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PM_{2.5} Filter Extraction Methods: Implications for Chemical and Toxicological Analyses

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Abstract

Toxicology research into the global public health burden of fine particulate matter (PM_{2.5}) exposures frequently requires extraction of PM_{2.5} from filters. A standardized method for these extractions does not exist, leading to inaccurate inter-laboratory comparisons. It is largely unknown how different filter extraction methods might impact the composition and bioactivity of the resulting samples. We characterized the variation in these metrics by using equal portions of a single PM_{2.5} filter, with each portion undergoing a different extraction method. Significant differences were observed between extraction methods for concentrations of elements and polycyclic aromatic hydrocarbons (PAHs) of the PM_{2.5} tested following its preparation for biological response studies. Importantly, the chemical profiles differed from those observed when using standard protocols for chemical characterization of the ambient sample, demonstrating that extraction can alter both chemical component amounts and species profiles of the extracts. The impact of these chemical differences on sensitive endpoints of zebrafish development was investigated. Significant differences in the percent incidence and timing of mortality were associated with PM_{2.5} extraction method. This research highlights the importance of and rationale for considering extraction method when making inter-laboratory comparisons of PM_{2.5} toxicology research.

Graphical Abstract

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Supporting Information (SI)

Details on SPE and derivatization methods, PAHs analyzed with abbreviations, elemental concentrations in SRM1649b, PAH surrogate concentrations following filter extraction, list of individual PAHs detected, list of additional compounds tested in blank filter extracts, estimated detection limits for PAHs and quantifiable limits for elements, raw zebrafish mortality and morphology data

Introduction

Toxicology research is essential to better understand the public health burden from fine particulate matter (PM_{2.5}) exposures which are associated with systemic health effects.^{1–3} In addition to exploring the full range of PM_{2.5} hazard potential, the use of an appropriate whole animal model can also identify toxic constituents and the molecular mechanisms underlying the associated systemic health effects.^{4–7} PM_{2.5} collected on filters can address the global variability in PM_{2.5} from a toxicological perspective, broadening the knowledge previously gained from fixed location testing and limited sample number.^{8,9} Use of spatially, temporally, and seasonally variable samples provides additional information on the toxic potential of PM_{2.5}.

Research groups currently use various filter extraction methods to prepare samples for these investigations,^{10–14} creating a potential toxicity bias from the extraction method, rather than from the actual PM_{2.5}-sample composition. The use of varying filter extraction procedures also complicates inter-laboratory comparisons and hence formation of a robust consensus of PM_{2.5} exposure hazard. Variability in extraction methods can misrepresent the toxic responses to specific PM_{2.5} samples because of the potential loss of a key toxic driver(s) during the extraction process.¹⁵ Few studies have compared PM_{2.5} filter extraction procedures but they indicate substantial differences between the actual and observed chemical components of PM_{2.5} post filter extraction.^{16,17} Not surprisingly, these differences are associated with similar discrepancies in sample oxidative potential¹⁸ and bioactivity.¹⁹ This previous research explored a single biological system and only compared two extraction methods, highlighting a clear knowledge gap in filter extraction impacts on chemical and toxicological analyses.

Recently, the zebrafish (*Danio rerio*) was utilized as an *in vivo* surrogate to evaluate particulate matter-induced toxicity.^{20–22} The developing zebrafish is highly sensitive to chemical perturbation. Advantages include embryo transparency, rapid external development (3 – 5 days post fertilization for most endpoints), and amenability to molecular and genetic techniques.^{23–25} These advantages enable rapid screening of PM_{2.5} samples with biological activity measurements spanning from overt toxicity (malformations and mortality), to subtle but important effects on behavior.^{20, 26} Thus far, PM_{2.5} extraction method-bioactivity studies have not been reported in zebrafish.

