUF extract (sample no. H09; Fuel 2 / hot start) and mutagenic activity in tester strain TA100 used with metabolic activation (+S9). Volume equivalent of extract represents ml of original SFE extract.

As previously mentioned, for the PUF sample extracts, the slope of the linear portion of the curve is reported as the specific mutagenic activity, or revertants per µl of extract collected by SFE. A summary of the specific mutagenic activities for the PUF samples from Fuel 1 and Fuel 2 exhaust is presented in Table 26. The total number of revertants for the entire PUF sample (per sample train) was calculated using the specific mutagenic activity and multiplying by the volume of SFE extract. This value reflects the vapor-phase mutagens collected from the secondary dilution tunnel. The total emissions were calculated by multiplying the total revertants per train (reported as total revertants per cycle) and a factor that accounts for dilution of the exhaust in the dilution tunnel. The value obtained represents the total emissions from a single cycle.

Table 26. Specific and total emissions of mutagenic activity equivalents from the PUF extracts. Emissions from Fuel 1 and Fuel 2. PUF extracts tested in the bioassay using Tester strain TA100 with S9 metabolic activation.

Sample ID	Fuel Type	Cycle/Start Type [a]	Specific Mutagenic Activity (TA100 revertants / µl) (+S9) [b]	Total PUF Revertants per Train	Total Revertant Emission (rev/hp-hr x 10^5)
H12	Fuel 1	FTP/Hot	1.180	2799	1.489
C13	Fuel 1	FTP/Cold/Single [c]	0.513	2389	1.314
H15	Fuel 1	FTP/Hot	0.805	1960	1.054
H17	Fuel 1	FTP/Hot/Single [d]	0.371	1833	0.984
H06	Fuel 2	FTP/Hot	2.440	6324	3.426
C08	Fuel 2	FTP/Cold/Single [c]	0.680	3181	1.852
H09	Fuel 2	FTP/Hot	1.201	2656	1.452
H11	No Diesel	Field Blank [e]	0.278	1433	--
T19A	No Diesel	Trip blank	0.274	1306	--
SFE - CO_2 Extraction blank			0.000	--	--

[a] All data are means of double FTP (Federal Test Procedure) runs based on our sampling system.
[b] Calculated from the linear portion of the dose-response curve for each sample extract
[c] Single cold start FTP.
[d] Single hot start FTP.
[e] Blank run = sample dilution tunnel without engine running.

Based on evaluation of the data, there is some variability in the bioassay values within each fuel type. This can be partly attributed to the potential loss of volatile mutagenic compounds during extraction, sample concentration, and bioassay preparation. Differences between PUF samples collected from the exhaust from both fuels need to be further tested and evaluated with multiple cycles. Emissions of PM for Fuel 1 were slightly higher and the total yield of mutagenic activity (particle and vapor-phase) may be higher for this fuel. The PUF sample

extracts were chemically fractionated by solid phase extraction (SPE) and subsequently tested for mutagenic activity.

6. Chemical analysis

a. Filter samples

For the Main Study, we analyzed larger quantities of sample and developed methods necessary for the quantitative chemical analysis of the diesel exhaust sample extracts. The particle and vapor-phase samples were collected from a dilution tunnel where filtered air is mixed with diesel exhaust. This dilution resulted in increasing sample collection volumes. The samples were collected at flow rates of approximately 40 LPM for the duration of the 20 min cycle.

Following solvent extraction and concentration, two (2) μL of each extract was injected into the GC/MS without further sample preparation. Each of these extracts was analyzed in triplicate. A summary of all filter sample extracts that were analyzed by GC/MS for PAHs is presented in Table 27.

Table 27. Summary of solvent-extracted filter samples from Fuel 1 and Fuel 2 that have been analyzed by GC/MS.

Sample ID	Fuel Type	Cycle/Start Type [a]	GC/MS Analyses	
			Substituted PAH	PAH
H12	Fuel 1	FTP/Hot	X	X
C13	Fuel 1	FTP/Cold/Single [b]	X	X
H14	Fuel 1	FTP/Hot	X	X
H15	Fuel 1	FTP/Hot	X	X
H16	Fuel 1	FTP/Hot [c]	field spike archived	
H17	Fuel 1	FTP/Hot/Single [d]	X	X
H04	Fuel 2	FTP/Hot	X	X
H05	Fuel 2	FTP/Hot	X	X
H06	Fuel 2	FTP/Hot	X	X
H07	Fuel 2	FTP/Hot	X	X
C08	Fuel 2	FTP/Cold/Single [b]	X	X
H09	Fuel 2	FTP/Hot	X	X
H10	Fuel 2	FTP/Hot [c]	sample archived	
H11	No Diesel	Field Blank [e]	X	X
T18A	No Diesel	Trip blank	X	X
T19A	No Diesel	Trip blank	[f]	X

[a] All data are means of double FTP (Federal Test Procedure) runs based on our sampling system.
[b] Single cold start FTP.
[c] Sample has been archived.
[d] Single hot start FTP.
[e] Blank run = sample dilution tunnel without engine running.
[f] Sample to be chemically analyzed.
X Sample has been extracted and chemically analyzed.

To provide a sufficient amount of sample for chemical analyses, two sets of diesel filter samples from the hot starts were extracted and pooled for each fuel type. Each pooled sample was chemically analyzed in triplicate by GC/MS and the concentrations of target PAHs detected are shown in Table 28.

Table 28. PAH Analyses of Pooled Diesel Filter Particulate Samples Collected from Hot Starts using Fuel 1 and Fuel 2.

	Concentration (μg PAH / hp-hr ± S.D.)[a]			
	Pooled Sample ID			
Compound	H05 + H06 (Fuel 2)	H07 + H09 (Fuel 2)	H12 + H14 (Fuel 1)	H15 + H17 (Fuel 1)
Phenanthrene	38.70 ± 0.30	36.32 ± 0.08	45.55 ± 0.33	47.38 ± 1.21
Anthracene	5.76 ± 0.92	5.53 ± 0.61	5.58 ± 0.26	6.41 ± 0.42
Fluoranthene	13.63 ± 0.55	14.30 ± 0.57	11.48 ± 0.26	12.81 ± 0.83
Pyrene	29.27 ± 0.58	28.86 ± 0.48	18.66 ± 0.09	18.50 ± 0.27
Benz[a]anthracene	4.63 ± 0.13	4.53 ± 0.11	4.01 ± 0.09	4.17 ± 0.18
Chrysene/Triphenylene	5.06 ± 0.14	4.79 ± 0.04	4.92 ± 0.03	4.89 ± 0.31
Benzo[b]fluoranthene[b]	3.61 ± 0.07	3.47 ± 0.03	2.98 ± 0.39	3.45 ± 0.04
Benzo[k]fluoranthene[b]	4.73 ± 0.14	4.63 ± 0.10	3.94 ± 0.54	4.62 ± 0.14
Benzo[e]pyrene	1.86 ± 0.02	1.88 ± 0.04	1.65 ± 0.02	1.61 ± 0.05
Benzo[a]pyrene	1.73 ± 0.02	1.70 ± 0.01	1.49 ± 0.02	1.42 ± 0.04
Perylene	0.21 ± 0.03	0.22 ± 0.03	0.15 ± 0.03	0.12 ± 0.02
Indeno[1,2,3-c,d]pyrene	1.73 ± 0.15	1.51 ± 0.04	1.62 ± 0.11	1.65 ± 0.07
Dibenz[a,h]anthracene	ND	ND	ND	ND
Benzo[g,h,i]perylene	1.67 ± 0.07	1.43 ± 0.07	1.41 ± 0.06	1.58 ± 0.20

[a] Each concentration based on triplicate analyses.
[b] Values are estimated due to coelution with benzo[j]fluoranthene.
ND = Not detected.

For each fuel type, the concentrations of PAHs present in the two pooled diesel filter samples appear to be similar and have rather a small range. This indicates that the method of pooling the filter samples was consistent. There appears to be a small decrease in concentration of particulate-associated phenanthrene in the Fuel 2 emission samples when compared with the Fuel 1 samples. The concentrations of all other measured PAH are elevated in the Fuel 2 emission samples, with an approximate 83% increase in particulate-associated pyrene concentrations over that detected for both sets of pooled Fuel 1 emission samples. Small increases in concentration were observed for the remaining target PAHs present in the Fuel 2 emission samples when compared with the Fuel 1 emission samples. For the cold start samples, only one set of filters were extracted for each fuel type. Each sample was chemically analyzed in triplicate by GC/MS and the concentrations of target PAH detected are shown in Table 29. Total PAHs were determined by summing the masses of the individual PAHs. The emissions values were converted by multiplying the total mass of PAHs by gram per horsepower-hour (g/hp-hr). The Fuel 2 emissions contained 111 μg/hp-hr, while the Fuel 1 emissions had 109 μg/hp-hr. Although the concentration of PAHs in the emissions from Fuel 1 was lower, the emissions were comparable to those from using Fuel 2, when compared on a g/hp-hr basis.

For the cold start diesel filter samples from Fuel 2 (sample ID C08), there appears to be a large decrease in the concentration of phenanthrene and an increase in the concentration of all other PAHs when compared to the cold-start filter samples collected from using Fuel 1 (sample ID C13). The concentration of phenanthrene detected on the filters collected from using Fuel 1 were similar for both hot and cold starts. The concentration of pyrene in filter samples using the Fuel 2 shows a substantial increase (almost double) over the levels detected in samples using Fuel 1 and is even higher than the concentration detected for the hot start using Fuel 2.

Table 29. PAH Analyses of Diesel Filter Particulate Samples Collected from Cold Starts using Fuel 1 or Fuel 2.

	Concentration (μg PAH / hp-hr ± S.D.)[a]	
	Sample ID	
Compound	C13 (Fuel 1)	C08 (Fuel 2)
Phenanthrene	32.94 ± 0.46	45.60 ± 0.24
Anthracene	5.53 ± 0.10	4.70 ± 0.57
Fluoranthene	19.29 ± 0.16	14.67 ± 0.53
Pyrene	42.07 ± 0.57	23.31 ± 0.43
Benz[a]anthracene	5.57 ± 0.24	5.15 ± 1.60
Chrysene/Triphenylene	5.99 ± 0.21	5.72 ± 0.36
Benzo[b]fluoranthene[b]	5.84 ± 0.09	4.59 ± 0.08
Benzo[k]fluoranthene[b]	6.03 ± 0.05	4.89 ± 0.02
Benzo[e]pyrene	2.39 ± 0.02	1.64 ± 0.22
Benzo[a]pyrene	2.38 ± 0.08	1.60 ± 0.01
Perylene	ND	ND
Indeno[1,2,3-c,d]pyrene	1.76 ± 0.04	1.28 ± 0.13
Dibenz[a,h]anthracene	ND	ND
Benzo[g,h,i]perylene	2.26 ± 0.05	1.72 ± 0.32

[a] Each concentration based on triplicate analyses.
[b] Values are estimated due to coelution with benzo[j]fluoranthene.
ND = Not detected.

Levels of PAHs in the trip blank were lower than in the tunnel blank. The levels of these components during a hot start are not noticeably different from the tunnel blank and should be considered part of the background of the dilution tunnel. These components appear to be much higher in the cold start sample extracts than in the blanks and are probably not due to tunnel artifacts. No cleanout of the tunnel was performed immediately prior to collecting the tunnel blank sample. The tunnel blank may be the upper limit to the background levels of the tunnel.

b. PUF and XAD samples

For the Main Study, we analyzed larger quantities of sample and developed the methods necessary for the quantitative chemical analysis of the diesel exhaust sample extracts. The particle and vapor-phase samples were collected from the dilution tunnel at flow rates of approximately 40 LPM for the duration of each 20 min cycle.

Following SFE, each extract was solvent exchanged into DCM. Two (2) μL of each extract was injected into the GC/MS without further sample preparation. Each of these extracts was analyzed in duplicate. A summary of all PUF and XAD sample extracts that were analyzed by GC/MS for PAHs and substituted PAHs is presented in Tables 30 and 31, respectively.

Analysis was expanded from PAHs to also include 20 substituted PAHs and PAH-like compounds to further chemically characterize the principal components found in diesel exhaust. Fuel 1 and Fuel 2 exhaust samples from both cold and hot starts, a tunnel blank, and trip blanks were analyzed for the presence of these compounds.

In the GC/MS analyses, there was significant coelution of acenaphthene and fluorene. To optimize separation of the vapor-phase PAHs, the temperature program was modified and the 30 m DB-5 column was replaced with a higher resolution 60 m DB-5 column. In subsequent analyses, the vapor-phase diesel exhaust samples were analyzed by GC/MS in the full scan mode. NIST library searches were conducted for the PUF and XAD extracts and the best matches from the library were reported.

Table 30. Summary of SFE extracts of PUF samples from Fuel 1 and Fuel 2 that have been extracted by SFE and analyzed by GC/MS.

Sample ID	Fuel Type	Cycle/Start Type [a]	GC/MS Analyses	
			Substituted PAH	PAH
H12	Fuel 1	FTP/Hot	X	X
C13	Fuel 1	FTP/Cold/Single [b]	X	X
H14	Fuel 1	FTP/Hot	X	X
H15	Fuel 1	FTP/Hot	X	X
H16	Fuel 1	FTP/Hot	field spike archived	
H17	Fuel 1	FTP/Hot/Single [c]	X	X
H04	Fuel 2	FTP/Hot	X	X
H05	Fuel 2	FTP/Hot	X	X
H06	Fuel 2	FTP/Hot	X	X
H07	Fuel 2	FTP/Hot	X	X
C08	Fuel 2	FTP/Cold/Single [b]	X	X
H09	Fuel 2	FTP/Hot	X	X
H10	Fuel 2	FTP/Hot	sample archived	
H11	No Diesel	Field Blank [d]	X	X
T18A	No Diesel	Trip blank	X	X
T19A	No Diesel	Trip blank	[e]	X
CO_2 Extraction blank		none	X	X

[a] All data are means of double FTP (Federal Test Procedure) runs based on our sampling system.
[b] Single cold start FTP.
[c] Single hot start FTP.
[d] Blank run = sample dilution tunnel without engine running.
[e] Sample to be chemically analyzed.
X Sample has been extracted and chemically analyzed.

Table 31. Summary of SFE extracts of XAD-4 samples from Fuel 1 and Fuel 2 that have been extracted by SFE and analyzed by GC/MS.

Sample ID	Fuel Type	Cycle/Start Type [a]	GC/MS Analyses	
			Substituted PAH	PAH
H12	Fuel 1	FTP/Hot	X	X
C13	Fuel 1	FTP/Cold/Single [b]	X	X
H14	Fuel 1	FTP/Hot	X	X
H15	Fuel 1	FTP/Hot	X	X
H16	Fuel 1	FTP/Hot	field spike archived	
H17	Fuel 1	FTP/Hot/Single [c]	X	X
H04	Fuel 2	FTP/Hot	X	X
H05	Fuel 2	FTP/Hot	X	X
H06	Fuel 2	FTP/Hot	X	X
H07	Fuel 2	FTP/Hot	X	X
C08	Fuel 2	FTP/Cold/Single [b]	X	X
H09	Fuel 2	FTP/Hot	X	X
H10	Fuel 2	FTP/Hot	sample archived	
H11	No Diesel	Field Blank [d]	X	X
T18A	No Diesel	Trip blank	X	X
T19A	No Diesel	Trip blank	[e]	X
CO_2 Extraction blank		none	X	X

[a] All data are means of double FTP (Federal Test Procedure) runs based on our sampling system.
[b] Single cold start FTP.
[c] Single hot start FTP.
[d] Blank run = sample dilution tunnel without engine running.
[e] Sample to be chemically analyzed.
X Sample has been extracted and chemically analyzed.

