Supporting Information

Toxicological Properties of Nanoparticles of Organic Compounds (NOC) from Flames and Vehicle Exhausts

L. A. Sgro, A. Simonelli, L. Pascarella, P. Minutolo, D. Guarnieri, N. Sannolo, P. Netti, A. D'Anna

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Details on sample collection and preparation

A recent article *(1)* describes the sample collection, preparation and characterization in detail, which is also summarized here for convenience to the reader. Water samples were collected from flames and vehicle exhausts. Combustion reactions burning all hydrocarbons produce water vapor as one of the main products. Cooling the combustion products below 100°C causes condensation of the combustion-generated water, which scrubs out also products that have some affinity for water. The samples for the vehicle exhausts were collected by driving the test vehicles on a chassis dynamometer, and flowing the exhaust through a 4m-long 50 cm ID stainless steel tube, cooled with a counter-flow water-cooled heat exchanger. The condensed combustion-generated water was drained and collected at the bottom of the tube. Laboratory flames consume fuel at a much lower rate than vehicles/engines so that very small amounts of water are collected by condensing extracted combustion products. To increase the sample volume and reduce breakthrough, we bubbled flame products through a two stage bubbling system (8 ml of bidistilled water in the first bubbler and 6 ml in the second) in addition to a water-cooled condenser. All combustion products that diffuse to the bubble-water interface and have a partial affinity for water should remain in the sample.

Earlier work showed that the UV-visible absorption spectra of material collected in water samples from premixed laminar ethylene air flames has the same spectral form as NOC measured in combustion conditions that form only these organic particles (and not larger more graphitic soot particles) *(2,3)*. NOC absorb and fluoresce only in the far UV (less than 300 nm), while soot particles have a continuous absorption and fluorescence throughout the UV-visible wavelength range (4-6). The concentration of NOC can be determined from the broadband UV absorption spectra in the wavelength range 200-300 nm if other interfering species (soot and gas phase organics) are not present. Even in more fuel rich flame conditions where the concentration of soot particles is equal or greater than that of NOC, only NOC remains dispersed throughout in the sample while the larger, more graphitic soot particles self-migrate to the boarders of these samples *(1, 7)*. Following these earlier works, we used water sampling

to isolate NOC from soot particles by collecting the sample from the center of the container after allowing the hydrophobic particles to migrate to the boarders *(1,8)*.

In addition to particles, CO_2 and semi-volatile organics (like benzene, napthalene, and substituted PAHs) are also collected in water samples, and UV extinction spectra show their presence as peaks overlaid on the broadband extinction attributed to NOC *(1,8)*. Gaseous, low-boiling point solutes are extracted by evaporation in a rotating vessel in two stages: a rapid boiling at 135-120 mbar absolute pressure and 45 °C temperature for about 10-20 s, followed by a slower liquid evaporation with a rate of about 55 ml/hour at 60 mbar. The sample volume (on the order of 500-1000 ml collected from vehicles over several New European Drive Cycles, which vary load and speed to simulate urban and extra-urban driving, and 200-500 ml collected from flames with different fuel:air mixtures) was reduced by 1-2 orders of magnitude during evaporation. The sample times to collect these volumes of water were on the order of 8 hours from vehicles and 12 hours from flames. For this study, we were mainly concerned with collecting large amounts of NOC for toxicological testing, and the samples contain concentrated amounts of NOC compared to ambient concentrations near combustion exhausts. UV extinction measurements before and after rotary evaporation show that the semivolatile organics are effectively removed from the samples. The removal of the volatile organic fraction from the samples by evaporation was further confirmed by Head Space/Solid Phase Microextraction followed by Gas Chromatography/Mass Spectrometry.

