EBPI Technical Document 1.3.2

Extraction techniques for mutagens and genotoxins in Particulate Matter (PM) from Filtered Air Samples

1.0 Purpose

This document aims to provide guidance for employees of EBPI and customers (if desired) on proper methodology for extraction of organic contaminants contained in particulate matter after initial filtration before testing these extracts using EBPI bacterial-based genotoxicity and mutagenicity products. Literature reviews will present available techniques that may be employed by clients based on their technical expertise but will detail two techniques that can be performed at the EBPI laboratory for this purpose. This document is meant to be used as a reference guideline and variations on stated techniques can be performed if desired.

2.0 Scope

This applies to all technical procedures performed by EBPI in their laboratory for the extraction of potential mutagens and genotoxins from air particulate or volatile organic compounds trapped on specialized filters including glass, quartz, teflon, or other approved materials. Employees of EBPI should follow these procedures strictly. If requested by scientists or personal outside of EBPI, the information in this document should be employed only as a guide.

3.0 Abbreviations

DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
EBPI	Environmental Bio-detection Products Incorporated
PAH	polyaromatic hydrocarbons
PCB	polychlorinated biphenyls
PM	particulate matter
EF	Erlynmeyer flask
RB	round bottom flask
RE	rotary evaporator
VF	volumetric flask
VOC	volatile organic compounds

4.0 Introduction:

To properly assess mutagenicity of air particulate, it is important to use an extraction technique that separates genotoxic materials from solid air filters without excessive artifacts or high background interference from the matrix. These materials must then be transferred to a compatible solvent with the Muta-Chromoplate™ SOS-Chromotest™ and UMU-Chromotest™ in a small concentrated volume to encourage a response and lower detection limits for the overall procedure. Adsorption of compounds by soil is influenced by diverse factors such as organic matter content, soil type, and physical-chemical properties of the contaminant like vapor pressure, water solubility and n-octanol-water partition coefficient.[1] Therefore when considering the appropriate method for extraction, one must understand the chemical properties of the compounds of interest, the appropriateness of the extraction solvent, equipment or laboratory capabilities as well as time and budget constraints.

Although many larger PAHs have lower volatility, they are adsorbed onto the surface of PM and can be carried to the absorptive surface in the lungs and contribute significantly to human exposure. In addition, to these PAHs, many compounds contained in air pollution have been classified by IARC as probable (group 2A), and possible (group 2B) carcinogens.

Identifying and quantifying human exposure to these genotoxic compounds is critical to accurately assess public health risk.[1] The diversity of toxicants contained in air pollution, and their potential interactions means that proper risk assessment cannot be adequately addressed using chemical analysis alone. Biological effect measurements, like mutagenicity testing, improve risk assessment by analyzing effects from unidentified substances, and can account for synergistic or antagonistic effects from interactions between individual pollutants. Academic research conducted in this area, has shown that PM from industrial and urban pollution caused significant positive responses in mutagenesis assays.[2,3]

Because of these considerations, researchers have employed various filter types and extraction techniques with variable levels of success that depend on pollutant source, particle size and extraction solvent. Quartz filters[4], membrane filters coated in Teflon[5] and glass fiber filters.[6] A review of the literature strongly suggests the use of quartz and Teflon coated filters based on extraction of PM2.5 efficiency and minimal test artifacts from filter material.[7] EBPI recommends these two filter types for air particulate extraction procedures. Please contact a representative at EBPI if there are any questions regarding the potential use of a filter material other than those listed above.

As many contaminants of interest like polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and heterocyclic polyaromatics contain non-polar, lipophilic groups, successful solvent choice has traditionally focused on non-polar organic solvents like dichloromethane (DCM), chloroform and acetone, as well as solvent mixtures with polar protic alcohols like

methanol to combine traits and extract contaminants with more polar groups (nitro, amide, hydroxyl)

Due to technical limitations at the EBPI laboratory, two extraction procedures, sonication and soxhlet extraction will be covered in detail for use in the lab. More recent commercial systems are available but extraction efficiencies often do not change significantly[8] and it goes against our general company testing strategy which promote reliable genotoxicity data acquisition with limited knowledge and expertise.

