Research Article

Rapid Communications in Mass Spectrometry

Received: 18 November 2011 Revised: 18 January 2012 Accepted: 18 January 2012 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2012**, *26*, 793–804
(wileyonlinelibrary.com) DOI: 10.1002/rcm.6170

Development of a liquid chromatographic method based on ultraviolet–visible and electrospray ionization mass spectrometric detection for the identification of nitrocatechols and related tracers in biomass burning atmospheric organic aerosol

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RATIONALE: Studying the chemical composition of biomass burning aerosol (BBA) is very important in order to assess their impact on the climate and the biosphere. In the present study, we focus on the characterization of some newly recognized biomass burning aerosol tracers including methyl nitrocatechols, nitroguaiacols and 4-nitrocatechol, but also on nitrophenols, methyl nitrophenols and nitrosalicylic acids, using liquid chromatography tandem mass spectrometry.
METHODS: For the purpose of their separation and detection in atmospheric aerosol, a new chromatographic method was initially developed based on reversed-phase chromatography coupled with ultraviolet/visible (UV/Vis) detection. The method was afterwards transferred to a liquid chromatography/electrospray ionization linear ion trap mass spectrometry (LC/ESI-LITMS) system in order to identify the targeted analytes in winter aerosol from the city of Maribor, Slovenia, using their chromatographic retention times and characteristic (−)ESI product ion (MS^2) spectra.
RESULTS: The fragmentation patterns of analytes obtained with LITMS are presented. Additional nitro-aromatic compounds (*m/z* 168 and 182) closely related to the targeted nitrocatechols and nitroguaiacols were detected in the aerosol. According to their MS^2 spectra these compounds could be attributed to methyl homologues of methyl nitrocatechols and nitroguaiacols.
CONCLUSIONS: The proposed LC/MS method results in a better separation and specificity for the targeted analytes. Several nitro-aromatic compounds were detected in urban BBA. The LC/MS peak intensity of the newly detected methyl nitrocatechols and nitroguaiacols is comparable to that of the methyl nitrocatechols, which also qualifies them as suitable molecular tracers for secondary biomass burning aerosol. Copyright © 2012 John Wiley & Sons, Ltd.

Biomass burning (BB) is considered as one of the greatest primary sources of organic aerosols in the atmosphere.[1] The chemistry of compounds present in biomass burning aerosol (BBA) is diverse and directly dependent on the chemical composition of the burning material and the combustion conditions.[2] A well-established tracer for primary BBA is levoglucosan (1,6-anhydro-β-anhydroglucose), which originates from the pyrolysis of cellulose or hemicelluloses.[2–8] Secondary BBA, which is formed after physical and chemical changes (aging) of primary BBA in the atmosphere, contains more oxidized and polar compounds. An important class of these secondary organic aerosol (SOA) compounds are nitrocatechols, which are strong absorbers of ultraviolet and visible light, and, therefore, could affect the radiative balance and climate of Earth. Recently, methyl nitrocatechols were proposed to be suitable tracers for highly oxidized secondary BBA.[9] These compounds are formed through photooxidation, in the presence of NO_x, of *m*-cresol, which is emitted at significant levels during BB.[9,10] Nitrophenols, structurally related compounds to nitrocatechols, have already been reported in the atmosphere.[e.g. 11–15] Their atmospheric emission can originate from primary sources (e.g. traffic, coal and wood combustion, industry) and predominates the secondary emission (reactions of monoaromatic compounds with OH radicals and NO_x), especially in urban areas.[11–15]

The influence of nitrocatechols on the Earth's climate and the biosphere has not been measured yet, nor has their toxicity toward living organisms (including humans) been studied. Several studies so far indicate that nitrophenols are phytotoxic[11] and can induce estrogenic, anti-androgenic or vasodilatory effects.[16–18]

The objectives of the present study were to characterize nitrocatechols and related compounds in ambient aerosol using mass spectrometric and chromatographic techniques with a view to develop in a further phase suitable quantitative methodology. The selected ambient aerosol samples were collected during a cold winter episode from an urban site in

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Maribor, Slovenia, which is influenced by heavy traffic and substantial emissions from residential wood burning for domestic purposes. In a first part of this study, we report the synthesis of a major reference compound, i.e. 3-methyl-5-nitrocatechol, and its structural characterization using nuclear magnetic resonance (NMR). In a second part, emphasis is given to selecting a suitable LC technique and optimizing the chromatographic conditions for separating methyl nitrocatechols and related nitro-aromatic compounds using diode array UV/Vis and subsequently (−)ESI-MS detection. In a third part, LC/(−)ESI-MS^2 data are presented and interpreted for the nitro-aromatic compounds that occur in ambient samples.