Description of methods used to asses the size, chemical nature, and mass of the samples

Several techniques were used to measure the amount and size of organic flame products in the water samples. The combined results show that after evaporation the main organic species remaining in the water samples is NOC with the same optical properties and size of incipient organic carbon particles that are routinely measured in flames burning hydrocarbons before the onset of larger more graphitic soot

particles *(1)*. That work also describes the comparison of measurements in water samples to *in situ* optical measurements (measured directly in the flames or engine exhaust from which the samples were collected) and off-line measurements of particles deposited on substrates by thermophoresis analyzed by Atomic Force Microscopy *(1)*. Here we provide a brief description of several analytical methods that were used to analyze the water samples for this study.

Good agreement between the mass concentrations estimated by three independent measurement methods (UV-vis light absorption, Total Organic Carbon (TOC) Analysis, and Electrospray-Differential Mobility Analysis (E-DMA) implies that most of the non-volatile OC material remaining in the samples after evaporation is NOC *(1)*. Three independent methods were also used to determine the size of material collected in the water samples: E-DMA, Fluorescence Correlation Spectroscopy (FCS), and Time Resolved Fluorescence Polarization Anisotropy (TRFPA). Agreement in the size determined by these three independent measurements indicates that the size of the non-volatile material measured by E-DMA is due to a species that fluoresces like NOC, again indicating that the main species remaining in the samples collected and treated as described above is NOC *(1)*. Here, we give a brief description and references for more detailed descriptions of these methods used to characterize the samples.

UV-visible Light Absorption

Light absorption gives the volume fraction (mass concentration with an assumed density) of absorbing species if their cross sections are known. We measured absorption in the UV-visible wavelength range using a broad band light source (wavelength, λ=200-500 nm) produced by focusing a Q-switched Nd:YAG laser operated at λ=1064 nm (200 mJ energy pulse) with an 8 mm focal-length high power lens in air at STP conditions (*9,10*). The measured extinction coefficient, $K_{ext}(\lambda)$, in water samples collected from flames and vehicles is limited to the far UV (λ<300 nm) and has a spectral form similar to NOC, which is the main species remaining after evaporation. For very small particles, absorption is equal to extinction, which is proportional to their volume fraction, f_v, in the water sample. Absorption in the wavelength range 240-300 nm in the water samples is due mainly to NOC, and this portion of the

spectra is well outside of an interfering absorption band at 200-230 nm found in vehicle exhausts that is barely present in the flame-generated water samples and is not removed by evaporation. The concentration of NOC was determined from the measured extinction at λ=266 nm. The proportionality constant to determine the volume fraction from the measured extinction coefficient was calculated from the optical properties of NOC (published in *(4-6)*), and is f_v/K_{ext}(266 nm)=2. 7x10^{-5}. Since the measured total extinction is a linear combination of the extinction due to all species in the probe volume, determination of the concentration of NOC from extinction measurements as described would overestimate its concentration if other absorbing species are present in relevant amounts. The mass concentrations of NOC was determined from f_v assuming unit density for NOC, ρ_{NOC}=1 g/cm^3.

TOC Analysis

The mass concentration of the sample was also measured using a Total Organic Carbon (TOC) Analyzer (Shimadzu TOC-5000A), and assuming that most of the OC material in the sample is in the form of NOC. The TOC analyzer first sparges the sample with high purity oxygen to eliminate inorganic carbon (mostly CO and CO2) for 8 minutes. Then, 2 ml of sample is introduced into a catalytic combustion tube, decomposed at 680 °C, and the resulting CO2 is measured with a non-dispersive infrared (NDIR) gas analyzer. The peak area measured by NDIR was calibrated with a standard solution of Oxalic acid to give the volume fraction, f_v, of TOC in the sample. The measurement is independent of the size of the OC species. Three measurements were taken and averaged for each sample.

