Final Report

Health Effects of Central Valley Particulate Matter

ARB Contract #09-330

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Disclaimer

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Abstract

The overall objective of this work was to better elucidate the toxic and inflammatory potential of urban and rural PM from the Central Valley on a suite of pulmonary, vascular and systemic endpoints in a mouse model. Specifically, mice received one intratrachael aspiration exposure to PM2.5, and effects were evaluated at post-exposure times of 1, 2 and 4 days to explore the temporal nature of different biological responses. PM2.5 samples were collected in a rural part of Davis that is surrounded by agricultural land and an urban part of downtown Sacramento near a major freeway interchange in order to obtain a comparison between the health effects elicited by PM that has different source mixtures. Sufficient PM was collected during a single winter collection campaign to allow animal exposures and chemical analysis using the same PM sampling filter. The results demonstrate (1) that the method of extraction of PM from the filter or impactor substrate has a substantial effect on the health effects elicited and the dose-response relationship; (2) some of the endpoints, especially the pulmonary ones, responded acutely to the PM, at 1 or 2 days post administration while other endpoints, especially systemic ones, responded at longer lag times, in agreement with epidemiological studies on cardiovascular responses to PM. The results have implications for design of future research studies, and help to explain some of the inconsistencies noted in previously published research.

1. Executive Summary:

Numerous epidemiological studies demonstrate a correlation between ambient particulate matter concentrations and increased morbidity and mortality with lags of 1 to 4 days (Smith et al., 2006; Ostro et al, 2007, Larrieu et al, 2009; Stieb et al, 2009), yet the mechanistic and causal links between health effects and particulate matter concentrations have eluded identification. A recent publication by our group (Smith et al 2006) showed the importance and informative nature of multiple sampling points following a laboratory exposure to coal fly ash, a particle composition that lacks the potency of concentrated ambient PM2.5. BALF (BronchoAlveolar Lavage Fluid) neutrophils increased prior to inflammatory cell changes in the blood.

During the 5 years of EPA funding for the PM Health Center at UC Davis (2006-2011), we exposed animals in the Fresno area to concentrated ambient particles. These exposure sites, in combination with a mobile trailer equipped with animal housing and exposure equipment, enabled us to compare the toxicity of urban and rural particulate matter (PM) for the lower San Joaquin Valley. In these studies, we found an association between health effects in mice exposed to concentrated ambient particles, but have not yet identified the proper metrics by which to determine either the peak or the persistence of particle-induced health effects. Such information is of critical importance to better elucidate the sources, sizes and chemical species of particles that produce adverse health outcomes. We have also noted the timing of response of the respiratory system does not always coincide with the timing of systemic effects due to particle exposure. This has been difficult to study in depth at remote sites and so in the work we chose two nearby sites for these time-sensitive studies. We investigated the biological responses of both the respiratory and vascular systems in mice exposed to PM using distinct lag times ranging from 1 to 4 days post-exposure to ambient particles encountered in an urban and a rural site in the Central Valley of California. This multi-investigator proposal employed a wide range of novel area of expertise to explore a complementary set of respiratory, vascular and systemic health effects endpoints.

The goals of the current project were to:

1) include time points both closer to and farther from the exposure so that we can better understand mechanisms and time sequence of effects that follow exposure,
2) investigate the effects of urban and rural winter high PM exposures that contain abundant PAH components, and
3) expand our previous studies of platelet effects to include studies of platelet activation and secretion.

This was accomplished using a fixed exposure time followed by various post-exposure sample collection times. The rationale and urgent need for this mechanistic approach is based on studies of

human populations, which suggest a different lag structure for pulmonary and cardiovascular health effects consequent to PM exposure.

This project investigated the pulmonary and systemic toxicity of PM in the Central Valley of California. PM2.5 samples were collected in a rural part of Davis that is surrounded by agricultural land and an urban part of downtown Sacramento near a major freeway interchange in order to obtain a comparison between the health effects elicited by PM that has different source mixtures. Sufficient PM was collected during a single collection campaign to allow us to instill mice with a sufficient mass both to elicit health effects and perform chemical analysis on the same PM samples.

Specifically, we

(a) Exposed mice to concentrated ambient particles collected in winter in an urban (Sacramento) and a rural (Davis) location.
(b) Employed a staggered set of post-exposure times to explore the temporal nature of different biological responses to concentrated ambient particles.
(c) Assessed markers of systemic and cardiovascular health effects induced by this PM.
(d) Assessed markers of pulmonary health effects induced by this PM.

The overall objective of this work was to better elucidate the toxic and inflammatory potential of urban and rural PM from the Central Valley on pulmonary, vascular and systemic health effects in a mouse model through the examination of effects at specific post-exposure times of 1, 2 and 4 days following aspiration of a single dose of PM.

There are a number of major conclusions from this work that have already been or will be submitted for publication:

- The method of extraction of PM from the filter or impactor substrate has a substantial effect on the health effects elicited. In this study, two different methods were employed that are very different in the fraction of PM that they remove from the substrates and how well they preserve semivolatile components as the extract is processed. When PM toxicity is tested, what is being tested is a complex combination of the toxicity of the large surface of the PM in combination with chemical components in the PM. But traditional toxicology is performed on a mass-based dose-response paradigm. For PM, the mass is the mass of the particulate matter administered. Consider a PM sample that contains toxic and benign components. If the extraction method concentrates the toxic component(s) relative to the benign one(s), the PM will appear more toxic. Conversely, if the method concentrates the benign component(s) relative to the toxic one(s), the sample will appear less toxic. This becomes even more complicated when multiple toxicity endpoints are tested for PM sample containing compounds that are toxic for some endpoints but benign for others. If the goal is to understand the toxicity of PM, which requires a dose response relationship, wherein the dose is characterized by the total mass of PM, then it is imperative that an extraction method is employed that extracts near 100% of the PM on the substrate and preserves this PM

throughout its processing before administration. Conversely, if the goal of the study is to focus on the toxic components of the PM, methods that preferentially accentuate these components may be preferred. Section 3 and 4 discuss these portions of the project.

- This work on extraction methods naturally leads into the next major component of the project which deals with the project's dose-response results. With multiple endpoints being tested, the appropriate dose is likely to be endpoint-dependent. The goal is to administer a dose that is not so low that it elicits no response yet not so high that the response has plateaued. And, as discussed above, the appropriate dose depends on how well the extraction and processing of the PM preserves both toxic and benign components. Yet another potentially confounding factor is the time delay between dose administration and response testing. Some responses have a very fast transient so may have already been restored to baseline by the time the response is tested, others may develop slowly so that they are not detectable until a longer time has passed since exposure. Despite all these complications, the PM collected in Sacramento elicited both pulmonary and systemic health effects. Sections 7 and 8 discuss the dose-response results from this project.

- The prior dose-response study set the stage for the final study in the project which addressed the time course of the responses. Endpoints were assessed at 1, 2 and 4 days post administration. It is possible that the PM elicited responses that then resolved prior to 1 d post-administration, but these rapid responses were not addressed in this study. As expected, (a) some of the endpoints, especially the pulmonary ones, responded acutely to the PM, at 1 or 2 days post administration and (b) other endpoints, especially systemic ones, responded with larger delays in agreement with epidemiological studies on cardiovascular responses to PM. These results are discussed in sections 9 and 10 of the report.

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2. PM Sample Collection, Extraction and Chemical Analysis

This chapter of the Final Report has been submitted for publication and has received positive reviews, but has not been accepted for publication yet.

2.1. Introduction

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 using various analytical techniques and/or toxicity using *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.

For toxicological studies, the primary objective of filter extraction is to conserve, as much as possible, 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 and this efficiency may be compositionally biased. Recently, an exhaustive, multi-solvent extraction (MSE) technique including sonication, liquid-liquid extraction, selective filtration and solvent removal was introduced, resulting in extraction efficiencies consistently exceeding 90% (*3*).

Chemical composition 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 potentially interfering species to eliminate matrix effects – i.e. in complex multi-component systems, the presence of certain components can interfere with the detection of others, either synergistically or antagonistically – 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 followed by acid digestion using a strong acid (*4*). The initial organic solvent extraction is necessary for most combustion generated aerosol and/or secondary organic aerosol (SOA) since *(i)* the trace metals are typically encapsulated by layers of organic compounds and *(ii)* 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. Similarly, there are several different sample preparation protocols for molecular speciation of particulate organic carbon via GC-MS that are based on organic solvent extraction followed by post-extraction cleanup steps to dissolve the organics into solution and separate them from the particle matrix (*5-10*).

Extrapolating the potential for toxicological matrix effects from the existence of chemical matrix effects is a new concept that is largely unstudied. For particle toxicity, the basic idea is that the sum of endpoint-specific toxicological responses to individual PM components may be different than the response to the composite of those components, i.e. the presence of endpoint-specific toxicologically inert PM components may interfere with the response to the toxicologically active PM components. This may further depend on the physical form in which the components are present; e.g., dissolved in solution, individual particles or particle aggregates. In this context, toxicological response may vary significantly depending on the filter extraction technique employed. A filter extraction technique designed according to one set of objectives may inadvertently alter the physical and/or chemical composition of the particle mixture in such a manner as to enhance or inhibit toxicological response relative to another technique designed with a different set of objectives. The current study was designed to test this hypothesis that the filter extraction technique influences the toxicological effects observed.

Separate filter extraction techniques commonly used in different laboratories and designed with different sets of objectives were used to extract ambient PM collected simultaneously from an urban and rural sampling site using high-volume $PM_{2.5}$ sampler systems. The extracted PM was exhaustively characterized both chemically and toxicologically using a suite of analytical techniques and toxicological endpoints. A comprehensive comparison between the two filter extraction techniques based on the chemical composition of extracted PM is presented in what follows. Results from the toxicological studies are published separately (*11*). Overall, and to the authors' knowledge, this is the first study to *(i)* provide an exhaustive chemical characterization of a single PM extract, *(ii)* analyze the same PM extracts as used in subsequent exposure studies and *(iii)* inter-compare different filter extraction techniques in terms of the chemical composition of extracted PM.

2.2. Methodology

2.2.1. PM Sampling

Field studies were conducted simultaneously during winter 2011 at two separate sampling sites representing an urban and rural environment using $PM_{2.5}$ high-volume sampler systems (Tisch Environmental Inc., TE-6070V-2.5-HVS) equipped with PM_{10} size-selective heads (Tisch Environmental Inc., TE-6001), operating at a flow rate of 40 cfm and loaded with aluminum foil substrates for collecting the coarse PM fraction ($PM_{10-2.5} = 2.5 < D_{p50} < 10$ µm) and Teflon coated borosilicate glass microfiber filters (Pall Corporation, TX40H120WW-8X10) for collecting the fine PM fraction ($PM_{2.5} = D_{p50} < 2.5$ µm). Aluminum foil substrates were pre-baked at 500º C for 24 hours and glass microfiber filters were pre-cleaned via successive sonication in milli-Q H_2O, dichloromethane (DCM) and hexane (Hx). Field blanks were included for all studies. The **urban** sampling site was located on the rooftop of a two story building at the northeast corner of T St. and

13th St. in downtown Sacramento, CA, surrounded by a mixture of residential, commercial and industrial sources and within a quarter mile of a major freeway interchange. The **rural** site was situated on top of a single story laboratory in the southeast corner of the Center for Health and the Environment complex on the south campus of U.C. Davis and surrounded by agricultural lands. $PM_{2.5}$ filter samples and field blanks from both sites were extracted using two different filter extraction techniques detailed below and the extracts subjected to an exhaustive chemical characterization using a range of analytical techniques, as discussed later.

2.2.2. Filter Extraction Techniques

Both filter extraction techniques used in this study have been described in detail elsewhere (*11-14*) so only a summary is provided here.

Multi-Solvent Extraction (MSE) is an exhaustive method comprised of a combination of sonication in multiple solvents, liquid-liquid extraction, microporous membrane filtration and detailed gravimetric analysis designed specifically to *(i)* maximize extraction efficiency, *(ii)* minimize compositional biases, *(iii)* minimize extraction artifacts and *(iv)* provide precise and accurate direct measurements of extracted PM mass. A comprehensive gravimetric characterization of this method showing that extraction efficiencies consistently exceed 90% for a wide range of PM samples was presented in a previous study (*12*). An outline of the various steps follows:

- Sonication in milli-Q H_2O (**H_2O Ex**)
- Liquid-liquid extraction of **H_2O Ex** in dichloromethane (**DCM soluble**) and hexane (**Hx soluble**)
- Microporous membrane filtration of **H_2O Ex, DCM soluble** and **Hx soluble** fractions
- Lyophilization of **H_2O Ex** followed by gravimetric analysis
- N_2 blowdown of **DCM soluble** and **Hx soluble** fractions followed by gravimetric analysis
- Sonication in dichloromethane (**DCM Ex**)
- Microporous membrane filtration, N_2 blowdown and gravimetric analysis of **DCM Ex**
- Sonication in hexane (**Hx Ex**)
- Microporous membrane filtration, N_2 blowdown and gravimetric analysis of **Hx Ex**
- Reconstitution of all fractions into single composite sample and final gravimetric analysis

The fractional distribution of total extracted PM mass among the various steps outlined above for the urban, rural and field blank filter samples is shown in Figure 1. The **Hx soluble** and **Hx Ex** fractions were below the minimum detection limit of the gravimetric analysis in all cases and thus are excluded from the figure. Extraction efficiencies of 95.4 ± 0.7% and 96.9 ± 0.5% were obtained for the urban and rural $PM_{2.5}$ samples, respectively, and residual filter material equaling less than 2% of total extracted PM mass was recovered during extraction of the field blank.

Spin-Down Extraction (SDE) was designed primarily to minimize the amount of contaminant filter material co-extracted with the PM. For both methods, filter glass microfibers (FGMs) are unavoidably shed from the filter during the sonication process and retained in the extracted PM either as freely suspended microfibers or agglomerated with PM. Although borosilicate glass is chemically inert, there is concern about potential interference effects induced by the size and

morphology of FGMs on the animals and cell cultures used in *in vivo* and *in vitro* studies (*15-17*). The microporous membrane filtration steps of the MSE method described above have been shown to selectively remove roughly 60-70% of FGMs by mass from the extraction of filter blanks but removal efficiencies have not been measured for the extraction of PM samples (*12*). The objective of the SDE method is to maximize FGM removal efficiencies in PM extracts.

As with MSE, sonication is the basic extraction method in SDE. However, SDE differs substantially by employing a microcentrifuge-based cellular homogenization technique serving as the selective filtration step separating FGM from the PM and soluble PM components. In cellular and molecular biology, homogenization refers to the mechanical shearing of higher-molecular weight cellular components to form a reduced viscosity, homogenous lysate after the initial cellular disruption step during RNA and DNA purification from cell and tissue samples. Centrifugal force provides the necessary pressure differential across the filter membrane to separate lower-molecular weight components from other cellular material. Applied to the extraction of PM, the concept is that the mechanical shear will break apart particle-particle and particle-FGM agglomerates to form a homogenous extract that can be selectively filtered to remove the FGM. The following is an outline of the SDE method:

- Top layer of filter membrane with PM deposit is removed, leaving filter backing behind
- Filter membranes added to top of QIAshredder® column (Qiagen, cat. # 79654)
- QIAshredder® column weighed to obtain extraction pre-weight
- 500 µL Dulbecco's PBS without $CaCl_2$ or $MgCl_2$ added to column
- Filter membranes probe sonicated for 5 seconds
- Collection tubes attached to column and centrifuged at 7,600 x g for 4 min
- Supernatant collected from tubes and transferred back to column
- Membranes sonicated in supernatant and then centrifuged; process repeated twice
- Final centrifuged PM sample resuspended in supernatant and filtered through clean column
- Supernatant lost during process replaced with fresh PBS to obtain 500 µL final volume
- Extracted membranes in original column washed with 500 µL distilled H_2O and centrifuged
- Extracted membranes and column dried in SpeedVac concentrator for 6 hours
- Extracted membranes and column weighed to obtain extraction post-weight
- Extraction pre- and post-weights subtracted to obtain extracted PM mass

The fundamental differences between the MSE and SDE methods are

- Extraction solvents: H_2O, DCM and Hx followed by solvent removal in MSE versus sonication directly into the PBS delivery vehicle for SDE
- Post-extraction cleanup steps: microporous membrane filtration for MSE versus centrifugal homogenization and filtration in SDE
- Gravimetric analysis: direct measurement of extracted PM mass for MSE versus difference between pre- and post-extracted filter mass for SDE

2.2.3 Sample Preparation and Chemical Characterization

The main objective of the current study is a compositional comparison between different filter extraction techniques. As a result, all sample preparation and subsequent chemical analyses were performed on the material extracted from PM and field blank filters rather than on the filters themselves. As will be shown later, this is a key distinction from the traditional approach to chemically characterizing exposure studies. Almost exclusively, collocated instrumentation running in parallel with the PM samplers are used to characterize the physical and chemical properties of the collected PM. Even in those cases where this instrumentation is also filter based, multiple filter types and sample preparation methods are required to cover the full spectrum of relevant PM components since different protocols have been developed independently for analyzing different PM components. Furthermore, these protocols vary significantly from those commonly used in toxicological laboratories to extract PM for in vitro and in vivo studies. Therefore, it is not clear that the chemical characterization is a true representation of the exposure. This is especially true since the analytical protocols have been optimized to be highly quantitative while the extraction efficiency standards of toxicological studies have as of yet not been defined. Instead, the focus of extraction techniques for toxicological studies is normalizing mass dose while assuming that the original PM composition distribution has been conserved, regardless of extraction technique or extraction efficiency. To the authors' knowledge, this is the first study to *(i)* provide an exhaustive chemical characterization of a single PM extract, *(ii)* analyze the same PM extracts as used in subsequent exposure studies and *(iii)* inter-compare different filter extraction techniques in terms of the chemical composition of extracted PM. As such, design and development of novel sample preparation methods was necessary. These methods are outlined according to chemical component and associated analytical technique in what follows.

All PM extracts were divided into multiple aliquots of known PM mass concentration in solutions specific to the various analytical techniques employed. Field blank extracts were divided into the same number of aliquots using the same solvent volumes as the PM extracts to ensure proper field blank correction. A process blank was also included for each method to quantify any potential sources of contamination during sample preparation. All PM composition data were field blank and process blank corrected. Measurement errors were propagated through all calculations to obtain 99% confidence intervals for the final reported compositional mass fraction data.

Trace Metals were measured via Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) according to the SOPs (Standard Operating Procedures), calibration standards, QA/QC and MDL/error analyses of the Interdisciplinary Center for Plasma Mass Spectrometry at U.C. Davis (http://icpms.ucdavis.edu/). The elements analyzed include Li, Be, Na, Mg, Al, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Ag, Cd, Cs, Ba, Tl, Pb and U. Sample preparation was accomplished by successive liquid-liquid extractions of the PM, field blank and process blank aliquots suspended in 3 mL milli-Q H2O using DCM and Hx to remove any organics. Concentrated nitric acid and milli-Q H_2O were then added to the solvent washed suspensions to obtain a 6 mL volume of 1M solution. These solutions were bath sonicated for ~ 1 hour and refrigerated until analysis

Water Soluble Inorganic and Organic Ions were measured via the combination of Ion Chromatography (IC), Automated Colorimetry (AC) and Atomic Absorption Spectrophotometry (AAS) according to the SOPs, calibration standards, QA/QC and MDL/error analyses of the Desert Research Institute (DRI) in Reno, NV (*18*). The ions analyzed include NH_4^+, Cl^-, NO_2^-, NO_3^-, SO_4^{-2}, PO_4^{-3}, Na^+, Mg^{+2}, K^+, Ca^{+2}, 17 different organic sugars and 9 organic acids. Sample preparation was accomplished by diluting the PM, field blank and process blank aliquots to a 10 mL volume with milli-Q H_2O, bath sonicating for 30 minutes and then filtering the solutions using a 0.2 μm pore size. Samples were refrigerated until analysis.

Molecular Organic Compounds, including 38 different polycyclic aromatic hydrocarbons (PAHs), 27 high molecular weight alkanes/alkenes, 10 iso/anteiso-alkanes, 2 methyl-alkanes, 3 branched alkanes, 5 cycloalkanes, 18 hopanes and 12 steranes, were measured via Thermal Desorption-Gas Chromatography Mass Spectrometry (TD-GCMS) according to the SOPs, calibration standards, QA/QC and MDL/error analyses of DRI (*18*).

The thermal desorption technique of DRI is designed for sample deposits on pure quartz filters so a novel method was developed during this study to deposit the PM and field blank extracts onto 25 mm Tissuquartz™ filters. The PM, field blank and process blank aliquots were diluted to 5 mL volume using MeOH (methanol) and then sonicated for ~ 30 minutes. Immediately following sonication, the MeOH suspensions were slowly dripped (~ 100 μL/min) onto a 25 mm Tissuquartz™ filter housed in a custom filter holder attached at the bottom to a filtration flask. A N_2 tank was attached to the vacuum port of the filtration flask and a 5 lpm reverse N_2 flow applied through the flask to *(i)* prevent solvent bleed-through and sample loss by providing back pressure at the bottom of the filter and *(ii)* accelerate MeOH evaporation from the filter.

Since the mass of the individual PM and field blank aliquots from the MSE method were directly measured, it was possible to quantify the mass transfer efficiency of the deposition process by taking the difference between the pre- and post-deposition masses of the Tissuquartz™ filters for these samples. The average mass transfer efficiency over all samples was 97 ± 8%, demonstrating quantitative transfer well within the detection limits of the analysis and thus validating the deposition technique.

Elemental Carbon and Organic Carbon (EC/OC) were measured via Thermal Optical Reflectance according to the SOPs, calibration standards, QA/QC and MDL/error analyses of DRI (*18*). Similar to TD-GCMS, the TOR measurements require sample deposits on pure quartz filters so the exact same procedures described above were also applied here. The mass transfer efficiencies for the EC/OC analysis were included in the TD-GCMS calculations.

2.3. Results and Discussion

2.3.1. Compositional Mass Fraction Data

Figures 2 through 6 show the fractional distribution of total extracted PM mass among the individual chemical components measured for the MSE and SDE extractions of the urban and rural PM

samples. Figure 2 shows (a) major and (b) trace metals, Figure 3 shows water soluble inorganic ions, Figure 4 (a) major and (b) minor PAHs, Figure 5 (a) major and (b) minor nonaromatic molecular organic compounds and Figure 6 organic carbon (OC), elemental carbon (EC) and total organic matter (OM), where OM was calculated using an OM-to-OC ratio of 1.6 ± 0.2 (*19*). These data have been filter blank and process blank corrected and the error bars represent 99% confidence intervals. The water soluble organics (i.e. sugars and acids) are not shown since almost all measured species were below detection limits for all extracts.

Since the SDE samples were extracted directly into PBS, which contains potassium chloride, potassium phosphate, sodium chloride and sodium phosphate, the sodium and potassium values in the metals data (Figure 2a) and sodium, potassium, chloride and phosphate values in the ions data (Figure 3) have been estimated from the MSE data using the average ratio of MSE to SDE data for the other metals and ions, respectively. This was done independently for the urban and rural samples and the calculated ratios showed surprisingly small spread.

In combination, there is a strikingly nonrandom difference in the compositional mass fraction data between the MSE and SDE techniques for all measured components in the urban and rural PM extracts. The MSE data are consistently higher than the SDE data. For example, the ratio of SDE to MSE mass fraction data for the urban and rural extracts are: 0.60 ± 0.08 and 0.67 ± 0.09 for total metals, 0.54 ± 0.04 and 0.52 ± 0.02 for total inorganic ions, 0.35 ± 0.05 and 0.8 ± 0.1 for total PAHs, 0.41 ± 0.04 and 0.32 ± 0.04 for total molecular organic compounds, and 0.8 ± 0.1 and 0.49 ± 0.06 for OM + EC. These trends indicate a significant amount of uncharacterized mass in the SDE extracts, as discussed next.

2.3.2. Mass Closure

A mass closure analysis was performed to further investigate the trends in the compositional mass fraction data discussed above. Figure 7 shows the results of this analysis for the MSE and SDE methods as the component sum of the compositional mass fraction data for the major PM components in the urban and rural PM extracts. Although the MSE data demonstrate good mass closure – 92 ± 8% and 95 ± 9% for the urban and rural extracts, respectively – it is immediately clear that a significant fraction of the PM mass extracted via SDE is unaccounted for, or missing, in both the urban (36 ± 7%) and rural (52 ± 4%) samples. Given that *(i)* the MSE extracts are well characterized by the measured chemical components and *(ii)* the total extracted PM mass was never directly measured in the SDE method but rather estimated by the difference in pre- and post-extraction filter weights, it is most likely that the unaccounted PM mass was actually lost somewhere in the SDE process. A retrospective mass reconciliation effort was made to test this hypothesis using archived SDE samples, as discussed next.

2.3.3. Retrospective Mass Reconciliation

The primary issue with directly measuring the mass of the SDE extracts is that the samples were extracted into PBS which contains high salt concentrations relative to the concentration of extracted PM. For example in the current study, a 500 μL volume of Dulbecco's PBS without $CaCl_2$ or $MgCl_2$

was used. It contains 5.28 mg of buffering salts compared to the ~ 4-5 mg of PM mass that can be extracted into this volume via the SDE method. Therefore, the accuracy of subtracting the volume-calculated mass of PBS salts from the total measured mass to obtain the mass of extracted PM not only depends on the accuracy of the gravimetric analysis but will be highly sensitive to the accuracy of the PBS volume measurements as well. To estimate the magnitude of this error, an archived aliquot of the SDE field blank extract was diluted with minimal MeOH, bath sonicated and evenly partitioned among six new aliquots. The MeOH and residual PBS H_2O content were evaporated under a N_2 atmosphere and the dry extract weighed using an analytical microbalance. The average percent difference between the measured mass and volume-calculated PBS mass for all six aliquots was 0 ± 6%, showing *(i)* excellent agreement between measured and calculated mass and *(ii)* excellent FGM removal efficiency for the SDE technique.

The exact same procedures described above were applied to archived aliquots of the SDE urban and rural extracts. The PBS-adjusted measured masses were then compared to the original pre- and post-extraction filter mass differences to show that, in fact, 44 ± 9% and 52 ± 8% of the extracted urban and rural PM mass, respectively, was lost during the SDE process. Using the adjusted masses in the mass closure analysis described previously increases the percent mass closure from 64 ± 4% to 110 ± 13% for the urban SDE extract and from 48 ± 3% to 100 ± 10% for the SDE rural extract, further substantiating the analysis and hypothesis. However, the relative distribution of PM mass among the measured components does not change since the data are simply scaled by a constant factor.
It is not immediately clear at this point what step(s) of the SDE method is responsible for the missing PM mass but the most likely sources include (*i*) any supernatant lost during the process, which was approximated to be roughly 10% based on the amount of makeup volume required at the end of the extractions, (ii) any PM lost during the final filtration through the clean QIAshredder® column, (iii) any PM retained in the original QIAshredder® column but removed upon final rinsing prior to post-extraction weighing and (iv) any organics and ammonium nitrate retained on the shredded filter membranes but lost to evaporation during SpeedVac drying.