We performed multiple extraction methods on portions of the same PM_{2.5} filter to determine the associated impacts on chemical recovery and bioactivity. Use of a single filter sample allowed for interpretation of data independent of physical and chemical properties that would vary from different collections of PM_{2.5}. From this we hypothesized that different filter extraction procedures on the same PM_{2.5} sample will impact the chemical and biological response data of these samples, introducing a methods bias. This research will guide selection of an extraction method that is best suited for bioactivity assessments using PM_{2.5} samples chemically representative of ambient samples.

Materials and Methods

Chemicals

PAHs and isotopically labeled standards information, including abbreviations and vendors, is provided in the Supporting Information (Table S1). Solvents including: methanol (MetOH), hexane, ethyl acetate (EA), acetonitrile (ACN), acetone (Ace), and dichloromethane (DCM); all optima grade were purchased from Thermo Fisher Scientific (Santa Clara, CA). Toluene, dimethylsulfoxide (DMSO), and N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) were purchased from Sigma-Aldrich (Milwaukee, WI).

PM_{2.5} Samples

Samples were donated by Keith Bein and collected in the winter in downtown Sacramento, CA from January 15–24, 2011 on PTFE-coated filters.¹⁶ The filter was cut into six equal portions for subsequent extraction. Blank PTFE-coated filters (Pallflex fiberfilm, 37 mm) that did not undergo PM_{2.5} collection were extracted to serve as methods controls.

Extraction Methods

Six different extraction methods were tested on ambient PM_{2.5} filters and blank control filters. The extraction process consisted of removal of particles from the filter piece, concentration of removed extracts, and reconstitution in DMSO (Table 1). Each extraction method is detailed below.

1. **Water:** The filter was sonicated in a waterbath sonicator (40 kHz, Branson) in 15 mL tubes with 6 mL of water. After sonication, the filter piece was removed and rinsed with water to remove any residual particles remaining on the filter. The sample was then concentrated via freeze drying and the dry PM_{2.5} was reconstituted in DMSO.

2. *Methanol*: The filter piece was sonicated and rinsed as described in method 1 but in methanol instead of water. The sample was concentrated by N₂ stream and then reconstituted in DMSO.
3. *DCM*: The filter piece was sonicated and rinsed as described in method 1 but in DCM instead of water. The sample was concentrated by N₂ stream and then reconstituted in DMSO.
4. *DMSO*: The filter piece was sonicated and rinsed as described in method 1 but in DMSO instead of water. The sample was solvent exchanged to ethyl acetate (EA), concentrated by N₂ stream, and then reconstituted in DMSO.
5. *Single Vial*: A single vial method was created in an effort to reduce sample loss, consumables, and sample process time. In this method, a single filter piece was placed into a 1.5 mL centrifuge tube and the same volume used for DMSO reconstitution in all other methods was used. The DMSO and filter were sonicated for 60 min in a waterbath sonicator, as occurred with the other sonication extraction methods.
6. *Pressurized Liquid Extraction (PLE)*: A filter piece was placed in a 33 mL cell (Dionex Accelerated Solvent Extractor 350) that underwent two cycles of pressurized liquid extraction: 1) DCM followed by 2) EA (1500 psi, 100 °C, 1 cycle, 240s purge). The sample was concentrated by N₂ stream and then reconstituted in DMSO.

All dry PM_{2.5} was reconstituted in an equal volume of DMSO, except for the single vial method which already contained the appropriate volume to result in a final concentration of 200 µg/mL. The soluble fraction from DMSO extraction was collected as previously described and selected for this research as it replicated the findings of the whole particle suspension, the insoluble fraction was not tested as it was previously shown to have negligible bioactivity compared to the soluble fraction for particulate matter samples.²⁰ The soluble fractions of PM_{2.5} and blank filter extracts resulting from the six different methods were split for chemical and biological testing.

