ABSTRACT

Carbonyl compounds exist in air, water, food, biological fluids and tissues, as well as nonbiological materials. Many methods have been developed to detect carbonyl compounds in the air, including numerous derivatization reagents to make carbonyl compounds more stable, multiple extraction methods, as well as multiple detection methods. These methods can be costly and time consuming, and also make use of toxic solvents. We sought a quick and inexpensive method to detect and quantify total carbonyls in air samples, specifically diesel exhaust samples, as well as extracellular media in biological samples,

We derivatized standard carbonyl compounds with 2,4-dinitrophenylhydrazine (DNPH); heptane was more efficient than pentane or hexane in extraction of the derivatives and leaves excess DNPH almost entirely in the aqueous phase. To test the usefulness of this technique carbonyls from diesel exhaust were collected on Sep-Pak DNPH-Silica cartridges, eluted, extracted with heptane, and analyzed by spectrophotometry and HPLC/UV/MS.

Linear regression analysis indicated a strong correlation (0.9821) between the UV absorbance (365 nm) of each sample and the total mass of carbonyl derivatives separated by HPLC and determined by MS. The usefulness of this technique was also tested on a biological sample. BEAS-2B cells were exposed to differing concentrations of residual oil fly ash (ROFA). UV absorbance increased with increasing ROFA concentrations, suggesting that carbonyl groups (products of lipid peroxidation) increased with increasing concentrations of ROFA. These results indicate that a quick and inexpensive method to detect and quantify total carbonyls in air samples, specifically diesel exhaust samples, as well as extracellular media in biological samples, may be achieved through the extraction of carbonyl derivatives with heptane and subsequent detection by spectrophotometry. In the future, there is potential to use this method to detect carbonyls in food and water samples.

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Spectrophotometric Determination of Carbonyls in Air and Biological Samples; A Faster, Cheaper Method to Quantify Total Carbonyls

By

Lars D. Perlmutt

1.0 BACKGROUND

1.1 Air Pollution

The history of air pollution and its deleterious effects on humans is well chronicled. As far back as 1306 King Edward stated "be it known to all within the sound of my voice, whosoever shall be found guilty of burning coal shall suffer the loss of his head," thus banning the use of coal (Hazucha, 2007). The serious consequences of exposure to high levels of ambient air pollution became evident as a result of several incidents in the 20th century. The area of Liege on the River Meuse in Belgium was once one of the most heavily industrialized areas of Europe, with steel mills, zinc smelters, glass manufacturing factories, as well as fertilizer and explosives plants. From December 1 to December 5, 1930, a thick fog covered the region. Beginning on December 3, 1930, hundreds began to experience respiratory symptoms, with over 60 people dying the next three days. The committee report on this fog concluded that morbidity and mortality was solely due to respiratory tract alterations (Nemery et *al.*, 2000). Also detailed for the first time is the role of temperature inversion and fog in air pollution. It was concluded that the burning of coal, both by factories and households was the main factor in morbidity and mortality (Nemery et *al.*, 2001).

In the United States, the first severe photochemical smog episode occurred in Los Angeles on July 26, 1943. The Los Angeles episode was different from the Meuse Valley

episode in that the episode occurred in the summer, yet was still influenced by an inversion layer, which trapped air pollutants at the surface. This inversion layer, in combination with low horizontal air movement and lack of turbulence, prevented contaminants from dispersing in the upper atmosphere. The principal health effects from the smog episode were temporary eye irritation, with the sources of irritation reported as unknown at the time (Senn et *al.*, 1948).

Perhaps the most famous air pollution disaster is the London Fog episode of 1952. With foggy weather, coal-burning homes, factories and power plants, a dense fog laid over London from December 5, 1952 to December 8, 1952. Official government reports indicate that the fog resulted in approximately 3000 more deaths than normal during the first three weeks of December. However, a reassessment of data by Bell et *al.* (2001) indicates that about 12,000 excess deaths occurred from December 1952 to February 1953 due to the acute, as well as persisting effects from the December 1952 fog episode. Mortality rates from this period were 50-300% higher than the previous year. Further, SO_2 levels (average concentration of 0.57 ppm) were 19 times greater than current US NAAQS standards for SO_2 (24-hr average 0.03 ppm) (Bell et *al.*, 2001). Finally, from December 3 to December 10, the relative risk for daily mortality and the previous day's pollution levels was 1.27 for a 0.10-ppm increase in the previous day's SO_2 level. Total suspended matter (TSM) was, at average concentrations of 1400 $\mu g/m^3$ from December 5-8, 1952, almost 10 times higher than the current U.S. NAAQS for PM_{10} (150 $\mu g/m^3$) (US EPA, 2009). For a 100 $\mu g/m^3$ increase in TSM from the previous days, the associated relative risk for mortality was 1.08 (Bell et *al.*, 2001).

Air pollution remains expansive throughout the world, representing one of the most widespread risks to population health. Some air quality projections have shown that in many locations, both in the developed and developing world, pollutant concentrations might not be

reduced significantly over the next 15 to 20 years (Craig et *al.*, 2008). Even in places where air quality standards are being met, extensive scientific data indicates a correlation between both short-term and long-term exposure to ambient air pollution and human health effects (Craig et *al.*, 2008). The World Health Organization estimates that air pollution is responsible for 1.4% of deaths worldwide, as well as 0.8% of disability-adjusted life years (WHO, 2002). In a study by Cohen et *al.* (2005), outdoor particulate matter (PM) air pollution in urban areas is estimated to be responsible for approximately 3% of adult cardiopulmonary disease mortality, 5% of trachea, bronchus, and lung cancer mortality, and about 1% of mortality in children as a result of acute respiratory infection. These figures amount to 0.80 million premature deaths (1.2%) and 6.4 million lost life years (0.5%) (Cohen et *al.*, 2005).

1.2 Sources of Air Pollution

Air pollutants can either be emitted directly into the atmosphere (primary pollutants) or may be formed within the atmosphere itself (secondary pollutants). Pollutants can be defined by their physical state (gaseous or particulate matter), as well as by their geographical scale (point, line, area, and regional sources). Primary pollutants include sulfur dioxide (SO_2), oxides of nitrogen (NO_x), carbon monoxide (CO), volatile organic compounds (VOC), carbonaceous particles, and non-carbonaceous particles. Particulate matter (PM) is classified by the diameter of the particle, and the EPA has standards for both PM_{10} (coarse particles), particles between 2.5 μm and 10 μm, and $PM_{2.5}$ (fine particles), for those particles smaller than 2.5 μm in diameter. Ultrafine particles ($PM_{0.2}$) are also a primary pollutant present in the atmosphere (US EPA, 2009). The only entirely secondary pollutant regulated by the US EPA National Ambient Air Quality Standards is ozone, but other secondary pollutants include secondary particulate matter, such as secondary

organic aerosols, which are oxidized organic compounds formed via reactions of VOC (Harrison, 2006).

The main source of SO_2 is the combustion of fuel sources containing sulfur, like coal and oil. Via combustion, sulfur in the source is almost converted quantitatively to SO_2 (Harrison et *al.*, 2006). The amount of sulfur in fossil fuel varies, and in developed countries, most is removed from motor fuels during the refining process. In Europe, the use of high sulfur coal for power generation and vehicle fuel has declined and thus the major source of SO_2 in the atmosphere have declined overall (Lipmann et *al.*, 2006). The advent of high chimney stacks in developed nations has also led to increases in SO_2 concentrations in rural areas, due to transport, with some rural areas having higher SO_2 concentrations than urban areas. The use of high-sulfur coal in developing nations is actually on the rise for power production, as well as its use in domestic cooking, and eating and ambient concentrations of SO_2 therefore remain at high concentrations (Lipmann, 2006). Finally, natural sources of SO_2 exist, such as volcanoes.

As with sulfur, the combustion of fuel containing nitrogen converts the nitrogen into oxides of nitrogen. Oil and gas contain a much lower nitrogen content, so coal is the main culprit in the formation of NO_x. But, NO_x are also formed when atmospheric nitrogen combines with oxygen during high-temperature combustion, which occurs in all high-temperature combustion processes and is thus a huge reason road traffic and power generation are the major sources of NO_x (Harrison et *al.*, 2006). On a global scale, however, natural emissions of NOx far outweigh anthropogenic sources. Such sources include volcanic and bacterial action, lightning, and intrusion of stratospheric nitrogen oxides. These global emissions, though, are dispersed throughout the Earth, and therefore background levels of NO_x are relatively low (Forastiere et *al.*, 2006).

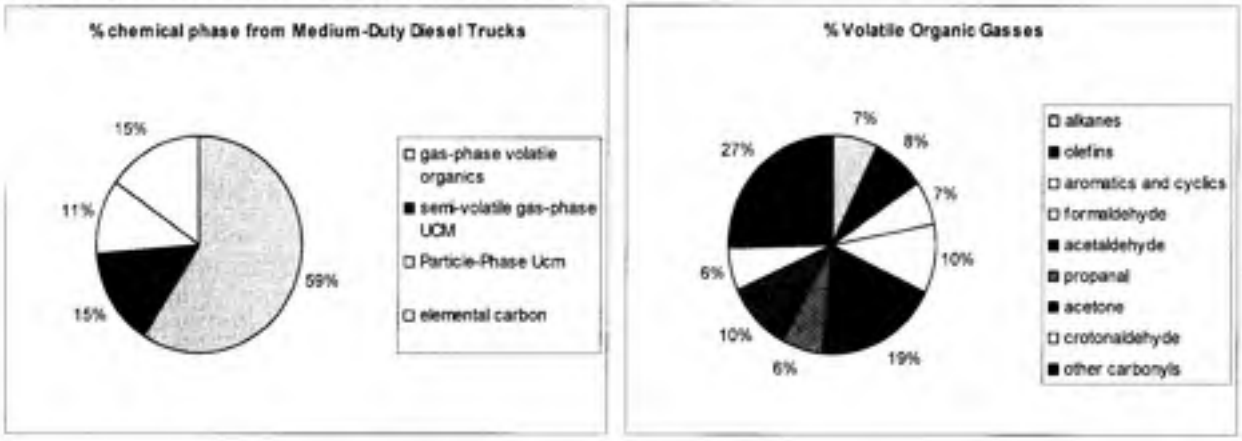
Volatile organic compounds (VOCs) include a wide range of hydrocarbons, oxygenates, halogenates, and other carbon containing compounds existing in the vapor phase in the atmosphere. VOCs, along with NO_x, are the precursors in the complex formation of tropospheric ozone. A major source of VOCs includes the combustion of fossil fuels. These processes contain unburned fuel fragments which are then emitted in the form of VOC (Harrison et *al.*, 2006).

1.3 Carbonyls As a Component of Air Pollution

The majority of carbonyls (i.e. aldehydes and ketones), types of VOC, in the atmosphere are considered secondary air pollutants with the potential for deleterious human health and environmental effects. Other carbonyls that are not volatile are primarily attached to particulate matter. A major source of primary carbonyls in the air is the combustion of fuel. Combustion of fuel is an oxidative process in which alkanes (such as those found in fossil diesel) are oxidized to alcohols, then carbonyls, next to carboxylic acids, then esters and finally to carbon dioxide (Guarieiro et *al.*, 2008). However, combustion of fossil fuels is not 100% efficient. Conditions are favorable for hydroxylation of fossil fuels when the fuel has been vaporized and adequately mixed with air before being burned. However, when burning ceases, the oxidation process halts at an intermediate state, leaving byproducts such as aldehydes (Boerlage et *al.*, 1936). In a typical medium-duty diesel truck the majority of byproducts as a result of combustion are gas-phase volatile organics (58.7%) by weight, while 14.7% are semi-volatile gas-phase unresolved complex mixture (UCM), 11.3% particle-phase UCM, and 15.2% elemental carbon. Of the gas-phase volatile organics (Figure 1.3.1), approximately 10.3% is formaldehyde, 19.3% acetaldehyde, 6.5% propanal, 6.2% crotonaldehyde, 25.6% other carbonyl compounds, 7.3%

alkanes, 8.0% olefins, and 6.5% aromatics and cyclics (Schauer et *al.*, 1999). These results are generated from medium duty diesel trucks driven through the hot-start Federal Test Procedure.

Figure 1.3.1 Chemical Phases of Diesel Exhaust and Proportion of Chemicals within Volatile Organic Gas Phase (Schauer et *al.*, 1999)



It is evident that carbonyls comprise a high percentage of the gas-phase volatile organics. With regards to particulate organic carbon emissions from heavy-duty diesel powered vehicles, depending on operating conditions, the carbonyl fraction of particulate matter is merely 3.3-3.9% (Destaillats et *al.*, 2007). These data, however, fail to include the contribution of formaldehyde and acetaldehyde to particulate organic carbon emissions. Jakober et *al.* (2006) notes that in general, emissions factors for carbonyls in the gas phase exceed those of the particulate phase, and for those compounds that are observed in both phases, gas-phase emissions are at least a factor of 10 or greater than those for the particulate phase.

Secondary carbonyl compounds in the atmosphere are formed via the following reactions (Carlier et *al.*, 1986):

(1) $R\cdot + O_2 \rightarrow RO_2$

(2) $RO_2\cdot + NO\cdot \rightarrow RO\cdot + NO_2$

(3) $RO\cdot + O_2 \rightarrow$ Carbonyl compound + HO_2

Thus, any organic radical, like hydroxyl radicals ($OH\cdot$), with the unpaired electron on a carbon atom reacts in the atmosphere to form carbonyl compounds.

Alkyl ($RONO_2$) and peroxyalkyl nitrates ($ROONO_2$) are thermally unstable, and can therefore be photolyzed, yielding more free radicals, and thus more carbonyl compounds are formed via the following reactions (Carlier et *al.*, 1986):

(4) $RO\cdot + NO_2\cdot \rightarrow RONO_2$

(5) $RO_2\cdot + NO_2\cdot \rightarrow ROONO_2$

At night, nitric oxide radical ($NO\cdot$) concentrations are low, so reaction (2) does not occur and RO_2 reacts mainly via reaction 5, but also via the following reactions (Carlier et *al.*, 1986):

(6) $RO_2\cdot + O_3 \rightarrow RO\cdot + 2O_2$

(7) $R_1O_2 + R_2O_2 \rightarrow R_1O\cdot + R_2O\cdot + O_2$

(8) $R_1O_2 + R_2O_2 \rightarrow R_1\text{-}O\text{-}O\text{-}R_2 + O_2$

(9) $R_1O_2 + R_2O_2 \rightarrow$ alcohol + aldehydes + O_2

In reactions 7-9, R_1 and R_2 refer to hydrogen or organic groups and when R_1 or R_2 equals H, only reaction 8 proceeds. When the organic group has a α-H, reaction 9 may proceed (Carlier et *al.*, 1986).

Secondary carbonyl groups are formed at the greatest rate during the day, with strong sunlight, and the production of carbonyls is determined by the formation of free radials, the hydroxyl radical, from organic compounds in the atmosphere. These reactions depend both on the concentration of the reactive species, like hydroxyl radicals, ozone (O_3), and nitrate (NO_3), as well as the rate constants between the reactive species and organic compounds of the troposphere (Carlier et *al.*, 1986).

Carbonyl compounds are also a crucial component in the formation of tropospheric ozone. Carbonyls undergo photolysis and react with nitrate ($NO_3\cdot$) and hydroxyl ($OH\cdot$) radicals. Formaldehyde gives hydroxyl radicals via photolysis. Formaldehyde also reacts with $OH\cdot$ radicals to produce H_2O and a formyl radical ($HCO\cdot$), which then subsequently reacts with O_2 to form HO_2 and CO (Seinfeld et *al.*, 1989):

$$OH + HCHO \rightarrow H_2O + HCO\cdot$$

$$HCO\cdot + O_2 \rightarrow HO_2 + CO$$

This is important in that HO_2, in the presence of NO leads to NO_2 and OH (Seinfeld et *al.*, 1989):

$$HO_2 + NO \rightarrow NO_2 + OH\cdot$$

NO_2 is thus created without using up a molecule of O_3, and thus enabling O_3 to accumulate in the atmosphere. NO_2 undergoes photolysis to produce NO plus O and O_3 is then produced when the OH radicals produced via the dissociation of aldehydes attack the hydrocarbons, forming organic peroxy radicals. The organic peroxy radicals, in the presence of O_2 and sunlight go onto form O_3 (Seinfeld et *al.*, 1989).

$$NO_2 + h\nu \rightarrow NO + O$$

$$RO_2 + O_2 + h\nu \rightarrow RO + O_3$$

Carbonyls have different degradation rates in the atmosphere, as well as seasonal differences in rates of degradation between summer and winter. Formaldehyde is photo-oxidized in sunlight to carbon dioxide, representing the major mechanism in which formaldehyde is removed from the atmosphere. The average half-life of this reaction is approximately 50 minutes (COMEAP, 2000). Photochemical degradation of formaldehyde, and other aldehydes, such as acetaldehyde, occurs via the interaction with reactive species like OH and NO_3 radicals

(COMEAP, 2000). It has been estimated by AEA Technology (1999) that the boundary layer lifetime of formaldehyde with respect to removal by OH radicals in the winter is on average 6.2 days and in the summer 1.2 days, and removal by NO_3 radicals is on average 80 days. The average boundary layer lifetime of acetaldehyde with respect to removal by reaction with OH radicals is, on average, 3.7 days in the winter, and 18 hours in the summer, and by reaction with NO_3 radicals, on average removal is 17 days (AEA Technology, 1999). Removal of formaldehyde and acetaldehyde in the winter average, by direct photolysis, 1.3 days and 67 days, respectively, while the summertime average via direct photolysis for formaldehyde is 6.3 hours and 5.2 days for acetaldehyde (AEA Technology, 1999). The large difference between summer and winter rates is due to the fact that in the summer months, more intense photoradiation and also as higher temperatures provide the proper conditions for the faster degradation of chemicals.

Outdoors, the most abundant carbonyls expressed as percentages of Los Angeles carbonyl air content collected in 1993 were formaldehyde (24%), acetaldehyde (18%), and acetone (7%) (Grosjean et *al.*, 1996). Other main carbonyls in ambient and indoor air, by concentration include acrolein, propanal, and benzaldehyde (Figure 1.3.1).

Generally, in remote, unpopulated regions of the world, ambient air concentrations of formaldehyde are less than 0.001 mg/m^3 (0.008 ppm) (COMEAP, 2000). In populated areas, though, concentrations are usually in the range of 0.001-0.02 mg/m^3 (0.008-0.016 ppm), but can be as high as 0.01 mg/m^3 (0.08 ppm) during periods of heavy traffic or during atmospheric inversion periods (IARC, 1995). In the outdoor environment, levels of formaldehyde are highest in urban areas during high temperature atmospheric conditions with low wind and therefore, little mixing. Further, in areas with high concentrations of vehicles lacking catalytic converters, formaldehyde concentrations will be higher (Vermont DOH, 2005). Exposure to tobacco smoke

is also a major source of exposure to formaldehyde and a pack-a-day smoker can inhale as much as 0.4 mg of formaldehyde (IARC, 2006). In the occupational setting, the highest continuous exposures to formaldehyde occur in particleboard mills, during the varnishing of furniture and wooden floors, where concentrations are often greater than 1 mg/m^3 (~8.0 ppm) [IARC, 2005]. In the non-occupational setting other indoor sources of formaldehyde include upholstery, carpets, cardboard and paper products, and urea formaldehyde foam insulation (The Office of Environmental Health Hazard Assessment, 2008). Non-workplace indoor air concentrations of formaldehyde average 0.0359 mg/m^3 in conventional residences, while mobile homes have an average formaldehyde concentration of 0.12 mg/m^3 (IARC, 2006). In Moncure, NC, formaldehyde levels in the air are particularly high, as a result of the manufacturing of particle board. Moncure has the sixth highest release in the United States of formaldehyde into the environment, based on 2002 rankings, with approximately 250,000 pounds released into the environment by the Sierrapine Factory, a factory producing composite board (Scorecard, 2005).