EXPERIMENTAL

Reagents, standards and standard solutions

Acetonitrile and methanol (Chromasolv gradient grade, for HPLC, ≥99.9%; Sigma-Aldrich, St. Louis, USA), 2-propanol (LiChrosolv gradient grade, for HPLC, ≥99.9%; Merck, Darmstadt, Germany), tetrahydrofuran (Chromasolv Plus, for HPLC, ≥99.9%, inhibitor-free; Sigma-Aldrich), and high-purity water (18.2 MΩ·cm), supplied by a Milli-Q water purification system (Millipore, Bedford, MA, USA), were used for the mobile phase and sample preparation. The following additives for mobile phase or sample preparation were used: glacial acetic acid (100% Suprapur; Merck), ammonium acetate (Fractopur; Merck), formic acid (98–100% analysis grade; Merck), ammonia solution (25% Suprapur; Merck), ammonium formate (Puriss p.a., eluent additive for LC; Fluka, Buchs, Switzerland), and ammonium bicarbonate (Fluka).

The following standard substances were used: 4-methyl-5-nitrocatechol (4M5NC, Santa Cruz Biotechnologies, USA), 3-methyl-4-nitrophenol (3M4NP, Acros Organics, Geel, Belgium), 4-nitrocatechol (4NC), 2-methyl-4-nitrophenol (2M4NP), 2-nitrophenol (2NP), 4-nitrophenol (4NP), 2,4-dinitrophenol (2,4DNP), 4-nitroguaiacol (4NG), 2-methoxy-5-nitrophenol (5-nitroguaiacol, 5NG), 3-nitrosalicylic acid (3NSA), 2-hydroxy-5-nitrobenzoic acid (5-nitrosalicylic acid, 5NSA) and 3-methylcatechol were purchased from Sigma-Aldrich. All standards had purity higher than 95% and were used without further purification. 3-Methyl-5-nitrocatechol (3M5NC) and 3-methyl-6-nitrocatechol (3M6NC) were synthesized from 3-methylcatechol by procedures described in the literature (details about the synthesis are provided in the Supporting information).[9,19,20] Individual standard stock solutions of the studied nitro compounds were prepared at concentrations of 500 mg L^{-1} in methanol. A composite standard solution with a concentration of 2 mg L^{-1} of each nitro compound in methanol/water (3/7; v/v) mixture containing 10 mM ammonium formate buffer pH 3 was initially used for the optimization of the chromatography of the nitro-aromatic compounds. For the optimization of the (−)ESI-MS/MS conditions on system 1 (see below) the individual dilute standards of 1 mg L^{-1} in methanol/water (30/70; v/v) mixture containing 10 mM ammonium formate buffer pH 3 were used. Before injecting aliquots into the LC or LC/MS systems, the standards were filtered through a PTFE membrane filter (pore size 0.2 µm, Iso-Disk, Supelco, Bellefonte, PA, USA). All standard solutions were stored at 4 °C and were stable for at least 1 month.

Instrumentation

Two different LC/MS configurations were used in this study: system 1 comprised an Agilent 1100 Series HPLC system (degasser, quaternary pump, autosampler and diode-array UV/Vis detector; Agilent Technologies, Waldbronn, Germany) coupled to an Applied Biosystems/MDS Sciex 4000 QTRAP LC/MS/MS system (Applied Biosystems/MDS Sciex, Ontario, Canada), while system 2 comprised a Surveyor Plus system (pump and autosampler) and a linear ion trap mass spectrometer (LXQ) equipped with an ESI source (Thermo Scientific, San Jose, USA).

