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Method Development for Selective and Non-Targeted Identification of Nitro-Compounds in Diesel Particulate Matter

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Abstract

Nitro-aromatic compounds are associated with a host of adverse human and ecological health outcomes, however current methods of detection are limited by the lack of accuracy for the non-targeted identification nitro-compounds. This manuscript describes the development of a novel, accurate, and selective method of identifying nitro-compounds, especially nitro Polycyclic Aromatic Hydrocarbons (PAHs), in complex soot mixtures. For the first time, high performance liquid chromatography (HPLC) was used in combination with Orbitrap Mass Spectrometry (MS) for the non-targeted identification of nitro-compounds. This method was validated on a mixture of 84 standard molecules containing 23 nitro-compounds, and then applied to a complex soot sample, the National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1650a, to successfully identify 105 masses, 62% of which were monosubstituted nitro-compounds. All nitro-compounds reported in the latest Certificate of Analysis (COA) for SRM 1650b were successfully identified except for 1,3-dinitropyrene and 1,6-dinitropyrene. Compounds not reported in the COA of the SRM 1650b, including but not limited to, 1,8-dinitropyrene, alkylated nitro-molecules of all masses in the COA of SRM 1650b, nitrophenynaphthalene isomers, dinitronaphthalene, nitro-phenols, nitro-keto-PAHs, nitrocarboxylic-PAHs, and other nitro partial polar compounds were also tentatively identified. Future work will consider a larger set of classes, including isomers. This will help identifying the chemical composition of mixtures in order to take proactive approaches to prevent health and environmental hazards.
Introduction

Diesel engine emissions are highly complex mixtures containing a wide range of organic and inorganic constituents in the gas and particulate phases. Organic compounds associated with the particulate phase include polycyclic aromatic hydrocarbons (PAHs) and their alkyl, nitrated, and oxygenated derivatives. Many of these polycyclic aromatic compounds are characterized by low water solubility and a tendency to partition into suspended and sediment organic matter in aquatic environments. The impact of nitro-PAHs emissions on human health can be severe given their mutagenic and carcinogenic effects and their widespread distribution.

There are various methods for mainly targeted analysis of nitro-PAHs in complex mixtures, however non-targeted identifications are fraught with difficulty. A recent study based on gas chromatography coupled to mass spectrometry (GC-MS) showed that the organic compounds in diesel exhaust particles belong to eleven families: alkane, alkene, alkyne, aromatic hydrocarbon, carboxylic acids, esters, ketone, alcohols, ethers, nitrogen-containing, and sulphur-containing compounds. Gas chromatography followed by negative ion chemical ionization coupled with MS (GC/NICI-MS) was used to determine selected nitro-PAHs in diesel standard reference materials, urban dust standard reference materials, and in natural ambient air samples. High-performance liquid chromatography (HPLC) coupled with atmospheric pressure chemical ionization (APCI), followed by Time of Flight (TOF) MS (HPLC-APCI-TOF-MS) was used by Schauer et al. to analyse selected nitro-PAHs in air particulate matter, soot and reaction product studies. Ultra-HPLC-APPI-MS/MS (APPI is Atmospheric Pressure Photolionization) was applied to analyse selected PAHs and nitro-PAHs in certified reference materials and real aerosol samples. All these methods were reported for targeted analysis of nitro-compounds in soot, air...
particles and laboratory fate evaluation studies of PAHs. However, the identification and
quantification of nitro-PAHs in complex mixtures remains a significant challenge. For example,
in soot samples, the quantity of compounds present is often insufficient for spectrometric
analysis using nuclear magnetic resonance (NMR) spectroscopy or infrared (IR) spectroscopy
\[15,16\]. Thus these mixtures are usually analysed by chromatography hyphenated with mass
selective detection with GC-MS applied in most cases \[16-18\]. The range of compounds analysed
successfully with GC-MS is restricted because of compound volatility and low thermal stability.
While HPLC-High Resolution MS (HPLC-HRMS) based methods may be used for structure
elucidation, the lack of elaborate spectral libraries remains a obstacle \[16,18\]. Thus, alternative
approaches need to be developed to exploit information gained from chromatography and MS for
the analysis of complex matrices.

Fourier Transform Ion Cyclotron Resonance MS (FT-ICR-MS) and Orbitrap have been
demonstrated to be effective for the analysis of partial polar compounds in complex mixtures \[19-\]
\[22\]. However, there are no previous reports about the utilization of HPLC-HR-MS for the non-
targeted analysis of nitro-compounds in diesel soot. This work aims to fill this gap by utilizing
HPLC-HR-MS to identify non-targeted nitro-compounds in complex soot mixtures. This is
important to identify unknown molecules in complex mixtures. This paper will first show the
validity of the developed method using a customized mixture of 84 standard molecules
containing 23 nitro-compounds, then, the applicability of the developed method by testing it on
the standard reference material (NIST SRM 1650a) issued by the National Institute of Standards
and Technology (NIST) for diesel particulate matter.
Methodology

Materials

The customized mixture used in this study was comprised of nitro-PAHs, nitro aromatic compounds, carboxylic acid-PAH, aldehyde-PAHs, quinones, hydroxyl-quinones, keto-PAHs, hydroxyl-PAHs, ketones, amides, amino-PAHs, and azaarenes. Table S-1 in the supplementary materials contains a detailed list, with supplier names of the standard molecules in the custom mixture. HPLC grade methanol and formic acid was from Merck, ammonium formate was from Sigma-Aldrich, and high purity water was obtained using a Seral-Pur Delta UV apparatus.

The soot used in this study was NIST SRM 1650a diesel particulate matter. This soot is typically used in evaluating analytical methods for the determination of selected PAHs and nitro-PAHs in complex matrices. The NIST SRM 1650a was prepared in year 2000 from the same bulk diesel particulate materials previously issued in 1985 as SRM 1650 .

