Project Report

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Figure 8B. DTT consumption rates in nanomoles/min/μg extract for the *NIST-DEP* sample extracts. Values represent mean of quadruplicate determinations ± SD. [a] MeOH-HRE significantly different than DCM-HRE, Tol-HRE, DMT-HRE, DMT-SE and H2O-SE, $p <0.05$

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Abstract

To study the effect of sample preparation on the toxicity of diesel exhaust particles (DEP), six different sample preparation techniques were chosen for toxicological screening via a multi-point standard assay panel. The DEP samples studied include a NIST standard and filter samples collected from the exhaust stream of a dilution tunnel during a chassis dynamometer study. These samples were prepared using two exhaustive (i.e. whole particle) and four selective (i.e. PM fractionation) extraction techniques. All sample extracts were toxicologically screened for ROS production via the acellular dithiothreitol assay, cellular inflammation via the cyclooxygenase-2 and interleukin-8 proteins, PAH response via cytochrome P450 1A1 expression and mutagenicity via the microsuspension Salmonella/microsome assay. Results demonstrated that: *(1)* selective extraction techniques consistently tend to enhance the effect of the more active DEP components by removing the less reactive matrix components that are included in exhaustive techniques, *(2)* nonpolar compounds elicit the greatest responses across all assays except for ROS production, which is largest for the more polar DEP components, *(3)* strong evidence exists for composite interference or toxicological matrix effects, where the sum of responses to individual components is greater than the response to the composite of those components and *(4)* although different sample preparation techniques appear to be equally repeatable across different assays, the techniques eliciting the most robust response are assay-specific and include cases where multiple techniques are equally effective.

Executive Summary

Background. In recent years, numerous advances have been made in the development of new engines, emissions control technologies, fuels and fuel blends with the aim of reducing the impact of transportation and vehicular emissions on human health and the environment. It is imperative that these new technologies and fuels are tested and the intended emissions and health impact reductions validated prior to releasing them to market for mass production and distribution. Numerous stakeholder agencies, such as the California Air Resources Board, routinely perform chassis dynamometer studies aimed at such investigations. In addition to measuring gas and particle emission factors, these studies also collect emissions, typically on filters and resin cartridges, for subsequent chemical and toxicological testing. Given the high throughput and low-cost requirements of these types of studies, standard assay panels have been developed using well known toxicity markers as an inter-comparative toxicity screening mechanism. An essential part of these efforts is the sample preparation technique used to pre-treat, extract and post-process the PM samples prior to toxicity screening. Numerous techniques are available and different groups employ different methods, potentially resulting in outcomes that are partially or wholly dependent on the technique employed and thus biasing inter-study comparisons. Remarkably, there has never been a systematic study that investigates this potential bias or compares the various techniques in terms of the elicited outcomes. This is the primary research objective here and the end goal is development of standard operating procedures for PM sample preparation that optimize the robustness, reliability and repeatability of each assay in the panel.

Objectives and Methods. The primary objective of this work is to investigate the effect of different sample preparation techniques on the outcomes of a standard assay panel for toxicity screening of PM emissions collected on filters during chassis dynamometer studies involving various combinations of engine and emission control technologies, driving conditions and fuels or fuel blends. For the current study, diesel exhaust particles (DEP) from a single chassis dynamometer study (*dyno-DEP*) were used and the assay panel held constant while the sample preparation technique was varied. A NIST diesel engine PM standard (NIST SRM 1650) provided as whole free PM (*NIST-DEP*) was subjected to the same sample preparation techniques and assay panel for comparative purposes. The sample preparation techniques included: *(1)* dichloromethane heat reflux extraction (DCM-HRE), *(2)* methanol heat reflux extraction (MeOH-HRE), *(3)* toluene heat reflux extraction (Tol-HRE), *(4)* serial DCM + MeOH + Tol heat reflux extraction (DMT-HRE), *(5)* water sonication extraction (H2O-SE) and *(6)* serial DCM + MeOH + Tol sonication extraction (DMT-SE). The multi-point assay panel included ROS production via the acellular dithiothreitol (DTT) assay, cellular inflammation via the cyclooxygenase-2 (COX-2) and interleukin 8 (IL-8) proteins, PAH response via cytochrome P450 1A1 (CYP1A1) expression and mutagenicity via the Ames assay. The null hypothesis states that for a given dynamometer study, the outcomes of the standard assay panel used to screen the toxicity of the PM emissions generated during that study are independent of the technique used to prepare – including pretreatment, solvent extraction and post-extraction processing – the PM sample, in whole or in part, for the assay panel. The core test hypotheses for this study include: *(1)* the outcome of a particular assay depends on whether the sample preparation technique is exhaustive or selective in nature; i.e. whether whole PM or solvent extractable PM components (fractionated PM) are used, *(2)* the outcome of a particular assay using either whole PM or fractionated PM depends on the solvent or combination of solvents used, *(3)* for assays conducted using fractionated PM, the sum of responses to individual PM

fractions will be significantly larger than the individual response to the composite of those PM fractions, i.e. the presence of assay-specific toxicologically inert PM components interferes with the response to the toxicologically active PM components, and *(4)* the sample preparation technique yielding the most robust, reliable and repeatable outcome will be assay dependent

Results. There were several general results consistently observed throughout this study. First, for a given sample preparation technique and across all assays, the response to *NIST-DEP* was significantly greater than *Dyno-DEP*, by more than an order of magnitude in some cases. This has been attributed to compositional differences in the DEP samples, as well as differences in how the DEP was sampled. Secondly, for both *NIST-DEP* and *Dyno-DEP*, and again across all assays, HRE tended to elicit significantly more activity than SE. The selective nature of the HRE technique compared to the exhaustive nature of SE gives rise to this trend. Thirdly, for HRE of *NIST-DEP* and *Dyno-DEP*, DCM and Tol generally elicited greater effects than MeOH while for SE, DMT was greater than H2O. Both trends relate to the toxicity of nonpolar versus polar DEP components. Lastly, DCM-HRE and MeOH-HRE of *NIST-DEP* were done in duplicate and all extracts blindly added to the sample collection measured by the assay panel to provide a blind metric of repeatability. Overall, good agreement between the duplicates was observed for both methods and all assays. Assay-specific results include *(1)* expression of CYP1A1 was largest for Tol-HRE and since Tol is most efficient at solvating polycyclic aromatic hydrocarbons (PAHs), this corroborates the notion that PAHs drive CYP1A1 response, *(2)* the highest expressions of IL-8 and COX-2 were elicited by multiple sample preparation techniques that were not significantly different from one another, e.g. DCM-HRE, Tol-HRE and DMT-HRE, *(3)* DCM-HRE of *Dyno-DEP* induced the most mutagenic activity, which is consistent with previous studies and *(4)* MeOH-HRE of *NIST-DEP* resulted in the largest DTT consumption rates suggesting polar DEP compounds, such as oxygenated organics, drive ROS production.

Conclusions. The most important conclusions of this work can be summarized as follows: *(1)* HRE consistently tends to enhance the effect of the more active DEP components by removing the less reactive matrix components that are included in SE, *(2)* nonpolar compounds elicit the greatest responses across all assays except for ROS production, which is largest for the more polar DEP components, *(3)* there is strong evidence for the existence of composite interference or toxicological matrix effects, where the sum of responses to individual components is greater than the response to the composite of those components and *(4)* although different sample preparation techniques appear to be equally repeatable across different assays, the techniques eliciting the most robust response are assay-specific and include cases where multiple techniques are equally effective. Recommendations for future research include a comprehensive chemical characterization of the sample extracts to fill the knowledge gap of the compositional differences driving the observed differential toxicity, which is necessary for a more informative description of the relationship between toxicity and chemical complexity. Despite the tendency to choose the method eliciting the greatest response, a candid conversation about whether selective or exhaustive extraction methods are the more appropriate evaluation metric is of critical importance given that complex particle mixtures, rather than fractionated PM components, more robustly characterize true human exposure. If a single sample preparation technique is preferred for all assays, then a hybrid of the DCM-HRE and MeOH-HRE methods is strongly recommended, and if an exhaustive method is preferred, then the hybrid HRE method without the post-extraction filtration steps should be tested and characterized.

1. Introduction

In chemistry, **sample preparation** is an integral component of almost all experimental, analytical, industrial and commercial applications and generally consists of three separate processes: (*1) pretreatment*, where the physical or chemical characteristics of the sample are altered prior to solvent extraction, (*2*) *solvent extraction*, where analytes, or compounds, are separated from a liquid, solid or semi-solid matrix and dissolved in a compatible solvent and (*3*) *post-extraction treatment*, where the sample extract is processed for a particular analyte of interest by removing matrix residues and interfering co-extractable compounds using filters, adsorbents such as silica or alumina and ion exchange interfaces, collectively referred to as solid phase extraction (SPE). Extractions can be characterized as either **exhaustive** – with the objective of maximizing the number and amount of compounds separated from the sample – or **selective**, where the objective is to isolate specific compounds and minimize the separation of all other compounds.

1.1. Solvent Extraction

For solid or semi-solid matrices, extraction is a diffusion based process where the solvent must diffuse into the matrix, dissolve mixtures of compounds and the solvated compounds then must diffuse from inside the matrix into the bulk solvent. Choice of solvent is perhaps one of most important extraction variables since it determines the types of compounds that will be dissolved and the extraction efficiency. This will be addressed in more detail in a following section. Other variables include temperature, pressure and mechanical energy. Elevated temperatures are generally used to accelerate the extraction kinetics by decreasing the viscosity of the solvent and increasing its diffusivity into the matrix. Elevated pressures or reflux condensers are often applied in these situations to maintain the solvent in the liquid phase. Mechanical energy can be supplied to the system to facilitate the breakdown of the matrix and thereby increase the surface area exposed to the solvent, increasing the rate and efficiency of the extraction. Several common extraction techniques are outlined below:

- **Heat reflux extraction (HRE)** is perhaps the simplest technique whereby the sample is placed directly into a solvent and the solvent is maintained at its boiling point for extended periods using a reflux condenser to keep the solvent, and other compounds, in the liquid phase. This technique is limited by the boiling point of the solvent and typically requires longer extraction times and more post-extraction processing but is a simple and cost-effective solution in small experimental applications.

- **Soxhlet extraction (SoxE)** is essentially identical to HRE with the addition of an apparatus called a Soxhlet extractor consisting of a glass chamber housing a thimble constructed from thick filter paper. The sample is placed in the thimble and the condensing solvent vapors slowly pass through the thimble during reflux to dissolve the compounds while keeping any solid matrix material from entering the bulk solution. This technique simply eliminates filtration from the post processing step.

- **Sonication extraction (SE)** is a process where ultrasonic energy is applied to a solvent to nucleate, grow and implosively collapse microscopic bubbles as a means of mechanically breaking down the sample matrix to increase the surface area

exposed to the solvent. This greatly increases the extraction efficiency and reduces extraction time. Although sonication is typically carried out at ambient pressure and temperature, these systems can be modified to accommodate elevated temperatures in sealed pressure vessels to further increase speed of extraction and reduce solvent.

- **Microwave-assisted extraction (MAE)** uses microwave radiation to heat the sample suspended in a solvent in a closed vessel. These systems are readily available and provide programmable temperature control and high throughput capabilities by batch processing multiple samples in parallel. Extractions can be performed relatively quickly and efficiently, reducing the amount of required solvent. They are more common in larger scale analytical laboratories handling large sample numbers.

- **Supercritical fluid extraction (SFE)** takes advantage of the hybrid gas- and liquid-like properties of supercritical fluids, which exhibit the dynamic motions of gases – resulting in lower viscosity and higher diffusivities – but can also dissolve compounds like a liquid solvent. Given the absence of surface tension and reduced viscosity, supercritical fluids can penetrate the sample matrix more efficiently and completely than liquids, thus significantly increasing the speed of extraction. By far the most commonly used supercritical fluid is carbon dioxide (CO_2). Since CO_2 is nonpolar and has limited dissolving power, modifiers such as low molecular weight alcohols are sometimes added as co-solvents to increase the range of compounds that can be extracted. Selective extraction can be achieved by varying the pressure and temperature, which alters the properties of the supercritical fluid. An additional benefit is that when the pressure is reduced after extraction, the extracted compounds precipitate out as the CO_2 enters the gas phase – which is either vented or recovered – eliminating the need for any solvent removal steps. However, the high pressure and special handling requirements of this technique make it considerably more expensive then liquid-based extractions and thus it has limited applicability.

- **Pressurized fluid extraction (PFE)**, also referred to as pressurized solvent extraction (PSE) or accelerated solvent extraction (ASE), is the most widely used technique in large scale, high throughput applications. It uses a combination of elevated temperature and pressure, typically in the range of 100-200°C at 1500 psi, to accelerate the extraction kinetics by decreasing the viscosity of the solvent. The high pressure not only keeps the solvent in the liquid phase but also tends to swell the matrix, increasing solvent penetration. This significantly increases extraction speed and reduces solvent requirements. Similar to MAE, numerous different systems are widely available with various levels of control, automation, throughput and post-processing capabilities, such as in-cell filtration and adsorbents.

The largest difference between all of the techniques outlined above is the amount of time and solvent required to complete the extractions, not necessarily the extraction efficiency in terms of the amount and types of compounds recovered which, as discussed next, depends more on solvent selection. Table 1 compares extraction times and solvent usage for several techniques based on extracting a single sample. For small-scale experimental studies with relatively low numbers of samples, longer extraction times and larger solvent requirements are an acceptable

tradeoff for the lower startup costs associated with simpler techniques such as HRE or SoxE. For large scale applications with high throughput, however, the initial startup costs of more sophisticated and automated techniques such as ASE is worth the investment.

Table 1. Estimates of extraction time and solvent usage for different extraction techniques

Technique	Extraction Times	Solvent Usage
Soxhlet	4-48 hrs	150-500 mL
Automated Soxhlet	1-4 hrs	50-100 mL
Sonication	0.5-1 hrs	150-200 mL
Supercritical Fluid	0.5-2 hrs	5-50 mL
Microwave	0.5-1 hrs	25-50 mL
Accelerated Solvent	0.2-0.3 hrs	5-200 mL

1.2. Solvent Selection

The properties of solvents that determine the types of compounds they can dissolve and the nature and stability of the solvation interaction include polarity, dipole moment, polarizability and hydrogen bonding. Solvents are categorized as either polar protic (e.g. water, methanol...), polar aprotic (e.g. dichloromethane, acetone...) or nonpolar (e.g. hexane, benzene...). In general, polar solvents best dissolve polar compounds (e.g. sugars, inorganic salts...) and nonpolar solvents are best at dissolving nonpolar compounds (e.g. oils, waxes...). Furthermore, anions are strongly solvated by protic solvents via hydrogen bonding while positively charged ions are strongly solvated by aprotic solvents via their negative dipole. Several common solvents are listed in Table 2 along with their relevant properties, including boiling point, density, dielectric constant (a measure of polarity), and dipole moment, as well as metrics of polar bonds (δP) and hydrogen bonding (δH) which form the basis of the Hansen solubility parameters.