Ambient sample characterization

An additional portion of the ambient PM_{2.5} filter used for all extraction method testing was used to determine the chemical constituents present on the filter following standard operating procedures (SOP) for chemical characterization, without the additional preparation steps required for toxicological research. PM_{2.5} was removed from the filter via pressurized liquid extraction followed by sample clean-up as previously described for PAH analyses.²⁷ For characterization of elements, a portion of the filter was sonicated in water for 60 min via water bath sonication (60 Hz). This extraction method has previously been shown to produce similar extraction efficiencies to liquid-liquid extraction methods with particulate matter samples.²⁸ This sample which underwent SOP characterization steps, without toxicology preparation steps (concentration and reconstitution), will be referred to as the “Ambient SOP Sample”.

Chemical Characterization

PAHs: Aliquots of the DMSO soluble fraction of PM_{2.5} and blank filter extracts were solvent exchanged to hexane via a TurboVap evaporation system (N₂ gas, 30 °C) followed by solid phase extraction (SPE) cleanup (SI). Samples were then solvent exchanged to EA and concentrated to 300 µL under a stream of N₂. Samples were spiked with isotopically labeled internal standards, hydroxy-PAH analysis was performed with an aliquot of the concentrated sample that was derivatized following addition of internal standards (SI). Organic compounds, specifically parent/methyl PAHs (n=19), and nitro- (n=22), oxy- (n=23), hydroxy- (n=36), and high molecular weight (MW > 302, HMW, n=14) PAHs, were quantitatively measured using Agilent 6890 gas chromatography (GC) coupled with an Agilent 5973N mass spectrometer (MS). Selected ion monitoring (SIM) was utilized with spectral data analysis performed with ChemStation software (V. E.02.02.1431, Agilent Technologies). Commercially available standards were used for all measured compounds (abbreviations and vendors available in S1) and all samples and controls were run in triplicate.

Elements: Aliquots of the DMSO soluble fraction of PM_{2.5} and blank filter extracts were added to ultrapure water, resulting in a 0.1 % DMSO concentration. Elements (n=14), were quantitatively measured using an Agilent 5110 inductively coupled plasma optical emission spectrometry (ICP-OES) system in axial view mode at the Central Analytical Laboratory at Oregon State University. Commercially available standards were utilized for all measured compounds and all samples and controls were run in triplicate.

Developmental Toxicity Screening

Zebrafish Husbandry: Standard procedures for fish care followed at Sinnhuber Aquatic Research Laboratory (SARL) were utilized with adult fish for a wildtype (Tropical 5D) that were maintained at 28±1 °C on a recirculating system, with a 14 h light/10 h dark cycle.²⁹ Embryos were collected from group spawns of adult zebrafish³⁰ and enzymatically dechorionated at 4 hours post fertilization (hpf).³¹ Embryos were then mechanically distributed into individual wells of a 96-well plate that contained 90 µl of embryo medium^{31, 32} and the soluble fraction of PM_{2.5} or vehicle (DMSO)/blank filter controls in embryo medium (10 µl) were added at 6 hpf. Final concentrations in all wells were 1 % DMSO in embryo medium. All experiments were conducted with fertilized embryos according to Oregon State University Animal Care and Use Protocols.

Developmental Toxicity Screen: Following embryo exposure at 6 hpf, the 96-well plates were sealed with Parafilm to prevent evaporation, wrapped in aluminum foil to prevent photodegradation, and placed on an orbital shaker at 235 rpm overnight to ensure gentle mixing after the exposure; plates were stored at 28 °C throughout the experiment.²⁹ Developmental toxicity was assessed at 24 and 120 hpf in all treatments and controls (n=32 embryos/group). Mortality and morphological outcomes (n=22 endpoints) were visually assessed using a dissecting microscope as previously described.²⁵