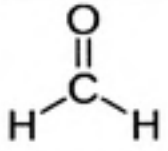
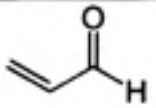
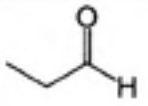
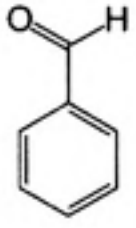
Acrolein is formed naturally in the atmosphere via photochemical oxidation of hydrocarbons, particularly from 1,3-butadiene, but has also been identified in emissions from pine forests in Europe and high altitude areas with little vegetation, like Nepal (COMEAP, 2000). Acrolein is also emitted from forest fires as a result of incomplete combustion of organic matter (Lipari et *al.*, 1984). Ambient air concentrations of acrolein range from about 0.004-0.016 mg/m^3 (0.002-0.007 ppm) in the United States (Grosjean et *al.*, 1990). Photolysis degradation of acrolein is minimal (Maldotti et *al.*, 1980) and the major degradation pathway occurs via reactions with hydroxyl radicals. Products of this reaction include carbon monoxide, formaldehyde, glycoaldehyde, ketene, and peroxypropenyl nitrate (WHO, 1992). Acrolein typically remains in the atmosphere for approximately one day (WHO, 1992). Major indoor sources of acrolein

include cigarette smoke, wood-burning stoves and fireplaces. Concentrations of acrolein in smoky public places have been measured as high as 0.23 mg/m^3 (0.1 ppm) [COMEAP, 2000]. Indoors, concentrations of acrolein in homes without environmental tobacco smoke average 2.2 μg/m^3, while those homes with environmental tobacco smoke have average acrolein concentrations of 3.0 μg/m^3 (Gomes et *al.*, 2002).

Acetaldehyde has been measured in once thought to be non-polluted areas, like the Arctic, and found to be present in concentrations of 0-0.00043 mg/m^3 [0-0.0003 ppm] (Cavanagh et al., 1969). In urban areas, acetaldehyde concentrations are as high as 0.063 mg/m^3 during episodes of moderate to severe photochemical pollution in the United States (Grosjean et *al.*, 1982). Indoor concentrations of acetaldehydes are dominated by non-combustion sources and include emissions from interior finish materials, like vinyl flooring and carpet, as well as wood-based products, like fiberboard and particleboard. Emissions concentrations from these products ranged from 4.6 to 26 μg/m^3 (2.6-14 ppb) (The Office of Environmental Health Hazard Assessment, 2008). Indoor concentrations of acetaldehyde range from 5-27 μg/m^3, while in a tavern with people smoking, the acetaldehyde concentration has been measured as high as 203 μg/m^3 (California EPA ARB, 1993).

It is thus evident that cigarette smoke is a large contributor of carbonyl groups in indoor air. On a per cigarette basis, acetaldehyde is the most prevalent, with a concentration of 709 μg/cigarette, followed by acrolein (82 μg/cigarette), formaldehyde (54 μg/cigarette), and crotonaldehyde (15 μg/cigarette). A side stream of smoke contains 12 times the concentration of acrolein than mainstream smoke (O'brien et *al.*, 2005).

Table 1.3.1 Main Carbonyl Compounds, By Concentration, In Ambient and Indoor air

Carbonyl Compound	**CAS Number**	**Molecular Weight**	**Structure**	**% of Gas Phase in Diesel Exhaust (Schauer, 1999)**
Formaldehyde (methanal)	50-00-00	30.02		10.0
Acetaldehyde (ethanal)	75-07-0	44.05		19.0
Acrolein (acraldehyde)	107-02-08	56.06		1.7
Propanal (propionaldehyde)	123-38-6	58.08		6.0
Acetone (propan-2-one)	67-64-1	58.08		10.0
Benzaldehyde	100-52-7	106.12		1.9

1.4 Other Sources of Carbonyls

Carbonyls are also produced endogenously during metabolism, a classic example being alcohol metabolism. Background levels of exocyclic propane-etheno-DNA adducts in tissues result from endogenous alkenals, hydroxyalkenals, dialdehydes, and alkanal products of lipid peroxidation decomposition (Bartsch et *al.*, 1999). Further, other lipid peroxidation aldehydes decomposition products are thought to be responsible for many endogenous protein adducts (O'Brien et *al.*, 2005). Other products ingested exogenously, like drugs or environmental agents, can undergo biotransformation, leading to reactive aldehydes metabolites or induction of lipid peroxidation. For example, in mice, increases in plasma and urinary aldehydes occur with iron overload (Bartfay et *al.*, 1999). Formaldehyde is also formed via oxidative stress, when polyamine is oxidized by reactive oxygen species (O'Brien et *al.*, 2005).

Madden and Ghio (1999) studied the effects of residual oil fly ash (ROFA), a metal rich component of ambient air particulate matter, on the endogenous production of carbonyls in the lungs. Instilled with differing concentrations of ROFA, when lavage fluids from rats were derivatized by DNPH, dose-dependent increases of acetaldehyde were observed. Further, increased formation of acetaldehyde was seen in BEAS-2B human airway cells incubated in vitro with ROFA. Soluble metals (V, Ni, and Fe) alone also induced an increase in acetaldehyde. The study concludes that metals on PM can induce lipid peroxidation and carbonyl formation (Madden et *al.*, 1999).

Aldehydes are also present in food sources. Methanol, present in fruit and vegetables, fruit juices, as well as fermented beverages, and ethanol, found in alcoholic beverages, are enzymatically converted to formaldehyde and acetaldehyde. Formaldehyde can be found in fruit, vegetables, meat, cheese and seafood. Acetaldehyde is a natural component of many fluids, with

concentrations in vegetables ranging from 0.2-400 μg/g, and in wine from 0.7-290 μg/g. During the cooking of fat-containing foods, formaldehyde, acetaldehyde and acrolein are all produced (Svensson et *al.*, 1999). Almonds and cherries contain benzaldehyde, while the unripe odor is caused by hexanal and 2-hexenal. Dietary aldehydes are more varied than environmental aldehydes, which are largely dominated by formaldehyde and to a lesser extent, acetaldehyde, acrolein, glyoxal, and methylglyoxal (O'Brien et *al.*, 2005).

Even perfume use, as well as use of nail polish removers and hardeners, is a source of carbonyls. Carbonyls are used extensively for their fragrant properties (Liu et *al.*, 2007).

1.5 Health Effects of Aldehydes

The Clean Air Act Amendments of 1990 as specified in section 112(b), list a number of carbonyls as Hazardous Air Pollutants (HAP), pollutants known to cause or reasonably thought to cause adverse human health and or environmental effects. Carbonyls included among the 188 specific pollutants include acetaldehyde, acetophenone, acrolein, 2-chloroacetophenone, formaldehyde, hydroquinone, isophorone, methyl ethyl ketone, methyl isobutyl ketone, beta-propiolactone, propionaldehyde, and quinone (US EPA, 2008).

1.5.1 Formaldehyde

Formaldehyde (CAS Number 50-00-0) can be inhaled directly from the vapor phase of air or absorbed via respiratory particles, and the dose supplied to the pulmonary compartment via inhalation of the vapor phase is orders of magnitude greater than the dose from the inhalation of particulates (Rothenberg et *al.*, 1989; Risby et *al.*, 1990). Acute exposure to formaldehyde yields different effects, depending on the concentration. At concentrations of 2.5-3.7 mg/m^3 (2-3 ppm), exposure to formaldehyde vapor initiates mild tingling in the eyes, nose and posterior pharynx (COMEAP, 2000), while concentrations greater than 7.4 mg/m^3 could affect the lower

airways (Imbus, 1985). Breathing difficulties and severe burning of the nose and throat occur at concentrations of 12-25 mg/m^3 (10-20 ppm) (Fassett et *al.*, 1963) while pulmonary edema, pneumonitis and pneumonia have occurred after inhalation of formaldehyde at concentrations of 60-120 mg/m^3 (50-100 ppm) (Proctor et *al.*, 1988). The WHO (1987) reports that death occurs at concentrations of 125 mg/m^3.

Formaldehyde is classified as a group 1 carcinogen by IARC based on sufficient evidence in human and animal studies (IARC et *al.*, 2006) and as a probable carcinogen by the US EPA (group B1) [US EPA IRIS, 1991], based on sufficient evidence in experimental animal studies with chronic exposure and limited evidence in humans. At inhalation concentrations of 8 μg/m^3, US EPA IRIS has identified squamous cell carcinoma risk at 1 in 10,000, risk of 1 in 100,000 at concentrations of 0.8 μg/m^3, and a 1 in 1,000,000 risk at inhalation concentrations of 0.08 μg/m^3 (US EPA IRIS, 1991). Case-control studies indicate a causal relationship between the highest categorized levels of formaldehyde exposure and the incidence of nasopharyngeal cancers (COMEAP, 2000), but, in cohort studies this association is limited by the relatively small numbers of cases that were observed.

As stated earlier, endogenous production of formaldehyde occurs as an intermediate, via the metabolism of other molecules, and occurs in all human cells. Blood concentrations of those not exposed to exogenous sources of formaldehyde are generally in the range of 2-3 mg/L and these concentrations are not altered greatly by inhalation exposure (Heck et *al.*, 1985). It has been suggested that this is due to the rapid metabolism of formaldehyde by respiratory tissues where formaldehyde initially comes into contact, as well as by erythrocytes. Formaldehyde is metabolized to formate by all tissues of the body and the reaction is primarily catalyzed by formaldehyde deyhdrogenase, for which the substrate is the formaldehyde-glutathione

hemiacetal (COMEAP, 2000). Formaldehyde is also an intermediate in synthesizing purines, thymidine, as well as amino acids, so inhaled formaldehyde can either be metabolized to formate and carbon dioxide, or may be biosynthetically incorporated into macromolecules (IARC, 2006).

In vitro models indicate that formaldehyde is genotoxic in exposed human and laboratory animals and that cellular proliferation increases markedly at concentrations greater than 6 ppm. Thus, IARC (2006) concluded that genotoxicity and cytotoxicity play crucial roles in the carcinogenesis of formaldehyde in nasal tissues and raised the classification of formaldehyde to a group 1 carcinogen.

1.5.2Acrolein

Studies in experimental animals show that acrolein (CAS Number 107-02-8) is typically retained almost exclusively within the respiratory tract due to its high reactivity, with little to no distribution to other organ systems (COMEAP, 2000). Acrolein reacts quickly with thiols, like reduced glutathione, and also with sulphydryl and amino groups (Esterbauer et *al.*, 1991).

Acrolein is extremely pungent and has a choking odor. It is an intense eye and respiratory irritant at concentrations of 0.1-0.2 mg/m^3, and accidental exposure to high levels has produced symptoms such as fever, dyspnea, coughing, cyanosis, pulmonary edema, and long lasting pulmonary symptoms like chronic pneumopathy, bronchitis, and emphysema (COMEAP, 2000). In controlled exposure studies, eye irritation is reported in humans at a concentration of 0.13 mg/m^3 (0.06 ppm) and nasal irritation at concentrations of 0.34 mg/m^3 [0.15 ppm] (WHO, 1992). Acrolein is categorized by IARC as a group 3 compound, meaning that it is not classifiable as to its carcinogenicity based on a lack of evidence in both human and animal studies. The US EPA classifies acrolein as a possible human carcinogen, group C, but this is based primarily on one oral toxicity study in rats (US EPA IRIS, 2003).

1.5.3 Acetaldehyde

Animal studies indicate that inhaled acetaldehyde (CAS Number 75-07-0) is absorbed via the lungs and distributed to other body tissues. Human studies suggest that 45-70% of inhaled acetaldehyde vapor is retained by subjects at exposure concentrations of 0.1-0.8 μg/mL. It has been suggested that due to the irritant effects of acetaldehyde at low levels, exposure to higher levels would normally be limited. At low levels, acute exposure leads to irritation of the eyes, skin and respiratory tract, with drowsiness occurring at longer exposure times. At higher doses, nausea, vomiting, headache, loss of consciousness, and pulmonary edema have all been exhibited (COMEAP, 2000). The odor detection threshold, as summarized by Amoore and Hautala (1983) is 0.09 mg/m^3.

Myou et al. (1993) have indicated that acetaldehyde, as a product of ethanol metabolism, could be a causative factor for alcohol induced brochoconstriction in asthmatics. Chronic toxicity of acetaldehyde has been shown to cause liver damage, but no data exist regarding the chronic effects of exposure to acetaldehyde vapor (COMEAP, 2000). ALDH2 polymorphism has a distinct effect on an individuals' susceptibility to aldehydes, with 5% of Asians having no activity (homozygous variants) and 40% of Asians having intermediate activity (heterozygous), relative to the predominant wild type (O'Brien et *al.*, 2005). Homozygous and heterozygous variants were associated with increased buildup of acetaldehyde after ethanol ingestion, and those who are alcoholics or heavy drinkers have been associated with an increased esophagus and lung cancer risk (Ginsberg et *al.*, 2002). Peak plasma levels of acetaldehyde in homozygous and heterozygous variants, in comparison to normal individuals, were 20 and 6 times higher, respectively, when consuming two alcoholic drinks (Ginsberg et *al.*, 2002). ALDH1 might also be associated with acetaldehyde metabolism, as plasma acetaldehyde levels one hour after

ethanol ingestion were four times higher in an ALDH2 heterozygote individual with low ALDH1 compared to other ALDH2 individuals with normal ALDH1 activity (Ginsberg et *al.*, 2002).

Acetaldehyde is classified by IARC as a Group 2B carcinogen, and the US EPA classifies the compound as a group B2 carcinogen on the basis of an increased incidence of nasal tumors in rats and laryngeal tumors in hamsters following inhalation exposure (US EPA IRIS, 1991). At inhalation concentrations of 5 $\mu g/m^3$, US EPA IRIS has identified nasal squamous cell carcinoma risk and adenocarcinoma risk at 1 in 10,000, a risk of 1 in 100,000 at concentrations of 0.5 $\mu g/m^3$, and a 1 in 1,000,000 risk at inhalation concentrations of 0.05 $\mu g/m^3$ (US EPA IRISb, 1991).

1.5.4 Propionaldehyde

No information is available on the acute effects of propionaldehyde (CAS Number 123-38-6) in humans. In animal studies, acute inhalation exposure to propionaldehyde at high levels caused anesthesia and liver damage. No information is available on the chronic effects of exposure to propionaldehyde, and the US EPA has not classified propionaldehyde for carcinogenicity, due to a lack of information in human and animal studies (US EPA, 2000).

1.5.5 Acetone

Acetone (CAS Number 67-64-1) is rapidly absorbed via the respiratory and gastrointestinal tracts of humans as indicated by the detection of acetone in the blood within 30 minutes of inhalation and 20 minutes of oral administration (WHO, 1998). Acetone is uniformly distributed among non-adipose tissues and exhalation is the major route of elimination for both acetone and its terminal metabolite, CO_2 (WHO, 1998). Acetone, in comparison to other industrial solvents, is relatively less toxic, but at high concentrations, acetone vapor can cause central nervous system depression, cardiorespiratory failure, and death. Acute exposures as high

as 4700 mg/m^3 (2000 ppm) have been shown to produce either no toxic effects, or minor effects, such as eye irritation. More severe effects, such as vomiting and fainting, were induced in workers at concentrations of 25,500 mg/m^3 (12,000 ppm) for approximately 4 hours of exposure, while at acute concentrations of 595 mg/m^3 (250 ppm), performance in neurobehavioral tests was altered. Finally, menstrual irregularities were reported in females exposed to acetone vapor concentrations of 2370 mg/m^3 (1000 ppm) [WHO, 1998].

1.5.6 Benzaldehyde

According to the US EPA IRIS profile on benzaldehyde (100-52-7) no determination has been made for either carcinogenicity assessment or reference concentration for chronic inhalation (US EPA, 2008). According to a material safety data sheet on benzaldehyde, acute exposure leads to irritation of the eyes, throat, and nose and can produce central nervous system depression with the possibility of respiratory failure. Skin exposure to benzaldehyde can lead to a rash, while long-term exposure can lead to contact dermatitis (Emerald Performance Materials, 2006).

1.6 Methods of Detecting Airborne Aldehydes

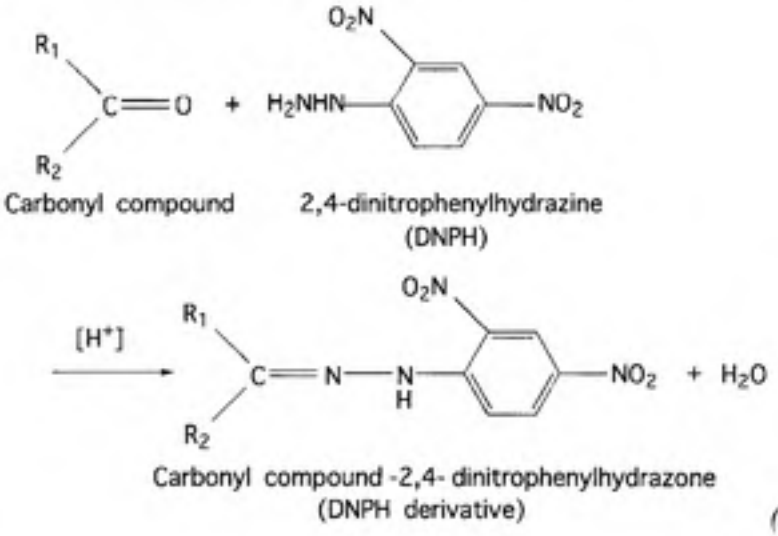
Methods for quantification of volatile carbonyls have been previously developed and these are discussed below. As volatile carbonyls are prone to evaporate and/or react, stabilization is achieved via chemical derivatization, with a large number of derivatization techniques existing. Derivatization can also reduce the volume of sample needed by reacting with capture reagents. Advantages and disadvantage of these techniques are discussed below.

1.6.1 Reagents Based On Nitroaromatic Hydrazines

Nitroaromatic hydrazines have been established as the most important derivatizing agents for the determination of carbonyl compounds and 2,4-dinitrophenylhydrazine (DNPH) is the

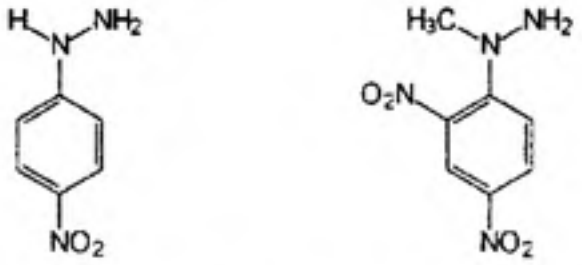
most popular derivatizing agent due to its rapid reaction with the analytes (Figure 1.6.1) (Vogel et *al.*, 2000). Other nitroaromatic hydrazines include 4-nitrophenylhydrazine (pNPH) and 1-methyl-1-(2,4-dinitrophenyl)hydrazine (MDNPH) (Figure 1.6.2).

Figure 1.6.1.1 Derivatization of carbonyl groups with DNPH



(Sakuragawa et *al., 1999)*

Figure 1.6.1.2 Molecular structure of nitroaromatic hydrazines pNPH and MDNPH



4-nitrophenylhydrazine (pNPH)

1-methyl-1-(2,4-dinitrophenyl)-hydrazine (MDNPH)

(Vogel et *al., 2000)*

A number of sampling techniques for air monitoring with derivatization by DNPH exist. Impingers filled with solutions of DNPH in different solvents are utilized, as well as DNPH-coated solid sorbents (Vogel et *al.*, 2000). Separation by HPLC and detection with UV is the most widespread analytical technique used with nitroaromatic hydrazones. However, it is only

possible to separate all peaks of monocarbonyls up to an alkyl chain length of three carbon atoms and for C_4 aldehydes, up to ten compounds with similar elution properties might be expected (Vogel et *al.*, 2000). Mass spectrometry has also been used to identify and quantify hydrazones and has expanded the selectivity of the method. Interferences do exist with this method, though. The reaction of ozone with DNPH results in the formation of several interfering peaks, as well as the possible depletion of the capture reagent, but may be avoided through the use of O_3 scrubbers. DNPH also reacts with nitrogen dioxide (NO_2) forming 2,4-dinitrophenylazide (DNPA), which can exhibit similar chromatographic properties as the formaldehyde dinitrophenylhydrazone (Vogel et *al.*, 2000). MDNPH could be a suitable alternative to DNPH in analysis of samples containing large amounts of oxidants, as MDNPH reacts with O_3 and NO_2 to form N-methyl-2,4-dinitroaniline, which can then be separated from the hydrazones of interest. However, MDNPH does not have a high reactivity with carbonyl compounds (Vogel et *al.*, 2000).