Apparatus and equipment

Soot Extraction and Clean-up

NIST SRM 1650a, 0.10 g, was extracted using accelerated solvent extraction (ASE 300, Dionex Corp., Sunnyvale, CA) with a mixture of dichloromethane:acetone at a volume ratio of 3:1 and dialyzed through a semipermeable membrane as described in details by Luebecke-von Varel et al. The final concentration used for the analysis was 100 mg extracted soot in 1 mL solvent.
Analysis of Nitro-PAHs by High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS)

The customized mixture and NIST SRM 1650a extract were dissolved in methanol for analysis. Chromatographic separations were performed using the Agilent series 1200 HPLC system. This system consists of a degasser, a high-pressure binary SL pump, an autosampler, and a column oven (Agilent Technologies, Santa Clara, CA, USA) controlled by the ChemStation software.

The customized mixture and NIST SRM 1650a extract were separated on an analytical C18 reversed-phase column (LC-PAH, 250 x 2.1 mm, 5 µm particle size, 120 Å; Supelco, Deisenhofen, Germany). Sample volumes of 5 µL were injected. Compounds were separated at 40°C at a flow rate of 200 µL/min with a gradient mixture of water containing methanol (5%, vol/vol) (A) and pure methanol (B) as the mobile phase. The elution program was set as follows: 0 min 50% B, ramped to 100% B by 40 min (0-40), held for 15 min (40-55), ramped back to initial conditions in 5 min (55–60) and finally held for 15 min (60–75) in order to equilibrate the column before the next injection. A blank (5% methanol in water) was injected before and after each sample to check the carryover effects in between injections. Detection was performed by a linear trap quadrupole (LTQ)-Orbitrap hybrid instrument (Thermo Fisher Scientific, Bremen, Germany), equipped with an APCI and controlled by the Xcalibur software. The use of the hybrid LTQ-Orbitrap not only provides high-resolution MS, but also high mass accuracy with less than 1 ppm error (see Table S-2), enabling an unambiguous determination of elemental composition. Ions with \( m/z \) ranging from 100 to 400 were scanned in the Orbitrap-FTMS with a resolution \( r = 30,000 \). The data dependent mode was activated to automatically switch between Orbitrap-FTMS and Ion Trap–MS/MS (IT-MS/MS) data acquisition. The detection limits in full scan MS were compound-dependent and, in the optimal ionization mode polarity, the detection
limits were below 50 pg on column\textsuperscript{20}. The three most intense ions were sequentially isolated for accurate mass measurements by an Orbitrap-FTMS. Collisionally induced dissociation (CID) was used for subsequent tandem MS fragmentation in the IT. The fragmentations of the standards and soot were acquired using CID (collision energy 35 %).

Chromatogram Visualization

The resulting chromatograms of the analysed diesel soot exhaust extract were complex, as shown in Figure S-1 (supplementary information). The open source software MZmine was used to visualize and process the chromatogram constituents with the noise level set to $5.0 \times 10^3$, the minimum peak height to $1 \times 10^4$, the mass resolution to 25,000, the m/z tolerance to 0.002 amu and the minimum peak duration to 0.2 min. An m/z tolerance of 0.003 amu and a retention time tolerance of 30 seconds were used for the custom database search. After peak detection with MZmine and exclusion of blank m/z values, the remaining constituents containing carbon, hydrogen, oxygen, and sulfur were selected for identification. The nitrogen rule was applied in order to select a probable empirical formulae.

Experimental Design

The methodology developed in this study was based on HPLC with tandem HPLC-APCI-HR-MS/MS. The chemical separation was achieved using HPLC with polymeric reversed phase (RP), RP-C18, stationary phase column. The ionization and detection was based on APCI-MS/MS, with mass spectrometric signals observed in both positive and negative modes. Nitro-PAHs are partially polar compounds and are not ionisable in solution making APCI a better choice for ionization rather than electrospray\textsuperscript{20,24,25}. 
The identification of NIST SRM 1650a components was based on processing the chromatogram constituents using an open source metabolomics deconvolution software (MZmine). The list of possible structures was then refined by comparison to standards prepared for 23 nitro-compounds, based on chromatographic and mass spectrometric classifiers.

Customized mixture and NIST SRM 1650a extract were analysed. To mimic the complexity of the NIST SRM 1650a, the customized mixture solution was prepared by mixing 1 part per million (ppm) of 84 molecules from a wide range of partially polar compounds.