Statistical Analysis

For chemical characterization data, histograms and statistical significance calculations (one- or two-way analysis of variance (ANOVA) tests and pairwise multiple comparison procedures (Hom-Sidak method) with significance set at $p < 0.05$) were completed with SigmaPlot 14.0 (San Jose, CA). All data was blank corrected using blank filter extracts for each method. For developmental toxicity screening, statistical significance was computed as previously reported.²⁹ Heatmaps were generated using RStudio (Boston, MA) with the gplots (heatmap.2 package)³³ and interaction plots were generated using the UpSet R package.³⁴ Significance of associations between chemical and toxicity data were computed in R 3.4.1 (Vienna, Austria) using the glm() function to obtain odd ratios (OR) and 95% Confidence Intervals (CI). To account for multiple testing, a Bonferroni corrected p-value was calculated, resulting in significance at $p < 0.000781$.

Results and Discussion

Chemical Characterization

PM_{2.5} elemental composition by extraction method is displayed in Figure 1a (ng/μL) with significant differences indicated ($p < 0.05$). No extraction method consistently resulted in the highest yield for all elements, eliminating a clear extraction method that would be ideal for increased removal of elements from PM_{2.5} collected on filters. For total elements detected in the PM_{2.5} extracts (concentration (ng/μL) $\pm$ s.d.), the order of extraction method yield from highest to lowest concentration was: water (5.90 ± 0.023), methanol (4.94 ± 0.020), DCM (1.41 ± 0.016), single vial (0.500 ± 0.027), PLE (0.350 ± 0.018), and DMSO (0.320 ± 0.009). Water and methanol were the most effective at extracting the detected PM_{2.5} associated elements from the filter. These results were corroborated by performing identical extraction procedures on the same filter material spiked with a National Institute of Standards and Technology (NIST) air particulate matter standard reference material of urban dust collected from Washington, DC (SRM1649b). Water and methanol had the highest extraction concentrations for the elements detected in this research (Table S2). Sonication in water or methanol were anticipated to be the most effective extraction methods for elements because these solvents are more polar than the others tested and many of the elements quantified are water soluble.³⁵ It should be noted that sonication has the potential to lead to the formation of radicals that could impact the observed chemical composition of PM_{2.5} samples in a solvent dependent manner,³⁶ this is an area for future investigations particularly due to the frequent use of sonication in PM_{2.5} toxicity studies.^{15, 37, 38}

When assessing the elemental profile from each extraction method and the profile from the Ambient SOP Sample filter that underwent standard elemental characterization procedure, there were notable differences (Figure 1b). Importantly, the differences in chemical profile between the Ambient SOP Sample and the extraction methods establishes that none of the methods tested replicated the ambient PM_{2.5} mixture. Additionally, 6 elements were only detected in the Ambient SOP Sample and these elements accounted for approximately 7% of the total elemental mass in the sample. Identifying that not only were the elemental profiles different in percent contribution but also that the number of elements detected was reduced for the samples prepared for toxicology research compared to the ambient sample. The

extraction methods tested here were designed for future toxicological analysis but might result in these elements not being present in the toxicological exposure medium. The total elemental concentration was 3–60 fold higher in the SOP (19.420 ± 0.071 ng/ μ L) than the extraction methods, indicating reduced recovery of elements throughout the extraction and toxicology preparation steps. These losses must be concerned and addressed in future PM_{2.5} toxicology studies.

Extraction method yields of PAHs are displayed in Figure 2a (pg/ μ L) as the sum of individual compounds in each class. Significant method dependent differences were observed in all PAH classes. Water extraction decreased parent, oxy-, hydroxy-, and HMW PAH yield compared to at least two of the other methods. Oxy-PAH yield was highest with methanol extraction. Total HMW PAH yield was highest with PLE. These results were corroborated by performing identical extraction procedures on the same filter material spiked with SRM1649b and isotopically-labeled or deuterated surrogate PAHs. Use of surrogate PAHs similarly demonstrated significant recovery differences between extraction methods (Figure S1). While the trends for the PAH surrogates were not identical to those observed in the extracted PM_{2.5} samples (i.e. oxy-PAH recovery was not significantly increased using methanol extraction) the surrogates only represented a subset of the 114 PAHs. The chemical behavior of compounds without representative surrogates may have differed in recovery due to structure and solubility differences. Additionally, matrix differences were present between the collected sample and laboratory replicated matrix (SRM1649b rubbed onto the same filter material) in the particle size of the samples and the impaction of the particulate onto the filter.