To determine the total levels of PAHs in the PUF and XAD extracts, the PAH and substituted PAH levels for each fuel type (hot starts only) were summed. This data is presented in Tables 32 and 33. In general, the PUF samples contained considerable amounts of naphthalene, acenaphthylene, fluorene, phenanthrene, and anthracene. Fluoranthene and pyrene were found at very low levels (see Appendix 4). PAHs containing four or more fused benzene rings were not detected in the PUF samples, but were present in the filter samples of PM. This indicates that the low sampling flowrate and limited sampling time minimizes loss of PAHs (4 rings or greater) from the filter to the vapor-phase.

Compared to the PUF sample, the XAD sample contained higher concentrations of naphthalene, but much lower levels of acenaphthylene, fluorene, phenanthrene, and anthracene, except for emissions from the hot starts from using Fuel 2 (see Appendix A). Compared to PUF, these XAD samples contained comparable levels of acenaphthylene, fluorene, and phenanthrene. Consistent with the PAHs present in the PUF sample, the XAD samples did not contain PAHs with four or more fused benzene rings.

The variability in the data for the hot-starts was mainly attributed to sample H06, which showed much higher PAH concentrations than samples H04 or H09. This same sample had higher mutagenic activity compared to either H04 or H09, which supports the hypothesis that some of the PAHs or substituted PAHs are responsible for this activity. The variability in sample H06 increases the uncertainty in the data for the Fuel 2 exhaust and has some impact on the comparison of the summed totals for PUF and XAD.

There appears to be quantitatively comparable levels of PAHs in emissions from both Fuel 1 and Fuel 2. The levels of 9,10-dihydrophenanthrene, 1-methylfluorene, 1-ethylnaphthalene, 2-ethylnaphthalene, 1-methylnaphthalene, and most of the dimethylnaphthalenes were very similar in the emissions from both fuels. Notable exceptions were 2,6- and 2,7-dimethylnaphthalene, 1,3-dimethylnaphthalene, and 1,2-dimethylnaphthalene, where levels were higher in the emissions from Fuel 1.

In general, the cold start Fuel 2 exhaust samples had lower levels of substituted PAHs than the exhaust from Fuel 1. As summarized in Table 32, the levels of PAHs were higher in the exhaust from Fuel 1 (sample ID C08) than in the exhaust sample from Fuel 2 (sample ID C13). For the PUF and XAD samples, there was little difference in the PAH levels. However, as seen in Table 33, there were higher levels of acenaphthylene, fluorene, phenanthrene, and anthracene in the exhaust samples collected using Fuel 1 compared to Fuel 2.

For the combined data from the PUF samples obtained under hot start conditions, the levels of PAHs were comparable in the emissions from both the Fuel 1 and Fuel 2 (see Appendix A). The following PAHs were present in order of highest to lowest concentration: naphthalene, phenanthrene, fluorene, acenaphthylene, anthracene, pyrene, and fluoranthene. The substituted PAHs, 9,10-dihydrophenanthrene, 1-methylfluorene, 2-methylanthracene, 1-methylphenanthrene, 9-methylphenanthrene, retene, 1-ethylnaphthalene, 2-ethylnaphthalene, 1-methylnaphthalene, and most of the dimethylnaphthalene compounds, were found in similar concentrations in the exhaust samples from both Fuel 1 and Fuel 2. However, 2,6- and 2,7-dimethylnaphthalene, 1,3-dimethyl-naphthalene, 1,2- dimethylnaphthalene were present in greater amounts in the emissions from Fuel 1.

For the combined data from the XAD extracts, the following PAHs are present in order from highest to lowest concentration: naphthalene, phenanthrene, acenaphthene, fluorene, and anthracene (see Appendix A). This trend was consistent for the emissions from both fuels. Naphthalene was found in the highest concentration of all PAHs. Since the PUF seems to have trapped most of the pyrene and fluoranthene, these compounds were not detected in the XAD.

Table 32. Chemical Analysis of Combined PUF and XAD Sample Data from Cold Start Samples C08 and C13.

Compound	Chemical Analyses of Cold Start Samples by Fuel Type	
	Fuel 1 Sample C13 (µg/hp-hr)	Fuel 2 Sample C08 (µg/hp-hr)
2-methylnaphthalene	810	500
1-methylnaphthalene	510	260
2-ethylnaphthalene	460	220
1-ethylnaphthalene	170	54
2,6- and 2,7-dimethylnapthalene	1100	320
1,3-dimethylnaphthalene	1400	420
1,4- and 2,3-dimethylnaphthalene	360	93
1,5-dimethylnaphthalene	240	68
1,2-dimethylnaphthalene	440	148
1,8-dimethylnaphthalene	26	11
9,10-dihydrophenanthrene	150	79
1-methylfluorene	240	120
2-methylanthracene	76	11
1-methylphenanthrene	81	43
9-methylphenanthrene	ND	ND
9,10-dimethylanthracene	ND	18
retene	ND	ND
naphthalene	810	970
acenaphthylene	92	91
acenaphthene	coelution	coelution
fluorene	240	95
phenanthrene	250	110
anthracene	46	32
fluoranthene	6.6	4.5
pyrene	6.6	4.5
benz[a]anthracene	ND	ND
chrysene	ND	ND
benzo[b]flouranthene	ND	ND
benzo[k]flouranthene	ND	ND
benzo[e]pyrene	ND	ND
benzo[a]pyrene	ND	ND
perylene	ND	ND

ND = not detected

Table 33. Chemical Analysis of Combined PUF and XAD Sample Data from Hot Start Samples H04, H06, H09 and H12, H14, H15.

Compound	Chemical Analyses of Hot Start Samples by Fuel Type: Fuel 1 Samples H12, H14, H15 (µg/hp-hr)	Fuel 2 Samples H04, H06, H09 (µg/hp-hr)
2-methylnaphthalene	740	700
1-methylnaphthalene	480	400
2-ethylnaphthalene	54	210
1-ethylnaphthalene	500	130
2,6- and 2,7-dimethylnapthalene	740	230
1,3-dimethylnaphthalene	430	300
1,4- and 2,3- dimethylnaphthalene	180	130
1,5-dimethylnaphthalene	140	55
1,2-dimethylnaphthalene	240	85
1,8-dimethylnaphthalene	NQ	NQ
9,10-dihydrophenanthrene	80	95
1-methylfluorene	140	130
2-methylanthracene	8.7	8.5
1-methylphenanthrene	35	30
9-methylphenanthrene	2.2	NQ
9,10-dimethylanthracene	ND	ND
retene	ND	ND
naphthalene	960	1040
acenaphthylene	76	140
acenaphthene	coelution	coelution
fluorene	120	94
phenanthrene	200	230
anthracene	26	28
fluoranthene	4.3	6.4
pyrene	4.3	8.5
benz[a]anthracene	ND	ND
chrysene	ND	ND
benzo[b]flouranthene	ND	ND
benzo[k]flouranthene	ND	ND
benzo[e]pyrene	ND	ND
benzo[a]pyrene	ND	ND
perylene	ND	ND

ND = not detected
NQ = not quantitated

Again, the PUF seems to have trapped most of the higher molecular weight substituted PAHs, relative to those trapped on XAD. Methylfluorene (M.W. = 180.25) was the highest molecular weight substituted PAH detected in the XAD extracts. Of the substituted PAHs detected in the XAD, the levels were generally higher in the emissions from Fuel 2. This trend is the opposite of that observed for the PUF extracts. This difference may be due to the large variability of the PAH and substituted PAH concentrations determined in the hot start XAD extracts from the exhaust from Fuel 2.

Levels of PAHs and substituted PAHs in the trip blank were lower than in the tunnel blank. However, 2-methylanthracene, 1-methylphenanthrene, 9-methylphenanthrene, fluoranthene, and pyrene, were found at similar levels in the tunnel blank PUF extract and in the hot start sample PUF extracts. This information suggests that the levels of these components during a hot start are not noticeably different from the tunnel blank and should be considered part of the background of the tunnel. These components appear to be much higher in the cold start sample extracts than in the blanks and are probably not due to tunnel artifacts. No cleanout of the tunnel was performed immediately prior to collecting the tunnel blank sample. The tunnel blank may be the upper limit to the background levels of the tunnel.

7. Summary

A sampling system was designed and used to collect diesel exhaust PM and vapor-phase compounds from a heavy-duty 1988 Detroit Diesel engine at the LACMTA engine dynamometer-dilution tunnel facility for the Main Study. The diesel sampling runs for the Main Study followed a transient Federal Test Procedure (FTP) for Engine Dynamometers and the following fuels were tested: 1) Fuel 1, a fuel that was obtained from a petroleum company and 2) Fuel 2, a fuel that was obtained from the stock supply at the MTA facility during the Main Study sampling period. Fuel samples were analyzed for sulfur, aromaticity, and polycyclic nuclear hydrocarbons by the ARB El Monte Laboratory.

a. Particulate matter

Prior to processing the filter samples collected during the Main Study, extraction conditions were established. Blank filters were extracted and tested for background mutagenicity in the bioassay and chemically analyzed for background contaminants.

The filter samples collected from the Fuel 1 and Fuel 2 exhaust were extracted by traditional solvent methods. The filter extracts were tested in the bioassay and were also used for chemical analyses. The specific mutagenic activity of the filter extracts were determined using the dose-response curves obtained from the bioassay.

Two sets of diesel filter samples from the hot starts were extracted and pooled for each fuel type. Each pooled sample was chemically analyzed in triplicate by GC/MS. For each fuel type, the concentrations of PAHs present in the two pooled diesel filter samples appear to be similar and have rather small standard deviations. There appears to be a small decrease in the concentration of phenanthrene in the Fuel 2 emission samples when compared with the Fuel 1 particulate samples. The concentrations of both fluoranthene and pyrene were elevated in the Fuel 2 emission samples, with an approximate 75% increase in pyrene concentrations over that detected for both sets of pooled Fuel 1 emission samples. Small increases in concentration were observed for the remaining target PAHs present in the Fuel 2 emission samples when compared with the Fuel 1 emission samples.

For the cold-start samples, only one set of filters were extracted for each fuel type. Each sample was chemically analyzed in triplicate by GC/MS. For the cold start diesel filter samples using Fuel 2, there appears to be a large decrease in the concentration of phenanthrene and a moderate increase in the concentration of fluoranthene when compared to the filter samples collected from using Fuel 1. The concentration of phenanthrene detected on the filters collected from using Fuel 1 was similar for both hot and cold starts. The concentration of pyrene in filter samples using the Fuel 2 showed a substantial increase

(almost double) over the levels detected in samples using Fuel 1 and is even higher than the concentration detected for the hot start using Fuel 2.

Any comparisons between cold and hot starts must be made with caution, since the results of the hot starts samples represent samples that were combined from 4 cycles, while there was only a single cycle cold start sample for each type of fuel. Also, because of variability between samples, caution must be taken when comparing results obtained for the two fuels. The emission results for both fuels indicate that most of the PAHs are present in higher amounts in the cold-start samples compared to the hot-start samples.

b. Vapor-phase - PUF and XAD

Prior to processing the PUF and XAD samples collected during the Main Study, SFE extraction conditions were established by examining a matrix of different extraction pressures, temperatures and time points. SFE recovery studies were conducted in triplicate for target and substituted PAHs that were spiked onto either PUF or XAD. Excellent recoveries of PAHs were obtained from the PUF, ranging from 104% for naphthalene (2 fused benzene rings, F.W. = 128) to 68.7% for chrysene (4 fused benzene rings, F.W. = 228). Excellent reproducibility in the recovery values were also obtained from three separate experiments. Compounds larger than chrysene, such as benzo[a]pyrene (5 fused benzene rings, F.W. = 252), had recoveries that were generally less than 30%. Excellent recoveries of substituted PAHs were obtained from the PUF, ranging from 84% for indan (1 benzene ring, F.W. = 118.17) to 105% for retene (3 benzene rings, F.W. = 234.32). Reproducible recovery values were obtained from three separate experiments. Very good recoveries of the substituted PAHs were obtained from the XAD as well for compounds from indan through 2-methylanthracene (F.W. = 192.26) from three separate SFE experiments with good reproducibility.

Blank adsorbents were extracted and tested for background mutagenicity in the bioassay and chemically analyzed for background contaminants. Based on the information obtained from

bioassay and chemical analyses, the SFE extraction conditions for both PUF and XAD-4 were established.

The PUF and XAD-4 samples collected from the exhaust from Fuel 1 and Fuel 2 were extracted by SFE. The SFE extracts of the PUF and XAD samples were tested in the bioassay. The XAD samples from each bioassay train were also used for chemical analyses. The specific mutagenic activity of the PUF extracts were determined using the dose-response curves obtained from the bioassay.

PUF samples from each chemical analysis train were extracted by SFE, while the corresponding XAD samples were archived. In general, the PUF samples contained considerable amounts of naphthalene, acenaphthylene, acenaphthene, fluorene, and phenanthrene, along with lower amounts of anthracene, fluoranthene, and pyrene. PAHs containing four or more fused benzene rings were not detected in the PUF samples, indicating that the sampling flowrate and sampling time minimizes loss of PAHs (4 rings or greater) from the filter to the vapor-phase. The XAD sample contained higher concentrations of naphthalene, but much lower levels of acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, and pyrene. The XAD samples did not contain PAHs with four or more fused benzene rings.

To determine the total levels of PAHs in the vapor-phase, the PAH and substituted PAH data for PUF and XAD from each fuel type from the hot starts were summed. There were slightly higher levels of PAHs in exhaust samples collected from Fuel 1 than from Fuel 2, as summarized in Table 33. The sum of PAHs measured in the emissions from using Fuel 1 was 2,440 ng/hp-hr, compared to 2,053 ng/hp-hr for emissions from using Fuel 2. This is a difference of approximately 16%. However, this difference was primarily due to the increase of selected dimethylnaphthalenes, such as 2,6- or 2,7- or 1,3- or 1,2-dimethylnaphthalene (Table 33). However, the cold start levels of PAHs, including both alkylated and parent PAH in the emissions from Fuel 1 were considerably higher as summarized in Table 32. The sum of PAHs measured in

the cold start was 3,495 ng/hp-hr for the emissions from using Fuel 1 and 1,652 ng/hp-hr for emissions from using Fuel 2.

