Protocol Development for Vehicle Emission Toxicity Testing for Particulate Matter

Keith Bein (PI), Chris Vogel and Norm Kado
University of California, Davis
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Motivation and Significance

- Continually evolving emissions and exposure landscapes (exposome)
  - Advancing technologies, changing practices, shifting regulations, emerging mitigation strategies…

- Constant need to evaluate for intended impact

- Main impetus is protecting human health
  - How do we assess?
    - Epidemiologically (correlative) versus toxicologically (causative)
  - What biological system do we use?
    - Animal model, cell culture, organoid…
  - How do we expose and at what dose?
    - Bolus versus inhalation, acute versus chronic, ambient versus concentrated, injection versus ALI deposition
  - How do we collect sample?
    - Field sampling versus laboratory generated
  - How do we extract and prepare samples?
    - Exhaustive versus selective
  - What endpoints do we test and at what timepoints?
    - Pulmonary, cardiovascular, neurological…
    - Inflammation, oxidative stress, mutagenicity…

- Outcome dependent upon variable selection but exactly how and why is not well understood
Research Objective and Hypotheses

- Investigate effect of PM sample preparation technique on outcome of standard assay panel using filter samples from dynamometer study
  - Single study → constant assay panel → vary PM preparation technique
  - Null hypothesis: outcomes independent of PM preparation technique
  - Test hypotheses
    i. Depends on nature of extraction
      - Exhaustive (whole PM) versus selective (fractionated PM)
    ii. Depends on extraction solvents
      - Polar protic versus polar aprotic versus nonpolar
    iii. Toxicological matrix effects
      - Sum of responses to fractionated PM > response to PM composite or whole PM
      - Antagonistic and/or synergistic effects
      - Toxicologically inert components interfere with response to active ones
      - Particle matrix inhibits bioavailability of active components
    iv. Most robust and reliable PM preparation technique is assay dependent
  - End deliverable: standard operating procedure for PM sample preparation
    - For DEP as a function of assay
Fundamentals

➢ Sample Preparation
   ❖ Three step process
     i. *Pretreatment* – physical/chemical characteristics altered prior to extraction
     ii. *Solvent Extraction* – components separated from liquid, solid or semi-solid matrix and dissolved in compatible solvent
        ▪ Several different extraction techniques currently available
     iii. *Post-extraction cleanup* – matrix residues and interfering co-extractable compounds removed
        ▪ SPE: Filtering, adsorbents (silica, alumina…) and ion exchange interfaces

❖ Exhaustive versus Selective
   • Exhaustive – maximize number and amount of components extracted
     ▪ PM extractions → whole PM
   • Selective – maximize specific components and minimize co-extraction of others
     • PM extractions → fractionated PM
Existing Extraction Techniques

- **Solvent Extraction** – diffusion based process (↔ matrix); *function*(solvent, energy, time)
  - **Heat Reflux Extraction (HRE)**
    - Reflux in solvent(s) for extended periods
    - Simple, cost-effective and small scale
  - **Soxhlet Extraction (SoxE)**
    - HRE with in-line filtration
  - **Sonication Extraction**
    - Ultrasonic energy breaks down matrix
    - ↑ extraction efficiency; ↓ extraction time
  - **Microwave-Assisted Extraction (MAS)**
    - Microwave radiation heats sample in solvent
    - High throughput and efficient (time + solvent)
  - **Supercritical Fluid Extraction (SFE)**
    - Hybrid gas-/liquid-like properties (↑ diffusivity + ↓ viscosity = ↑ penetration)
    - Special handling requirements → expensive → limited applicability
  - **Pressurized Fluid Extraction**
    - ↑ pressure + temperature → ↓ viscosity → ↑ extraction kinetics
    - High throughput, fast and efficient (time + solvent)

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## Solvent Selection

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Sample Collection & Extraction Objectives

Filter Extractions

- PM sampled from environment by drawing air across collection substrate
  - Ambient atmosphere, smokestack, fence line, interiors, lab generated exhaust
  - Numerous collection substrates including various filter types
- Sampled PM extracted from collection substrates for subsequent studies
- Extraction objectives depend on study
  - In vitro studies
    - Conserve original physical and chemical properties of PM
    - Represent true population exposure as much as possible
  - Chemical characterization
    - PM chemically complex → extraction depends on analytical technique
    - Maximize selective extraction and minimize co-extraction/interference
  - In vivo studies
    - Assays designed for specific endpoints; e.g. inflammation, ROS, mutagenicity…
    - Elicit most robust, reliable and repeatable response for given assay
    - Best extraction technique likely assay specific
Particulate Matter Source