The US EPA has established two published methods utilizing DNPH to identify and quantify carbonyl groups name TO-5 and TO-11. In US EPA method TO-5 (1984), ambient air is drawn through an impinger containing 2N HCl, 0.05% 2,4-dinitrophenylhydrazine (DNPH) and isooctane. Aldehydes and ketones in the ambient air readily form stable 2,4-dinitorphenylhydrazones (DNPH derivatives). The derivatives are recovered by removing the isooctane layer, and extracting the aqueous layer with hexane/methylene chloride. One concern with using hexane/methylene chloride is that methylene chloride is classified as a Group 2B carcinogen by IARC (1999) and a Group B2 carcinogen by the US EPA (1995). Hexane/methylene chloride (organic layer) is dried under a stream of nitrogen redissolved in

methanol. The DNPH derivatives are determined using reversed phase High Performance Liquid Chromatography (HPLC) coupled with an ultraviolet absorption detector operated at 370 nm.

EPA Method TO-11 (US EPA, 1999) modifies EPA Method TO-5 (1984) by adding a coated adsorbent. A known volume of ambient air is drawn through a pre-packed cartridge coated with acidified DNPH. After sampling, the sample cartridges and field blanks are capped, stored in a 4°C container and returned to the lab for analysis, or the cartridges may immediately be washed via gravity feed elution with CH_3CN into a graduated test tube. The eluate is diluted to a known volume and refrigerated until analysis. The DNPH-derivative is determined using isocratic reverse phase HPLC coupled with a UV absorption detector operated at 360 nm. C_1-C_7 carbonyl compounds are measured effectively to less than 0.5 ppbv.

Dye et *al.* (1998) indicates that in a conventional HPLC column, the risk for HPLC coelutions of carbonyl compounds increases with the number of possible isomers. The separation of a standard mixture containing 10 different C_4 carbonyl compounds is difficult to achieve with conventional HPLC columns. However, with the use of HPLC/MS with quadrupole ion trap mass spectrometers and atmospheric pressure chemical ionization in the negative ion mode (APCI), one can distinguish between mono- and dicarbonyl-DNPHs as well as aldehydes and ketones. Further, aromatic, saturated, unsaturated branched, or straight-chain structures may be differentiated, whereas with UV diode-array spectra, differentiation between aliphatic, aromatic and dicarbonyl structures is only achievable. The authors thus conclude that APCI (-) mass spectrometry is the most suitable ionization technique for the detection and quantification of DNPH carbonyl derivatives.

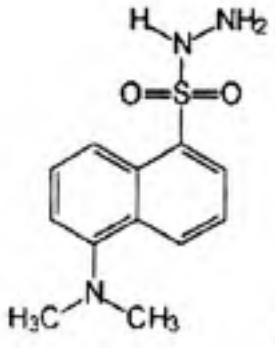
The disadvantages of utilizing the DNPH derivatization coupled with detection and quantification via HPLC/UV VIS include interferences with airborne oxidants, as well as limited

sensitivity. Detection via HPLC/MS with quadrupole ion trap mass spectrometers and APCI in the negative ion mode can increase the sensitivity, but the hardware used can be quite costly, and sampling time lengthy, with each sample requiring 80 minutes. The advantages of identifying carbonyls based on DNPH-hydrazones, however, include a wide range of applications of this method, as well as the stability of both the reagent and derivatives (Vogel et *al.*, 2000).

1.6.2 1-Dimethylaminonaphthalen-5-sulfonyldydrazide (Dansyl hydrazine or DNSH)

DNSH (Figure 1.6.2.1) has been used in the determination of ultratrace levels of carbonyls in air samples. Via fluorescent detection, after separation with HPLC, quantification of DNSH and the respective C1-C9-hydrazones can be measured in the picogram, range (Schmied et *al.*, 1989). However, interferences exist with water, creating unstable hydrazones (Vogel et *al.*, 2000). Further, a significant amount of time is required for each sample separated by HPLC, as well as the use of multiple solvents.

Figure 1.6.2.1 DNSH molecular structure



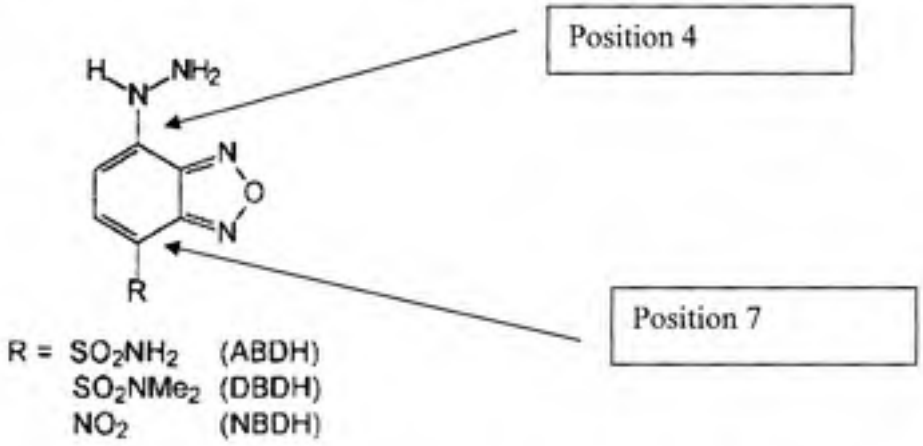
(Vogel et *al.*, 2000)

1.6.3 Reagents Based On Benzooxadiazole Structures

In the benzooxadiazole reaction with carbonyls, the benzooxadiazole backbone (Figure 1.6.3.1) is substituted in positions 4 and 7, with most applications focusing on the substitution of a fluoro or chloro functionality in position 7 by an amine or amino acid as analyte, therefore

leading to strong fluorescent products, which can be separated via HPLC (Vogel et *al.*, 2000). NBD hydrazine (NBDH) is a functional reagent based on the benzooxadiazole backbone.

Figure 1.6.3.1 Molecular structure of benzooxadiazole backbone (Vogel et *al.*, 2000)

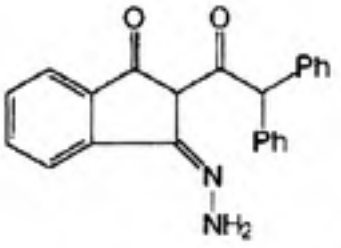


While detection limits are low for alkyl chain lengths with three or more carbons, this method cannot detect formaldehyde or acetaldehyde (Vogel et *al.*, 2000). Also, NBDH is sensitive to light, which leads to degradation products (Uzu et *al.*, 1990). Using the N-methylated reagent *N*-Methyl-4-hydrazino-7-nitrobenzofurazan (MNDBH), the derivatives have weak fluorescence properties, but the UV/vis spectroscopic limits of detection are two times less than those of DNPH derivatives. For example, Buldt et *al.* (1999) shows that the detection limit for MNDB-hydrazones versus DNP-hydrazones for formaldehyde is $3x10^{-8}$ mol/L and $6x10^{-8}$, respectively. Moreover, Buldt et *al.* (1999) indicates that the velocity of the reaction between the reagent and the aldehydes is comparable to that of DNPH, while just as with MDNPH, the *N*-methylated form of NBDH is not limited by interferences with oxidants. However, like previous methods, time is required to separate each sample via HPLC. Further, this method is not specific to two of the most abundant carbonyls in the air, formaldehyde and acetaldehyde.

1.6.4 2-diphenylacetyl-1,3-indandione-1-hydrazone (DAIH)

2-diphenylacetyl-1,3-indandione-1-hydrazone (DAIH) (Figure 1.6.4.1) bas been used for years as a derivatizing agent to quantify carbonyl groups.

Figure 1.6.4.1 Molecular structure of DAIH



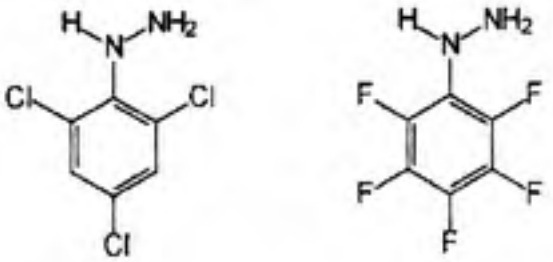
(Vogel et *al.*, 2000)

Sensitive detection of formaldehyde and acetaldehyde in gas phase analysis is possible via DAIH-coated silica-gel cartridges. After carbonyls are collected, they are separated and quantified via HPLC and fluorescence spectroscopy. Detection limits of formaldehyde and acetaldehyde for this method are lower than the traditional DNPH method. For formaldehyde, the detection limit per cartridge using DAIH is approximately 18 ng, versus approximately 33 ng utilizing DNPH. For acetaldehyde, the detection limit per cartridge when using DAIH is 24 ng versus 51 ng with DNPH (Possanzini et *al.*, 1997). As with previous derivatization methods, separation is accomplished via HPLC, which again, requires a significant amount of time per sample (~80 minutes). Another disadvantage of this method is that it has limited applicability for quantifying ketones, as the reaction rates with ketones are much slower than aldehyde derivatization rates (Vogel et *al.*, 2000).

1.6.5 Halogenated Aromatic Hydrazine Reagents

2,4,6-trichlorophenylhydrazine (TCPH) and pentafluorophenylhydrazine (PFPH) (Figure 1.6.5.1) are halogenated phenylhydrazine reagents that are particularly useful in identifying carbonyl groups via GC/MS or GC/ECD.

Figure 1.6.5.1 Molecular structure of TCPH and PFPH



TCPH PFPH

(Vogel et *al.*, 2000)

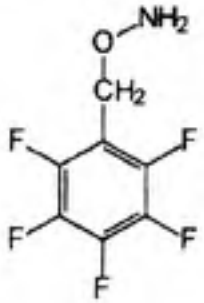
TCPH is volatile enough to be used with gas chromatography without thermal decomposition, as is the case with DNPH (Lehmpuhl et *al.*, 1996). Detection limits for formaldehyde with electron capture detection were 0.1 ppb and in the range of 0.02-0.03 ppb for other carbonyls (Lehmpuhl et *al.*, 1996). Due to slow reaction rates in liquid phases, though, this method is not useful when applying samples to impingers (Vogel et *al.*, 2000). In the absence of an acid catalyst, TCPH only requires 6 minutes for complete reaction with low molecular mass carbonyls, thus eliminating the need carry strong acids during field sampling (Lehmpuhl et *al.*, 1996). This method is also quite specific for aldehydes C1-7, as well as benzaldehyde and acetone (Lehmpuhl et *al.*, 1996). Like HPLC, separation by mass spectrometry requires time, as well as requiring more of an expense, in comparison to spectrophotometry.

1.6.6 O-Alkylated Hydroxylamines

Hydoxylamines, like O-methylhydroxylamine, O-benzylhydroxylamine, O-(p-nitrobenzyl)hydroxylamine, and O-pentafluorobenzylhydroxylamine (PFBOA) (Figure 1.6.6.1)

all react with carbonyls to form stable derivatives, which can then be easily separated with gas chromatography (Vogel et *al.*, 2000).

Figure 1.6.6.1 Molecular Structure of PFBOA



Using PFBOA absorbed on solid-phase microextraction fibers, the detection limit of formaldehyde was calculated to be 4.6 ppb when separated via GC and detected via flame ionization (FID) (Martos et *al.*, 1998).

1.7 Rationale For Project and Hypothesis

Carbonyls exist in air, water, food, biological fluids and tissues, as well as nonbiological materials. Current methods to detect carbonyls, described above, involve separation and quantification instruments that are quite expensive, as well as the use of multiple costly solvents. Moreover, these separation methods are time consuming. The total time required, after sample exposure, extraction, and analysis by HPLC/UV and or HPLC/MS is a number of days, depending on how many samples and standards must be analyzed. HPLC/UV/MS, alone, requires 80 minutes per sample. Further, some of these methods are more specific in detecting specific carbonyls than others, and sensitivity differences exist, as well. Also, some of the methods require the use of toxic solvents, like methylene chloride, used as an extraction solvent, and classified as a Group 2B carcinogen by IARC (1999). The time required for upkeep of these instruments can be extensive, as well as potential costs of training instrument operators. Finally,

these instruments are available only to labs with analytical chemistry capabilities, thus limiting laboratories that can detect carbonyl groups.

Exposure to pollutants can lead to biological responses and health effects. One hypothesis is that oxidative stress from pollutant exposures leads to responses and health effects. Screening of oxidative stress induced by exposure to pollutants, like particulate matter for relative potencies, is important for hazard identification, and ultimately for improving public health. Biological oxidative stress may be measured through production of lipid peroxidation products. Such products include carbonyls, produced in cells, as well as outside of cells. Detection of free carbonyls, not macromolecule adducts, are thus one oxidative stress candidate to measure. A fast method to quantify carbonyls could lead to faster screening of pollutants, like particulate matter for relative potencies.

There is thus a need for a method that can be used in most common labs, a method that is inexpensive, as well as quick. This method must have good specificity and some low sensitivity to measure at least total carbonyls. Therefore, the following methodology was developed to meet the above criteria via DNPH derivatization, organic extraction, and basic spectrophotometry. DNPH is chosen due to its specificity of reaction with carbonyl groups, stability of the formed derivative, and the ability to be analyzed by an LC/MS system. The method was developed initially with carbonyl dinitrophenylhydrazone standards, and then applied to samples of diesel exhaust generated in a controlled exposure chamber and cells undergoing oxidative stress induced by a metal rich PM source.

The potential use of spectrophotometry to quantify total carbonyls is not only less expensive, but it is also much quicker and is available in most laboratories. Further, the following project looks at the efficacy of hexane versus two other solvents, pentane and heptane in extracting

dinitrophenylhydrazone standards into the organic layer, while leaving excess DNPH in the aqueous layer. Moreover, while spectrophotometry cannot determine, nor quantify specific carbonyls simultaneously, it is hypothesized that a correlation exists between the total spectrophotometric absorbance and the total mass of carbonyls separated and quantified via HPLC/MS. If a strong correlation exists, spectrophotometry can thus be used as a quick, inexpensive method to quantify total carbonyls in a sample.

MATERIALS AND METHODS

2.1 Materials

2.1.1 Biochemicals and Reagants

All chemicals were of analytical grade unless otherwise mentioned. 2,4-dinitrophenylhydrazine (DNPH) (CASRN 119-26-6) and cis-11-hexadecenal (CASRN 53939-28-9) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Submicron filtered pentane (CASRN 109-66-0), hexane (CASRN 110-54-3), and heptane (CASRN 142-82-5), as well as methanol (MEOH) [CARN 67-56-1] and acetonitrile (CH_3CN) [CASRN 75-05-8], were purchased from Fisher Scientific (Fairlawn, NJ). Carbonyl standard stock AE00043 was purchased from Accustandard (New Haven, CT) and contained DNPH-derivatized carbonyls acetaldehyde, acetone, acrolein, benzaldehyde, butanal, crotonaldehyde, hexanal, methacrolein, methyl ethyl ketone, p-tolualdehyde, pentanal, propionaldehyde at a concentration of 20 µg/mL and formaldehyde at a concentration of 40 µg/mL in CH_3CN. Glassware was purchased from Kimble Chase Life Science and Research Products (Vineland, NJ) and had Teflon lined screw caps.

2.1.2 Particle Source and Collection

Diesel exhaust (DE) was introduced into controlled chambers at the US EPA Human Studies Facility (HSF) in Chapel Hill, NC. DE was introduced into the chamber after approximately 1:30 dilution with purified and humidified air. Ambient air was purified by HEPA filter and charcoal scrubbing. DE was generated via an idling six-cylinder, 5.9 L-displacement diesel engine (Cummins, Columbus IN) that was mounted in a vehicle located outside the HSF facility and burned certified diesel fuel (Chevron Phillips Chemical Co., Borger, TX; 0.5 LS Certification Fuel, type II, sulfur content approximately 347 ppm). Fuel

specifications are listed in Appendix A. DE particulate levels were feedback-controlled by an exhaust dilution manifold via use of real-time measurements given by a tapered element oscillating microbalance (ThermoFisher Scientific, Franklin, MA) and monitored on a DataRAM aerosol monitor (ThermoFisher Scientific, Franklin, MA). Particle size was measured on a scanning mobility particle sizer (TSI Inc., Shoreview, MN) and $PM_{2.5}$ exposure concentration was determined on a versatile air pollutant sampler (VAPS) (URG, Chapel Hill, NC) (Sobus et al., 2008). Carbonyls in the exhaust were collected on Waters Sep-Pak DNPH-Silica Cartridge 50-105 µm (Part # WAT037500) (Milford, MA) at a flow rate of 0.1 L/min over two hours, the equivalent of 12 L collected over 2 hours. A 0.1 µm filter was placed in line to remove PM before the gas phase exhaust reached the DNPH cartridge.

2.1.3 Analytical and Experimental Instruments

Sorvall RT 6000D Centrifuge (Waltham, MA), Savant Speedvac Concentrator (Farmingdale, NY), Beckman DU 640 B Spectrophotometer (Fullerton, CA), Zymark Turbovap LV Evaporator (Hopkinton, MA), Waters Xterra MS C_{18} 5 µm 3.9 150 mm analytical column (Milford, MA) with a C_{18} 50µm Supelco guard column (Sigma-Aldrich, St-Louis, MO), Waters Assoc, ZMD model MS detector coupled with a 2487 Dual Wavelength Absorbance Detector (UV) (Milford, MA), and a Waters Assoc., 2695 HPLC separation Module (Milford, MA) were used.

2.1.4 In Vitro Exposures

BEAS-2B human airway epithelial cell line (courtesy of Dr. Curtis Harris from the National Institutes of Health), Keratinocyte Growth Media (KGM; Clonetics, San Diego, CA), 25 cm^2 flasks (Costar, Cambridge, MA), Synthetic Residual Oil Fly Ash (Courtesy of Andy Ghio, US EPA) with high concentrations of Fe, V, and Ni; the synthetic ROFA is similar in

composition to ROFA collected from a Florida power plant burning low sulfur number 6 residual oil (Madden, 1999). Phosphate Buffered Saline (PBS; Invitrogen, Carlsbad, CA), Mammalian Protease Inhibitor Cocktail and Hanks' Balanced Salt Solution (HBSS) from Sigma-Aldrich (St. Louis, MO), Cytotox 96 radioactive cytotoxicity assay from Promega (Madison, WI), Bio Tek Instruments Ceres UV 900 HDi (Winooski, VT), Costar 96-well plate (Cambridge, MA) were used.

2.2 Extraction Solvent Validation

DNPH, in the presence of carbonyls, reacts to form 2,4-dinitrophenylhydrazones (DNPH derivatives). DNPH has been chosen as a derivatizing agent as a result of the unique stability of the formed derivative, specificity of reaction, as well as the ability to analyze the derivatives with an LC-MS system (Sakuragawa et al., 1999).

DNPH (0.125% DNPH) in CH_3CN was prepared. Blanks included DNPH alone, and solvents (no DNPH solution) alone; the dinitrophenylhydrazone standard (AE00043) undiluted and with dilutions was used for initial experiments. Blank solutions (1.0 mL) and standard solutions (50 µL) were added to 5.0 mL dH_2O and 950 µL CH_3CN was placed in each standard solution tube, for a final volume of 6.0 mL and solutions were vortexed (approximately 10 seconds).