The choice of solvent clearly depends on the objective of the extraction. For exhaustive extractions, using several different solvents spanning the polarity range in series will yield the best results. For selective extractions, however, a single solvent is often chosen that is most compatible with the compound of interest but also minimizes co-extraction of interfering, or unwanted, compounds. Additional considerations include the cost, required volume, toxicity and boiling point of the solvent, as well as its compatibility with equipment and system design. In most applications, the solvent will be removed, or solvent exchanged, post-extraction via N_2 blow-down or an evaporator system to recover the extracted compounds or prepare them for subsequent analysis via analytical techniques such as GC-MS. In these cases, the boiling point of the solvent is an important parameter to consider in terms of the temperatures, pressures and time required to remove the solvent and the stability of the extracted compounds in that environment. For example, in an exhaustive extraction of a sample of unknown composition that may contain semi-volatile compounds or compounds with low boiling points it is essential that a solvent with the lowest possible boiling point be chosen to minimize the loss of these species during solvent removal. This will be considered further in a following section.

1.3. Filter Extractions

In air quality science, particulate matter (PM) is commonly sampled from an environment – e.g. the ambient atmosphere, smokestacks, building interiors and laboratory generated exhaust

streams – by drawing air across a filter, or some other type of substrate, to separate the PM from the gases. PM collected on filters can then be analyzed for composition and/or toxicity using various analytical techniques and in vivo and in vitro systems. In almost all cases, the PM must be removed, or extracted, from the filter prior to analysis. Depending on the objective, the filter extraction process can be exhaustive – i.e., maximizing the amount of total PM removed from the filter – or selective, i.e. extracting only certain PM components or compound classes.

Table 2. Common solvents and their relevant solubility parameters.

Solvent	Boiling Point (°C)	Density (g/mol)	Dielectric Constant	Dipole Moment	δP Polar Bonds	δH Hydrogen Bonding
Non-Polar Solvents						
Hexane (hx)	69	0.655	1.88	0.00 D	0.0	0.0
Benzene (Bz)	80	0.879	2.30	0.00 D	0.0	2.0
Toluene (tol)	111	0.867	2.38	0.36 D	1.4	2.0
Diethyl ether (eth)	35	0.713	4.30	1.15 D	2.9	4.6
Chloroform (chl)	61	1.498	4.81	1.04 D	3.1	5.7
1,4-Dioxane	101	1.033	2.30	0.45 D	1.8	9.0
Polar Aprotic Solvents						
Ethyl acetate	77	0.894	6.02	1.78 D	5.3	7.2
Tetrahydrofuran (THF)	66	0.886	7.50	1.75 D	5.7	8.0
Dichloromethane (DCM)	40	1.327	9.10	1.60 D	7.3	7.1
Acetone (ace)	56	0.786	21.00	2.88 D	10.4	7.0
Acetonitrile (MeCN)	82	0.786	37.50	3.92 D	18.0	6.1
Dimethylformamide (DMF)	153	0.944	38.00	3.82 D	13.7	11.3
Dimethyl sulfoxide (DMSO)	189	1.092	46.70	3.96 D	16.4	10.2
Polar Protic Solvents						
Acetic acid	118	1.049	6.20	1.74 D	8.0	13.5
n-Butanol	118	0.810	18.00	1.63 D	5.7	15.8
Isopropanol	82	0.785	18.00	1.66 D	6.1	16.4
n-Propanol	97	0.803	20.00	1.68 D	6.8	17.4
Ethanol (EtOH)	79	0.789	24.55	1.69 D	8.8	19.4
Methanol (MeOH)	65	0.791	33.00	1.70 D	12.3	22.3
Formic acid	101	1.210	58.00	1.41 D	10.0	14.0
Water	100	1.000	80.00	1.85 D	16.0	42.3

For most *in vivo* toxicological studies, the primary objective of filter extraction is to conserve the physical and chemical properties of the PM as it originally existed in the atmosphere or exhaust stream – including particle size, number concentration, morphology and individual particle compositional and structural integrity – so that the results of these studies are representative of true population exposure. Currently, the most widely applied filter extraction technique involves sonication in ultra-pure water followed by lyophilization to remove the water and recover dry PM (*1, 2*). The PM is then suspended in the delivery vehicle and sonicated and/or vortexed immediately prior to instillation or aspiration. Extraction efficiencies – i.e. the mass of PM removed by extraction relative to the mass of PM collected on the filter – on the order of 60-70% are commonly reported for this technique. Recently, a more exhaustive, multi-solvent process based on a combination of sonication, liquid-liquid extraction, selective filtration and solvent

removal was introduced, resulting in extraction efficiencies consistently exceeding 90% and often times approaching 100% (*3*).

Compositional studies, however, require a suite of analytical techniques given the chemical complexity of PM and each technique measures a certain class of compounds; e.g. metals via Inductively Coupled Plasma Mass Spectrometry (ICP-MS), inorganic ions via Ion chromatography (IC) or polycyclic aromatic hydrocarbons (PAHs) via Gas Chromatography Mass Spectrometry (GC-MS). In this case, the goal of filter extraction is to selectively extract certain compounds while minimizing co-extraction of potential interfering species and thus each analytical technique typically requires its own filter extraction protocol. For example, trace element analysis via ICP-MS requires an initial organic solvent extraction (usually acetone or DCM) followed by acid digestion using a strong acid such as nitric or hydrofluoric acid (*4*). The initial organic solvent extraction is necessary for most combustion generated aerosol and/or secondary organic aerosol (SOA) since *(a)* the trace metals are typically encapsulated by layers of organic compounds and *(b)* most organic compounds are hydrophobic and thus are not likely removed from the filter to any significant degree by water alone. Once the organic layers are removed, acid digestion dissociates the metal oxides and salts, bringing the metal ions into solution for analysis.

There are several different sample preparation protocols for molecular speciation of particulate organic carbon via GC-MS analysis as well, which are based on organic solvent extraction using common solvents such as DCM and hexane followed by some kind of post-extraction cleanup step (*5*-*10*). These will not be reviewed in any detail here but consider, as an example, one of the most recent protocols for PAH analysis using ASE (*11*) or PFE (*12*). In the former, the filter is placed in a pressure cell with DCM and extracted at 100°C and 1500psi for 30 minutes. The extract is then pumped through acidic alumina and an in-cell filter to remove any residual matrix material. The solvent is evaporated using N_2 blow-down and solvent exchanged to hexane prior to GC-MS analysis. The PFE technique is very similar except that the DCM extraction is followed by a DCM/benzene mixture and then subjected to a mini column cleanup containing silica prior to solvent exchanging to hexane and GC-MS analysis.

In vitro studies – where the PM is administered to cell cultures and different assays are used to assess cellular response for various endpoints such as reactive oxygen species (ROS) via the heme oxygenase-1 (HO-1) enzyme, inflammation via the expression of cyclooxygenase-2 (COX-2) enzyme and interleukin 8 (IL-8) chemokine or interleukin 6 (IL-6) cytokine and PAHs via cytochrome P450 1A1 (CYP1A1) expression – are commonly used as a high throughput toxicity screening mechanism since they are considerably faster and less expensive than *in vivo* studies. Similar to *in vivo* studies, however, the PM must still be extracted from the filter prior to testing. Typically, the objective of the extraction for toxicity screening is to elicit the most robust, reliable and repeatable response for a particular assay. Since different assays are designed for specific endpoints and since these endpoints may respond solely to the presence of particles, regardless of composition, or may be more sensitive to specific PM components, the appropriate extraction technique may vary depending on the assay and thus may be exhaustive, as it is for *in vivo* studies, or selective, as is the case for compositional analysis. For example, inflammation may simply be a response to the presence of particles. It may be exacerbated by the presence of certain compounds in the particle but may also be undetectable in the presence of PM

components not in particulate form; i.e. dissolved in the cell media. In this case, an exhaustive extraction that aims to conserve the physical and chemical properties of the particles may elicit the greatest effect. Conversely, the presence of particles and unrelated particle components may interfere with the response of an assay designed for a specific compound class such as PAHs so that a selective extraction that minimizes co-extraction of interfering species may be best. Similarly, the cellular response to ROS obviously depends on ROS production, which many studies have shown depends on the presence of particulate trace metals such as iron and copper. If these trace metals are fully encapsulated by organic layers and these layers are not removed during extraction, or dissolved by the cell media, then this may inhibit ROS production and obscure the response of the assay. In this case, a more sophisticated extraction that dissolves the organic layers and then suspends and disperses the remaining particulate core matrices into the cell media may elicit the most robust response.

1.4. Diesel Exhaust Particles

The composition and toxicity of PM emitted by diesel engines, commonly referred to as diesel exhaust particles (DEP) in the toxicology literature, have been studied for over four decades, beginning with the inception of the U.S. EPA's diesel emissions research program by the Office of Research and Development in 1979 (*13, 14*); epidemiological studies date back to the 1950s (*15*). Since the chronic exposure studies on the carcinogenic effects of DEP and assay based mutagenicity tests of the 1980s (*16-36*), there has been an exponential increase in the sophistication, scope and resolution of biological toxicity screening, including: *(a)* assays – e.g. reverse transcription polymerase chain reaction (RT-PCR) and ultra-sensitive enzyme-linked immunosorbent assays (ELISA), *(b)* animal models – e.g. transgenic and knockout mice, *(c)* cell lines – e.g. human lung epithelial cells, macrophages, cardiomyocytes and stem cells, *(d)* endpoints – e.g. pulmonary, cardiovascular and neurological, *(e)* injury metrics – e.g. protein levels and gene expression and regulation, *(f)* and exposure scenarios – e.g. real-time concentrated ambient particle studies (CAPS), source-oriented toxicity, adjuvant effects and multipollutant considerations. Studies are also becoming increasingly mechanistic rather than causal in nature.

Diesel emissions (DE) have been more heavily researched than any other source of air pollution and, as a result, there is currently an exhaustive body of literature on the subject (*37-41*). In fact, the need for additional health effects studies on diesel emissions has come in to question based on the premise that enough is already known to both merit and guide regulatory policy and emission reduction strategies (*42*). However, with the recent advent of new engine and emission control technologies, fuels and fuel blends, the field is quickly reemerging as regulatory agencies and other stakeholders try to determine how mass implementation of these technologies might change the emissions and toxicology landscape. Dynamometer studies are clearly an available and reproducible approach to begin investigating this since experimental conditions can be precisely controlled so that variable dependencies are accurately determined and parameterized. Given the rate of technological advancement and the societal need for quick implementation of that technology, dynamometer studies also offer an efficient venue for conducting this research. This is further facilitated by the continued development of real-time instruments for validating intended emissions reductions and high throughput assays to screen those emissions for unintended changes in toxicity.

A primary requirement of high-throughput toxicity screening is designing an assay panel that most accurately represents the severity-weighted range of potential health effects using robust indicators that can elicit statistically significant measures of differential toxicity with confidence. Maximizing dynamic range in assay response is a viable strategy for pursuing this. One of the key factors in assay response is the methodology used to prepare the PM sample prior to testing. As stated above, there is an exhaustive literature on the health effects of diesel emissions and accompanying that literature is a fairly large range of sample preparation techniques as well (*43-96*). It is evident in this literature that assays respond differently to different techniques so that determining those techniques that elicit the most robust and reliable response should be a major objective. For most studies, however, it has been sufficient to simply demonstrate cause and effect and any effort to maximize the metric of that causal relationship has been unnecessary. As a result, there is a general paucity of studies that inter-compare the various sample preparation techniques in terms of the responses they elicit from different assays. This is, in fact, the main objective of the work conducted here.

Although a systematic review of the DEP extraction literature will not be given here, it is obvious that all techniques naturally fall into one of two categories: whole particle or solvent extractable PM component (PM fractionation). Whole particle extractions can be further categorized by whether the DEP is suspended and dispersed directly in the cell culture medium or extracted by sonication in a solvent followed by solvent removal and resuspension in medium. Solvent sonication can be further divided by the type and number of solvents used. Similarly, PM fractionation can be categorized by extraction technique, type and number of solvents and post-processing cleanup. An organizational chart showing the current state of science in extracting DEP for subsequent toxicological studies is shown in Figure 1.

Essentially all *in vitro* based DEP studies in the literature begin with a whole PM sample either purchased as a standard from an agency like the National Institute of Standards and Technology (NIST) or gifted from another research group with an existing dynamometer facility. For the latter, the PM sample is either scraped off some part of the heat exchange or dilution system or sampled from the dilution exhaust onto a filter; typically Teflon bound glass microfiber filters. As a result, and for comparative purposes, the study being described here included both a NIST standard and a DEP sample collected via filter deposition during a dynamometer study.

2. Materials and Methods

For the study described here, six different techniques were employed to prepare DEP samples for a multi-point standard assay panel. The DEP samples studied include a NIST diesel engine PM standard (NIST SRM 1650) provided as whole free PM (*NIST-DEP*) and a filter sample collected from the exhaust stream of a Heavy Duty Vehicle (HDV) connected to a dilution tunnel during a chassis dynamometer study (*Dyno-DEP*). A filter blank from that same study (*FB*) was also included. The outcomes of the standard assay panel were statistically analyzed to address the test hypotheses outlined in a following section. Details on the various sample preparation techniques and toxicological assays used in this study follow as well.

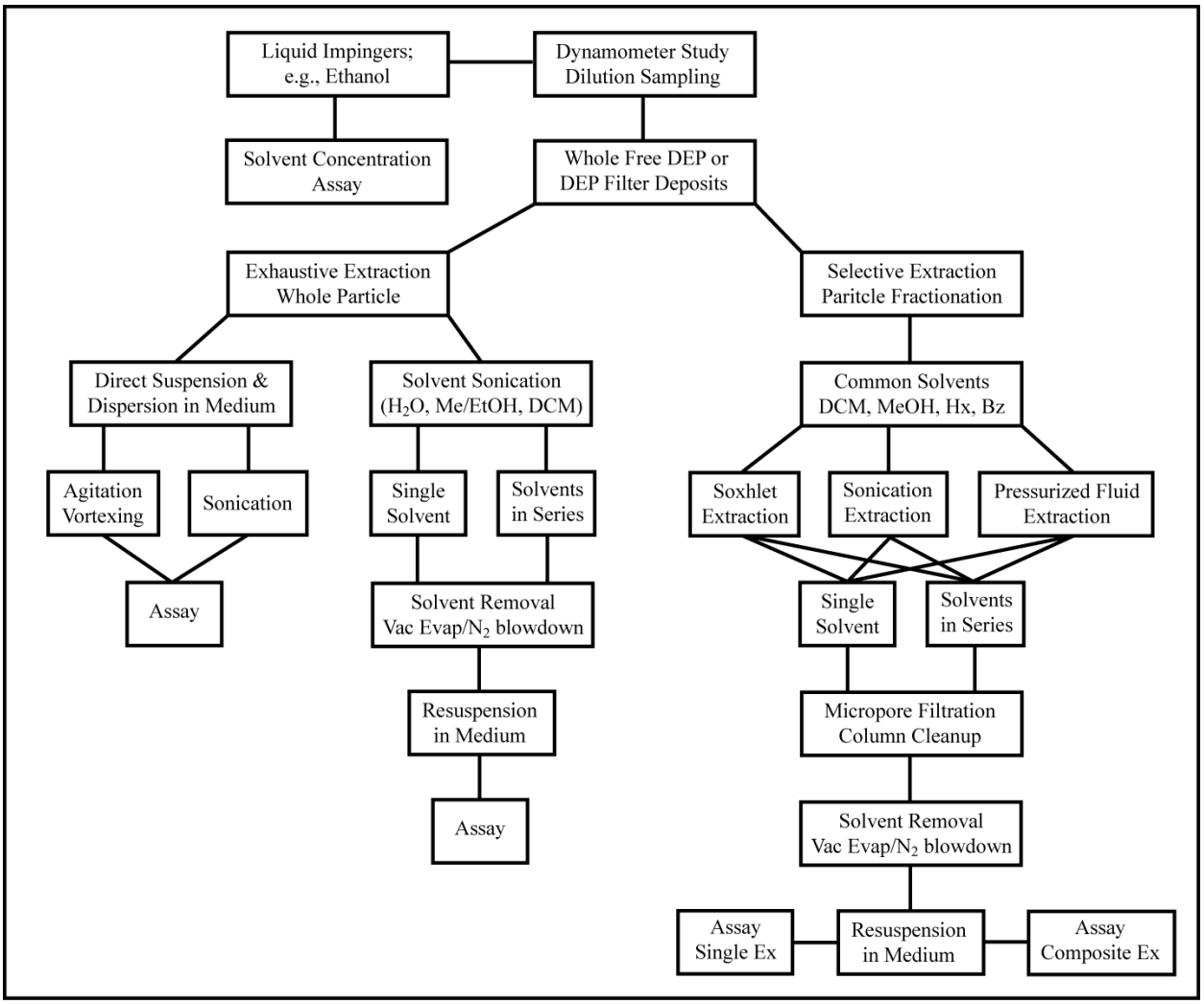

Figure 1. Organizational chart showing the current state of science in extracting DEP for subsequent toxicological analysis; see references in the text.