As a final analysis, the enrichment factors of the measured chemical components in the SDE extracts relative to the MSE extracts were calculated using the adjusted PM masses. These results are shown in Figure (8) for both the urban and rural PM samples. Values close to one indicate that the component was lost in constant proportion to the total loss of PM mass and thus was not enriched or depleted in the SDE extract while values above/below one indicate enrichment/depletion relative to the MSE extract. It is clear from the mass closure analysis that inorganic ions and organic matter constitute the largest sources of missing mass for both samples simply because they represent the largest fraction of total PM mass. However, the enrichment factors vary widely from component to component and between samples. For example, the urban sample is highly depleted in PAHs and inorganic ions and only slightly depleted in metals while the rural sample is highly enriched in PAHs and metals and neither enriched nor depleted in ions. Similarly, both samples are highly depleted in molecular organic compounds and highly enriched in EC but show opposite trends in OC. This represents a severe compositional bias between the two extraction techniques that could potentially manifest as differences in toxicological response and underscores the importance of characterizing and standardizing the filter extraction process.

2.3.4. Compositional Differences between Urban and Rural PM

To investigate the compositional differences between the urban (U) and rural (R) PM samples used in the time-delay studies, a statistical analysis was performed on the compositional mass fraction data shown in Section 4 to determine specific PM components demonstrating statistically significant differences between the two samples. These data are shown in Figures 9-13 as the percent difference between the urban and rural PM samples as a function of PM component, including: metals in Figure 9, inorganic ions in Figure 10, polycyclic aromatic hydrocarbons in Figure 11, nonaromatic organic compounds in Figure 12 and particulate carbon in Figure 13. Only those component comparisons with p-values less than 0.05 are included in the figures. For this analysis, percent difference (%D) was defined as

$$\%D \equiv 100[([U]-[R])/(1/2([U]+[R]))]$$

where [U] and [R] are the mass fractions of a specific PM component in the urban and rural PM samples, respectively. Using this definition, PM components enhanced in the urban sample relative to the rural sample will be positive and those enhanced in the rural relative to the urban will be negative. From the combination of these figures, it is clear that the urban sample is enhanced in metals, PAHs, nonaromatic organics and organic matter while the rural sample is enhanced in non-metallic inorganic ions and elemental carbon.

2.4. Conclusions

Separate filter extraction techniques commonly employed by different laboratories were used in the current study to extract $PM_{2.5}$ samples collected simultaneously at an urban and rural sampling site. A comprehensive inter-comparison of the extracted PM showed significant compositional variance between the two techniques attributed to a process-based loss of PM mass from the SDE method. This was confirmed retrospectively using detailed gravimetric analyses. To the authors' knowledge, this is the first study to demonstrate *(i)* an exhaustive chemical characterization of a single PM extract, *(ii)* the importance of chemically characterizing the extracted PM rather than relying on parallel measurements used in toxicological studies, *(iii)* the relevance of directly measuring extracted PM mass rather than using pre- and post-extraction differences in filter weights, *(iv)* the existence of substantial compositional biases between different filter extraction techniques and *(v)* the importance of standardizing filter extraction objectives and procedures to avoid introducing study bias into toxicological studies.

A detailed toxicological inter-comparison of the MSE and SDE extracted urban sample, published separately (*11*), revealed an unintuitive trend opposite that of the chemical characterization described here. With the exception of PAH response, the SDE-extracted PM sample consistently elicited the largest and most robust toxicological response for almost all endpoints tested. This is especially intriguing considering that the mass reconciliation of the SDE method occurred long after the toxicology studies were conducted such that incorrect PM masses were used to determine dose. This means that the elicited responses were observed at roughly half the intended SDE dose. In combination, these two studies present a paradox. The data presented here clearly demonstrate that the MSE method best conserves the original chemical composition of the sampled PM while the largest and most robust toxicological responses were generally elicited from the SDE method. The obvious question becomes which method is more appropriate. It may be the case that toxicological

matrix effects are being observed; i.e. SDE removed a large fraction of toxicologically inert components, thus amplifying the response to the active components, while MSE maximized extraction of all components and inert ones diluted the response to active ones. Conversely, it may be the case that MSE altered the physical composition of the PM – e.g., particle size distribution, internal distribution of chemical components or inclusion of FGM – which inhibited the bioavailability of the extracted PM. Although the current study sets the stage for this discussion, further research is required to fully address these issues and answer this question.

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2.6. Figures

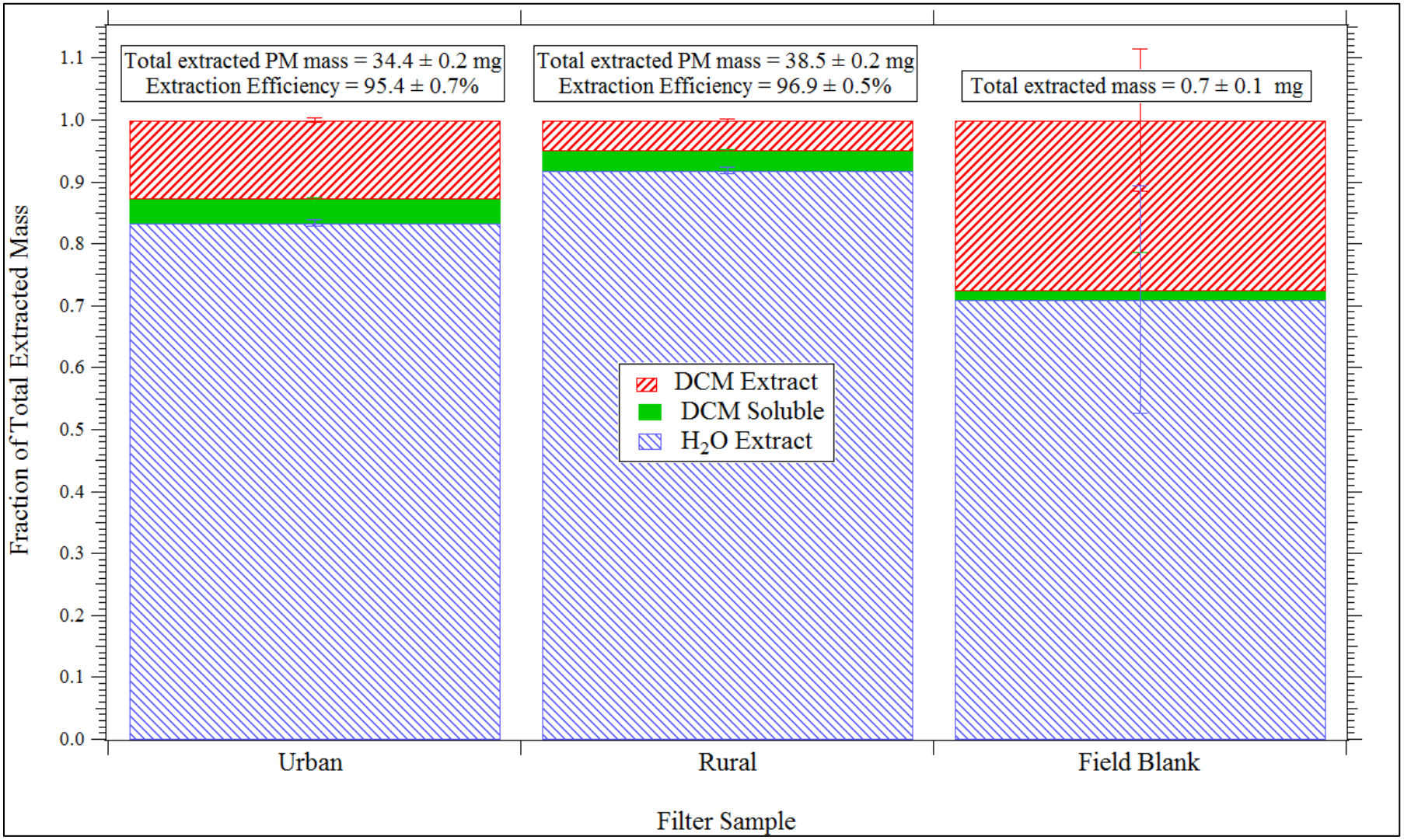


Figure 1. The fractional distribution of total extracted mass among the various steps of the multi-solvent extraction (MSE) for the urban, rural and field blank filter samples; see text for discussion.

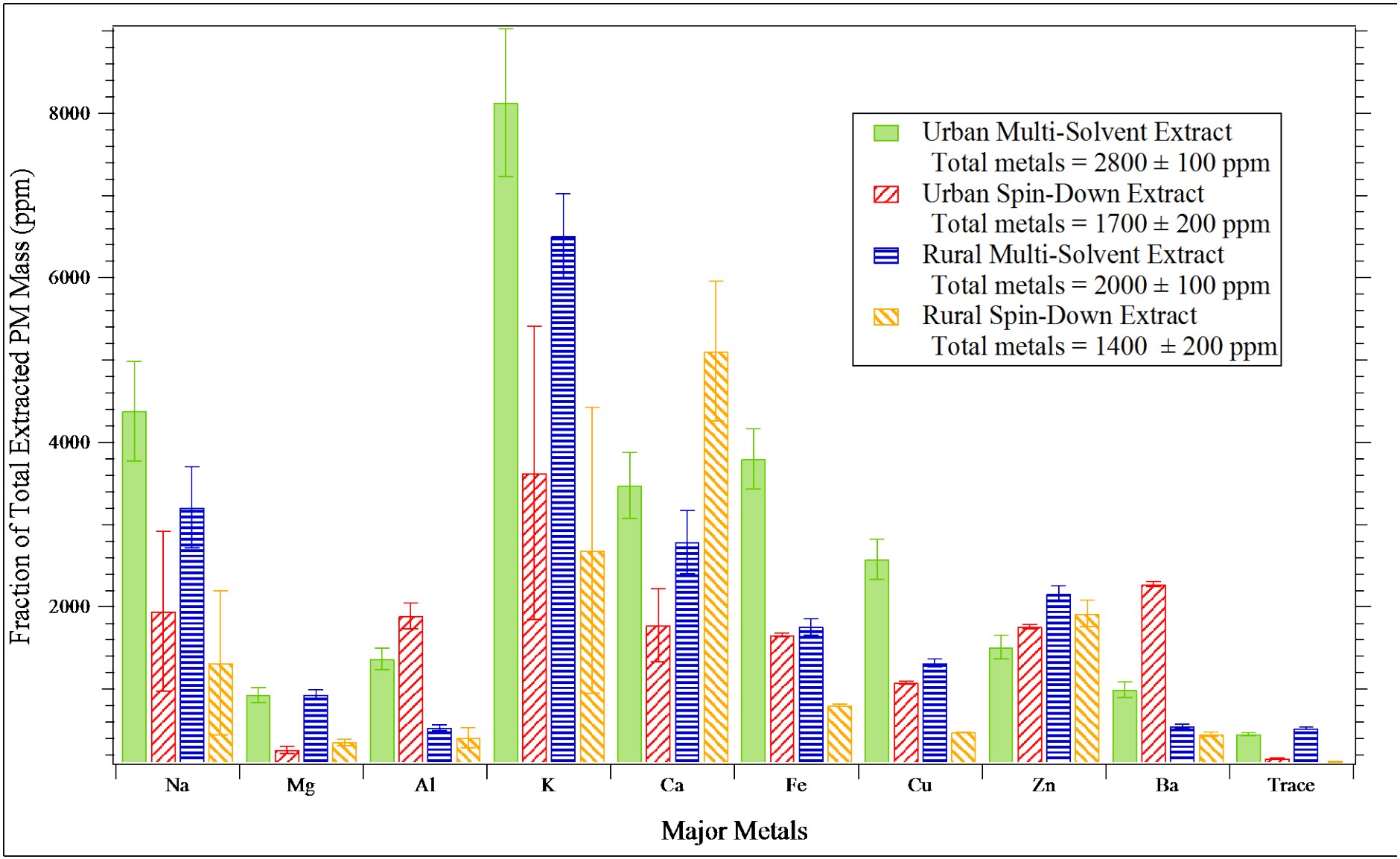


Figure 2a. Fraction of total extracted PM mass accounted for by major metals detected during ICP-MS analysis of the urban and rural PM samples extracted via MSE and SDE; error bars represent 99% confidence intervals.

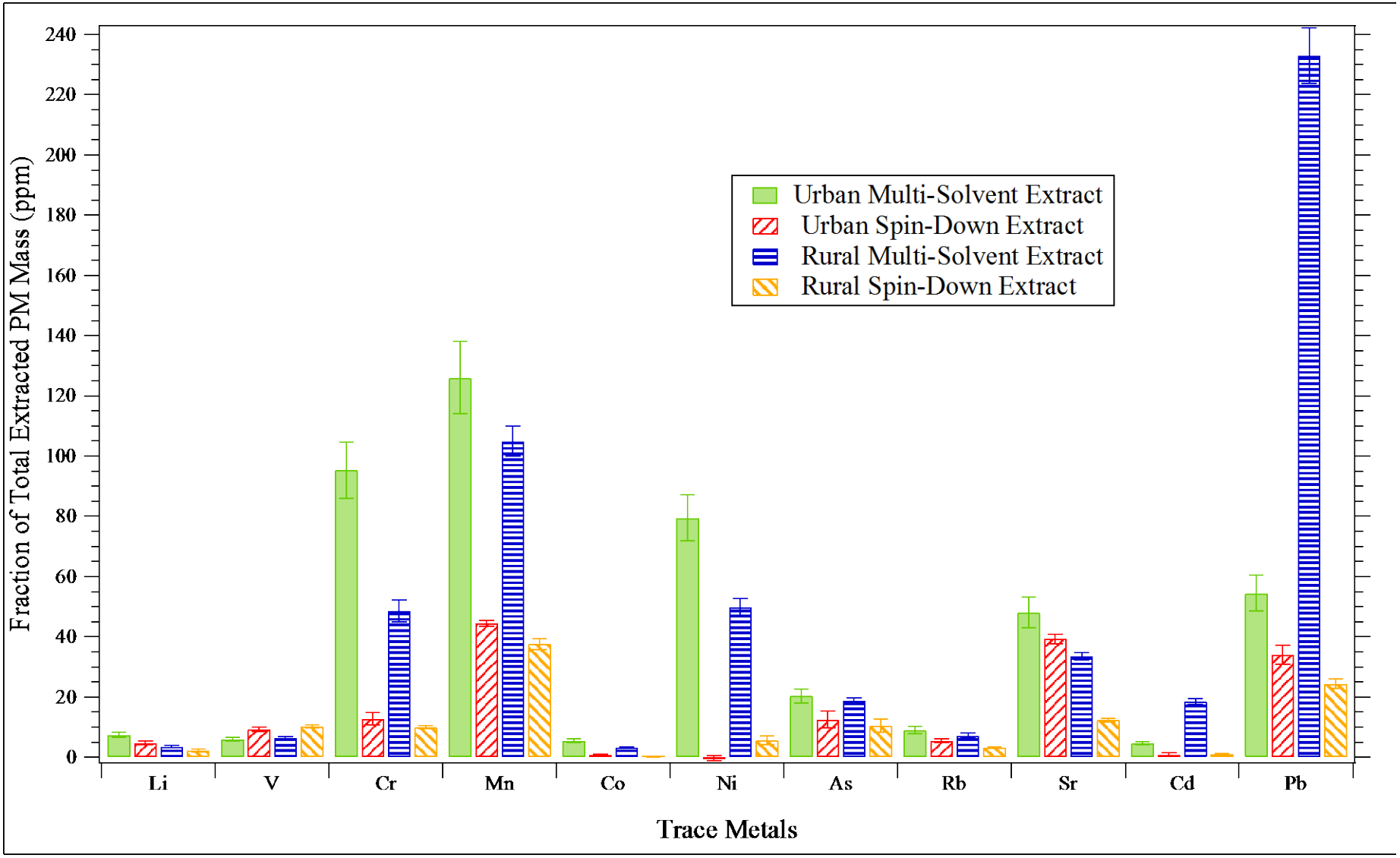


Figure 2b. Fraction of total extracted PM mass accounted for by trace metals detected during ICP-MS analysis of the urban and rural PM samples extracted via MSE and SDE; error bars represent 99% confidence intervals.

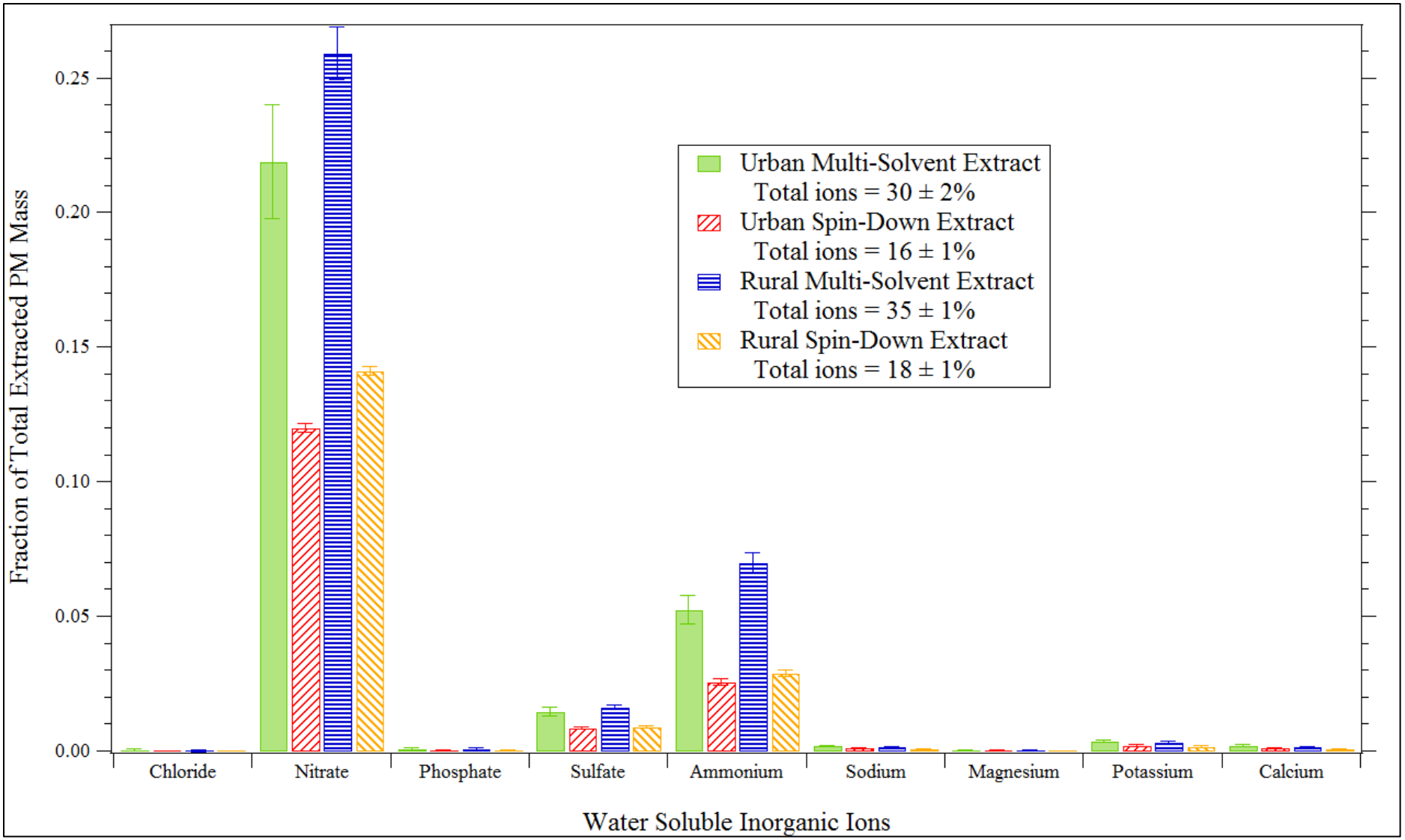


Figure 3. Fraction of total extracted PM mass accounted for by the water soluble inorganic ions detected during IC, AC and AAS analysis of the urban and rural PM samples extracted via MSE and SDE; error bars represent 99% confidence intervals.

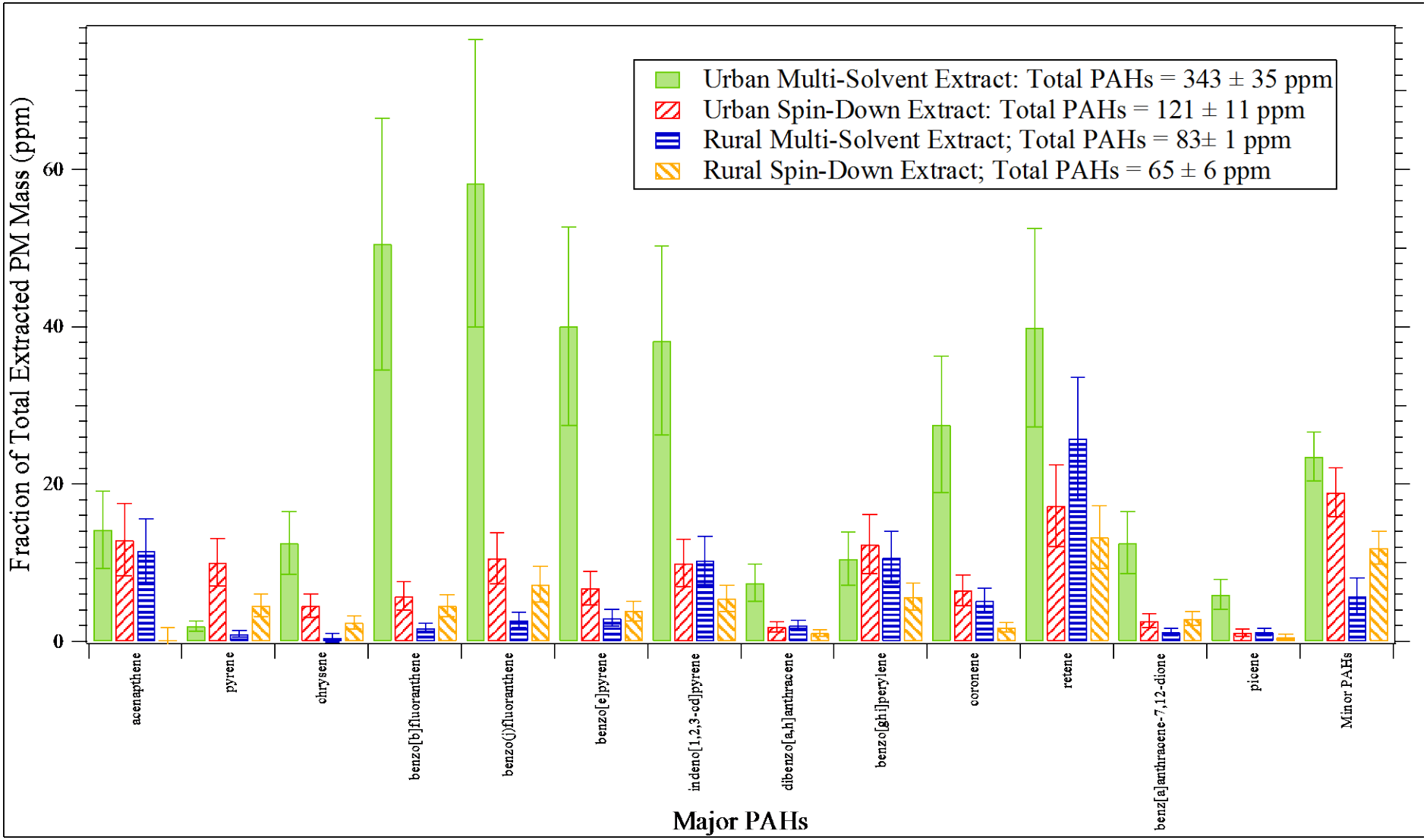


Figure 4a. Fraction of total extracted PM mass accounted for by major PAHs detected during TD-GCMS analysis of the urban and rural PM samples extracted via MSE and SDE; error bars represent 99% confidence intervals.

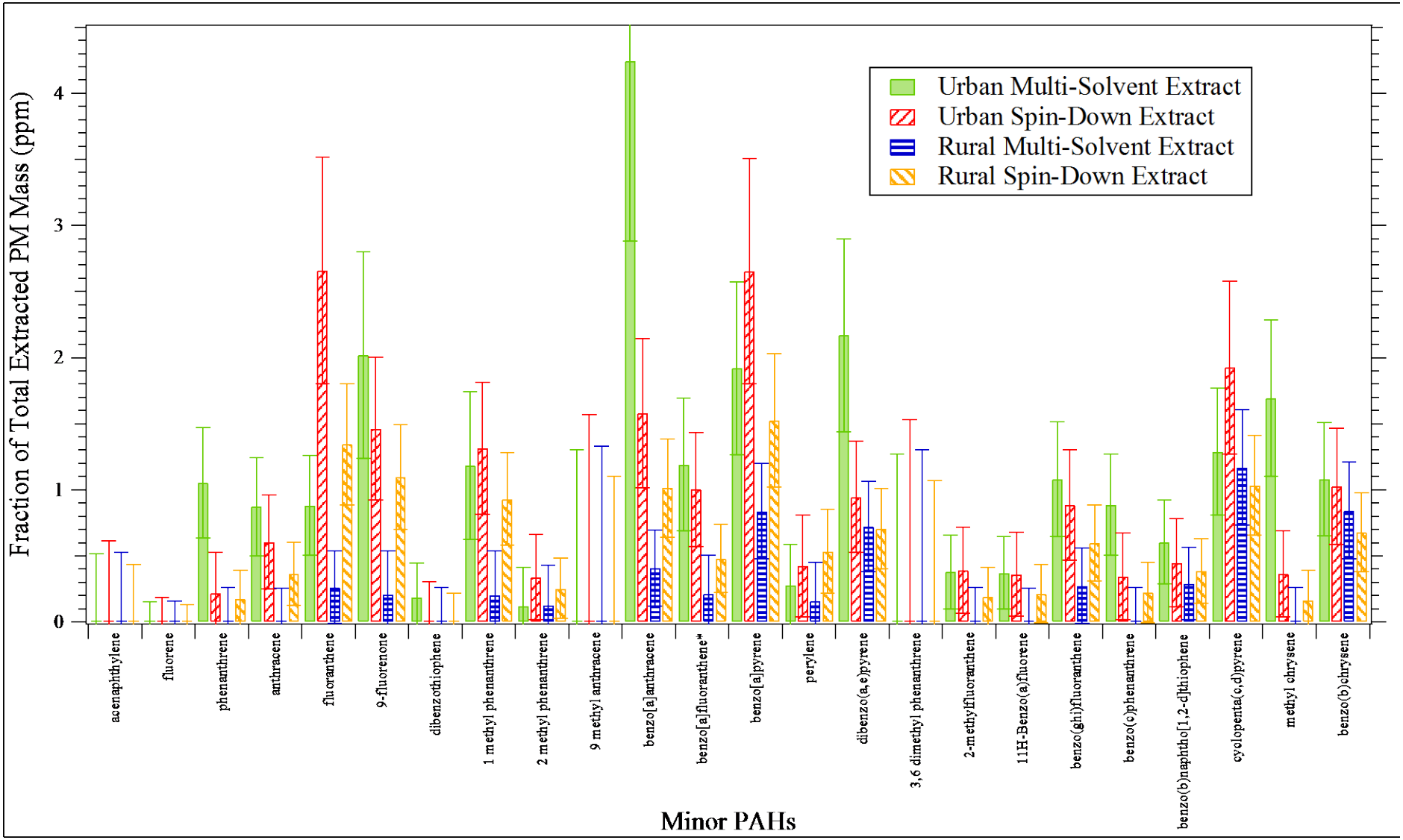


Figure 4b. Fraction of total extracted PM mass accounted for by minor PAHs detected during TD-GCMS analysis of the urban and rural PM samples extracted via MSE and SDE; error bars represent 99% confidence intervals.