The standard solutions, in addition to blank solutions were then extracted with hexane, pentane, or heptane (2 mL extraction solvent: 6 mL standard solutions). Solutions were centrifuged (500 x g) for 5 minutes at room temperature. The organic layer (1.0 mL) was removed and the standards were re-extracted 2 more times in a similar manner (but with 2.0 mL organic layer removed) after centrifugation, and the organic extracts combined (approximately 5 mL total). In some experiments, standard solutions were extracted 5 times with hexane to

compare the completeness of extractions. A portion of the aqueous layer (approximately 3 mL) was removed and placed into glass test tubes. Organic samples were placed in a Savant Speedvac Concentrator and centrifuged under vacuum at room temperature until a volume of approximately 500 µL remained. Samples were removed from the Speedvac, placed in a water bath, and dried to completion under nitrogen at 40°C using a Zymark Turbovap LV evaporator.

For spectrophotometric analysis organic samples were redissolved in 200 µL CH_3CN and aqueous samples take directly from the tubes containing the aqueous layer. For organic samples, CH_3CN was used as a blank and the absorbance of organic samples was measured at 365 nm. Samples were diluted with CH_3CN if needed to stay on scale (below 3.0 rau). For analysis of aqueous samples, the spectrophotometer blank was dH_2O. The absorbance of each aqueous sample was measured at 365 nm.

Organic and aqueous samples dried under nitrogen at 40 °C. Once organic and aqueous samples were dried, samples were redissolved with 180 µL of CH_3OH/CH_3CN (50:50 mixture) and 20 µL cis-11-hexadecenal as an internal standard (0.2 µg/mL). Solutions were mixed and an aliquot was placed into the respective HPLC autosampler vial for injection and analysis by UV and MS.

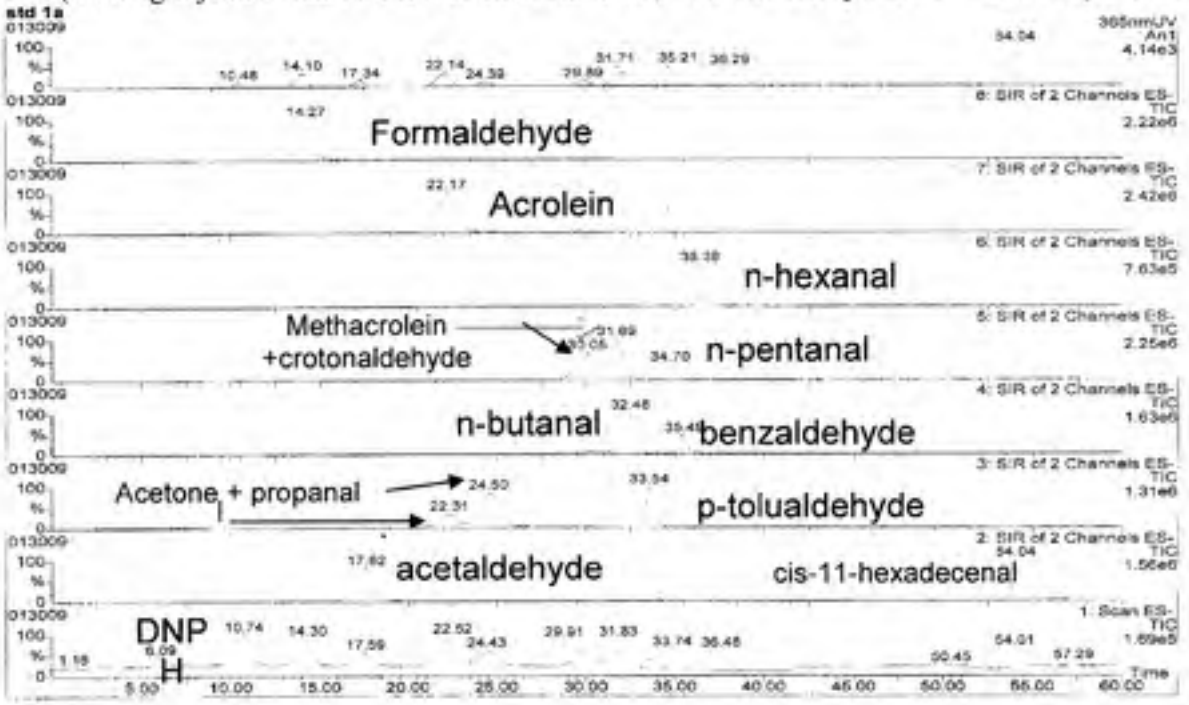
Carbonyl-DNPH derivatives were measured by using a reverse phase HPLC separation utilizing a Supelco 50 µm guard column and a Waters Xterra MS C_{18} 5 µm 3.9x150 mm analytical column. One pump (pump A) delivered dH_2O/MeOH (9/1) with 0.01% formic acid solution. A second pump (pump B) delivered CH_3CN/MeOH (9/1) with 0.01% formic acid solution under the following gradient program (Table 2.2.1).

Table 2.2.1 HPLC System Gradient Program

Time (min)	Pump A dH_2O/MeOH (%)	Pump B CH_3CN/MeOH (%)
0	44.4	55.6
10	44.4	55.6
10.10	11.1	88.9
40	11.1	88.9
40.10	0	100.0
60	0	100.0
60.10	44.4	55.6
80	44.4	55.6

Solutions of samples or standards (20 µL) were injected and the dinitrophenylhydrazones were detected first with a 2487 Dual Wavelength Absorbance Detector (UV) at an absorbance of 365 nm and subsequently with a Waters Assoc., ZMD model MS detector. The MS was operated in ESI negative mode with a capillary voltage of 2kV, cone voltage of 10V, extractor voltage of 3V, source block temperature of 150°C, desolvation temperature 350°C, nitrogen gas flow ~ 500 L/hr and rf lens of 0.3. Selected ion monitoring was performed at 209, 223, 235, 237, 249, 251, 265, 279, 285, 299, and 417, which correspond to the parent ions for formaldehyde, acetaldehyde, acrolein, propanal + acetone, methacrolein + crotonaldehyde, butanal, n-pentanal, n-hexanal, benzaldehyde, p-tolualdehyde, and IS (Madden et *al.*, 2003). The total time per sample required via separation and detection by HPLC/UV/MS was 80 minutes. An example of an undiluted standard (AE00043) chromatogram is shown below.

Figure 2.2.1 AE00043 Standard Chromatogram
Undiluted AE00043 standard chromatogram after separation by HPLC and detection by UV and MS (0.36 ug injected mass online of each standard, with the exception of formaldehyde, 0.72 ug)



2.3 Experimental Design for Environmental Sample Extractions Plus Analysis

Waters Sep-Pak DNPH-Silica Cartridges were placed in a human study chamber and diesel exhaust was collected at 0.1 L per minute for 120 minutes (~ 12 L total after 2 hour exposure) with a target particle concentration of 100 $\mu g/m^3$. Actual PM concentrations ranged from 76-92 $\mu g/m^3$, with an average PM concentration (n=6) of 84.1 $\mu g/m^3$ ($\pm$ 5.75 $\mu g/m^3$) based on filter weight (Appendix B). Average total hydrocarbon concentrations through the six runs were 2.9 ppm ($\pm$ 0.1 ppm) (Appendix B). Other gaseous parameters, including CO, NO_2, and NO concentrations can also be found in Appendix B. DE particulate levels were feedback-controlled by an exhaust dilution manifold via use of real-time measurements given by a tapered element oscillating microbalance (ThermoFisher Scientific, Franklin, MA) and monitored on a

DataRAM aerosol monitor (ThermoFisher Scientific, Franklin, MA). Carbonyls were collected on the cartridges from three separate days with 2 runs each day. After a 2 hour collection samples were stored in a 4°C refrigerator until analysis.

The carbonyl derivatives trapped on the Sep-Pak DNPH-Silica cartridges were eluted with MeOH (3 mL), then with CH_3CN (3 mL). The elutant was placed in a glass tube. MeOH (3 mL) and CH_3CN (3 mL) were passed through unused Sep-Pak DNPH-Silica cartridges as blanks. The samples and blanks were transferred to a Savant Speedvac concentrator and dried by rotation at room temperature. Each sample was redissolved in 200 µL 1:1 CH_3CN/MeOH solution. Aliquots of the redissolved sample (200 µL) were removed that corresponded to ½, ¼, 1/8, and 1/16th of the sample (i.e. 100, 50, 25, and 12.5 µL). All samples were adjusted to a volume of 200 µL with 1:1 CH_3CN/MeOH (Appendix C).

A portion of the sample and blanks (100 µL) was added to de-ionized water (5 mL), and 900 µL CH_3CN, and vortexed (approximately 10 seconds). Samples were extracted with heptane (2 mL x 3 extractions) as previously detailed in section 2.2. All test tubes with the organic phase were dried by rotary evaporation in a Speedvac concentrator. Samples were redissolved with 200 µL CH_3CN and absorbance at 365 nm was then measured. Of the remaining 100 µL from the subsequent aliquots (1/2, 1/4, 1/8, 1/16), 54 µL was placed into HPLC autosampler vials with 6 µL IS. Standards (AE00043) with IS were also placed in HPLC autosampler vials, and a blank containing CH_3CN plus IS also analyzed.

Chromatograms for each standard and sample were integrated and the total peak area of each specific dinitrophenylhydrazone was derived using Micromass Masslynx version 3.2 (Manchester, UK). Derivative values were normalized to the value of the IS. A standard curve for each dinitrophenylhydrazone was generated using Microsoft Excel 2003, and from the

standard linear curve, the mass per sample was calculated, and the mass of each dinitrophenylhydrazone was converted to nanomoles.

2.4 Biological Sample Extractions and Analysis

A saturated DNPH solution was made by mixing 100 mg DNPH in 100 mL KBM, heated at 37 °C for 1 hour, and followed by filtration with a sterile 0.2 µm filtration apparatus to remove the undisolved DNPH. BEAS-2B cells (passages 60-90) were cultured in 25 cm^2 flasks and grown at 37 °C with 5% CO_2 in KGM. BEAS-2B cells are an immortalized line of normal human bronchial epithelium. Cells were grown to 95% confluence, media removed, and cells then rinsed with 2 mL PBS. The PBS was removed and then the saturated DNPH solution (9 mL) plus 1 mL of either ROFA solution or KBM placed in each respective flask. Thus, cells were exposed to final ROFA concentrations of 0, 50 or 200 µg/mL. Cells were exposed for 2 hours, when supernatant (extracellular media) was removed and placed in microfuge tubes. One mL protease inhibitor solution (PIS; diluted 1/10 in HBSS) was placed in each flask, cells scraped, and fluid placed into respective microfuge tubes. Cells scraped a second time by the addition of 1 mL PIS to each respective flask. The flasks with supernatant were placed in a centrifuge and spun at 500 x g for 10 minutes at 4 °C. Approximately 9 mL supernatant was removed, taking care to not remove the pellet, and placed in separate respective microfuge tubes. Cells were sheered through 27.5 gauge needle (Becton Dickinson, Franklin Lakes, NJ). Samples (900 µL supernatant or cell lysate or blank) were extracted with heptane in the same manner as in section 2.2. Samples were dried, and redissolved in 200 µL CH_3CN, then analyzed via spectrophotometer (section 2.2).

A colorimetric, coupled enzymatic assay (CytoTox 96; Promega, Madison, WI) was used to measure lactate dehydrogenase (LDH) activity, which was reflected in the conversion of a

tetrazolium salt to a red formazan product. Each sample was run in triplicates and placed in a 96-well plate, with 50 µL of sample plus 50 µL of binding substrate (from cytotox 96 non radioactive cytotoxicity assay). As a positive control, 10 mL of KBM plus 2 µL LDH enzyme (from cytotox 96 non radioactive cytotoxicity assay) was adequately vortexed, and 50 µL placed in 96-well plate, in addition to 50 µL binding substrate. The 96-well plate was placed in an incubator (37 °C) for 30 minutes and upon removal, 50 µL stop solution was placed in each well and the bubbles popped with a syringe. The plate was placed in the Bio Tek Ceres UV 900 HDi plate reader and the absorbance was measured at 490 nm.

Using Graphpad Prism Version 4 software (La Jolla, CA), one-way ANOVA and nonparametric comparisons, followed by a Dunnett's Multiple Comparison test, was completed, to test whether absorbance values for cellular and extracellular media were significantly different from each other. Finally, one-way ANOVA and nonparametric comparisons, followed by Neuman-Keuls Multiple Comparison Test was used to determine whether a statistically significant difference between differing concentrations of ROFA and measured absorbance as a sign of LDH activity.

3.0 RESULTS

3.1 Extraction of Dinitrophenylhydrazones By Different Solvents

The effect of the number of extractions on the recovery of the dinitrophenylhydrazones was determined using the AE00043 standard solution. The spectrophotometric absorbances of the samples extracted 3 times versus 5 times in hexane are listed in Table 3.1.1. The absorbances for each sample are totaled and the percent in organic layer and percent in aqueous layer is calculated. The results indicate the absorbance (rau), when corrected for volume, in both the aqueous and organic layers. The blank absorbances indicate that absorbance is minimal in the organic layer, in that the majority of measurable absorbance occurs in the aqueous layer. When 0.125% saturated DNPH in acetonitrile was extracted in duplicate, a high percentage (greater than 86%) of DNPH remains in the aqueous layer in both the 3X and 5X extractions. This means that unreacted DNPH will remain in the aqueous layer. Moreover, the 3X extraction actually shows better extraction capabilities as 89% of standard 1 is found in the organic layer, versus only 25% with a 5X extraction. However, it is evident that an error occurred, as a high initial aqueous reading in the standard solvent of the 5X extraction, followed by the correction factor, created a skewed result. Thus, 3X extraction yields similar results to a 5X extraction, suggesting that for future extractions, 3X is adequate for optimal recovery.

Table 3.1.1 Absorbance of a 3X hexane extraction versus a 5X hexane extraction of a mixture of dinitrophenylhydrazones

3X Extraction	*Aq Layer Abs* (RAU)*	*Org Layer Abs* (RAU)*	*Total*	*% in Aq Layer*	*% in Org Layer*
Solvent Blank	0.790	-0.021		100.0	0.0
DNPH 1 (1250 µg)	59.2	6.88	66.0	89.6	10.4
DNPH 2 (1250 µg)	63.0	9.59	72.6	86.8	13.2
Std undiluted (14 µg total)	0.610	4.92	5.53	11.1	88.9
Std 1/10	-1.68	0.823		0.0	100.0
Std 1/100	-1.760	-0.006			

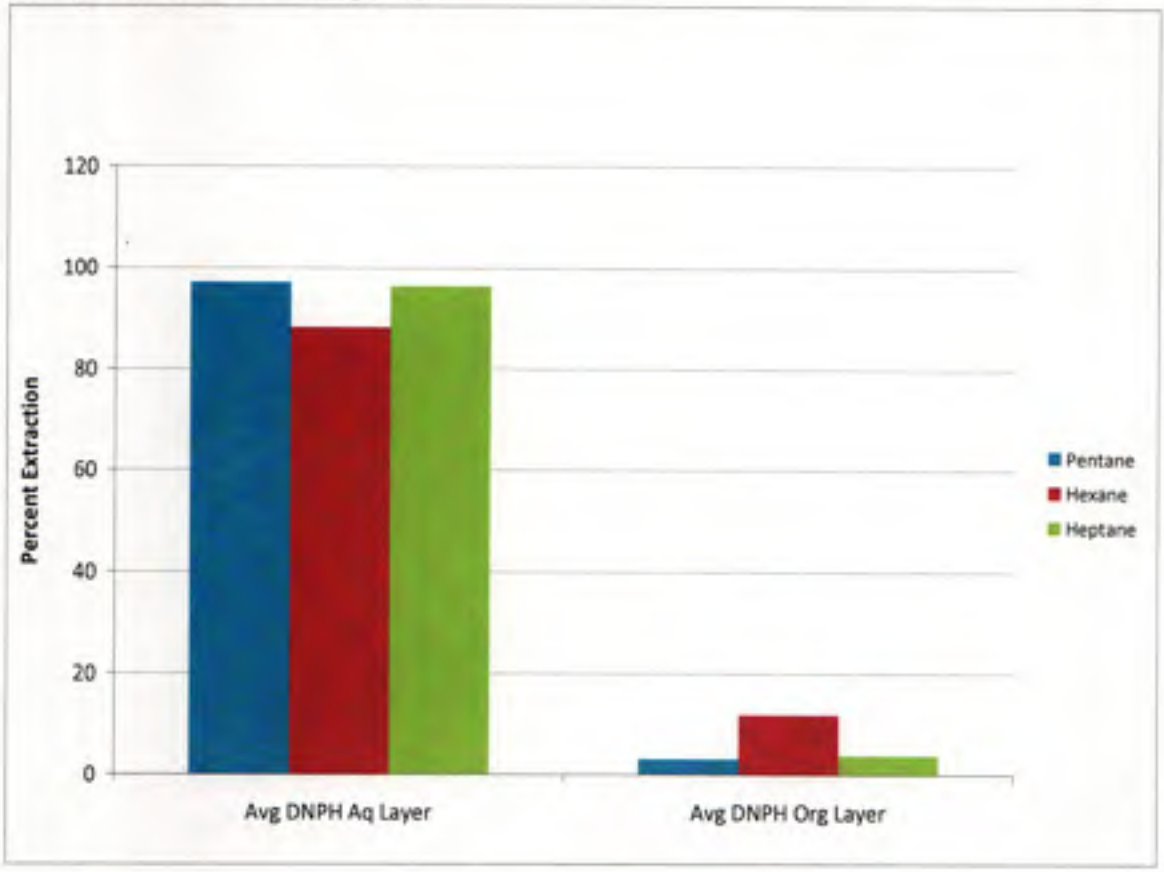
5X Extraction	*Aq Layer Abs *(RAU)*	*Org Layer Abs *(RAU)*	*Total*	*% in Aq Layer*	*% in Org Layer*
Solvent Blank	0.815	0.092	0.907	89.9	10.1
DNPH 1 (62.5 µg)	251.1	31.5	282.6	88.9	11.1
DNPH 2 (62.5 µg)	253.4	7.42	260.8	97.2	2.80
Std undiluted (14 µg total)	2.81	0.951	3.76	74.7	25.3
Std 1/10	0.415	0.193	0.608	68.3	31.7
Std 1/100	0.480	0.006	0.486	98.8	1.20

*Values corrected for dilution factor

The effect of extraction solvent on the recovery of DNPH was determined using the DNPH standard solutions. The absorbance of each DNPH sample was measured via spectrophotometry and the percent DNPH in the aqueous layer versus the organic layer was calculated. Figure 3.1.1 shows the average percent extraction of DNPH samples (n=2) in the aqueous layer and the organic layer. Each DNPH sample contained 1250 µg of DNPH. The data suggests that heptane and pentane are comparable solvents, leaving a larger percentage of DNPH

in the aqueous layer than in the organic layer, in comparison to hexane. Extracting into pentane, 96.96% of DNPH remains in the aqueous layer, extracting into hexane, 88.18% of DNPH remains in the aqueous layer, while using heptane as an extraction solvent, 96.28% remains in the aqueous layer.