2.1. Dynamometer Diesel Exhaust Particles (Dyno-DEP)

The chassis dynamometer diesel exhaust particles (Dyno-DEP) provided for this study were collected from the emissions of a 2000 Freightliner Truck equipped with a 2000 Caterpillar C15 engine. The vehicle was run on California certified Ultra-Low Diesel Fuel (CARB Diesel Fuel) and tested using the Urban Dynamometer Driving Schedule (UDDS). The testing was part of a study of Biodiesel fuel emissions (*12, 97*) where the CARB diesel fuel served as a control baseline fuel.

Tests were conducted at the California Air Resources Board (CARB) Heavy-Duty Engine Emissions Testing Laboratory (HDEETL) located at the Metropolitan Transit Authority facility in Los Angeles, California. This facility is equipped with a Schenck-Pegasus chassis dynamometer with a single 72 inch diameter roller. The dynamometer is driven by a direct current 675 horsepower motor that can absorb up to 600 horsepower. Criteria pollutant emissions

were tested using a full flow constant volume sampler (CVS) dilution system as previously reported (*98*).

Samples were drawn from the dilution tunnel using a stainless steel probe positioned in the same plane as the PM probes used for Code of Federal Regulations (CFR) PM measurements. The sampler for the bioassay analyses consisted of a 1.5" O.D. stainless steel probe connected to an 8" x 10" High-Volume (Hi-Vol) filter unit. The 8x10 inch filters were Teflon (Zefluor, Pall Life Sciences, Ann Arbor, MI). Flow rates were nominally 450 liters per minute (Lpm) for the samplers. Flows were calibrated on the inlet side using a calibrated laminar flow element (CME, Davenport, IA, USA). Two back-to-back UDDS test cycles were run for each filter collection representing approximately 35 min and 11 miles driven.

Hi-Vol filter samples were stored at – 20°C until shipment to the University of California, Davis. Samples for shipments were placed in insulated containers, packed with blue ice, and shipped using overnight delivery. Two separate Hi-Vol filter samples were used for the analyses. Each filter was cut into small measured rectangles based on PM mass. **Filter 1** size cuts were 35.5 x 21.3 mm each while **Filter 2** size cuts were 27.1 x 22.5 mm. Each piece was estimated to contain 200 ug PM. Sets of **Filter 1** and **Filter 2** pieces were placed together representing 400 ug PM for each of the six sample preparation techniques, described below.

2.2. Sample Preparation

In total, and based on existing techniques for extracting DEP (Figure 1), two exhaustive, or whole particle, extractions were performed and four selective, or PM fractionation, extractions were performed on the *NIST-DEP*, *Dyno-DEP* and filter blank (*FB*) samples. To account for any potential systematic errors in repeated applications of the same sample preparation technique without having to run additional assays, each technique was repeated independently on 2 different filter pieces for the *Dyno-DEP* and *FB* samples (**Filter 1** and **Filter 2** described above) and these extracts pooled into one composite extract for subsequent toxicity screening so that any variability associated with systematic error is explicitly inherent in the composite extract. Although this variability is likely negligible, especially when compared to the relatively large random error inherent in biological systems, it was included as a matter of robustness.

It is important to note that, although perhaps more biologically relevant in terms of modeling conditions in the human lung, using aqueous physiologic fluids such as surrogate lung fluids for extracting PM in the current study is not appropriate for cellular based assays. Surrogate lung fluids have typically been used in cell-free laboratory solutions that examine the effects of antioxidants and metals on the formation of reactive oxygen species. In those investigations, a PM extract (previously extracted with water, for example) is added to a cell-free lung solution. Also, surrogate lung fluids typically contain antioxidants that could complicate interpretation of results if directly used for extraction. The extraction methods used here were designed to obtain maximum, reproducible, and concentrated extracts for testing in cellular systems.

It is also important to note that this study focuses solely on the toxicity of DEP and extractable DEP components. There is clearly a large vapor phase component to diesel exhaust emissions and these vapors likely elicit toxicity. However, the vapor phase emissions were not sampled during the chassis dynamometer study used here and are not offered as a standard reference

material by NIST. Furthermore, the methodologies used here for extracting PM and exposing in vitro systems to the extracts cannot be used on vapor phase samples. An entirely new methodology would have to be designed, developed and characterized for that effort.

2.2.1. Exhaustive (Whole Particle) Extraction

Sonication extraction (SE) is by far the most widely used technique for extracting particles from filters, segregating agglomerated PM and dispersing extracted PM into solution, including delivery vehicles and cell culture media; see references in section **1.4**. As such, and for comparative purposes, SE was also used in the current study.

2.2.1.1. Single Solvent (SS-SE)

For this technique, the *NIST-DEP*, *Dyno-DEP* and *FB* samples were placed in milli-Q water in glass vials sealed with PTFE-lined screw caps and sonicated in a bath style ultrasonic sonicator for ~ 2 hours. The water was removed via distillation and the mass of dry PM extracts determined gravimetrically. No post-extraction processing was employed for this technique with the exception of simply removing and rinsing filter pieces from the sonicated medium for the *Dyno-DEP* and *FB* extractions. The extracted DEP samples were stored in sealed vials in a -20°C freezer until use. In the discussions that follow, these extracts will be abbreviated as H2O-SE.

2.2.1.2. Serial Multiple Solvent (SMS-SE)

All samples (*NIST-DEP*, *Dyno-DEP* and *FB*) were sonicated in series using three separate solvents spanning the polarity range, including dichloromethane (DCM; polar aprotic), methanol (MeOH; polar protic) and toluene (Tol; non-polar). DCM and MeOH are by far the most commonly used solvents in the literature and thus were chosen here for comparative purposes, as well as having low boiling points; see Table 2. The use of non-polar solvents is significantly less common but when included Hexane (Hx) and Tol are among the most frequently cited, the latter of which was chosen for the current study given its exceptional ability to solvate cyclic organic compounds such as polycyclic aromatic hydrocarbons (PAHs). Sequential sonications were performed in the order of the lowest to highest boiling point of the solvent to help minimize the loss of any semi-volatile species during the solvent removal process; i.e., DCM followed by MeOH and then Tol.

After each solvent sonication, the solvent was removed via vacuum distillation to minimal volume and then blown to dryness under a nitrogen (N_2) stream. Each sonication fraction was weighed using an analytical microbalance and then sequentially added into a composite DEP extract in the reverse order as sonication – i.e. Tol followed by MeOH and then DCM – removing the solvent after each addition. The final composite DEP extract was weighed and then immediately stored in sealed vials in a -20°C freezer until use. In the discussion that follow, these extracts will be abbreviated as DMT-SE.

2.2.2. Selective (PM Fractionation) Extraction

A vast majority of sample preparation techniques in the literature use solvent reflux conditions to perform selective extractions, most notably Soxhlet Extraction (SoxEx) and Heat Reflux Extraction (HRE), and thus HRE was chosen for the current study for comparative purposes. A

few recent studies report using more sophisticated, high-speed techniques such as PFE or ASE but for reasons stated in section **1.1** these techniques were incongruous here.

In brief, during HRE each sample (*NIST-DEP*, *Dyno-DEP* and *FB*) was suspended in solvent and the solvent brought to its boiling point using a heated paraffin oil bath. A reflux condenser was attached with a sheath flow of ice water to maintain the solvent and any semi-volatile species in the liquid phase; a picture of the reflux setup is shown in Figure 2. The solvent was allowed to reflux for ~ 24 hours and then the extract solution filtered using a glass syringe, stainless steel syringe filter holder with PTFE gasket and seals and 0.2μm pore size unlaminated PTFE filter discs; a picture of the syringe filtration setup is shown in Figure 3. The solvent was removed via vacuum distillation to minimal volume, blown to dryness under a nitrogen (N_2) stream and the dry sample weighed using an analytical microbalance. The same solvents chosen for the whole particle sonication extraction were also used here for comparative purposes; i.e. DCM, MeOH and Tol. In the discussions that follow, these extracts will be abbreviated as DCM-HRE.

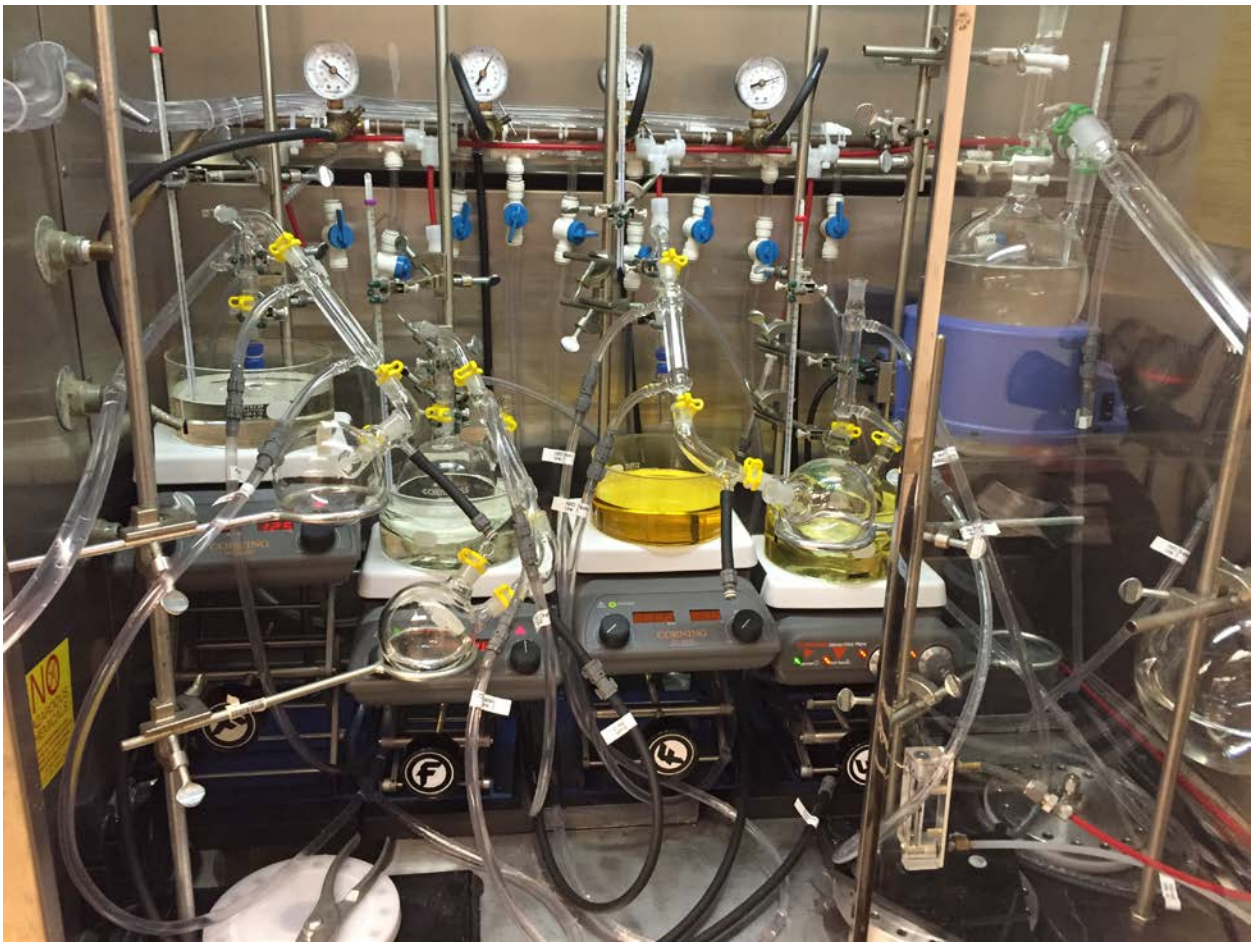

Figure 2. A picture of the heat reflux and solvent distillation apparatuses; see text for discussion.

2.2.2.1. Single Solvent (SS-HRE)

HRE was applied to each sample using all of the solvents individually and a new sample was used for each solvent, resulting in a total of nine independent extracts: (DCM-HRE, MeOH-HRE, Tol-HRE) × (*NIST-DEP*, *Dyno-DEP*, *FB*). Each extract was weighed and then immediately stored in sealed vials in a -20°C freezer until use.

2.2.2.2. Serial Multiple Solvent (SMS-HRE)

HRE was applied to each sample using all of the solvents individually, same as above, but in this case the same sample was used for each solvent and the solvent extracts combined into a composite extract, resulting in only 3 independent extracts: (DCM/MeOH/Tol-HRE) × (*NIST-DEP*, *Dyno-DEP*, *FB*). This was accomplished by transferring the original sample and/or filter from one solvent extraction to the next and adding back in any material filtered out of the extract solution between solvents. The same solvent order used for the whole particle sonication extraction was also used here; i.e. lowest to highest boiling point during extraction and the reverse order for reconstituting the composite sample. Composite samples were weighed and then immediately stored in sealed vials in a -20°C freezer until use. In the discussions that follow, these extracts will be abbreviated as DMT-HRE.