Florescence Correlation Spectroscopy (FCS) and

Time Resolved Fluorescence Polarization Anisotropy (TRFPA)

The two methods utilizing fluorescence, FCS and TRFPA, measured the size of species in the samples directly in the water samples without drying. FCS determines size from the measured translational diffusion of fluorescing species moving by Brownian motion in the waters samples. The FCS measurements were performed placing the samples on the stage of a confocal microscope (Zeiss

LSM510Confocor2), using an 8-well chamber slide (LabTek-Nunc). The fluorescence was excited by an argon laser (λ=488nm), and the emission signals were collected between λ=530-600nm.

Time Resolved Fluorescence Polarization Anisotropy (TRFPA) was used to determine particle size by measuring the rotational diffusion coefficients of particles in the water sample. The TRFPA measurements used the second harmonic (λ≈ 400 nm) of a 100 fs Ti:Sapphire laser to excite fluorescence, and the emitted radiation was collected in the band λ=430-560 nm with a lens and a band-pass filter. A Glan-Thompson polarizer alternatively selected the perpendicular and parallel fluorescence components relative to the exciting field. The time resolved fluorescence signals were detected with a Streak Camera at a very high temporal resolution (10 ps). More details about the experimental techniques and set-up can be found in previous works *(11,12)*.

E-DMA

E-DMA measures the size distribution (size and amount) of all non-volatile material present in the sample, independent of its chemical composition *(13-15)*. The sample was dispersed into tiny droplets, dried and singly charged by charge-neutralized electrospray. The electrical mobility spectra of the charged residues were measured with a DMA. To minimize errors associated with impurities, we generated very small droplets (with a main drop diameter of about 50 nm) by raising the conductivity of the solution to 1 S/m with ammonium acetate addition and by feeding the spray with extremely low flow rates (1nl/s) *(14,15)*. Clustering of sample molecules is avoided by reducing the sample concentration by dilution to the concentration below which only one or no particles will be present within a droplet, $C_{monomer}$. For these dilute concentrations, the electrical mobility, Z, is related to the diameter, d, of the singly charged particles or macromolecules in the sample by $Z = 0.441 \cdot e\left(k_B T / m\right)^{1/2} / p\left(d + d_0\right)^2$, where e is the electron charge, k_B is the Boltzmann constant, and *T, m, p*, and *do* are the temperature, mass, pressure, and effective diameter of the particle-free sheath gas that transports the charged aerosol through the electrostatic classifier (EC) of the DMA *(16)*. The DMA set up uses a shortened 'Vienna'

electrostatic classifier *(17)*, which was modified to allow higher laminar flow rates to reduce diffusional losses and increase resolution *(18)* equipped with a turbulent mixing condensation nucleus counter detector that grows the 1-10 nm classified particles to a size that is large enough to be detected by an optical particle counter (d>300 nm) *(19)*. The carrier gas flow through the EC was recirculated and run at a constant flow rate of 285 lpm. This DMA setup is able to measure charged particles and ions smaller than 10 nm, and distinguish the singly charged monomer peak of a pure solution with a molecular weight as small as 400 amu.

E-DMA spectra of pure molecular standards with a single known molecular weight give a series of separate peaks that correspond to different molecular clusters, according to their charge and aggregate state. Holding the spray variables constant and increasing the level of neutralization of the drying spray products causes multiply charged peaks to reduce and eventually disappear from the spectra while the peak height of singly charged peaks increases. Singly charged peaks correspond to a series of peaks; each peak is a discrete molecular cluster comprised of an integral number of analyte molecules, which depends on how many molecules were in each drop during electrospray drying. Reducing the concentration in the spray solution reduces the number of molecules/drop and the signal of larger cluster peaks until eventually only the monomer peak remains (formed from droplets containing a single analyte monomer). For concentrations below $C_{monomer}$, the peak height of the singly charged monomer is proportional to the concentration in the spray solution. We used two pure standards (Lysozyme, Molecular Weight = 14000 Da) and Angiotensin (1000 Da) to determine the neutralization level of the spray that insures that only singly charged peaks are present and to calibrate peak height with concentration for dilute solutions below $C_{monomer}$. These two standards gave similar calibration curves.

