ENVIRONMENTAL HEALTH

Effect of Three Extraction Techniques on Submitochondrial Particle and Microtox® Bioassays for Airborne Particulate Matter

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The effect that three extraction techniques (e.g., Soxhlet, ultrasound and microwave-assisted extraction) have on the toxicity, as measured by submitochondrial particle (SMP) and Microtox® assays, of organic extracts was compared from three sources of airborne particulate matter (APM). The extraction technique influenced the toxicity response of APM extracts and it was dependent on the bioassay method, and APM sample source. APM extracts from microwave-assisted extraction (MAE) were similar or more toxic than the conventional extraction techniques of Soxhlet and ultrasound, thus, providing an alternate extraction method. The microwave extraction technique has the advantage of using less solvent volume, less extraction time, and the capacity to simultaneously extract twelve samples. The ordering of APM toxicity was generally urban dust > diesel dust > PM10 (particles with diameter < 10 µm), thus, reflecting different chemical composition of the samples. This study is the first to report the suitability of two standard *in-vitro* bioassays for the future toxicological characterization of APM collected from Puerto Rico, with the SMP generally showing better sensitivity to the well-known Microtox® bioassay.

Key words: Air particulate matter, Submitochondrial particles, Microtox®, Extraction techniques, Toxicity

Airborne particulate matter (APM) of anthropogenic origin is a major health concern because it contains a mixture of toxic sorbed chemical compounds like polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and inorganic elements (1-4). The complexity of APM makes determining their chemical composition difficult, so researchers are relying more on toxicological bioassays. Most of the toxicological characterization of APM has been conducted with the Ames Salmonella mutagenicity test (1, 5-18). Understanding that the extraction technique of APM could significantly influence the result of the mutagenicity test, different conventional extraction techniques such as Soxhlet and ultrasound, with different organic solvents, have been compared (14-18). For instance, Morin et al. (15) obtained consistent mutagenic activity of airborne particles with ultrasound extraction when compared to Soxhlet. An alternate extraction technique for the removal of organic extracts from APM is the microwave assisted extraction (MAE). However, MAE has been mainly used for the quantitative recovery of known environmental organic chemical compounds (19, 20). Its efficiency in removing organic extracts of toxicological interests in comparison to other conventional methods has not been documented for APM.

Although APM is known to contain mutagenic chemical compounds, its organic extract may include chemicals with different modes of toxic actions (2, 21). Therefore, toxicological assays that measure toxic responses other than mutation need to be evaluated. The submitochondrial particle (SMP) and the Microtox® are two short-term in-vitro bioassays tests that measure the effect of toxicants on energy-coupled enzymatic reactions taking place in the membrane (22). The median effective concentration that causes 50% inhibition (EC50) in each test is used for toxicity comparisons. In the Reverse Electron Transfer

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(RET) variant of the SMP test, used in this study, a biochemical reaction produces nicotinamide adenine dinucleotide (NADH) that is measured by a spectrophotometer (23, 24). Toxicity to SMP is indicated by a decrease in the rate of NADH formation. In the Microtox® assay, toxicity is expressed by a decrease in bacteria (*Vibrio fisheri*) bioluminescense (25). Both tests are easy to use and correlate well with in-vivo bioassays, obtaining wide applications for testing water, soils, and sediments, as well as pure chemical compounds (22, 23, 25).

Despite their broad environmental applications, no publication describes the use of either method to evaluate the toxicity of APM, and on how the extraction technique affects their toxic responses. The purpose of this study is to compare the effect that three extraction techniques (Soxhlet, ultrasound and MAE) have on the toxicity, as measured by SMP and Microtox® assays, of organic extracts from three sources of APM in order to apply it to future toxicological characterization of APM collected from Puerto Rico.

Materials and Methods

Sampling. APM samples consisted of standard reference materials (SRMs) 1649 "urban dust", and 1650 "diesel particulate matter", referred in this study as diesel dust (National Institute of Standards & Technology, Gaithersburg, MD). In addition, APM with diameter < 10 µm (PM10) obtained from urban Guaynabo, Puerto Rico, were included after collection over a 24-hr period in November 2000 using a high-volume sampler at 1 m^3/min by the Puerto Rico Environmental Quality Board. Two composites were prepared for PM10. One PM10 composite consisted of five quartz filters sampled during the 24-hr collection period, while the other composite was from five unused quartz filters to be used as a laboratory blank. PM10 filters were cut into eight strips, individually weighed, wrapped in aluminum foil and stored in a freezer at -20°C, until further analysis.