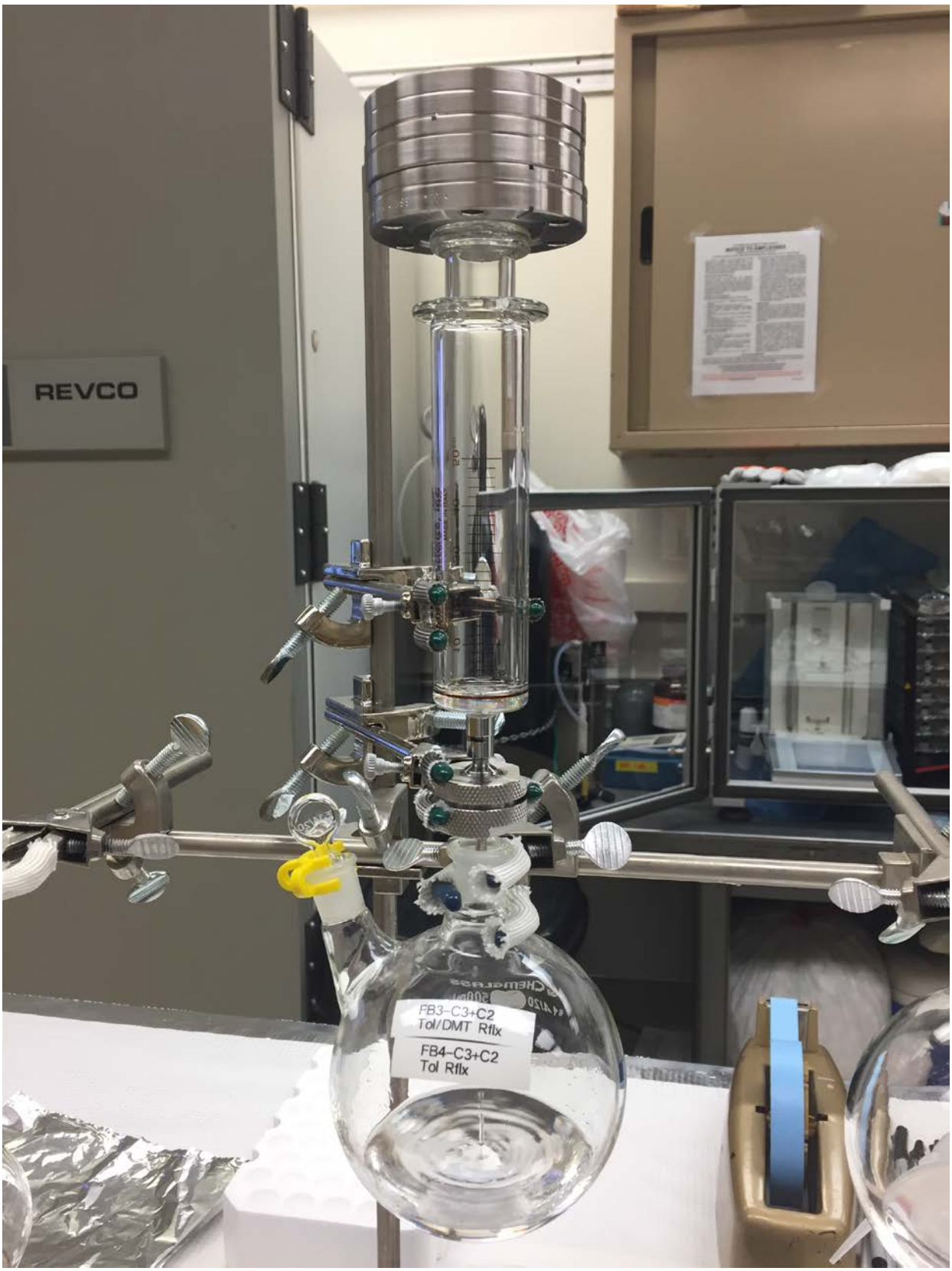

Figure 3. A picture of the syringe filtration apparatus; see text for details.

2.3. Test Hypotheses

The six sample preparation techniques outlined above were designed to test four core hypotheses and were selected based on an extensive literature review of what is most commonly being employed in the scientific community. These hypotheses are outlined below along with the corresponding sample preparation techniques required to test each one.

1. *The outcome of a particular assay depends on whether the sample preparation technique is exhaustive or selective in nature; i.e. whether whole PM or solvent extractable PM components (fractionated PM) are used*

    - Assay response to the sonication extracts (H2O-SE and DMT-SE) is compared to that of the heat reflux extracts (DCM-HRE, MeOH-HRE, Tol-HRE and DMT-HRE) for all assays in the panel to test this hypothesis.

2. *The outcome of a particular assay using either whole PM or fractionated PM depends on the solvent or combination of solvents used*

    - The SE and HRE extractions are intercompared as a function of assay to test this hypothesis.

3. *For assays conducted using fractionated PM, the sum of responses to individual PM fractions will be significantly larger than the individual response to the composite of those PM fractions, i.e. the presence of assay-specific toxicologically inert PM components interferes with the response to the toxicologically active PM components*

    - The sum of assay responses to the individual single solvent heat reflux extracts (DCM-HRE + MeOH-HRE + Tol-HRE) is compared to the individual assay response to the serial multiple solvent heat reflux extract (DMT-HRE) for all assays in the panel to test this hypothesis. Previous studies of diesel exhaust particles have shown that different PM components elicit different responses from a given toxicological assay and, more interestingly, that the sum of responses to individual PM components is significantly larger than the response to the composite of those components (*55, 99*). Further evidence of this composite interference was recently observed in an in vivo study also funded by CARB (*100, 101*)

4. *The sample preparation technique yielding the most robust, reliable and repeatable outcome will be assay dependent.*

    - The combination of assay responses to all extracts and for all assays in the panel are compared to test this hypothesis.

2.4. Standard Assay Panel

All sample extracts (18 in total; see Table 3) were subjected to a multi-point assay panel measuring ROS production via the acellular dithiothreitol (DTT) assay (*102, 103*), cellular inflammation via the cyclooxygenase-2 (COX-2) and interleukin 8 (IL-6; IL-8) proteins (*104-107*), PAH response via cytochrome P450 1A1 (CYP1A1) expression (*108-110*) and

mutagenicity via the microsuspension *Salmonella*/microsome (Ames) assay (*85, 111-113*). Details of these assays have been described extensively in the literature so will not be repeated here. For the cellular assays, U937 macrophages will be used (*114-116*) and expression detected via quantitative real-time Reverse Transcription-Polymerase Chain Reaction (qPCR).

2.4.1. U937 Macrophages

Cell culture and transient transfection Human U937 monocytic cells were obtained from the American Tissue Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium containing 10% fetal bovine serum (Gemini, Woodland, CA), 100 U penicillin, and 100 μg/ml streptomycin supplemented with 4.5 g/L glucose, and 1 mM sodium pyruvate. Cell cultures were maintained at cell concentrations between 2×10^5-2×10^6 cells/ml. For differentiation into macrophages, U937 cells were treated with 12-0-tetradecanoyl-phorbol-13-acetate (TPA; 3μg/mL) and allowed to adhere for 48 hr in a 5% CO_2 tissue culture incubator at 37°C, after which they were fed with TPA-free medium. Differentiation state was assessed by attached cell phenotype and increased expression of MAC-2.

Alveolar macrophages are key effectors in diesel PM-induced lung injury. The human U937 macrophage model is a well-established cell bioassay to test inflammatory responses and induction of CYP1A1 as biomarkers after treatment with PM and extracts from various sources. In a recent study, we showed that AhR-mediated responses after chemical exposure of U937 derived dendritic cells are comparable with responses in primary human dendritic cells derived from blood monocytes (*117*). Macrophages and dendritic cells have many similar characteristics with regards to responses after chemical exposure.

2.4.2. Quantitative Real-Time PCR (qPCR)

Total RNA was isolated from U937 cells using a high-purity RNA isolation kit (Roche) and cDNA synthesis performed as described previously (*118*). Quantitative detection of β-actin and differentially expressed genes were done with a LightCycler Instrument (LC480 Roche Diagnostics, Mannheim, Germany) using the QuantiTect SYBR Green PCR Kit (Qiagen) according to the manufacturer's specifications. DNA-free total RNA (1.0 μg) was reverse-transcribed using 4 U Omniscript reverse transcriptase (RT; Qiagen) and 1 μg oligo$(dT)_{15}$ in a final volume of 40 μL. The primers for each gene were designed on the basis of the respective cDNA or mRNA sequences using OLIGO primer analysis software provided by Steve Rozen and the Whitehead Institute/MIT Center for Genome Research (*119*) so that the targets were 100–200bp in length. PCR amplification was carried out in a total volume of 20 μL containing 2 μL cDNA, 10 μL 2 × QuantiTect SYBR Green PCR Master Mix and 0.2 μM of each primer. The PCR cycling conditions were 95°C for 15 min followed by 40 cycles of 94°C for 15 sec, 60°C for 20 sec, and 72°C for 10 sec. Detection of the fluorescent product was performed at the end of the 72°C extension period. Negative controls were run concomitantly to confirm that the samples were not cross-contaminated. A sample with DNase- and RNase-free water instead of RNA was also examined concomitantly for each of the reaction units described above. To confirm the amplification specificity, the PCR products were subjected to melting curve analysis. Data analysis was done with the LightCycler analysis software.

2.4.3. Mutagenicity Bioassay

The mutagenicity measurements of all sample extracts followed procedures as previously reported (*98*). Briefly, a simple modification of the *Salmonella*/mammalian microsome plate incorporation test (*16*) called the "microsuspension" procedure (*120*) that is approximately at least 10 time more sensitive than the standard assay was used throughout. Tester strain TA98 was used throughout. To account for possible metabolism or metabolic activation required of the test substance or complex mixture, a liver homogenate (referred to as S-9) is used. A 3-point dose-response curve was developed for each sample preparation technique with each dose tested in duplicate. All Environmental Health and Safety procedures were closely followed.

S-9 was used throughout in the mutagenicity assay. Activity minus S9 was not tested due to limited sample material, especially for Dyno-DEP. With limited sample, the choice was to use +S9 since both direct-acting (-S9) activity and compounds that require metabolic activation (+S9) could be detected. The contribution of –S9 activity to the + S9 activity would not be known without testing these separately. Ideally, both +S9 and –S9 activities should be tested if sample was not limited. However, historically, mutagenic activity for these types of diesel PM emission samples have been reported with the addition of metabolic enzymes and correlate with direct-acting (-S9) activity.

2.4.4. Dithiothreitol (DTT) Assay

The production of Reactive Oxygen Species (ROS) by DEP was measured using the Dithiothreitol (DTT) assay, which is based on the consumption of DTT by reaction with ROS. The method of Kumagai et al. (*84*) and Cho et al. (*121*) were followed throughout this study. In general, the method involves tracking the consumption of DTT based on the reaction of remaining DTT with dinitrothiobenzene. The method is colorimetric and involves multiple time points. All samples were tested in duplicate over a period of 35 min at 37° C. All sample extracts were diluted with DMSO to a final concentration of 10 μg/μL and were distributed for the DTT analyses in quadruplicate. Blank DMSO background DTT consumption levels were routinely tested in parallel to the extracted samples. The consumption of DTT over time resulted in a linear curve. A DEP control extract was kindly provided by the UCLA group and served as positive control for the DTT assay. The activity of this positive control was consistently in the range of what the UCLA group have reported (*121*).

2.4.4. Measurement Matrix

A 3-point dose response (DR) curve was generated for each combination of assay and DEP sample extract. The mass doses that were tested ranged from 1-25 μg depending on the specific assay and according to knowledge of relevant dosing ranges from previous DEP studies. A dose was then chosen from a linear part of the DR curve with the most robust response and each assay was repeated in duplicate at the chosen dose for all sample extracts; [(6 *NIST-DEP* extracts + 6 *Dyno-DEP* extracts + 6 *FB* extracts) × 3 (DR + duplicate)] = 54 data points per assay. The measurement matrix is outlined in Table 3. All mass doses for the DR curves and subsequent duplications were determined by direct gravimetric measurement of the actual dry mass extracted from the *Dyno-DEP* and *NIST-DEP* samples, rather than the original masses or differences between the pre- and post-extraction filter masses. This is to ensure that all comparisons between different sample preparation techniques are normalized to an equal mass dose basis. The *FB*

extracts were handled identically to their associated *Dyno-DEP* extracts on an equal volume basis; i.e. the same volume of delivery vehicle used to create the *Dyno-DEP* doses was also used to create the *FB* doses. This ensures that the *FB* and *Dyno-DEP* comparisons for each sample preparation technique are normalized to extraction of equal filter surface areas.

2.5. Statistical Considerations

All sample extracts will be prepared and randomly labeled so that the toxicological screening is done as a blind study. For each combination of sample extract and assay, the initial value from the DR curve and subsequent tests in duplicate will be used to calculate average assay response. These data will be checked for normality using the Shapiro–Wilk test and, if necessary, log transformed via the generalized log transformation. Two-way ANOVA will be applied to the filter blank sample extracts to assess any statistically significant differences between sample preparation controls as a function of assay. Assay response to the *Dyno-DEP* sample extracts will be blank corrected by the associated response to filter blank sample extracts and then the entire data matrix (*NIST-DEP* + blank corrected *Dyno-DEP*) subjected to two-way ANOVA to analyze for statistical significance between assays for a given technique, between techniques for a given assay, and between *NIST-DEP* and *Dyno-DEP* as a function of assay and technique. Simple two-point comparisons will be made using **Student's *t*-test** with $p < 0.05$. The alternate hypotheses laid out in the **Project Objectives** section of this proposal will be tested using the results of these analyses.

Table 3. Measurement matrix showing number of data points per assay and sample preparation technique; DR = dose response

Assay	Study	Numbe of assay data points for each sample prepartion technique applied to each sample																	Totals
		H2O-SE			DMT-SE			DCM-HRE			MeOH-HRE			Tol-HRE			DMT-HRE			
		NIST	Dyno	FB	NIST	Dyno	FB	NIST	Dyno	FB	NIST	Dyno	FB	NIST	Dyno	FB	NIST	Dyno	FB	
	3-pt DR		3			3			3			3			3			3		18
	Test Dose	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	36
	3-pt DR		3			3			3			3			3			3		18
	Test Dose	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	36
	3-pt DR		9			9			9			9			9			9		54
	Test Dose	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	108
Totals All		6	15	6	6	15	6	6	15	6	6	15	6	6	15	6	6	15	6	162

3. Results and Discussion

Figure 4 shows a picture of the *Dyno-DEP* filter pieces both pre- and post-extraction and the PTFE syringe filter discs used in the post-extraction cleanup steps of the heat reflux extractions. The following general observations were made during sample preparation: *(1)* the extraction efficiency as a function of solvent in terms of mass of DEP extracted is DCM > Tol > MeOH > H_2O for both SE and HRE, *(2)* HRE was more efficient than SE for extracting DEP from filters, *(3)* approximately 30-60% of the extracted DEP was removed during the post-extraction filtration step of HRE, *(4)* the removal of suspended DEP from the HRE extracts during syringe filtration appeared to vary significantly from sample to sample, and (5) the extracted mass from each of the DEP filter pieces was very small, < 100 μg in most cases, resulting in large measurement uncertainties during gravimetric analysis; i.e. two very large masses (pre- and post-weights of the sample vials) were subtracted to obtain a very small mass. Results from the assay panel for the various sample extracts are detailed in what follows.