Analytical procedures used for LC/ESI/MS

For each test we mixed by agitation 5µl of an aqueous solution of peptide at 1µg/µl with 540µl of sample and incubated for 18 hours at 37°C, to mimic body temperature. The solutions were dried and successively dissolved in a solution of 0.1% formic acid. LC/ESI/MS analyses were performed using an Agilent 1100 series HPLC system (Palo Alto, CA, USA) and a LCQ^{TM}_{DECA} (Thermo) ion trap mass spectrometer, equipped with an ESI source. A Jupiter C18 (250mm x 2.0mm, 5µm, 300Å) column (Phenomenex, St. Torrance, CA, USA) was used for HPLC separations; HPLC grade solvents were obtained from Carlo Erba (Milan, Italy). We used the following solvents: solvent A = 0.1% aqueous formic acid; solvent B = 0.1% methanolic formic acid. The chromatographic process was characterized by a linear gradient of solvent B from 5% to 95% in 95 minutes, with a flow rate of 0.2 ml/min. The effluent was directly connected to the electrospray ion source of the mass spectrometer (heated capillary temperature = 300 °C). Data were acquired and processed using the Xcalibur program (version 1.1, ThermoQuest) in the range *m/z* 50-2000.

Cell culture growth and treatment for observation by optical and confocal microscopy

Mouse embryo fibroblasts, NIH3T3 cells were maintained in 10mm diameter Petri dishes in Dulbecco Modified Eagle's Medium (DMEM; Gibco, Life Technologies) supplemented with glutamine (Euroclone), penicillin-streptomicin (Euroclone) and 10% Fetal Bovine Serum (FBS; Gibco, Life Technologies). Medium was changed every 2-3 days and NIH cells were split every 7 days. Cells were treated with trypsin for 5 min at 37°C, harvested in a fresh tube and centrifuged at 1000 rpm for 5 min. 10^5 cells were seeded in a 24-well for the Alamar Blue viability assay, and on a 12 mm diameter coverslip for confocal microscope observations. Finally, the cells were incubated with 0.5 ml of particle suspension, ranging from 0 to 30µg/ml in DMEM- 10% FBS without phenol red at 37°C for 1 and 3 hours.

Graphical Resuts of LCI/ESI/MS

Here, we show some spectra, which show the complex nature of the samples tested, the interaction of peptides with the samples, and some repetitive mass spectral peaks consistently observed in the tested samples. Adjacent chromatographic peaks in the NOC samples often contained species with a spacing of 44 Da. Fig. S1 shows an example of a NOC sample collected from a gasoline powered vehicle exhaust. The mass spectra of species eluting at 29.91 min from the gasoline NOC sample showed mainly m/z = 283.1 and 300.1, and the adjacent peak eluting at 34.48 min showed m/z =327.1 and =344.1 (Fig. S1). Fig. S2 shows the same spacing noted in adjacent chromatographic peaks with higher retention times. These patterns were observed in the size range of about 300 to 2000 Da. Figs. S1 and S2 show the complexity of the tested samples. Each chromatographic peak corresponds to more than one substance, as noted in the corresponding mass spectra. The mass spectra for chromatographic peaks at larger retention times become increasingly more complex compared to those at lower retention times.

While LCI/ESI/MS could not improve our knowledge of the chemical structure of NOC, it showed clearly that the peptides effectively interacted with the samples. For example, Fig. S3 shows the chromatographic profile of Angiotensin I, and the NOC sample collected from gasoline powered vehicle exhaust before and after incubation with the peptide. For all of the peptides tested, the peptide peak was clearly absent in the sample after incubation. However, we could not attribute changes in the samples before and after incubation with the peptides to a simple interaction or discern adducts.