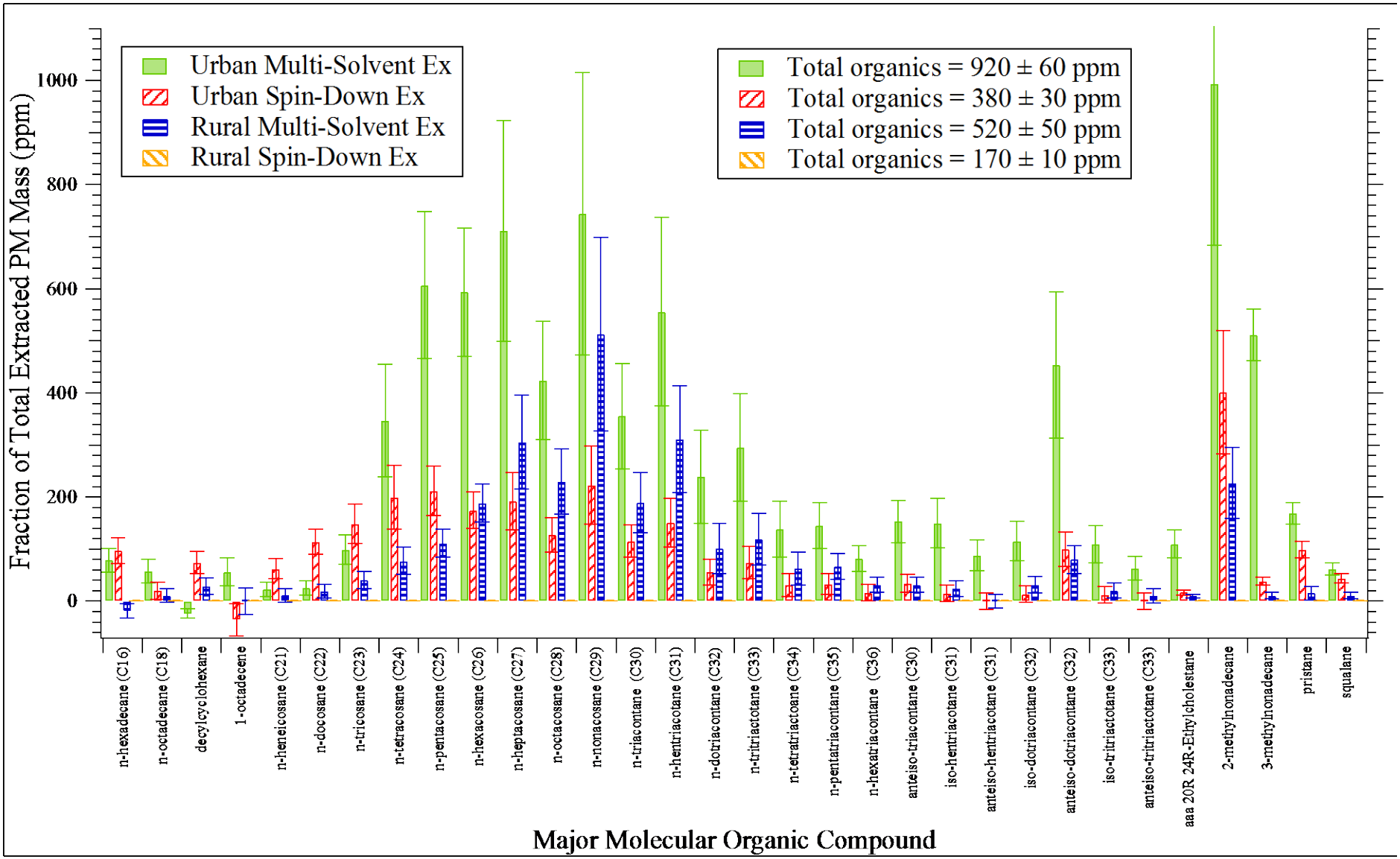


Figure 5a. Fraction of total extracted PM mass accounted for by major molecular organic compounds detected during TD-GCMS analysis of the urban and rural PM samples extracted via MSE and SDE; error bars represent 99% confidence intervals.

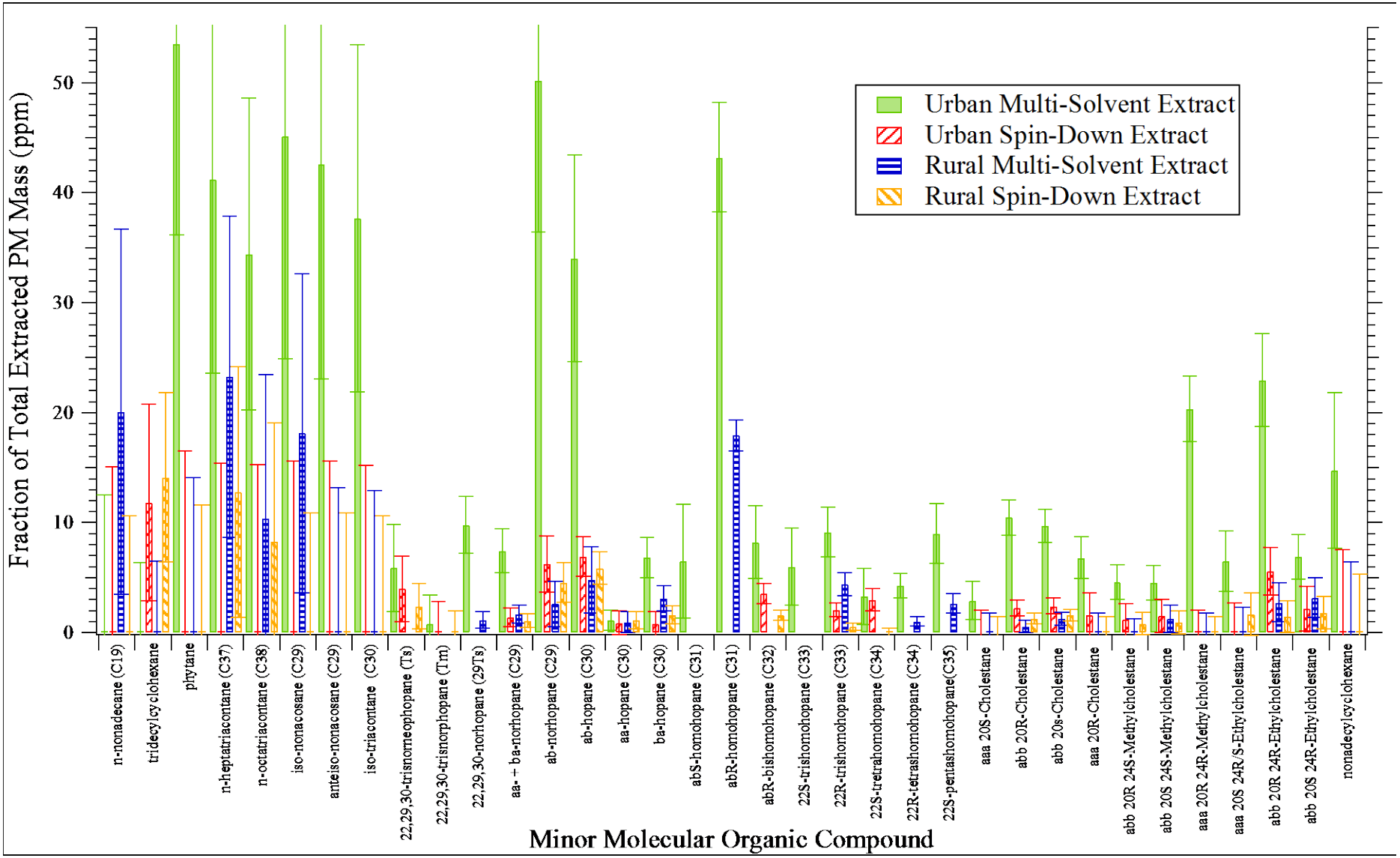


Figure 5b. Fraction of total extracted PM mass accounted for by minor molecular organic compounds detected during TD-GCMS analysis of the urban and rural PM samples extracted via MSE and SDE; error bars represent 99% confidence intervals.

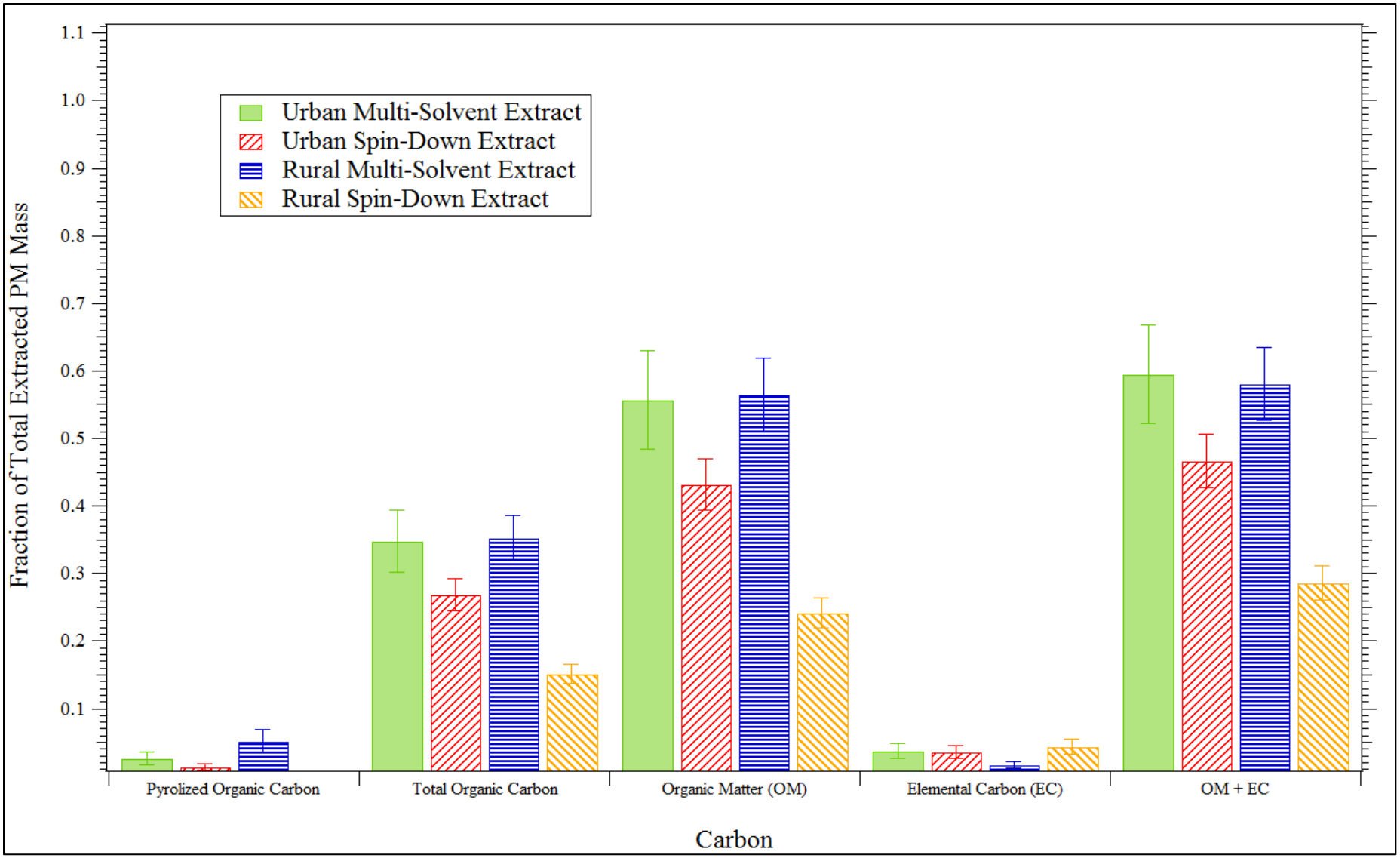


Figure 6. Fraction of total extracted PM mass accounted for by various carbon fractions determined from TOR measurements of the urban and rural PM samples extracted via MSE and SDE; error bars represent 99% confidence intervals.

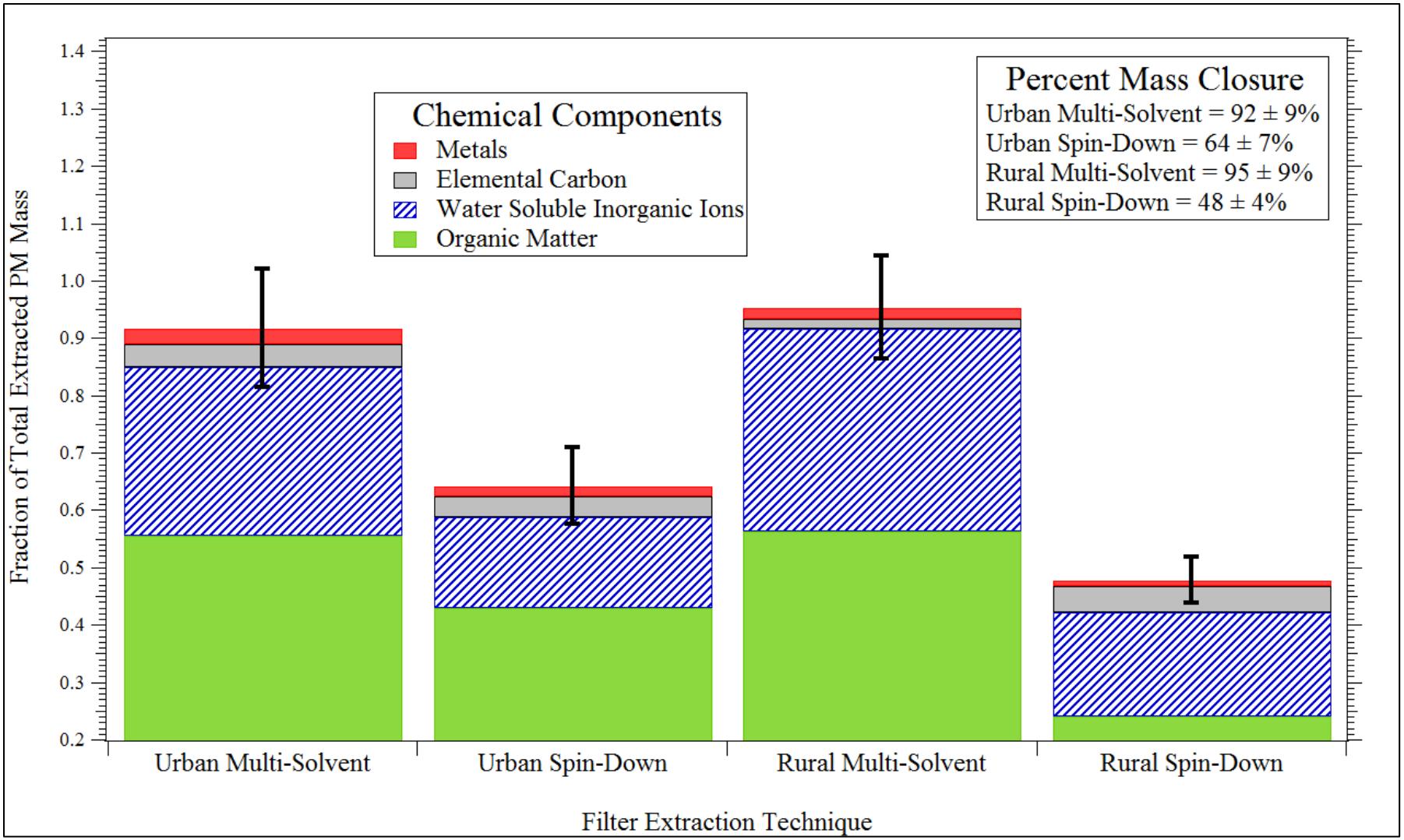


Figure 7. Fractional distribution of total extracted PM mass among the chemical components measured for the rural and urban PM samples extracted via MSE and SDE; error bars represent 99% confidence intervals for the component sum.

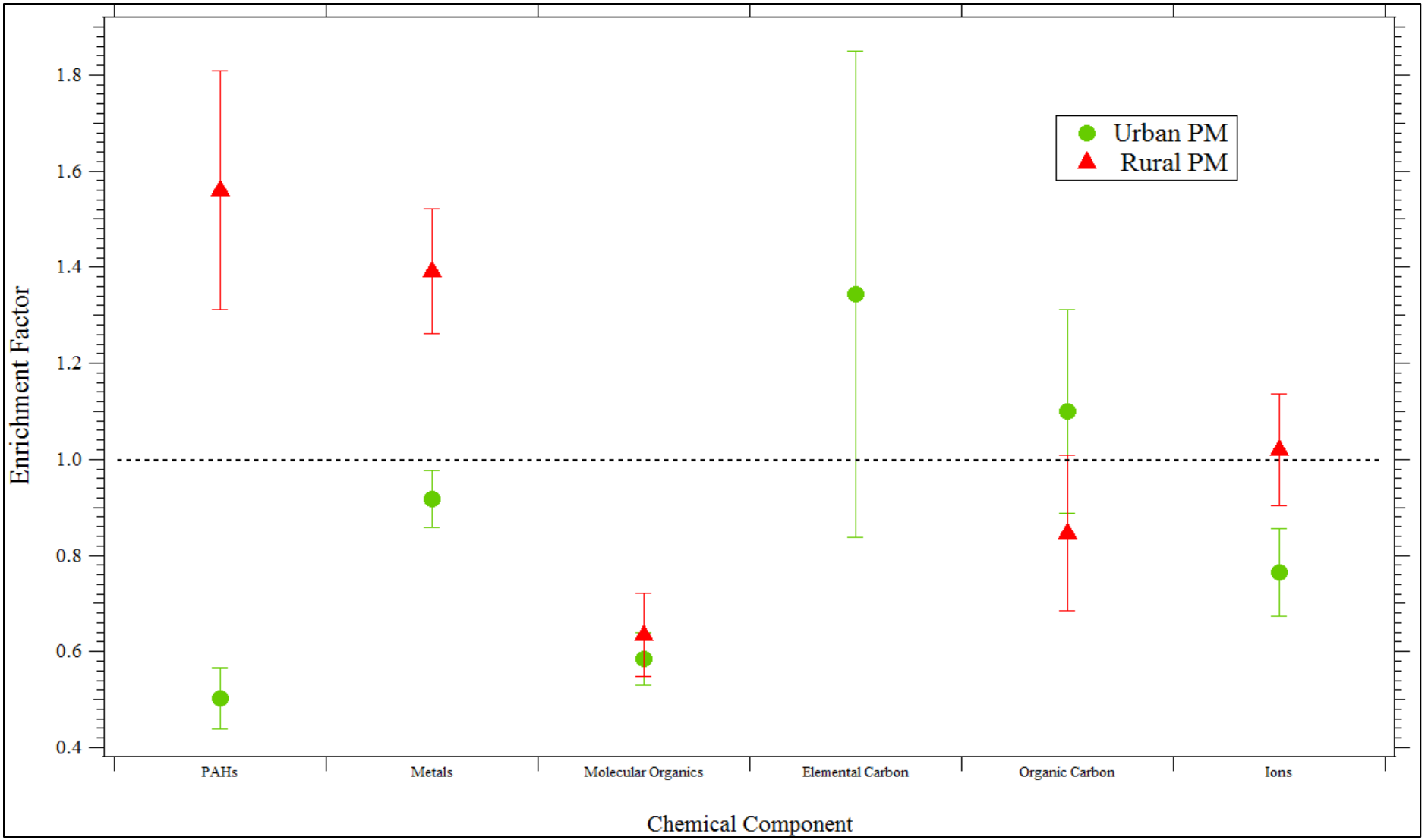


Figure 8. Enrichment factors for the various chemical components in the SDE extracted urban and rural PM samples relative to the MSE samples after adjusting the SDE data for the true mass of extracted PM; see text for discussion.

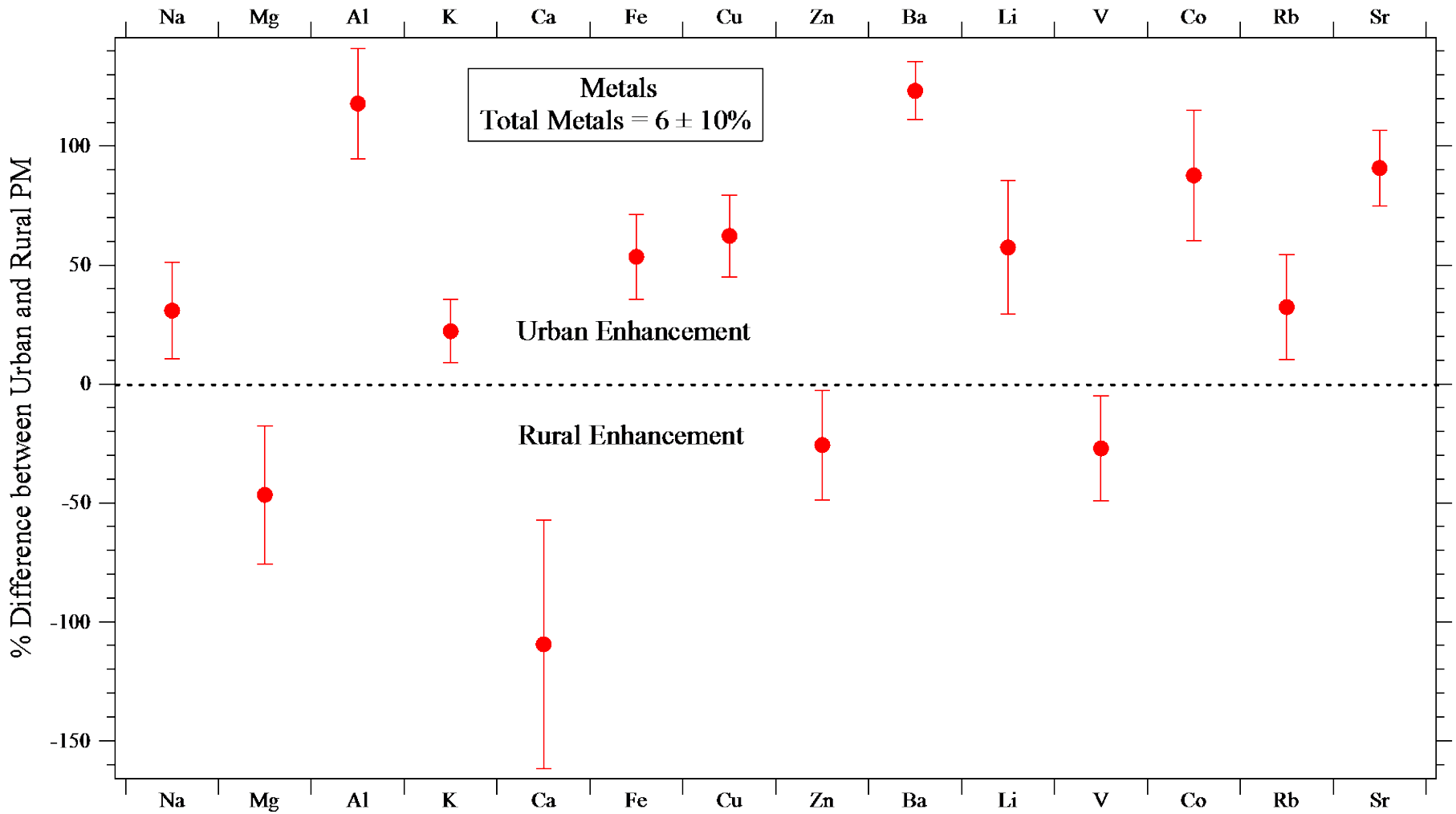


Figure 9. Percent difference between the urban and rural PM samples for trace metals determined via statistical analyses of ICP-MS data; see text for definition of percent difference used in this analysis. Only those metals showing statistically significant differences with p-values < 0.05 are included in the figure. The error bars represent 99% confidence intervals.

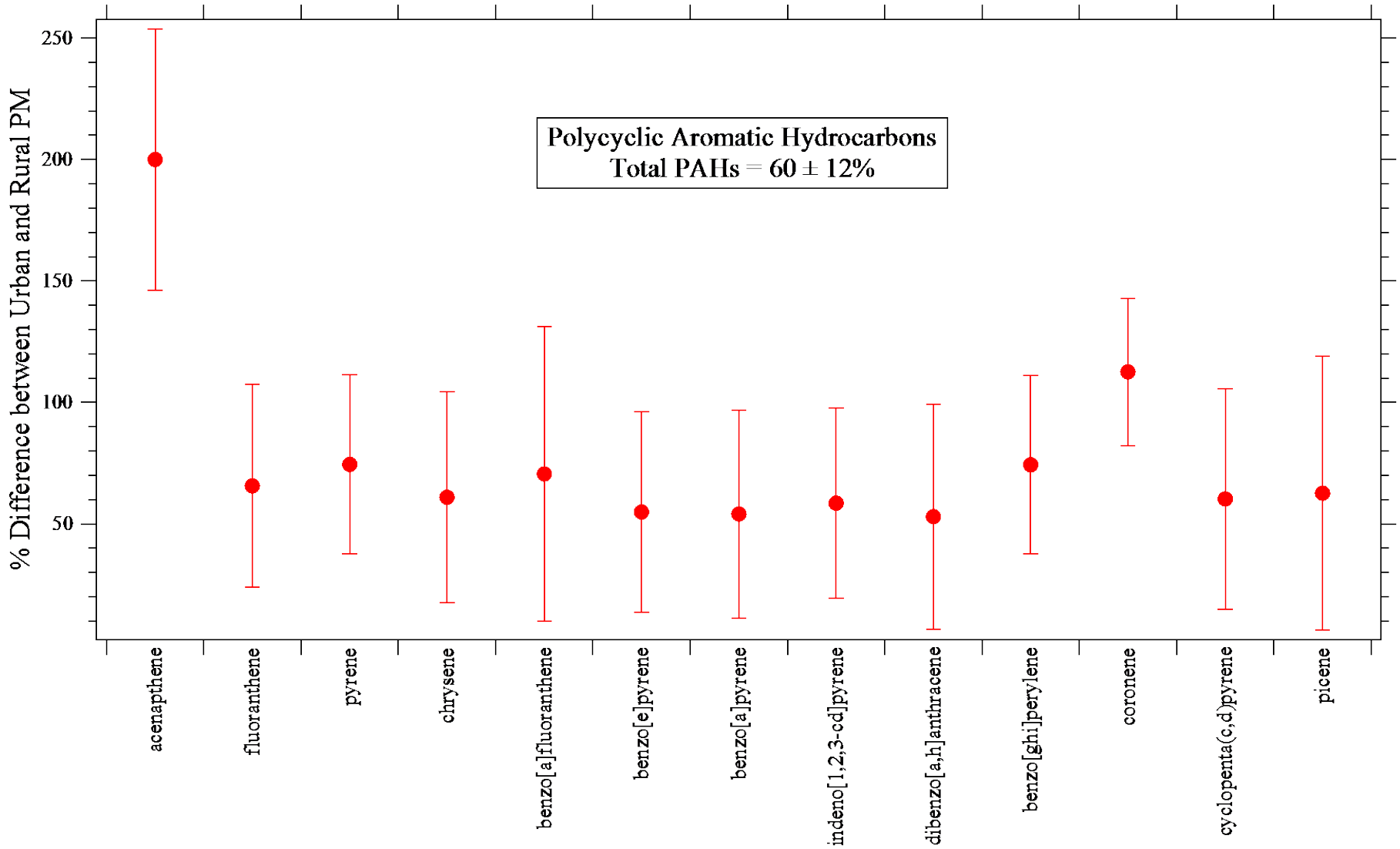


Figure 10. Percent difference between the urban and rural PM samples for polycyclic aromatic hydrocarbons (PAHs) determined via statistical analyses of TD-GCMS data; see text for definition of percent difference used in this analysis. Only those PAHs showing statistically significant differences with p-values < 0.05 are included in the figure. The error bars represent 99% confidence intervals.