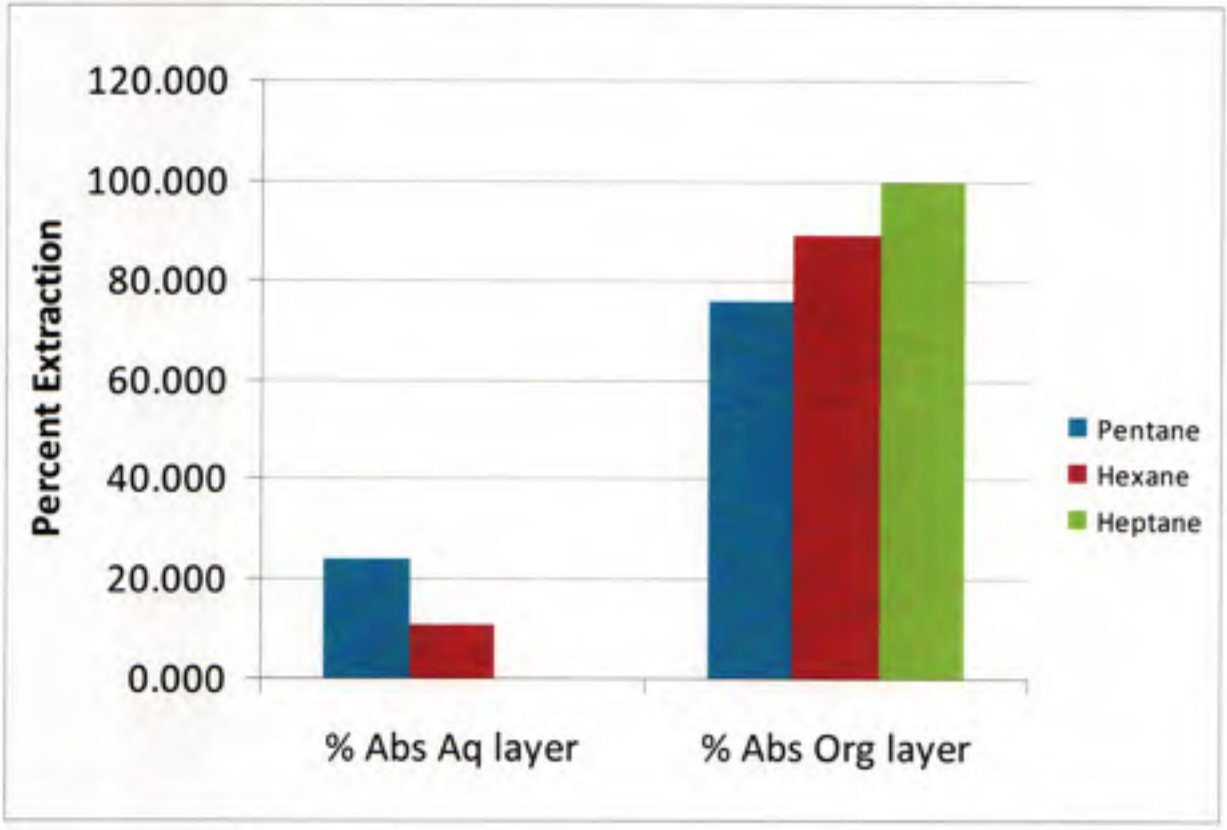
Figure 3.1.1 Average Percent Extraction by Pentane, Hexane, or Heptane of DNPH only samples based on Spectrophotometric Absorbances
DNPH only samples (n=2) were extracted (3X) by pentane, hexane, or heptane, spectrophotometric absorbances measured and the extraction percentages in each phase were averaged (DNPH = 1250 ug/mL)



The effect of extraction solvent on the recovery of dinitrophenylhydrazones was determined using AE00043 standard solutions. As with DNPH standard solutions, the absorbances of standard solution is measured in aqueous and organic layers, and the percent of standard in the organic layer versus the aqueous layer of 14 µg of standard solution is calculated. Figure 3.1.2 indicates that 75.8% dinitrophenylhydrazones are extracted into the organic layer

with pentane, 88.9% with hexane, and 100% with heptane. Thus, the percent recoveries based on spectrophotometric absorbance for DNPH and solvent standards suggest that heptane is the most effective extraction solvent.

Figure 3.1.2 Percent Extraction by Pentane, Hexane, or Heptane of Standard Samples (280 ug/mL) based on Spectrophotometric Absorbances



Next, the DNPH solutions analyzed via spectrophotometry, were then prepped and separated via HPLC and detected by UV (section 2.2), to confirm that heptane was a more effective extraction solvent than pentane. Pentane and Heptane were compared by HPLC/UV because their percent extraction percentage by spectrophotometric analysis of DNPH samples was similar, as well as higher than hexane. The aqueous layer was diluted (1:10 dilution), as the undiluted sample was above the limit of detection for UV. Dilution of the organic layer was not necessary. Table 3.1.2 lists the UV peak area of both the aqueous layer and the organic layer for one DNPH sample.

The data indicate that 68% of DNPH remains in the aqueous layer via heptane extraction, while only 56% remains in the aqueous layer via pentane extraction. Thus, the data suggest that via UV detection, heptane is a more effective extraction solvent.

Table 3.1.2 UV Detection of DNPH After Extraction With Pentane or Heptane

Extraction Solvent	*DNPH Aq Peak Area**	*DNPH Org Peak Area*	*Total Peak Area*	*% in Aq Layer*	*% in Org Layer*
Pentane	2810	2173	4983	56.4	43.6
Heptane	9510	4470	13980	68.0	32.0

*Values corrected for dilution factor

Lastly, the solvent standards that were extracted with pentane and heptane are compared. The aqueous layer and organic layer are separated via HPLC and individual diphenylhydrazones are measured by MS, as outlined in section 2.2. The total peak area of each diphenylhydrazone was determined after normalization with the IS (Table 3.1.3). In comparing MS values of the undiluted AE00043 standard extractions, heptane extracted a greater percent of several individual diphenylhydrazones into the organic fraction than pentane. With regards to formaldehyde, 51% is extracted into the organic layer via heptane extraction, versus 46% for pentane. Acetaldehyde also is better extracted into the organic layer with heptane (51%) versus pentane (39%). The trend continues with each subsequent increase in carbon number. The data thus suggest that by HPLC separation and measurement by MS, heptane is more effective in extracting dinitrophenylhydrazones, which is thus consistent with the data from spectrophotometric analysis.

Table 3.1.3 Comparison of Undiluted AE00043 Standard Extractions of Pentane versus Heptane As Measured by MS

Pentane	*Aqueous Peak Area*	*Organic Peak Area*	*Total Peak Area*	*% in Aq. Layer*	*% in Org. Layer*
Formaldehyde	87700	56500	14400	60.8	39.2
Acetaldehyde	39100	27200	66300	59.0	41.0
Acrolein/Acetone	33200	94500	12800	26.0	74.0
Propanal	1360	32500	33900	4.10	95.9
Methacrolein/Crotonaldehyde	0	80400	80400	0.00	100
Butanal/Methylethylketone	4420	68800	73200	6.10	93.9
Benzaldehyde	4370	123000	12700	3.40	96.6

Heptane	*Aqueous Peak Area*	*Organic Peak Area*	*Total Peak Area*	*% in Aq. Layer*	*% in Org. Layer*
Formaldehyde	69000	72000	141000	48.8	51.2
Acetaldehyde	35000	29000	64000	54.9	45.1
Acrolein/Acetone	34000	131000	165000	20.4	79.6
Propanal	1580	38000	40000	3.90	96.1
Methacrolein/Crotonaldehyde	0	93000	93000	0.00	100
Butanal/Methylethylketone	2170	72000	74000	2.91	97.1
Benzaldehyde	7500	146000	153000	4.90	95.1

Making use of 3 different concentrations (undiluted, ½ dilution, and ¼ dilution) of the standard AE00043, the molar contribution to UV absorption is examined for each of the individual dinitrophenylhydrazones found in the standard (Appendix D). Based on the average of the three dilutions, the molar contribution, expressed as a dinitrophenylhydrazone, the more polar derivatives (formaldehyde and acetaldehyde) are similar to one another, while the less polar derivatives are similar to one another (Figure 3.1.3). As the standard is separated by HPLC and detected by UV, the solvent composition is changing with the gradient program. Thus, the solvent composition changes the degree of absorbance at a fixed wavelength to some extent.

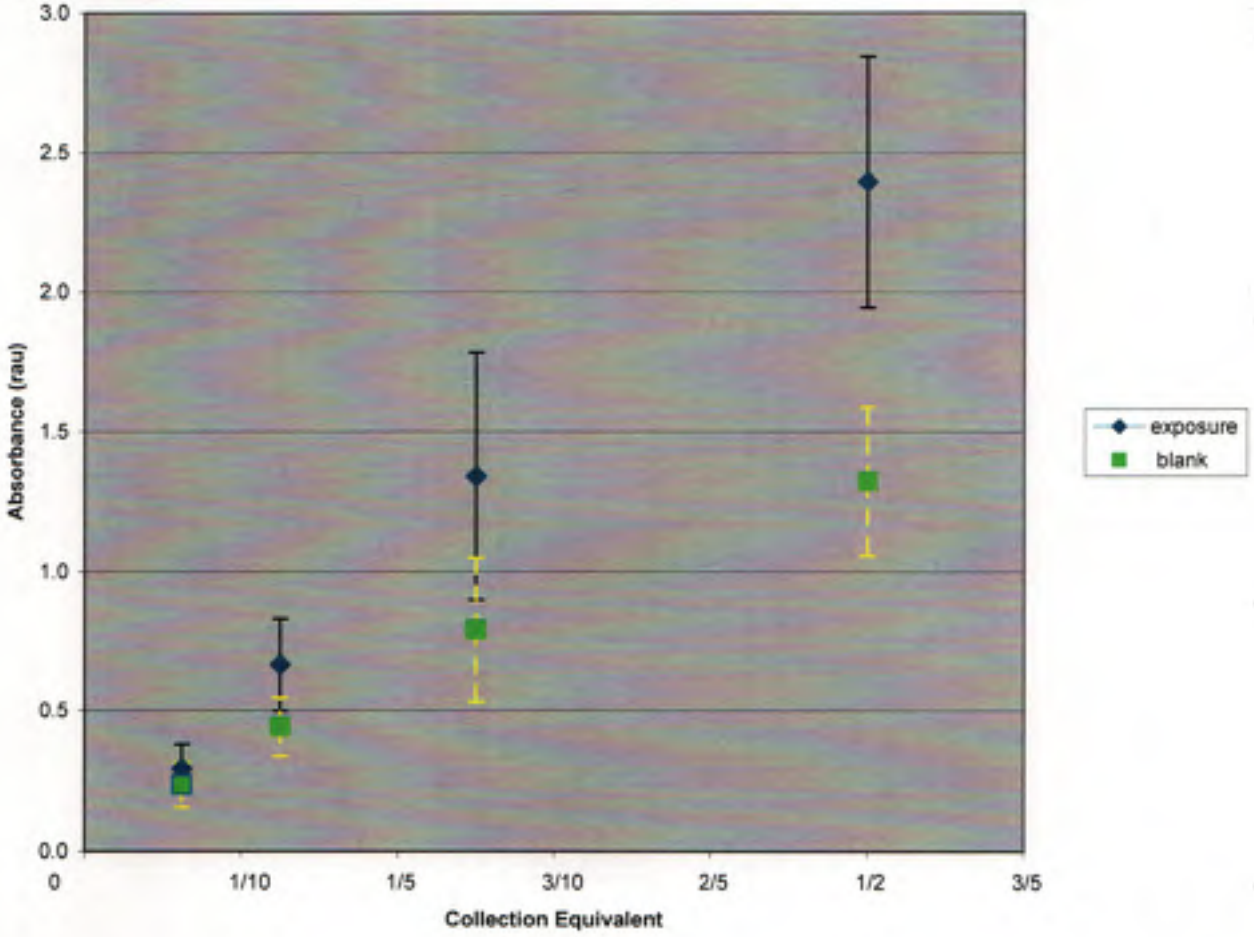
Table 3.1.4 Individual dinitrophenylhydrazone molar contribution to UV absorption
The average of molar contribution to UV absorption, expressed as a dinitrophenylhydrazone, of 3 standard sample dilutions is calculated after separation by HPLC and detection by UV (365 nm).

	peak area/umol	peak area/nmol
carbonyl diphenylhydrazone derivative	185137.78	185.14
formaldehyde	147433.75	147.43
acetaldehyde	505784.63	505.78
acrolein	29334.34	29.33
acetone + propanal (masses added together)	92241.68	92.24
methacrolein + crotonaldehyde (mass added together)	168706.69	168.71
n-butanal	335667.41	335.67
p-tolualdehyde	284504.31	284.50
n-pentanal	322037.33	322.04
benzaldehyde	268131.52	268.13

3.2 Extraction and Analysis of Gaseous Diesel Exhaust

Carbonyls were collected in a diesel exhaust chamber on a C_{18} Sep-Pak DNPH-Silica cartridge and the absorbance of ½, ¼, 1/8, and 1/16th collection exposure equivalents were measured after extraction of the eluate with heptane (Section 2.3). Figure 3.2.1 shows the mean absorbance of each of the six exposure cartridges at the respective collection exposure equivalents, as well as the six blank cartridges and their respective exposure equivalents. The data show that the largest collection equivalent (i.e. ½) had the greatest absorbance for both exposure and blank cartridges (2.394 ± 0.450 and 1.324 ± 0.2668), while the smallest equivalent (i.e. 1/16th) had the least absorbance for the exposure and blank cartridge (0.290 ± 0.0882 and 0.234 ± 0.0788). Therefore, a greater portion of carbonyls is collected in the exposure cartridges versus the blank cartridges. Moreover, the range, particularly in the ½ collection exposure aliquot is greatest, whereas the range in the 1/16 exposure aliquot is the least. Absorbance data (Appendix E) indicates that a positive linear dose response relationship does exist, with a decreasing absorbance as dose decreases. Blank cartridges are expected to contain dinitrophenylhydrazones including formaldehyde, acetaldehyde, and acetone, as Waters Corporation includes the levels of these contaminants in each lot manufactured.

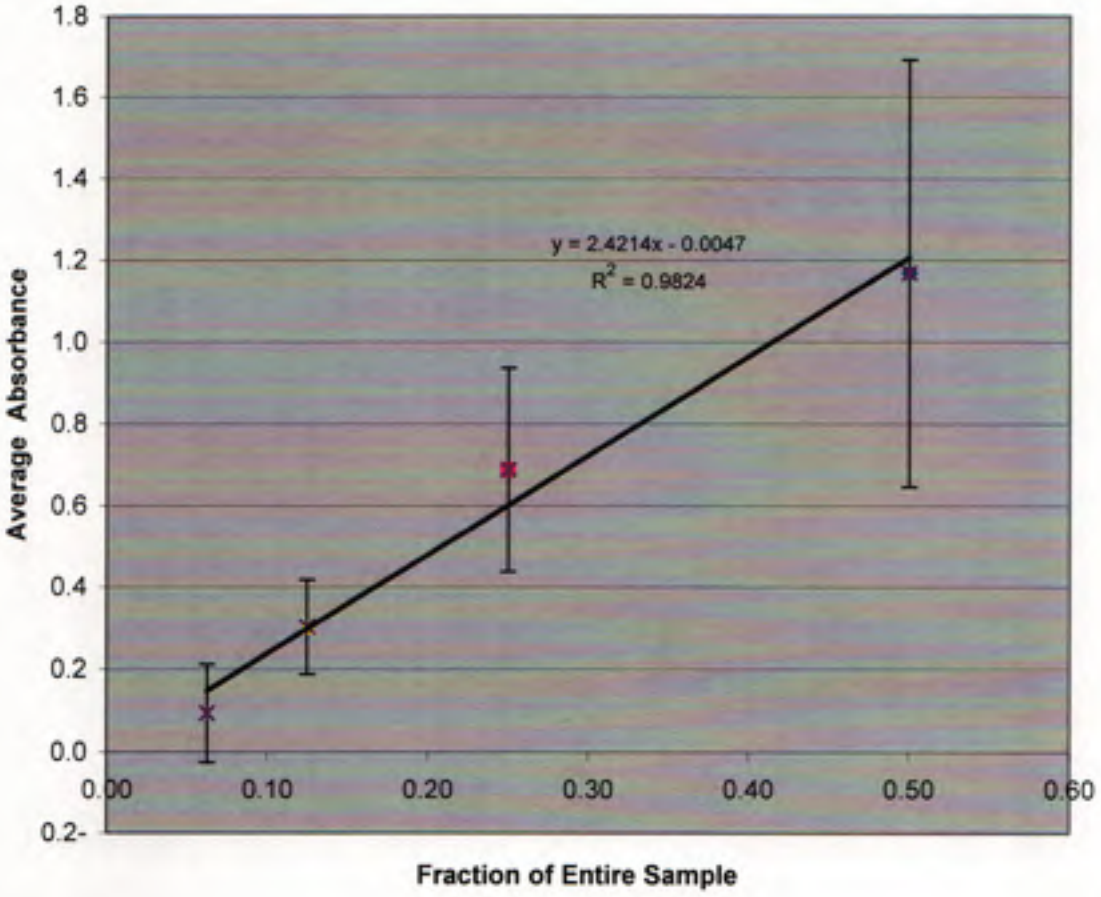
Figure 3.2.1 Spectrophotometric absorbance of increasing fractions of diesel exhaust samples.
Blank and exposure samples of experiments 1-6 were extracted with heptane and spectrophotometric absorbance was measured at 365 nm.



In Figure 3.2.2, the average spectrophotometric absorbances of blank equivalents are subtracted from the average spectrophotometric absorbances of exposure equivalents. Here, experiment 4 is excluded, as negative values occur when subtracting blank equivalents from exposure equivalents (Appendix E). The data indicate that a greater proportion of carbonyls

collected exist in the exposure cartridges than in the blank cartridges. Waters states that the cartridges contain carbonyl contaminants and their amounts vary by lots. As the collection fraction increases, the variation in absorbance also increases, leading to larger standard deviations when calculating the average absorbencies for each respective collection fraction. Figure 3.2.2 thus illustrates a positive dose response relationship between collection fraction equivalent and spectrophotometric absorbance with a slope of 2.421 and a strong correlation of 0.9824.

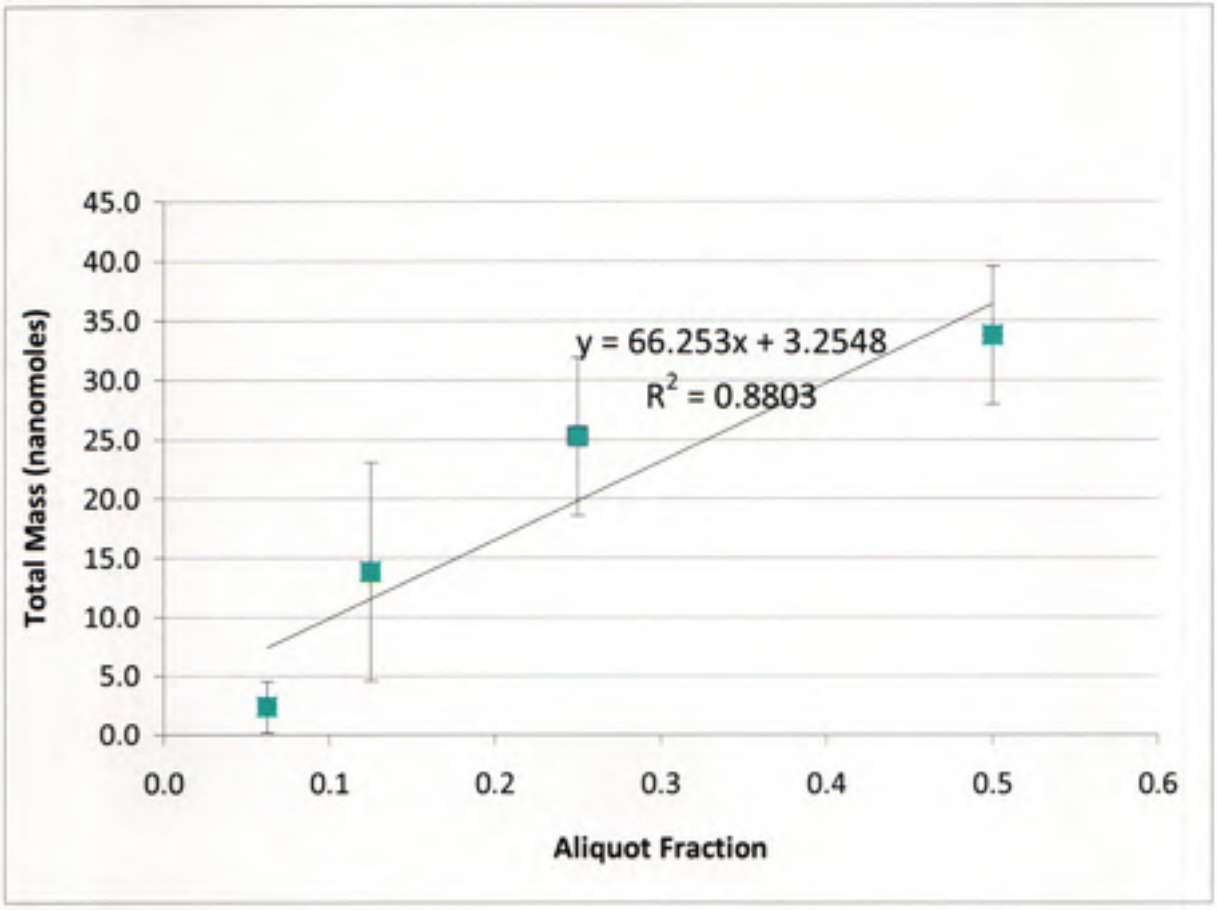
Figure 3.2.2 Spectrophotometric absorbance of increasing fractions of diesel exhaust sample
Blank and exposure samples of experiments 1-6 were extracted with heptane and spectrophotometric absorbance was measured at 365 nm. Blank absorbances were subtracted from their respective exposure fraction absorbance, and absorbances of experiments 1-6, with the exception of experiment 4, were averaged.