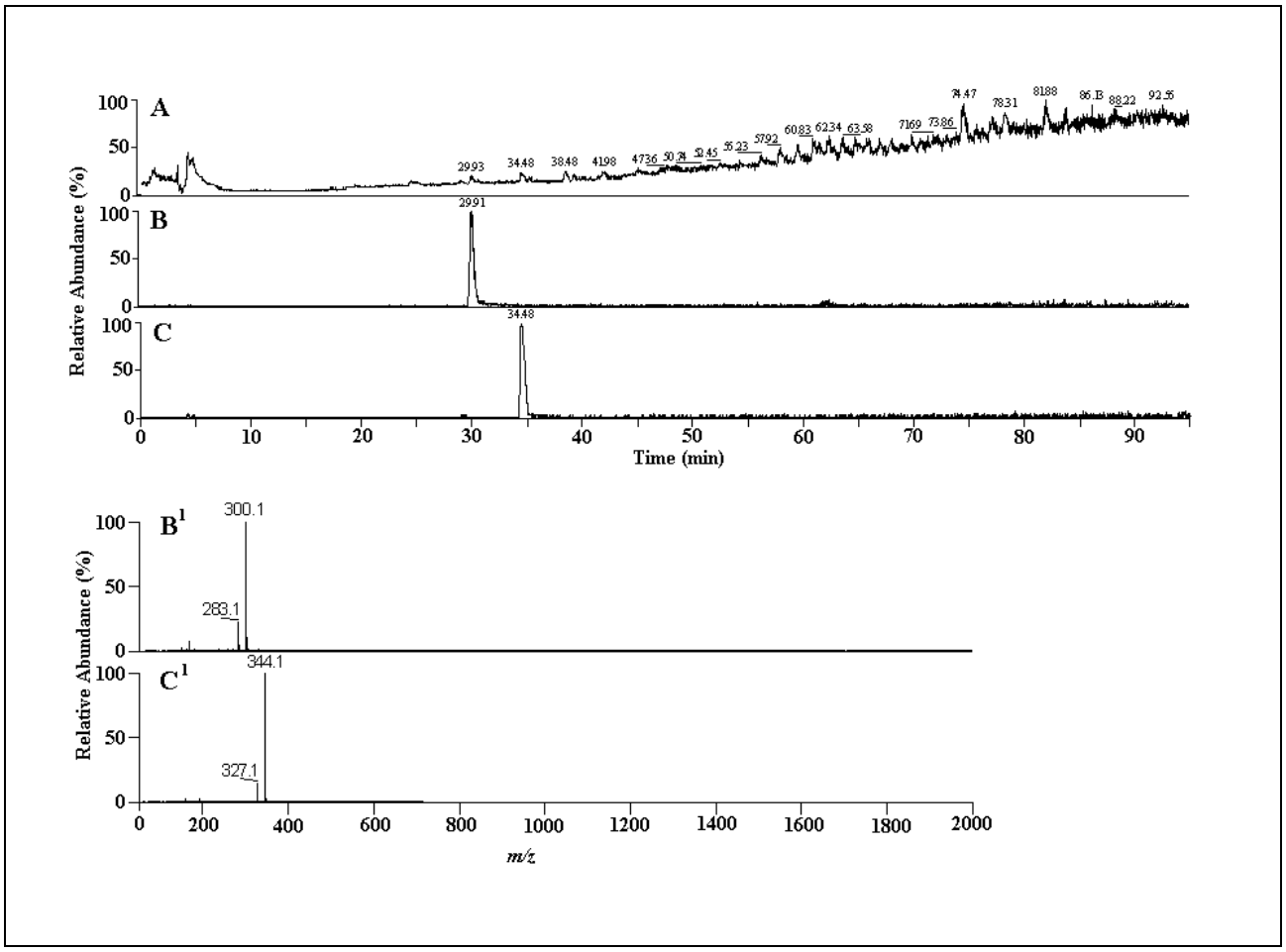

Figure S1. Chromatographic profile of a NOC sample collected from gasoline powered vehicle exhaust before incubation (A). Chromatographic profiles of the extracts of the total ion current (TIC) of A for the ions with m/z 300 and 344 (B and C), and the full mass spectra for these peaks (B^1 and C^1).