3.1. Expression of CYP1A1

The expression of CYP1A1 was significantly up-regulated by all DEP extracts tested after treatment for 24 h (Fig. 5A) compared to vehicle control. Similarly, with the exception of H2O-SE, the expression of CYP1A1 was significantly upregulated by all *Dyno-DEP* extracts relative to their associated *FB* extracts. In all cases, the expression of CYP1A1 for a given sample extraction technique was greater for *NIST-DEP* compared to *Dyno-DEP*. For both *Dyno-DEP* and *NIST-DEP*, CYP1A1 expression was greater for *(1)* HRE compared to SE and *(2)* DMT-SE compared to H2O-SE. The highest increase of CYP1A1 expression by over 400-fold was observed after treatment with Tol-HRE of *NIST-DEP* followed by a 320-fold increase for DMT-HRE of *NIST-DEP*. Interestingly, DCM-HRE and MeOH-HRE of *NIST-DEP* had a similar effect on CYP1A1 expression but were significantly different for *Dyno-DEP*.

3.2. Expression of IL-8 and COX-2

Similar to CYP1A1, and as shown in Figure 5B, the highest increase of IL-8 expression of 12-fold compared to vehicle control for *NIST-DEP* was Tol-HRE, followed by DCM-HRE (10.5-fold), DMT-HRE (10-fold), and then MeOH-HRE (10-fold). Only a relatively small 3-fold induction of IL-8 above vehicle control was found after treatment with H2O-SE. Again, the following observations hold true: *(1)* for a given sample preparation technique, *Dyno-DEP* had a smaller effect on IL-8 induction compared to *NIST-DEP*, *(2)* for *NIST-DEP* and *Dyno-DEP*, HRE had a larger effect on IL-8 expression relative to SE, and DMT-SE was greater than H2O-SE, and *(3)* H2O-SE of *Dyno-DEP* was not significantly greater than the corresponding *FB* extract.

The expression of the inflammatory enzyme COX-2, as shown in Figure 5C, was significantly induced above vehicle control for all DEP extracts except for H2O-SE of *Dyno-DEP*.

Interestingly, only DCM-HRE and DMT-HRE of *Dyno-DEP* were significantly elevated above their respective *FB* controls. For *NIST-DEP*, the expression of COX-2 was induced about 6-fold by treatment with DCM-HRE, Tol-HRE and DMT-HRE compared to vehicle control, while MeOH-HRE led to a 5-fold increase. General observations described above for IL-8 expression (*1* and *2*) also apply here.

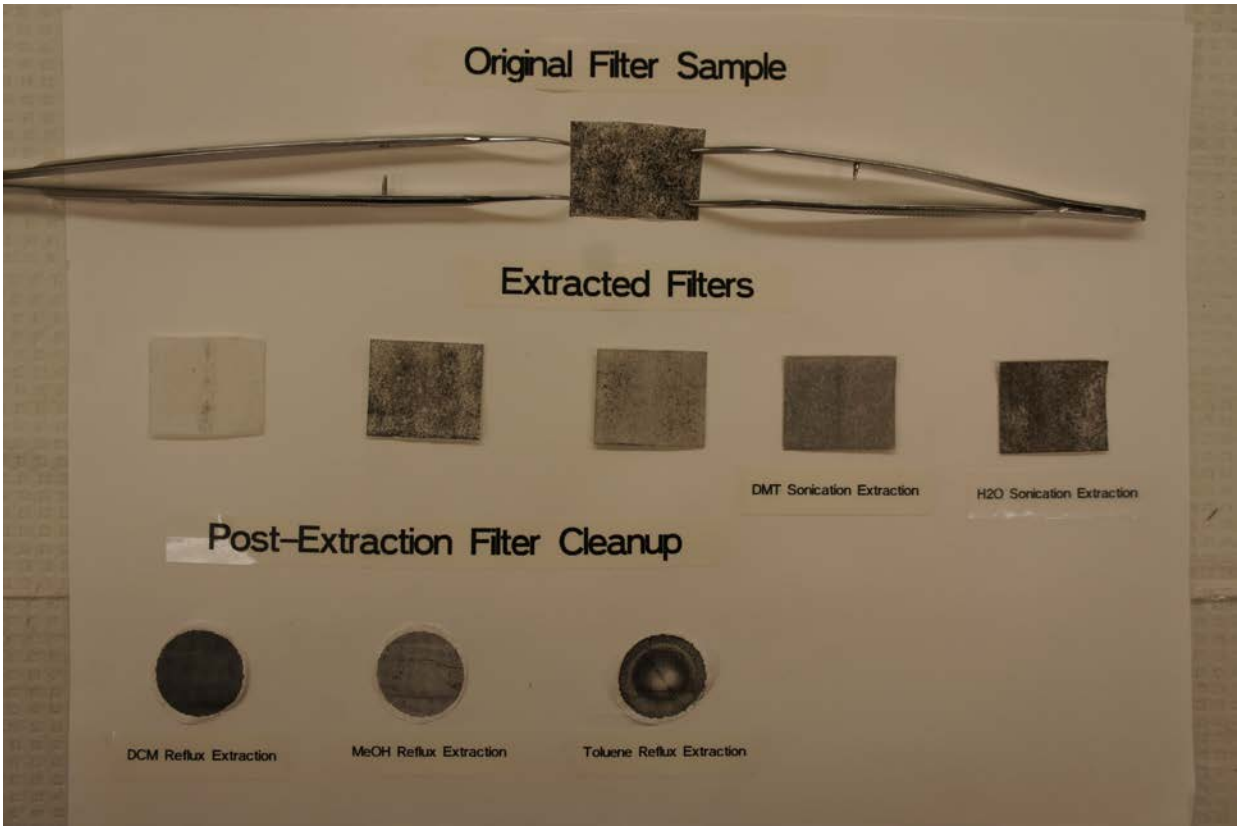

Figure 4. A picture of the *Dyno-DEP* filter pieces both pre- and post-extraction and the syringe filter discs used in the sample cleanup steps of the heat reflux extractions; see text for details.

3.3. Dose-dependent induction of CYP1A1

The dose-dependent effect of all sample extracts from *NIST-DEP* and DMT-HRE of *Dyno-DEP* on the expression of CYP1A1 in macrophages is shown in Figure 6A. All DEP extracts clearly show a dose-dependent effect on the expression of CYP1A1. Similar to the discussions above, the highest increase of CYP1A1 of about 310-fold above vehicle control was found for the 5 μg/mL dose of Tol-HRE of *NIST-DEP*, although the effect of 5 μg/mL was not significantly different than the 2.5 μg/mL dose. This was followed by a 300-fold increase from DMT-HRE of *NIST-DEP* and then a 220-fold increase for DCM-HRE, both at the 5 μg/mL dose. Again, similar to the discussions above, the expression of CYP1A1 for DMT-HRE of *Dyno-DEP* was significantly smaller than DMT-HRE of *NIST-DEP*, with the effect of the largest *Dyno-DEP* dose (5 μg/mL) smaller than even the smallest *NIST-DEP* dose (1.25 μg/mL).

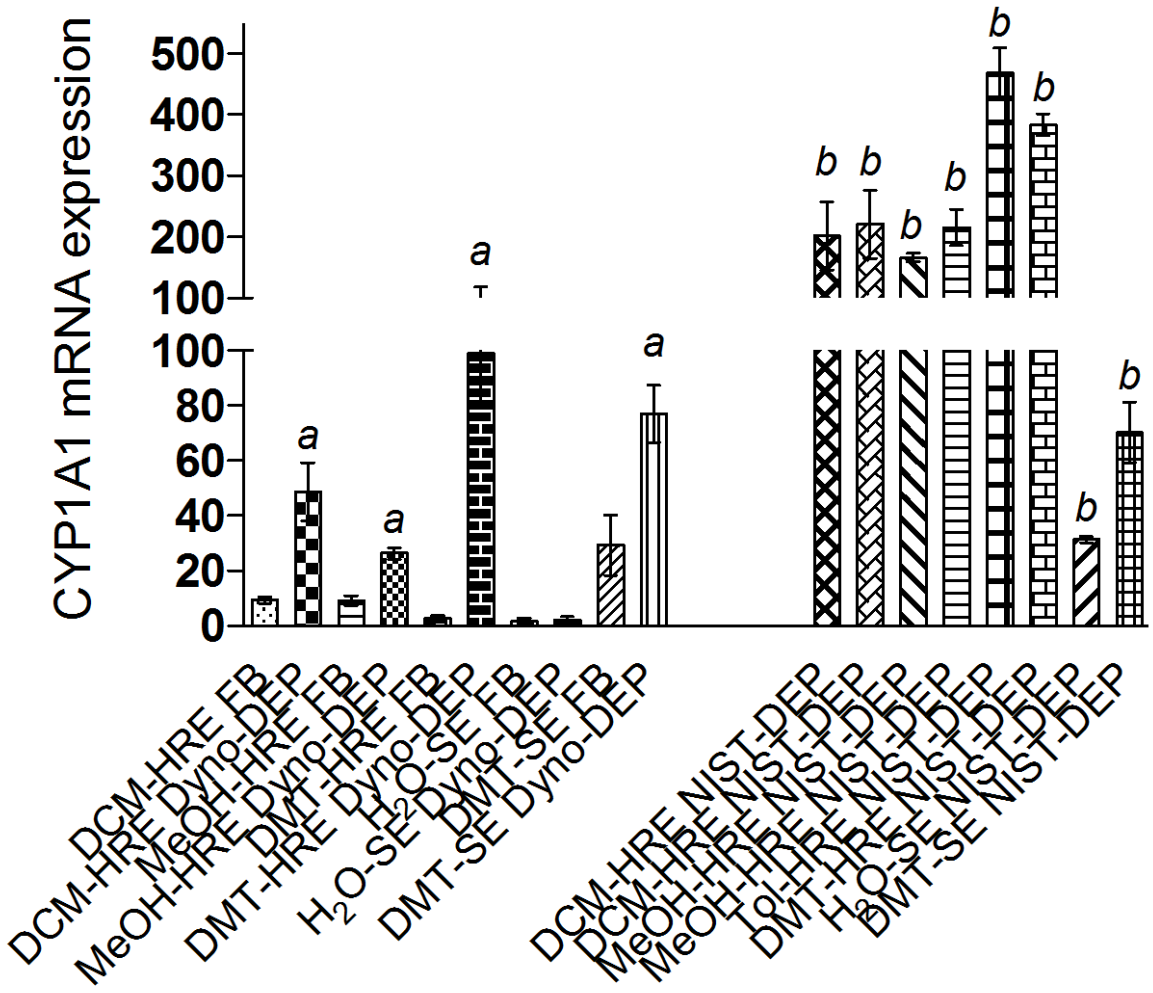

Figure 5A. Induction of CYP1A1 mRNA expression after treatment with various extracts reported as fold-increase relative to vehicle control. U937-derived macrophages were treated for 24h with 5 µg/mL extract. CYP1A1 expression was analyzed using qPCR and is corrected versus β-actin used as a housekeeping gene. Mean and SEM of three experiments are shown.
[a] significantly different from corresponding FB, $p < 0.05$; [b] significantly higher than corresponding Dyno-DEP extract, $p < 0.05$

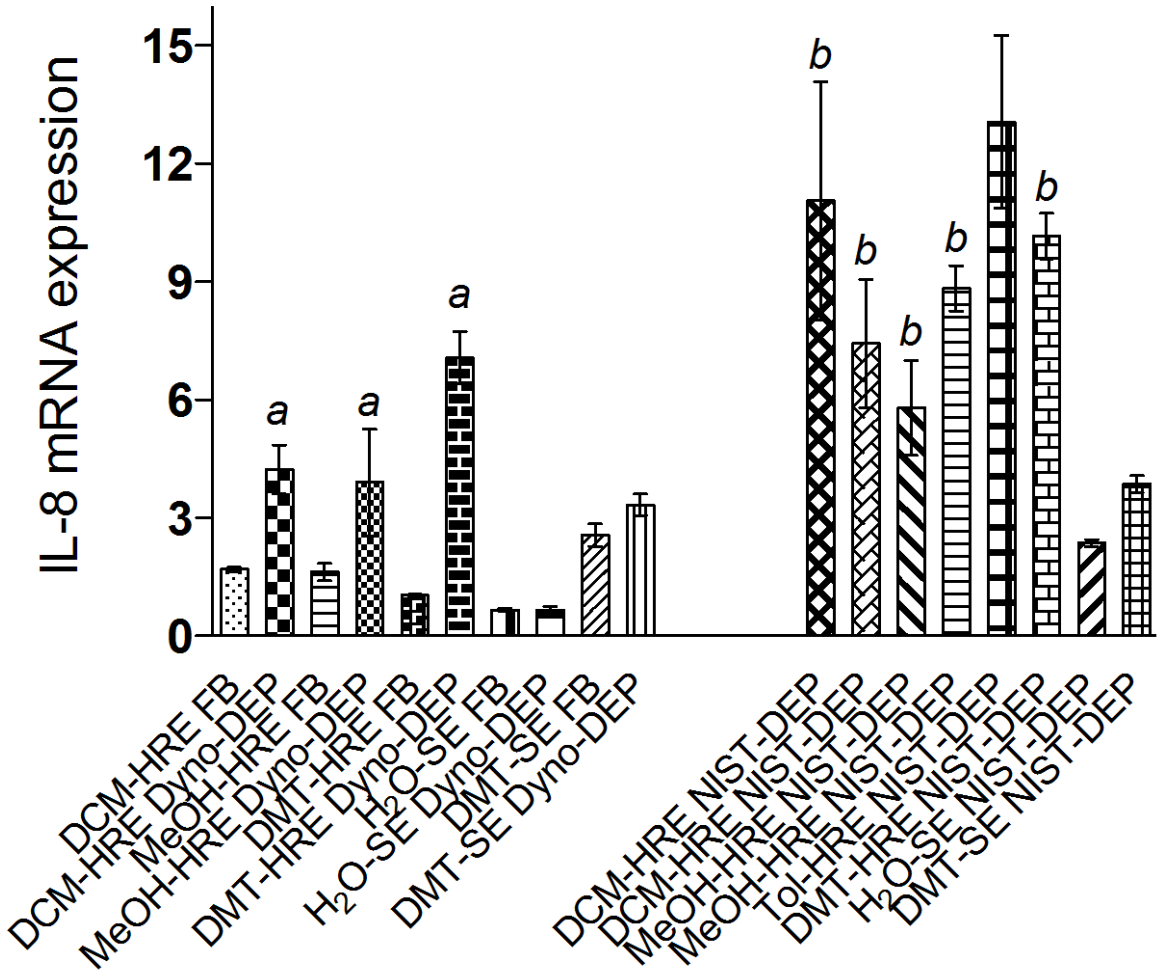

Figure 5B. Induction of IL-8 mRNA expression after treatment with various extracts shown as fold-increase relative to vehicle control. U937-derived macrophages were treated for 24h with 5 µg/mL extract. Expression of IL-8 was analyzed using qPCR and is corrected versus β-actin used as a housekeeping gene. Mean and SEM of three experiments are shown. [a] significantly different from corresponding FB, $p < 0.05$; [b] significantly higher than corresponding Dyno-DEP extract, $p < 0.05$