If there are any questions regarding the procedures detailed in the technical document, please refer to the references given in section **8.0**. The two procedures detailed in this document were chosen based on their simplicity and efficiency for extracting complex mixtures of non-polar organics, PAHs and PM contaminants from filtered air samples. For adequately filtered air samples from contaminated sources with the use of correct solvents, tests can detect contaminants with high efficacy.

5.0 Safety

It is the personal responsibility of every employee of EBPI to be fully aware of the hazards involved with storage, handling and dispensing of all chemical reagents as well as the safe operation of the proper laboratory machinery. If you are at all concerned with any procedures highlighted in this document, consult the laboratory manager or Dr. Aaron Witham or Mr. Will Lush before proceeding.

5.1 General Safety Concerns

1. Never eat, drink, or smoke while working in the laboratory.
2. Read all labels carefully.
3. Do not use any equipment unless you are trained and approved as a user by your supervisor.
4. Wear safety glasses or face shields when working with hazardous materials and/or equipment.
5. Wear gloves when using any hazardous or toxic agent.
6. Clothing: When handling dangerous substances, wear gloves, laboratory coats, and safety glasses. Shorts and sandals should not be worn in the laboratory at any time. Shoes are required when working in the machine shops.
7. If you have long hair or loose clothes, make sure it is tied back or confined.
8. Keep the work area clear of all materials except those needed for your work. Coats should be hung in the hall or placed in your office. Extra books, purses, etc. should be kept away from equipment.
9. Disposal - Students are responsible for the proper disposal of used material if any in appropriate containers.

10. Equipment Failure - If a piece of equipment fails while being used, report it immediately to the laboratory supervisor
11. If leaving a lab unattended, turn off all heat sources and lock the doors.
12. Never pipette anything by mouth.
13. Clean up your work area before leaving.
14. Wash hands before leaving the lab and before eating.

5.2 Chemical Safety

1. Treat every chemical as if it were hazardous. Make sure to read MSDS sheets carefully before attempting to use any chemical or solvent
2. Make sure all chemicals are clearly and currently labeled with the substance name, concentration, date received and name of the recipient.
3. Never return chemicals to reagent bottles. (Try for the correct amount and share any excess.)
4. Comply with fire regulations concerning storage quantities, types of approved containers and cabinets, proper labeling, etc. If uncertain about regulations, contact the building coordinator.
5. Use volatile and flammable compounds only in a fume hood. Procedures that produce aerosols should be performed in a hood to prevent inhalation of hazardous material.
6. Never allow a solvent to come in contact with your skin. Always use gloves.
7. Dispose of waste and broken glassware in proper containers.
8. Clean up spills immediately.
9. Do not store food in laboratories

5.3 Bacterial Safety

1. Although bacteria used in the laboratory is non-pathogenic, always be careful to ensure sterilization whenever possible.
2. Use aseptic techniques when dispensing all bacteria-related reagents, growth media etc.
3. Always sterilize work space using alcohol prior to any procedure
4. Maintain proper storage conditions for lyophilized bacteria and reagents at all times.
5. Dispose of all reagent bottles, plates, reagent boats, tubes and pipette tips in the proper biohazard bag
6. Autoclave all garbage prior to disposal to ensure sterility

6.0 Sample Collection. Preservation and Handling

Various source and ambient air sampling systems is beyond the scope of this protocol. If more information on specific sampling systems is required contact EBPI for more information.

6.1. Ambient air sampling

Ambient air sampling techniques include the standard high-volume (Hi-Vol) samplers, massive-volume samplers,[9] medium-volume samplers, low-volume samplers and ultra high-volume samplers.[10]

The sampling of PM in ambient air is usually performed with the objective of determining the particle size distribution and the nature and concentrations of individual components at various points in the environment. The selection of sampling sites plays an important role in sample collection. Sampling sites are determined to evaluate the following: (1) characterization of rural or urban background levels, (2) assessment of health hazards to people in the vicinity, (3) determination of source effects and (4) establishing of transport mechanisms.