As per the procedure in section 2.3, the dinitrophenylhydrazone total mass, in nanomoles, was calculated for exposure equivalent and blank equivalent cartridges (1/2, ¼, 1/8, 1/16th) (Appendix F). As experiment 4 data was not included in the spectrophotometry section, it will

also be excluded in calculating the average total dinitrophenylhydrazone mass. Further, as the total mass for experiment 1 is so much greater than experiments 2,3,5, and 6, a Grubbs' test was performed and data from experiment 1 were calculated to be outliers at the $p < .05$ significance level (Appendix G). Thus, Figure 3.2.3 illustrates data for n=4 experiments, with the following carbonyl derivatives quantified and their total mass summed for each collection equivalent: formaldehyde, acetaldehyde, acrolein, propanal, methacrolein, butanal, pentanal, hexanal, benzaldehyde, and p-tolualdehyde (Appendix H). Figure 3.2.3 shows that the largest collection fraction equivalent (1/2) also has the greatest total dinitrophenylhydrazone mass (33.74 nanomoles), while the smallest collection fraction equivalent had the smallest total dinitrophenylhydrazone mass (2.342 nanomoles). Moreover, the Figure indicates the standard deviations of each collection fraction equivalent, with the largest deviation occurring at the $1/8^{th}$ collection fraction equivalent and the smallest calculated from the $1/16^{th}$ collection fraction equivalent. Finally, Figure 3.2.3 indicates that as the collection fraction equivalent increases, the total mass of dinitrophenylhydrazones also increases. Thus, a positive linear dose-response relationship exists, with a slope of 66.25 and a correlation of 0.88.

Figure 3.2.3 Mass of carbonyls recovered from sample of diesel exhaust
The average dinitrophenylhydrazone total mass (n= 4 experiments) of increasing fractions of diesel exhaust, when subtracting blank exposure equivalents from collection exposure equivalents, is calculated after extraction by heptane and HPLC separation followed by MS detection



Of the total mass of carbonyl derivatives collected, it is important to note that formaldehyde and acetaldehyde make up the majority of carbonyls collected in the exposure equivalents. For the ½ exposure collection equivalent, formaldehyde as a percent of the total mass of carbonyls collected, ranges from 53% to 93%, while acetaldehyde ranges from 3% to 30% (table 3.2.1).

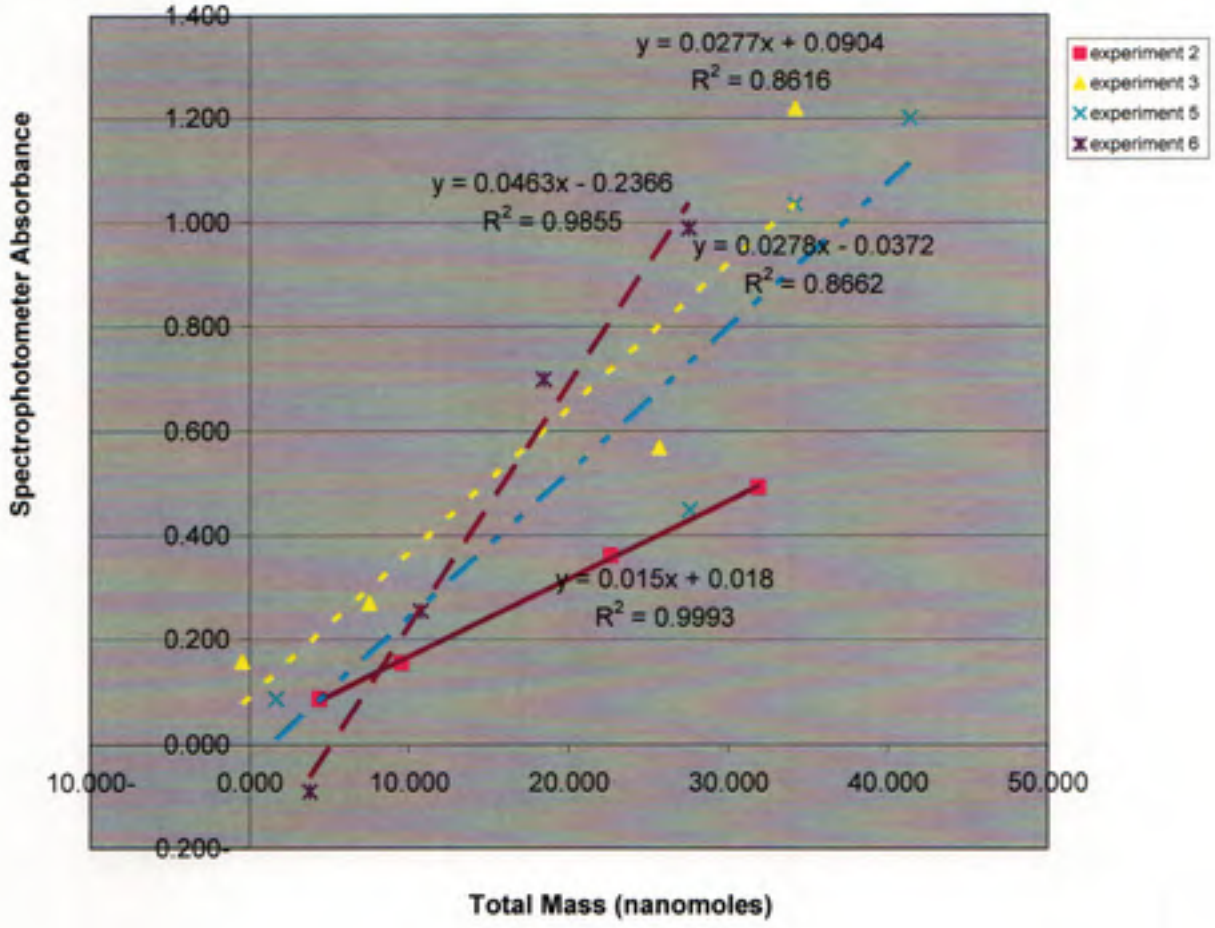
Table 3.2.1 Percent of Formaldehyde and Acetaldehyde from Total Carbonyls Measured

Experiment	*Formaldehyde mass (nanomoles)*	*Acetaldehyde mass (nanomoles)*	*Total Mass of Carbonyls (nanomoles)*	*% Formaldehyde*	*% Acetaldehyde*	*% Formald + Acetald*
1	319.0	12.9	340	93.8	3.80	97.6
2	27.4	13.2	46.4	58.9	28.4	87.4
3	41.8	1.72	56.1	74.6	3.08	77.6
4	101.0	37.4	164.9	61.2	22.7	83.9
5	31.4	17.7	58.3	53.9	30.4	84.4
6	32.4	13.8	52.2	62.1	26.4	88.6

As a positive linear relationship exists between spectrophotometric absorbance and collection fraction equivalent, as well as between dinitrophenylhydrazone total mass and collection fraction equivalent, to be determined next was whether or not a positive linear relationship existed between spectrophotometric absorbance and dinitrophenylhydrazone total mass at each respective collection equivalent. Figure 3.2.4 shows the spectrophotometric absorbance versus the total dinitrophenylhydrazone mass at each collection aliquot for n=4 experiments. A positive linear relationship exists with each experiment, but with varying degrees (varying slopes). Experiment 2 and Experiment 6 have the best correlated data points, with respective R^2 values of 0.9993 and 0.9855.

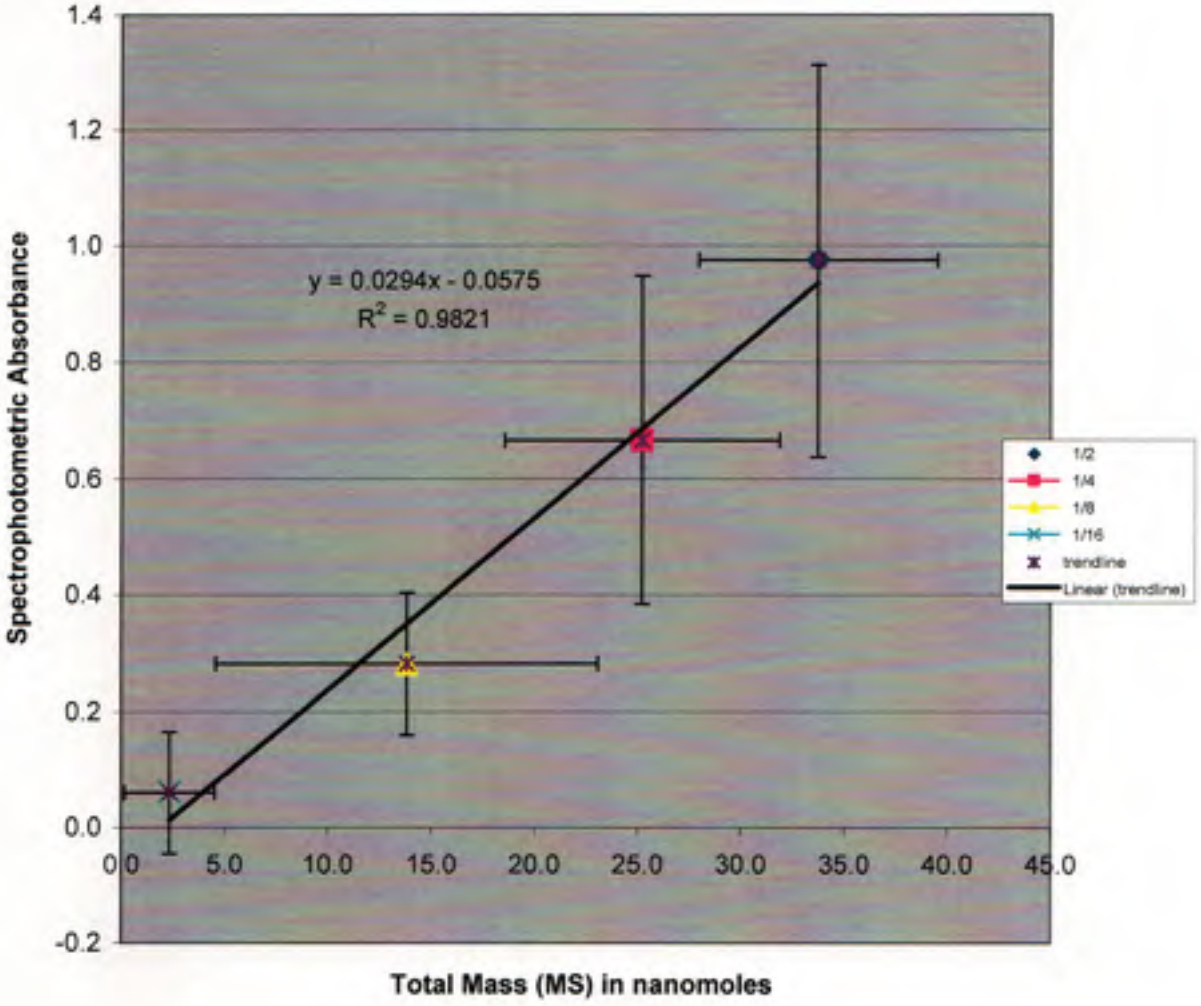
Figure 3.2.4 Spectrophotometric absorbance versus mass of carbonyls recovered from diesel exhaust

Spectrophotometric absorbance (determination at 365 nm) versus the total mass of dinitrophenylhydrazones after separation by HPLC and determination by MS of 4 experiments is plotted below.



The average total spectrophotometric absorbance versus the average dinitrophenylhydrazone mass at each respective collection equivalent for the four experiments plotted in Figure 3.2.4 is plotted next (Figure 3.2.5). The greatest collection fraction (i.e. ½ equivalent) has the highest absorbance value, as well as the highest dinitrophenylhydrazone total mass, while the smallest collection fraction ($1/16^{th}$ equivalent) has the smallest absorbance and lowest dinitrophenylhydrazone total mass. Therefore, a strong correlation exists between absorbance (determined by spectrophotometry at 365 nm) and dinitrophenylhydrazone total mass (determined by MS), with a slope of 0.029 and an R^2 value of 0.9821.

Figure 3.2.5 Average Spectrophotometric absorbance versus average mass of carbonyls recovered from diesel exhaust
The spectrophotometric absorbance (determination at 365 nm) of 4 experiments is averaged and plotted against the average dinitrophenylhydrazone mass (determination by MS) after extraction by heptane.



The airborne concentration in the diesel exhaust chamber of specific aldehydes, using the ½ exposure collection equivalent of the previously used four experiments, was calculated with results shown in table 3.2.2. This table indicates the specific concentration of a particular aldehyde (formaldehyde, acetaldehyde, acrolein, and propionaldehyde) in the diesel exhaust

chamber, when normalized for air flow (0.1 L/min for 2 hours) and collection fraction equivalent (1/2 equivalent). The aldehyde with the highest concentration is formaldehyde with an average concentration of 255.4 $\mu g/m^3$ ($\pm$ 110.1 $\mu g/m^3$), followed by acetaldehyde, with a concentration of 164.3 $\mu g/m^3$ ($\pm$ 51.431 $\mu g/m^3$).

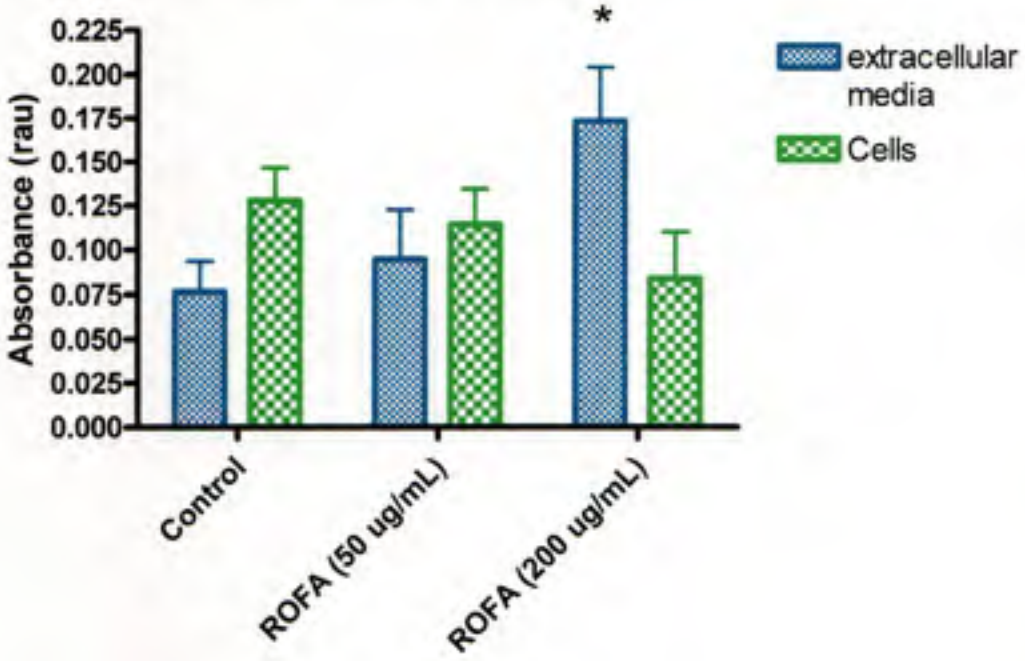
Table 3.2.2 Airborne Concentration in Diesel Exhaust Chamber of Specific Aldehydes using the ½ exposure collection equivalent (blank subtracted)

	ug/m^3	Standard deviation	Corresponding PPM
formaldehyde	255	110	0.210
acetaldehyde	164	51.4	0.091
acrolein	0.487	0.270	0.000210
Propionaldehyde	21.7	10.4	0.009

3.3 Extraction and Analysis of Biological Sample

Carbonyls were collected from a biological sample after exposure of BEAS-2B cells to a control and varying concentrations of ROFA, followed by extraction with heptane, and the absorbance was measured (Section 2.4). As previously mentioned, ROFA exposure of BEAS-2B cells induces an increase in total carbonyls (Madden et *al.*, 1999), primarily detected as acetaldehyde by HPLC/UV/MS. Absorbances of both the cellular media, as well as the extracellular media were measured for four experiments and the average absorbances are shown in Figure 3.3.1.

Figure 3.3.1 Average spectrophotometric absorbance of cellular and extracellular media The absorbance of cellular and extracellular media is measured at 365 nm after exposure of cells to differing concentrations of ROFA for 2 hours, followed by extraction by heptane (3X) and the absorbance values of 4 experiments are averaged.

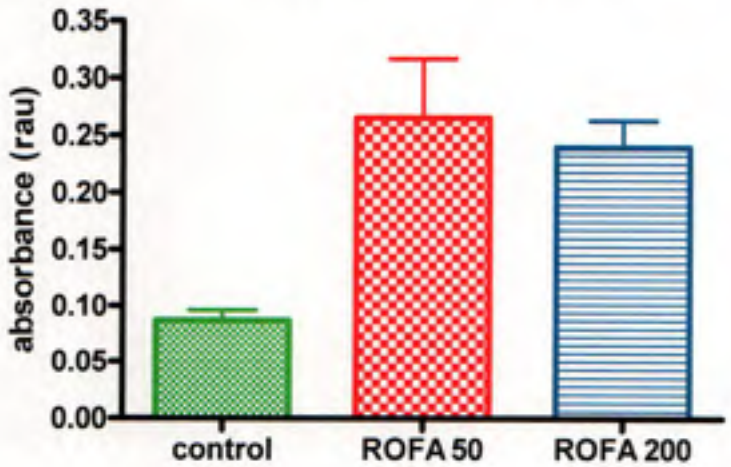


The absorbance of the control (0.127 ± 0.038) and ROFA (50 μg/mL) (0.114 ± 0.040) are higher in cellular media than in extracellular media (0.076 ± 0.034 and 0.094 ± 0.056, respectively), but the absorbance of ROFA (200 μg/mL) is higher in extracellular media (0.173 ± 0.61) than in cellular media (0.084 ± 0.053). What is also clear is that in the extracellular media, absorbance increases with an increase in ROFA concentration, while the opposite is true in the cellular media. There is a statistically significant different between the average control absorbance and the average ROFA 200 absorbance in extracellular media ($P < 0.05$). There was no difference between the control and ROFA 50 or ROFA 200 samples in the cellular lysate values.