- **Diesel Exhaust Particles (DEP)**
  - Most heavily studied source of air pollution
  - Greater than four decades of research and an exhaustive literature
    - Epidemiological studies date back to 1950s
    - US EPA diesel emissions research program established in 1979
    - Carcinogenic/mutagenic chronic exposure studies of the 1980s
    - Characterization studies of the 1990s and 2000s
      - Stationary, mobile, roadside, tunnel, dynamometer, laboratory generated
      - Detailed physical and chemical characterization; emission factors and rates
  - Exponential ↑ in sophistication, scope and resolution of toxicity testing
    - Assays; e.g. reverse transcription polymerase chain reaction (RT-PCR)
    - Animal models; e.g. transgenic and knockout mice
    - Cell lines; e.g. human lung epithelial cells, macrophages, stem cells…
    - Endpoints; e.g. pulmonary, cardiovascular and neurological
    - Injury metrics; e.g. protein levels, gene expression and regulation
    - Exposure scenarios; e.g. CAPS, source-oriented toxicity; adjuvant effects
Common DEP Extraction Techniques

- Liquid Impingers; e.g., Ethanol
  - Solvent Concentration Assay

- Dynanometer Study Dilution Sampling
  - Whole Free DEP or DEP Filter Deposits

- Exhaustive Extraction Whole Particle
  - Direct Suspension & Dispersion in Medium
    - Agitation Vortexing
    - Sonication
  - Solvent Sonication (H₂O, Me/MeOH, DCM)
    - Single Solvent
    - Solvents in Series
    - Solvent Removal Vac Evap/N₂ blowdown
    - Resuspension in Medium
    - Assay

- Selective Extraction Particle Fractionation
  - Common Solvents DCM, MeOH, Hx, Bz
    - Soxhlet Extraction
    - Sonication Extraction
    - Pressurized Fluid Extraction
      - Single Solvent
      - Solvents in Series
      - Micropore Filtration Column Cleanup
      - Solvent Removal Vac Evap/N₂ blowdown
      - Assay
    - Resuspension in Medium
      - Assay
    - Single Ex
    - Assay Composite Ex
Experimental Design

3 samples × 6 sample preparation techniques × 5-pt assay panel

- Chassis Dynamometer DEP Filter Sample (*Dyno-DEP*)
  - CARB Heavy-Duty Engine Emissions Testing Laboratory
  - 2000 Freightliner Truck w/2000 Caterpillar C15 engine
  - California certified Ultra-Low Diesel Fuel
  - Urban Dynamometer Driving Schedule (UDDS)
  - Samples drawn from dilution tunnel
  - High-Volume filter unit loaded with Teflon filter sheets (8”x10”)
  - Collection time ~ 35 minutes (2 UDDS test cycles)

- Chassis Dynamometer Filter Blank (*FB*)

- NIST Standard SRM 1650 (*NIST-DEP*)
Experimental Design

3 samples × 6 sample preparation techniques × 5-pt assay panel

➢ Two exhaustive (whole particle) extractions
  • Single solvent sonication extraction (SS-SE)
    - Samples sonicated in water
  • Serial multiple solvent sonication extraction (SMS-SE)
    - Samples sonicated in series using DCM, MeOH and Tol
    - Reconstituted into composite DEP extract

➢ Four selective (PM fractionation) extractions
  • Single solvent heat reflux extraction (SS-HRE)
    - Samples extracted individually in DCM, MeOH and Tol
  • Serial multiple solvent heat reflux extraction (SMS-HRE)
    - Samples extracted in series using DCM, MeOH and Tol
    - Reconstituted into composite DEP extract

➢ Independent replications pooled to account for systematic errors in prep
Experimental Design

3 samples × 6 sample preparation techniques × 5-pt assay panel

• U937 Macrophages + qPCR
  • Reactive oxygen species (ROS) production
    • Acellular dithiothreitol (DTT) assay
  • Inflammation
    • Cyclooxygenase-2 (COX-2) and interleukin 8 (IL-8)
  • Cellular response to PAHs
    • Cytochrome P450 1A1 (CYP1A1) expression
  • Mutagenicity
    • Ames assay
• 3-point dose response curve for each combination of assay and extract
  • Repeat in duplicate at linear part of dose response curve
# Measurement Matrix

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<th>Assay</th>
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<th>H2O-SE</th>
<th>DMT-SE</th>
<th>DCM-HRE</th>
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Extraction Apparatus