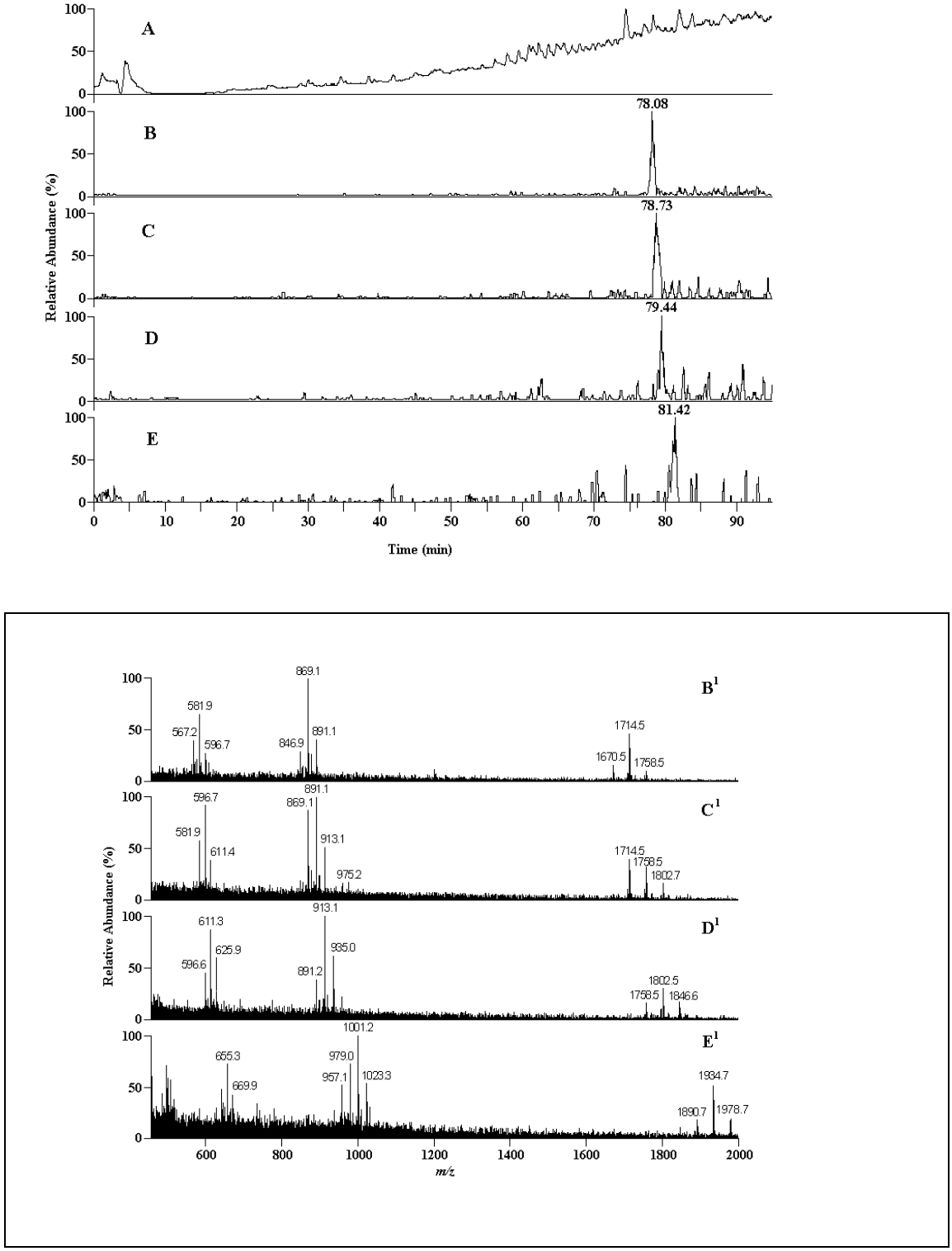

Figure S2. Chromatographic profile of a NOC sample collected from gasoline powered vehicle exhaust before incubation (A). Chromatographic profiles of the extracts of the total ion current (TIC) of A for the ions with m/z 1714.5, 1758.5, 1802.5 e 1890.7(B, C, D, E), and the full mass spectra for these peaks (B^1, C^1, D^1, E^1).