For the combined PUF samples obtained under hot start conditions, the levels of PAHs were comparable in the exhaust from both Fuel 1 and Fuel 2. However, 2,6- and 2,7-dimethylnaphthalene, 1,3-dimethylnaphthalene, 1,2-dimethylnaphthalene were present in greater amounts in the exhaust from Fuel 1 than in the exhaust from Fuel 2. The PAHs in the XAD extracts were present in higher levels in the exhaust from Fuel 2 than in the exhaust from the Fuel 1. The PUF seems to have trapped most of the higher molecular weight substituted PAHs. Methylfluorene (M.W. = 180.25) was the highest molecular weight substituted PAH detected in the XAD extracts. Of the substituted PAHs detected in the XAD, the levels of these compounds were generally higher in the exhaust from Fuel 2. Levels of PAHs and substituted PAHs in the trip blank were lower than in the tunnel blank.

Any comparisons between cold and hot starts must be made with care, since the results of the hot starts samples represent an average of 3 replicates, while there was only a single cold start sample for each type of fuel. Also, because of variability between samples, caution must be taken when comparing results obtained for the two fuels. The results for both fuels indicate that most of the PAHs and substituted PAHs are present in higher amounts in the cold start sample extract for the emissions from Fuel 1.

C. Diesel Exhaust Marker Evaluation

The final task of the chemical analyses involved fractionating the chemically complex filter, PUF and XAD extracts. In our previous work, both the filter and SFE extracts from diesel exhaust were fractionated by HPLC and these fractions were subsequently tested in the microsuspension bioassay. Since the fraction rich in substituted PAHs had the highest mutagenic activity and the general composition of the diesel exhaust from the present samples is similar to that found in our previous work, our analytical methods initially focused on the characterization of fractions obtained

using HPLC. The HPLC fractions that were the most mutagenic were then analyzed by GC/MS to identify those compounds that are potentially responsible for the mutagenicity. NIST library searches were also used to tentatively identify the components present in the HPLC fractions. Authentic standards were used, whenever possible, for positive identification.

To establish the HPLC conditions for fractionation, model mixtures of nitro-PAHs, alkyl-PAHs, and other substituted PAHs were prepared to evaluate different HPLC columns and running solvents. The column most suitable for separating out the model mixture was a silica column using a hexane:DCM gradient. In a preliminary experiment, we fractionated the CO2BS diesel filter sample and the SFE extract of the CO2BS PUF sample obtained from a cold-start run using Fuel 2. Briefly, approximately 200 μL of the CO2BS filter or PUF extract was injected into the HPLC and separated using a hexane:DCM gradient program. Seven fractions were collected based on the retention times of the model compounds. Each fraction was concentrated down to a final volume of 1 ml in a precleaned 4 ml amber vial. However, chemical recoveries were low for the model PAHs.

1. SPE Fractionation

a. Filter Samples

The use of HPLC was originally proposed to fractionate the filter extracts. However, model mixtures consisting of PAHs and substituted PAHs were not easily recovered by HPLC, with chemical recoveries around 40%. To improve chemical recoveries, fractionate mixtures of compounds into general chemical classes, and to provide a convenient method for fractionation, we evaluated a solid phase extraction (SPE) technique using C-18 extraction cartridges. This technique is described in the Materials and Methods section. Briefly, model mixtures of the PAHs, substituted PAHs were spiked onto the C-18 cartridges. The extracts that were eluted from the cartridges were solvent exchanged into DCM and then concentrated with nitrogen. The overall recoveries dramatically increased for the substituted PAHs and ranged from 70 to 90%. Since the

results of this recovery study were acceptable, we proceeded to fractionate the filter extracts. The filter sample extracts that were fractionated are shown in Table 34.

For each filter sample extract, five (5) fractions were collected. The first SPE fraction was eluted with methanol, the second fraction with methanol:acetonitrile (50:50), the third fraction with acetonitrile, the fourth fraction with DCM, and the fifth fraction was eluted with hexane. Each fraction was then chemically analyzed by GC/MS and tested for mutagenicity.

Table 34. Filter Samples from Hot-Start Emissions that were Pooled and Fractionated by Solid-Phase Extraction (SPE).

Fuel 1 Filter Samples	Fuel 2 Filter Samples
H12	H05
H14	H06
H15	H07
H17	H09

b. PUF SFE Samples

The SFE extracts from the PUF samples were also fractionated by SPE, using the same procedure that was used to fractionate the filter sample extracts. The PUF sample extracts that were fractionated are shown in Table 35.

Table 35. PUF Samples Fractionated by Solid-Phase Extraction (SPE).

Sample ID	Fuel Type	Start Type
C13	Fuel 1	Cold
H12	Fuel 1	Hot
H14	Fuel 1	Hot
H15	Fuel 1	Hot
C08	Fuel 2	Cold
H04	Fuel 2	Hot
H05	Fuel 2	Hot
H09	Fuel 2	Hot
H11	---	Tunnel Blank
T19	---	Trip Blank

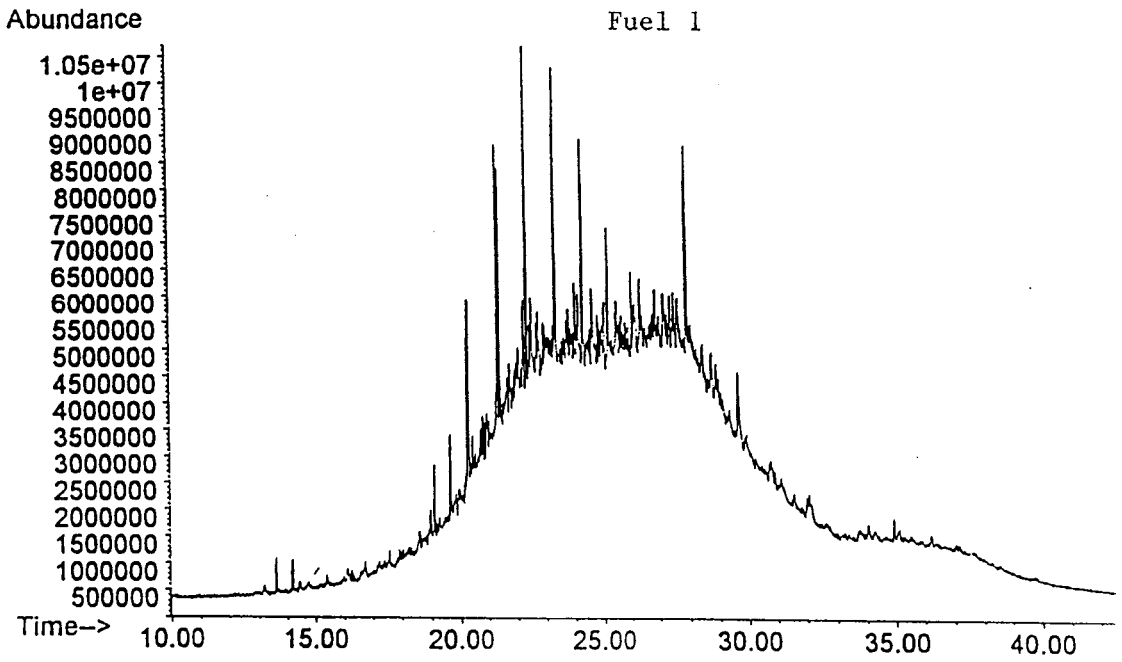
For each PUF sample extract, five (5) fractions were collected. The first SPE fraction was eluted with methanol, the second fraction with methanol:acetonitrile (50:50), the third fraction with acetonitrile, the fourth fraction with DCM, and the fifth fraction was eluted with hexane. Each fraction was then chemically analyzed by GC/MS and tested for mutagenicity.

2. GC/MS Analysis

a. Filter SPE Fractions

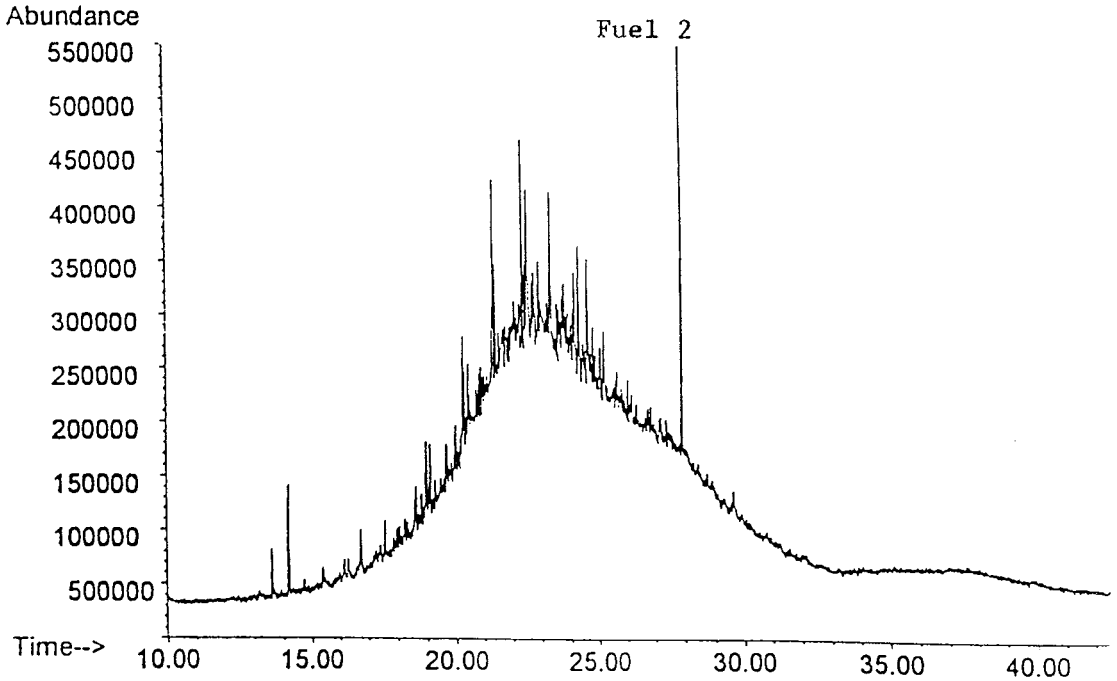
An aliquot from each filter SPE fraction was removed and further concentrated using a stream gentle of nitrogen to a volume of approximately 20 µl. A 1 µl aliquot was analyzed by GC/MS run in full scan mode. Figures 12 through 16 show the total ion chromatograms for each of the SPE fractions for the exhaust emissions from using Fuel 1 and Fuel 2. For comparison, each figure shows the total ion chromatogram for each fuel emission from like SPE fractions. NIST library searches were conducted on the larger peaks in each chromatogram and the results are detailed in Appendix A. To further characterize the fractions, extracted ion chromatograms for compounds of interest were generated and the mass spectra of tentatively identified compounds are shown.

SPE MeOH fraction of diesel particulate emissions of Fuel 1



a)

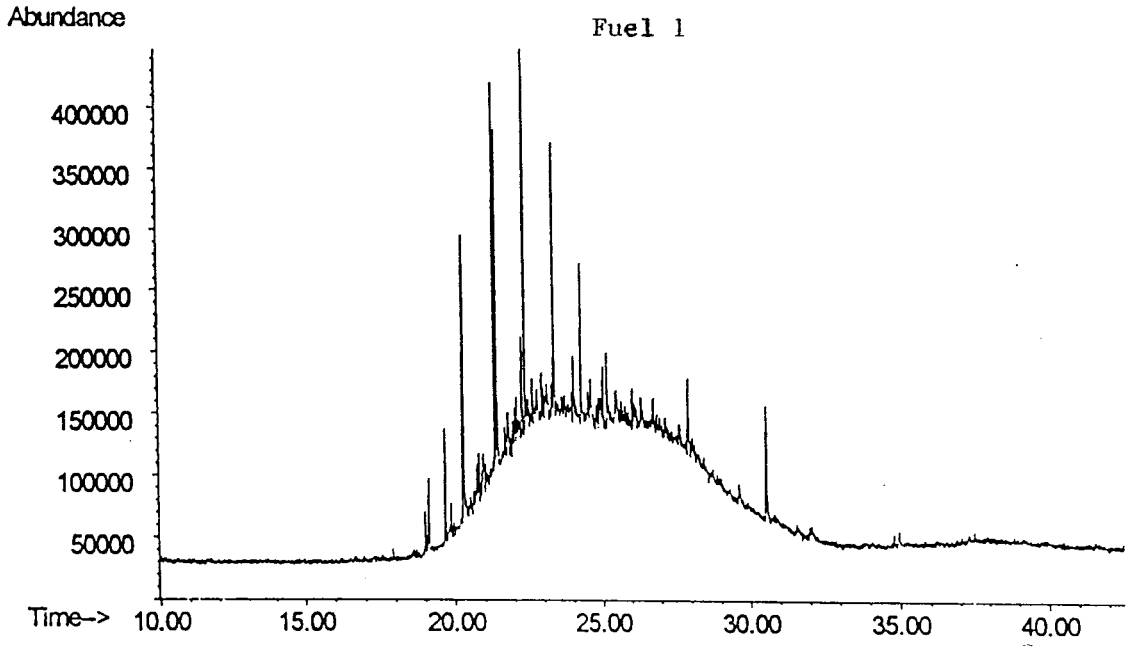
SPE MeOH fraction of diesel particulate emissions of Fuel 2



b)

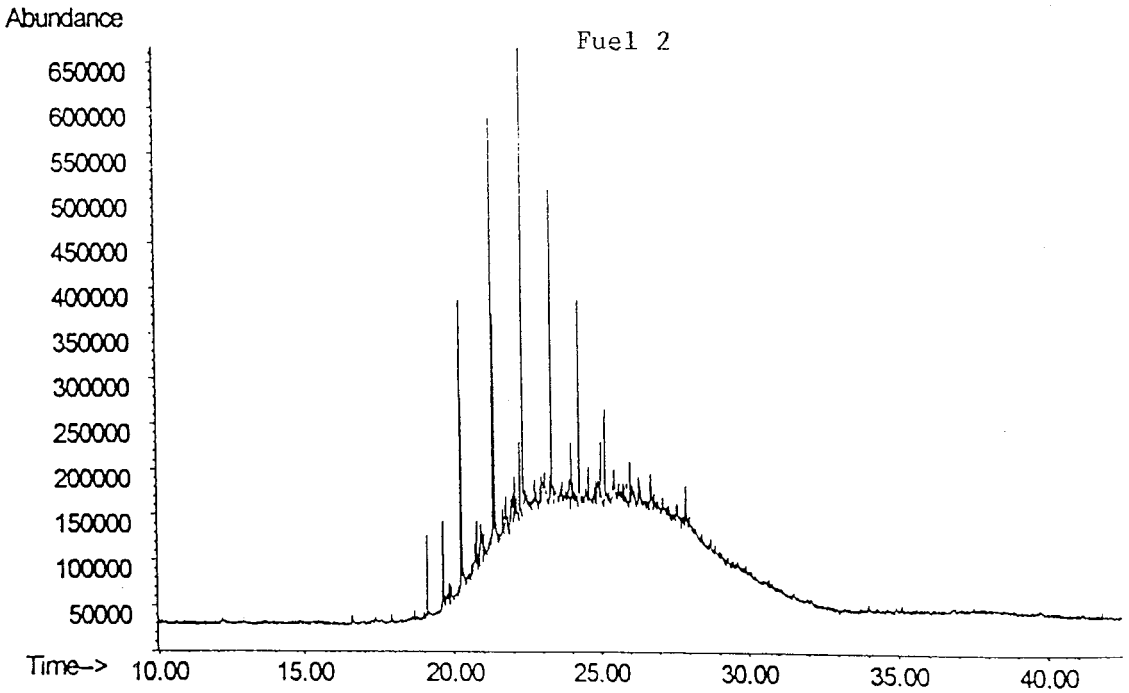
Figure 12. GC/MS Total Ion Chromatograms of SPE Fraction #1 (methanol) from a) Fuel 1 emissions and b) Fuel 2 emissions.