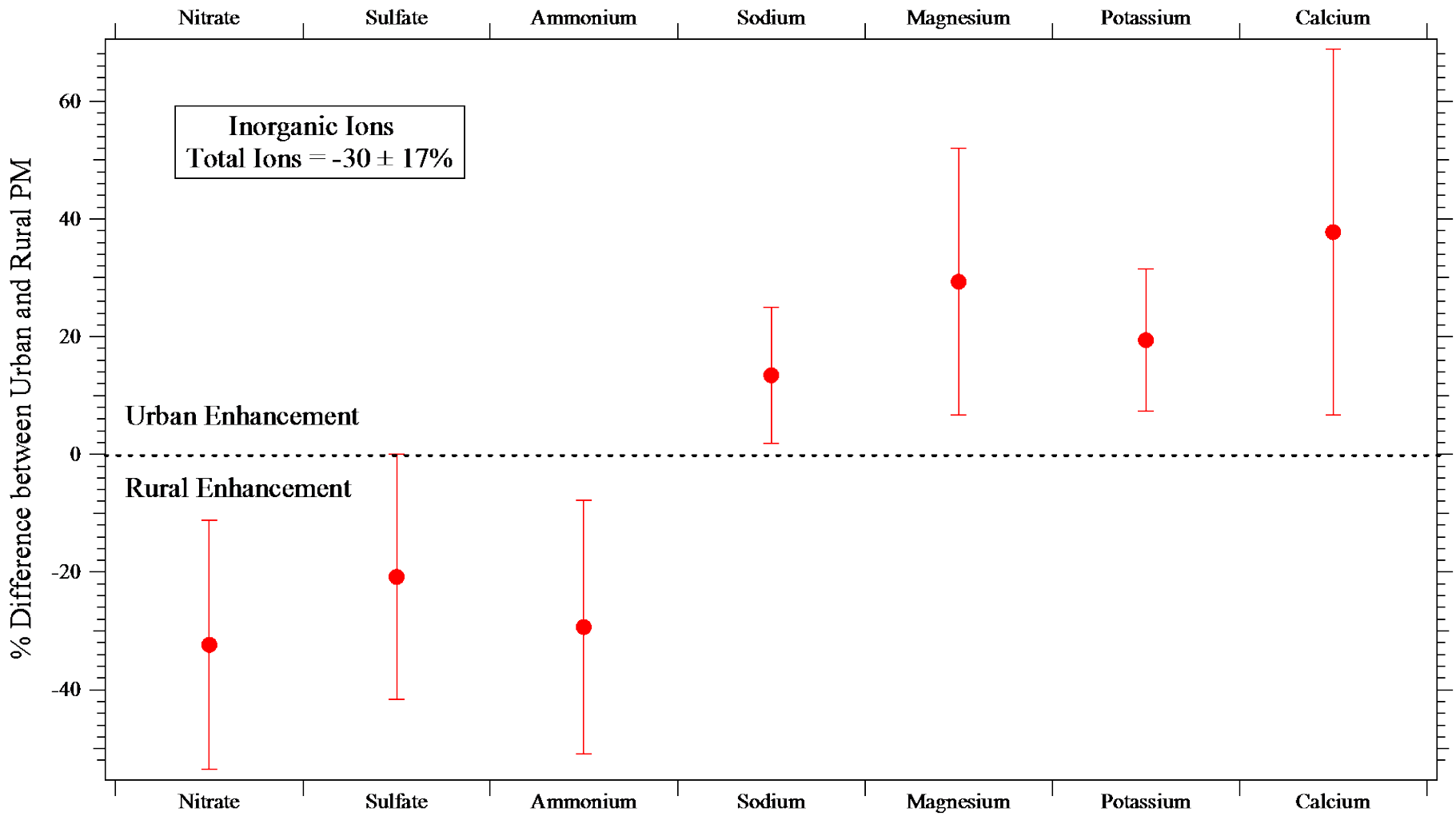


Figure 11. Percent difference between the urban and rural PM samples for inorganic ions determined via statistical analyses of IC, AC and AAS data; see text for definition of percent difference used in this analysis. Only those ions showing statistically significant differences with p-values < 0.05 are included in the figure. The error bars represent 99% confidence intervals.

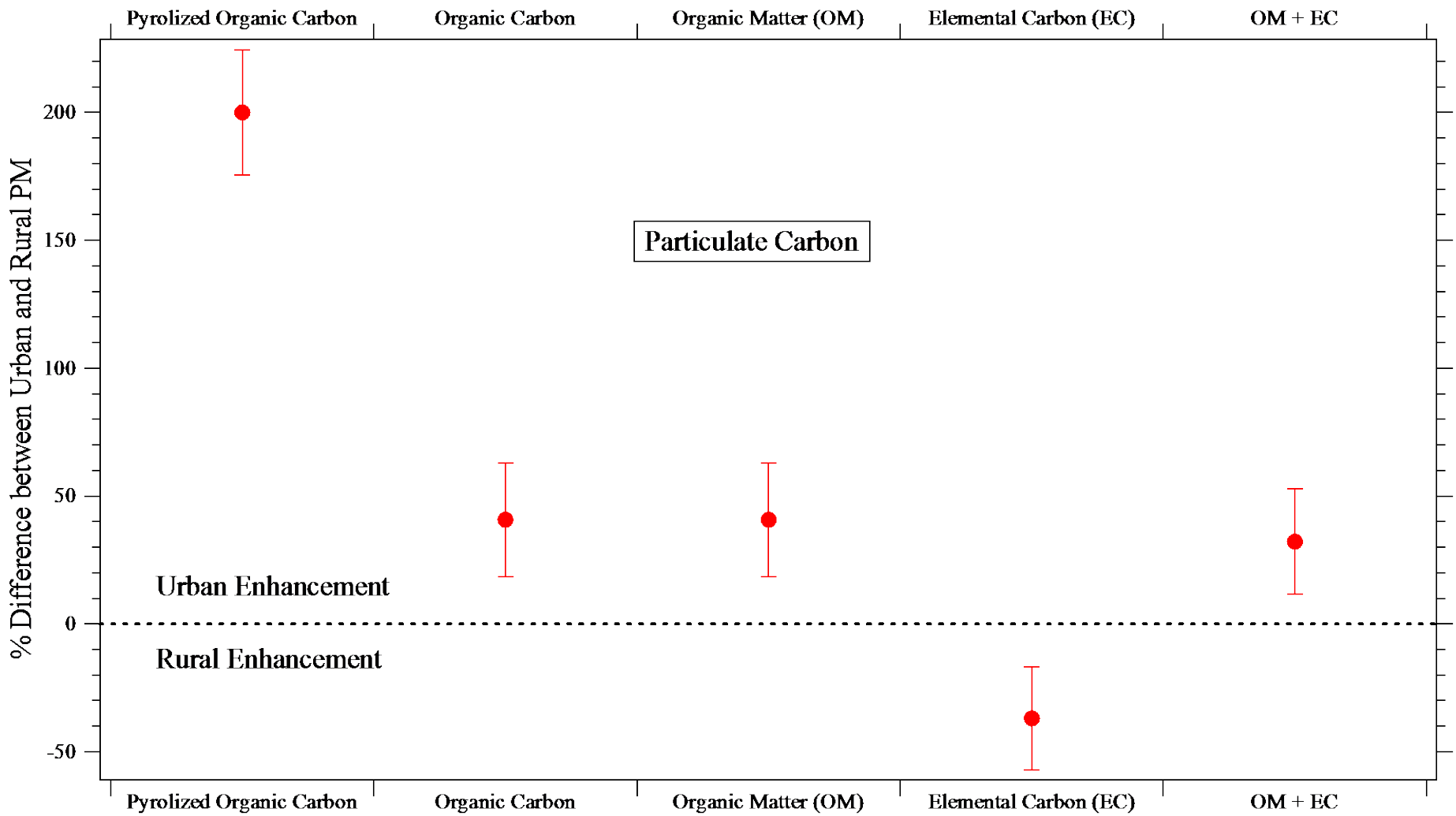


Figure 12. Percent difference between the urban and rural PM samples for particulate carbon determined via statistical analyses of TOR data; see text for definition of percent difference used in this analysis. Only those components showing statistically significant differences with p-values < 0.05 are included in the figure. The error bars represent 99% confidence intervals.

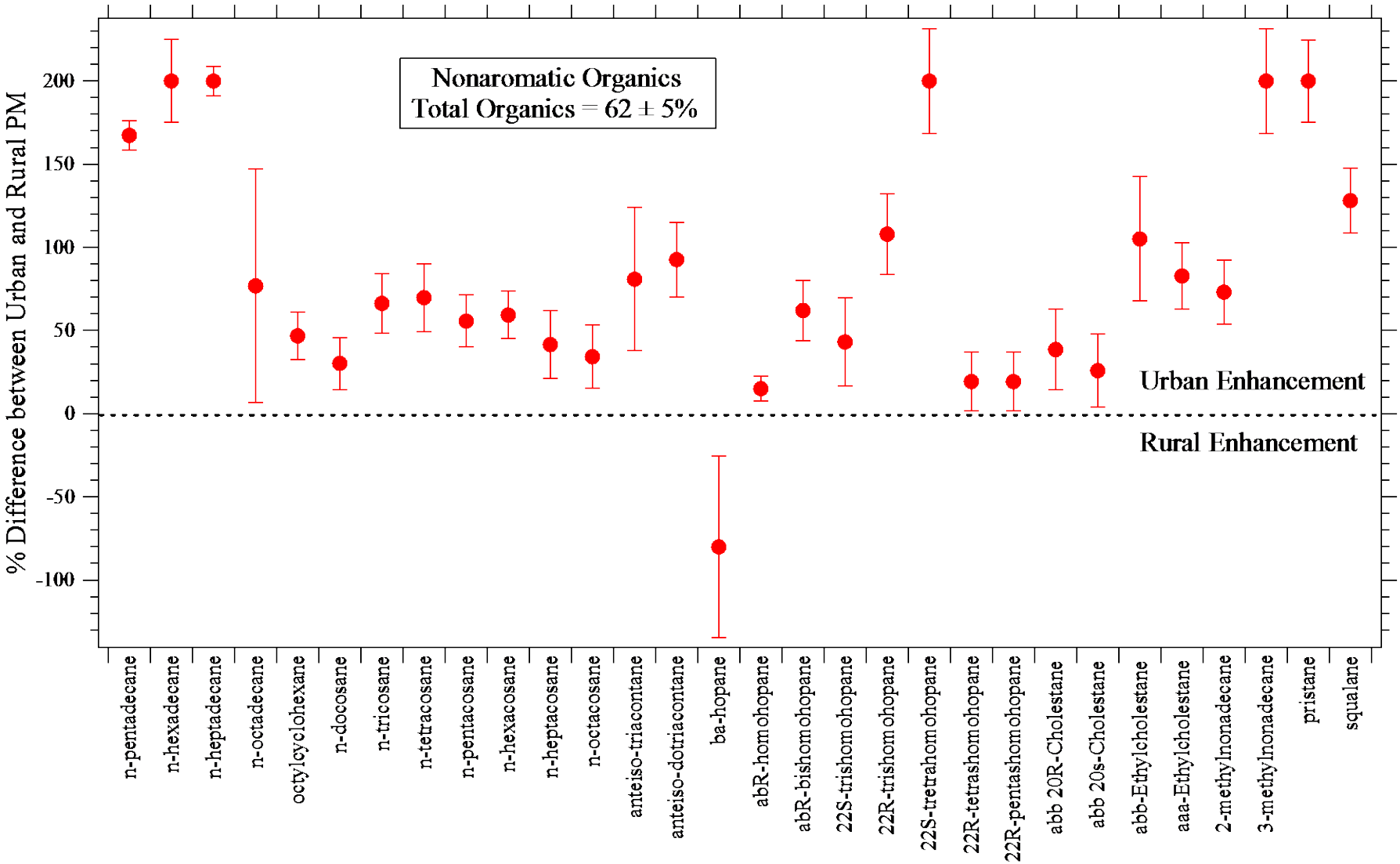


Figure 13. Percent difference between the urban and rural PM samples for nonaromatic organic compounds determined via statistical analyses of TD-GCMS data; see text for definition of percent difference used in this analysis. Only those organics showing statistically significant differences with p-values < 0.05 are included in the figure. The error bars represent 99% confidence intervals.

3. Animal Exposure Studies

Following a full characterization of the particle extract composition, as detailed in the previous section, we conducted two studies of the bioactivity of the extracts. The first study (called "dose response") was a dose response characterization of the Urban PM extracts with the rationale that we needed to select a dose that would give a moderate response so that urban and rural could be readily compared. With this study we also wanted to compare the acute effects of the two particle extract preparation methods. Once this study was completed we selected a dose and a particle type for the second study ("time delay"). The rationale for this study was to increase our understanding of the temporal pattern of response in both the pulmonary and vascular system following a single acute exposure for the reasons delineated in Section 1. The results of the dose response study are discussed first *en toto* and this is followed by discussion of the time delay data in a subsequent section. For all exposures, PM and filter blank extracts were resuspended in PBS, sonicated and administered via oropharyngeal aspiration. Control mice were treated with 50µl suspensions of the filter blank extracts and treated mice were given 50µl suspensions of the PM extracts.

3.1. Animal Exposure Methods

Dose Response: Eight week reproductively capable adult male BALB/c mice (Harlan Laboratories, Hayward, CA) were allowed to acclimate in filtered air (FA) for 7 days. BALB/c mice were selected because they have robust inflammatory responses to environmental exposures and are useful for studies involving respiratory challenges. Mice were provided with Laboratory Rodent Diet (Purina Mills, St. Louis, MO) and water ad libitum. All animal procedures followed approved institutional animal care and use protocols. Animals were exposed to the same volume of particle extracts (50 µL) via oropharyngeal aspiration under light 2.5% isoflurane anesthesia (De Vooght et al. 2009). Instillation has been used for many years to expose animals to PM and is the only way to perform controlled, dose-response exposures required for this study. Six mice were used for each time point and exposure dose (N=6 per group). Different doses of the extract were used with the goal of identifying equivalent dosing regimens for the two extracts in relation to neutrophil recruitment to the bronchoalveolar lavage fluid, BALF. For comparison of sample preparation methods, doses were chosen to be 5.5, 27.5 and 55 µg of the spin-down extract and 10, 50 and 100 µg of the multi-solvent extract to yield similar dose response profiles of % neutrophils as an indicator of acute inflammation.

Time-Delay: Eight week reproductively capable adult male BALB/c mice (Harlan Laboratories, Hayward, CA) were allowed to acclimate in filtered air (FA) for 7 days. Mice were provided with Laboratory Rodent Diet (Purina Mills, St. Louis, MO) and water ad libitum. All animal procedures followed approved institutional animal care and use protocols. Animals were exposed to the same volume of particle extracts (50 µL) via oropharyngeal aspiration under light 2.5% isoflurane anesthesia (De Vooght et al. 2009). Six mice were used for each time point and exposure dose (N=6 per group). Both Davis and Sac PM were given at the same dose (50ug) with the particles extracted using the spin-down extraction method to optimize particle potency for endpoints involved with inflammation. Animals were necropsied at 1, 2 and 4 days after exposure. Sham extracted filter blanks run as controls at all time points.

Necropsy protocol: All animal experiments were performed under protocols approved by the University of California Davis IACUC (Institutional Animal Care and Use Committee) in accordance with NIH guidelines. All animals were euthanized through intraperitoneal injection of an overdose of pentobarbital (150 mg/kg). At necropsy, tracheas were cannulated, the thorax was opened and lung removed *en bloc* for processing. Other procedures were as described in the following section.

3.2 Toxicological Assay Methods

3.2.1. BALF Differentials and Total Protein

The left lobe was tied off and only the right lung lobes were lavaged with approximately 0.6-0.7 mL of 0.9% sterile saline 2 times. The resultant bronchoalveolar lavage fluid (BALF) was collected into 5 mL round bottom tubes and kept on ice. BALF was centrifuged at 2000 rpm at 4°C for 10 minutes to pellet cells. The BALF supernatant was transferred to a 1.5 mL microfuge tube for total protein determination. The cell pellet was resuspended in 0.5ml sterile 0.9% saline. Viable and total leukocyte counts were performed using Tyrpan Blue exclusion assay. A cytospin slide was prepared from the resuspended cell pellet. Slides were fixed with methanol and stained with DiffKwik Differential Stain kit (Mastertech, Lodi, CA). BALF cell profile was determined by counting 500 cells per animal using a light microscope. Total protein in the BALF was determined using a Bradford protein assay (Biorad) performed in a standard 96 well plate using manufacturer's protocol. All samples were assayed in triplicate. The plate was read in on a SpectraMax plate reader (Molecular Devices).

3.2.2. Quantitatitve RT-PCR

Dose response: Lung compartmental RNA was isolated from microdissected intrapulmonary airways and surrounding parenchymal tissue from RNAlater (Ambion, Austin, TX) stabilized lung tissue using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) as previously described (Baker et al. 2004). RNA purity was confirmed through spectrophotometric absorbance at 260/280 nm. Quantification of CYP1A1 (Mm00487218_m1), CYP1B1 (Mm00487229_m1), IL1B (Mm00434228_m1), and the reference gene RPL13A (Mm01612987_g1) in the airway and parenchymal compartments were performed using inventoried Taqman probes and primers (Applied Biosystems, Foster City, CA) as previously described (Baker et al. 2004; Stelck et al. 2005). CYP1A1 and CYP1B1 are genes that code for cytochrome P450 monooxygenase enzymes that are linked to metabolism of chemicals. IL-1B is a gene that codes for IL-1B precursor protein that is cleaved by caspase 1 to form the mature cytokine IL-1B. This cytokine is an important part of the inflammatory response and is also involved in cell proliferation, differentiation and apoptosis. IL-1B is also a component of autoimmunity cascades involving the NLRP3 receptor and inflammasomes. Results were calculated using the comparative Ct method (Livak and Schmittgen 2001). Results are expressed as a fold change in gene expression relative to sham (filter extract exposed) animals.

Time-Delay Lung Inflammation and Oxidant/Antioxidant Gene expression: Lung compartmental RNA was isolated from microdissected intrapulmonary airways and surrounding parenchymal tissue from RNAlater (Ambion, Austin, TX) stabilized lung tissue using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) as previously described (Baker et al. 2004). RNA purity was confirmed using spectrophotometric absorbance at 260/280 nm and the reference gene RPL13A in the airway and parenchymal compartments were performed using inventoried Taqman probes and primers (Applied Biosystems, Foster City, CA) as previously described (Baker et al. 2004; Stelck et al. 2005). Results are expressed as a fold change in gene expression relative to filtered air exposed animals of the same age, unless otherwise stated. The following genes were analyzed using qRT-PCR on lung parenchymal tissue because they are part of cytokine or growth factor mediated inflammatory signaling cascades : CCL11, Ccl20, Csf2, Cxcl1, Cxcl2, Cxcl9, Cybb, IL10, IL12b, IL13, IL1b, IL 4, INFg, PDGFb, Sele, TNF. Genes analyzed for oxidant/antioxidant effects were CYP1A1, CYP1B1, CYP2F2, GCLC, GCLM, GSTa, GSTm1, GSTp1, Hmox1, and Prdx6.

3.2.3. Immunohistochemistry

Paraffin sections from the left lung lobe of 3 mice per treatment group were immunostained for rabbit anti CYP1A1 antibody (Xenotech) and rabbit anti CYP1B1 as described (Chan et al. 2013). An avidin-biotin peroxidase (Vectastain ABC, Vector Laboratories, Burlingame CA) kit was used to detect primary antibody binding sites. Nickel–enhanced 3’,3’-diaminobenzidine tetrahydrochloride (Sigma Chemical, St Louis Mo) was used as the chromagen. Controls included the substitution of primary antibody with PBS. A series of dilutions were used to determine optimal antibody concentration. Sections from all groups were run together to minimize run to run variability.

3.2.4. Histologic Evaluation

Histopathologic evaluation of lung lesions was done on two hematoxylin and eosin-stained sections of lung representing short and long axial pathways of the left lung lobe from each animal in each treatment group. Slides were evaluated in random order without knowledge of treatment group assignment. All terminal bronchiole-alveolar duct junctions in each section were examined and a subjective severity score assigned relative to airway epithelial changes, inflammation in alveolar ducts and surrounding parenchyma and periarteriolar inflammation and medial hypertrophy in the terminal branches of the pulmonary artery. Similarly, an overall severity score was assigned to each specimen. Lesions were scored from 0-4 with 4 being the most severe changes. Results were tabulated and average group scores calculated with statistical analysis by the Kruskal Wallace non-parametric ranking test with a $p<.05$ significance level. A similar approach was used in the time course study, subjective scores for the extent of inflammation were determined for every terminal bronchiole in each section without knowledge of group assignment. An overall inflammation score was calculated as the average of individual terminal bronchiole (TB) scores. Each section was assigned an overall severity score relative to changes in airway epithelium, arteriolar inflammation and medial hypertropy and parenchymal inflammation. All filtered extract-treated animals were combined for statistical analysis (Kruskal Wallace non parametric test).

3.2.5. Platelet alpha granule proteins and integrins

Mouse platelet activation was analyzed in whole blood by flow cytometry using the following anti-mouse antibodies, along with appropriate isotypes for each: a biotin conjugated monoclonal antibody to the a_{2b} subunit for the major platelet integrin $a_{2b}b_{3a}$ (CD41, BD Pharmingen) followed by Streptavidin Alexa 633 (in Vitrogen), a FITC-conjugated monoclonal antibody to P-selectin for alpha granule secretion (CD62P, BD Pharmingen), and a PE-conjugated monoclonal antibody to LAMP-1 for lysosomal granule secretion (CD107a, eBioscience). A resting (unstimulated) sample and samples stimulated with either 10mM ADP or 0.1U/ml thrombin were examined for each animal. After stimulation of whole blood, platelets were labeled with the preceding antibodies for one hour and fixed in 1% (final) paraformaldehyde prior to analysis by flow cytometry (FC500, Beckman-Coulter, Miami, FL). Platelets were defined by forward scatter characteristics (FSC), and side scatter characteristics (SSC) and ten thousand events were collected within the platelet gate for each animal and each condition.

3.2.6 Bioplex analysis of lung and serum cytokines (Time-Delay study only)

Serum and lung cytokine protein assays were done on a subset of 3 animals per each time point (one and four days) and treatment. A total of 32 cytokines were assayed with a fluorescent bead based conjugated antibody assay (Bioplex, Biorad) using standard mouse cytokine kits (mouse 9plex and mouse 32plex cytokine assay). These assays included probes for IL-1a, IL1-b, IL-5, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IL-18, Eotaxin, G-CSF, GM-CSF, IFN-g KC, MCP-1, MIP-1a, MIP-1b, Rantes, TNFa, bFGF LIF M-CSF MIG MIP-2 PDGF and VEGF. Significant differences between groups were assayed by ANOVA.

3.2.7. Statistics

All data are reported as mean ± standard error of the mean (SEM) unless otherwise stated. Statistical outliers were eliminated using the extreme studentized deviate method (Graphpad, La Jolla, CA). Within treatment group comparisons for continuous data were performed using a one-way ANOVA followed by PLSD (Protected Least Significant Difference) post hoc analysis using StatView (SAS, Cary, NC). Lesion scoring data was analyzed using a Kruskal-Wallis one way ANOVA test. P values of < 0.05 were considered statistically significant. Since the ANOVA was only done on the within type exposures (i.e. all Davis or all Sacramento), there were a small number of comparisons so the chance of a false negative due to multiple comparisons is small.

3.3 Dose-Response Results

3.3.1. Results

As expected, the multi-solvent extraction technique resulted in higher PAH content in the sample. The total PAH content of extracted PM was 330 +/- 30 and 120 +/- 10 ppm by mass for the multi-solvent and spin-down techniques, respectively.

The spin-down extract was approximately 2 fold more potent than the multi-solvent extract at both induction of total cells into the BALF and increasing the neutrophil response (Figure 1A and B) with the mid and high dose of both preparations increasing neutrophils in the BALF. Notably the multi-solvent sham filter extract also significantly increased total cells in the BALF compared to the PBS control. The reason for this is not clear but may be related to persistence of glass microfibers in

the multi-solvent extracted samples. Lung injury was modest with only one dose (high dose) from each preparation inducing an increase of protein into the BALF (Figure 1C).

At 24 hrs after dosing, the spin-down preparation induced more apparent pathology at the terminal bronchiole alveolar duct junction at all doses tested (low, medium and high) compared to the multi-solvent extracted sample (Figure 2). Spin-down extract treated mice had bronchiolar epithelial hyperplasia, accumulation of neutrophils and macrophages in alveolar ducts and periarteriolar inflammation. Multi-solvent extract treated mice had less inflammation and fewer neutrophils. Arteriolar inflammation was minimal in multi-solvent extract treated mice. To quantify these changes, pathology scoring was utilized (Figure 3). Overall lesion scores indicated a dose responsive severity of injury in the spin-down extract exposed group. The lesion scores for the multi-solvent extract exposed group peaked at the mid dose and were approximately half as severe as the maximal response in the spin-down extract exposed group (Figure 3A). Notably the response in the spin-down extract exposed group involved fairly similar inflammation in the vessels, the parenchyma and the bronchioles, but the inflammation in the multi-solvent extract exposed groups was exhibited in the vessels and parenchyma and not the bronchioles (Figure 3B).

Since both preparations appeared to affect the inflammation patterns at the conducting airway level based on pathology, an additional study was performed to separate conducting airway responses from those of the whole lung. RNAlater™ preserved lung tissue (Baker et al. 2004) was microdissected to isolate the conducting airways. The remainder of the lung lobe (whole lung lobe) was analyzed intact for mRNA expression of genes involved in detoxification and toxification of PAHs. CYP1A1 (Figure 4A and B), CYP1B1 (Figure 4C and D) and IL-1B (Figure 4E and F) were examined. IL-1B, a gene associated with inflammation and macrophage activation, has been documented to be upregulated in previous studies of PAH containing PM (den Hartigh et al. 2010). Airway CYP1A1 mRNA was significantly decreased by exposure to the spin-down sample in the high dose only (Figure 4A). Within the lung lobe CYP1A1 was increased by the mid dose of the multi-solvent extract sample, but not the high dose (Figure 4B). CYP1B1 was increased in both the airways and the whole lung by the spin-down extract but only whole lung mRNA expression was increased by the multi-solvent extract (Figure 4C and D). Both sample preparations induced the expression of IL-1B (Figure 4E and F) but the spin-down extract was more potent, inducing an increase at the mid dose in whole lung (Figure 4F). Gene expression at a single time point gives only a partial picture of the temporal pattern of changes as well as the distribution and abundance of the corresponding proteins within the tissue itself. As a result, we also examined the abundance of two key P450s (CYP1A1 and CYP1B1) in the terminal bronchioles for the highest doses tested (Figure 5). CYP1A1 protein was induced in both of the extract preparations with the multi-solvent extract exposure exhibiting greater induction (Figure 5D). Increased expression of CYP1A1 was noted in both the epithelium of the terminal bronchiole as well as in interstitial macrophages. Conversely, exposure to both extracts reduced CYP1B1 protein expression (Figure 5 G and H) at 24 hrs after dosing.