Chemical profiles in classes of PAHs differed between extraction methods and from the Ambient SOP Sample (Figure 2b). Across the extraction methods, the percent contribution of quantifiable hydroxy-PAHs was greatly increased relative to the Ambient SOP Sample. This may be due to an increased extraction efficiency for these compounds and ineffective extraction of other PAH classes, altering the overall distribution observed. The removal and analysis procedures for PAH identification and quantification used on the Ambient SOP Sample³⁹ were not specifically directed at removing more polar PAHs, including hydroxy-PAHs, and this may have affected the lower percent contribution. Nitro-PAH recovery was low or non-existent in the extraction methods, except for PLE recovering these compounds but below the estimated limits of detection (Figure S1). The low recoveries resulted in deviation from the Ambient SOP Sample where the percent contribution of nitro-PAHs was 13.5 % of the total PAHs quantified compared to the extraction methods at 0 – 0.1 %. When assessing nitro-PAH surrogate recovery (Figure S1), differences were not observed between 4 of the 6 methods, including PLE. Variation between nitro-PAH recovery in the surrogates and samples could be due in part to altered ability to recover particle-bound PAHs in samples with the extraction methods used⁴⁰ and the lack of overlap between the nitro-PAH surrogates used (d₇-1-nitronaphthalene, d₁₁-6-nitrochrysene) and the compounds detected in the samples (2-nitrofluorene, 9-nitroanthracene, 3-nitrophenanthrene, 2-nitrofluoranthene, 1-nitropyrene, 2,8-dinitrodibenzothiophene, 7-nitrobenz[a]anthracene, 1,6-dinitropyrene, and 1,8-nitropyrene). The PAH chemical profiles presented were created following preparation for toxicological analysis and thus the exact step(s) in the extraction and preparation process that altered these profiles cannot be quantitatively determined. But, the preparation steps for

toxicological treatment were identical in all of the extraction methods, except for single vial. The single vial method deviated from the others because it did not require a concentration or re-suspension step and therefore was anticipated to have increased yield due to reduced losses from additional preparation steps. This was not observed and may be due to the reduced volume of solvent used during the sonication step and tight filter packing into the small volume/collection tube, reducing the effectiveness of the initial sonication step.

Comparison of Detected Constituents between Methods: Overlap of detected compounds between extraction methods was assessed for elements (Figure 3a) with a majority (8 of 12) of elements being detected in all methods. While there was consistency in element detection it should be noted that the concentrations differed significantly between methods (Figure 1a). When we examined individual PAHs, significant yield differences were associated with extraction method (Table S3). Assessment of PAH structural overlap by the different extraction methods (Figure 3b) showed that a core of only 7 out of 114 PAHs were recovered by each of the 6 extraction methods. The best performance (highest number of detected compounds) was from PLE where 50 total PAH structures were recovered with 19 unique PAHs detected using this method. Methanol extraction was the second best method with 35 PAH structures recovered, but only 2 that were unique to this extraction method. We noted that 1,2-naphthoquinone, perhaps one of the most developmentally toxic of the compounds to zebrafish that we assessed,⁴¹ was only recovered by PLE. 1,2-naphthoquinone was also detected in the Ambient SOP Sample (SI). Inefficient extraction of this and other compounds may grossly misrepresent the complexity of human exposure to PM_{2.5}. The limited overlap in PAH recovery among these methods, especially between PLE and the other extraction methods, highlights our concern that extraction methods can greatly skew the picture of chemical composition and, by extension, any assessment of sample bioactivity.