SPE ACN:MeOH fraction of diesel particulate emissions of Fuel 1



a)

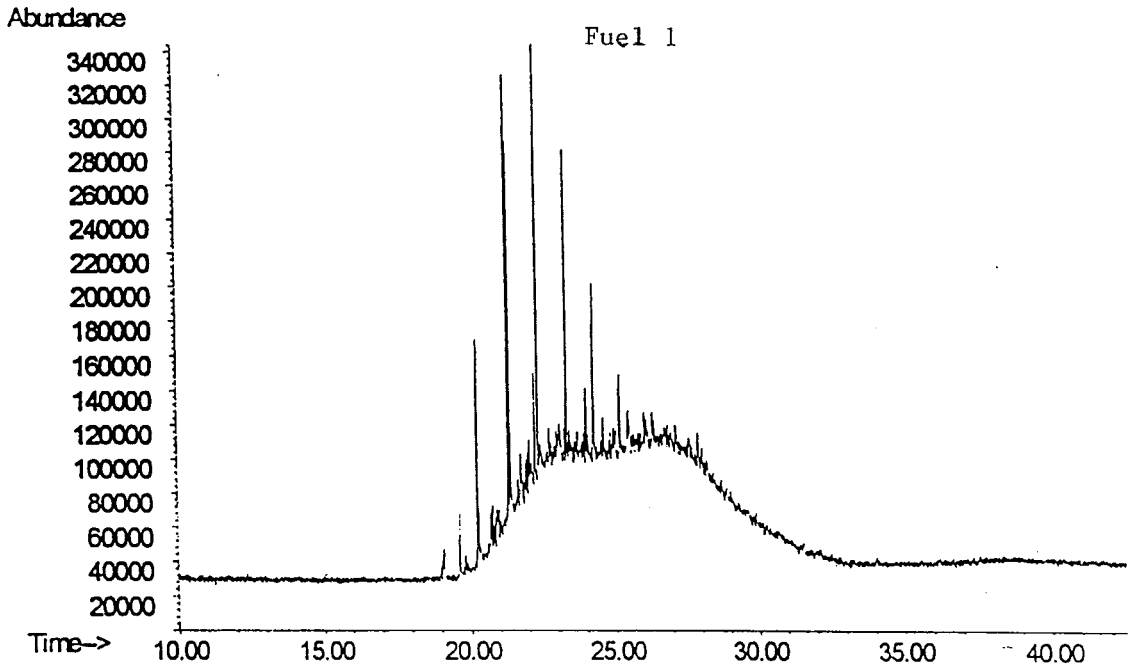
SPE ACN:MeOH fraction of diesel particulate emissions of Fuel 2



b)

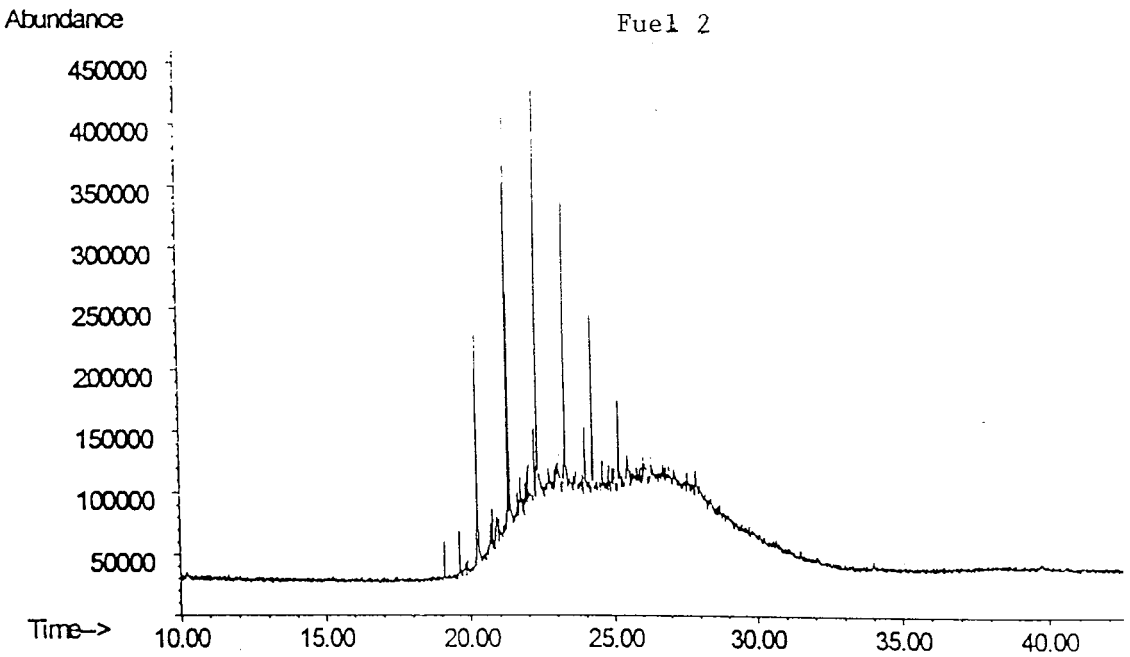
Figure 13. GC/MS Total Ion Chromatograms of SPE Fraction #2 (acetonitrile:methanol) from a) Fuel 1 emissions and b) Fuel 2 emissions.

SPE ACN fraction of diesel particulate emissions of Fuel 1



a)

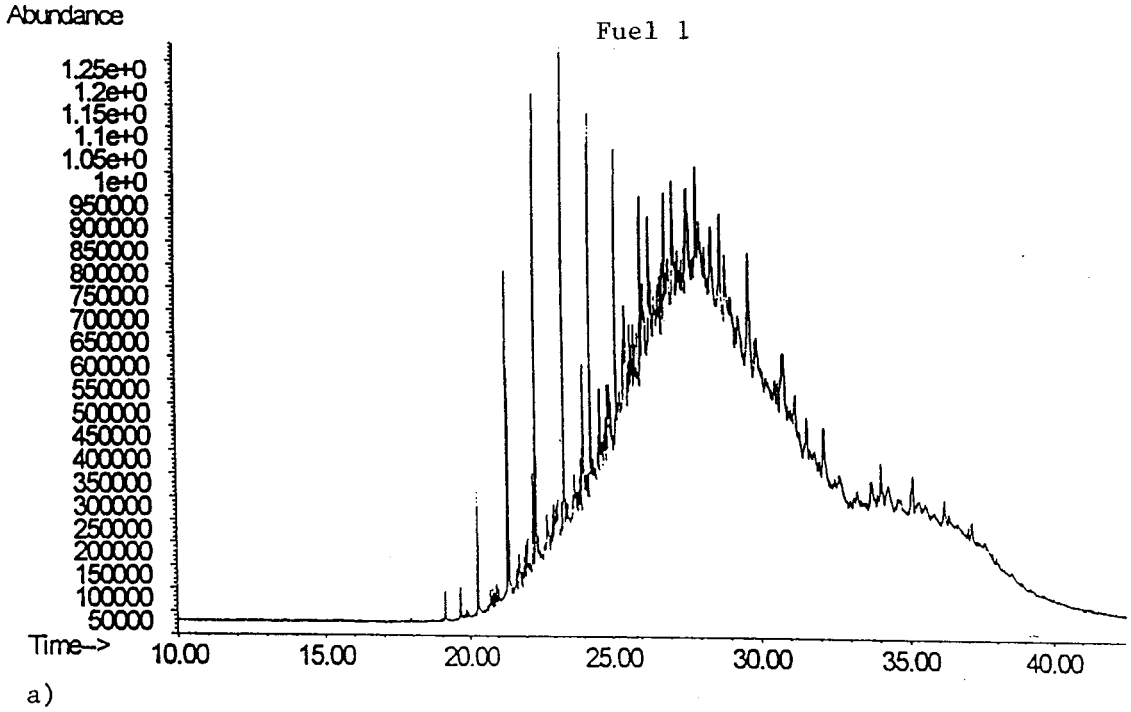
SPE ACN fraction of diesel particulate emissions of Fuel 2



b)

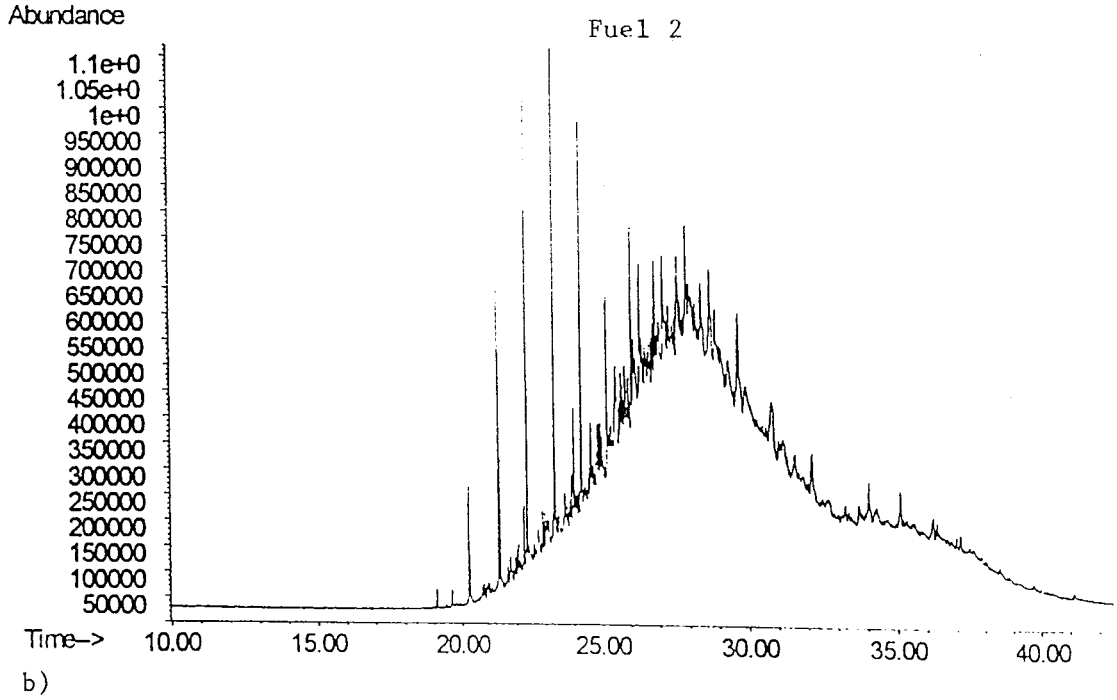
Figure 14. GC/MS Total Ion Chromatograms of SPE Fraction #3 (acetonitrile) from a) Fuel 1 emissions and b) Fuel 2 emissions.

SPE DCM fraction of diesel particulate emissions of Fuel 1



a)

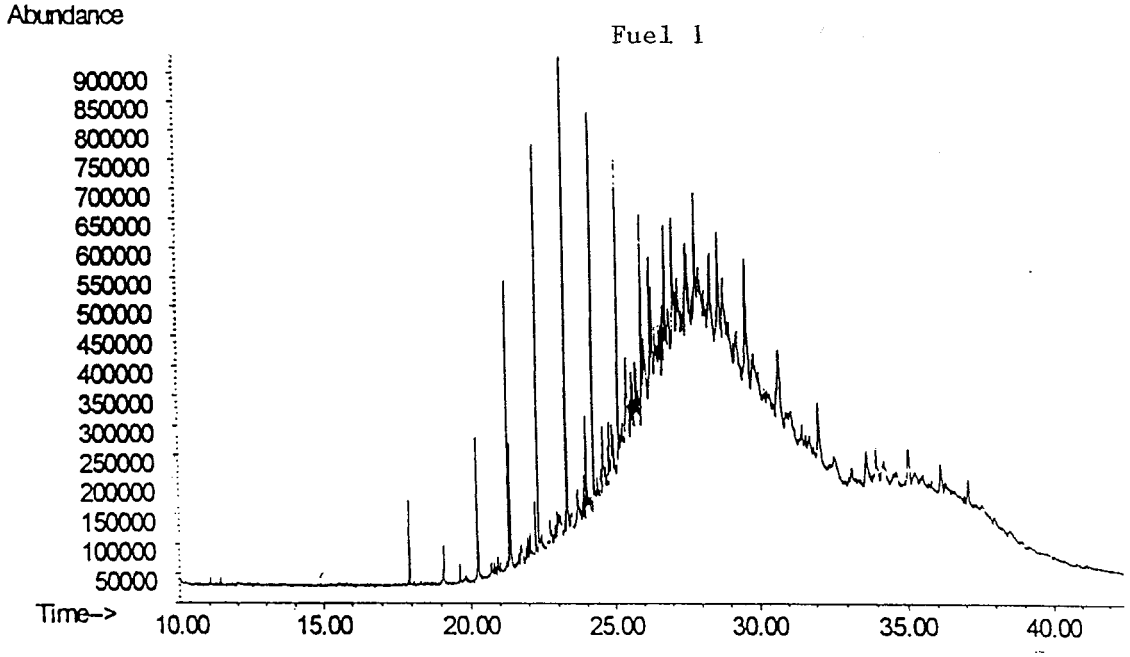
SPE DCM fraction of diesel particulate emissions of Fuel 2



b)

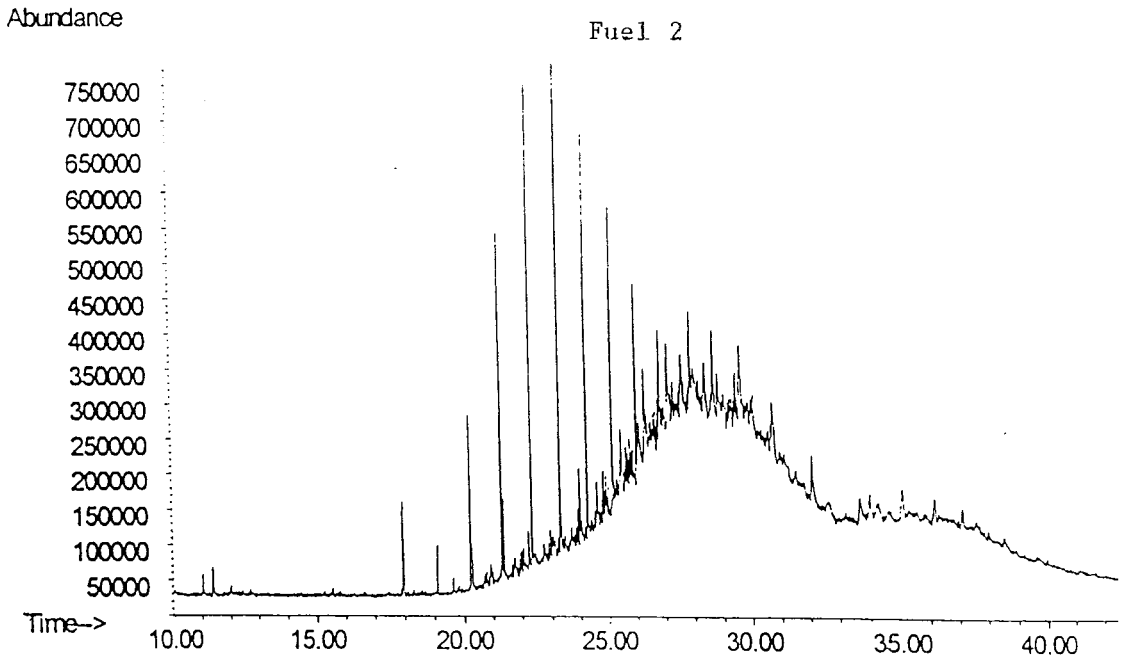
Figure 15. GC/MS Total Ion Chromatograms of SPE Fraction #4 (DCM) from a) Fuel 1 emissions and b) Fuel 2 emissions.