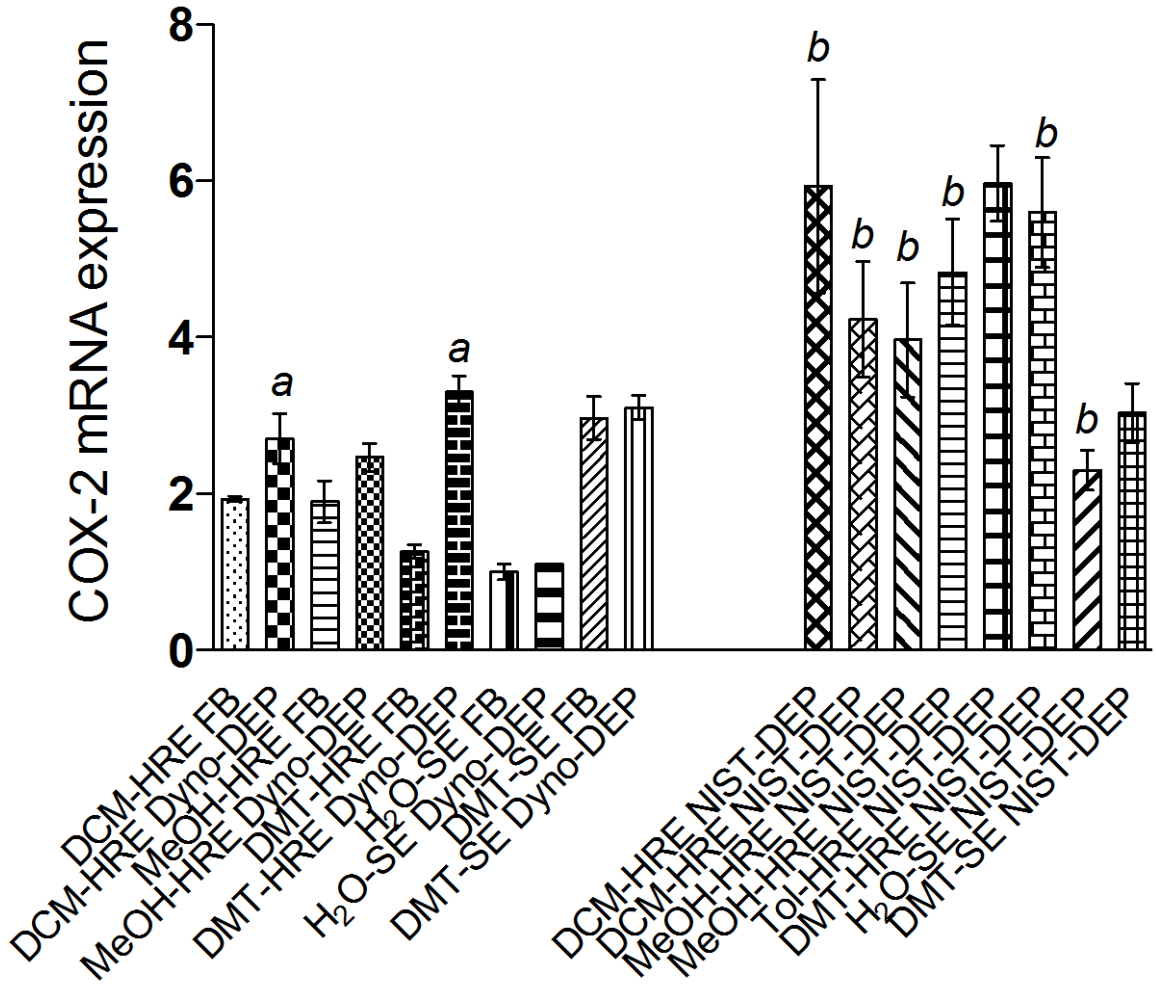

Figure 5C. Induction of COX-2 mRNA expression after treatment with various sample extracts shown as fold-increase relative to vehicle control. U937-derived macrophages were treated for 24h with 5 µg/mL extract. Expression of COX-2 was analyzed using qPCR and is corrected versus β-actin used as a housekeeping gene. Mean and SEM of three experiments are shown. [a] significantly different from corresponding FB, $p < 0.05$; [b] significantly higher than corresponding Dyno-DEP extract, $p < 0.05$

3.4. Dose-dependent induction of IL-8 and COX-2

The expression of IL-8 was dose-dependently induced by treatment with the DCM-HRE, MeOH-HRE and DMT-HRE extracts of *NIST-DEP*, as shown in Figure 6B. Although all doses were significantly above vehicle control, the trends for Tol-HRE of *NIST-DEP* and DMT-HRE of *Dyno-DEP* increased from the lowest to the middle dose but then decreased for the highest dose, and no dose was significantly different than any other dose. For the *NIST-DEP* extracts at the 5 μg/mL dose, the ability to induce IL-8 expression was largest for DCM-HRE at an 11-fold increase over vehicle control, followed by a 9-fold increase for MeOH-HRE and Tol-HRE and an 8-fold increase for DMT-HRE. Interestingly, when comparing DMT-HRE of *Dyno-DEP* and *NIST-DEP*, the highest dose was significantly greater for the latter by more than a factor of 2 but there were no significant differences between the two at the lower doses.

Very similar trends were observed for the expression of COX-2 compared to IL-8: *(1)* all doses were significantly above vehicle control, *(2)* dose-dependency was observed for DCM-HRE, MeOH-HRE and DMT-HRE of *NIST-DEP*, *(3)* the trends for Tol-HRE of *NIST-DEP* and DMT-HRE of *Dyno-DEP* were much weaker compared to the others and most doses were not significantly different from each other, and *(4)* DMT-HRE of *NIST-DEP* was significantly greater than *Dyno-DEP* by more than a factor of 2 at the highest dose but was not significantly different at the lower doses.

3.5. Mutagenicity

The Specific Mutagenic Activity (SMA) of the *Dyno-DEP* extracts is illustrated in Figure 7A and expressed as Revertants per μg extract. DCM-HRE, DMT-HRE and DMT-SE had activities significantly different than their corresponding *FB* extracts, while MeOH-HRE and H2O-SE did not. DCM-HRE exhibited the highest SMA that was significantly different than all others, followed by DMT-HRE and DMT-SE, which were not significantly different from each other.

The SMA's of the *NIST-DEP* sample extracts are presented in Figure 7B. The highest activities were observed for DCM-HRE, Tol-HRE and DMT-HRE, although none of these values were significantly different from each other. MeOH-HRE was significantly different than all other values but only exhibited SMA approximately ½ that of the other HRE data. There are several general observations in the mutagenicity data that are congruent with the cellular markers discussed previously, including: *(1)* for a given sample preparation technique, the response to *NIST-DEP* is significantly greater than *Dyno-DEP*, by more than an order of magnitude in this case, *(2)* for *NIST-DEP* and *Dyno-DEP*, HRE tends to elicit significantly more activity than SE and *(3)* for HRE of *NIST-DEP* and *Dyno-DEP*, DCM and Tol generally elicit a greater effect than MeOH while for SE, DMT is greater than H2O.

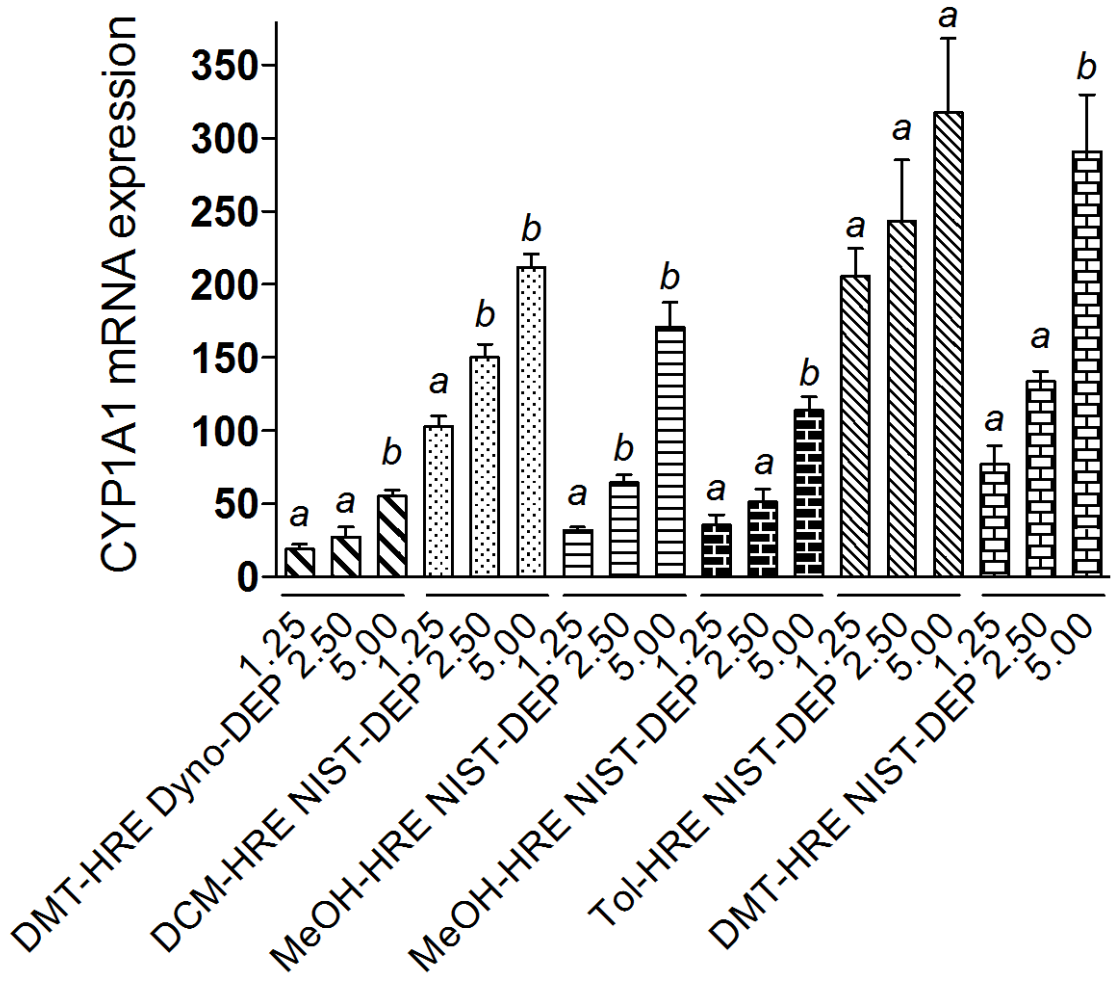

Figure 6A. Dose-response study of CYP1A1 induction after treatment with various extracts shown as fold-increase over vehicle control. U937-derived macrophages were treated for 24 h with 1.25, 2.5, and 5 μg/mL extract. CYP1A1 expression was analyzed using qPCR and is corrected versus β-actin used as a housekeeping gene. Mean and SEM of three experiments are shown. ^{a} significantly different from filter blank (FB), $p < 0.05$; ^{b} significantly higher than lower concentration, $p < 0.05$

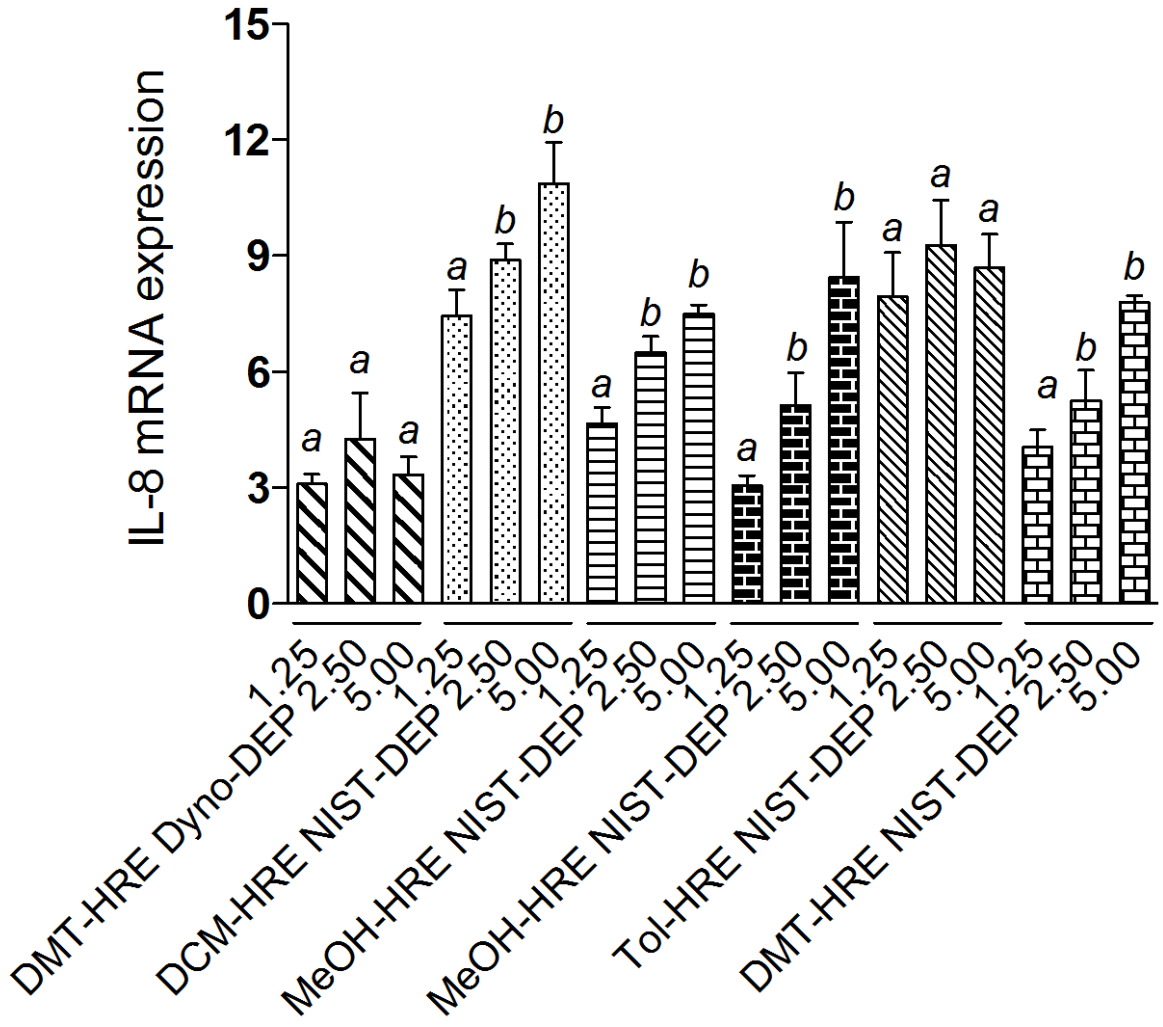

Figure 6B. Dose-response study of IL-8 induction after treatment with various extracts shown as fold-increase above vehicle control. U937-derived macrophages were treated for 24 h with 1.25, 2.5, and 5 μg/mL extract. IL-8 expression was analyzed using qPCR and is corrected versus β-actin used as a housekeeping gene. Mean and SEM of three experiments are shown.
[a] significantly different from filter blank (FB), $p < 0.05$; [b] significantly higher than lower concentration