The ultimate objective when extracting PM_{2.5} for use in toxicological analyses is to obtain an extraction solution that is representative of what humans are exposed to (the bioavailable compounds in the ambient mixture). However, this is challenging both from a chemical and physical property standpoint. Previous research used a combination of extraction methods to increase efficiency of removal,⁴² however there is limited research on direct chemical comparison from PM_{2.5} prepared for toxicological analyses with an ambient collected sample. The present work provides a considerably more extensive comparison between PM_{2.5} collected on a filter and the same PM_{2.5} following preparation for toxicological analyses. Significant chemical differences occurred based on the filter extraction method selected and this must be considered for future research when selecting a method as well as when comparing results between studies that use different methods. Furthermore, the alterations to the chemical and physical properties of ambient PM_{2.5} compared to material collected onto a filter is poorly understood. There is a clear need for further research into these questions to ensure that the preparation methods currently adopted are representative of the chemical mixtures present in the environment. Ideally, a standardized method for the field will be adopted with awareness of the potential chemical recovery biases that result from the selected protocol.

Developmental Toxicity Screening

Percent incidences of mortality and morphology following developmental exposure to PM_{2.5} extracts from the six methods are displayed in Figure 4. For all extraction methods, developmental exposure to PM_{2.5} resulted in 80% or higher mortality by 120 hpf, except for the single vial method which had 34% mortality. Abnormal morphological endpoints were only observed following exposure to PM_{2.5} from the single vial method or the blank filter extracts as no animals survived to measure endpoints beyond mortality in the other treatment groups. Significant mortality was associated with samples from each method, though the bioactivity differed significantly between methods at 24 hpf ($p = 0.001$, one-way ANOVA) and 120 hpf ($p = 0.001$, one-way ANOVA). This emphasizes that the chemical composition differences due to extraction method also impact sample bioactivity. It should be noted that gravimetric analysis of PM_{2.5} was not conducted following extraction and preparation of the soluble fraction from DMSO extraction and therefore differences in the compounds relative to total mass (ng compound / μg PM_{2.5}) likely differed between the extraction methods. Previous research has shown that filter extraction methods can impact the toxicity observed from PM_{2.5} exposure,¹⁹ however, this is the first study to assess bioactivity differences associated with multiple, commonly used filter extraction techniques. Because zebrafish were recently demonstrated as a model to study PM_{2.5} exposures,^{20–22} there is now a unique opportunity to establish a rapid standardized filter extraction-hazard assessment protocol in a whole animal model. This would eliminate much of the current inability for inter-laboratory comparisons of PM_{2.5} toxicity due extraction procedure and model differences.

Another important finding from this research was that filters not exposed to PM_{2.5} (“blank filters”) resulted in significant mortality and morphological changes, demonstrating that the filter extraction procedure alone releases toxicants detected using the sensitive zebrafish platform. Following the discovery of the blank filter effects, extensive chemical characterization (over 200 compounds - including elements, PAHs, and fluorinated compounds, Table S4) was conducted, but none of these compounds were detected in the blank filter extracts. The two methods that had the highest bioactive response to the blank extracts (DCM sonication and PLE) both used DCM as a solvent, however, based on unpublished research from our group we concluded that potential, residual DCM from the sonication or PLE methods alone was not responsible for the observed toxicity. The responses were only observed in combination with a blank filter suggesting that an interaction between the solvent and filter material may be occurring, this could potentially be extraction of the PTFE coating from the filter and its subsequent exposure to zebrafish. Yet over 50 fluorinated compounds were analyzed, including derivatives of PTFE, and none were detected in the blank filter extracts. While the specific compound(s) driving the observed developmental toxicity are currently unknown, it is apparent that the filter extraction method can have an impact on the developmental responses as seen by the elevated sublethal effects following exposure to the blank filter extracts. Adding collected PM_{2.5} to these already toxic methods makes it likely that the observed 100% mortality represented a shift of the sublethal blank filter effects to mortality, though we cannot be certain. Further research beyond the conducted chemical characterization, including non-targeted analysis, will identify the specific compound(s) that play a role in the blank effects. Identification of these drivers is necessary as the methods, particularly PLE, have strong,