SPE Hexane fraction of diesel particulate emissions of Fuel 1



a)

SPE Hexane fraction of diesel particulate emissions of Fuel 2



b)

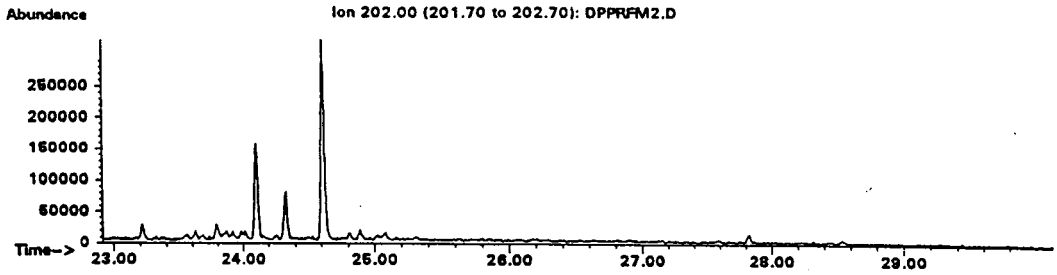
Figure 16. GC/MS Total Ion Chromatograms of SPE Fraction #5 (hexane) from a) Fuel 1 emissions and b) Fuel 2 emissions.

The SPE methanol extracts (Fraction #1) from both Fuel 1 and Fuel 2 emissions contained alkylated phenanthrenes, anthracenes, fluoranthenes and pyrenes. Extracted ion profiles of these compounds are presented in Figures 17 through 20. Comparable levels of alkyl phenanthrenes and anthracenes are also found in SPE Fraction #1 for the emissions from using both fuels. Levels of C1 and C2 pyrenes and fluoranthenes in the emissions from Fuel 2 appear to be higher than those from using Fuel 1. Based on this analyses and the amount of sample extracted, nitro-PAHs, dihydrophenanthrenes, and alkyl dihydrophenanthrenes were not detected in any of the SPE fractions from either fuel. Nitro-PAHs are probably present in these fractions, but at levels too low to be detected by GC/MS run in full scan mode.

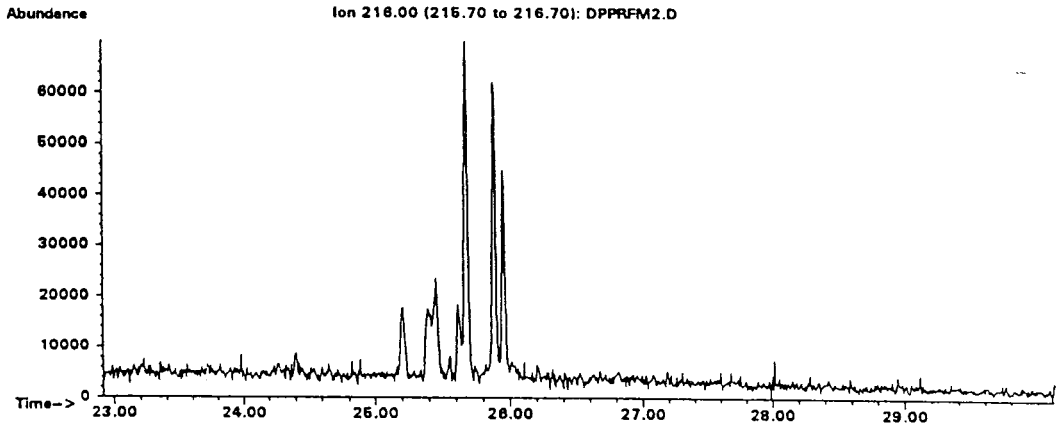
The library searches of Fraction #2 (acetonitrile:methanol), Fraction #3 (acetonitrile), Fraction #4 (DCM) and Fraction #5 (hexane) indicate that they contain high levels of hydrocarbons. These hydrocarbons might interfere with the mass spectral identification of other classes of compounds present in these fractions.

Comparisons of the total ion chromatograms from Fuel 1 and Fuel 2 emission SPE fractions show that each fraction is chemically complex. Separation of trace components cannot be achieved with the high resolution capillary column that was used in our analysis. Additional steps, or a subfractionation procedure, is needed to remove the hydrocarbons. Although individual trace components cannot be identified, inspection of the total ion chromatograms does reveal some differences between the SPE fractions from the emissions from using each fuel.

a)



b)



c)

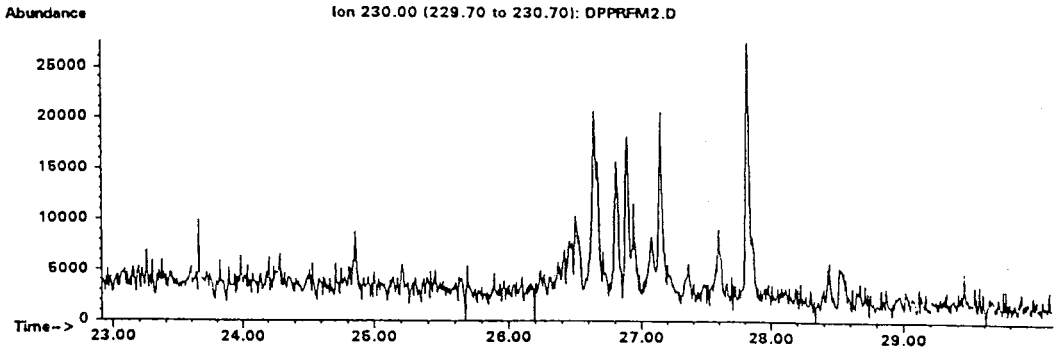


Figure 17. Fuel 2 Emission Extracted Ion Profile from SPE Fraction #1 (Methanol) of a) Fluoranthene, b) Pyrene, and c) Alkyl Pyrenes and Alkyl Fluoranthenes. Ions 216 and 230 are Molecular Ions for C1 and C2 Pyrenes and Fluoranthenes.

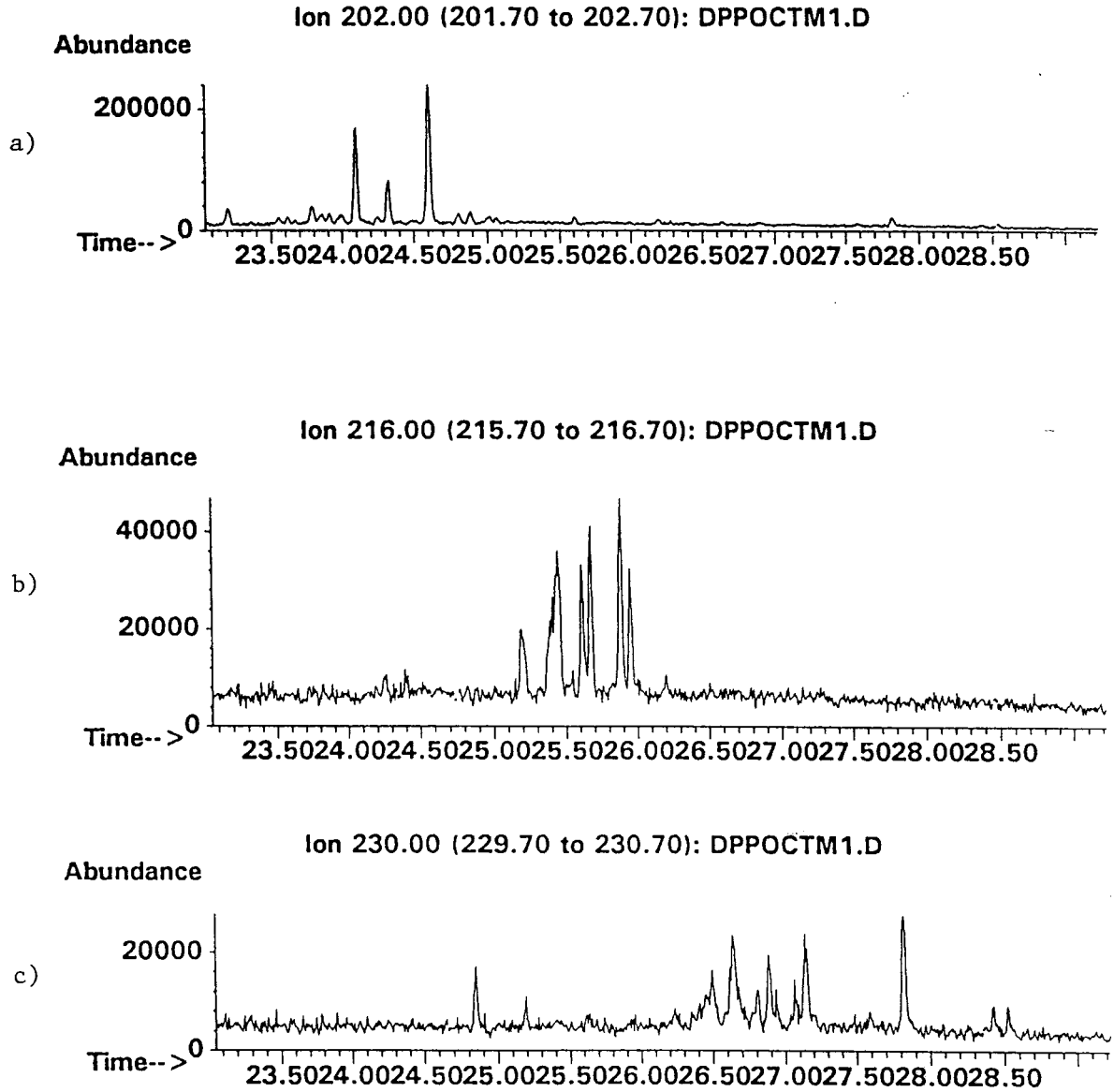


Figure 18. Fuel 1 Emission Extracted Ion Profile from SPE Fraction #1 (Methanol) of a) Fluoranthene, b) Pyrene, and c) Alkyl Pyrenes and Alkyl Fluoranthenes. Ions 216 and 230 are Molecular Ions for C1 and C2 Pyrenes and Fluoranthenes.

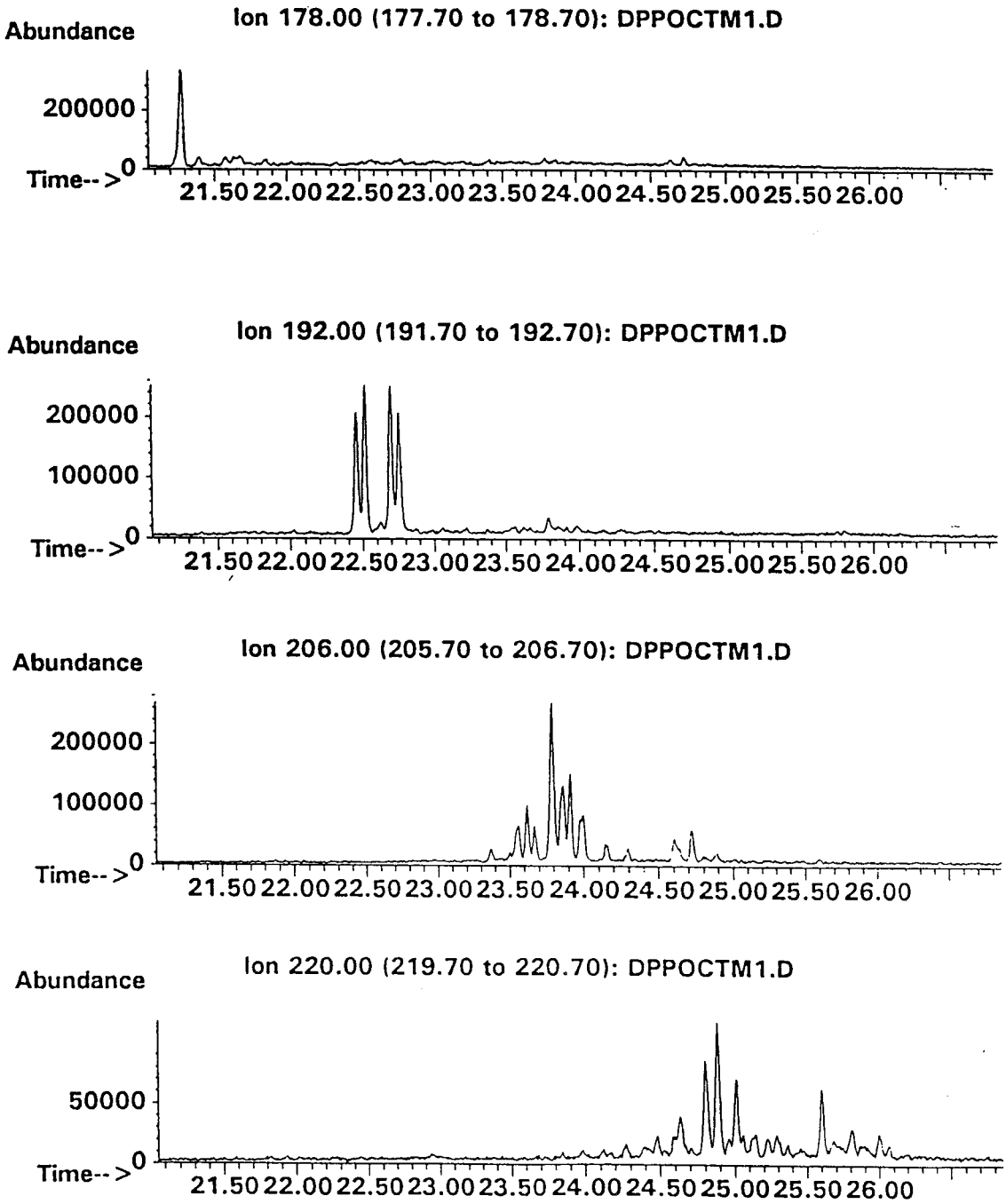


Figure 19. Fuel 1 Emission Extracted Ion Profile from SPE Fraction #1 (Methanol) of Phenanthrene, Alkyl Phenanthrenes, and Anthracenes. Ions 192, 206 and 220 are Molecular Ions for C1, C2, and C3 Phenanthrenes and Anthracenes.