Due to the apparent correlation of (some) gene expression responses with tissue scores for inflammation as well as increased neutrophils in the BALF, Pearson linear correlations between these endpoints were determined. Positive correlations were only noted for the spin-down samples. Airway IL-1B gene expression had a positive, and significant correlation with tissue inflammation (r=0.523165) and neutrophils in BALF (r=0.682310), but whole lung IL-1B gene expression only

had significant correlation with neutrophils in the BALF (0.607316). Interestingly, CYP1B1 gene expression in both the airway (r=0.464732) and the whole lung (r=0.560052) also correlated with neutrophils in BALF. A stronger correlation was found for CYP1B1 gene expression in the whole lung and the histology scores (r=0.689086).

The number of circulating platelets and blood hemoglobin content were measured (Figure S1). Hemoglobin content did not vary between groups and thus indicated no change in blood volume by group. The number of platelets in the blood was increased by exposure to the multi-solvent extract at all doses, although all values were within normal limits. In contrast, when platelet response to the potent physiological agonist thrombin was studied, the spin-down preparation was approximately twice as potent as the multi-solvent extract (Figure 6). Platelet activation can be measured by the surface expression of alpha (P-selectin) and lysosomal (LAMP-1) granule proteins, both of which are secreted during activation. However, it should be noted that platelets may secrete more of one type of granule than another. Spin-down extracts induced a strong response to thrombin stimulation, as evidenced by both the number of cells that were positive for the platelet alpha granule protein P-selectin (Figure 6A), and those positive for lysosomal granule membrane protein -1 (LAMP-1). (Figure 6B). Additionally, unstimulated platelets from animals given the spin-down extract had high levels of the major platelet integrin CD41b, as would be expected in a normal healthy population of cells (Figure 6E). The multi-solvent extract resulted in an inability of platelets to respond to physiological stimuli such as thrombin, either by the expression of P-selectin (Figure 6A) or LAMP-1 (Figure 6B); solvent-extracted PM did not result in this inability.

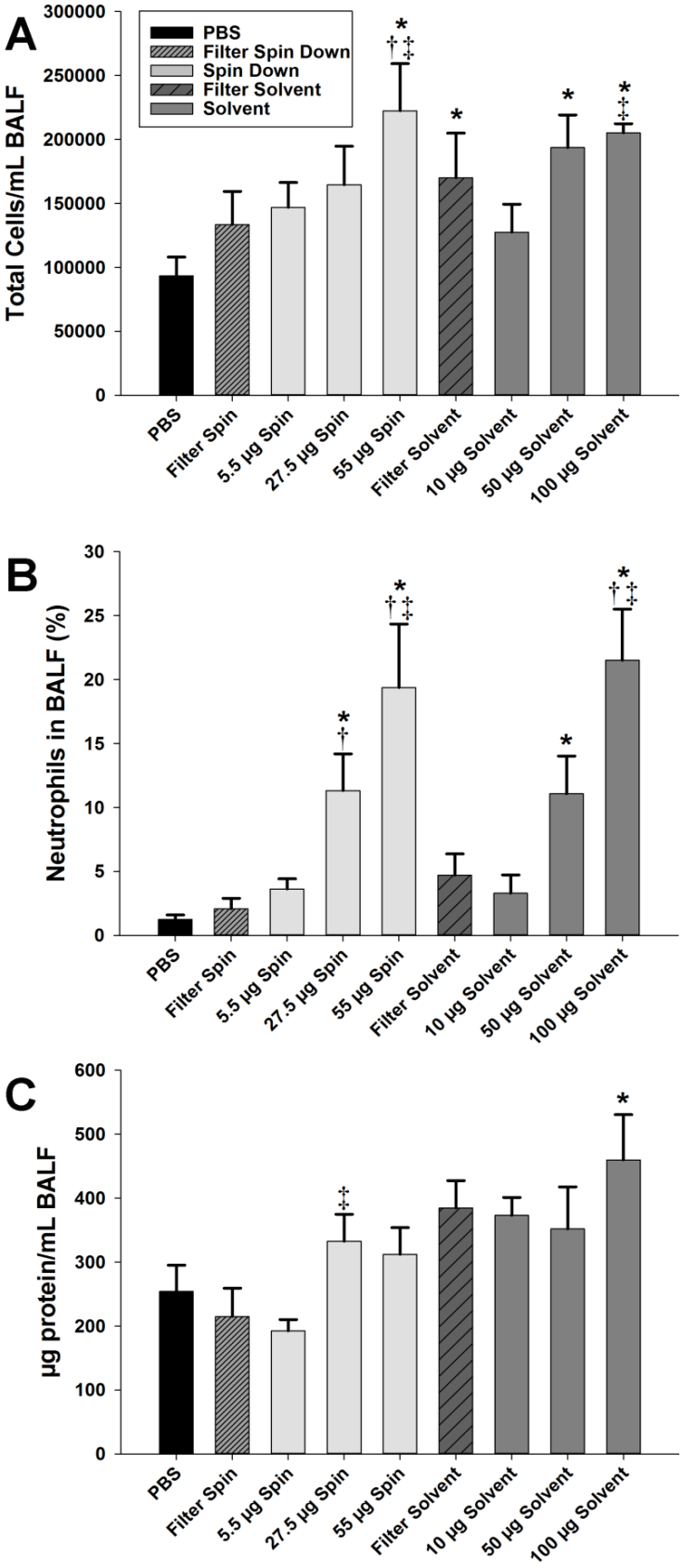
3.3.2. Figures

Figure 1: Bronchoalveolar lavage fluid was analyzed for total cellular infiltrates (A), percent of neutrophils (B) and total protein (C) as an indication of potential lung injury at 24 hours following SacPM administration. Two preparations were compared (spin-down "spin" vs multi-solvent "solvent") and the dose response that yielded equivalent neutrophil infiltration is shown. To examine whether there was a possible effect of the filter blank preparations (filter son and filter extract) an additional group was administered PBS as a control. N= 6/group

* significantly different from PBS control.

† significantly different from respective filter control.

‡ significantly different from lowest dose group of same treatment. P< 0.05.



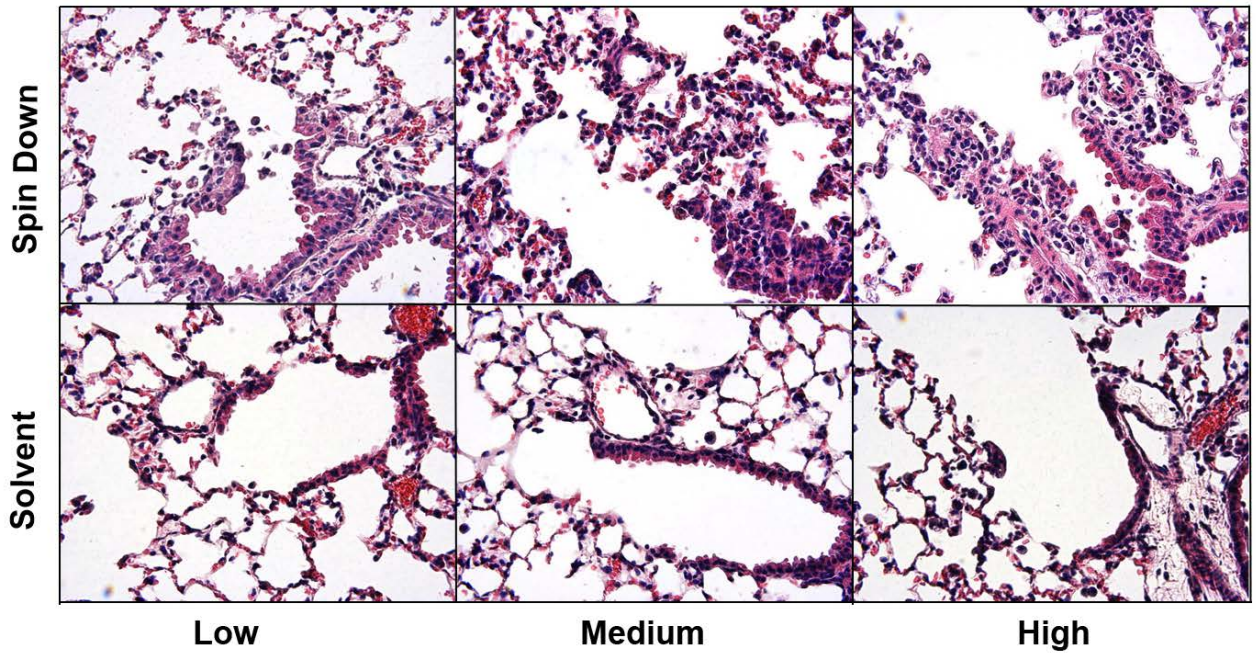


Figure 2: Lung lesions in mice given collected ambient particulate matter prepared by spin-down or multi-solvent extraction. PM was administered by oropharyngeal aspiration at doses determined to be low, medium or high based on induction of inflammatory cells in BALF. Multi-solvent extract PM treated mice were administered 25, 50 or 100 µg while spin-down treated mice were given 5.5, 27.5 and 55 µg. Spin-down treated mice had bronchiolar epithelial hyperplasia, accumulation of neutrophils and macrophages in alveolar ducts and periarteriolar inflammation. Mice treated with multi-solvent extracted PM had less inflammation, demonstrated by fewer neutrophils.

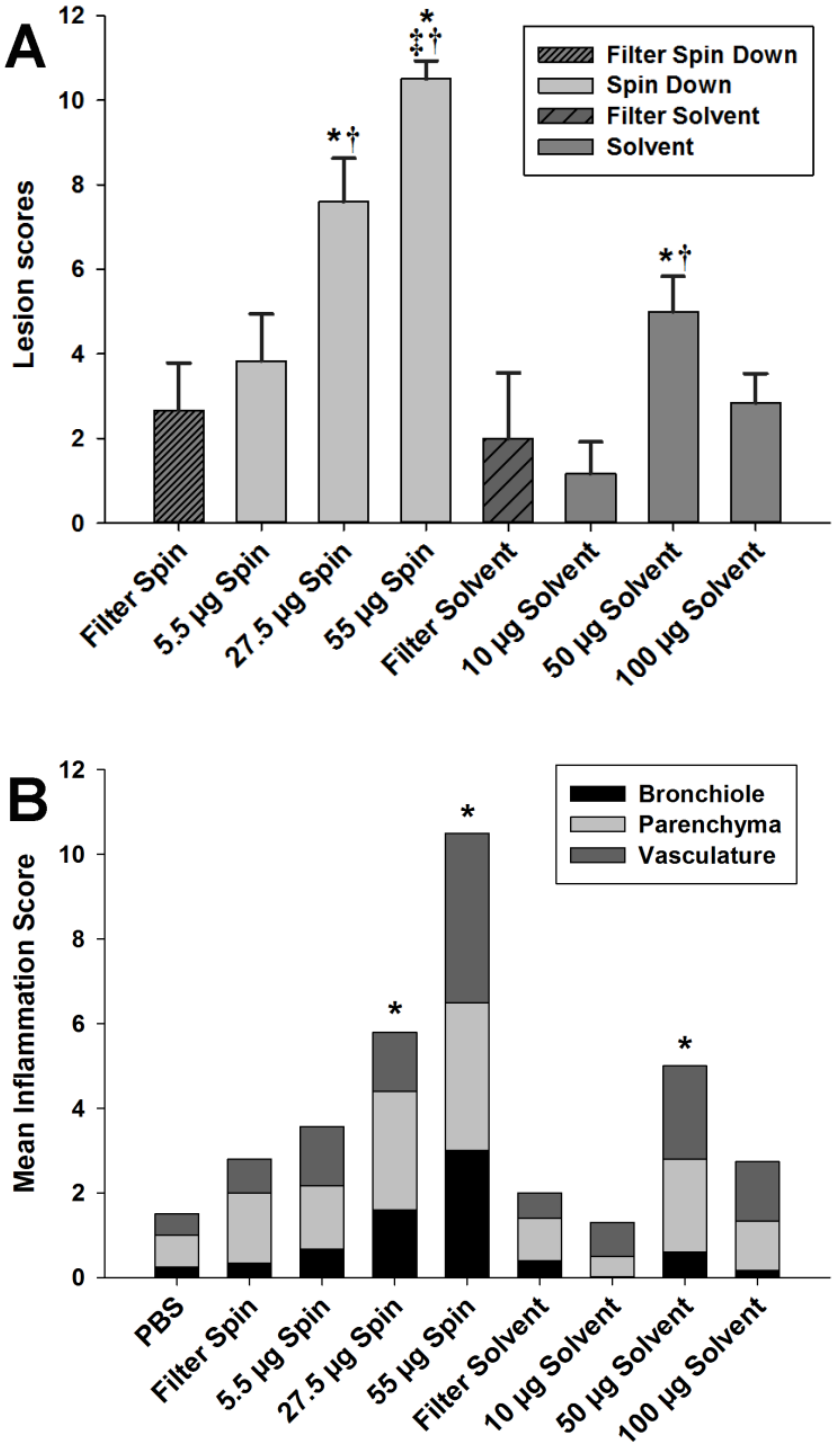


Figure 3: All terminal bronchiole-alveolar duct junctions in each section were examined and an overall subjective severity score assigned relative to airway epithelial changes, inflammation in alveolar ducts and surrounding parenchyma and periarteriolar inflammation and medial hypertrophy in the terminal branches of the pulmonary artery. Similarly, an overall severity score was assigned to each specimen. Lesions were scored from 0-4 with 4 being the most severe changes. Results

were tabulated and average group scores calculated with statistical analysis by the Kruskal-Wallace non-parametric ranking test with a $p<.05$ significance level. N= 6/group.

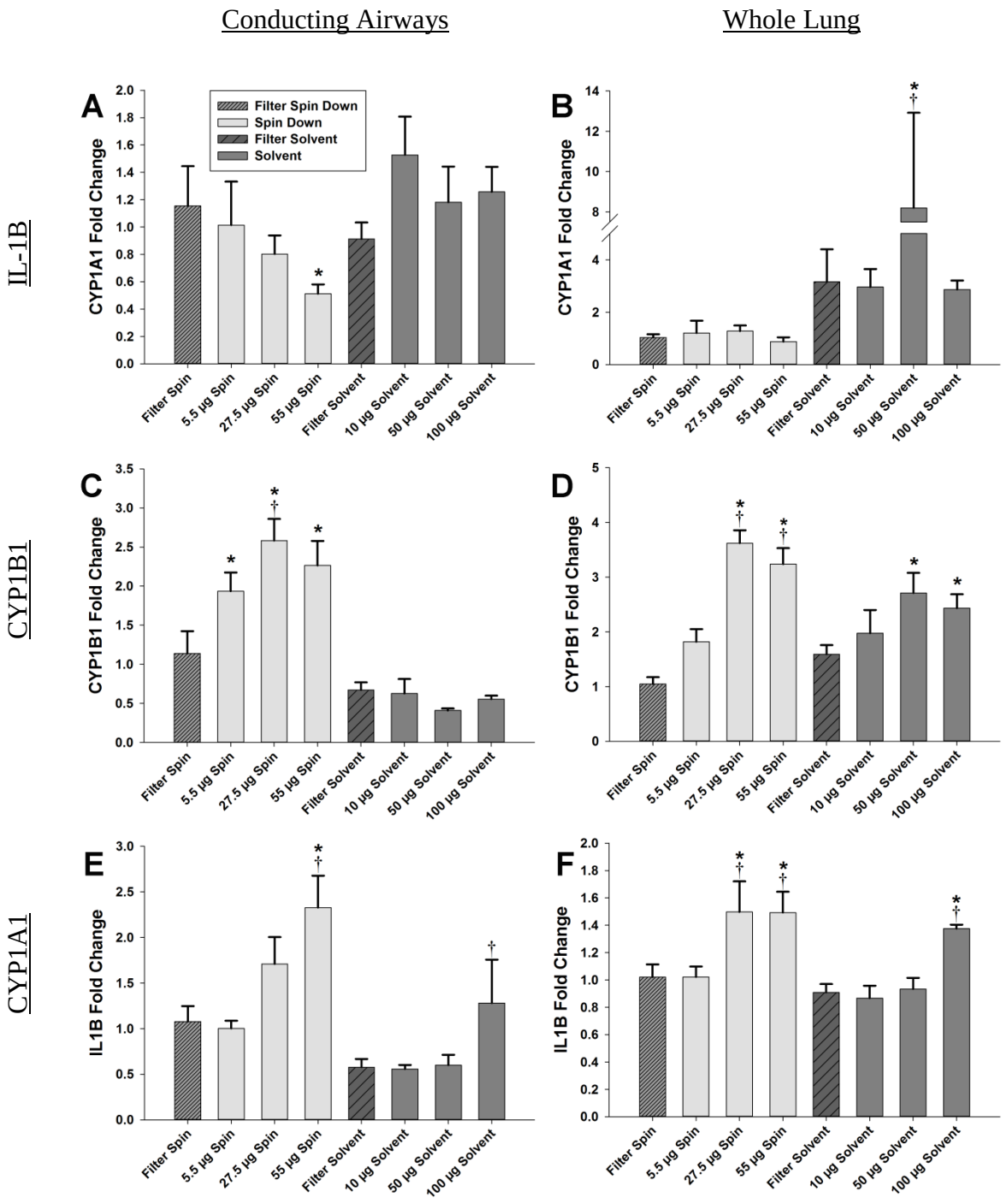


Figure 4: Fold change in gene expression for CYP1A1 (A and B), CYP1B1 (C and D) and IL-1B (E and F) in the conducting airways (A, C, E) and whole lung (B, D, F) 24 hours after exposure to SacPM. All fold change was calculated relative to the spin-down filter control after normalizing to RPL13A reference gene. N= 6/group * significantly different from respective filter control. † significantly different from lowest dose group of same treatment. P< 0.05

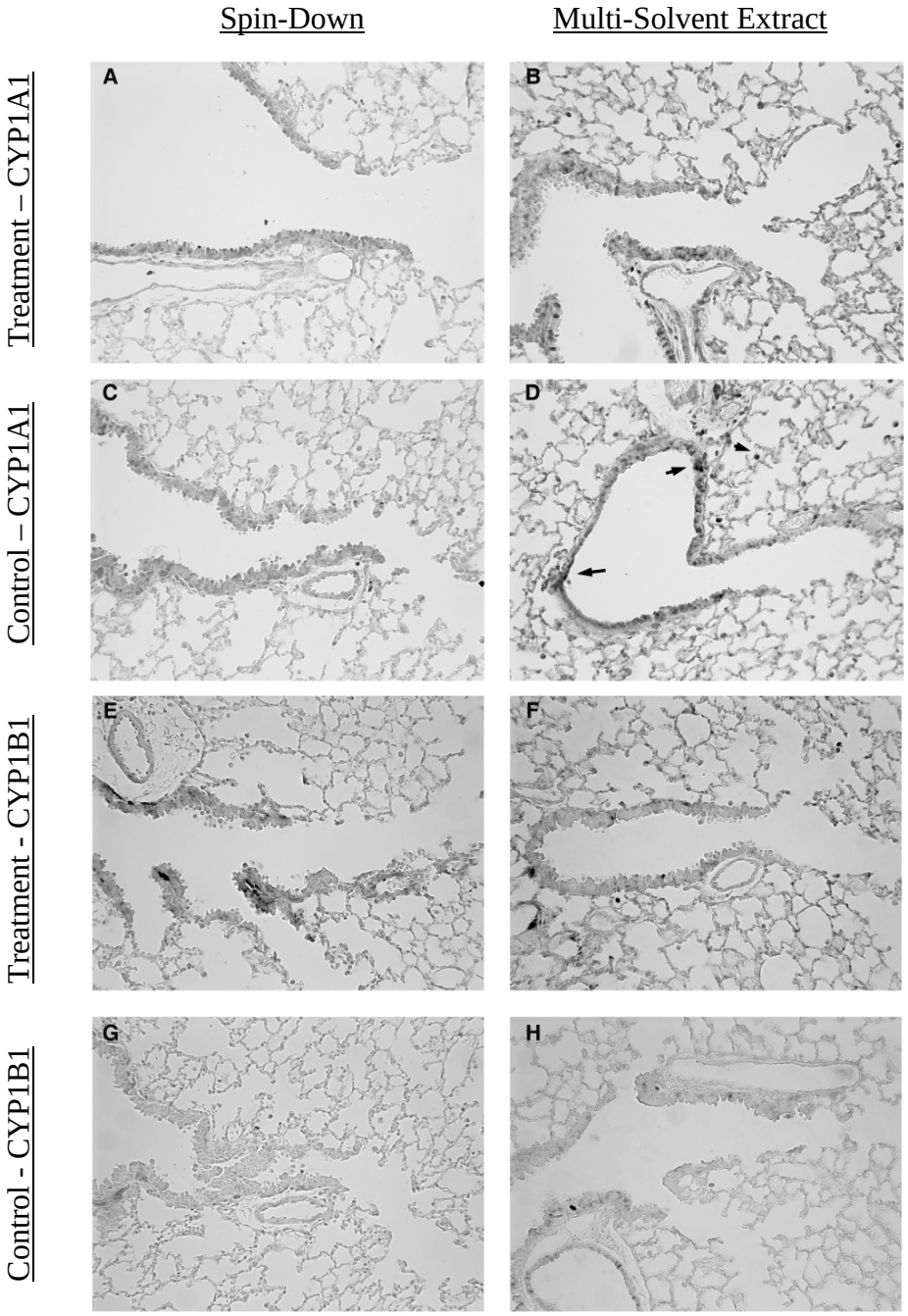


Figure 5 Immunohistochemical localization of CYP1A1 and CYP1B1 protein in the lungs 24 hrs following treatment with the high dose of SacPM. CYP1A1 expression in control mice treated with the spin-down filter control preparation (A) or the multi-solvent extract filter control (B). CYP1A1 expression in the airways treated with either the spin-down (C) or the multi-solvent extract (D). Arrows indicate focal regions of the airways with high levels of expression (D). CYP1B1 expression in control mice treated with the spin-down filter control preparation (E) or the multi-solvent extract filter control (F) or in the lungs of mice following treatment with either the spin-down (G) or the multi-solvent extract (H). CYP1A1 protein is induced in both of the extract preparations containing SacPM with the multi-solvent extract exposure exhibiting greater induction. Conversely, exposure to SacPM reduced CYP1B1 protein expression, regardless of extraction method. 3 mice/group were examined for immunohistochemical staining. Gene expression data is N= 6/group.

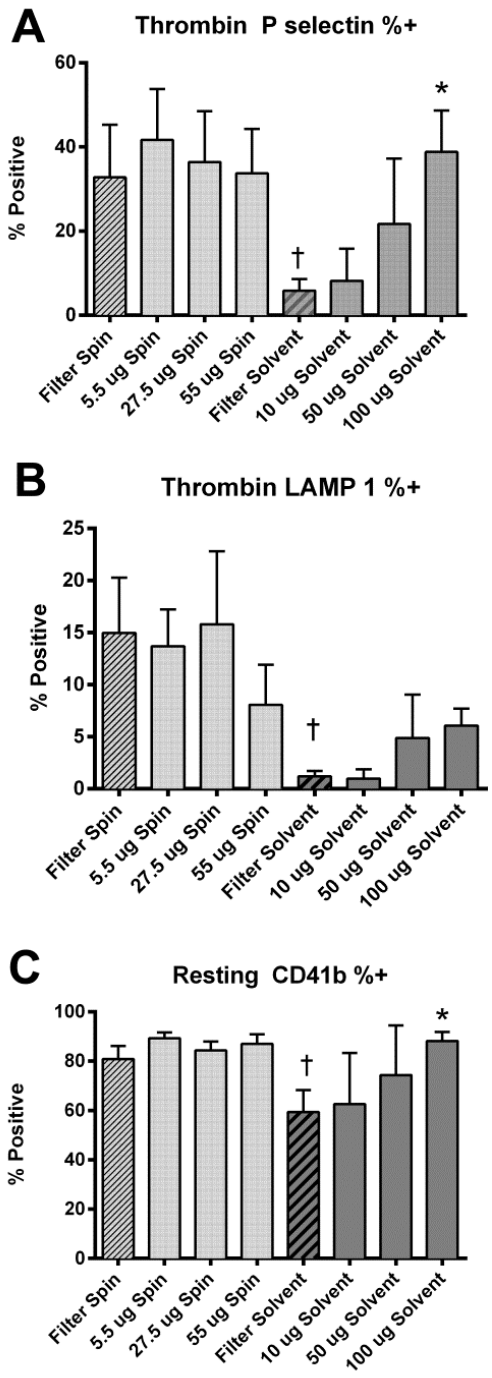


Figure 6 Flow cytometric analysis of expression of platelet alpha granule membrane protein P-selectin (A) and lysosomal granule protein (LAMP-1) (B) in platelets stimulated by the potent physiological agonist thrombin. Platelets from animals exposed to the multi-solvent extract are unable to respond to agonist stimulation by secretion of their lysosomal granules. There are high levels of expression of the integrin CD41b on the resting platelet surface from spin-down extracts, while it is significantly decreased in multi-solvent extracts (C). † significantly different from spin-

down filter control group. * significantly different from filter control group same extraction method. N= 6/group.

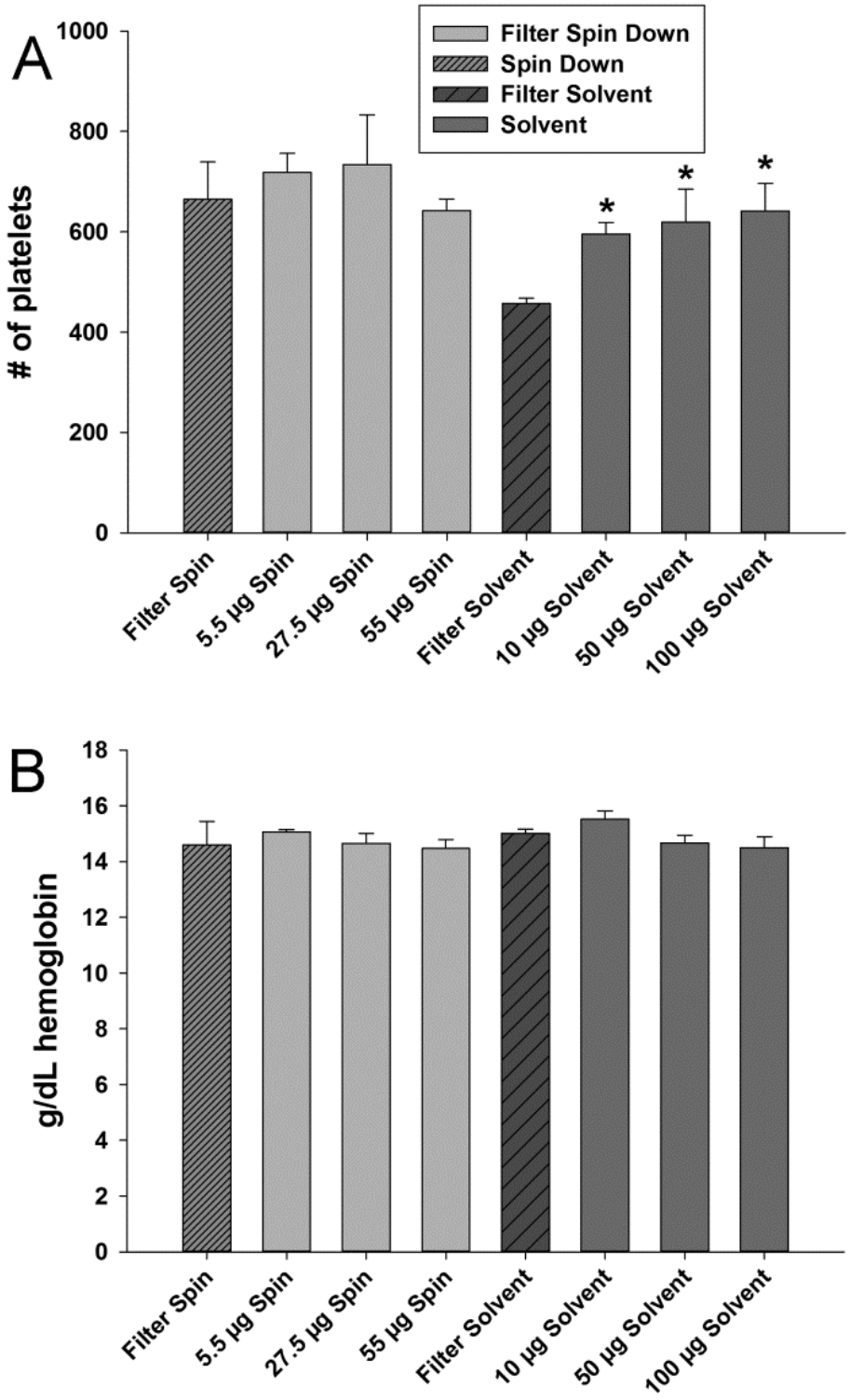


Figure S1: Blood values for platelet numbers (A) and hemoglobin levels (B) in mice 24 hrs after exposure to SacPM prepared either by spin down (spin) or multi-solvent (solvent) extraction. N=6/group. * = significantly different from filter control at $P<0.05$.