low bias recovery of bioactive compounds present in ambient PM_{2.5}, including PAHs. Once the toxic drivers are identified, we can either identify alternative filter materials that remain inert throughout the extraction procedures, or include a compound-specific removal step that allows continued use of the PTFE filter methods.

Associations between the measured chemical constituents in the PM_{2.5} extracts and mortality outcomes in developing zebrafish were reported as odds ratios (Table 2). The odds ratios for exposure to quantified elements and mortality were not statistically significant. Significant associations were observed for 15 individual PAHs and total hydroxy-PAHs to mortality, indicating that chemical composition, specifically PAHs, played a role in the observed developmental toxicity. The chemical profiles resulting from the different extraction methods in this study all had an elevated contribution of hydroxy-PAHs compared to the Ambient SOP Sample. The higher hydroxy-PAH burden may have contributed to the higher toxicity of the associated exposures. Previous studies have shown developmental toxicity to hydroxy-PAHs⁴³ but these studies do not take into account the complex mixture present in PM_{2.5} exposures. While the observed associations align with individual chemical exposures and the limited epidemiology research with well-characterized ambient samples^{44, 45} or individual exposure data,^{46, 47} future investigation of the exposures to ambient PM_{2.5} mixtures in concentrations representative of these exposures is necessary.

We used the soluble fraction of PM_{2.5} which eliminated particle agglomeration concerns but precluded our measuring the bioactivity of particle-sorbed chemicals and potential alterations in bioavailability. This research used a single hi-volume PM_{2.5} filter sample from an urban, traffic-dominated location in the winter. The translation of these findings may differ when using alternative sampling designs, filter types, and collection of PM_{2.5} with different source contributions. Further research into the impacts of filter extraction methods is crucial to the field to enable accurate inter-laboratory comparisons from representative ambient mixtures.

PM_{2.5} that is representative of the complex mixtures that occur in human exposures is essential to better understand the biological outcomes and mechanisms associated with these exposures. We have demonstrated through characterization of elements and PAHs that commonly used extraction procedures do not maintain chemical compositions that are representative of the ambient mixture. Further research into the recovery of elements and PAHs as well as additional components of PM_{2.5} (additional organic and inorganic compounds, salts, etc.) will provide PM_{2.5} samples that are more representative of the ambient mixture and thus enable improved toxicology research. Similar studies of PM_{2.5} samples with differing source contributions will give a broader description of how well these exposures recapitulate the ambient mixture composition, as well as provide insight into improving current extraction methods. For example, using multi-step extraction methods to recover a broader range of compounds.¹⁶ A balance between creating representative mixtures and cost-efficiency is required to achieve this goal. For our current research goals, we have selected sonication in methanol for future studies due to its low cost, comparatively small blank filter effect, ability to extract hydrophobic and hydrophilic compounds, and already frequent use in PM_{2.5} toxicology studies.^{10, 48, 49}

The limitations of the extraction method selected must be considered when interpreting results, particularly during inter-laboratory comparisons where the extraction methods may be influencing observed differences, regardless of the actual differences in PM_{2.5}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Concentrations and Percent Contributions of Quantifiable Elements. Concentrations of detected elements (ng/ μ L, a) are reported for each extraction method following blank filter correction for the corresponding extraction method. Elements that were below detection limits are indicated by the color-specific symbol “”. A statistically significant difference (one-way ANOVA, $p < 0.05$) from all other extraction methods for concentrations of an element is signified by *. For significant differences between specific extraction methods, lettering of methods (a–f) is used to indicate a significant difference. Elemental contributions (b) are presented for each extraction method as well as the Ambient SOP Sample which was measured using standard procedures for chemical characterization directly from the filter without preparation steps for toxicology studies, these samples are indicated as “Ambient.” The term “Other” refers to quantified elements that were detected in the Ambient SOP

Sample but in none of the extraction method samples and include: As, Cd, Cr, Li, Na, Ni, Sr, and V. “SV” indicates the single vial extraction method.