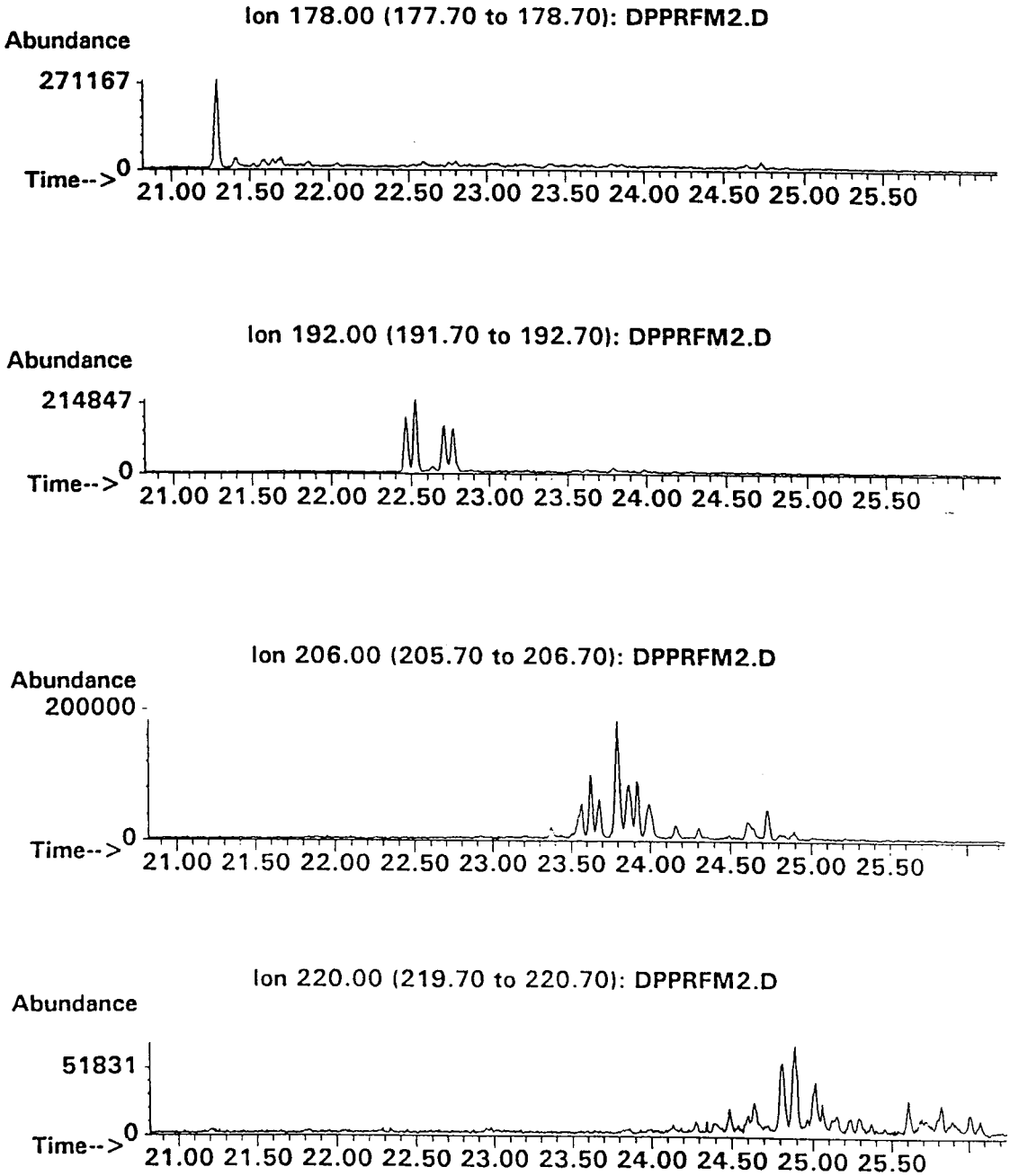


Figure 20. Fuel 2 Emission Extracted Ion Profile from SPE Fraction #1 (Methanol) of Phenanthrene, Alkyl Phenanthrenes, and Anthracenes. Ions 192, 206 and 220 are Molecular Ions for C1, C2, and C3 Phenanthrenes and Anthracenes.

For SPE Fraction #1 (methanol) from Fuel 1 there was almost a two- fold increase in the amounts of compounds present when compared to Fuel 2, based on total peak areas from the total ion chromatograms. In addition, there were more late eluting peaks of higher abundance in the Fuel 1 emissions. In SPE Fraction #2 (acetonitrile:methanol) the Fuel 1 emissions showed chromatogram peaks of lower abundance than in the Fuel 2 emissions. In general, the chromatograms for SPE Fraction #3 (acetonitrile) from both fuel emissions, appear to be very similar to those from SPE Fraction #4 (DCM). The chromatograms for SPE Fraction #5 (hexane) from both fuel emissions were similar and Fuel 1 emissions had a slightly higher abundance of peaks. Inspection of mass spectra from SPE Fraction #5 showed a marked increase in the presence of hydrocarbons and other late eluting compounds that were not present in any of the other SPE fractions. The chromatograms for SPE Fraction #5 (hexane) from the emissions from using both fuels were almost identical to those obtained for SPE Fraction #4 (DCM). The abundances of the peaks in the chromatogram of SPE Fraction #5 (hexane) from Fuel 1 emissions are slightly higher those for the Fuel 2 emissions.

b. PUF SPE Fractions

The H09 And H14 PUF SPE hot start fractions were analyzed by GC/MS in the full scan mode. Since the H09 and H14 methanol:acetonitrile (Fraction #2) and hexane (Fraction #5) fractions exhibited elevated levels of mutagenicity, these fractions were further analyzed by GC/MS. Library searches were conducted on each of these fractions to determine which compounds or classes of compounds might be present in these fractions. Although 5 separate fractions were collected by SPE, each fraction was chemically complex. Even with the use of a high resolution capillary column, many of the peaks were poorly resolved and resulted in poor mass spectra, especially when compared with the NIST library of 54,000 mass spectra.

Most of aromatic compounds were found in the methanol (Fraction #1) and the methanol:acetonitrile (Fraction #2) fractions. Table 36 lists the general classes of compounds that were identified in SPE Fractions #1 and #2.

Chemical analyses of these fractions indicate that the exhaust from Fuel 2 contains higher levels of C_1-, C_2-, and C_3-tetrahydronaphthalenes. The partially hydrogenated naphthalenes are found mainly in the methanol:acetonitrile fraction (Fraction #2), while PAHs and substituted PAHs are found in both the methanol and methanol:acetonitrile fractions (Fractions #1 and #2, respectively). The major components in the acetonitrile, DCM and hexane fractions (Fractions #3, #4, and #5) appear to be saturated hydrocarbons. Minor components were not sufficiently resolved to obtain good mass spectra and could not be identified. Further subfractions would be required to obtain more detailed information about these fractions. However, due to the limited amount of sample obtained, we were not able to subfractionate these fractions.

Table 36. Principal components identified in SPE Fractions #1 and #2.

Phenol
Methylphenols
Naphthalene
Methylnaphthalenes
C_2-naphthalenes
C_3-naphthalenes
Tetrahydronaphthalenes
C_1-tetrahydronaphthalenes
C_2-tetrahydronaphthalenes
C_3-tetrahydronaphthalenes
Indans
C_1-indans
Biphenyl
Methylbiphenyls
Fluorene
Methylfluorenes
Phenanthrene
Anthracene
Methylphenanthrenes/anthracenes

3. Mutagenicity Measurements

a. Filter SPE Fractions

The SPE fractions derived from filter sample extracts were each tested for mutagenic activity. The extract was a pool from three filters for each fuel type. Filter extracts from the tunnel blank were also pooled and fractionated and tested in parallel. All five fractions were tested in tester strain TA98 with and without the addition of metabolic enzymes. Volume equivalents ranging from 3.3 μl to 15 μl of the original fraction were tested per tube. Fraction 1 (methanol) was mutagenic for both fuel types and is illustrated in Figures 21 and 22 for Fuel 1 and Fuel 2, respectively. The dose-response curves for both fuel types with and without metabolic activation are linear and nearly identical. The specific mutagenic activities (based on volume of each fraction) were 26.2 and 27.5 revertants per ml for Fuel 1 and Fuel 2, respectively, with metabolic enzymes added. The specific mutagenic activities without metabolic enzymes were lower at 15.7 and 15.3 revertants per ml for Fuel 1 and Fuel 2, respectively.

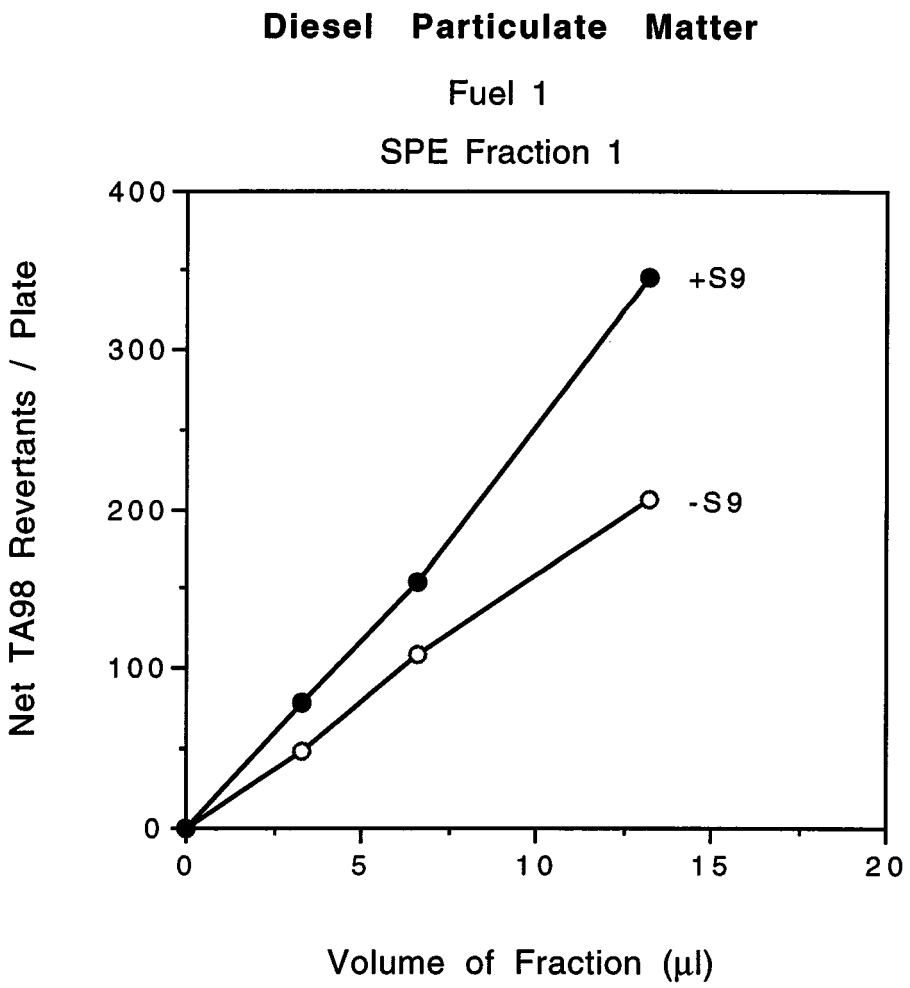


Figure 21. Dose-response relationship of SPE Fraction #1 (methanol) from pooled diesel particulate extract from Fuel 1. Tester strain TA98 with and without the addition of metabolic enzymes (S9).

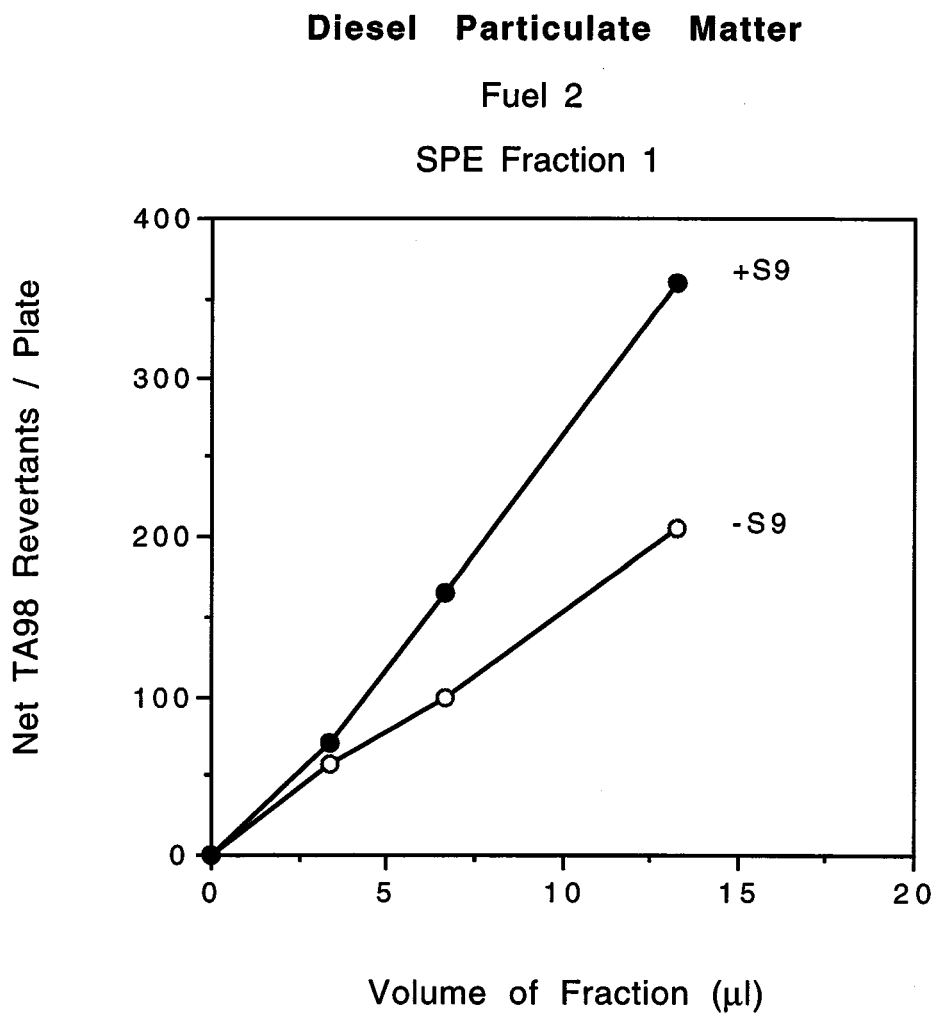


Figure 22. Dose-response relationship of SPE Fraction #1 (methanol) from pooled diesel particulate extract from Fuel 2. Tester strain TA98 with and without the addition of metabolic enzymes (S9).

