**Endotoxin, unidentified material, and low amounts of defined toxicants are confounding factors when using the air pollution Standard Reference Material (SRM) 2786 in biological experiments.**

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**Abstract**
The National Institute of Standards and Technology (NIST) provides the reference sample SRM 2786 for use in analytical studies of particulate air pollution. The sample originated from an atypical source and possibly contains materials that are unrelated to air pollution. Investigators conducting studies with SRM 2786 should note the relatively low amounts of toxic components listed in the certificate of analysis as well as a large amount of uncharacterized material. The presence of endotoxin in SRM 2786 is reported here. Endotoxin and other uncharacterized material in SRM 2786 may influence results and conclusions for biological studies using this reference material.

**Introduction**
Airborne particulate matter (PM) is complex and variable in its composition and has been linked to adverse health effects (1). Consequently, particulate matter of 10 microns (PM10) and 2.5 microns (PM 2.5) in the air are monitored to protect health. The US EPA and the World Health Organization have established standards of 12 ug/m$^3$ and 5 ug/m$^3$, respectively, as acceptably safe levels of PM2.5 in air (2, 3). Airborne PM is regulated by its size rather than its composition and toxicological properties. For example, PM2.5 in the air might come from an anthropogenic source such as engine exhaust, or natural sources such as wind-blown soils and biological agents such as bacteria and viruses. Particulates from these different sources produce different effects when inhaled, but they all are considered PM2.5 for the purpose of regulation.

A particulate matter sample, SRM 2786, was prepared and partially characterized by the National Institute of Standards and Technology (NIST) of the USA (4). This material was intended to represent urban PM2.5 air pollution. But rather than being directly isolated on standard air sampling media, this material was obtained from “an air intake filtration system of a major exhibition center in Prague, Czech Republic.” This source of material is problematic because the air filter type, usage time, and amount of air filtered are unspecified. Commercial air filters that are in place for extended periods of time can be contaminated with non-airborne material and provide a source for microbial growth. Thus, SRM 2786 may not be solely representative of urban PM2.5 air pollution. SRM 2786 was not specifically prepared or characterized for use in biological or toxicological experiments. The material from the commercial air filter was fractionated by size to < 4 um and then partially characterized for numerous elements and organic molecules as described in its certificate of analysis (4). A summary of these analytical categories and their amounts are shown in Table 1 below. Low amounts of potentially toxic metals and

organics were measured in this sample. Seventy-eight percent of the sample (by weight) is unknown, and about 20% consists of elements (mostly sodium, magnesium, calcium, iron, aluminum, and chlorine). Some of the metal components of SRM 2786 will not remain PM2.5 when placed in aqueous or biological environments since they likely exist as soluble salts. Additionally, SRM 2786 was characterized for 51 specific polycyclic aromatic hydrocarbons (PAHs), 7 nitro-PAHs (N-PAHs), 6 polybrominated diphenyl ethers (PBDEs), 17 chlorinated dioxins and dibenzofurans (Dioxins), 3 hexabromocyclododecane isomers (HBCDs), 3 anhydrosugars (levoglucosan, mannosan, glactosan) and moisture content. The polycyclic aromatics and halogenated organic compounds collectively constitute <0.1% of SRM 2786.

Table I. Summary of the analyte groups in SRM 2786 (g/kg)*.

| Elements | PAHs | N-PAHs | PBDEs | Dioxins | HBCDs | Anhydrosugars | Moisture | Uncharacterized |
|---|---|---|---|---|---|---|---|---|
| 203 | 0.1 | 0.0014 | 0.00025 | 0.000013 | 0.0006 | 0.6 | 17 | ~780 |

*Summarized from the SRM 2786 certificate of analysis (4).

Hill, et al used SRM 2786 as a representative PM2.5 urban air pollution source to induce inflammation in a murine model of lung cancer (5). While the authors attributed the inflammatory tumor-promoting effects to the particulate nature of this air pollution reference material, no unsized control material was used for purposes of comparison. Consequently, it is unknown if the particulate nature of SRM 2786 is required for the inflammatory effects. The large amount of uncharacterized material and the lack of appreciable amounts of characterized toxic substances raises the question of the identity of bioactive components in SRM 2786.

**Research methods and results**

Endotoxin is a potent, natural, and well-known initiator of inflammation (6). Due to the origin of SRM 2786, one possibility for its inflammatory effects is that it contains endotoxin. To test this idea, one ml of sterile water was added to approximately 100 mg SRM 2786 to produce a final concentration of 100 mg/ml. The sample was vortexed extensively to dissolve as much of the material as possible. A Pierce chromogenic endotoxin test kit (ThermoFisher A39552) was used as directed to detect the presence of endotoxin. After the reaction was stopped, the assay plate was allowed to stand for 1 hour and then 150 ul of the assay supernatant was pipetted into a new 96-well plate. This transfer eliminated the interfering sedimented material at the bottom of the assay plate. The SRM 2786 sample was incubated with the chromogenic substrate alone (no *Limulus* enzyme) to verify that there was no spontaneous activity. Spectrophotometric absorbance readings were taken on the wells of the new plate at 405 nm to detect nitroanilide, the indirect product of the bioassay that indicated the presence of endotoxin. Known amounts of endotoxin were compared different concentrations of SRM 2786 to determine the amount of endotoxin present (Table 2).

Table 2. Endotoxin assay and levels in SRM 2786

| Endotoxin units, standard curve | Abs. 405 nm (+/- S.D.) | SRM 2786 (ug) | Abs. 405 nm (+/- S.D.) | Endotoxin (units/ug SRM 2786) |
|---|---|---|---|---|
| 1 | 0.95 (0.05) | 50 | Out of range | - |
| 0.33 | 0.78 (0.11) | 16.7 | 0.94 (0.04) | ~ 0.06 |
| 0.1 | 0.54 (0.05) | 5.5 | 0.74 (0.03) | ~ 0.06 |
| 0 | 0 (background subtracted) | - | - | - |

Extrapolations from the endotoxin standards indicated SRM 2786 sample contains approximately 0.06 endotoxin units per ug of material. Based on previous studies, these amounts of endotoxin could produce inflammatory responses and cytokine expression in the lungs (7-8). The SRM2786 sample was also tested for viable microorganisms. Five mg SRM 2786 in water was spread onto tryptic soy agar plates and compared with a vehicle control (water alone). Plates were incubated at 22 and 37 $^{o}$C for 5 days. No microbial growth was detected from the SRM 2786 material. Thus, SRM 2786 may lack viable microbes, but it contains considerable endotoxin. Endotoxin can be present in the air as a natural microbial product or may have come from microbial growth on the original air filter that provided the starting material for SRM 2786. The latter possibility becomes probable if the commercial air filter source was used for an extended period in an environment that allowed exposure to sufficient humidity and other factors that favored microbial growth.

**Summary**

SRM 2786 is a complex material that was partially characterized for specific chemical components. It is suitable as a reference standard for analytical studies of PM2.5 but should be used with caution in biological systems since it contains endotoxin and considerable amounts of uncharacterized material. No experiments have been performed to discern whether the particulate nature of SRM 2786 is required for its biological effects. Environmental studies of PM2.5 tend to focus on anthropogenic air pollution sources which are regulatable. As levels of anthropogenic PM2.5 decline due to increased pollution control, natural sources will become a greater proportion of PM2.5 in air. Endotoxin, microbes, and other natural materials can be abundant bioactive components of PM2.5 and may produce strong inflammatory responses (6-9). Natural agents as well as anthropogenic ones should be accounted for when conducting studies on health effects of air pollution.

**References**

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