The approach developed in this work for the identification of nitro-compounds in complex matrixes is illustrated in the schematic block diagram below. Tentatively identified compounds are those which could not be confirmed with a reference standard and thus were not detected with certainty.

```
Accelerated Solvent Extraction (ASE); Solvents: DCM, Acetone

Accelerated membrane-assisted cleanup

HPLC-APCI-HR-MS/MS analysis

Chromatogram visualization using deconvolution software (MZmine)

Generate empirical formulae from the exact mass

Select only the formulae that match with nitro-compounds

Filtering the retrieved hit by MS spectral and chromatographic classifiers

Positive/Tentative Identification
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Results and Discussion

It is essential to understand 1) the chromatographic behavior on the polymeric C18 column, and 2) the characterization of the ionization, the in-source fragmentation, and the fragmentation during tandem MS/MS in order to characterize the constituents in the NIST SRM 1650a. The sections below cover the chromatographic behavior of the nitro-compounds on the used columns, the characterization of the ionization and the fragmentations patterns, then the identification of the nitro-compounds for the validation of the method, and finally the application of this method for the determination of non-targeted nitro-compounds in the NIST SRM 1650a.

Chromatography Behavior of the Model Nitro-Compounds

The 23 nitro-compounds in the customized mixture were mostly compounds containing at least one aromatic ring or a larger polyaromatic structure substituted with at least one nitro group (collectively referred to as nitro-compounds in this work). The nitro compound standards cover a wide range of octanol-water partition coefficients (log $K_{ow}$ 1.1-6.6) as obtained from the PubChem database $^{28}$. Table S-2 lists the names of the model nitro-compounds and their exact masses, calculated m/z, measured m/z, ppm error, log P, maximum length to breadth (L/B) ratio and retention time.

In this study, the 23 standard nitro-compounds in the customized mixture eluted between 6.81 and 45.56 minutes (see Table 1). As shown in Figure 1, the shape of each of the peaks is symmetric with a narrow peak width of less than one min, meaning that the separation is efficient even for isomers like 1,3-dinitropyrene, 1,6-dinitropyrene and 1,8-dinitropyrene.
Figure 1: RP-HPLC/APCI-HR-MS extracted ion chromatograms of some selected model compounds in the customized mixture. In case of isomers, only one structure is shown.

The separation of nitro-PAHs, including their isomers on the polymeric stationary phase, is based on their hydrophobicity expressed as log P, intramolecular steric hindrance, and the L/B ratio. For the standard molecules covered in this study, it was observed that on polymeric RP-C18 the retention times for nitro-PAHs exhibited a regular increase corresponding to the increase in the number of carbon atoms, and a systematic decrease in the presence of keto functional groups. For example, the elution time of 9-nitroanthracene (three fused rings) is 32.05 min while it is 40.44 min for 6-nitrochrysene (four fused rings). The 2-nitrofluorene (three rings) and 2-
nitrofluorenone (three rings and a keto functionality) were eluted at 30.93 min and 25.58 min, respectively. Figure S-2 shows a direct correlation between the retention time and Log P (which is effected by the number of carbons) with a high $r^2$ value of 0.95. Unlike what has been expected based on observations in reference 19, the correlation between the retention time and L/B ratio is obvious only for a subset of the molecules, but not for all 23 standard molecules.

One of the strengths of the methodology used in this study is the ability to separate isomers of nitro-PAHs. In a previous study 19, using the same method, it was shown that the retention time of structural isomers was most influenced by (1) the presence of substituents at the bay position, (2) the number of H atoms peri to the substituent group and (3) the L/B ratio of the molecule. In this study, it was observed that the retention times for non-sterically hindered isomers, with similar Log P, are longer for structures with greater L/B ratios. For instance, the isomer 1,6-dinitropyrene (L/B=0.9) was eluted at 38.85 min while 1,8-dinitropyrene (L/B=1.2) eluted at 39.29 min.

Characterization of the Ionization, and Fragmentation by Tandem MS for Standard Nitro-Compounds

As outlined in Table 1 and 2, the dominant signals for standard nitro-compounds were observed in the negative ionization mode. In the presence of acidic hydrogens (e.g. phenols) and with partially aromatic structures (e.g. 2-nitrofluorene), the formation of deprotonated molecules [M-H] was observed. This is in accordance with results found in previous studies 19,29,30. All molecules, except for compounds with no acidic hydrogen and partial aromatic structures, give radical anions [M$^-$]. The neutral loss of NO was observed for all molecules in the negative mode
of in-source fragmentation. In the positive mode, all compounds, except for those with an acidic hydrogen, and methylated poly-nitroaromatic were detected protonated ions [M+H]+.

In the negative ion mode, the tandem MS for all compounds showed the neutral loss of NO as a diagnostic fragment. This characteristic serves as unique fingerprint which can be used for the selective tracking of the nitro-substituted compounds in NIST SRM 1650a. In the negative ion mode, other minor fragments such as H2CN, NO2, CHO, CH3, 2NO and OH were also lost. In the positive ion mode, the tandem MS showed the main fragment ions as [M+H-OH]+, [M+H-NO2]+. In the positive ion mode, other minor fragments such CNO, CNO2, NO, HNO, H2NO, H2CN, H2O, CH3 and HNO2 were also observed.