Extraction. Triplicate portions of the urban (100 mg each) and diesel dusts (25 mg each), and duplicate portions of PM10 (100 mg each) were used for each extraction technique. The extraction solvent was 1:1 (v:v) hexane:acetone, a proportion recommended by the United States Environmental Protection Agency (USEPA) for solid materials because it efficiently extracts polar and non-polar compounds, while generating less toxic waste than dichloromethane (26). Table 1 summarizes the conditions for each extraction technique. The Soxhlet extraction was performed following the method described by Reyes et al. (2). For the ultrasound extraction, a cell disrupter (Virsonic, model 16-850) was used according to USEPA Method 3550B, except that in order to minimize sample loss during solvent transfer, samples were extracted once with 200 mL of solvent instead of twice with 100 mL. The MAE extraction was performed with a MSP-1000 microwave sample preparation system (CEM Corp., Matthews, NC) according to the method of Lopez-Avila et al. (19). For quality control, laboratory blanks consisted of unused filters, clean Soxhlet thimbles, and clean extraction solvents.

Table 1. Extraction Conditions.

Extraction method	Solvent volume (mL)	Extraction time	Temperature (°C)	Pressure	Power
Soxhlet*	175	24 hr	60	ambient	n/a
Ultrasound†	200	3 min	25	ambient	0.56%§ 0.35%
MAE‡	30	3 min (stage 1)	80	100 psi	100 %
		21 min (stage 2)	115	100 ps	100 %

*Corresponding blanks were thimble plus solvent, extraction solvent and cleaned quartz filters
† Corresponding blanks were extraction solvent, and cleaned quartz filters
‡ Corresponding blanks were extraction solvent and cleaned quartz filters
§ Output
Input
n/a not applicable

Extracts of urban and diesel dusts were transferred to 50-mL Teflon tubes, then centrifuged for 5 min at 3000 rpm (4°C) to remove suspended solids. PM10 sample extracts appeared clear and did not need to undergo such treatment. Each sample extract was reduced to about 10 mL using a rotary evaporator at 50°C, then filtered through a 0.45 µm-PTFE filter and collected in 15-mL borosilicate centrifuge tubes. Extracts were further concentrated to less than 1 mL under nitrogen, and then transferred to 1.2-mL pre-weighed amber vials. Centrifuge tubes were rinsed twice with hexane:acetone, and rinsates transferred to corresponding amber vials. Extracts, including blanks, were dried under nitrogen, weighed and then reconstituted with 500 µL of hexane:acetone (1:1). Finally, 300 µL aliquots were transferred into dimethyl sulfoxide (DMSO) by solvent-exchange for toxicological analyses.

Bioassays. SMP and Microtox® materials and experimental protocols were obtained from Harvard Bioscience (24) and Strategic Diagnostics (25), respectively. For the SMP test, serial dilutions were made in duplicates for each APM organic sample extract by mixing 30 µL of sample with 30 µL of DMSO. Aliquots (12 µL) of each dilution were added to 1.2 mL amber vials containing 928 µL of distilled deionized water in order to obtain a 1% DMSO solution in the final assay medium. The RET procedure of the SMP assay was conducted in

96-well microplates. In each well, 235 µL of diluted sample was mixed with 45 µL of SMP/Concentrated Reaction Mixture (CRM) (24, 27). The RET reaction was initiated with the addition of 20 µL of 0.5 µmol adenosine 5'-triphosphate (ATP), and the rate of NADH production recorded at 340 nm with a Bio Rad (Richmond, CA) Ultramark Microplate Imaging System. Tests of a positive control (sodium azide) yielded an EC50 within recommended control limits. Toxicity is indirectly proportional to the formation rate of NADH.

For the Microtox® analyses, 60 µL of sample extract was mixed with 5.94 mL of Microtox® diluent providing a 1% DMSO solution. Then, serial dilutions (all with 1% DMSO) were prepared according to the test supplier's recommendation for solubilizing organic compounds (25). Tests were run following the standard protocol using a model M500 Microtox® analyzer, and a toxicant exposure of 15 min. A positive control of zinc sulphate was included in every run. Average EC50s of duplicate samples were calculated using Azur Microbics software (25). Mean EC50s were used in comparisons among extraction techniques, and between SMP and Microtox® bioassays by Scheffe's F-test ($p < 0.05$), calculated with the statistical program StatView (SAS Institute Inc., Cary, NC).