Heat Reflux, Vacuum Distillation and N₂ Blowdown

Syringe Filtrations
Extraction Results

- Extraction efficiency as function of solvent for both HRE and SE
  - DCM > Toluene > MeOH > H₂O
- HRE more efficient than SE
- 30-60% of extracted DEP removed by post-extraction filtration
CYP1A

- Significantly upregulated by all DEP extracts relative to vehicle control
- NIST-DEP expression greater than Dyno-DEP for given extraction
- For Dyno-DEP and NIST-DEP
  - HRE > SE
  - HRE-DMT largest
  - DMT-SE > H2O-SE
IL-8

- NIST-DEP expression greater than Dyno-DEP for given extraction
- For Dyno-DEP and NIST-DEP
  - HRE > SE
  - DMT-HRE largest
  - DMT-SE > H2O-SE
- Dyno-DEP H2O-SE not significantly different that FB extract
COX-2

- All extracts significantly above control
- Only DCM-HRE and DMT-HRE of Dyno-DEP significantly above FB
- *NIST-DEP* expression greater than *Dyno-DEP* for given extraction
- For *Dyno-DEP* and *NIST-DEP*
  - HRE > SE
  - DMT-HRE largest
  - DMT-SE > H2O-SE
- *Dyno-DEP* H2O-SE not significantly different that FB extract
CYP1A1
Dose Response
IL-8 Dose Response
COX-2
Dose Response
Mutagenicity

- **NIST-DEP activity >> Dyno-DEP**
- **HRE activity > SE for both DEP**

- **DMT-SE > H2O-SE**
- **H2O-SE not significant**
### Reactive Oxygen Species Production

- **DTT consumption generally low**
- **MeOH-HRE of FB largest**
- **Data noisy and uninformative**

#### DTT Consumption

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- **NIST-DEP consumption >> Dyno-DEP**
- **MeOH-HRE largest**
- **Repeatability issue for DCM-HRE**
Evaluation of Test Hypotheses

➢ Outcomes dependent upon extraction technique; i.e. exhaustive versus selective
  • Observed in all data for both Dyno-DEP and NIST-DEP
  • HRE (selective) consistently greater than SE (exhaustive)
  • HRE amplifies effect of active components by removing inactive matrix

➢ Outcomes dependent upon solvent system
  • Observed in both Dyno-DEP and NIST-DEP
  • DMT-SE consistently greater than H2O-SE
  • CYP1A1 greatest for Toluene; most efficient at solvating PAHs
  • DTT consumption greatest for MeOH; polar compounds driving ROS

➢ Toxicological matrix effects; component sum greater than composite
  • Not possible to evaluate on equal mass dose basis
  • Inferred from NIST-DEP dose response curves for HRE
    - DCM + MeOH + Toluene (lowest dose) >> DMT at highest dose

➢ Robustness and reliability of sample preparation technique assay dependent
  • DTT consumption greatest for MeOH-HRE
  • CYP1A1 expression greatest for Tol-HRE
Conclusions

- For given extraction technique, response to *NIST-DEP* >> *Dyno-DEP* for all assays
  - Attributed to compositional differences in DEP
    - *NIST*: collected in 1984; several uncontrolled diesel engines; scraped of heat exchanger surfaces; higher sulfur and aromatic content in fuel
    - *Dyno*: collected from single modern (2000) diesel engine; sampled onto filter from dilution tunnel; ultra-low sulfur fuel

- HRE consistently enhances response of active components by removing inactive matrix components included in SE extracts

- Nonpolar compounds elicit greatest response across all assays except ROS production, which is largest for more polar DEP components

- Strong evidence for existence of composite interference or toxicological matrix effects

- Although different techniques appear equally repeatable across assays, those eliciting most robust response are assay-specific
Recommendations

- **Chemical Characterization**
  - Are compositional differences driving observed differential responses?
  - Explore relationship between PM compositional complexity and toxicity

- **Selective versus Exhaustive Extraction**
  - Fractionated DEP extracts consistently elicit greatest response
  - Complex particle mixtures better characterize true human exposure
  - Which method is the more appropriate evaluation metric?

- **Standardization**
  - Single method for all assays: DCM-HRE
  - Assay specific methods
    - MeOH-HRE for DTT
    - DCM-HRE for molecular markers and mutagenicity
    - Tol-HRE for CYP1A1
  - Exhaustive method
    - Compare HRE without post-extraction filtration to SE

- **Dyno-DEP Filter Samples**
- **Labware Selection**
Questions?

- Keith Bein (kjbein@ucdavis.edu)
- Christoph Vogel (cfvogel@ucdavis.edu)
- Norman Kado (nykado@ucdavis.edu)