3.4 Dose-Response Conclusions

3.4.1. PM extraction and P450

In this study we have established that, for studies of $PM_{2.5}$ extracted from glass microfiber filters, the method of particle removal from the filters has a substantial and significant effect on biological response. This may be due to modification of inherent, or bulk, particle properties during the filter extraction process. Further, we show that application of two different filter extraction protocols also results in particles with a difference in potency, as measured by neutrophil recruitment potential. We normalized the doses based on degree of neutrophil infiltration into the BALF. We set the high doses for each extraction method so that the same level of neutrophil recruitment was attained for each extract at the high dose. The extraction method that was optimized to retain particle bound and solvent extractable nonvolatile PAHs (multi-solvent extraction), as well as particle mass from the filters, stimulated expression of CYP1A expression in the airways (at the mid dose) but did not increase CYP1A1 expression significantly in whole lung. On the other hand, inflammatory responses, including circulating platelet responses, which may be a key link between PM exposures and observed incidence of heart attacks and stroke, were greater in animals treated with the spin-down extract preparation.

CYP1A1 and CYP1B1 are two prototype CYPs that are abundant in lung tissue and can be induced by exposure to PAHs in the lung via activation of the Aryl hydrocarbon receptor AhR (Gebremichael et al. 1996; Choudhary et al. 2003; Chan et al. 2013). CYP1A1 is most abundant in alveolar type II cells and the endothelium (Forkert et al. 1996) and CYP1B1 is most abundant in airway epithelium (Chan et al. 2013). Exposure of mice by inhalation to concentrated ambient Fresno California PM was found to increase CYP1A1 gene expression in lung tissue including pulmonary vasculature, parenchyma and airways (Tablin et al. 2012). A study of Milano winter PM_{10} and $PM_{2.5}$ removed from filters by sonication found that aspiration of either size fraction resulted in increased expression of CYP1B1 in the airways and alveoli of BALB/c mice (Farina et al. 2011). Our data indicate that the PAH content of the material is quite important for local lung responses, especially P450 mediated responses as the multi-solvent extracted material had approximately 2.75 fold more total PAH than the spin-down preparation at the same dose. This may explain the large induction of CYP1A1 gene expression in the whole lung lobe exposed to the mid-level dose of the multi-solvent extracted particles. However, a higher dose of the same preparation did not result in an increase in gene expression for CYP1A1 (but did increase protein expression). Further, the high dose of the spin-down preparation resulted in a significant decrease in CYP1A1 gene expression. The explanation may be that gene expression is a rapid, and transient, phenomena and so the increase may have occurred at a different time point than 24 hrs after exposure. For instance, gene expression may have been increased at 2 hrs after exposure but may appear unchanged at 24 hrs after exposure if a rapid response was needed to detoxify and excrete PAHs associated with a very high dose. We feel that this is a likely explanation for the high dose multi-solvent extract data that shows no change while the mid dose does show a change in response to PM exposure. CYP1B1 was very responsive to the spin-down extract in conducting airways and alveoli, and this correlated with tissue and BALF inflammation markers, but the multi-solvent extract did not produce a response in the airways and produced only a small positive response at the highest dose in the whole lung.

It is curious that the two extract preparations differed substantially in their ability to stimulate P450 gene expression, even when derived from the same PM starting material and also acting through a similar mechanism of upregulation. Some of this may be due to different degrees of agglomeration of the material as it was delivered, but underscoring the importance of the extraction mechanism in determining the nature and the degree of the biological response. The extraction method likely alters interaction of components in the complex mixture ambient PM, thereby contributing to differences in biological responses. These interactions may involve both synergism and inhibition of toxicological effects. An illustration of this interaction has been shown for diesel exhaust where the sum of responses to individual components of the exhaust are significantly larger (by an order of magnitude) than the responses to the whole mixture, i.e. the whole exhaust (Hayakawa et al. 1997; DeMarini et al. 2004), suggesting potential interference effects for complex multi-component mixtures. Importantly, laboratory studies of mechanisms of PM-induced lung disease need to consider the composition of the sample and how it may be altered by processing, particularly of PAHs. Several recent human studies suggest a key role of PAHs, particularly the larger PAHs, in air-pollutant associated childhood asthma (Gale et al. 2012) as well as systemic inflammation (Delfino et al. 2010). Because these responses may be time-dependent, future studies should consider evaluating CYP and other gene expression responses at additional time points. Further, the location of these responses in the lung tissue will be important as our data shows that isolated conducting airways have different response profiles than whole lung.

3.4.2. Inflammation and Systemic Effects

Both preparations of SacPM were capable of inducing inflammation in the lung with the mid-level and high doses of both preparations causing a significant, dose dependent increase in BALF neutrophils. However, since the high dose of the spin-down extract was approximately half that of the multi-solvent extract, the spin-down preparation was more potent at inducing inflammation in the lung. This is also supported by a significant increase in IL-1B gene expression at the mid dose of the spin-down extract for whole lung (Figure 4F) and by greater changes in the lung tissue pathology. IL-1B is an acute response cytokine that can be released by alveolar macrophages exposed to PM and can, in turn, stimulate lung epithelial cells to produce other proinflammatory mediators (Ishii et al. 2004). The spin-down preparation induced IL-1B gene responses in the microdissected conducting airways and in the lung as a whole. Further, IL-1B did to a limited extent parallel BAL inflammation, suggesting a positive link. Involvement of the lung was greater on a histologic basis for the spin-down preparation versus the multi-solvent preparation and included increased inflammation in 3 compartments (bronchioles, parenchyma and vasculature). Notably the mid dose of the multi-solvent extract also had a significant effect on these parameters, although in general the multi-solvent extracted PM was half as potent that the spin-down extract.

This study provides additional evidence that inhaled PM2.5 can have systemic effects. While there have been numerous animal studies examining the effects of PM2.5 exposure on cardiovascular endpoints (Tong et al. 2010), few studies have evaluated the effect of PM on platelets (Tablin et al. 2012). Platelets are key players in heart attacks and strokes which have been shown to be increased following exposure to PM. The evidence from our study shows that platelet activation depends on how the test particles are prepared. The multi-solvent extract exposed cells had significantly less CD41 in the resting population. CD41 is constitutively expressed on the platelet

surface. When platelets are activated CD41 can be internalized and recycled or it may be shed in platelet microvesicles. Reduced CD41 suggests that the exposed cells were already activated, which was most likely the reason that they did not respond to thrombin. Further, the multi-solvent extract (even from the filter sample) might have damaged and/or activated the platelets such that they were unable to secrete lysosomal granules and had only limited alpha granule release upon agonist stimulation. Previous studies have found that when mice were exposed by inhalation to concentrated ambient particles over a two week period, particularly from rural sources, platelets are up-regulated and show an even greater response to agonist stimulation, than in this study (Wilson et al. 2010). However, since most of the epidemiologic data on chronic effects of urban PM2.5 indicates increases in mortality due to cardiovascular events (Dockery et al. 1993; Laden et al. 2006) and a lag time of one day or longer (Rosenthal et al. 2008), future studies should examine a time course of platelet response and the specific differences in urban and rural PM on platelet responses.

A key point to consider is why two extracts of the same PM give differences in response. The goal of this study is not necessarily to demonstrate that one extraction technique is "better" than another but rather that different extraction techniques designed with different sets of objectives can elicit differential toxicity and thus standardizing exposure objectives and extraction procedures to avoid introducing study bias is important. From the results of this work, it is perhaps reasonable to argue that the spin-down technique is "better" since it consistently elicits the largest toxicological response for almost all endpoints tested. However, a comparison of these two techniques based on the chemical composition of the extracted PM demonstrates that the multi-solvent technique best conserves the original composition of the sampled PM (Bein and Wexler, under review). An alternate argument could be presented that the multi-solvent technique is "better" since the primary objective of filter extraction is to conserve, as much as possible, the physical and chemical properties of the PM as it originally existed in the atmosphere. However, we cannot rule out that either extraction method may have also altered the properties of PM components that were removed from the filter and this may explain why control groups were different between the extracts. All PM samples used in this study were extracted from Teflon coated borosilicate glass microfiber filters. Glass microfibers can be shed from the filter during the extraction process. These fibers can be retained in the extracted PM either as freely suspended microfibers or agglomerated with PM. The two extraction techniques include different methods to minimize the amount of microfibers retained in the extract. The multi-solvent technique uses microporous membrane filtration while the spin-down technique uses a microcentrifuge-based cellular homogenization method. Based on direct gravimetric analysis of the extract controls, the spin-down technique does a better job of removing the microfibers than the multi-solvent technique (Bein and Wexler, under review) and this may account for the discrepancies between the toxicological responses to the controls. Overall the variance in the responses by particle extraction method presents a cautionary tale about how identical PM prepared using two different extraction methods can give different results.

This study was a pilot study performed to select the particle extraction method and exposure dose for the study described in the following sections, which examined biological responses to PM from different locations, and investigated the lag structure of different endpoints.. It is notable in the dose-response portion of this work that all responses were observed just 24 hours after dosing and so may continue to either increase (expected for some of the peripheral blood responses) or decrease (possibly some of the acute inflammation responses, especially for neutrophils) over time. One goal

of this study was to set an optimal dose for the follow-up study. The optimal doses for the spin-down and multi-solvent preparations were 27.5 and 50ug, respectively. This is based on ability to recruit PMN at that dose as well as the lesion scores and gives room in any comparisons of different particle types to show an increase or a decrease over time. In conclusion this study shows that extraction method for removal of particles from filters critically influences the biological responses observed following administration to the respiratory tract.

3.5 Time-Delay Results

3.5.1. Results

Histology

Typical lesions in mice given urban source PM by oropharyngeal aspiration are shown in Figure 7, section 9.2. Lesions were most prominent at the terminal bronchiolar-alveolar duct junction and included accumulation of PM in alveolar ducts and adjacent interstitial tissue, accumulation of mixed inflammatory cells including PMNs and monocytes, perivascular edema and arteriolar contraction. PM could be visualized both intracellularly in macrophages and free in alveolar lumens (Figure 8). Lesion scores for overall inflammation were elevated at day one for both urban and rural source PM and persisted for all three days in the urban source treated animals (Figure 9). Rural source animals maintained significant inflammation at day 2 but this was largely resolved by day 4 (Figure 9). Urban source PM had a higher density of PM visually evident in sections and this density persisted throughout the 4 day study (Figure 10). Rural source PM densities were lesser and not statistically significant but trended markedly towards scores from filter extract treated animals by day 4 (Figure 10). Evaluation of the relative contribution of neutrophils to the inflammatory exudate showed they were a large component of the one and two-day responses to urban source PM but were largely absent by 4 days despite the persistence of inflammation due to mononuclear cells (Figure 10). The presence of intravascular platelets in arterioles was also subjectively evaluated with some evidence of increased numbers at day 4 in the urban source treated animals. This later finding was unexpected and considered somewhat equivocal due to the difficulty in identifying these very small cells in histologic section.

Platelets

As was the case for the dose response studies, platelets were evaluated in the resting (unstimulated) and thrombin stimulated state. Three markers were used for evaluation of activation: P-selectin,(CD62P), a marker of alpha granule secretion; LAMP-1(CD107a) a marker of lyosomal granule secretion and CD41b (the integrin $a_{2b}b_{3a}$). Ten thousand events were counted for every marker and the percentage of platelets which expressed the marker (percent positive) and the mean fluorescence intensity (MFI; number of molecules on the platelet surface) were measured for every variable. Platelets were examined at 24, 48 and 96 hr time points.

Resting Platelet Evaluation: Platelets (control, Davis or Sacramento PM exposed) showed no significant differences in P-selectin (either percent position or MFI) at 24, 48 or 96 hours, data not shown. LAMP-1 staining revealed no differences between control, Davis or Sacramento platelets either for percent positive or MFI for both 24 and 48 hours. However, at 96 hours there were significant differences ($p<0.05$) between the Sacramento platelets and control platelets (Figure

11). Lastly, we examined the expression of the major platelet integrin $a_{2b}b_{3a}$ on the platelet surface. Similar to the other platelet markers, there were no significant differences in any of the conditions at either 24 or 48 hours. However at 96 hours there were significant differences in the expression of CD41b on the surface of the platelets (Figure 11). Control platelets expressed greater amounts of this integrin, while both Sacramento and Davis platelets expressed less of the integrin ($p<0.05$) indicative of integrin shedding and/or recycling – both indicators of activation.

Thrombin Stimulated Platelet Evaluation: Stimulation of platelets by thrombin, a potent physiological agonist virtually always results in platelet activation. We evaluated the same three membrane proteins for this portion of the study. As was the case for resting platelets there were no changes in P-selectin – either percent positive or MFI, for 24, 48 and 96 hours. Lamp-1 percent positive and MFI showed a very similar pattern, data not shown. Similar to the patterns we observed in resting platelets, CD41b expression – either as percent positive or MFI was not significantly different at 24 and 48 hours, data not shown. However, at 96 hours, there were significant differences between the three groups regarding MFI (Figure 12). MFI in control platelets was significantly greater than that of the Sacramento group and there was a similar trend in the Davis platelets.

Lung and Serum Cytokines

Protein: A total of 32 cytokines were assayed using a multiplex bead based assay (Bioplex, BioRad). Serum and lung tissue cytokine protein assays were measured on a subset of 3 animals at the 1 and 4 day time points. While most assays had results within the standard curve of the assay, only those illustrated in Figure 13 had significant differences between groups when assayed by ANOVA. Supporting a systemic effect of pulmonary exposure the following cytokine proteins had a significant difference from control or by source in serum: PDGF, IL12p40 and Eotaxin (Figure 13). Exposure to Sac PM caused a significant decrease in PDGF at 1d compared to filtered air control, while Davis (rural) PM caused a significant decrease only at 4 days following exposure. IL12p40 was decreased in the serum 4d after exposure to Sac PM. A slightly different profile emerged for lung tissue where the proteins VEGF, IL-9 and also eotaxin all exhibited changes with exposure (Figure 13). VEGF in lung was significantly increased by exposure to Davis PM at 1d. Lung IL-9 was increased by Sac PM at 4 days following exposure. The only cytokine that exhibited changes in both lung and serum was the CC chemokine eotaxin, which is a potent eosinophil chemoattractant. However, the abundance of this protein was differentially affected by PM exposure in serum vs lung tissue. In serum, eotaxin decreased acutely following exposure to Sac PM, while it increased in the lung for both urban and rural PM at 4 d following exposure. All remaining cytokines did not show significant differences.

Genes: Lung compartmental RNA was isolated from microdissected intrapulmonary airways and surrounding parenchyma. The following genes were analyzed using qRT-PCR on lung parenchymal tissue: CCL11, Ccl20, Csf2, Cxcl1, Cxcl2, Cxcl9, Cybb, IL10, IL12b, IL13, IL1b, IL 4, INFg, PDGFb, Sele, TNF. Detection of many genes was at low levels and limited the significance of some of the genes examined as many samples were below the limit of detection. However, the expression of two key genes related to inflammatory cell recruitment, particularly of neutrophils to lung tissue, NADPH oxidase and KC (keratinocyte chemoattractant) were significantly increased by exposure to PM (Figure 14). Only Davis (rural) PM caused a large (>700

fold) and significant increase in NADPH oxidase mRNA 1d following exposure (Figure 14A). In contrast, the urban PM (Sacramento) was more effective at inducing KC mRNA expression, increasing it greater than 20 fold (Figure 14B).

Bronchoalveolar lavage (BALF): Total cell number and the abundance of cell types were measured in the BALF (Figure 15). Of the cells that make up the BALF, only % neutrophils is shown (Figure 15B) as all other cell types were not significantly different from control with PM exposure. Both sources caused modest decreases in the total number of cells recovered. The Davis (rural) PM caused a significant decrease in total BALF cells only at 2 days following exposure and returned to steady state by 4 days post exposure. In contrast, Sacramento PM (urban) caused a decrease in total cells at all time points examined. There was a significant neutrophil influx in response to both sources of PM with the rural Davis PM resulting in only a transient increase at 1d, while Sacramento urban PM resulted in longer lasting neutrophilic elevation in the BALF. Lactate dehydrogenase activity and total protein were also measured in the BALF fluid as markers of acute cytotoxicity. These markers either did not change or decreased with exposure to PM indicating a lack of direct acute cytotoxicity from these PM preparations (data not shown).

Oxidant/antioxidant expression: Because expression of these genes and proteins can vary substantially by lung region, we microdissected intrapulmonary airways and surrounding parenchyma to probe each compartment for gene expression. Genes analyzed for oxidant/antioxidant effects in both airways and parenchyma were CYP1A1, CYP1B1, CYP2F2, GCLC, GCLM, GSTa, GSTm1, GSTp1, Hmox1, Prdx6. Heme oxygenase (HMOX1) gene expression in microdissected conducting airways was significantly increased 1 d after exposure to either Davis or Sacramento PM (Figure 16). Gene expression of phase II antioxidant enzyme, Glutathione S transferase pi (GSTpi) was significantly increased 1 d following exposure to urban Sacramento PM. Antioxidant and phase 2 metabolism genes were also altered in the parenchymal compartment following exposure to PM (Figure 17). In contrast to the airway specific analysis, GSTpi did not change in the parenchyma after exposure to either Davis or Sacramento PM. The 1-cys peroxiredoxin 6 (prdx6) was increased only at 1d following exposure to Sacramento PM but did not change in response to Davis PM. A component of the rate limiting enzyme in glutathione synthesis, glutamyl cysteine ligase-modifier subunit (GCLM) was increased at 2d following exposure to Sacramento PM. Two P450s involved in PAH bioactivation (CYP1A1 and CYP1B1) were also assessed using regional microdissection of lung subcompartments (Figures 18 and 19). While CYP1A1 gene expression in the conducting airway decreased as much as 2 fold following exposure to PM, this decrease was not significant (Fig 18A). In contrast to the conducting airway response, there was a great deal of variance in the parenchymal CYP1A1 gene response, including in the sham treated controls. While both urban (Sac) and rural (Davis) PM caused increases in CYP1A1 gene expression (more than 2 fold) in the parenchyma, there were different temporal pattern by particle type. The urban particles caused a significant increase at 2 d following exposure which then decreased back towards control levels at 4d. Davis PM in contrast, did not show a significant increase until 4d following exposure (Figure 18). In contrast to CYP1A1, CYP1B1 was increased following exposure to PM (Figure 19). However, only Sacramento PM caused a

significant increase in CYP1B1 and this occurred at 1d following exposure and only in the conducting airways.

3.5.2. Figures

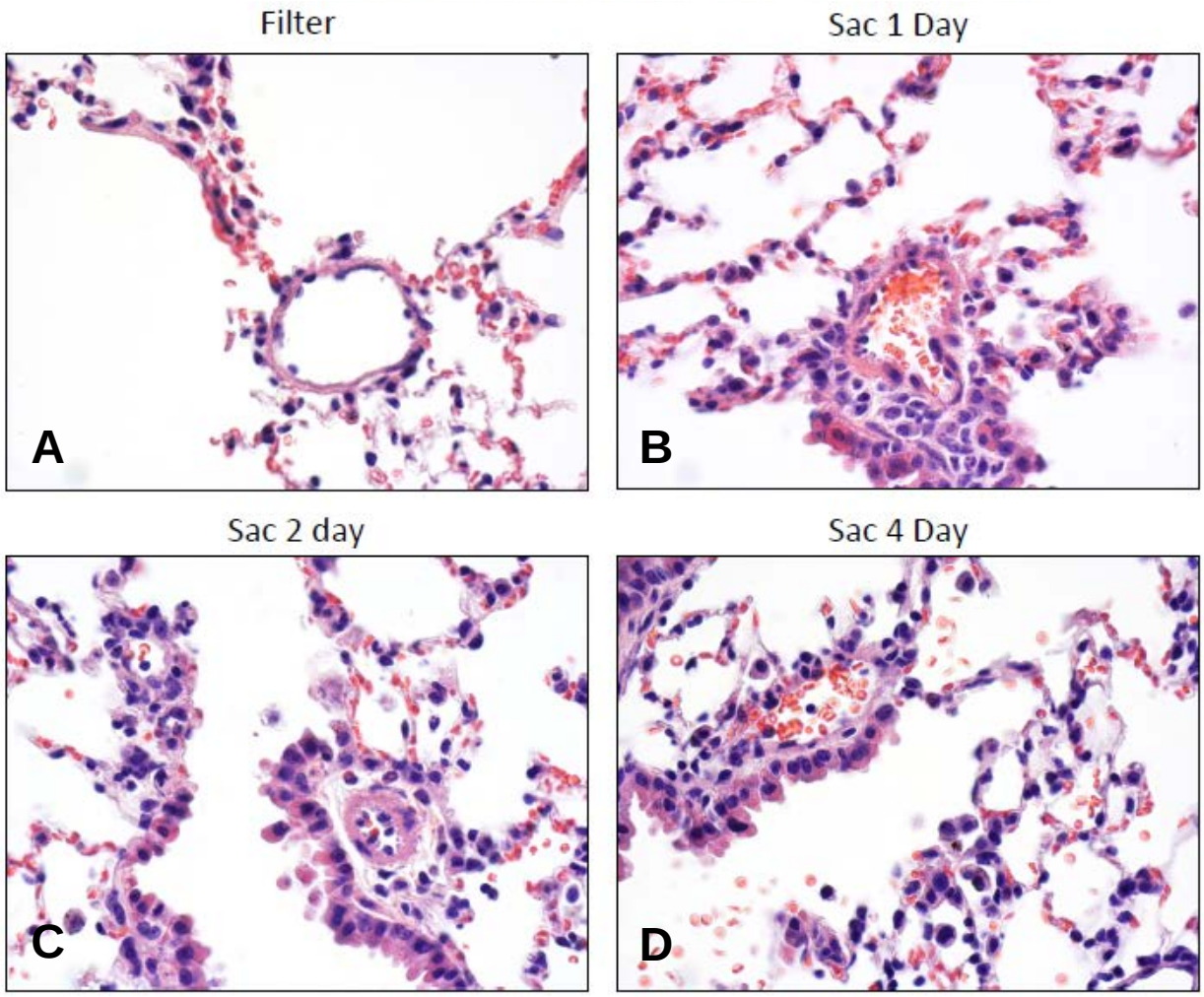


Figure 7: *Histologic changes at the terminal bronchiolar junction in mice exposed to urban source PM by oropharyngeal aspiration*. Compared with controls treated with filter extract only (A), lungs of mice one day after PM instillation (B) had hypertrophy of terminal bronchiolar epithelium, peribronchiolar and periarteriolar accumulation of monocytes and neutrophils, and accumulation of macrophages containing heterogeneous particulate matter in alveolar ducts. These changes persisted 2 days post instillation (C) with more evident arteriolar medial hypertrophy. At 4 days post-instillation (D), monocytic inflammation and epithelial hypertrophy persisted but neutrophils were less evident. Intracytoplasmic PM was also evident in 4 day post-instillation mice.

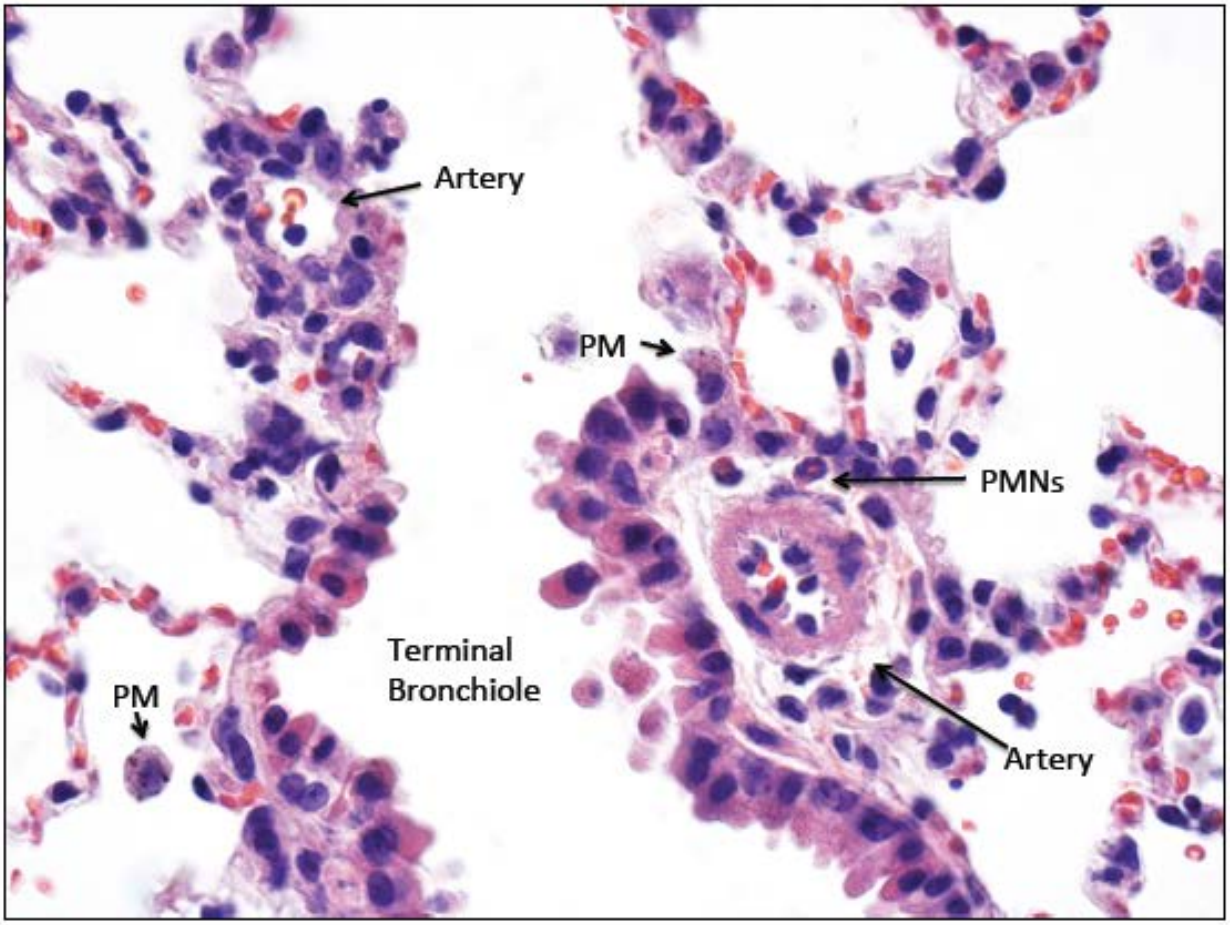


Figure 8: *Higher magnification demonstrating changes used in assigning lesion scores in the time course study of oropharyngeal aspiration.* This terminal bronchiole from a mouse treated for 2 days with urban source PM has PM present in intra-alveolar macrophages in peribronchiolar and alveolar duct regions. Periarteriolar inflammation includes neutrophils (PMNs) and mononuclear cells. There is marked increase in the arteriolar media implying either contracture or hypertrophy.