Results

Average weights of APM organic sample extracts ranged from 3.17 mg to 9.08 mg after correcting for weights of corresponding blanks (Table 2). Substantial amounts of residual mass (1.1 mg to 2.2 mg) were obtained from thimbles and clean quartz filter blanks after ultrasound and Soxhlet extractions. Although there was measurable toxicity in some of the blanks yielding solid residue, inhibition was below 50%. Diesel dust extracted with the Soxhlet technique represents duplicates instead of three replicates because of a sample loss during the extraction.

Table 2. Average Weights (mg) ± Standard Deviation of Sample Extracts after Correcting for Blanks.

APM samples	Ultrasound	Soxhlet	MAE
Urban dust (SRM 1649)	5.49±0.22(3)	8.60±1.79(3)	9.08±1.3(3)
Diesel dust (SRM 1650)	7.97±2.16(3)	5.04±0.052(2)	5.53±0.57(3)
PM10	4.94±0.14(2)	6.56±0.14(2)	3.17±0.10(2)

The measured toxicity of APM samples varied significantly both with the extraction technique, the bioassay method, and the APM sample source (Figure 1). The ordering of APM toxicity was generally urban dust > diesel dust > PM10. The toxicity measured by Microtox® to PM10 extracts showed significant differences, with MAE obtaining the most toxic response when compared to Soxhlet and ultrasound. The toxic response of SMP to PM10 extracts was the same regardless of the extraction technique. Extracts of diesel dust resulting from MAE and Soxhlet extractions were significantly more toxic than ultrasound to both bioassays. The SMP toxicity of urban