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Figure 2.

Concentrations and Percent Contributions of Quantifiable Classes of PAHs. Concentrations of detected summed classes of PAHs (pg/uL, a) are reported for each extraction method following blank filter correction for the corresponding extraction method. Nitro-PAHs are excluded from visual representation in Figure 1a as only PLE had quantifiable compounds (2.517 ± 0.0828 pg/uL), nitro-PAHs in all other extraction methods were below detection limits. Classes of PAHs that were below detection limits are indicated by the color-specific symbol “”. Statistical significance (one-way ANOVA, $p < 0.05$) from all other extraction methods for concentrations of a class of PAHs is signified by *. For significant differences between specific extraction methods, lettering of methods (a–f) is used to indicate a significant difference. PAH class contributions (b) are presented for each extraction method as well as the Ambient SOP Sample measured using standard procedures for chemical

characterization directly from the filter without preparation steps for toxicology studies, these samples are indicated as “Ambient”. “SV” indicates the single vial extraction method.

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Figure 4.

Heatmap of Percent Incidence of Mortality and Morphological Changes Following Developmental Exposure to PM_{2.5} or Blank Filter Extracts. The x-axis lists the extraction method used and whether the treatment was to PM_{2.5} or blank filter extracts (n=32 animals/treatment group). Y-axis indicates measured outcome, all outcomes are at 120 hpf unless noted as 24 hpf. Increasing color intensity indicates an increase in the percent of animals with the specific outcome. Groups with near 100 % mortality (dark blue shading) did not have morphological endpoints as no viable animals were present to assess these sublethal outcomes.

Table 1.Description of Different PM_{2.5} Extraction Methods

	1: Water	2: MeOH	3: DCM	4: DMSO	5: Single Vial	6: PLE
Removal	Sonication in Water	Sonication in MeOH	Sonication in DCM	Sonication in DMSO	Sonication in DMSO	Pressurized Liquid Extraction (DCM:EA)
Concentration	Freeze Drying	N ₂	N ₂	Solvent exchange N ₂	N/A	N ₂
Reconstitution	DMSO	DMSO	DMSO	DMSO	N/A	DMSO

Table 2.

Chemical Exposure and Biological Response Association Table

Compound	Odds Ratio	p-value
Parent PAHs		
Retene	2.754	6.10E-07
Benzo(a)pyrene	1.372	5.28E-05
Benzo(b)fluoranthene	1.257	4.96E-04
Benz(e)pyrene	1.210	7.18E-05
Benzo(k)fluoranthene	1.131	3.55E-04
Indeno-1,2,3-cd-pyrene	1.066	1.06E-06
Chrysene/Triphenylene	1.046	3.68E-06
Benzo(a)anthracene	0.879	2.97E-10
Benzo(ghi)perylene	0.775	5.04E-05
Oxy-PAHs		
Benzanthrone	1.339	4.78E-05
Hydroxy-PAHs		
1-Hydroxynaphthalene	1.467	5.02E-05
3-Hydroxyfluorene	1.147	1.43E-04
1-Hydroxyphenanthrene	1.082	5.96E-11
2-Hydroxyphenanthrene	1.072	1.69E-10
4-Hydroxyphenanthrene	1.070	1.85E-05
2-Hydroxy-9-fluorenone	1.053	3.21E-10
3-Hydroxyphenanthrene	1.049	4.05E-06
Total Hydroxy-PAHs	1.005	1.13E-04