The triple quadrupole – linear ion trap hybrid mass spectrometer of system 1 (4000 QTRAP LC/MS/MS system) was equipped with a TurboIonSpray (TIS) source, which is a variation of an ESI source. A Harvard 11 plus syringe pump (Harvard Apparatus, Holliston, MA, USA) was used for direct infusion of standards into the TIS source at a flow rate of 10 µL min^{-1}. For the MS system, a central supply of high-purity nitrogen was used as nebulizer, drying and collision gas. To obtain the MS^2 product ion spectra of the deprotonated molecules $[M - H]^-$ of 4M5NC and 3M5NC in negative ion ESI, the following values for the MS parameters were used: –4500 V for the TIS capillary voltage, 10.0 psi for the curtain gas, 'high' setting (vacuum: 4.5–5.0 × 10^{-5} Torr) for the collision gas, 20.0 psi for the nebulizer gas, –49.0 V for the declustering potential, and −5.0 V for the collision cell exit potential. The spectra recorded for each compound were summed spectra obtained by ramping the collision energy from −120 V to −5 V using 1 V per spectrum step. This approach for spectra recording was followed in order to obtain a final spectrum that is rich in characteristic product ions. A Chemstation for LC 3D systems Rev. B.03.02 (Agilent Technologies) and Analyst 1.5 Software (Applied Biosystems/MDS Analytical Technologies Instruments) were used for acquisition and analysis of the LC and (−)ESI-MS/MS data.

System 2 also incorporated a data system using Xcalibur version 2.0 software (Thermo Scientific). The LXQ linear ion trap was operated under the following conditions: sheath gas flow (nitrogen), 0.75 L min^{-1}; auxiliary gas flow (nitrogen), 1.5 L min^{-1}; source voltage, –4.5 kV; capillary temperature, 350 °C; and maximum ion injection time, 200 ms. The ion optics of the LXQ instrument were optimized for maximum $[M - H]^-$ signal intensity using direct introduction of a solution of 4-nitrocatechol in methanol/water (3/7; v/v) containing 10 mM ammonium acetate (pH 3) of 50 µg mL^{-1} at a flow rate of 200 µL min^{-1}. For collision-induced dissociation (CID) experiments (MS^2) on system 2, the ions of interest were activated by applying a fraction of an 80-V supplementary a.c. (alternating current) potential to the exit rods of the linear ion trap at the resonance frequency of the selected ion. The instrument settings were as follows: activation q value, 0.25; normalized collision energy, 35%; isolation width, 2 *m/z* units; and activation time, 30 ms. Helium was introduced as damping and collision gas at a flow rate of 1.0 mL min^{-1}.

The chromatographic columns used were two Atlantis T3 (150 mm long, 3 µm particle size, with 3.0 mm or 2.1 mm i.d.) and an XBridge BEH Amide (100 mm × 3.0 mm i.d., 3.5 µm particle size), and were all purchased from Waters (Milford, MA, USA). An external thermostated water bath was used to keep the column temperature constant.

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^{1}H-NMR, ^{13}C-NMR and 2D correlation spectra of the synthesized 3M5NC were recorded on a Varian Unity Inova 300 MHz NMR spectrometer using acetone-d_6 as solvent. For ^{1}H spectra, 100 scans were acquired with relaxation delay of 10.0 s. For ^{13}C spectra, 4000 scans and relaxation of 5.0 s were used.

The pH of the aqueous buffers used during the LC method optimization and application was measured using a pH meter (Mettler Toledo MP 220), which was calibrated with commercially available buffer solutions with pH 4.0 and pH 7.0 (Merck).

ACD/LC simulator (Advanced Chemistry Development, Inc.) software was used to aid the reversed-phase (RP) chromatography method development for the selected nitro-aromatic compounds.