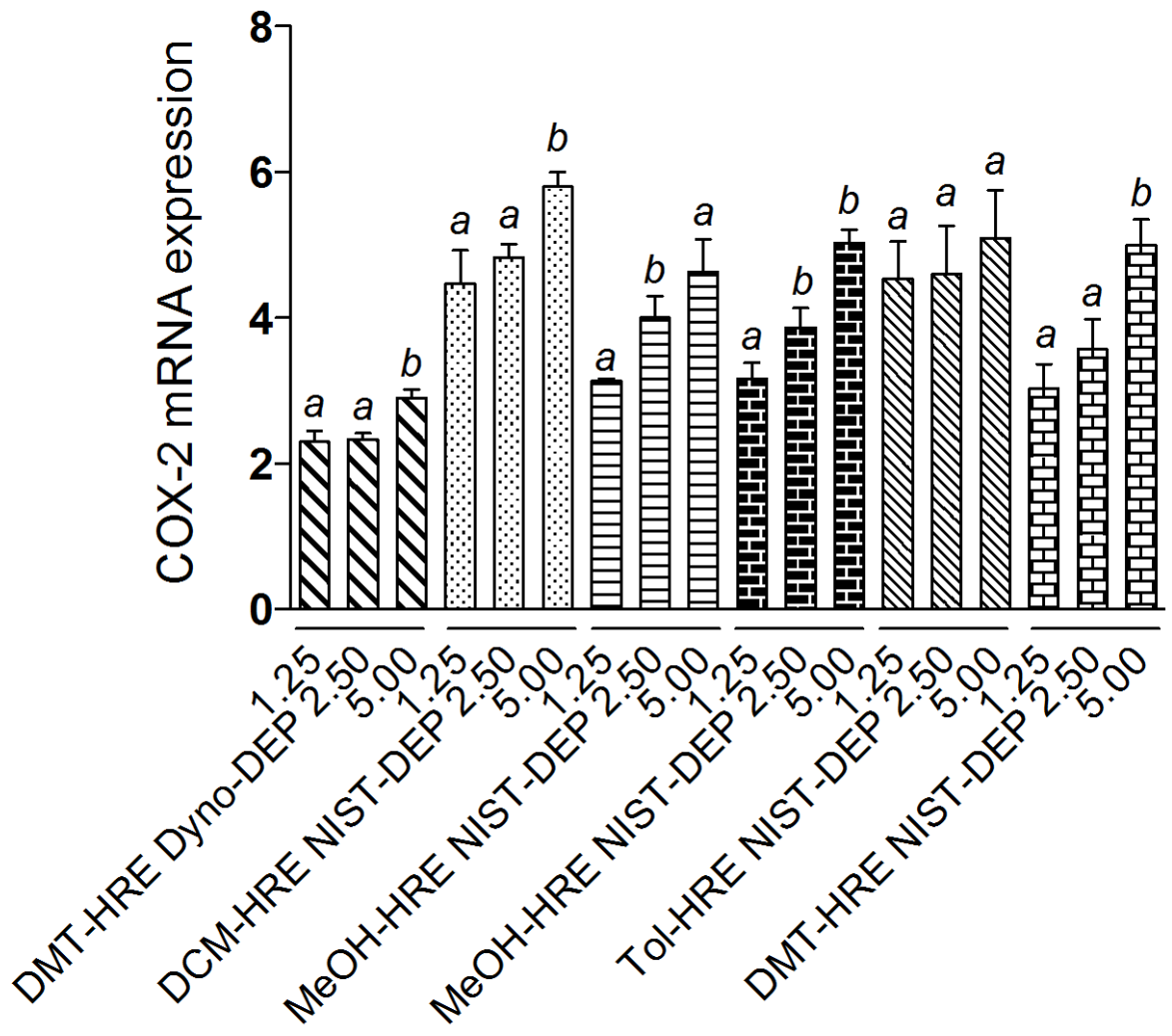

Figure 6C. Dose-response study of COX-2 induction after treatment with various extracts shown as fold-increase above vehicle control. U937-derived macrophages were treated for 24 h with 1.25, 2.5, and 5 μg/mL extract. COX-2 expression was analyzed using qPCR and is corrected versus β-actin used as a housekeeping gene. Mean and SEM of three experiments are shown. [a] significantly different from filter blank (FB), $p < 0.05$; [b] significantly higher than lower concentration

3.6. ROS Production via DTT

Figure 8A depicts the DTT consumption rates in nanomoles/min/µg extract for the *Dyno-DEP* sample extracts and their corresponding *FB* extracts. Only the DCM-HRE and DMT-HRE data were significantly above their FB controls. Although the MeOH-HRE and H2O-SE consumption rates were the largest, their corresponding FB rates were approximately equal to the sample rates. It is not immediately clear why this is the case but it is possible these samples were somehow contaminated prior to, or during, the DTT measurements. As a result, DMT-HRE has the highest relative consumption rate followed by DCM-HRE. Overall, the consumption rates for all Dyno-DEP extracts were quite low, although measurable, and this may be attributed to insufficient starting mass for the DTT assay given the limited availability of Dyno-DEP sample.

The DTT consumption rates for the *NIST-DEP* sample extracts are shown in Figure 8B. Like the other assays, the *NIST-DEP* data are markedly more elevated than the corresponding *Dyno-DEP* data. Unlike the other assays, MeOH-HRE exhibited the highest consumption rate, which was significantly different than all other values. This was followed by DCM-HRE, DMT-SE and Tol-HRE, which were not significantly different from each other, and then H2O-SE and DMT-HRE.

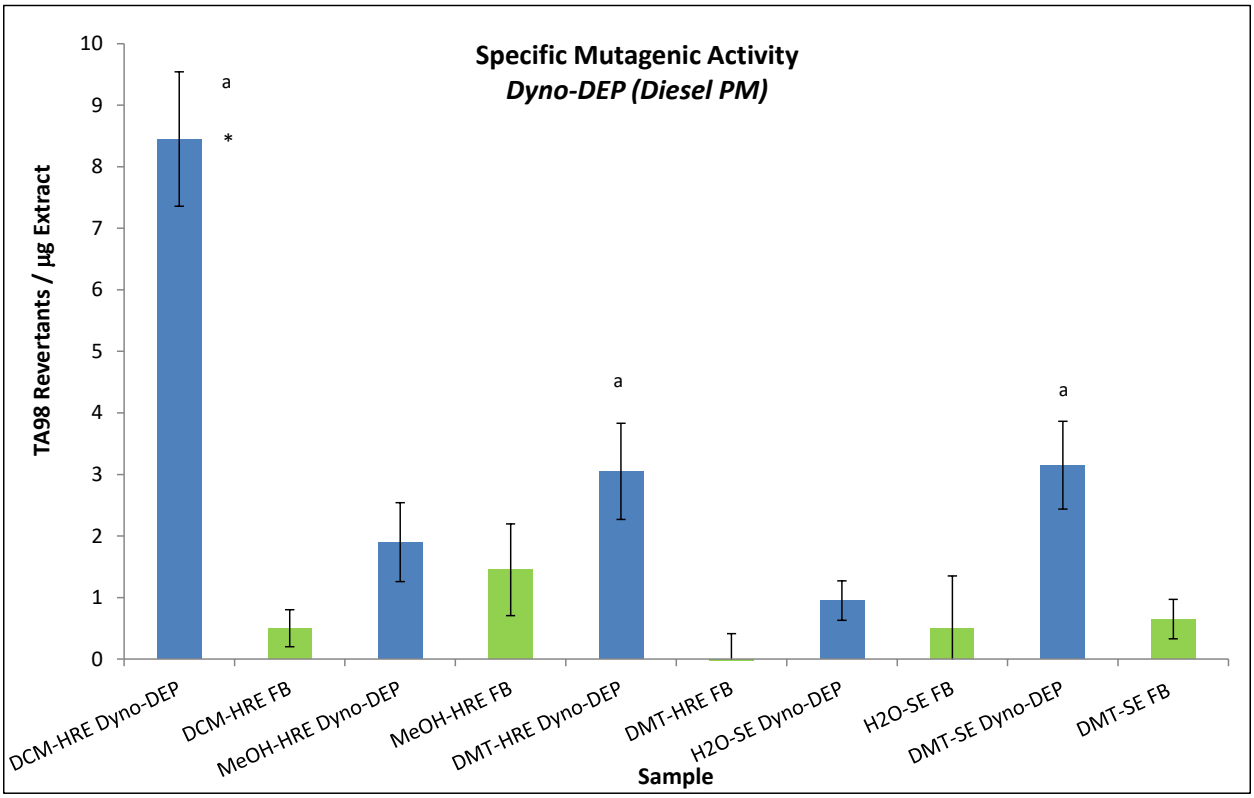

Figure 7A. Specific mutagenic activity (Revertants per µg of extract) for the *Dyno-DEP* sample extracts and their corresponding *FB* extracts. Values are reported as mean ± SD of quadruplicate specific mutagenic values. [a] Significantly different from corresponding *FB*, $p<0.05$; * DCM-HRE significantly different than MeOH-HRE, DMT-SE, H2O-SE and DMT-HRE, $p<0.05$.

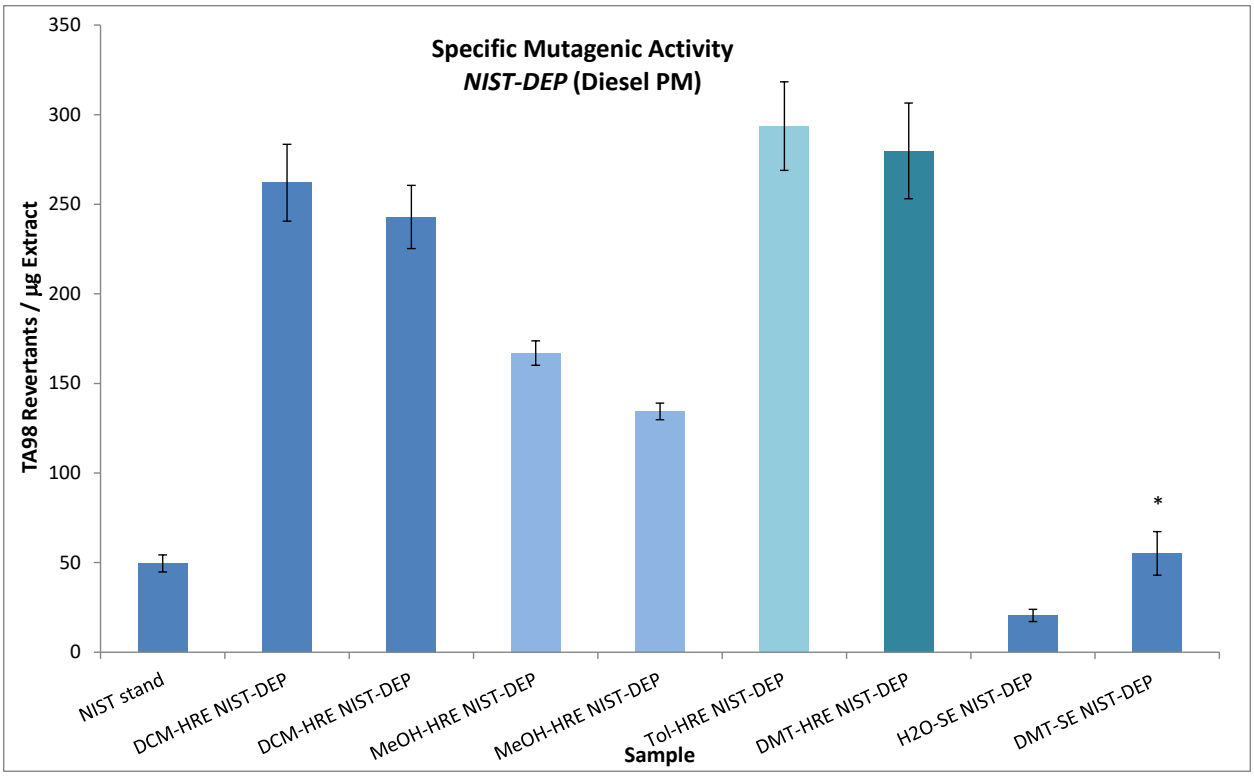

Figure 7B. Specific mutagenic activity (Revertants per µg of DEP extract) for the *NIST-DEP* extracts. Values are reported as mean ± SD of quadruplicate specific mutagenic values. [a] Significantly different from corresponding *FB*, $p<0.05$; * DMT-SE significantly different than DCM-HRE, MeOH-HRE, Tol-HRE, DMT-HRE and H2O-SE, $p<0.05$

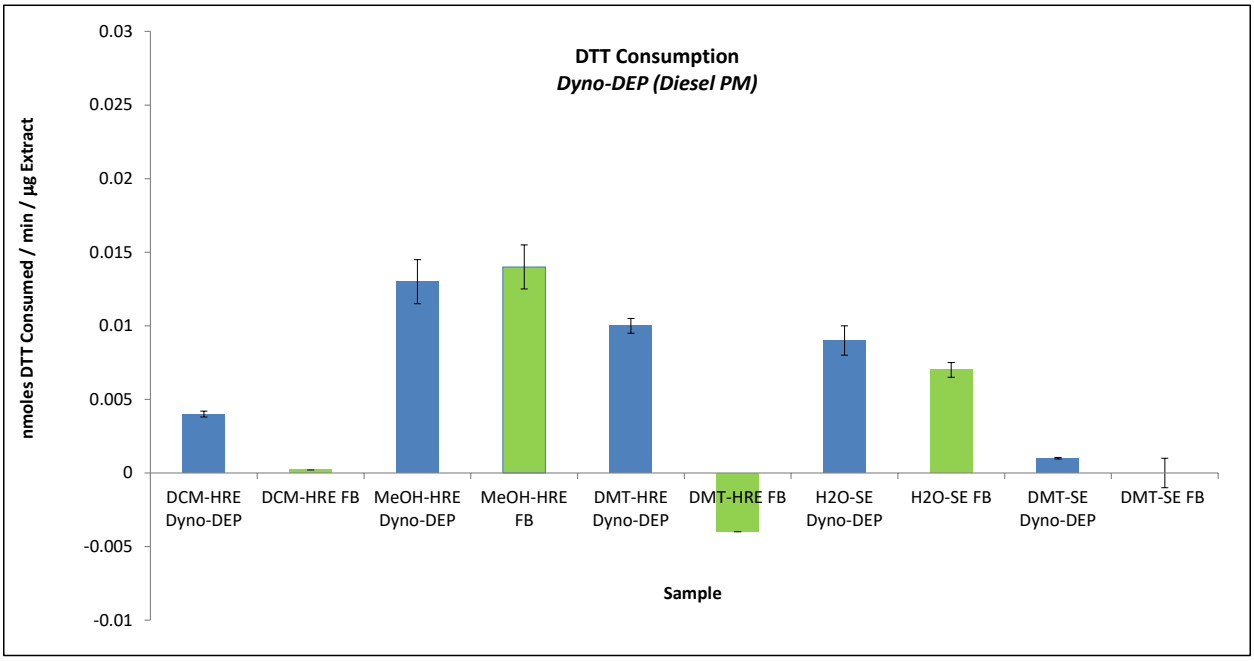

Figure 8A. DTT consumption rates for the *Dyno-DEP* sample extracts and their corresponding FB extracts expressed in nanomoles/min/µg extract. Values represent mean of quadruplicate determinations ± SD. [a] DMT-HRE significantly different from corresponding *FB*, $p < 0.05$.