In addition to the selection of a preferable method and site, certain other factors must be adequately taken into consideration during ambient sampling. The height of the sampler intake above ground level and the local topography and climate influence the data obtained. For example, the influence of summer-like temperature on losses of benzo(a)pyrene from airborne particles has been studied during real high-volume atmospheric samplings.[11] Seasonal variation in the specific surface areas and densities of suspended particulate matter has been observed.[12] The effect of wind on the collection efficiencies of particulate matter has also been demonstrated.[13] Other factors, such as sampling rate and time, are also important in the overall sampling strategy. The total amount of samples to be collected usually depends on the amount necessary to perform the bioassay. The optimum amount of air particulates needed for both bioassay and chemical analyses is approximately 2 g. However, it is possible to do the genotoxicity assays from EBPI with much less (≤ 100 mg of starting material).

The filters must be cleaned and dried completely before the analysis. They should arrive clean but should be dried in an oven at 130 °C prior to use. Filters should be weighed on the analytical balance prior to the test after testing we will measure as well and the mass difference, coupled with the particle counter should give appropriate measures of particle composition. Ensure that the filter intake volume is compatible with the filter of choice. May need to adjust the flow of the intake or customize the filter device to match up.

6.2 Preservation and Handling

After sampling, filters are folded into quarters (sample touching sample), held in glass-lined bags or glass containers during transport to the laboratory from the field. Ensure that the container has a good seal to minimize sample loss during transport

The filters must be equilibrated to laboratory temperature and humidity for 4 hours and are then weighed by a microbalance to determine the total weight of air particulate.

Place the filters back into their original container and store at -20 °C until solvent extraction procedure.

7.0 Equipment Needed

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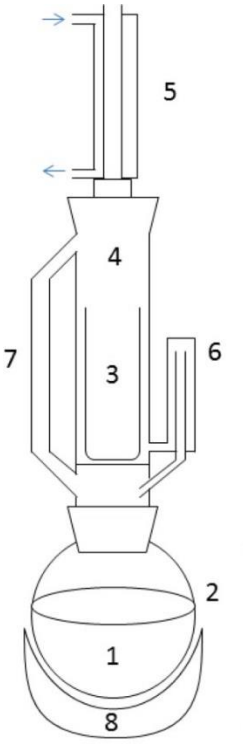
Soxhlet Extaction (7.1)

A) RB flasks of increasing sizes 100 mL to 500 mL
B) Soxhlet thimble
C) Soxhlet extractor
D) Condenser with cold water available
E) Siphon
F) Side arm adapter (lagged with glass wool)
G) Heating Mantle
H) Oven
I) Dessicator
J) Sodium sulphate (anhydrous)
K) Sand
L) Extraction solvent
M) Rotary evaporator

Figure 1: Soxhlet extraction apparatus diagram (taken from[14])



1 – Solvent (ethanol)
2 – Round bottom flask
3 – Soxhlet thimble
4 – Soxhlet extractor
5 – Condenser with running water
6 – Siphon
7 – Side arm (lagged with glass wool)
8 – Isomantle (heat source)

Sonication Extraction (7.2)

A) EF of different sizes (50 to 250 mL)
B) Homogenizer
C) Sodium sulphate (anhydrous)
D) RB flasks of different sizes (100 to 250 mL)
E) Sonication bath
F) Vacuum filtration apparatus with filter (0.45 µm)
G) Rotary evaporator
H) Extraction solvents
I) Reconstitution solvent (DMSO)

7.0 Extraction Procedures

7.1 Soxhlet Extraction

Based on EPA method 3541[15]