Aerosol sample collection and preparation

The filter samples containing particulate matter PM_{10} (particles with aerodynamic diameter below 10 μm) and control blank filters were provided by the Environmental Agency of the Republic of Slovenia. PM_{10} samples were collected on quartz fibre filters (QMA 47 mm diameter, Whatman) at an urban location in Maribor (ca. 110 000 inhabitants), Slovenia, using low-volume sampling. The measurement site has the typical character of a street canyon and is located on the main road leading to the city centre. Due to heavy traffic, the chosen location is one of the most polluted ones in Slovenia. The air flow through the sampler was 2.3 $m^3 h^{-1}$ and the sampling time was 24 h. Prior to sampling the filters were heated at 500 °C for 3 h to remove organic contaminants. The weighing of the filters was done before and after sampling, after conditioning for 48 h at a relative humidity of 50 ± 5% and a temperature of 20 ± 1 °C. Samples from winter (February) 2010 were chosen for this study. The average daytime temperature during February was about −1 °C. PM_{10} filters and blank filters were stored at −18 °C until analysis. Before extraction, they were equilibrated to room temperature under controlled ambient conditions. The filters were extracted following the aerosol sample preparation procedure described in our previous article.[21] Briefly, the filters were extracted three times with 15 mL of methanol in an ice-cooled ultrasonic bath for 15 min. The total extract was then evaporated to dryness at 25 °C using a gentle stream of nitrogen. The residue was redissolved in 2 mL of methanol/water (30/70; v/v) mixture containing 10 mM ammonium formate buffer pH 3 (the same buffer type used in the final method; see below).

RESULTS AND DISCUSSION

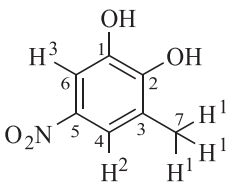
Synthesis and identification of methyl nitrocatechols by NMR and direct (−)ESI-MS^2 techniques

Three different positional isomers are possible for the methyl nitrocatechols, i.e. 4M5NC, 3M5NC, and 3M6NC, which all occur in ambient atmospheric aerosol with 3M5NC being present at high relative abundance (see below). Of these methyl nitrocatechols, 4M5NC was commercially available, while 3M5NC was synthesized in pure form, and 3M6NC was also obtained synthetically as a minor product together with 3M5NC following reported procedures.[9,20] As there was ambiguity with regard to the identification of 3M5NC and 3M6NC, the identification of the major synthesized product as 3M5NC (and not 3M6NC) by NMR techniques is also discussed here. In addition, we present direct (−)ESI-MS^2 data for 3M5NC and 4M5NC, which could be obtained in pure form, and compare them with data obtained for an ambient winter PM_{10} sample from Maribor, Slovenia.

Nitration of 3-methylcatechol in aqueous solution using a $NaNO_2/H_2SO_4$ system (the synthesis procedure is given in the Supporting information) led to the preferential formation of 3M5NC (as reported previously by Palumbo *et al.*[20]). The product was isolated as a yellow solid and its structure determined by ^{1}H-NMR, ^{13}C-NMR and 2D correlation spectroscopy. By careful inspection of the H-2 splitting pattern which appears as an apparent doublet of doublets in ^{1}H-NMR at 7.63 ppm we were able to deduce a long-range ^{4}J coupling constant between H-1 and H-2 with a value of 0.7 Hz (Table 1). In addition, the ^{4}J coupling constant between H-2 and H-3 has a value of 2.7 Hz which falls within the range of *meta*-coupling in aromatic protons. Accordingly, proton H-3 which resonates at 7.60 ppm appears as an apparent doublet with a ^{4}J coupling constant of 2.7 Hz. ^{13}C-NMR analysis reveals a quaternary carbon C-5 bearing the nitro group which resonates at 140.7 ppm and C-6 which resonates at 108.9 ppm. Carbon C-4 is slightly more deshielded in respect to C-6 and resonates at 119.0 ppm (Table 1). This structural assignment of 3-methyl-5-nitrocatechol is additionally supported by HSQC 2D correlation spectra. Analysis of COSY spectra clearly displays a correlation between aromatic H-2 and aliphatic H-1 protons due to long-range coupling. Based on the HMBC correlation from H-2 to C-3, C-5 and C-6 and from H-3 to C-5 and C-4, the quaternary carbon C-5 bearing a nitro group is connected to C-4 and C-6 (Supplementary Fig. S1, see Supporting information).