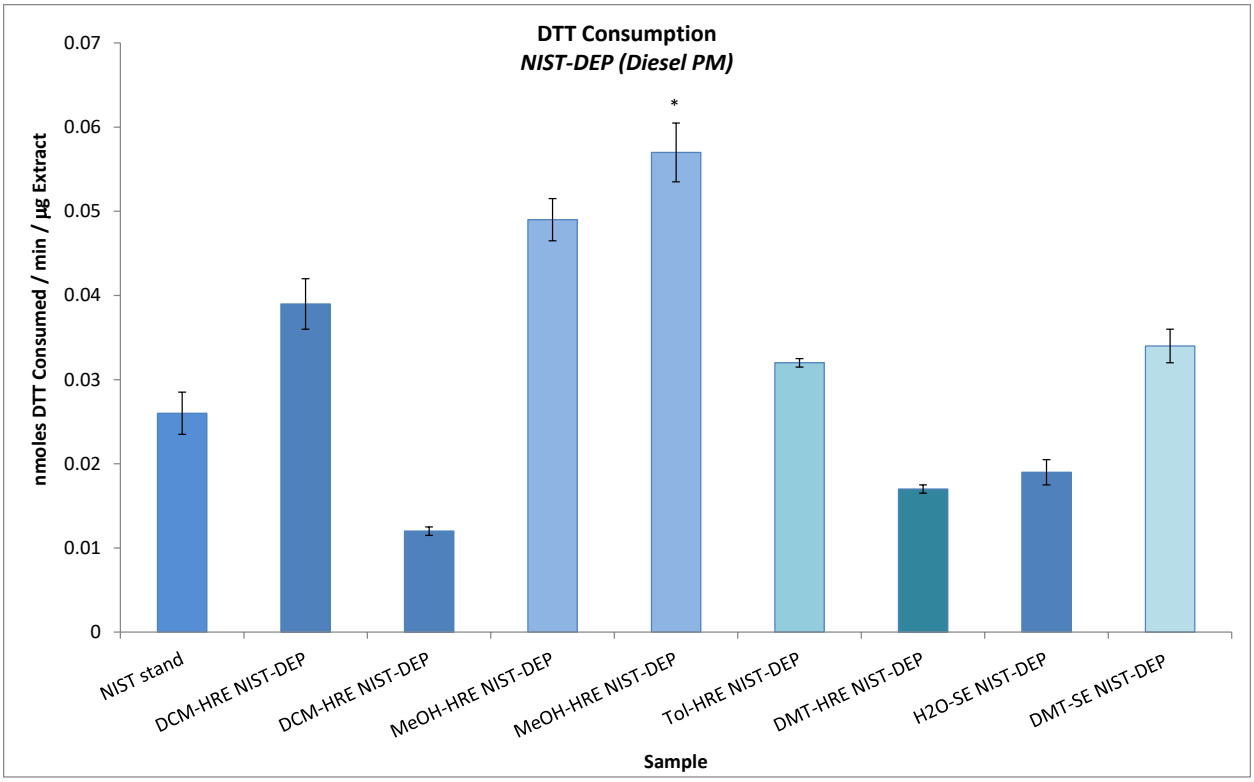

Figure 8B. DTT consumption rates in nanomoles/min/µg extract for the *NIST-DEP* sample extracts. Values represent mean of quadruplicate determinations ± SD. [a] MeOH-HRE significantly different than DCM-HRE, Tol-HRE, DMT-HRE, DMT-SE and H2O-SE, $p < 0.05$.

3.7. Repeatability

The DCM-HRE and MeOH-HRE of *NIST-DEP* were performed in duplicate and all sample extracts blindly added to the collection of samples measured by the assay panel. This was done to provide a blind metric of repeatability for this study. Overall, there was good agreement between the duplicates for both DCM-HRE and MeOH-HRE across all assays. For example, the CYP1A1 data shown in Figures 5A and 6A, the COX-2 dose response curves shown in Figure 6C and the mutagenicity data shown in Figure 7b were in excellent agreement. In essentially all other cases – for example the IL-8 data shown in figures 5B and 6B, the COX-2 data in Figure 5C and the MeOH-HRE data in Figures 7B and 8B – there were apparent differences between the duplicates but those differences were not statistically significant. A noticeable exception here are the DCM-HRE consumption rates shown in Figure 8B, which are significantly different from each other. The source of this discrepancy is not immediately clear.

3.8. Evaluation of Test Hypotheses

The core test hypotheses laid out in section 2.3 are evaluated in what follows using the assay data presented and discussed above.

1. *The outcome of a particular assay depends on whether the sample preparation technique is exhaustive or selective in nature; i.e. whether whole PM or solvent extractable PM components (fractionated PM) are used*

    - This is clearly visible in almost all comparisons between HRE (fractionated) and SE (whole particle) data for both *NIST-DEP* and *Dyno-DEP*, and is a major finding of this work. Since all assays were performed on an equal mass dose basis, the implication is that the HRE technique is amplifying the effect of those DEP components that are eliciting the greatest responses by removing the matrix components that elicit no, or low, responses. The SE technique, on the other hand, includes everything so the relative distribution of active components to nonactive components on a mass basis is reduced compared to HRE.

2. *The outcome of a particular assay using either whole PM or fractionated PM depends on the solvent or combination of solvents used*

    - Again, this is clearly evident when intercomparing the various solvent systems used in the SE and HRE techniques for both the *Dyno-DEP* and *NIST-DEP* samples. As mentioned previously, the most consistent and significant observation of this is the comparison of the DMT-SE and H2O-SE data across all assays. The DMT combination of solvents is consistently more efficient at removing the DEP compounds that elicit the more significant responses compared to H2O, which removes the polar compounds but not the nonpolar ones. This implies that the nonpolar compounds are eliciting the greater responses, as corroborated by the HRE data. The HRE results, however, need to be evaluated on an assay-by-assay case. For example, MeOH-HRE of *NIST-DEP* induces the largest DTT consumption rates suggesting that it is the polar DEP compounds, such as oxygenated organics, that are driving ROS production. The expression of CYP1A1, however, was largest for Tol-HRE. Since Tol is the most efficient solvent for solvating polycyclic aromatic hydrocarbons (PAHs), especially larger molecular weight PAHs, this confirms that these compounds are driving the CYP1A1 response.

3. *For assays conducted using fractionated PM, the sum of responses to individual PM fractions will be significantly larger than the individual response to the composite of those PM fractions, i.e. the presence of assay-specific toxicologically inert PM components interferes with the response to the toxicologically active PM components*

    - It is not possible to evaluate this hypothesis for those HRE samples that were tested on an equal mass dose basis. Therefore, it must be inferred from the dose response curves for *NIST-DEP* shown in Figures 6A-6C. For all three molecular markers – CYP1A1, IL-8 and COX-2 – the sum of the DCM-HRE, MeOH-HRE and Tol-HRE responses at the lowest dose (1.25 μg/mL) is considerably larger than the DMT-HRE response at the highest dose (5 μg/mL). Stated differently, the sum of responses to individual PM components is significantly larger than the response to the composite of those components; strong evidence for composite interference or toxicological matric effects, as has been observed previously (*101, 122*)

4. *The sample preparation technique yielding the most robust, reliable and repeatable outcome will be assay dependent.*

    - Evaluation of reliability and repeatability is limited to the duplicate DCM-HRE and MeOH-HRE measurements for the *NIST-DEP* samples. As discussed previously, both showed generally good agreement across all assays, which would be contrary to the stated hypothesis. Robustness, however, did show a tendency to be assay specific. For example, MeOH-HRE of *NIST-DEP* elicited the largest DTT consumption rates while Tol-HRE induced the largest CYP1A1 expression and DCM-HRE of *Dyno-DEP* produced the most mutagenetic activity. The highest expressions of IL-8 and COX-2, on the other hand, were shared by multiple sample preparation techniques that were not significantly different from one another; e.g. DCM-HRE, Tol-HRE and DMT-HRE of *NIST-DEP*.

4. Summary and Conclusions

In summary, six separate sample preparation techniques involving various combinations of different solvents in either heat reflux extraction or sonication extraction – DCM-HRE, MeOH-HRE, Tol-HRE, DMT-HRE, H2O-SE and DMT-SE – were applied to filter samples of diesel exhaust particles collected during a chassis dynamometer study and the associated filter blanks (*Dyno-DEP* and *FB*), as well as a NIST standard (*NIST-DEP*). All sample extracts were subjected to a multi-point assay measuring ROS production via the acellular DTT assay, cellular inflammation via COX-2 and IL-8 expression, PAH response via CYP1A1 expression, and mutagenicity via the Ames assay. Dose response curves were generated for each combination of extraction technique and assay; measurements were performed in duplicate at the chosen dose.

Perhaps the most consistent result in this study has been the observation that for a given sample preparation technique, the responses elicited by *NIST-DEP* have been significantly larger than those elicited by *Dyno-DEP*, which holds true across the entire assay panel. The most likely explanation is compositional differences in the DEP samples, as well as differences in how the DEP was sampled. First, *NIST-DEP* was collected in 1984 from several uncontrolled diesel engines while *Dyno-DEP* was collected from a single, more modern (2000) diesel engine. Secondly, *NIST-DEP* was scraped of the heat exchanger surfaces and provided in powder form while the *Dyno-DEP* was sampled from the dilution tunnel air via Hi-Vol samplers and provided as filter samples, which effects compositional extraction efficiencies. Lastly, the fuel consumed during the collection of *NIST-DEP* had significantly higher sulfur and aromatic content than that used for collecting the *Dyno-DEP*, which was ultra-low sulfur fuel.

The most important conclusions of this work include *(1)* HRE consistently tends to enhance the effect of the more active DEP components by removing the less reactive matrix components that are included in SE, *(2)* nonpolar compounds elicit the greatest responses across all assays except for ROS production, which is largest for the more polar DEP components, *(3)* there is strong evidence for the existence of composite interference or toxicological matrix effects, where the sum of responses to individual components is greater than the response to the composite of those components and *(4)* although different sample preparation techniques appear to be equally

repeatable across different assays, the techniques eliciting the most robust response are assay-specific and include cases where multiple techniques are equally effective.

5. **Recommendations**

Chemical Characterization – It is clearly evident from this work that the choice of sample preparation technique can significantly impact the outcome of toxicological assays. However, the knowledge gap remains as to the compositional differences between sample extracts driving the observed differential responses. It is highly recommended, as a follow-on study, to provide a comprehensive chemical characterization of the different sample extracts for both *Dyno-DEP* and *NIST-DEP*. These data can then be correlated to the toxicological data to provide a more informative description of the relationship between compositional complexity and toxicity.

Selective versus Exhaustive Extraction – A major conclusion of this work was that fractionated DEP extracts derived via the selective HRE method consistently elicited greater toxicological responses than whole particle extracts derived via the exhaustive SE method. Continued discussion of which method is the more appropriate evaluation metric is of critical importance. It is perhaps instinctive to conclude that the methods yielding the greatest response are best. However, exposure is better characterized by complex particle mixtures than fractionated PM components, such that the latter may be misleading about, or oversimplify, the true biological response to the complexity of particulate pollution.

Standardization – If a single sample preparation method is preferred for all assays and the issue of selective versus exhaustive extraction is not a concern, then the DCM-HRE method is recommended given its efficiency, consistency and versatility. If an exhaustive approach is preferred, then it is recommended that the DCM-HRE method without the post-extraction filtration step be compared to the SE methods and a decision made based upon those results. Recommendations for assay-specific methods would include MeOH-HRE for DTT and DCM-HRE for the molecular markers and mutagenicity, or a hybrid combination of the two methods for all assays. Again, if an exhaustive approach is preferred, then it is recommended that, similar to DCM-HRE, the MeOH-HRE method without the post-extraction filtration step be compared to the SE methods and a decision made based upon those results; the hybrid should be tested too.

Dyno-DEP Filter Samples – The mass of *Dyno-DEP* on the filter pieces made available for this research were insufficient to produce the desired robustness and statistical strength in the gravimetric analyses and toxicological measurements, and eventually resulted in the reduction of the originally proposed measurement matrix due to mass limitations. Only two filter pieces (**Filter 1** and **Filter 2**) were available for each sample preparation technique for a combined pre-extraction DEP mass of ~ 400 μg per method. With extraction efficiencies as low as 20-30% for some methods, the amount of sample extract available for the full assay panel was on the order of 100 μg. Repeated manipulation of these small quantities tend to be lossy and easily

contaminated. Furthermore, the uncertainties in the gravimetric analyses grow rapidly with decreasing sample size since two very large masses (pre- and post-weights of the sample vials) are being subtracted to obtain a very small mass and the accuracy of the analytical balance is a function of the measured mass. It is highly recommended that future studies begin with significantly more starting mass on the filter samples. A general rule of thumb is to start with an amount that is at least an order of magnitude larger than what is ultimately needed, trying carefully to estimate and account for all potential sources of sample loss throughout the process.

Post-Extraction Filtration – A single, 0.2 μm pore size, unlaminated PTFE filter disc was used during the post-extraction syringe filtration steps associated with the HRE methods. This resulted in an apparent variability in filtration efficiency depending on the solvent used, although this could not be directly measured. It was clear, however, that a nontrivial fraction of DEP and matrix residues remained in solution after syringe filtration. It is highly recommended that multiple filter discs be used in series during this step to ensure more robust filtration results.

Labware Selection – For all stages of sample preparation, it is highly recommended that all labware surfaces contacting the sample extracts and associated solvents at any point be solely composed of glass, stainless steel and PTFE, or comparable fluoroelastomer. A significant amount of time was spent in process characterization testing a wide range of different materials. Contaminating residues were consistently observed in gravimetrically measurable quantities for all cases except those where only the aforementioned materials were present.

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Glossary of Abbreviations

DEP = Diesel Exhaust Particles

Dyno-DEP = chassis Dynamometer Diesel Exhaust Particles

NIST-DEP = NIST standard Diesel Exhaust Particles

FB = Filter Blank

DCM = Dichloromethane

MeOH = Methanol

Tol = Toluene

DMT = Dichloromethane + Methanol + Toluene

HRE = Heat Reflux Extraction

SE = Sonication Extraction

DCM-HRE = Heat Reflux Extraction in Dichloromethane

MeOH-HRE = Heat Reflux Extraction in Methanol

Tol-HRE = Heat Reflux Extraction in Toluene

DMT-HRE = serial Heat Reflux Extraction in Dichloromethane, Methanol and Toluene

H2O-SE = Sonication Extraction in water

DMT-SE = serial Sonication Extraction in Dichloromethane, Methanol and Toluene