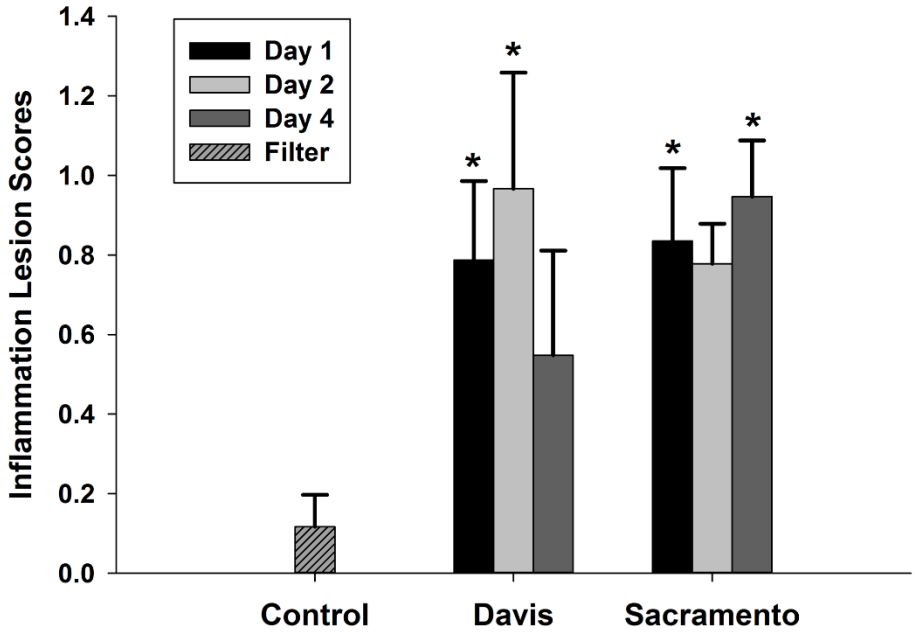


Figure 9*: Subjective lesion scores for inflammation in the lung*. Inflammation scores for mice treated with urban or rural source PM for 1,2 or 4 days. Significant inflammation was present in urban (Sac) and rural (Davis) source mice at day 1 post instillation. Inflammation persisted through day 4 in urban source treated mice but lessened in rural source treated mice by day 4. (* $p<.05$ Kruskal Wallace analysis).

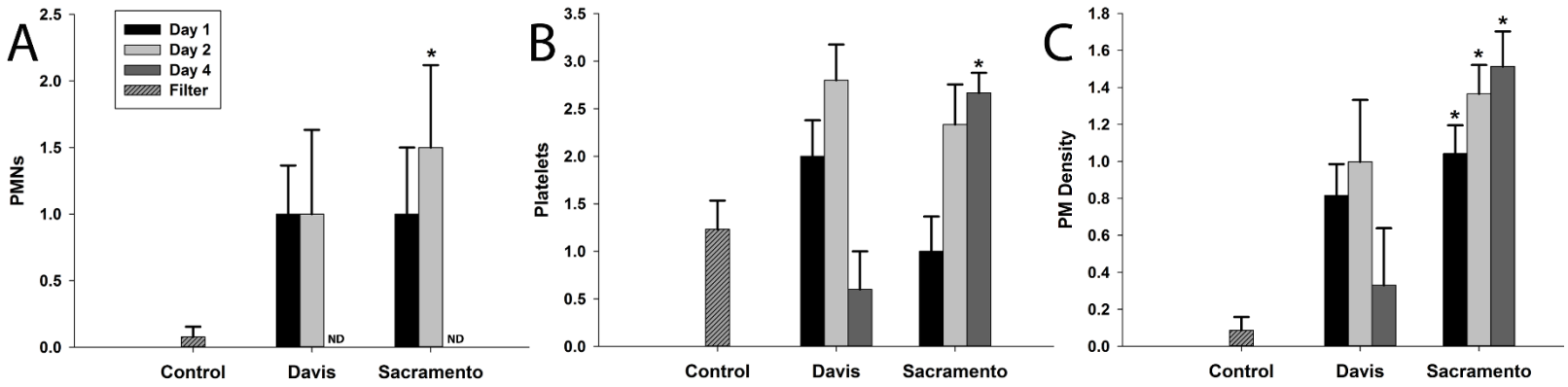
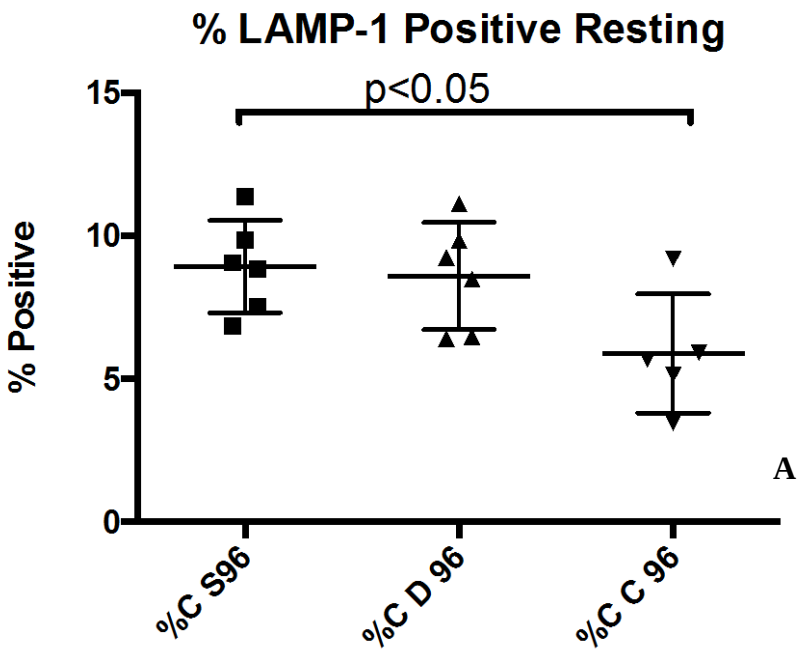


Figure 10: *Histologically defined components of inflammation from pathologic analysis of lung sections from mice treated with urban or rural source PM for 1, 2 or 4 days.* A Neutrophils (PMNs) were evident at 1 and 2 days but were a much smaller component of inflammation in mice treated with either source PM at 4 days. ND = not detected B The presence of platelets in intrapulmonary microvasculature trended towards increased density in treated mice and was statistically significant at day 4 in urban source treated mice. C The density of histologically evident PM was similar between the two sources at day 1 but rural source treated mice had markedly less PM evident at 4 days post instillation while PM persisted in the lungs of urban source treated mice at 4 days.



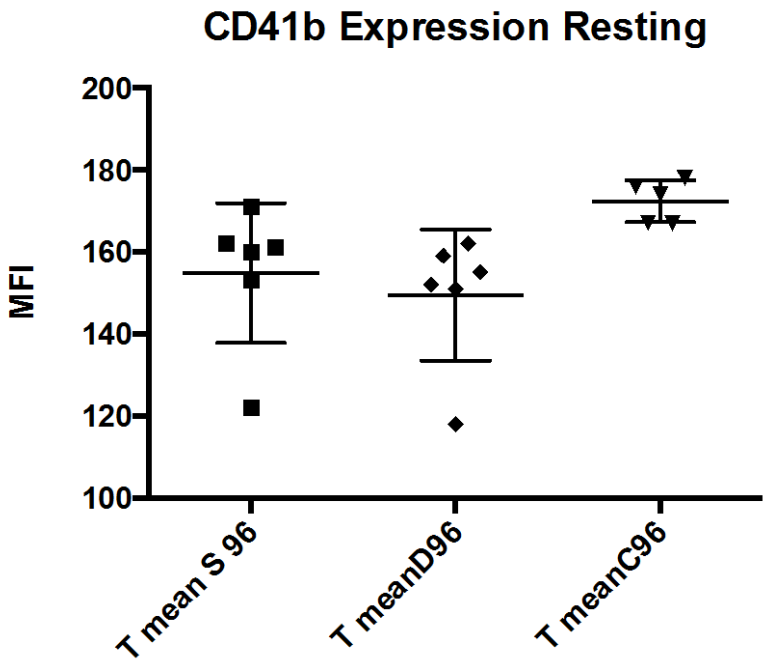


Figure 11 *Flow cytometric evaluation of resting platelets:* Platelets were evaluated for LAMP-1 (C), P-selectin (E) and the integrin CD41b (part of the $a_{2b}b_{3a}$ complex(T)). Plate A: Evaluations included the number of cells which were positive for expression of the protein of interest (%positive) as well as, Plate B, the number of receptors of interest present on the cell surface (mean fluorescence intensity MFI). Data are presented as means +/- standard deviations. At 96 hours, platelets from Sacramento PM exposed animals (CS96) showed significantly greater expression of LAMP-1, an activation marker, than those of control platelets. In addition, there was a decrease in expression of CD41b in Sacramento and Davis PM treated platelets, another indicator of activation. This decrease was not statistically significant.

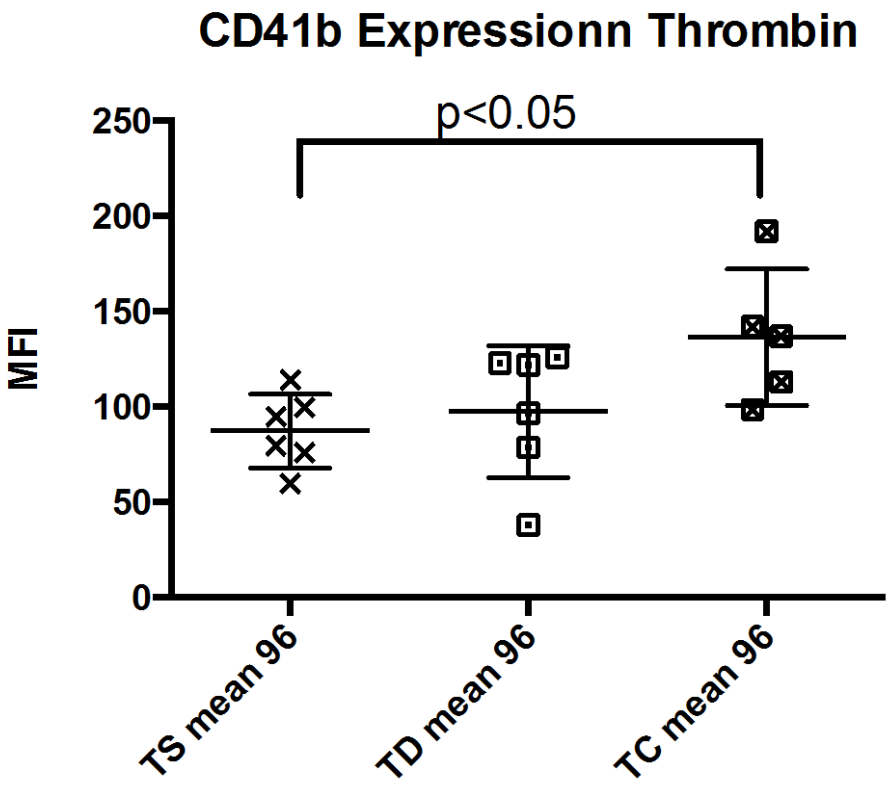


Figure 12: Flow cytometric evaluation of thrombin stimulated platelets: At 96 hours there were no significant differences in either LAMP-1 or P-selectin expression. There were, however, significant decreases in CD41b expression (a marker of activation) in the Sacramento PM (TS) exposed platelets, when compared with control platelets (TC) and Davis platelets (TD).

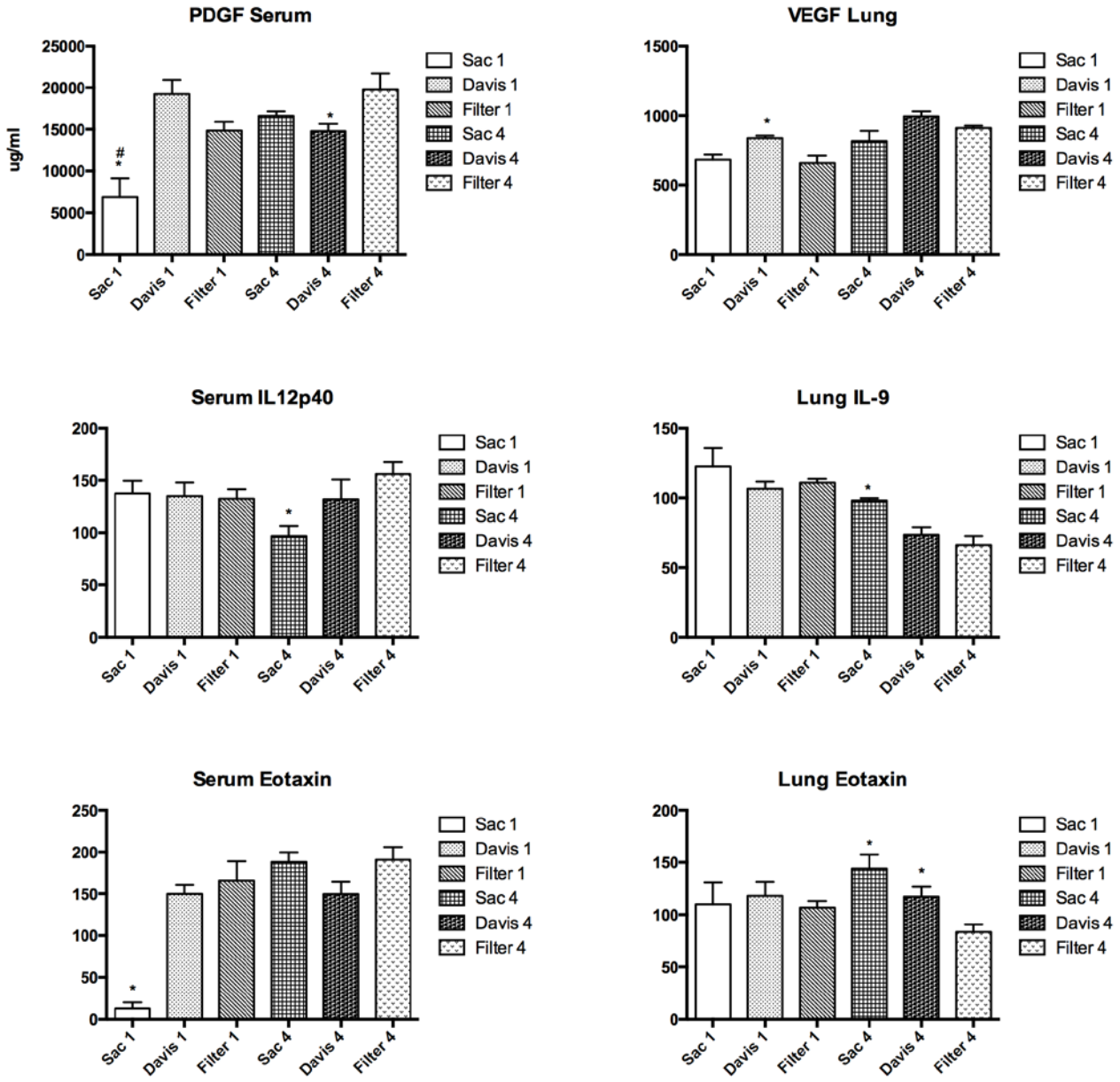


Figure 13: *Results of multiplex cytokine assays in serum and lung from mice given collected ambient PM by oropharyngeal aspiration*. Groups represent days post dosing with urban source (Sac) or rural source (Davis) PM. * significant difference from filter control. # significant difference between sources. $P<0.05$.

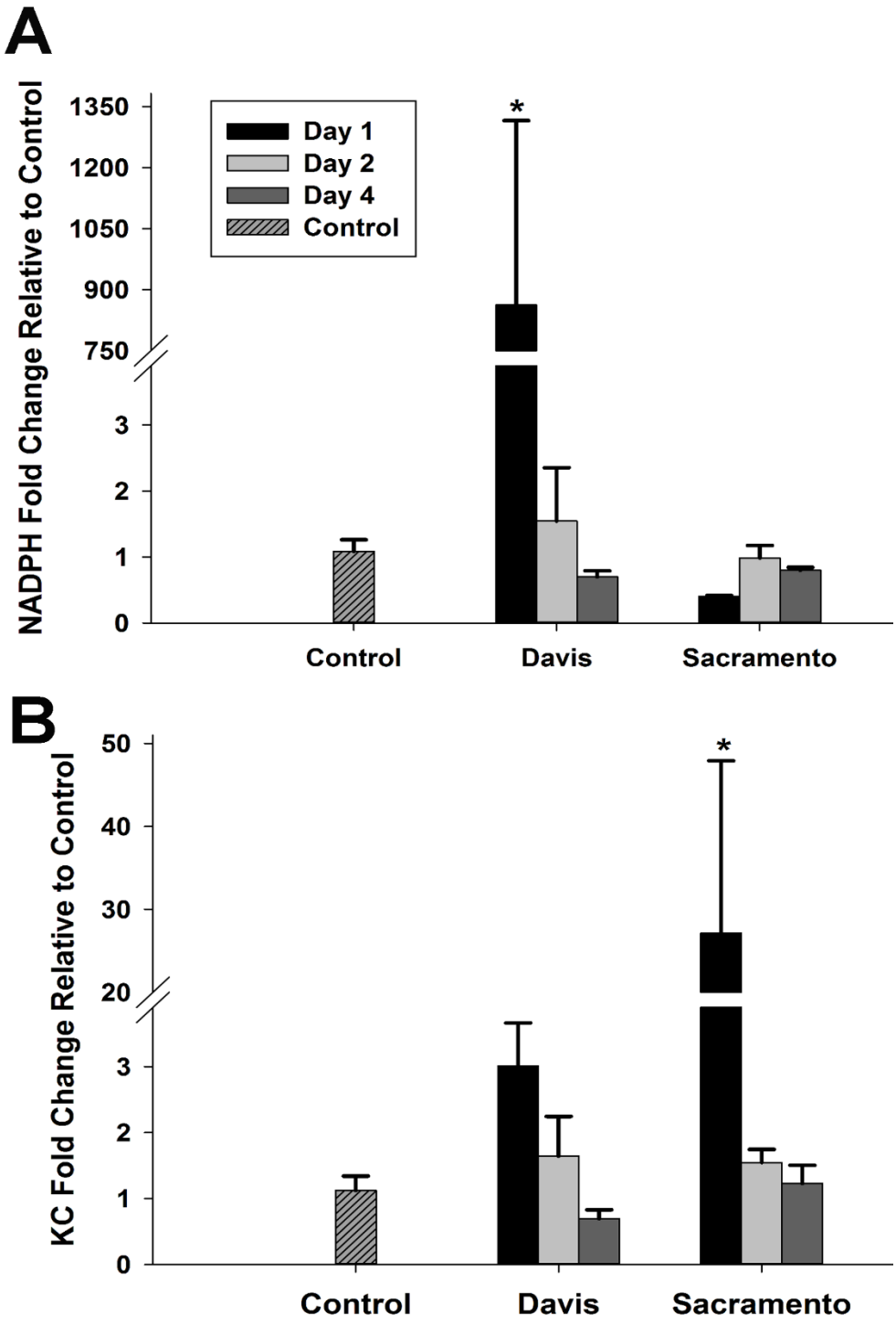


Figure 14 *Expression of key genes involved in inflammatory cell recruitment to lung tissue.* NADPH oxidase (A) and KC (B) fold in lung parenchyma following exposure to Sac, Davis PM or sham controls. A. Only Davis (rural) PM caused a larger (>700 fold) and significant increase in NADPH oxidase mRNA 1d following exposure B. In contrast only Sacramento PM caused a significant increase (>20 fold) in KC mRNA expression at 1 d following exposure. Control data was not different by time point and so was pooled for this analysis. * = significantly different from control. $P < 0.05$.

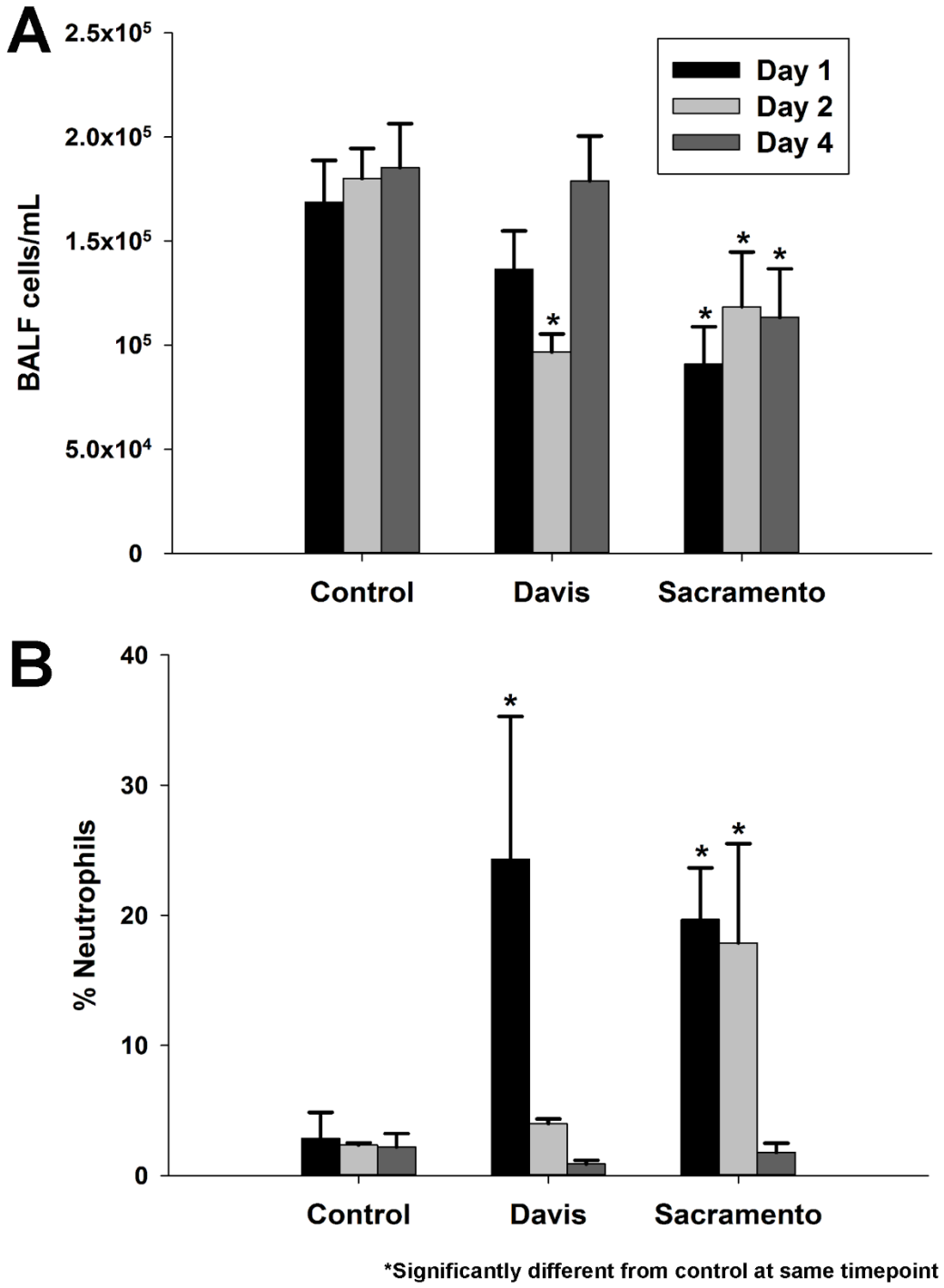


Figure 15 *Bronchoalveolar (BALF) cell counts for total cell numbers (A) and % neutrophils (B).* Sham treated control data as well as data from animals exposed to Sac or Davis PM at 1, 2, or 4 days previously. A. Davis (rural) PM caused a significant decrease in total BALF cells 2 days following exposure. Sacramento PM (urban) caused a decrease in total cells at all time points examined. B. Both Davis and Sac PM caused an increase in neutrophils in the BALF with Davis PM causing only an acute increase at 1d and Sac PM causing an increase at both 1 and 2 days. * = significantly different compared to time matched control group. $P < 0.05$.

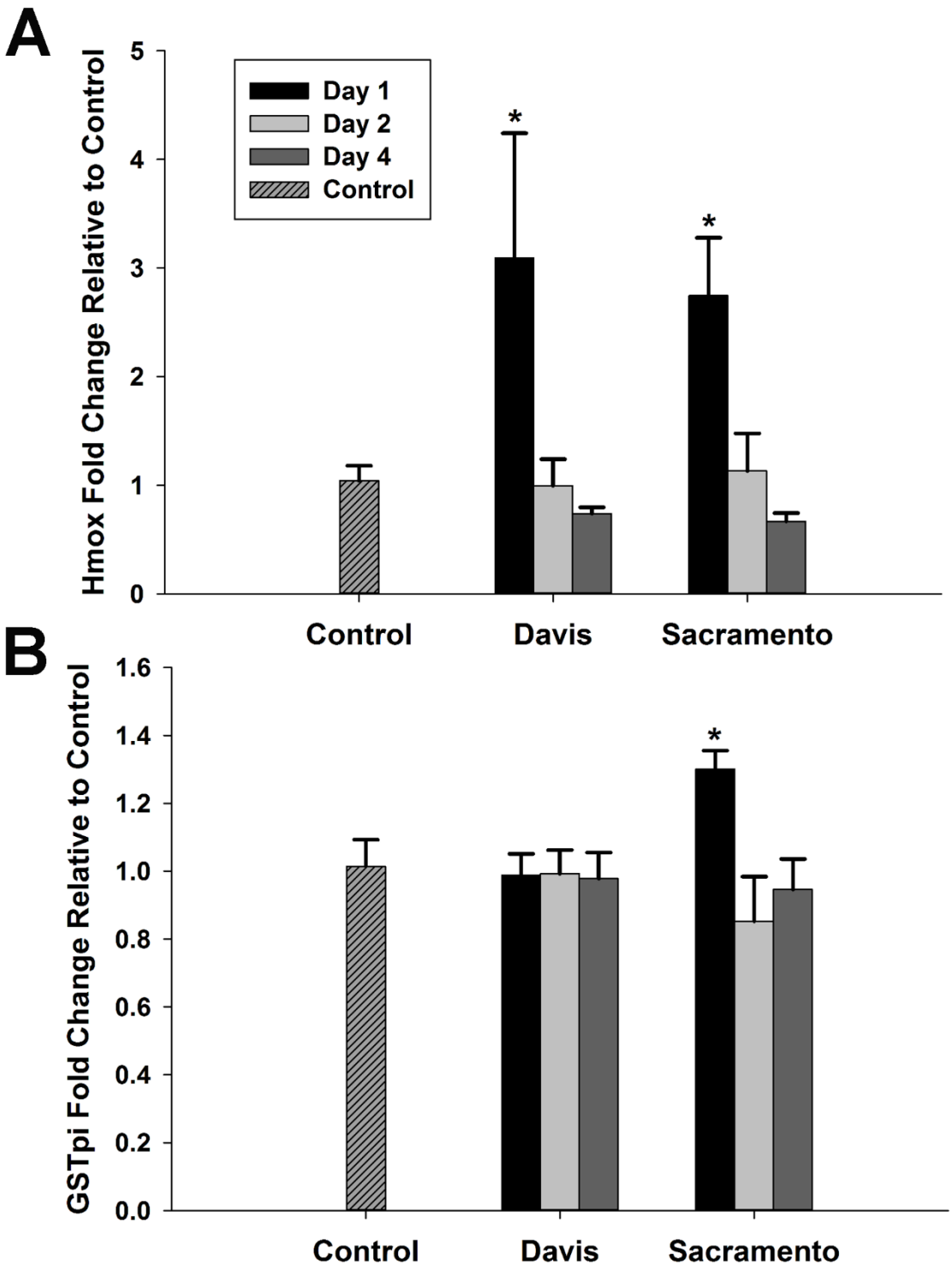


Figure 16 *Conducting airway expression of key antioxidant and phase 2 metabolism genes was altered by exposure to PM.* A. Heme oxygenase (HMOX1) gene expression in microdissected conducting airways was significant increased 1 d after exposure to either Davis or Sacramento PM. B. Glutathione S transferase pi (GSTpi) was increased 1 d following exposure to Sacramento PM. Control data was not different by timepoint and so was pooled for this analysis. * = significantly different from control. $P < 0.05$.