Figure 1 presents the *m/z* 168 product ion MS^2 spectra for 3M5NC and its positional isomer 4M5NC, which were obtained using direct infusion on system 1. It can be seen that both product ion spectra contain ions corresponding to the neutral loss of NO (*m/z* 138), the combined loss of NO and a

Table 1. NMR data of 3-methyl-5-nitrocatechol in acetone-d_6



Observed J's
^{4}J (H-1, H-2) = 0.7 Hz
^{4}J (H-2, H-3) = 2.7 Hz

Carbon	^{1}H-NMR: δ_H [ppm], mult, *J* [Hz]	^{13}C-NMR: δ_C [ppm]
C-1		144.8
C-2		151.1
C-3		125.7
C-4	7.63, app dd, 0.7, 2.7	119.0
C-5		140.7
C-6	7.60, app d, 2.7	108.9
C-7	2.28, s	16.0

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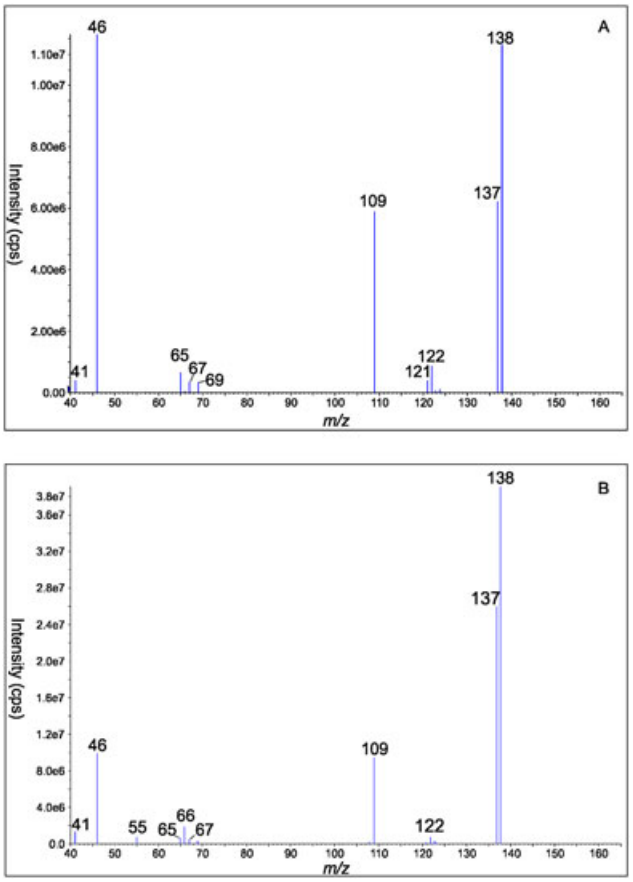

Figure 1. Direct infusion ESI – MS^2 product ion spectra of *m/z* 168 for 3-methyl-5-nitrocatechol (A) and 4-methyl-5-nitrocatechol (B) standards obtained on the 4000 QTRAP from system 1.

hydrogen radical (*m/z* 137), the loss of NO_2 (*m/z* 122), the combined loss of NO and CO (*m/z* 109) from the deprotonated molecule, as well as an ion at *m/z* 46 (NO_2^-), although with different relative abundances. The same *m/z* 168 product ions were also obtained for the PM_{10} sample (not shown), indicating that methyl nitrocatechols are present; however, at this stage the isomeric composition could not be inferred with certainty. In addition, the *m/z* 168 product ion spectrum of the ambient sample contained very weak ions at *m/z* 153, 123 and 95 that are characteristic of nitroguaiacols, which have been reported in ambient aerosol.[9] Thus, in order to confirm the presence or absence of certain isomeric methyl nitrocatechols or nitroguaiacols an adequate chromatographic separation is needed. Hence, an effort was made to develop systematically a suitable chromatographic method for the separation of isomeric methyl nitrocatechols and related nitro-aromatic compounds that are expected to be present in ambient BBA.

Development of a LC method for separation of methyl nitrocatechols and related compounds