Table 1: Standard nitro-compounds investigated in this study with retention times (RT), observed mass spectrometric signals, and positive (+) and negative (-) modes tandem MS/MS. The tandem mass spectral signals of highest relative intensities are in bold. n.d. implies not detected.

<table>
<thead>
<tr>
<th>Name</th>
<th>Formula</th>
<th>RT (min)</th>
<th>Measured m/z (+ mode)</th>
<th>Observed Signal (+ mode)</th>
<th>MS/MS (+ mode)</th>
<th>Diagnostic Fragments (+ mode)</th>
<th>Measured m/z (- mode)</th>
<th>Observed Signal (- mode)</th>
<th>MS/MS (- mode)</th>
<th>Diagnostic Fragments (- mode)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Nitrofluorene</td>
<td>C10H7NO2</td>
<td>30.93</td>
<td>210.0561, 211.0639</td>
<td>[M-H], [M]^+</td>
<td>181, 195, 164</td>
<td>NO, CH3, NO2, 212.0711</td>
<td>[M-H]^+</td>
<td>195, 166, 170, 180, 197, OH, NO2, CNO, HNO, CH4</td>
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<td></td>
</tr>
<tr>
<td>9-Nitroanthracene</td>
<td>C10H7NO2</td>
<td>32.05</td>
<td>223.0639</td>
<td>[M-H]^+</td>
<td>193, 195</td>
<td>NO, H2CN, 224.0711</td>
<td>[M-H]^+</td>
<td>207, 178, 193, OH, NO2, HNO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Nitropyrene</td>
<td>C8H7NO2</td>
<td>40.44</td>
<td>247.0639</td>
<td>[M]^+</td>
<td>217</td>
<td>NO, H2O, 248.0711</td>
<td>[M-H]^+</td>
<td>202, 231, NO2, OH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-Nitrochrysene</td>
<td>C10H7NO2</td>
<td>40.44</td>
<td>273.0795</td>
<td>[M-H]^+</td>
<td>243</td>
<td>NO, H2O, 274.0868</td>
<td>[M-H]^+</td>
<td>257, 228, 244, 216, 232, OH, NO2, CNO, CNO</td>
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<td></td>
</tr>
<tr>
<td>Nitrobenz[a]anthracene</td>
<td>C11H7NO3</td>
<td>41.9</td>
<td>273.0795</td>
<td>[M]^+</td>
<td>243, 226</td>
<td>NO, HNO2, 274.0868</td>
<td>[M-H]^+</td>
<td>257, 228, 244, 227, OH, NO2, CNO, NO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,3-Dinitrobenzene</td>
<td>C12H7NO3</td>
<td>36.88</td>
<td>292.049</td>
<td>[M]^+</td>
<td>262</td>
<td>NO, HNO2, 293.0562</td>
<td>[M-H]^+</td>
<td>276, 247, 237, OH, NO2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,6-Dinitrobenzene</td>
<td>C12H7NO3</td>
<td>38.85</td>
<td>292.049</td>
<td>[M]^+</td>
<td>262</td>
<td>NO, HNO2, 293.0562</td>
<td>[M-H]^+</td>
<td>276, 247, 237, OH, NO2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,8-Dinitrobenzene</td>
<td>C12H7NO3</td>
<td>39.29</td>
<td>292.049</td>
<td>[M]^+</td>
<td>262</td>
<td>NO, HNO2, 293.0562</td>
<td>[M-H]^+</td>
<td>276, 247, 237, OH, NO2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6- Nitrbenzo[a]pyrene</td>
<td>C12H7NO3</td>
<td>45.56</td>
<td>297.0795</td>
<td>[M]^+</td>
<td>267</td>
<td>NO, HNO2, 298.0868</td>
<td>[M-H]^+</td>
<td>281, 252, 267, 268, OH, NO2, HNO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitro-keim-PAHs</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>3-Nitrobenzanthrone</td>
<td>C10H7NO2</td>
<td>34.69</td>
<td>275.0588</td>
<td>[M-H], [M-H]^+</td>
<td>245, 247, 246</td>
<td>NO, CO, CHO, 276.066</td>
<td>[M-H]^+</td>
<td>230, 246, 259, 218, OH, NO2, CO, CNO</td>
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<td></td>
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<tr>
<td>Nitro Compounds</td>
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<td></td>
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<td></td>
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<tr>
<td>2-Nitrotoluene</td>
<td>C8H7NO2</td>
<td>14.49, 15.54</td>
<td>136.0404</td>
<td>[M-H]^+</td>
<td>106, 92 (very week signal)</td>
<td>NO, HNO2, 138.055</td>
<td>[M-H]^+</td>
<td>120, 92, 110, 106, 121, 163, H2O, NO2, H2O, CN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,4,6-Trinitrotoluene</td>
<td>C8H7NO4</td>
<td>11.44</td>
<td>227.0184</td>
<td>[M-H], [M-H]^+</td>
<td>210, 197, 180, 167, 137, 151, 198</td>
<td>NO, HNO, 210, 197, 180, 167, 137, 151, 198</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Methyl-2, 6- nitrotoluenol</td>
<td>C8H7NO4</td>
<td>14.08, 15.61</td>
<td>152.0535</td>
<td>[M-H], [M-H]^+</td>
<td>122</td>
<td>NO, HNO, 191.0451</td>
<td>[M-H]^+</td>
<td>151, 149, 194, 117, 173, 174, NO, NO2, OH, H2O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Nitroquinone N-oxide</td>
<td>C8H7NO4</td>
<td>6.81</td>
<td>190.0384</td>
<td>[M-H], [M-H]^+</td>
<td>168, 173, 142, 142, 132, 116</td>
<td>NO, HNO, 168, 173, 142, 142, 132, 116</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Chloro-2,4- dinitrobenzene</td>
<td>C8H7NO4Cl</td>
<td>13.54</td>
<td>201.9787</td>
<td>[M-H], [M-H]^+</td>
<td>172, 156, 185</td>
<td>NO, HNO, 283.9876</td>
<td>[M-H]^+</td>
<td>252, 243, 235, 232, C8H7Cl, C8HCl, NOCl, Cl</td>
<td></td>
<td></td>
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<tr>
<td>Nitrobenzene</td>
<td>C8H7NO2Cl</td>
<td>31.91</td>
<td>282.9808</td>
<td>[M-H], [M-H]^+</td>
<td>138, 212, 217, 238, 247, 254, 253, 252</td>
<td>NO, HNO, 283.9876</td>
<td>[M-H]^+</td>
<td>328, 243, 235, 232, C8H7Cl, C8HCl, NOCl, Cl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Table 2: Summary of MS classifiers for the standard nitro-compounds in the customized mixture (aggregated in classes) based on ion formation during APCI ionization including in-source fragmentation, and tandem MS/MS fragmentation (the bold font indicates major fragments). n.d. = not detected.

<table>
<thead>
<tr>
<th>Classes of Molecules</th>
<th>Observed Signals</th>
<th>Diagnostic Fragments APCI (-)</th>
<th>Diagnostic Fragments APCI (+)</th>
</tr>
</thead>
</table>

Identification of the Nitro-PAHs in the Customized Mixture