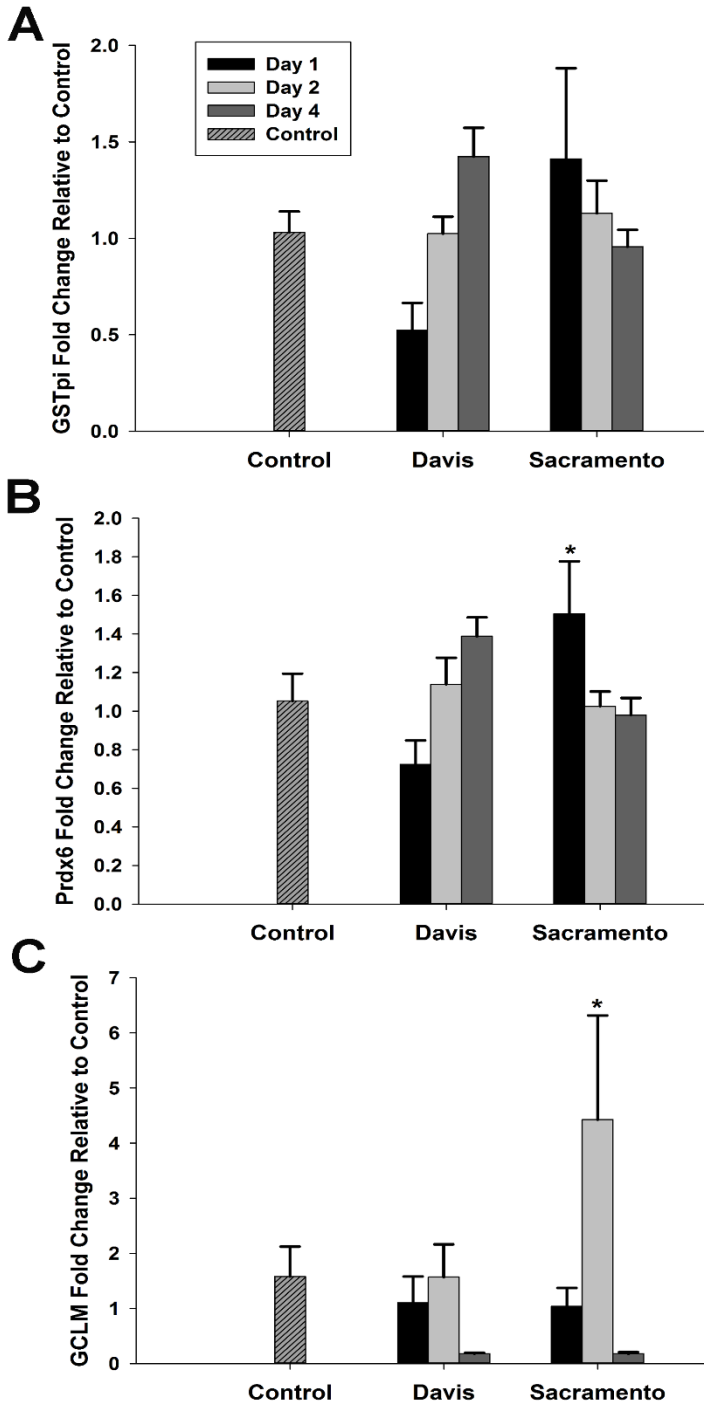


Figure 17 *Parenchymal expression of key antioxidant and phase 2 metabolism genes was altered by exposure to PM.* A. GSTpi did not change in the parenchyma after exposure to either Davis or Sacramento PM. B. Peroxiredoxin 6 was only increased at 1d following exposure to Sacramento PM. C. Glutamyl cysteine ligase-modifier subunit was increased at 2d following exposure to Sacramento PM. Control data was not different by time point and so was pooled for this analysis. * = significantly different from control. $P < 0.05$.

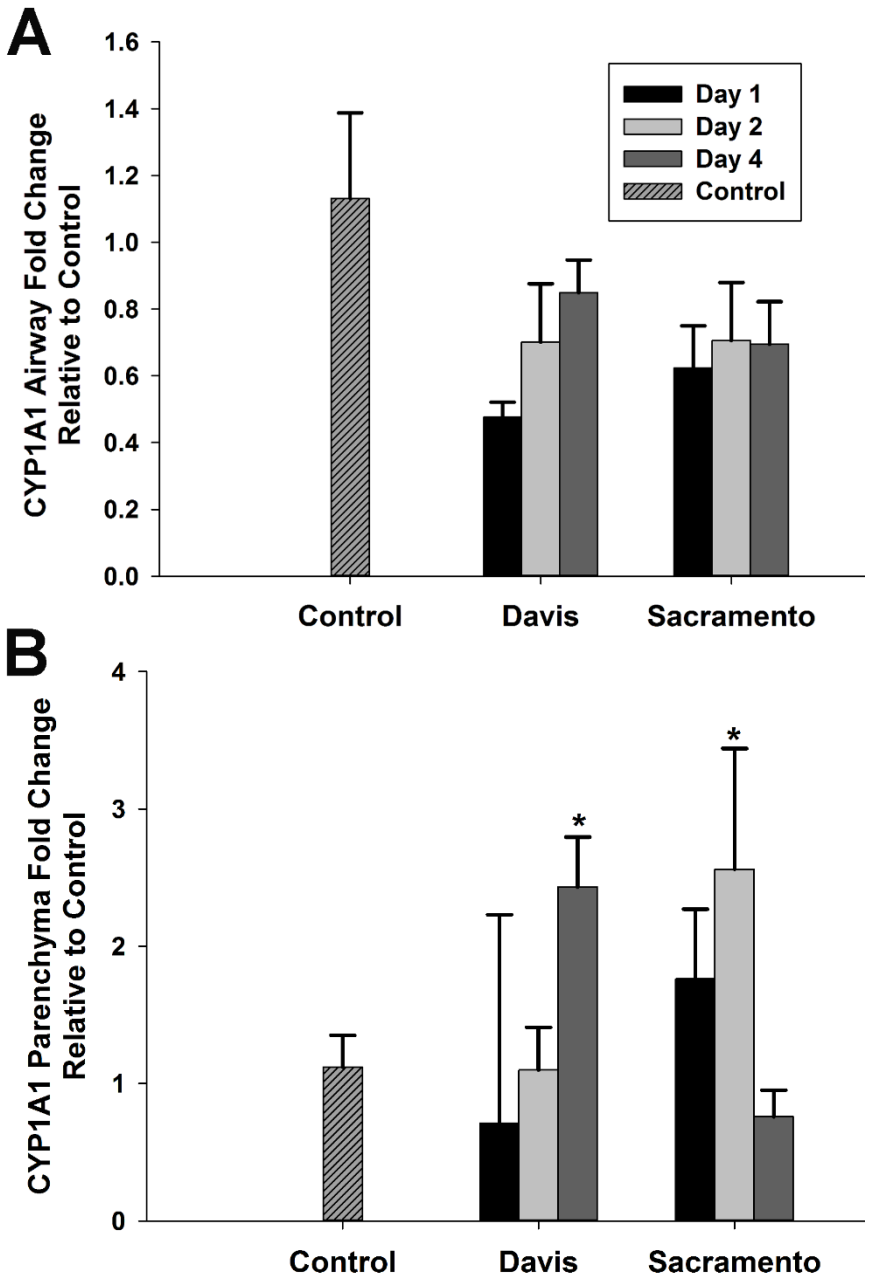


Figure 18 *Expression of CYP1A1 mRNA in microdissected lung subcompartments; conducting airway (A) and parenchyma (B).* A. While CYP1A1 gene expression in the conducting airway decreased as much as 2 fold following exposure to PM, this decrease was not significant. B. There was a great deal of variance in the parenchymal CYP1A1 gene response. Gene expression increased more than 2 fold at 4d following exposure to Davis PM. Sacramento PM exposure caused a significant increase at 2 d following exposure. Control data were not different by time point and so was pooled for this analysis. * = significantly different from control. $P < 0.05$.

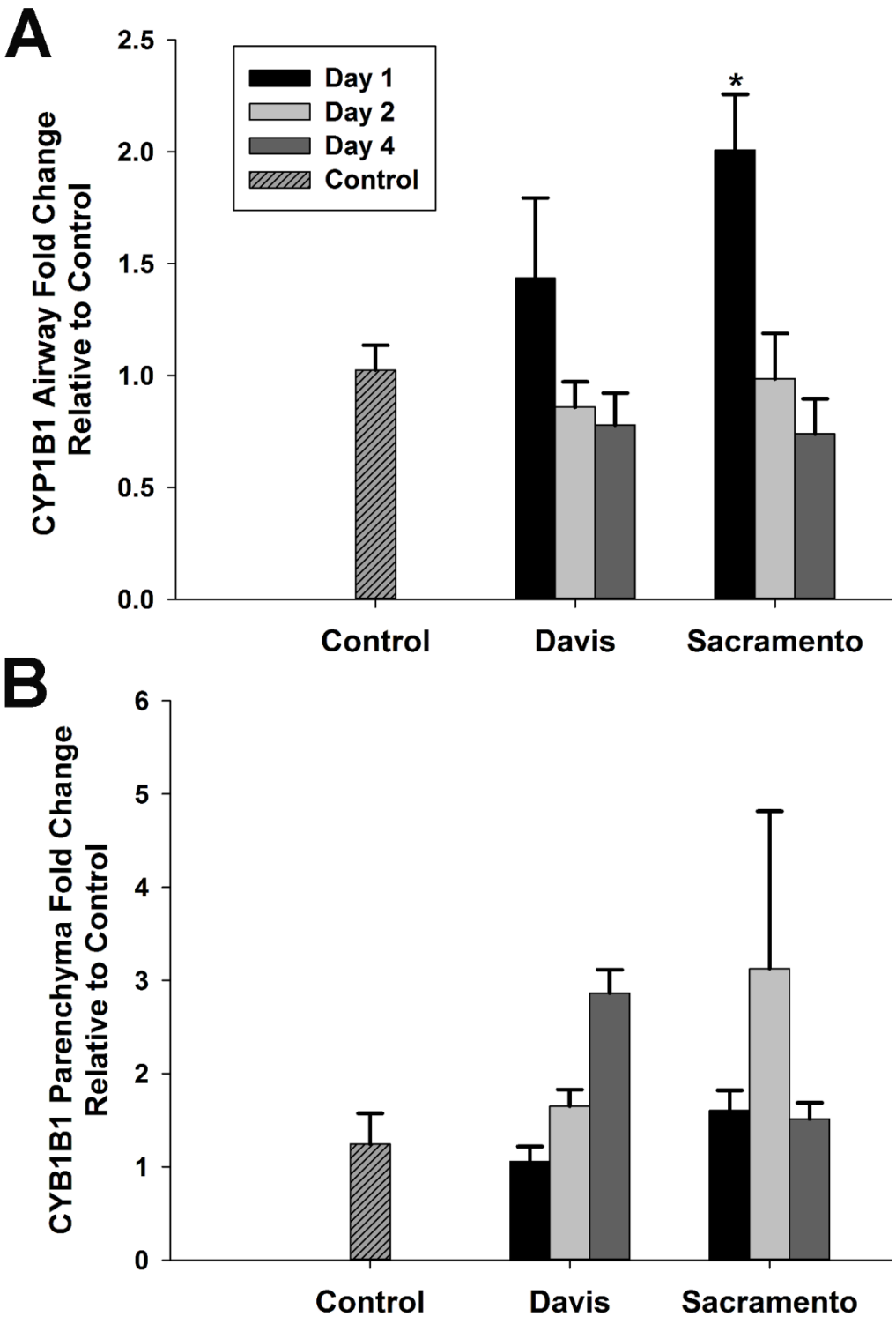


Fig 19 *Expression of CYP1B1 mRNA in microdissected lung subcompatments; Conducting airway (A) and parenchyma (B).* A. Sacramento PM caused a significant increase in CYP1B1 at 1d following exposure. B. While the trend was for parenchyma CYP1B1 to increase following exposure to PM, this was not statistically significant. Control data were not different by timepoint and so was pooled for this analysis. * = significantly different from control. $P < 0.05$.

3.6 Time-Delay Conclusions

3.6.1. Inflammation

In this study we exposed Balb/c mice to two types of PM: urban PM from downtown Sacramento near a major freeway interchange and rural PM from Davis. Both sources of PM were collected in the same manner onto filters during a similar time period in the winter. Based on our previous study, we chose to remove the particles from the filters using the spin down sonication method which we showed previously was optimal at preserving the capability of the particles to produce inflammation in lung tissue, in BALF and in peripheral blood platelets. We found that both urban and rural PM produced lung tissue inflammation and increases in the percentage of PMNs in BALF. However, the two sources of PM also had substantial differences in the timing of inflammation associated responses with urban PM providing longer lasting effects on lung cell infiltration than rural PM. Both sources of PM were effective at activating platelets.

The histopathologic lesions clearly support recruitment of inflammatory cells quickly to the lung, within the first 24 hrs. However, this was not accompanied by an increase in any markers of lung cell cytotoxicity such as increased protein or LDH activity in the BALF. This supports the concept that these particles are not directly toxic to lung cells but instead activate signaling molecules to influence responses. Because our first time point was at 1 day after exposure, we cannot exclude the possibility that cytotoxicity occurred earlier.

An influx of inflammatory cells is a common response in the lung following exposure to PM (Dye et al. 2001; Bachoual et al. 2007). While our data does show an increase in % neutrophils with PM exposure, it does not show a corresponding increase in total cells in lavage. This is in contrast to our previous dose response data that did show an increase in total cells in BALF, although only at the highest dose. The reason we do not see this increase in total cells in this study may be due to different baseline values in the filter treated controls with higher values reported for controls in the time delay study. A lack of increased cells in lavage may be attributed to an inability to extract cells that were adhering to tissue, as illustrated by our histopathology results. NADPH oxidase is a key component of phagocytes oxidative capability. The large increase in NADPH oxidase in the lung tissue exposed to Davis PM (Figure 14A) correlates well with the % neutrophils recovered from lavage for the Davis PM (Figure 15B). However, Sacramento PM which had a prolonged increase in neutrophils in BALF did not show an increase in parenchymal levels of NADPH oxidase gene expression. Some of this may be due to differences in the sites of neutrophil influx between the two particle types as BALF is especially good at sampling/representing cells from the conducting airways. Further, we should point out that neutrophil influx is an area of agreement between the time-delay and dose response study for Sacramento PM responses. Another reason there may be a mismatch is that the signaling for neutrophil influx, KC, may have occurred later (at one day) in the animals exposed to urban Sacramento PM than in the Davis PM (where a KC increase may have occurred prior to the 24 hr sampling point). This would be consistent with the temporal pattern of changes observed in some animal models following exposure to PM where changes in gene expression involving signaling molecules are a very early event, occurring as soon as 2 hrs after exposure (Timblin et al. 2002; Chan et al. 2011; Chan et al. 2013).

Signaling molecules such as chemokines and growth factors are thought to play a major role in choreographing cellular responses to PM. This is thought to involve both local and peripheral

responses (Nemmar et al. 2006; Alfaro-Moreno et al. 2007; Uski et al. 2012). We investigated this by surveying a panel of proteins in both the blood serum and in the lung tissue. Admittedly there should be some overlap in this data as serum proteins present in the blood will likely also be in the lung sample although as only a small component. Surprisingly, very few molecules were significantly changed by the exposure and only one, eotaxin, was altered in both tissues. Several factors may account for this result including the small number of mice assessed, the variance in the data and the timing of sampling. Signaling molecule changes, such as those associated with neutrophil influxes to lung tissue, usually occur soon following exposure and our first time point, at 1 d, may have been too late to catch the peak of the change. Interestingly, several of these molecules were significantly decreased at 4 days following exposure to Sac PM, including IL12p40 in serum and lung IL-9, possibly indicating involvement in late responses to PM, such as platelet activation. Exposure of mice to PM has been shown to induce a TNFalpha dependent increase in PAI-1 and IL-6 activation of coagulation in the periphery (Budinger et al. 2011), however we did not find changes in IL-6. Again, this may be due to timing of the sampling and the short duration of the single acute exposure. One has to wonder why Sac PM caused late changes in IL12p40 and IL-9 but Davis PM did not. The physicochemical characteristics of particles may again play a role. Previous studies have demonstrated that innate immunity is differentially affected by particles of different sizes (Samuelsen et al. 2009). Also, particles containing high levels of PAH have been shown to cause greater biological effects than particles with lower levels of PAH. In the current study we do not know if the effects we saw are due to PAHs that are still attached to the particle.

More interestingly, however, was the fact that serum eotaxin had a substantial and significant decrease 1 day following exposure to Sac PM. This may indicate a rebound effect from an earlier increase or possibly indicates that circulating eotaxin may be suppressed by exposure to PM. Curiously, both sources of PM caused an increase in lung tissue eotaxin expression compared to filtered air controls at 4 days following exposure, perhaps this is a direct sequelae of the suppression in blood serum eotaxin. We did not find any increases in BALF eosinophils at this point but it would be interesting to investigate later time points in future studies.

The composition of the urban, Sacramento, PM was significantly increased for organic carbon, organic matter, aluminum and acenaphthene. It is not possible to determine which of these components contributed to the increased inflammation seen following exposure to urban vs rural PM. Further, for endpoints where there was not a major difference between urban and rural PM (such as the HMOX1 response) it is not clear whether that indicates that these are, in turn, not contributing to that result. Further it cannot be determined whether instead of one causative substance instead what takes place is inhibition of the biologic response by a second component of the mixture.

Finally, numerous studies have found cardiovascular health effects, especially strokes and heart attacks, and also asthma, following exposure to PM. However, there has been a recent focus on the importance of “lag time” following an acute exposure on human health. A recent study in Denver of both cardiovascular and respiratory hospital admissions found that the relative risks were generally larger with shorter time lags for total cardiovascular admissions and at longer lags for respiratory admissions, especially asthma (Kim et al. 2012). A study of human exposure to PM on platelet aggregation found that exposure to PM10 increased platelet aggregation between 72 and 96 hrs after exposure (Rudez et al. 2009) . Our current data, which found an increase in markers of

platelet activation at 4 days following exposure as well as changes in eotaxin at this timepoint (which can be related to eosinophilic inflammation in asthma) agrees with these previous observations of a lag time to effect. While some epidemiologic studies have linked acute exposure to traffic to cardiovascular effects, in the current study both PM sources (urban and rural) were equally capable of inducing platelet activation, an outcome that is linked to thrombogenic events.

3.6.2. Oxidant/Antioxidants

Particulate matter can also cause effects through direct oxidant effects on lung tissues. While the PM extraction method we used in this study was optimized for inflammation effects, some effects on the redox systems in the lung tissue were also noted. Heme oxygenase is an enzyme that is regulated by the cytoplasmic transcription factor, Nrf2, which can be induced by electrophilic stress. Hmox1 is widely reported in the literature as a broad based lung tissue response to PM that indicates oxidative stress, and possibly inflammation, in the tissue (Li et al. 2003; Farina et al. 2011). Both urban and rural PM exposures resulted in similar increases in Hmox1 gene expression in the airways. However, only Sacramento PM induced an increase in the most abundant GSH transferase in the conducting airways, GSTpi. GSTpi is a key phase II metabolism enzyme that detoxifies oxidative chemicals with glutathione (GSH). Importantly, humans have polymorphisms in GST pi and these have been associated with traffic related health effects including asthma (Salam et al. 2007; Melen et al. 2008). Our data indicate that increases in lung tissue GSTpi gene expression are involved with urban PM exposures, but that rural PM does not have this effect.

Glutathione synthesis is an important detoxification route for the lung and GCL is the rate limiting enzyme in this synthesis pathway. Urban Sac PM was able to induce gene expression of this enzyme, an important change that increases detoxification of oxidant chemicals in the lung. It is puzzling that an increase was not seen in the conducting airways, especially since GSTpi had an increase. However, again, the explanation for this may be that we missed a key time point where this increase occurred.

CYP1A1 is involved in bioactivation of PAHs which can be present in the vapor or particulate phase of atmospheres containing vehicle exhaust. In this case, we only examined the water soluble or particle bound PAHs in our sample due to the choice of particle extraction techniques. We did not see an effect of PM exposure on airway CYP1A1 gene expression. However, the parenchymal compartment which includes vessels and alveoli, did have an increase in CYP1A1 gene expression following exposure to either source of PM. Some of this variance in response may be due to how the PM dispersed once it was aspirated into the lung, although oropharyngeal aspiration is thought to be an effective method for delivery (De Vooght et al. 2009), particle agglomeration may have had an effect on response (Mercer et al. 2008). Particles were more visible in the lung tissue following exposure to urban Sacramento PM but this may be due to either particle agglomeration or to a difference in the native particle color in the tissue rendering these particles more detectable than the rural Davis particles. The efficiency of the extraction for PAHs may have also impacted the distribution of response. We should note that we only took one lung lobe (right cranial) to investigate gene specific effects and so differences in dispersion of the aspirated PM may have affected the response. CYP1B1 was significantly increased by exposure to Sacramento PM at 1 day in the airways and this likely reflects the higher content of PAH in the

urban PM. This result is in agreement with the results from our previous dose-response study using this Sacramento PM.

3.7. Overall Conclusions

In conclusion these studies underscore a number of important points that may influence how PM exposure studies in animals are conducted and how previous and future results are interpreted. First our dose response study shows that the extraction method for removal of particles from filters critically influences the biological responses observed following administration to the respiratory tract with some responses being larger for different extraction approaches. Specifically, much of the pathology and inflammation associated changes were more potent with the spin down approach and most of the PAH related responses were more potent with the solvent extraction method. Second, our time lag study found that there are significant shifts in the temporal pattern of response based on particle type. In general, Sac PM had a longer period of BALF inflammation and also pathology associated with lung tissue inflammation than Davis rural PM. Thirdly, there were intriguing differences by lung region in terms of both the antioxidant responses and the chemokine responses in the lung. When the lung was microdissected, airways and parenchyma had markedly different responses to PM exposure. This underscores the importance of evaluating site-specific responses when evaluating toxic effects in the lung. If we had not dissected the lung tissue into compartments, we might have seen little effect at all because the airway specific effects are only 5% of the whole lung volume and so would have been swamped by the influence of the larger mass of parenchymal tissue. Finally, we report a novel increase in eotaxin in the lung tissue following a significant lag time after exposure. This latter point is quite important in light of reports in the literature on long lag times following PM exposure being associated with the onset of asthma. Eosinophilic inflammation in the lung, attracted by eotaxin, is one of the hallmarks of asthma exacerbations.

We have a number of recommendations for future work. Future studies that include gene expression data would be wise to include very short time points after exposure (2-6 hrs) because of the potential for rapid changes in gene expression. Another key element that should be included in future studies is study of the peripheral blood cells counts, circulating progenitor cells and possibly microparticles in addition to platelets. This would enable studies of the eotaxin effects, which would also require longer time points and incorporation of pulmonary function measures in a sensitive animal model of asthma, such as the rhesus monkey model of childhood asthma or the mouse OVA model. Follow up studies should be conducted with the alternate extraction method, solvent extraction, to assess the influence of PAHs on specific lung regions, particularly in light of the strong effect of the high PAH containing urban Sacramento PM on GST pi. Inclusion of a tighter acute time course and tissue compartment specific measurement of GSH depletion and repletion could be quite informative regarding which sites/cells in the lung are most susceptible to PM. Because there is a link with some of our findings to childhood asthma as well as a commonality with markers that have been shown to be differentially regulated following PAH exposure in neonatal rats, a follow on study in an animal model of childhood asthma would be important with a specific focus on the role of oxidant/antioxidant enzymes in the response. This study was designed to investigate pulmonary endpoints and some peripheral inflammation, yet strong evidence exists that shows that PM affects

many more organs besides the lung. The heart and the vasculature as well as the brain and possibly behavior can be modified by exposure to PM. Future studies should address these issues using the approaches we have outlined in this study. There is much to be gained by considering the impact of both the time course and magnitude of responses following exposure to PM from different sources. Finally, to complete the loop on the platelet data it would be fascinating to have a better understanding of how platelets are signaled to have responses to PM and also to correlate measures of platelet activation with later biological effects relative to clinical conditions mediated by at least in part through thrombosis such as stroke, acute coronary syndrome (myocardial infarction and unstable angina), and deep venous thrombosis.

This report is expected to have a number of impacts on future experimental design, especially for studies using extracted PM. First, this study underscores the importance of characterizing how representative particle extracts are of the native PM on the filters. This requires rigorous chemical characterization of both the filters and the extracts for each site, as recovery of various components may be altered by the extraction methods used and determination of mass balance. This is rarely done. An additional impact of this report will likely be on the concept that not all extracts increase even similar responses equally. For instance in the dose response study, both extract methods increased BALF inflammation but only one extract had a major impact on peripheral inflammation in terms of platelet activation. Further, in this study we propose the concept that the complex nature of PM may include substances that both promote and inhibit biological responses even in the same mixture and the idea that the most bioactive extract may not necessarily be the most chemically representative one. We would hope that an impact of this study would be to point out that if one wants to study biological effects due to PAHs on PM, it is quite important to establish that the PM you are using has that as a major component. Finally, our study provides important data on how site specific evaluation of biologic responses in lung tissue can provide increased sensitivity of detection of response. These methods have been published for over 20 years and yet many labs have not adopted them because they are labor intensive. We believe our study shows that the results make it worth the effort. Future study designs would benefit from carefully considering evaluation of target sites within the lung using site specific methods when evaluating biologic responses to PM. Future studies can build on this data to explore directly which components of the PM are responsible for which biologic responses in direct testing.

3.8. References

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