Selection of the separation mode

Initially, the study was focused on some selected nitro-aromatic compounds, i.e. 4NP, 2NP, 4NC, 2,4DNP, 4M5NC, 3M5NC and 3M6NC. In a first series of experiments hydrophilic interaction liquid chromatography (HILIC)[22] was evaluated for their separation. 4NC and methyl nitrocatechols are weak acids in aqueous solution with the first pKa values between 6 and 8,[19] while 2NP, 4NP and 2,4DNP have pKa values of 7.23, 7.15 and 4.07, respectively.[23] All these compounds are polar and can be transformed into a more polar state by dissociation to monoanions, i.e. nitrocatecholate or nitrophenolate, respectively. These dissociated forms should be suitable for separation under HILIC conditions. This hypothesis was first tested using an XBridge Amide BEH column and a mobile phase consisting of acetonitrile and aqueous ammonium bicarbonate buffer of pH 9.0 and 10 mM concentration in the final mobile phase. 4NC standard and the LC part of system 1 were used for the development of the separation mode. Under HILIC conditions, 4NC gave a retained, very broad and tailing peak, suggesting that these conditions are not optimal for its chromatography, even when the chromatographic conditions were further optimized. In the next experiments, a mobile phase consisting of acetonitrile/ammonium acetate buffer pH 5.0 or acetonitrile/ammonium formate buffer pH 3.0 was tested for HILIC of 4NC. Neither sufficient symmetrical peak shape nor better retention was achieved; the loss of retention at pH 3.0 and 5.0 for 4NC is logical and can be explained by the presence of analyte molecules in undissociated form on the column. As a conclusion, HILIC using the amide stationary phase is not suitable for nitrocatechol analysis. It is worth mentioning that previous studies also reported difficulties for obtaining proper peak shape and retention of catecholamines (biogenic compounds that are structurally related to nitrocatechols) under HILIC conditions using a bare silica column.[24,25] However, a satisfactory separation of several catecholamines on the same HILIC column as employed in our study (XBridge Amide BEH) was recently reported.[25] Dos Santos Pereira *et al*.[26] suggested a new approach for overcoming the difficulties with the separation of catecholamines under HILIC using so-called 'per-aqueous LC' (or PALC). Briefly, PALC uses a 100% aqueous mobile phase and a polar column stationary phase (like bare silica). The suggested mechanism of retention is a reversed-phase (RP)-like interaction of the undissociated polar analytes from the mobile phase and siloxane bridges on the surface of the silica packing. The retention of the analytes lowers as the percentage of the organic modifier (added to the aqueous mobile phase) increases (a typical RP mechanism of retention). A very good and improved separation of the catecholamines under PALC using a silica column and aqueous 50 mM ammonium formate buffer as mobile phase could be obtained. The findings of dos Santos Pereira *et al*.[26] prompted us to evaluate the PALC separation technique. Using aqueous buffers (ammonium acetate pH 5.0 and ammonium formate pH 3.0) as mobile phase and the amide column in PALC gave better results for 4NC only when ammonium formate pH 3.0 was used as mobile phase. However, even though the retention factor (k) of 4NC was <1, its peak showed significant tailing and thus smaller separation efficiency, which was substantially influenced by the ionic strength of the buffer in the mobile phase. A higher ionic strength of the buffer (e.g. 50 mM) gave better peak shape and efficiency. Together with the column temperature it remains the only parameter that can be optimized under PALC conditions in order to obtain proper retention or separation. Addition of methanol or acetonitrile to the aqueous mobile phase significantly decreased the retention

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of the 4NC peak under these conditions. Taking into account the structural similarity of the nitrocatechols, nitrophenols, and methyl nitrocatechols, together with the limited chromatography parameters that could be changed under PALC to further optimize their separation, we did not proceed with further experiments using this mode. Hence, in a next series of experiments reversed-phase chromatography (RPC) was evaluated and optimized for the separation of nitrocatechols, nitrophenols and methyl nitrocatechols. Previous studies on nitro-aromatics have shown the suitability of RPC in the analysis of nitrophenols,[e.g. 11,13–16] nitrocatechols and 4-nitroguaiacol.[9,13,14]