Table 1 summarizes the list of nitro-compounds identified in the customized mixture. The molecules were classified in three categories: nitro-PAHs, nitro-keto-PAHs and nitro-aromatics. All 23 nitro-compounds, including the various isomers of nitropyrene and dinitropyrene, were distinctly identified from the customized mixture of 84 standard molecules (as shown in Table 1). For graphical illustration, Figure S-3-A and B, show that the 9-nitroanthracene standard was eluted (in the positive and negative modes) at 32.05 min. In the negative mode, it was detected as [M]− with the dominant loss of neutral NO and a minor fragment of H₂CN (See Figure S-3-A1). In the positive mode, the [M+H]+ showed the loss of OH as a dominant fragment, and NO₂, NO and HNO as minor fragments (See Figure S-3-B1). In Figure S-3-C and C1, the 1-nitropyrene standard was eluted at 38.51 min and was detected as a [M]− in the negative mode with the only loss of NO. In the positive mode, protonated ion [M+H]+ showed the loss of OH and as a dominant fragment, and NO₂, NO and HNO₂ as minor fragments (See Figure S-3-D and D1).

These results from the customized mixture will be compared, in the following section, with those from the NIST SRM 1650a.

Method Evaluation on NIST SRM 1650a
The certificate of analysis (COA) of the latest SRM 1650b, issued by NIST in July 17th 2013, reports that the sample contains eight nitro-PAH masses with a total of a 22 isomers (see Table 3) \(^{31}\). The following nitro-PAHs were reported in the COA: 1-nitronaphthalene, 2-nitronaphthalene, 2-nitrobi phenyl, 3-nitrobi phenyl, 5-nitroacenaph thene, 2-nitrofluorene, 9-nitroanthracene, 3-nitrophenanthrene, 4-nitrophenanthrene, 9-nitrophenanthrene, 1-nitropyrene, 4-nitropyrene, 1-nitrofluoranthene, 2-nitrofluoranthene, 3-nitrofluoranthene, 8-nitrofluoranthene, 6-nitrochrysene, 7-nitrobenz[a]anthracene; 1,3-dinitropyrene, 1,6-dinitropyrene, 6-nitrobenzo[a]pyrene and 3-nitrobenzo[e]pyrene. These molecules were determined using GC with negative ion chemical ionization mass spectrometry (GC/NICI-MS) and high-resolution mass spectrometry using negative chemical ionization (GC/NCI-HRMS) \(^{11,12,31}\). The extraction method used in the NIST analysis was based on Pressurized Fluid Extraction (PFE) using dichloromethane (DCM), toluene, or a toluene/methanol mixture at two extraction temperatures (100 °C and 200 °C).

As mentioned earlier, SRM 1650b is an equivalent of NIST SRM 1650a. Our results for the NIST SRM 1650a are in good agreement with the COA of SRM 1650b.

All nitro-compounds present in the soot except two were either positively or tentatively identified as shown in Table 3. The two missing compounds in the analysis are 1,3-dinitropyrene and 1,6-dinitropyrene. This could be due to the conditions used in the extraction method, or extremely low concentrations. However, the isomer 1,8-dinitropyrene (which was not reported in the the COA) was positively identified with our method. It is worth reminding the readers that using our method resulted in a successful and distinct separation of the three isomers of dinitropyrene in the standard mixture.

LC-MS performance of individual molecules will be provided below.
Table 3: Nitro-PAHs identified in the present study in NIST SRM 1650a compared with NIST SRM 1650b.

<table>
<thead>
<tr>
<th>Identified nitro compounds according to COA July 2013</th>
<th>Exact mass</th>
<th>Formula</th>
<th>Log P</th>
<th>Observed m/z</th>
<th>Isomers positively or tentatively identified in 1650a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Nitronaphthalene</td>
<td>173.0477</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;7&lt;/sub&gt;NO&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>3.3</td>
<td>173.0482</td>
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<td>3.7</td>
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<td>no standard; tentative identification</td>
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<td>3.9</td>
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<td>no standard; tentative identification</td>
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<td>247.0639</td>
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</tr>
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</tr>
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<td>4.7</td>
<td>292.049</td>
<td>n.d.</td>
</tr>
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<td>C&lt;sub&gt;16&lt;/sub&gt;H&lt;sub&gt;8&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;</td>
<td>4.7</td>
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<td>3-Nitrobenzo[e]pyrene</td>
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<td>C&lt;sub&gt;20&lt;/sub&gt;H&lt;sub&gt;13&lt;/sub&gt;NO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>6.3</td>
<td>297.0795</td>
<td>no standard; tentative identification</td>
</tr>
</tbody>
</table>
Nitroanthracene, nitrophenanthrene and isomers

The 9-nitroanthracene molecule in the NIST SRM 1650a was eluted at the same retention time as the standard in the customized mixture, 32.05 min. It also had the same ionization behavior as the standard in the customized mixture, i.e. [M+H]$^+$ and [M$^-$. In the tandem MS, the radical anion [M$^-$] showed the loss of NO as a dominant fragment in addition to the minor fragments of $\text{H}_2\text{CNO}$, CNO, $\text{H}_2\text{CN}$, CH$_3$ and OH (see Figure 2, top). The protonated ion [M+H]$^+$ for the 9-nitroanthracene showed the loss of OH as the dominant fragment, and the loss of NO$_2$, H$_2$CN, HCNO, NO, CNO, H$_2$O and CH$_3$ as minor fragments (see Figure 2, bottom).  