1. Place boiling chips into solvent vessel (RB flask or cylindrical flask)
2. Dry solvent flask in a drying oven at 110 °C for one hour
3. Fill the silica gel into the dessicator and insert the dessicator plate allow the dessicator to cool to room temperature (30 minutes)
4. Weigh the solvent vessel with boiling chips to 0.001 g
5. Fold filter into a bundle to prevent sample loss and introduce it into the thimble. Filter can also be cut into small pieces directly into the thimble to increase surface area.
6. Extraction thimble is weighed to the nearest 0.001 g
7. The extraction thimble is closed with fat-free cotton wad and placed into the Soxhlet extractor. If extractor has a spigot for sample draining, close the spigot. Ensure setup appears like figure 1 and ask supervisor to inspect before proceeding.
8. Fill the solvent into the solvent vessel and extract at temperatures of 110-130 °C for 20 -10 extraction cycles depending on both the nature of the sample and extraction solvent.
9. Open the spigot and drain the solvent into a clean, dry container and continue heating until all the solvent has gone through evaporation and condensation
10. Evaporate solvent in the sample thimble by RE and further drying in the oven
11. Dry completely and allow to cool to room temperature in dessicator
12. Weigh the dried extract and redissolve in minimal DMSO
13. Clean up area properly and wash all glassware.
14. Perform mutagenic analysis with extract on EBPI genotoxicity tests.

7.2 Sonication Extraction

Based on EPA method 3550B[15]

1. Fold filter into a bundle to prevent sample loss and weigh the filter to the nearest 0.01 g on an analytical balance.

2. Filter can also be cut into small pieces directly onto analytical balance prior to weighing to increase surface area.

3. Introduce filter or filter pieces into a clean DRY VF

4. Add extraction solvent (10 mL)

5. Manually homogenize the sample in the solvent (if possible) and ultrasonicate the sample for 20 minutes at room temperature in a sonicator bath.

6. Swirl occasionally to ensure solvent is mixed sppropriately and to keep filter pieces off the sides of the flask

7. Filter extraction solvent through a 0.45 µm filter using vacuum filtration.

8. Ensure filtrate is dry beforehand by drying with anhydrous sodium sulphate. Alternatively, filtrate can be passed directly through sodium sulphate on top of filter

9. Repeat the extraction 2 more times in the same solvent.

10. Combine the extracts in a large RB flask that has been pre-weighed and dried in the oven

11. Evaporate the solvent to dryness using RE and an oven

12. Mass the vial again and use differential masses to measure the mass of organic extract

13. Redissolve the organic extract in minimal DMSO (1-5 mL)

14. Clean up area properly and wash all glassware

15. Perform mutagenic analysis on redissolved extract with EBPI genotoxicity and mutagenicity assays

7.3 Storage of samples

All extracted samples should be immediately stored on ice or in a refrigerator. DMSO will freeze at 19 °C and it is important that the sample be stored cool to prevent evaporation of volatile organic compounds from the extract.

Long term storage should occur in a fridge or freezer at 4 °C to -20 °C in properly labelled containers with date extracted, mass of extract and technician clearly stated. Mutagenicity and genotoxicity assays should be run as soon as possible after extraction has been completed. Maximum storage time from extraction date and sample run date should not exceed 2 weeks (14 days). Tests run after this time period will be considered compromised.

7.4 Solvent selection

The extraction solvent can be individually chosen but the most popular solvents based on the literature are

- DCM[13].
- Ethyl acetate[4]
- Acetonitrile[14]
- Acetone[15]
- Ethanol[16]
- Hexane:Acetone 1:1[17]

7.5 Documentation

All documentation should be kept according to EBPI's records **(form 1.3.1a)**. Proper experimental write up should be done for all extraction procedures. Justification for solvent use, and drying procedures should be discussed with laboratory manager prior to experiment start.

Final masses of extracts, quality checks, storage dates and technician information should be recorded.

Lab manager must sign off on documentation before completion of extraction will be recorded.

7.6 Limitations

This method has some limitations. Samples with appreciable moisture content will not undergo proper extraction and separation, will have erroneous masses of final extract and will not be completely evaporated at the conclusion of the extraction. Water should be strictly avoided until after extraction is complete and sample has been redissolved into DMSO.

All glassware, filters and solvents should be bought in dry format, or carefully dried using appropriate solvent drying procedures. All glassware should be dried in an oven prior to use and cooled to room temperature under an inert atmosphere if possible.

Portable sonicators can be used however, sonicator baths will work better and will decrease contamination from hand held devices.

8.0 References

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