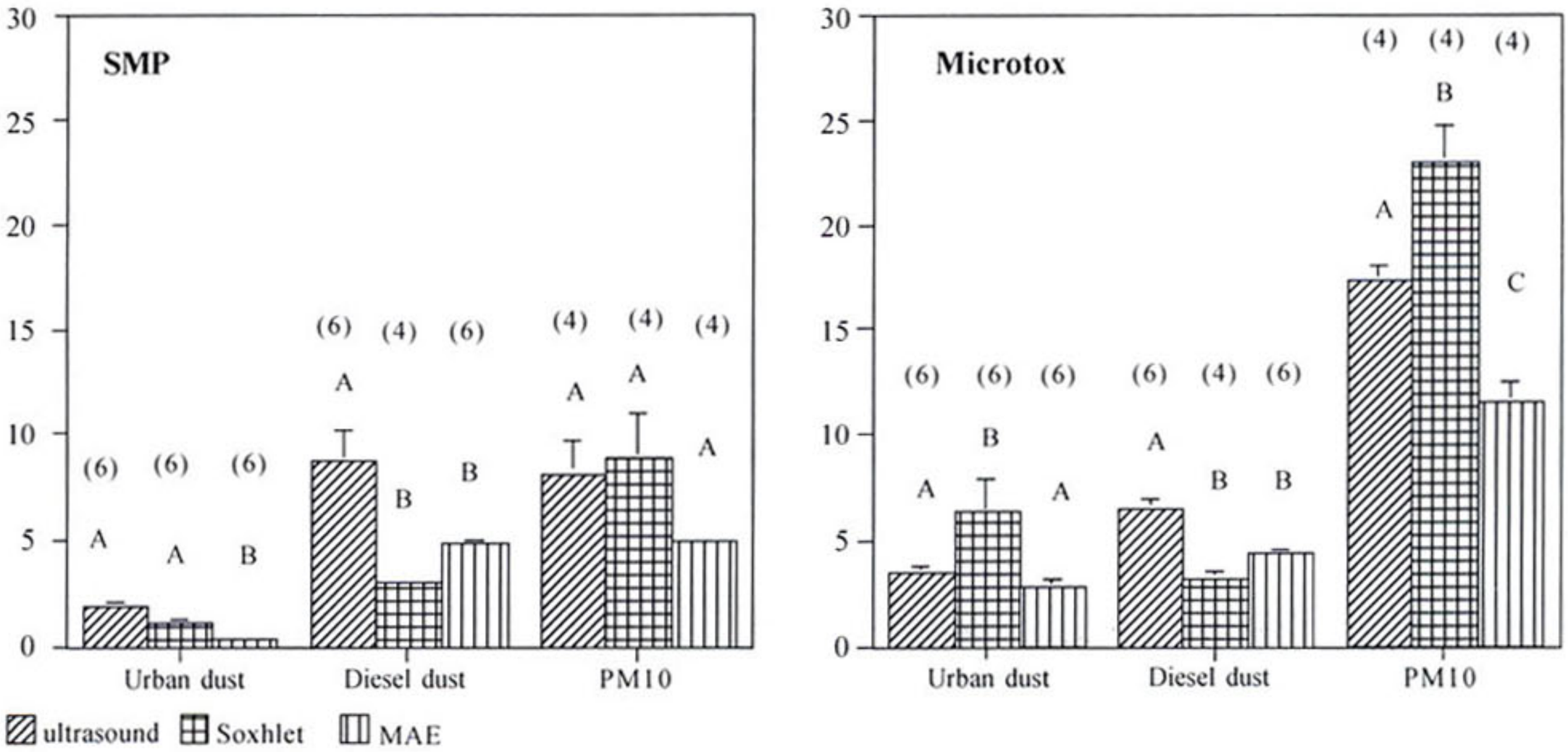

Figure 1. The effect of the extraction technique on EC50 (mg/L) of SMP and Microtox® tests. Average EC50 ± SE followed by the same letter are not statistically significant ≥ (0.05) using Scheffe's F test. Values in parenthesis are number of replicates.

dust extracts obtained by MAE were 3.5 and 5.4 times greater than Soxhlet and ultrasound methods, respectively. In contrast, the Microtox® toxicity test of extracts from urban dust were significantly more toxic using MAE and ultrasound extraction techniques than Soxhlet.

Discussion

The different pattern in toxicity among APM samples was probably due to their chemical composition. The diverse content of chemical constituents of urban dust probably made this sample more toxic. Urban dust (SRM 1649) has been chemically characterized as having PAHs, PCBs, chlorinated pesticides, and inorganic elements (4). In contrast, diesel dust (SRM 1650), representing APM from heavy-duty diesel engine emission, was mainly composed of PAHs (28) which could exert its toxicity by photoactivation (29). PM10 from urban Guaynabo, Puerto Rico, has been partially characterized to contain phthalates, malathion, 4-morpholinepropamine, 6-undecylamine, 1-[3-methyl-4-(4-morpholinyl)-1-oxo-2,2-diphenylbutyl]-pyridine, and tridecane (2).

In general, the SMP test was more sensitive ($p<0.05$) than the Microtox® to extracts from urban dust and PM10, regardless of the extraction technique used, whereas both bioassays responded similarly to extracts of diesel dust. That SMP was generally more sensitive to Microtox® could be explained by the fact that the former consists of vesicles of inner mitochondrial membrane that were more accessible to toxicants. In contrast, the bacterium *Vibrio fischeri* contains a cell wall that toxicants must traverse to reach potential sites of action (22, 24, 25).

In conclusion, results showed that the choice of the extraction technique influenced the toxicity of APM samples. In terms of toxicity, APM extracts obtained by MAE were, as toxic or more toxic, than extracts obtained with the conventional extraction techniques of Soxhlet and ultrasound. MAE has advantages of requiring less organic solvent, shorter extraction times, and the capacity to extract twelve samples simultaneously. This study is the first to report the suitability of two standards *in-vitro* bioassays for the future toxicological characterization of APM collected from Puerto Rico, with the SMP generally showing better sensitivity to the well-known Microtox® bioassay.

Resumen

En este estudio se comparó el efecto que tienen tres técnicas de extracción (ej. Soxhlet, ultrasonido y microondas) en la toxicidad causada por tres fuentes de material particulado respirable utilizando los bioensayos de partículas submitocondriales (SMP, por sus siglas en inglés) y de Microtox® para medir la toxicidad. Se encontró que la técnica de extracción influyó la respuesta tóxica de los extractos provenientes del material particulado atmosférico (APM, por sus siglas en inglés) y que el grado de toxicidad dependía del método de bioensayo y de la fuente del APM. Los extractos de APM obtenidos por el método de microondas (MAE, por sus siglas en inglés) fueron igual o más tóxicos que los extractos obtenidos por técnicas convencionales de extracción como el Soxhlet y el de ultrasonido. La extracción por MAE tiene las ventajas de que usa menos volumen de disolvente, toma menos tiempo para la extracción y tiene la capacidad de procesar doce muestras simultáneamente. El grado de la toxicidad del APM fue: APM urbano > APM diesel > PM10 (partículas con diámetro < 10 µm), lo que refleja diferencias en la composición química de las muestras. Este estudio es el primero en reportar la aplicabilidad de dos métodos de bioensayos para la futura caracterización toxicológica de APM colectados en Puerto Rico, demostrando mayor sensitividad el método de SMP sobre el de Microtox®.

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