**Figure 2:** Tandem MS/MS spectrum for 9-nitroanthracene in NIST SRM 1650a sample (m/z 223; RT 32.22 min for both modes). The top figure is for [M$^-$] in the negative mode, and the bottom figure is for [M+H]$^+$ in the positive mode.
The NIST COA reported the presence of three isomers of nitrophenanthrene: 3-nitrophenanthrene, 4-nitrophenanthrene and 9-nitrophenanthrene. In the HPLC chromatogram of the NIST SRM 1650a, nine peaks were observed for the m/z of 223.0639 at 20.70, 29.36, 31.97, 34.03, 34.44, 34.95, 36.41 and 38.65 min (see Figure 3). The three peaks that eluted at 34.03, 34.44, 34.95 min were observed as \([\text{M}^-]\), \([\text{M-H}]^-\) and \([\text{M+H}]^+\), which is in agreement with the mass spectral characterization of nitro-PAHs (see Table 1). In the tandem MS, the \([\text{M}^-]\) signal for the three peaks showed the loss of NO, \(\text{H}_2\text{CN}\), and \(\text{H}_2\text{CNO}\) as dominant fragments, and the loss of \(\text{CNO}, \text{H}_2\text{O}, \text{and CH}_3\) as minor fragments. The tandem MS for the \([\text{M-H}]^-\) signal showed the loss of NO as dominant fragment, and the loss of \(\text{H}_2\text{CNO}, \text{H}_2\text{CN}, \text{CH}_3\) and \(\text{H}_2\text{O}\) as a minor fragments. These fragmentation patterns are commonly observed for nitrophenanthrene and nitrophenanthrene. Thus using retention times, mass spectral data and tandem MS fragmentation pathways, it is suggested that these peaks correspond to isomers of nitroanthracene and nitrophenanthrene.

**Figure 3:** Extracted ion chromatograms (in the negative ion mode) for m/z 223.0639 in NIST SRM 1650a.
Alkyl- nitroanthracene and nitrophenanthrene isomers

In the NIST SRM 1650a analysed (as shown in Figure 4), between m/z of 237.0792 and 307.1573, there is a systematic trend of a difference accounting for a CH₂ group. This suggests the presence of nitro-PAHs, namely nitroanthracene and nitrophenanthrene, with alkyl group(s). Thus, several isomers of C₁₋₆ alkyl-nitro anthracene and phenanthrene were detected. All alkyl-isomers of nitroanthracene and nitrophenanthrene were detected as radical anions and protonated ions. As shown in Figure 4, an increase in the degree of alkylation delays progressively the retention time in the chromatogram by roughly 2 min. The tandem MS for all isomers as radical anions showed the loss of NO as a dominant fragment, and the protonated ions showed the loss of OH and NO₂ as dominant fragments (see Figure S-4). These reassure the tentative identification of alkyl-nitro anthracene and phenanthrene.

In the NIST SRM 1650a, as shown in Figure 4 and Figure S-5, the trend of the peak elutions in both the APCI⁺ and APCI⁻ are equivalent, suggesting that the peaks in the positive and negative correspond to the same formula. This observation can be used as a powerful tool in confirming the identity of the molecules tentatively identified.
Figure 4: Extracted ion chromatograms in the negative mode from the analysis of NIST SRM 1650a. The chromatograms show isomers of nitroanthracene and nitrophenanthrene with a net number of zero to six carbons as alkyl groups.

Nitropyrene, nitrofluoranthene and isomers

In the NIST SRM 1650a, 1-nitropyrene eluted at the same retention time as the standard (38.51 min), and was detected with similar signals in the negative, i.e. as [M]⁻, and positive, i.e. as [M+H]⁺, modes. In the NIST SRM 1650a, the tandem MS fragmentation of the radical anion corresponded to the major loss of neutral NO, and the minor loss of H₂CNO, CNO, H₂CN, and OH. However, as shown in Figure 5, in the standard sample, only the major loss of NO was observed, with no minor fragmentations. This could be a consequence of the complexity of the existing matrix in the standard soot sample and incomplete separation of isomers. This observation holds true for all identified molecules in the NIST SRM 1650a.
Figure 5: Mass spectrum from the negative ionization mode of 1-nitropyrene ([M]-, m/z 247) in the customized mixture (top) and the NIST SRM 1650a (bottom).

For the signals observed in the positive ionization mode, 1-nitropyrene in the NIST SRM 1650a showed the loss of OH as a dominant fragment (exactly as observed in the standard); and NO₂, NO, HCNO and CH₃ as minor fragments. In the customized mixture, however, 1-nitropyrene [M+H]+ signals showed the minor loss of only NO₂ and HNO. Again, the discrepancy in the peaks of the minor fragments between the NIST SRM 1650a and the customized mixture could be a result of the complexity of the matrix in the standard soot or the incomplete separation of isomers.

In the HPLC chromatogram of the NIST SRM 1650a, three peaks were observed for the m/z of 247.0635 at 38.55 min (ranging from 38.1 to 38.9 min), 39.29 min (ranging from 38.9 to 39.6 min) and 40.40 min (ranging from 40.0 to 40.5 min) (see Figure S-6A). The maximum peak width is about 0.7 min.

In the customized mixture, there were four molecules with identical m/z, three of which are isomers of nitropyrene and the fourth is 3-nitrofluoroanthene. However, only three peaks appeared in the chromatogram namely at 38.51 min (ranging from 38.00 to 38.82 min), 38.95
min (ranging from 38.82 to 39.39 min) and 40.44 min (ranging from 40.17 to 40.62 min) (see Figure 1). The maximum peak width is also about 0.7 min, just like in the observation of the soot sample. This suggests that the three peaks observed with the standard soot for the same m/z of 247.0635 could represent more than a total of three molecules/isomers.

In comparison with the SRM 1650b, the COA reports two isomers of nitropyrene (1-nitropyrene, 4-nitropyrene), and four isomers of nitrofluoranthene (1-nitrofluoranthene, 2-nitrofluoranthene, 3-nitrofluoranthene and 8-nitrofluoranthene), i.e. a total of six molecules with m/z = 247.0635.

Based on the m/z (247.0635), clearly separated retention times (38.55, 39.29 and 40.40 min), types of ions formed ([M⁻] and [M+H]⁺), and tandem MS fragmentation patterns discussed above, we could positively identify, among the six molecule reported in the COA of SRM 1650b, 1-nitropyrene, 4-nitropyrene and 3-nitrofluoranthene. The remaining 1-nitrofluoranthene, 2-nitrofluoranthene and 8-nitrofluoranthene which were tentatively identified according to the m/z and the expected retention time based on the similarity of their structures with the positively identified isomers and log P (4.3, 5.0 and 5.0, respectively).