For the remaining fractions, only Fraction 2 had a slight increase in mutagenic activity over the background levels of activity. The other fractions did not have increased mutagenic activity over the spontaneous number of revertants. The tunnel blank did not have any elevated mutagenicity in any of the five fractions tested at the same dose equivalents as the samples.

The specific mutagenic activity of the fractions and the total volume of the fractions were used to calculate the total number of revertants per fraction. The resulting mutagenicity profile is illustrated in Figure 23 for the particle extract from Fuel 1. Approximately 97% of the mutagenic activity is present in Fraction 1. The mutagenicity profile for the particle extract from Fuel 2 is illustrated in Figure 24. The profile is nearly identical to the profile from Fuel 1, with approximately 96% of the mutagenic activity (+S9) and 98% (-S9) of all fractions is present in Fraction 1.

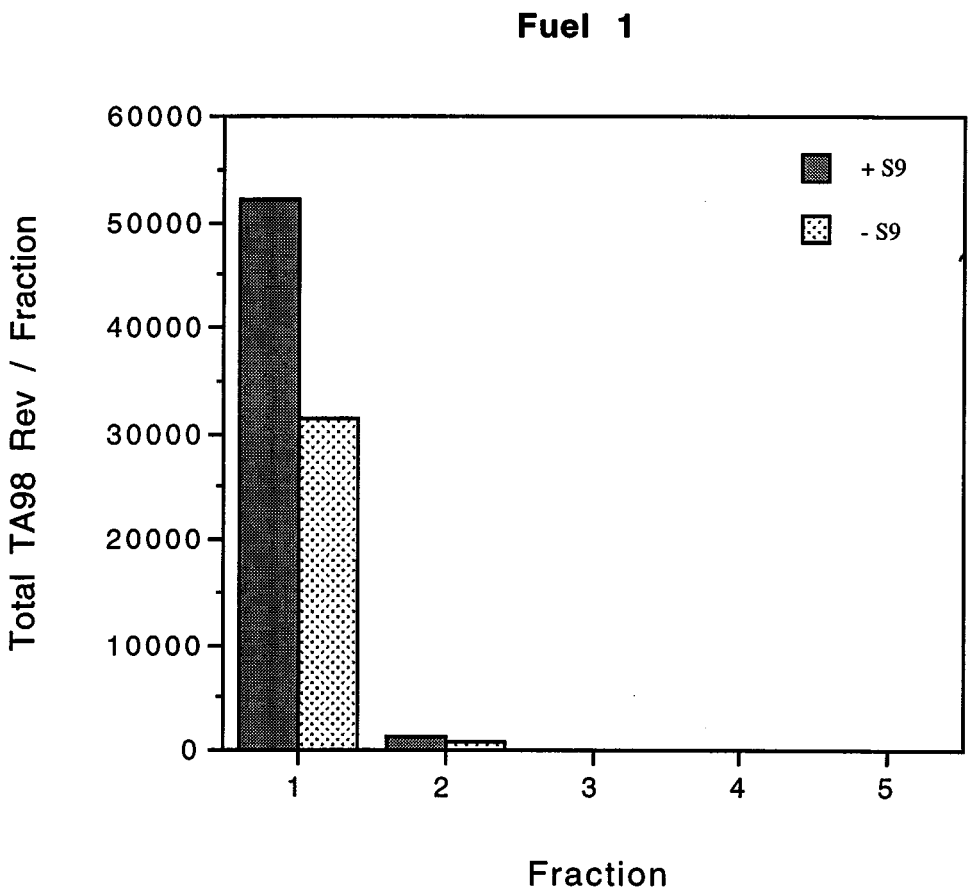


Figure 23. Mutagenicity profile of SPE Fractions from pooled diesel particulate extract from Fuel 1. Tester strain TA98 with and without the addition of metabolic enzymes (S9).

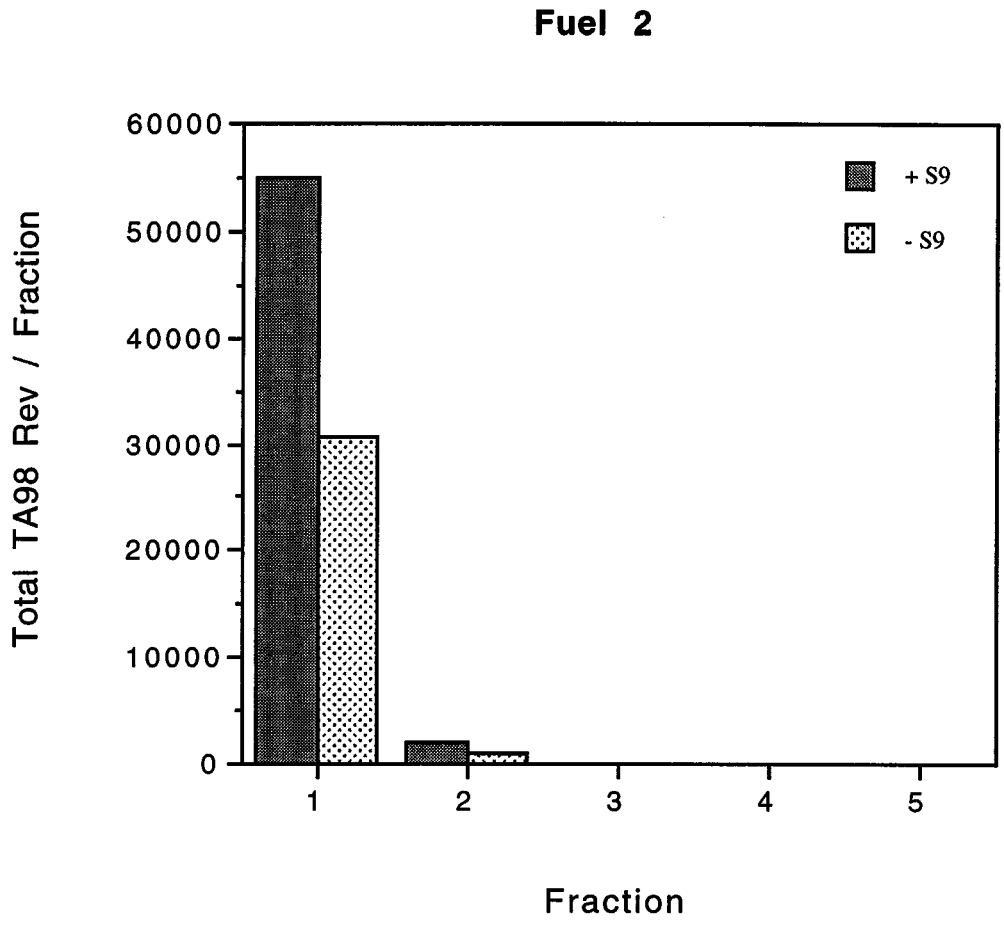


Figure 24. Mutagenicity profile of SPE Fractions from pooled diesel particulate extract from Fuel 2. Tester strain TA98 with and without the addition of metabolic enzymes (S9).

b. PUF SPE Fractions

The PUF sample SFE extracts that were pooled and fractionated by SPE were subsequently tested for mutagenic activity to develop a mutagram, or mutagenicity profile. The bioassay dose-response results for the five SPE fractions that were tested are illustrated in Figures 25 through 29. As illustrated in Figure 25, SPE Fraction #1 (methanol) from Fuel 1 and Fuel 2 exhaust PUF sample extracts have slightly elevated levels of mutagenicity, with an indication of toxicity in the exhaust from the Fuel 2 PUF sample at the highest dose of extract tested.

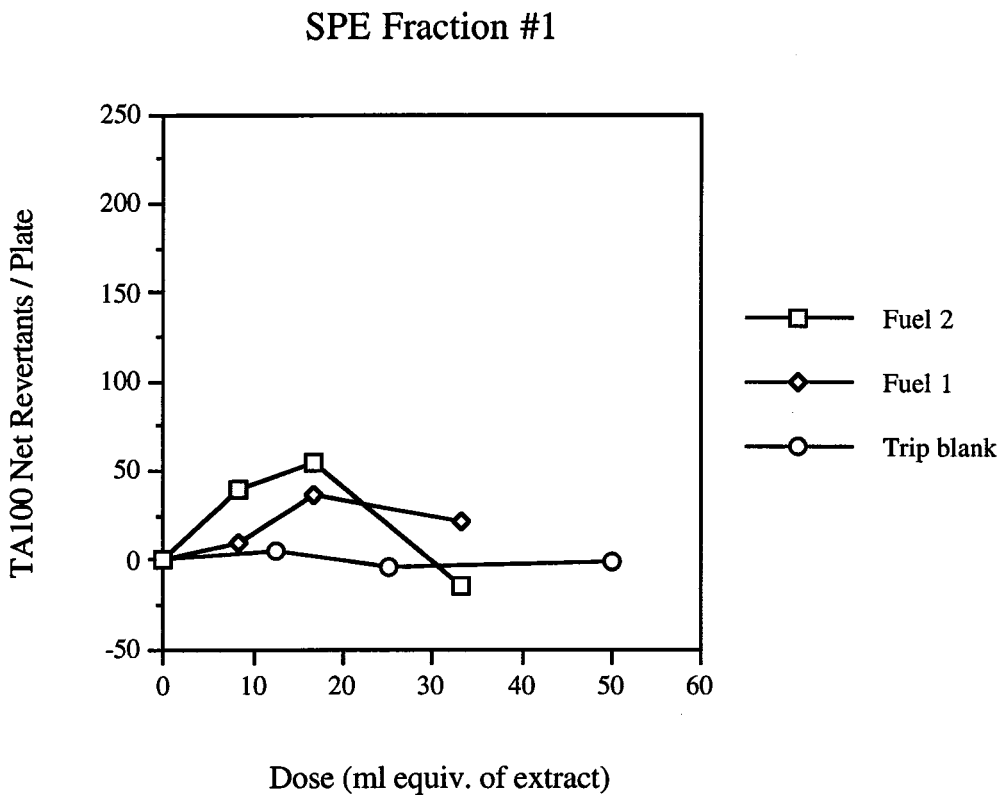


Figure 25. Dose-response relationship of SPE Fraction #1 (methanol) from combined PUF hot start extracts for Fuel 1 and Fuel 2 as tested in tester strain TA100 with metabolic activation (+S9). Volume equivalent of extract represents mL of original SPE extract.

Elevated levels of mutagenicity were also observed in SPE Fraction #2 (methanol:acetonitrile), as illustrated in Figure 26, with toxicity observed in the Fuel 1 PUF exhaust sample. There were no indications of mutagenicity for any sample in either SPE Fraction #3 (acetonitrile) or #4 (DCM), as illustrated in Figures 27 and 28.

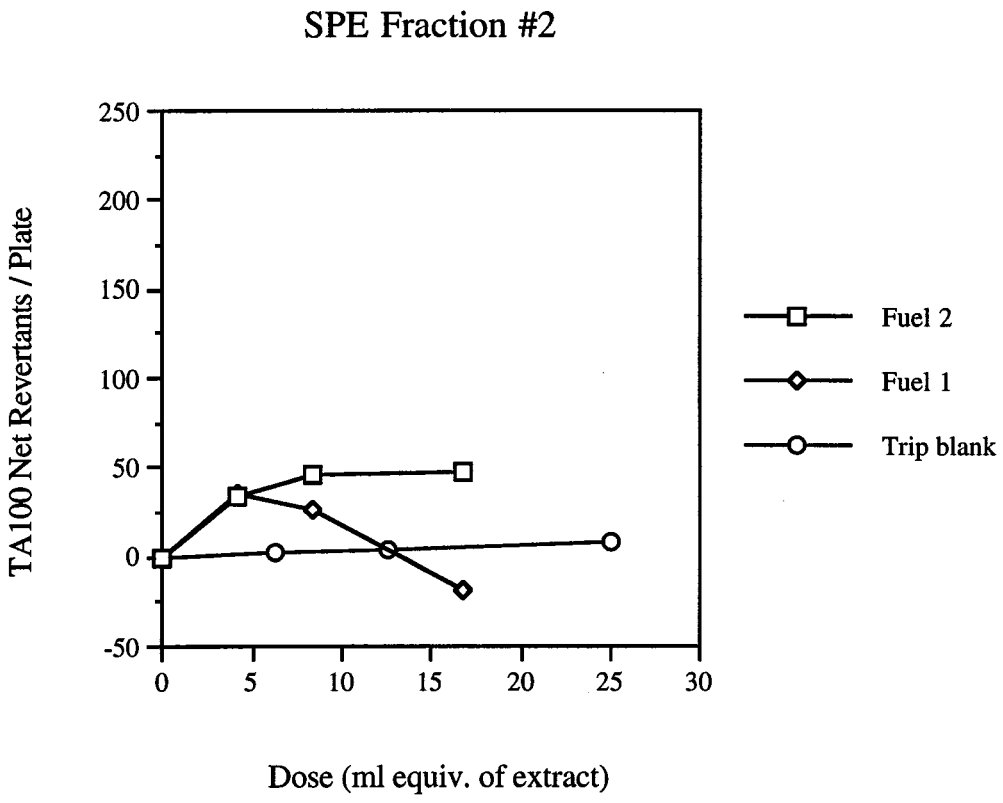


Figure 26. Dose-response relationship of SPE Fraction #2 (methanol: acetonitrile) from combined PUF hot start extracts for Fuel 1 and Fuel 2 as tested in tester strain TA100 with metabolic activation (+S9). Volume equivalent of extract represents mL of original SPE extract.