After exposure to particles, the extracellular media was analyzed for LDH activity, a viability assay. The average of 4 viability assays for each of the three exposed samples is shown

in the Figure 3.2.2. An increase in absorbance represents an increase in LDH activity. An increase in absorbance, and therefore LDH activity, occurs between the control (0.086) and ROFA 50 sample (0.265), but absorbance values are lower in the ROFA 200 sample (0.239) in comparison to the ROFA 50 sample. There is a statistically significant difference between the control and ROFA 50 and ROFA 200 samples ($P < 0.01$). However, there is not a statistically significant difference between each ROFA concentration ($P > 0.05$). Therefore, administration of ROFA to cells leads to LDH activity in extracellular media.

Figure 3.3.2 Average absorbance of extracellular media as a measure of LDH activity
The absorbance (at 490 nm) of extracellular media after 2 hour exposure of cells to differing ROFA concentrations of 4 experiments was averaged.



4.0 DISCUSSION AND CONCLUSIONS

This study had a primary objective of determining whether or not a statistically significant correlation exists between spectrophotometric absorption of carbonyls collected and total mass of carbonyls detected via HPLC/UV/MS. In examining this objective, part of the methods development studied the efficiency of hexane, versus pentane and heptane, as a suitable method for extracting carbonyl groups.

Results of this study indicate that heptane was more efficient in extracting dinitrophenylhydrazones than pentane or hexane. Results from the current study indicated that heptane not only extracted a higher percentage of carbonyls into the organic group, it was also more efficient in not extracting DNPH. The present study looked at the extraction capabilities of pentane, hexane, and heptane via detection by spectrophotometric absorbance. In a 3X extraction of standard solutions (AE00043), heptane extracted approximately 100% of DNPH derivatives into the organic aliquot, while hexane extracted 75.8%, and pentane extracted 88.9% of dinitrophenylhydrazones, as shown in Figure 3.1.2. Heptane was also more effective in leaving a greater amount of DNPH in the aqueous phase than hexane, and equally effective as pentane. Heptane left 96.3% of DNPH in the aqueous phase, and pentane left 97.0% in the aqueous phase, while hexane left 88.2% of DNPH in the aqueous phase, when measuring spectrophotometric absorbance (Figure 3.1.1). These results are important in that they indicate that in measuring total dinitrophenylhydrazones via spectrophotometric absorbance, heptane is the most optimal extraction solvent. 3X extraction versus 5X extraction was examined, but due to technical difficulties, the results could not be compared. The likely cause of error occurred in the aqueous phase analysis, as a high initial absorbance reading in the aqueous phase of the standard samples, followed by a large correction factor, skewed the results, so that a larger

percentage of the standard appeared to remain in the aqueous phase than extracted into the organic phase.

Since heptane was more efficient than pentane in extracting standard solutions into the organic phase, and just as efficient as pentane in leaving DNPH in the aqueous phase, efficiencies of the two solvents were examined next via separation by HPLC and detection by UV or MS. In the detection of DNPH by UV (table 3.1.2), ~56% of DNPH is left in the aqueous phase when using pentane, while ~68% of DNPH is left in the aqueous phase upon utilization of heptane as an extraction solvent. Heptane also is more efficient at extracting individual DNPH derivatives. After detection via MS, heptane, particularly with regards to formaldehyde and acetaldehyde, is more efficient in extracting these two carbonyl groups into the organic layer, than pentane. Heptane extracts 51.2% of formaldehyde and 45.1% of acetaldehyde into the organic layer, while 39.2% of formaldehyde and 31.0% of acetaldehyde are extracted into the organic layer by pentane (table 3.1.3). For carbonyl groups with 3 or more carbons, extraction recoveries exceeded 95% for both solvents. What is disconcerting, though, is that the recoveries of formaldehyde and acetaldehyde are not higher. Fung and Grosjean (1981) have shown that using hexane/methylene chloride as an extraction solvent yields recoveries of greater than 96% for formaldehyde and acetaldehyde. The higher recoveries are likely due to their use of methylene chloride in conjunction with hexane, but their data do not show the effect of this extraction solvent on DNPH. However, due to the highest recoveries of standard solutions by extraction with heptane as measured by spectrophotometric absorbance it was determined that heptane would be used in future experiments to capture carbonyl groups.

The minimization of extraction of DNPH into the organic layer via heptane is crucial. By initially decreasing the amount of DNPH extracted into heptane, one may minimize the

interference of DNPH in absorbance readings via spectrophotometer. This is crucial in that the goal of measuring spectrophotometric absorbance is to measure and quantify total carbonyl groups. By using a solvent (heptane) that initially decreases DNPH extraction into the organic layer most efficiently, one is minimizing the risk of interference from the derivatizing agent, which also absorbs light at the same wavelength at which the carbonyls being quantified also absorb light.

Conversely, it is also important to optimize extraction of contaminants that absorb at 365 nm into the organic phase to minimize interferences. In sampling diesel exhaust, the interferences by other compounds that absorb UV light at 365 nm are minimized. Large molecular weight polyaromatic hydrocarbons are mainly found on the diesel exhaust particles, but those found in the gas phase (typically two ringed or less; Sobus et *al.*, 2008) should pass through as DNPH is specific to derivatizing carbonyl groups. In the cell exposure system, however, cellular constituents leaving the cells, as a result of increased cell membrane permeability could interfere with spectrophotometric absorbance at 365 nm. Such constituents include non-polar molecules that have carbon double bonds or are aromatic, such as polyunsaturated fats or amino acids with an aromatic group. The likelihood of extraction of relatively polar amino acids into heptane is minimal, however more hydrophobic lipids potentially may pose a problem as artifacts. Furthermore, by increasing the efficiency of the dinitrophenylhydrazone derivatives into heptane, one can achieve a more accurate quantification of total carbonyls, by spectrophotometric absorbance as well as via HPLC/UV/MS.

Individual dinitrophenylhydrazones and the molar contribution to absorbance at 365 nm was examined. Evident is that the more polar dinitrophenylhydrazones, like formaldehyde and acetaldehyde, contribute to absorption, on a molar basis, similarly, while less polar

dinitrophenylhydrazones tend to contribute equally to absorption. Less polar dinitrophenylhydrazones actually tend to contribute more to absorption, based on the peak area/nmol dinitrophenylhydrazone. In terms of measuring total carbonyls via spectrophotometry at 365 nm, this means that in a mixture of equal concentrations, on a molar level, those that are less polar contribute more to absorption than more polar carbonyl groups. Moreover, acrolein contributes the highest value (Appendix D), on a molar level, to absorbance at 365 nm, meaning that even small changes in the concentration of acrolein can greatly alter the absorbance measured via spectrophotometry.

We used a controlled diesel exhaust system to capture carbonyl groups and to test whether or not a significant correlation exists between spectrophotometric absorbance and total dinitrophenylhydrazone mass, as determined by HPLC/MS. What is clear is that from six experiments, formaldehyde and acetaldehyde make up the majority of total carbonyls measured, ranging from 77% to 97% with an average across six experiments of 86.58% (± 6.61%) (Table 3.2.1). These values are comparable to previously published literature. For instance, Siegl et *al.*, (1999) indicates that formaldehyde and acetaldehyde make up about 74% of the total carbonyl emissions in experiments with diesel exhaust. Further, the percent of formaldehyde and acetaldehyde is 53.7% and 20.4%, respectively, in the study by Siegl et *al.*, while in our study, the average formaldehyde percent is 67.46% (± 14.61%) and the average acetaldehyde percent is 19.14% (± 12.43%).

Of concern, however, is that only about half of formaldehyde and acetaldehyde was recovered with heptane as an extraction solvent. Previous studies have used hexane/methylene chlorine as an extraction solvent, leading to higher recoveries of formaldehyde and acetaldehyde. However, what these studies fail to show is data on the fate of DNPH in the sample. Adding

methylene chloride might extract more excess DNPH into the organic phase, which would thus lead to even greater interferences with spectrophotometric absorbance readings at 365 nm and skew quantification of carbonyls via spectrophotometric absorbance measurement. Carbonyls with 3 carbons or greater are recovered in excess of 95% so it is thus evident that the molecular weight of a carbonyl might determine the extent of recovery via heptane extraction. By only recovering half of formaldehyde and acetaldehyde, it is possible that the spectrophotometric absorbance, as well as total mass derived from MS is underestimated by half, too.

When the spectrophotometric absorbance of environmental samples was compared to the total mass of carbonyls derived from HPLC/MS, linear regression analysis indicated that the correlation was quite strong. Utilizing four experiments, because of 2 outliers, the correlation between absorbance and total mass was 0.9821 with a slope of 0.0294 (Figure 3.2.5). Using these values, one could conceivably measure the absorbance via spectrophotometer of a portion of collected carbonyls and determine the total mass of carbonyls present. This is important in that utilizing spectrophotometry not only requires a lot less time than HPLC/MS analysis (in our studies, the time required for spectrophotometry was one day, while the time required for HPLC/UV/MS analysis was multiple days), but it also requires fewer chemical solvents, as well as a lesser volume of solvents. In conclusion, the data suggest that detection via spectrophotometry is a viable method of quantifying total carbonyl groups in an environmental sample.

In calculating the average airborne concentration of formaldehyde (255 $\mu g/m^3 \pm 110.1$ $\mu g/m^3$ or 0.21 ppm), based on the ½ exposure collection equivalent of four experiments, the concentration is comparable to other values found in literature. In the study by Pepelko and Peirano (1983), chamber studies are used to evaluate the harmful effects of diesel engine

emissions and with a target particle concentration of 6 mg/m^3, the formaldehyde concentration was 0.106 ± 0.029 ppm, while with a target particle concentration of 12 mg/m^3, the formaldehyde concentration was 0.251 ppm ± 0.059. In a study by McDonald et *al.* (2004), a single-cylinder 5500-watt diesel engine was run at 100% rated load, and emissions analyzed. In the McDonald et *al.* study, the average formaldehyde concentration is 0.229 ppm (± 0.023 ppm). The McDonald et *al.* (2004) study also characterizes concentrations of other carbonyl compounds from diesel exhaust; the acetaldehyde concentration was 0.0671 ppm (± 0.0067 ppm), whereas in our study the average concentration of acetaldehyde based on the ½ exposure collection equivalent of four experiments was 0.091 ppm. Finally, the published concentration of propionaldehyde (McDonald et *al.*, 2004) was 0.0724 ppm ± 0.0072 ppm, whereas the concentration of propionaldehyde in the air based on the ½ exposure collection equivalents of our four experiments was 0.00912 ppm. While the concentrations of these three carbonyl compounds compare similarly to previously published concentrations, the concentration of acrolein (0.00021 ppm) was significantly lower than the McDonald et *al.* (2004) value of 0.0105 ppm. Even though standard curve data for acrolein (Appendix I) indicates adequate recovery concentrations, through each experiment, acrolein concentrations were consistently low, meaning that the HPLC/MS instruments were not the source of error.

It was calculated earlier that data from experiment one in the diesel exhaust experiments was considered an outlier via a Grubbs' test for outliers (Appendix F). It must be noted that the particle concentration of experiment one (76.7 μg/m^3), while it is the lowest particle concentration of the six runs, as well as furthest away from the average particle concentration (84.1 μg/m^3), it is also not an outlier. Furthermore, hydrocarbon concentrations for each of the six runs were similar, meaning that this could not be the source of error in experiment 1 data.

The methodology developed in this study was applied to analyze carbonyls in biological samples. BEAS-2B cells were cultured and when confluent, exposed to differing concentrations of residual oil fly ash (ROFA). After 2 hours of exposure, the supernatant was collected, and the cells scraped and collected. Results of experiments with ROFA exposure (Figure 3.3.1) show that in supernatant media, average spectrophotometric absorbance increases with increases in ROFA concentration, while the opposite is true with regards to cellular media and average absorbance. The increase in supernatant media was due to increases in products of lipid peroxidation. As ROFA concentration increases, the products of lipid peroxidation increase, thus increasing the absorbance at 365 nm. The lower levels of total carbonyls in the cells can be explained in a couple of ways. First, it is possible that the trapping method (saturated DNPH in media) did not adequately trap and derivatize the carbonyl groups fast enough in the cells, or even make it into the cells. This would lead to lower cellular values than actually produced. Second, cellular processes are also very complex, with different metabolic pathways, as well as binding of molecules to other molecules. Catabolism could therefore be a reason as to why the decrease in absorbance with increasing concentrations of ROFA is seen in Figure 3.3.1. At the cellular level, as the cells are exposed to greater concentrations of ROFA, formation of aldehydes followed by further metabolism could thus lead to decreases in total absorbances. Also, ROFA may have altered the cellular catabolism of carbonyls, by increasing the release of carbonyls into the supernatant. Also, ROFA treatment may make it easier for carbonyls to leave, which could be possible via increased cell permeability.

In analyzing LDH production of extracellular media (Figure 3.3.2), an increase in LDH is seen from control to the sample with a ROFA concentration of 50 μg/mL, followed by a decrease in LDH in the sample with a ROFA concentration of 200 μg/mL. This can be explained by the

fact that LDH is released only after an increase in cellular membrane permeability. The production of LDH may reach a maximum level, which could thus be a reason why the absorbance of the 200 μg/mL flask is not greater than the 50μg/mL flask. The increased LDH release supports the notion of the increase in extracellular carbonyls due to an alteration of cell permeability.

The results of analyzing carbonyls indicate that the method may be applied to extracellular media in a biological sample. The carbonyl products of lipid peroxidation, as previously mentioned, can have deleterious health effects on human health. By using the methodology established in these studies, it is possible to quantify total carbonyls in extracellular media, and therefore can be used to screen for products of lipid peroxidation. Furthermore, as mentioned before, the method is fast, and inexpensive.

From measurement of carbonyls in vitro as a sign of oxidative stress, measurements of biomarkers of effect can also be measured in vivo by blood, urine, and breath samples. Malondialdyde is an aldehydic biomarker of lipid peroxidation that may be measured in plasma (Block, 2002). Furthermore, 8-isoprotane may be measured in exhaled breath as a sign of lung oxidative stress, as well as being detectable in urine as a surrogate tissue fluid (Montuschi, 2000). Recently it was reported that exhaled breath levels of malondialdehyde were associated with lung function decrements in asthmatic children residing in relatively heavily polluted Mexico City (Romieu et al., 2008).

In conclusion, the data from our current experiments suggests that heptane is more effective than pentane and hexane in extracting standard solutions into the organic phase, while keeping DNPH in the aqueous phase. The method developed was then tested on diesel exhaust samples and in the comparison of spectrophotometric absorbance versus total mass of aldehydes

derived from HPLC/MS, a strong correlation exists. The data thus suggest that spectrophotometric absorbance may be used as a suitable method in quantifying the total carbonyl concentration in an air sample, and results are also applicable to the extracellular carbonyls when applying the method to a biological sample. Although this method is not specific for carbonyl groups, this finding is important for a number of reasons. As it does not require expensive, analytical instruments, one merely needs a spectrophotometer and the ability to complete extractions, thus increasing the number of labs that may test for the presence of total carbonyls in the air. This could help in monitoring local air, e.g. in Moncure, NC, where emissions of formaldehyde into the environment are quite high, due to the approximate release of 250,000 pounds of formaldehyde into the environment by a composite board factory, the sixth highest release of formaldehyde in the nation (Scorecard, 2005). Second, this method uses a lesser volume of chemical solvents. Finally, this method is much quicker than analysis by HPLC/UV/MS. Analysis with HPLC/UV/MS, including time required for extractions, as well as sample prep of samples and standards for HPLC analysis, followed by separation by HPLC and detection by UV or MS (80 minutes/sample), can take a number of days, depending on the number samples. However, with instant absorbance readings via spectrophotometry, the total time required to analyze a group of samples is approximately one work day. This method can thus be used as a quicker, cheaper way to quantify total carbonyls in the air and in extracellular media of biological samples and can then be followed by HPLC/MS/UV if more specific results are necessary. Future applications of this method may be applied to food sources, as well as water sources.

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Appendix A Chevron Phillips Chemical Co., Borger, TX; 0.5 LS Certification Fuel, type II, sulfur content approximately 347 ppm

Chevron Phillips Chemical Company LP

DATE OF SHIPMENT

CUSTOMER PO NO.

SALES ORDER NO.

MFG. DATE: 11-2004
SHELF LIFE: UNDETERMINED

CERTIFICATE OF ANALYSIS

DIESEL .05 LS CERT FUEL (#2)
LOT 4KP01201

TESTS	RESULTS	SPECIFICATIONS	METHOD
Specific Gravity, 60/60	0.8436	0.8398 - 0.8654	ASTM D-4052
API Gravity	36.23	32 - 38	ASTM D-1250
Corrosion, 50°C, 3 hrs	1A	3 Max	ASTM D-130
Sulfur, ppm	346.9	300 - 500	ASTM D-5453
Flash Point, °F	148.5	130 Min	ASTM D-93
Pour Point, °F	-15	0 Max	ASTM D-97
Cloud Point, °F	-2	10 Max	ASTM D-2500
Viscosity, cs 40°C	2.53	2.2 - 3.2	ASTM D-445
Carbon wt%	86.76	Report	ASTM D-3343
Hydrogen wt%	13.20	Report	ASTM D-3343
Carbon Density (gm/gal)	2770	2750 - 2806	Calculated
Net Heat of Combustion BTU/LB	18455	Report	ASTM D-3338
Particulate Matter, mg/l	0.6	15 Max	ASTM D-2276
Cetane Index	47.6	46 - 48	ASTM D-976
Cetane Number	46.4	46 - 48	ASTM D-613
DISTILLATION, °F			ASTM D-86
IBP	358.0	340 - 400	
5%	389.8		
10%	409.8	400 - 460	
20%	437.5		
30%	459.3		
40%	479.8		
50%	498.7	470 - 540	
60%	517.3		
70%	536.5		
80%	559.8		
90%	590.0	560 - 630	
95%	620.1		
EP	645.8	610 - 690	
Loss	0.3		
Residue	1.0		
HYDROCARBON TYPE, VOL%			ASTM D-1319
Aromatics	29.4	28 - 31	
Olefins	1.2	Report	
Saturates	69.4	Report	
SFC Aromatics, wt%	31.59	Report	
Polynuclear Aromatics, wt%	7.40	Report	