Influence of buffer type and pH under reversed-phase chromatography

In order to obtain the best conditions concerning symmetrical peak shapes and a high efficiency and resolution among peaks, different buffers and pH values were evaluated initially for the separation of nitro-aromatics. Ammonium acetate buffer pH 5.0 used in the mobile phase together with methanol gave non-symmetrical and badly tailing peaks for nitrocatechols, while ammonium formate at pH 3.9 and 3.4 gave fronting peaks for nitrocatechols and symmetrical peaks for nitrophenols. The reasons for peak fronting could be different, i.e. formation of column void, buffer/pH mismatch between the injected solutions and the mobile phase, a thermally non-equilibrated column, overloading of the column, or eventually a wrongly chosen mobile phase buffer and/or pH conditions.[27] Formation of column void as a cause for the observed fronting was ruled out because all the peaks should have been equally affected, which was not the case. In a next experiment, the column was thermally equilibrated at constant temperature (25 °C) and the final standard solutions were prepared in the mobile phase (methanol/water/200 mM ammonium formate buffer; 30:50:20; v/v/v) in order to exclude the possibility for peak fronting owing to a thermally non-equilibrated column or a buffer/pH mismatch. The overloading of the column was ruled out as the cause for peak fronting, because the mass of the analytes injected on column was around 100 ng. The only remaining logical explanation for the observed peak effect is the non-optimal buffer and/or pH used in the final mobile phase. Indeed, when ammonium formate buffer pH 3.0 was used in the mobile phase, the problem with fronting nitrocatechol peaks was alleviated. Peak symmetry and column efficiency were slightly influenced by the ionic strength of the final mobile phase. It was found that for optimal peak shape and efficiency, a concentration of 40 mM ammonium formate is needed in the final mobile phase.

Subsequently, 0.1% (w/w) formic and 0.1% (w/w) acetic acid were evaluated as additives in the mobile phase. The pH values of the aqueous 0.1% formic and 0.1% acetic acid are 2.7 and 3.3 (at 25 °C), respectively. Figure 2 shows the chromatograms of a standard mixture containing nitrophenols, 4-nitrocatechol and methyl nitrocatechols studied, obtained under the same chromatographic conditions, differing only in the acid used. When formic or acetic acid was used in the mobile phase (with final mobile phase concentration of 0.1% w/w, see Figs. 2(A) and 2(B)) there was no change in selectivity and all peaks showed significant tailing, which was more pronounced when acetic acid was used. The