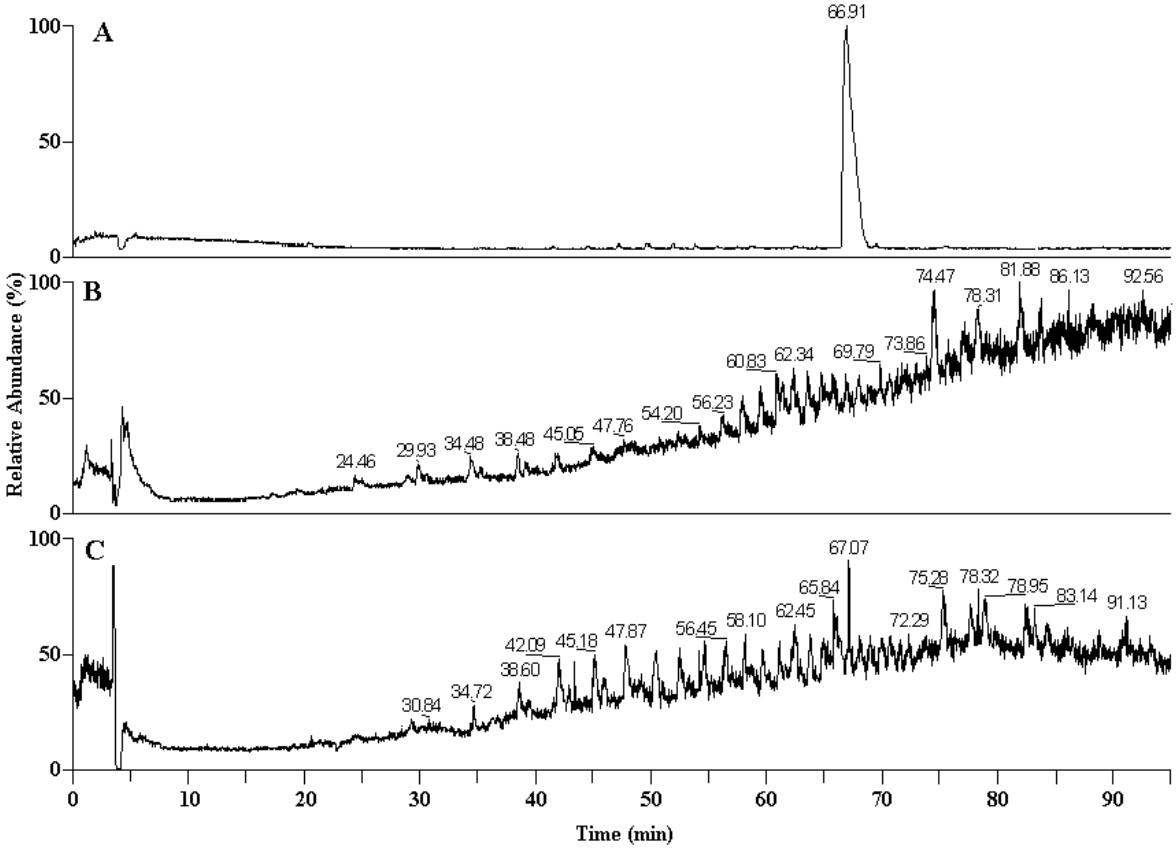

Figure S3: Chromatographic profile of the model peptide Angiotensin I (A), a NOC sample collected from gasoline powered vehicle exhaust before incubation (B), and the sample after incubation with Antiotensin I (C).

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