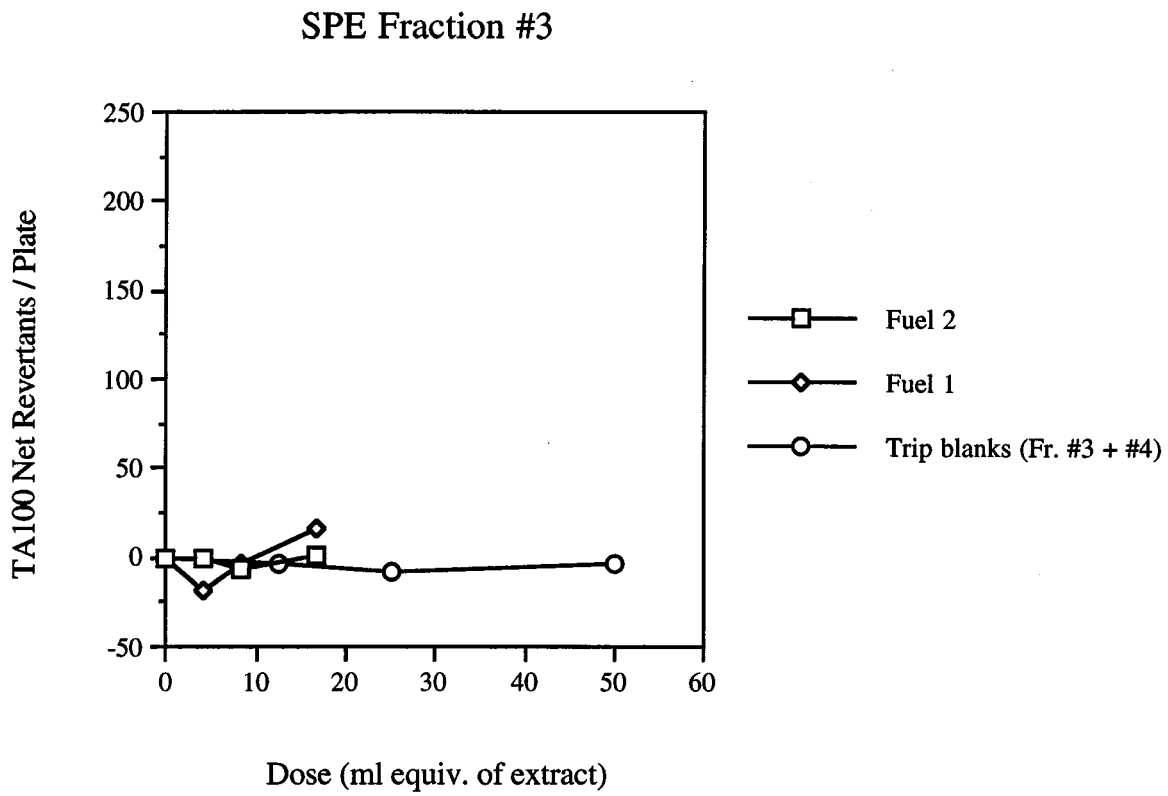


Figure 27. Dose-response relationship of SPE Fraction #3 (acetonitrile) from combined PUF hot start extracts for Fuel 1 and Fuel 2 as tested in tester strain TA100 with metabolic activation (+S9). Volume equivalent of extract represents mL of original SPE extract.

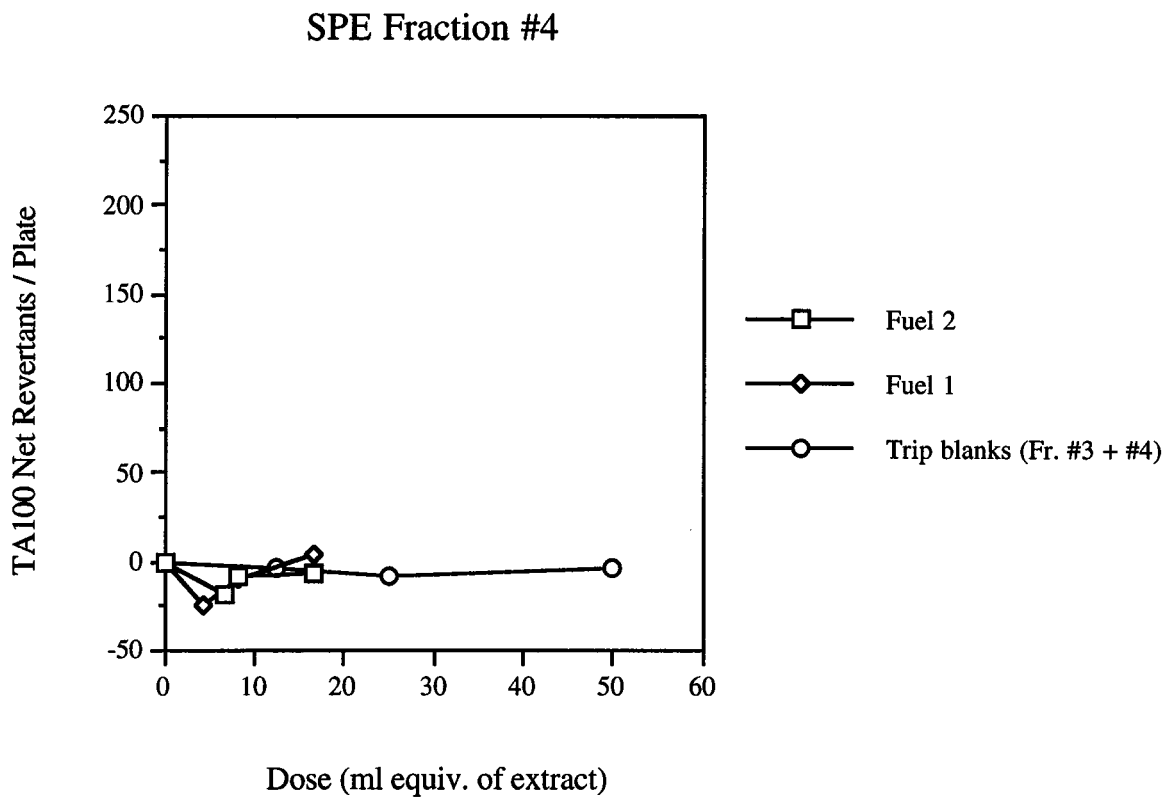


Figure 28. Dose-response relationship of SPE Fraction #4 (DCM) from combined PUF hot start extracts for Fuel 1 and Fuel 2 as tested in tester strain TA100 with metabolic activation (+S9). Volume equivalent of extract represents mL of original SPE extract.

However, significantly elevated levels of mutagenicity were observed for both Fuel 1 and Fuel 2 PUF exhaust samples in SPE Fraction #5 (hexane). The levels of mutagenicity was much higher than that observed in either SPE Fractions #1 or #2.

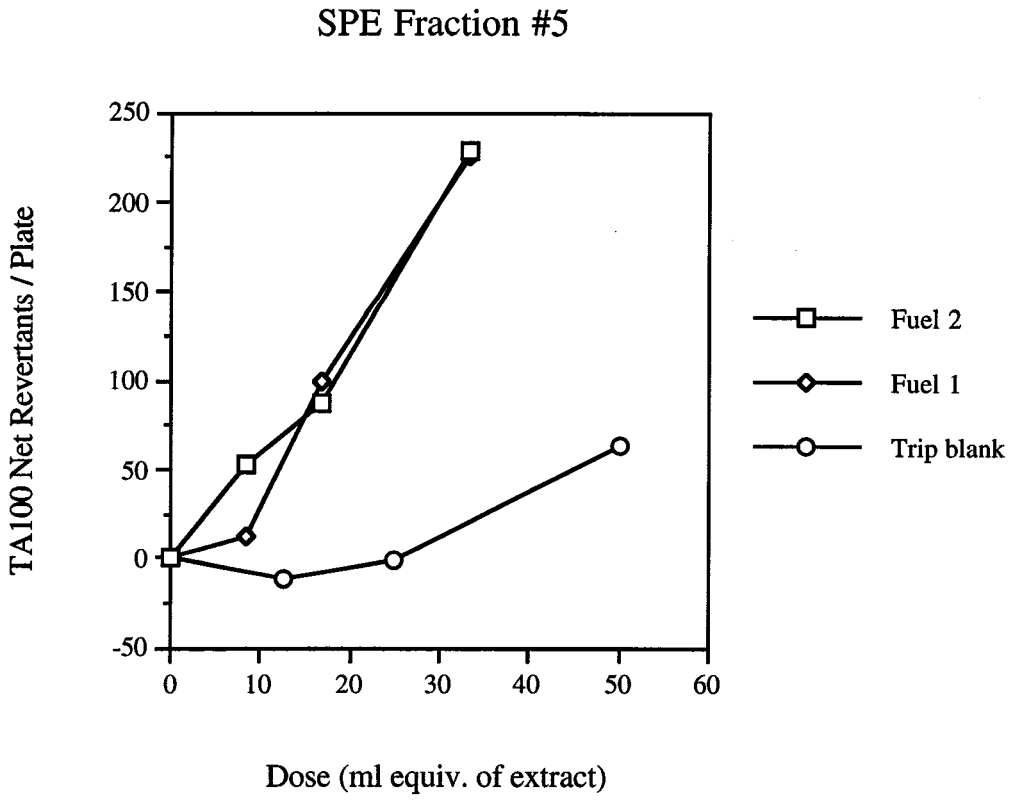


Figure 29. Dose-response relationship of SPE Fraction #5 (hexane) from combined PUF hot start extracts for Fuel 1 and Fuel 2 as tested in tester strain TA100 with metabolic activation (+S9). Volume equivalent of extract represents mL of original SPE extract.

Based on the information obtained from the dose-response curves for the individual SPE fractions from the pooled PUF sample extracts, a mutagram was constructed as illustrated in Figure 30. The mutagram shows the approximate total number of TA100 revertants as extrapolated back to the PUF sample extract prior to SPE fractionation. Similar to the dose-response curves, there were significantly elevated levels of mutagenicity observed for both the Fuel 1 and Fuel 2 PUF exhaust samples in Fraction #5 (hexane). Based on this mutagram, there was an approximate 15 to 20% increase in total mutagenic activity (calculated from the sum of all fractions) for the emissions from Fuel 1 compared to those from Fuel 2.

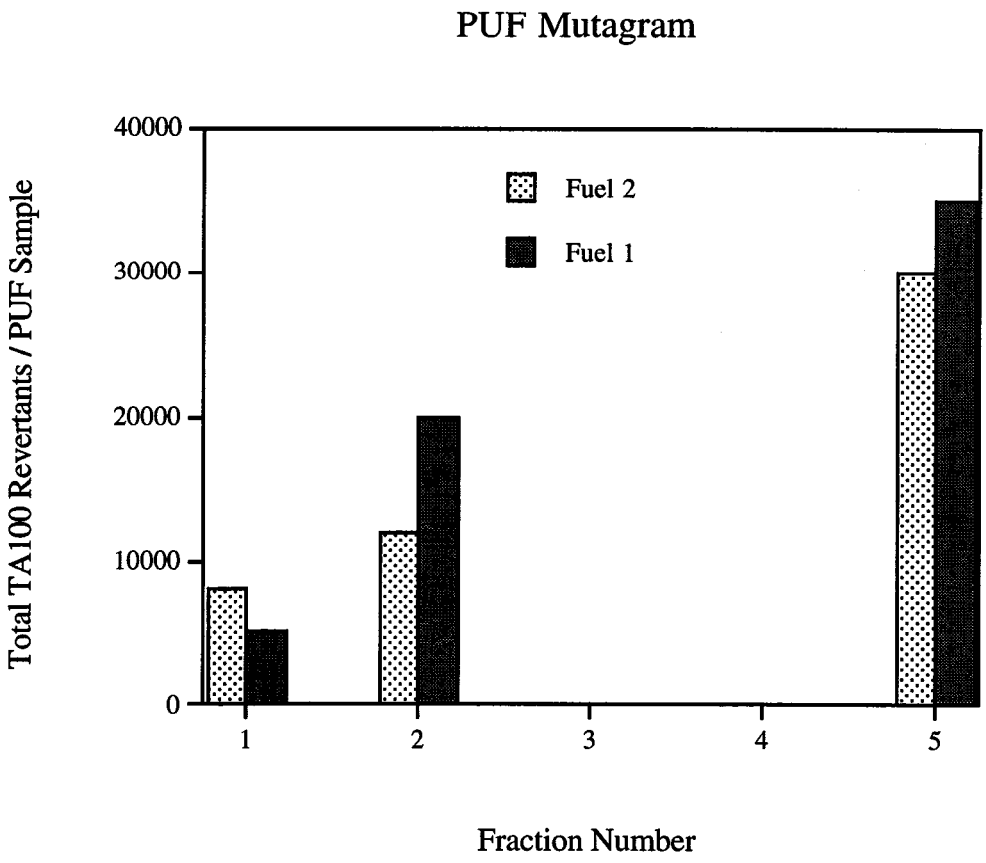


Figure 30. Mutagram of SPE Fractions from combined PUF hot start extracts for Fuel 1 and Fuel 2 as tested in Tester strain TA100 with metabolic activation (+S9).

4. Summary

HPLC was originally proposed to fractionate the filter extracts and the SFE extracts from the PUF samples. Unfortunately, model mixtures consisting of PAHs and substituted PAHs were not easily recovered when injected onto the HPLC, with chemical recoveries around 40%. To improve chemical recoveries, a solid phase extraction (SPE) technique was used. Model mixtures of the PAHs, substituted PAHs were spiked onto the C-18 cartridges. Compared to recoveries using HPLC, the overall recoveries using SPE increased for the substituted PAHs and

ranged from 70-90%. Since the results of this recovery study were acceptable, we proceeded to fractionate the filter extracts and the SFE extracts from the PUF samples using this technique.

For each filter or PUF sample extract, five (5) fractions were collected. Each fraction was chemically analyzed by GC/MS and tested for mutagenicity. Each fraction was a complex mixture of compounds and many of the peaks were poorly resolved, resulting in poor mass spectra. Most of aromatic compounds were found in Fraction #1 (methanol) and in Fraction #2 (methanol:acetonitrile). Chemical analyses of these fractions indicate that the exhaust from Fuel• 2 contained higher levels of C_1-, C_2-, and C_3-tetrahydronaphthalenes. The partially hydrogenated naphthalenes were found mainly in Fraction #2, while PAHs and substituted PAHs were found in both Fractions #1 and #2, respectively. The major components in Fractions #3, #4, and #5 appear to be saturated hydrocarbons. Further fractionation of these fractions would be required to obtain more detailed chemical information. However, due to the limited amount of sample obtained, we were not able to subfractionate these fractions.

The filter sample extracts that were pooled and fractionated by SPE were subsequently tested for mutagenic activity. Linear dose-response curves were developed for the fractions with and without the addition of metabolic enzymes. The specific mutagenic activities based on the slope of the dose-response curve were used to determine the total mutagenic activity per fraction. Mutagenic activity profiles were developed for the particle extracts from each fuel. Almost all of the mutagenic activity was observed in Fraction 1 for both fuel types. The mutagenicity profiles were nearly identical for the exhaust from Fuel 1 and Fuel 2.

The PUF sample SFE extracts that were pooled and fractionated by SPE were subsequently tested for mutagenic activity. There appears to be slightly elevated levels of mutagenicity in SPE Fraction #1 from Fuel 1 and Fuel 2 PUF exhaust sample extracts, with possible toxicity in the Fuel 2 PUF exhaust sample at the highest concentrate tested. Elevated levels of mutagenicity were also observed in SPE Fraction #2, with toxicity observed in the Fuel 1

PUF exhaust sample. There were no indications of mutagenicity for SPE Fractions #3 or #4 at the doses tested. However, significantly elevated levels of mutagenicity were observed for both Fuel 1 and Fuel 2 PUF exhaust samples in SPE Fraction #5. The levels of mutagenicity was much higher than that observed in either SPE Fractions #1 or #2. Chemical analysis of Fraction #5 (hexane fraction) was completed and was dominated by saturated hydrocarbons. The total levels of mutagenic activity per PUF sample were calculated by summing the activity of all fractions from each fuel type. The total levels of mutagenic activity were approximately 15 to 20% higher in the emissions from the Fuel 1 than in those from Fuel 2.