Alkyl- nitropyrene and nitrofluoranthene isomers

In analogy with what was observed with nitroanthracene and nitrophenanthrene, multiple isomers of C₁₋₆ (i.e. methyl to hexyl) alkyl- nitropyrene and isomers of C₁₋₆ alkyl-nitrofluoranthene (m/z: 261.0795, 275.0947, 289.1104, 303.1260, 317.1417, 331.1573) were also detected at retention times that increase systematically by roughly 2 min as the alkyl chain increases by one carbon (see Figure S-7A).
Nitrobiphenyl, nitroacenaphthene and isomers

The certificate of analysis reported the presence of 2-nitrobiphenyl and 3-nitrobiphenyl in SRM 1650b. However, these molecules were not available to be included in the set of standards considered in this study. Nitrobiphenyl has an exact m/z of 199.0633. This m/z was detected in the NIST SRM 1650a, and it is eluted in the chromatogram at eight retention times, namely 22.60, 25.4, 25.91, 26.48, 27.63, 28.75, 30.74, and 31.28 min (see Figure S-6B). The nitrobiphenyl mass was observed as [M-] and [M-H] in the negative mode, and as [M+H]+ in the positive mode.

The tandem MS for the [M-] signal, which was eluted at 25.91 min, showed the loss of H2CNO, NO, H2CN, H2O, OH and CNO; while the tandem MS for the [M+H]+ ion showed the loss of HCNO, OH, H2CN and NO2 (See Figure S-8A and B). All these fragmentations are probable for nitrobiphenyl and support the tentative identification of nitrobiphenyl.

Nitroacenaphthene has the same m/z of 199.0633 as nitrobiphenyl. This molecule has a partial aromatic structure which, based on the spectral nitro-PAHs characterization in Table 1, should be detected as a deprotonated ion. The chromatogram did actually show [M-H]- signal at 25.84, 26.86 and 28.11 min with m/z of 198.0572 which corresponds tentatively to nitroacenaphthene isomers (see Figure S-6B). As discussed above, for the same m/z of 199.0633, [M-] and [M+H]+ signals were observed, which further match the characterization displayed in Table 1.

Multiple isomers of C10-7 alkyl- nitrobiphenyl and nitroacenaphthene (m/z: 213.0795, 227.0949, 241.1105, 255.1262, 269.1418, 283.1575, 297.1731) were also detected (See Figure S-7B).

Nitronaphthalene isomers
Based on similar arguments provided above, the molecule 1-nitronaphthalene (m/z 173.0482) was positively identified as reported in Table 3. Three other isomers of nitronaphthalene were tentatively identified for the m/z of 173.0482 as four peaks at RT of 21.28, 21.82, 23.27 and 26.28 min eluted from the column. Various isomers of methyl, dimethyl and trimethyl-nitronaphthalene (m/z 187.0638, 201.0795, 215.0951) were also detected at multiple retention times suggesting again numerous isomers.

Positive identification of other molecules reported in the COA of SRM 1650b

According to arguments listed above, i.e. m/z, retention times, tandem MS fragmentation patterns and ionization forms, the molecule 2-nitrofluorene was positively identified and several isomers of C$_1$-5 alkyl-nitrofluorene (m/z 225.0793, 239.095, 253.1106, 267.1263, 281.1419) were also detected. Similarly, 6-nitrobenzo[a]pyrene was positively identified (with a tentative identification of the 3-nitrobenzo[e]pyrene). The molecules 6-nitrochrysene and 7-nitrobenz[a]anthracene were also positively identified, and several isomers of C$_1$-3 alkyl-nitrochrysene and nitrobenz[a]anthracene (m/z 287.0951, 301.1108, 315.1264) were detected.

Potential of Prediction and Tentative Identification of Unknowns Using HPLC Orbitrap Mass Spectrometry

This section will show the potential of the method proposed in this study for the identification of molecules from complex soot samples. From the analysis of the NIST SRM 1650a, 105 masses related to nitro-substituted aromatic and nitro-PAH compounds were identified. Each mass refers to several isomers that were eluted at different retention times as shown in Table S-3. From the graph of the peak area vs. m/z (Figure 6, left side), it is clear that only a few molecules had high peak areas, in other words, only very few molecules had exceptionally...
high concentrations, while the rest of the molecules were present in relatively equal amounts within a narrow range of concentrations. The retention time vs. m/z graph (Figure 6, right side) clearly shows that the nitro-compounds in the standard soot were successfully resolved in the chromatography column.

Figure S-9 shows that mono-substituted nitro-compounds (NO₂) were the most abundant molecules, they compose 62% of the sample analysed, based on the sum of the peak areas in the chromatogram. The abundance of molecules mono-substituted with NO₄ (e.g. nitroquinone, nitro lactone, nitro benzofuran and nitro carboxylic acid) is 16%. The abundance of molecules mono-substituted with NO₃ (e.g. nitro-keto, nitro-hydroxy and nitro-phenol) is 12%. The remaining 10% of the sample consisted of molecules with N₂O₄ (e.g. dinitro and alkyl-nitro-quinoline oxide).

Figure 6: 2D view of the nitro-compounds masses identified in NIST SRM 1650a.

In the negative mode, a m/z of 152.0353 was detected and it was eluted after 14.76 and 17.05 min (see Figure 7-A upper trace). The mass was detected as protonated ion in the positive ion mode at the same retention times. This indicates that 152.0353 is a deprotonated ion with the C₇H₆O₃N empirical formula. The tandem MS for the deprotonated ion for the two eluted isomers showed a diagnostic neutral loss of NO (see Figure 7-A1).