D.G. Doerr

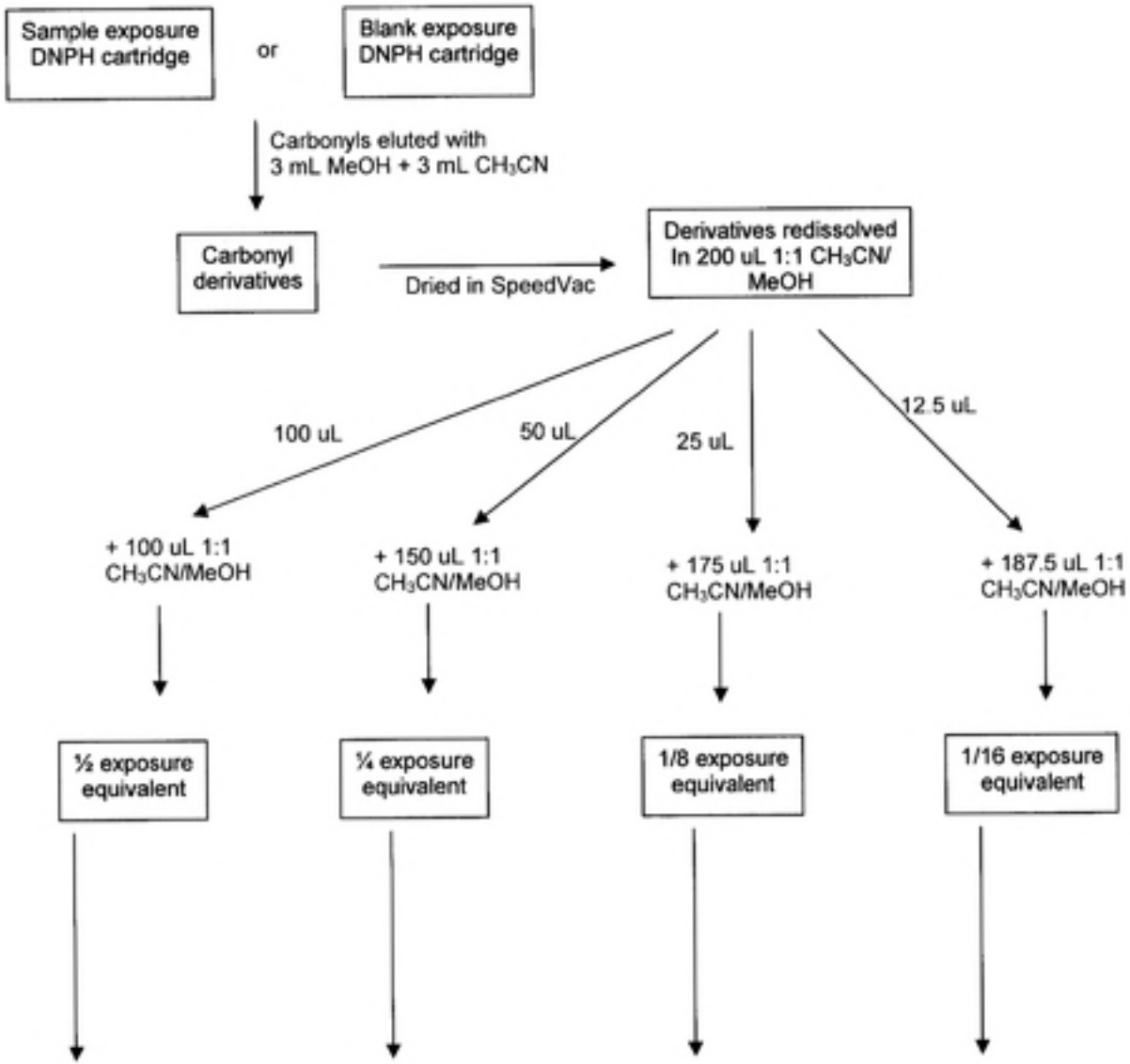
D.G. Doerr
Fuels Unit Team Leader

EJN: teh
02/05/07

Appendix B: Gas Phase and PM Parameters of Diesel Exhaust after 1:30 dilution with humidified and purified air

collection #	Date	Run #	CO (ppm)	NO (ppm)	NO2 (ppm)	HC (ppm)	SO2 (ppm)	PM (ug/m3)
1	12/1/2008	1	3.3	3.4	0.9	2.9	0.04	76.7
2	12/1/2008	2	3.2	3.5	0.9	3	0.04	78.8
3	12/10/2008	1	3.3	3.9	0.9	2.9	0.05	89
4	12/10/2008	2	3	3.6	0.8	3.1	0.05	91.5
5	12/17/2008	1	3.6	3.8	0.9	2.9	0.05	85.7
6	12/17/2008	2	3	3.4	0.8	2.8	0.05	82.9
average			3.2	3.6	0.9	2.9	0.0	84.1
standard deviation			0.2	0.2	0.1	0.1	0.0	5.8

Appendix C: Experimental Design for Diesel Exhaust Sample Extractions

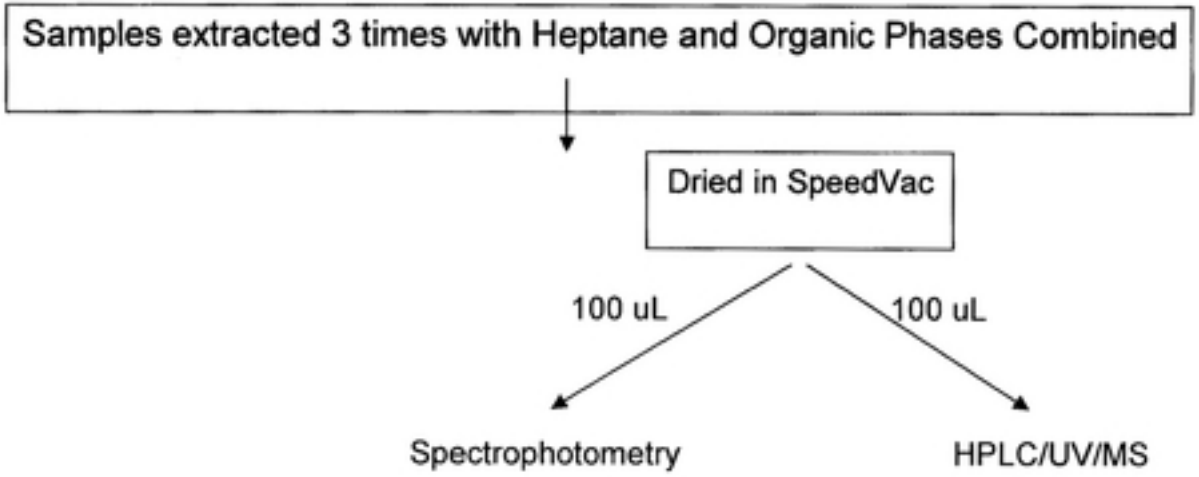


100 uL sample + 5 mL dH_2O + 900 uL CH_3CN

100 uL sample + 5 mL dH_2O + 900 uL CH_3CN

100 uL sample + 5 mL dH_2O + 900 uL CH_3CN

100 uL sample + 5 mL dH_2O + 900 uL CH_3CN



Appendix D Individual dinitrophenylhydrazone molar contribution to UV absorption

The chart shows the contribution of each aldehyde derivative found in the AE00043 standard and its molar contribution to UV absorption, based on the molecular weight of its corresponding dinitrophenylhydrazone.

std 1 carbonyl

carbonyl diphenylhydrazone derivative	injection mass (ug)	molecular weight (ug/umol)	inj mass umol (ug/mw)	peak area (UV at 365 nm)	peak area/umol	peak area/nmol
formaldehyde	0.72	209.92	0.00343	911.00	265607.11	265.61
acetaldehyde	0.36	223.95	0.00161	318.00	197822.50	197.82
acrolein	0.36	235.96	0.00153	1117.00	732131.44	732.13
acetone + propanal (masses added together)	0.72	237.98	0.00605	283.00	46769.68	46.77
methacrolein + crotonaldehyde (mass added together)	0.72	249.99	0.00576	646.00	112148.29	112.15
n-butanal	0.36	252.01	0.00143	131.00	91703.64	91.70
p-tolualdehyde	0.36	300.10	0.00120	526.00	438479.44	438.48
n-pentanal	0.36	266.03	0.00135	503.00	371703.03	371.70
benzaldehyde	0.36	286.02	0.00126	518.00	411551.00	411.55
n-hexanal	0.36	280.06	0.00129	436.00	339183.78	339.18

Std 2 carbonyl

carbonyl diphenylhydrazone derivative	injection mass (ug)	molecular weight (ug/umol)	inj mass umol	(ug/mw)	peak area (UV at 365 nm)	peak area/umol	peak area/nmol
formaldehyde	0.36	209.92	0.00171		353.00	205838.22	205.84
acetaldehyde	0.18	223.95	0.00080		78.00	97045.00	97.05
acrolein	0.18	235.96	0.00076		439.00	575480.22	575.48
acetone + propanal (masses added together)	0.36	237.98	0.00303		36.00	11899.00	11.90
methacrolein + crotonaldehyde (mass added together)	0.36	249.99	0.00288		238.00	82635.58	82.64
n-butanal	0.18	252.01	0.00071		160.00	224008.89	224.01
p-tolualdehyde	0.18	300.10	0.00060		221.00	368456.11	368.46
n-pentanal	0.18	266.03	0.00068		208.00	307412.44	307.41
benzaldehyde	0.18	286.02	0.00063		219.00	347991.00	347.99
n-hexanal	0.18	280.06	0.00064		193.00	300286.56	300.29

Std 3 carbonyl

carbonyl diphenylhydrazone derivative	injection mass (ug)	molecular weight (ug/umol)	inj mass umol	(ug/mw)	peak area (UV at 365 nm)	peak area/umol	peak area/nmol
formaldehyde	0.18	209.92	0.00086		72.00	83968.00	83.97
acetaldehyde	0.09	223.95	0.00040		0.00	0.00	0.00
acrolein	0.09	235.96	0.00038		80.00	209742.22	209.74
acetone + propanal (masses added together)	0.18	237.98	0.00151		0.00	0.00	0.00
methacrolein + crotonaldehyde (mass added together)	0.18	249.99	0.00144		118.00	81941.17	81.94
n-butanal	0.09	252.01	0.00036		68.00	190407.56	190.41
p-tolualdehyde	0.09	300.10	0.00030		60.00	200066.67	200.07
n-pentanal	0.09	266.03	0.00034		59.00	174397.44	174.40
benzaldehyde	0.09	286.02	0.00031		65.00	206570.00	206.57
n-hexanal	0.09	280.06	0.00032		53.00	164924.22	164.92

Appendix E: Spectrophotometric Absorbance (365 nm) of Sep-Pak DNPH-Silica Cartridges exposed to diesel exhaust. Cartridges were exposed to diesel exhaust for 2 hours at a flow rate of 0.1 L/min and then extraction by heptane (3X). Blank, unexposed cartridges were similarly extracted and absorbances measured in a similar manner

Spectrophotometry Absorbance (rau)	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 6
½ exposure aliquot	3.033	1.839	2.436	2.021	2.266	2.769
¼ exposure aliquot	1.453	1.068	1.260	0.696	1.581	1.981
1/8 exposure aliquot	0.789	0.607	0.698	0.361	0.714	0.822
1/16 exposure aliquot	0.429	0.268	0.349	0.173	0.251	0.270
½ blank aliquot	1.089	1.346	1.218	1.442	1.067	1.781
¼ blank aliquot	0.677	0.707	0.692	0.837	0.547	1.283
1/8 blank aliquot	.404	0.453	0.428	0.527	0.266	0.567
1/16 blank aliquot	0.202	0.182	0.192	0.304	0.166	0.360
½ exposure-blank	1.944	0.493	1.218	0.579	1.199	0.988
¼ exposure-blank	0.775	0.362	0.568	-0.141	1.034	0.698
1/8 exposure-blank	0.385	0.154	0.270	-0.167	0.448	0.255
1/16-exposure-blank	0.228	0.087	0.157	-0.131	0.086	-0.090

Appendix F: Total Mass of Carbonyls in Diesel Exhaust Collected on DNPH Cartridges and Blank Cartridge Values as determined by detection by HPLC/UV/MS.

Total Mass (nanomoles)	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 6
½ exposure aliquot	340.237	46.379	56.097	164.963	58.264	52.176
¼ exposure aliquot	238.341	28.979	32.799	45.957	43.635	28.493
1/8 exposure aliquot	153.975	12.762	10.210	35.549	30.012	15.930
1/16 exposure aliquot	70.141	5.322	0.300	13.276	3.422	4.295
½ blank aliquot	51.574	14.510	21.924	8.608	16.884	24.650
¼ blank aliquot	36.581	6.329	7.141	7.640	9.481	10.058
1/8 blank aliquot	35.748	3.257	2.702	11.273	2.438	5.207
1/16 blank aliquot	29.482	0.953	0.746	3.484	1.744	0.526
½ exposure – blank	288.663	31.869	34.173	156.355	41.379	27.526
¼ exposure – blank	201.760	22.650	25.658	38.317	34.154	18.435
1/8 exposure-blank	118.227	9.505	7.508	24.276	27.575	10.723
1/16 exposure-blank	40.659	4.369	-0.446	9.792	1.678	3.769

Appendix G: Grubbs' Test for Outliers for Total Carbonyl Mass

1/2 collection eq.	nanomoles	Z value	Significant outlier. P
exp 1	288.663	1.78713	< 0.05
exp 2	31.869	0.46315	
exp 3	34.173	0.44296	
exp 5	41.379	0.37981	
exp 6	27.526	0.50121	

1/4 collection eq.	nanomoles	Z value	Significant outlier. P
exp 1	201.760	1.78411	< 0.05
exp 2	22.650	0.47855	
exp 3	25.658	0.44055	
exp 5	34.154	0.33322	
exp 6	18.435	0.5318	

1/8 collection eq.	nanomoles	Z value	Significant outlier. P
exp 1	118.227	1.76304	< 0.05
exp 2	9.505	0.53201	
exp 3	7.508	0.57416	
exp 5	27.575	0.15056	
exp 6	10.723	0.5063	

1/16 collection eq.	nanomoles	Z value	Significant outlier. P
exp 1	40.659	1.77802	< 0.05
exp 2	4.369	0.32696	
exp 3	-0.446	0.60625	
exp 5	1.678	0.48305	
exp 6	3.769	0.36176	

Appendix H: Average concentration (n=6) of specific carbonyl groups for Sep-Pak DNPH-Silica Cartridges exposed to diesel exhaust for 2 hours and blank, unexposed Sep-Pak DNPH-Silica cartridges after separation by HPLC, detection by MS, and concentration derivation via standard curves for specific carbonyls

average (ug/sample)	Formaldehyde	Acetaldehyde	Acrolein	Acetone+Propanal	Methacroelin +Crotonaldehyde	Butanal	n-Pentanal	n-hexanal	benzaldehyde	n-heptanal	p-tolualdehyde	n-octanal	n-nonanal
1/2 collection exposure equivalent	2.769	0.710	0.004	0.163	0.010	0.349	0.208	0.060	0.031	2.834	0.011	0.730	0.679
1/4 collection exposure equivalent	1.720	0.340	0.003	0.078	0.006	0.139	0.076	0.023	0.010	1.604	0.004	0.571	0.672
1/8 collection exposure equivalent	1.084	0.200	0.002	0.042	0.004	0.055	0.044	0.012	0.007	1.101	0.003	0.323	0.382
1/16 collection exposure equivalent	0.391	0.079	0.003	0.019	0.002	0.019	0.012	0.001	0.003	0.451	0.001	0.132	0.316
1/2 blank equivalent	0.344	0.214	0.004	0.068	0.003	0.219	0.153	0.039	0.016	3.924	0.001	0.707	0.725
1/4 blank equivalent	0.229	0.101	0.004	0.037	0.002	0.062	0.071	0.017	0.007	1.449	0.001	0.460	0.160
1/8 blank equivalent	0.185	0.051	0.004	0.021	0.001	0.040	0.036	0.011	0.004	0.996	0.001	0.391	0.422
1/16 blank equivalent	0.125	0.028	0.004	0.013	0.001	0.016	0.020	0.002	0.001	0.491	0.000	0.148	0.303

standard deviation (ug/sample)	Formaldehyde	Acetaldehyde	Acrolein	Acetone+Propanal	Methacroelin +Crotonaldehyde	Butanal	n-Pentanal	n-hexanal	benzaldehyde	n-heptanal	p-tolualdehyde	n-octanal	n-nonanal
1/2 collection exposure equivalent	3.441	0.515	0.013	0.076	0.003	0.258	0.196	0.053	0.028	4.037	0.011	1.078	3.442
1/4 collection exposure equivalent	2.467	0.165	0.011	0.016	0.001	0.086	0.028	0.012	0.004	2.435	0.002	0.908	2.505
1/8 collection exposure equivalent	1.634	0.126	0.012	0.018	0.002	0.024	0.037	0.011	0.004	1.611	0.002	0.456	1.124
1/16 collection exposure equivalent	0.784	0.049	0.011	0.011	0.002	0.017	0.024	0.004	0.003	0.674	0.001	0.218	0.615
1/2 blank equivalent	0.420	0.136	0.006	0.014	0.002	0.155	0.054	0.020	0.006	5.625	0.001	0.960	4.006
1/4 blank equivalent	0.338	0.049	0.007	0.008	0.002	0.038	0.024	0.004	0.003	2.106	0.001	0.705	1.866
1/8 blank equivalent	0.371	0.032	0.007	0.007	0.001	0.028	0.019	0.005	0.003	1.568	0.000	0.561	1.637
1/16 blank equivalent	0.336	0.024	0.007	0.011	0.002	0.014	0.020	0.003	0.003	0.865	0.001	0.194	0.730

***n-heptanal, n-octanal, n-nonanal are not used in quantifying total aldehydes as they are not included in the standard solution AE00043**

Appendix I: Average peak area (n=4) of specific carbonyls after HPLC separation and MS determination of differing masses of standard AE00043

Average Peak Area (n=4) Normalized to IS

MS Standard Curve Injection Mass (micrograms)	Formaldehyde	Acetaldehyde	Acrolein	Acetone+Propanal	Methacrolein +Crotonaldehyde	Butanal	n-Pentanal	n-hexanal	benzaldehyde	n-heptanal	p-tolualdehyde	n-octanal	n-nonanal
0.3600	4317.671	2609.503	6097.664	2585.356	2776.586	2066.316	712.539	700.445	1306.773	1.738	898.376	3.020	7.249
0.1800	3025.982	1740.745	4265.320	1726.595	2288.069	1802.767	579.399	588.118	1070.960	1.963	703.746	3.830	10.276
0.0900	1951.886	1050.733	2437.997	937.808	1573.887	1239.410	412.012	438.869	845.822	2.953	582.609	9.124	12.286
0.0450	1030.079	557.183	1229.883	495.159	885.206	711.573	257.780	261.938	559.656	2.467	356.096	4.920	9.839
0.0225	613.283	324.992	674.942	261.821	484.372	383.115	157.819	156.089	384.961	2.450	225.911	4.702	12.132
0.0113	381.670	199.203	325.963	138.330	234.648	204.360	89.543	85.317	201.174	2.539	133.649	3.983	13.327
0.0056	244.541	129.544	159.879	74.897	112.778	103.370	50.811	46.122	115.860	2.710	70.317	4.038	12.414
0.0028	193.782	102.272	88.574	45.266	57.290	57.844	37.184	25.602	58.878	4.162	38.885	5.453	11.153
0.0000	118.265	63.363	1.418	16.554	0.665	13.381	3.758	2.074	2.446	2.353	0.676	3.389	9.373

Standard Deviation

MS Standard Curve Injection Mass (micrograms)	Formaldehyde	Acetaldehyde	Acrolein	Acetone+Propanal	Methacrolein +Crotonaldehyde	Butanal	n-Pentanal	n-hexanal	benzaldehyde	n-heptanal	p-tolualdehyde	n-octanal	n-nonanal
0.3600	2703.654699	1610.545163	3770.6482	1615.800553	1442.419073	1021.988	355.64095	345.384	716.7906273	0.894204	446.7468658	2.865996	1.862912
0.1800	1400.606858	813.4046031	1888.8096	718.4299019	800.5144908	724.1127	237.40947	233.4773	418.1881396	0.5074638	287.9398202	4.567052	5.451208
0.0900	875.6662873	514.4901916	1275.5118	460.3113559	641.4836404	556.8153	174.27147	183.8847	306.6358735	1.0684138	248.5656729	9.236696	1.875748
0.0450	482.4592818	254.808289	660.1021	230.8912183	387.4957716	364.6653	122.09401	111.5664	270.0619096	1.0335488	173.1923638	3.227927	4.235628
0.0225	300.0102024	163.9447011	319.36228	110.3413941	237.897338	192.3647	80.170281	75.29641	173.2757343	0.8610885	111.6960784	3.892598	7.384889
0.0113	167.184857	88.58578539	126.56245	47.98211542	87.24666742	87.32323	42.912899	38.59823	85.7056904	1.1669921	55.37067881	5.216755	10.65011
0.0056	123.3415713	66.85434801	70.349771	35.37691268	49.52865318	48.7827	24.066602	19.77942	52.80791178	1.1833076	32.00005691	4.819207	3.886325
0.0028	97.43515164	51.97316546	44.764875	18.26956772	22.74865846	27.87542	13.721755	11.48135	25.78663362	3.9536292	16.18079587	10.82623	5.695268
0.0000	67.43059082	38.3585324	2.0500327	5.485105112	0.771683082	5.143868	4.355486	1.00056	1.289307151	0.8714473	0.520132857	6.778485	3.300632 *

Both acetone/propanal and methacrolein/crotonaldehyde co-elute in this system