The model standard 4-methyl-2-nitrophenol was eluted after 15.61 min, it was detected as deprotonated ion (Figure S-10-A upper trace), and the tandem MS showed the loss of NO (Figure S-10-A1). It is thus suggested that the measured m/z 152.0353 corresponds to two isomers of methyl-nitrophen-ol. Noguchi et al. reported the identification of some methylated nitro phenols in diesel exhaust particulate extract. The molecule 2-nitro-9-fluorenone was eluted in the NIST SRM 1650a at the same retention time, and showed similar detection as the standard (See Figure 7-A lower trace, Figure S-10-A lower trace). Other nitro-fluorenone isomers were detected at 15.3, 17.4, 19.5, and 24.9 min. In the tandem MS, all isomers radical anion showed the dominant fragment, which is the loss of NO, and in addition there were other fragments of H$_2$CNO, H$_2$CN, and H$_2$O (see Figure 7-A2). The protonated ion for the 2-nitro-9-fluorenone in NIST SRM 1650a was detected with a weak signal, so the tandem MS/MS was not recorded.

An m/z of 242.0455 was detected as deprotonated and protonated ions (Table S-3). The mass was eluted in the chromatogram after 32.20, 35.90, 36.10 min (see Figure 8 A, B). The peak shape showed back tailing in the chromatogram. The tandem MS for the deprotonated ion showed a dominant loss of NO, and other minor fragments of H$_2$CNO, H$_2$CN, H$_2$O, and CH$_3$ (See Figure 8-A1). The protonated ion in tandem MS showed dominantly the loss of OH and NO$_2$ (see Figure 8-B1).

A model compound of 2-naphthoic acid was eluted after 27.14 min as deprotonated and protonated ions. The chromatographic peak of the 2-naphthoic acid showed back tailing due to dual retention mechanism on the chromatographic column. It is thus suggested that the m/z 242.0455 is a molecule with an acidic functional group. Based on the chromatography, spectral and tandem MS fragmentation, the m/z of 242.0455 is likely a nitro carboxylic-PAHs.
Based on m/z (292.0490), retention time (39.29 min from the standard) and ionization products ([M⁻] and [M+H⁺]), the 1,8-dinitropyrene was positively identified. The peak for this molecule (m/z 292.0490) in the NIST SRM 1650a eluted at 39.36 min which is in great agreement with what has been found in the customized mixture.

There are many examples of tentatively identified molecules. All alkylated isomers of the eight masses reported in the COA of SRM 1650b were tentatively identified.

Nitro-phenyl-naphthalene isomers were detected in the soot sample as [M⁻] and [M+H⁺]. The radical anion (m/z 249.0791) and the protonated ion (m/z 250.0858) showed similar fragmentations to what was summarized in Table 1. Several isomers of C₁₋₄ alkyl-nitrophenyl-naphthalene (m/z 263.0948, 277.1104, 291.1265, 305.1421) were also detected.

Dinitronaphthalene (m/z 218.0331), dinitroacenaphthene and dinitrobiphenyl (m/z 244.0485), dinitrofluorene (m/z 256.0490), dinitroanthracene and dinitrophenanthrene (m/z 268.0490) were also tentatively identified. There were isomers of dinitro-biphenyl in NIST SRM 1650a. Methyl, dimethyl, trimethyl-dinitronaphthalene (m/z: 232.0490, 246.0646, 260.0803), methyl, dimethyl, trimethyl-dinitrofluorene (m/z: 270.0646, 284.0803, 298.0959), and methyl-dinitroanthracene/dinitrophenanthrene (m/z: 282.0646) were also tentatively identified.
Figure 7: Selected extracted ion chromatograms showing isomers for some selected identified masses in NIST SRM 1650a, and their tandem MS/MS. (A) upper trace: EIC m/z 152.0353 [M-H], lower trace: m/z 225.0431 [M+]. (A1) MS/MS spectrum; m/z 152; [M-H], (Rt =17.05), and (A2) MS/MS spectrum; m/z 225; [M+], (Rt =24.99 min).
Figure 8: extracted ion chromatograms and tandem MS/MS spectrum for m/z 242.0455 in NIST SRM 1650a. (A) EIC m/z 242.0455, (- ion mode; [M-H]) (A1) MS/MS spectrum m/z 242; [M-H]⁻ (Rt = 36.10), (B) EIC m/z 244.0601, (+ ion mode; [M+H]) (B1) MS/MS spectrum m/z 244; [M+H]⁺ (Rt = 36.10).
Conclusions

The importance of this study is in accurately and comprehensively identifying nitro-compounds which have deleterious impact on health of humans and the safety of the environment. This study presents a newly developed innovative method for identifying unknown nitro-PAHs and nitro-compounds in NIST SRM 1650a. This method consists of the use of high performance liquid chromatography in combination with high resolution Orbitrap mass spectrometry. Before the identification of non-targeted nitro-compounds, we exploited information gained from APCI/HR-MS/MS and chromatographic behavior (e.g. neutral loss of NO) of model compounds in a customized mixture.

Nine molecules, namely 1-nitronaphthalene, 2-nitrofluorene, 9-nitroanthracene, 1-nitropyrene, 4-nitropyrene, 3-nitrofluoranthenes, 6-nitrochrysene, 7-nitrobenz[a]anthracene and 6-nitrobenzo[a]pyrene, among all molecules reported in the COA of SRM 1650b, were positively identified. The molecules 2-nitronaphthalene, 2-nitrobiphenyl, 3-nitrobiphenyl, 5-nitroacenaphthenes, 3-nitrophenanthrene, 4-nitrophenanthrene, 9-nitrophenanthrene, 1-nitrofluoranthenes, 2-nitrofluoranthenes, 8-nitrofluoranthene; and 3-nitrobenzo[e]pyrene, were tentatively identified. Having standards will help the positive identification of these molecules. While two molecules of the SRM 1650b, namely 1,3-dinitropyrene, 1,6-dinitropyrene, were not identified, the 1,8-dinitropyrene isomer (which is not listed in the COA of SRM 1650b) was positively identified clearly demonstrating the predictive strength of proposed methodology. Moreover, this method was capable of identifying 98 additional nitro-compounds masses and their isomers. Various classes of nitro-compounds were identified, e.g. nitro-PAHs, alkylated-nitro-PAHs, dinitro-PAHs, alkylated-dinitro-PAHs, nitro-phenolic compounds and nitro partial polar-PAHs. Mono substituted nitro-compounds (NO₂) was the abundant group with 62% based in the sum of the peak areas in the chromatogram. The second most abundant group was NO₄ with 16% followed by NO₃ with...
12% and $\text{N}_2\text{O}_4$ with 10%. Overall, this study is a step forward in non-target identification of unknown nitro-compounds in complex matrices such as the SRM 1650b soot matrix, and can be applied for other mixtures e.g. diesel soot and sediment fractions.

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NIST SRM 1650a

Loss of NO