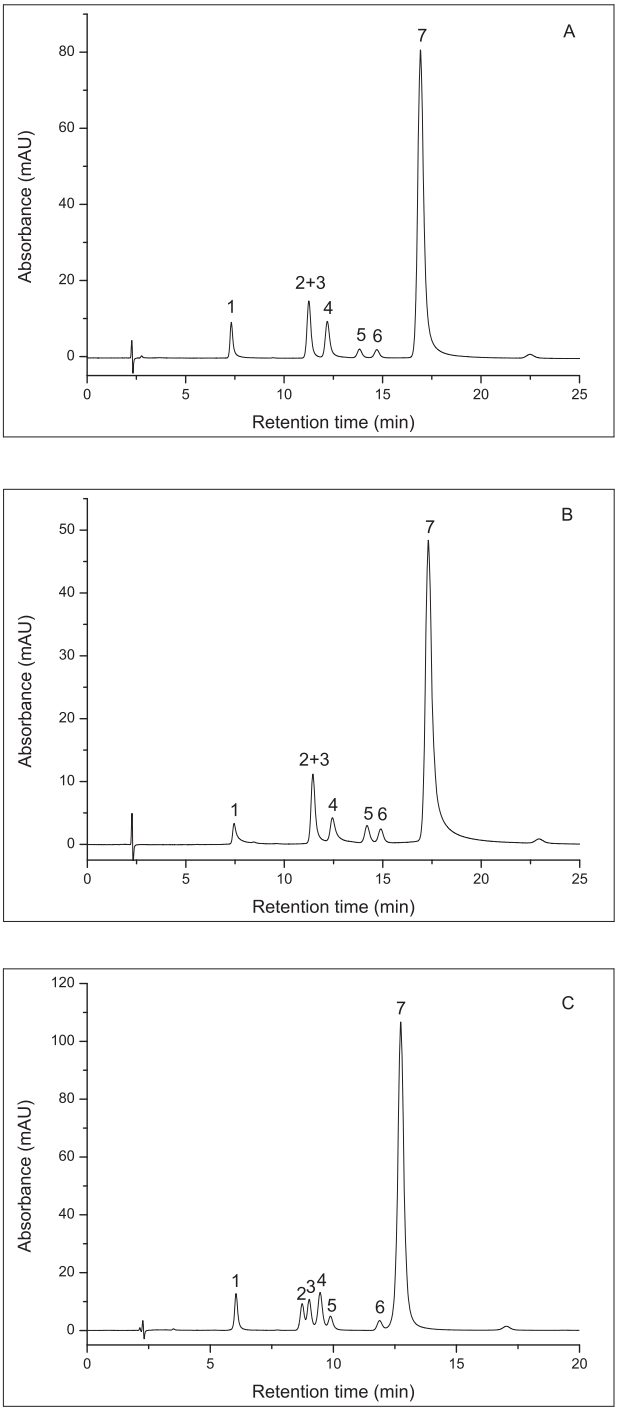

Figure 2. Chromatograms of standard containing: 4-nitrocatechol (**1**), 4-methyl-5-nitrocatechol (**2**), 4-nitrophenol (**3**), 3-methyl-6-nitrocatechol (**4**), 2,4-dinitrophenol (**5**), 2-nitrophenol (**6**) and 3-methyl-5-nitrocatechol (**7**), obtained under following conditions: methanol/water/1.0% formic acid = 44/46/10 (v/v/v) (A), methanol/water/1.0% acetic acid = 44/46/10 (v/v/v) (B), methanol/water/200 mM ammonium formate pH 3.0 = 44/36/20 (v/v/v) (C). For all chromatograms – column: Atlantis T3 (150 x 3.0 mm i.d, 3 µm particle size), flow rate: 0.4 mL min^{-1}, column temperature: 30 °C, injection volume: 5 µL, detection: UV (345 nm).

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chromatogram obtained using a 40 mM final mobile phase concentration of ammonium formate buffer pH 3.0 (Fig. 2(C)) shows a different selectivity and a much better separation and peak shape for the analytes compared to the previous ones (Figs. 2(A) and 2(B)). Increasing the concentration of acetic or formic acid in the final mobile phase from 0.1% to 0.2%, 0.3% or 0.5% gradually improved the peak shape to some extent, but did not completely alleviate the problem with peak tailing. Hence, ammonium formate buffer pH 3.0 was chosen for the RPC optimizations reported in the next section.

Optimization of isocratic separation using methanol as organic modifier

Isocratic mobile phases with a fixed ionic strength (40 mM) of ammonium formate pH 3.0, but a different percentage of methanol in the final composition (30, 40, 50, and 60%), were used at four different temperatures (25, 30, 35, and 40 °C) to obtain the retention data for all compounds. The resolution map for the software-aided simultaneous optimization of % organic modifier B (methanol) and column temperature is given in Supplementary Fig. S2 (see Supporting information).

In order to obtain a better prediction for analyte retention and separation, only the theoretical points within the rectangular area limited and populated by the experimental points on the map were taken into consideration. Within this area, the theoretical points that show sufficient separation among peaks and reasonable run times (< 20 min) have 'coordinates' around 45% for methanol and around 30 °C for column temperature. The most optimal separations according to the software predictions and the real experiment are shown in Supplementary Fig. S3 (see Supporting information). As a conclusion, the best separation for the targeted analytes can be achieved using isocratic elution with 44% of methanol and ammonium formate pH 3.0 (final mobile phase concentration of 40 mM) and at temperature of 32 °C. A wintertime PM_{10} sample analyzed under these optimized conditions is shown in Fig. 3(A). For the purpose of identification, retention times (RTs) and UV/Vis spectra (range: 200–800 nm) of the sample peaks were compared with those of standards. Several nitro-aromatic compounds were detected in the winter PM_{10} sample from Maribor, Slovenia (Fig. 3(A)), i.e. 4NC, 4M5NC, 3M5NC, and 4NP. The peak assigned to 2,4DNP, based on chromatographic RT matching, turned out to be a different compound because its UV/Vis spectrum significantly differed from that of 2,4DNP (not shown).

Change of selectivity by addition of different organic modifiers

A substantial change in the selectivity of separation can be accomplished by a change in the composition of the stationary phase and/or mobile phase, as well as of the column temperature.[27–29] Because only one stationary phase was used in our experiments and taking into account the narrow optimal temperature range, changing the composition of the mobile phase should be the strongest tool for altering the selectivity. For ionizable analytes, changing the mobile phase pH could substantially influence the selectivity of separation.[29] However, the change of pH and buffer type is very limited because of the peak shape problems encountered when the pH is greater than 3. Hence, a practical tool for altering the selectivity could be the change of the mobile

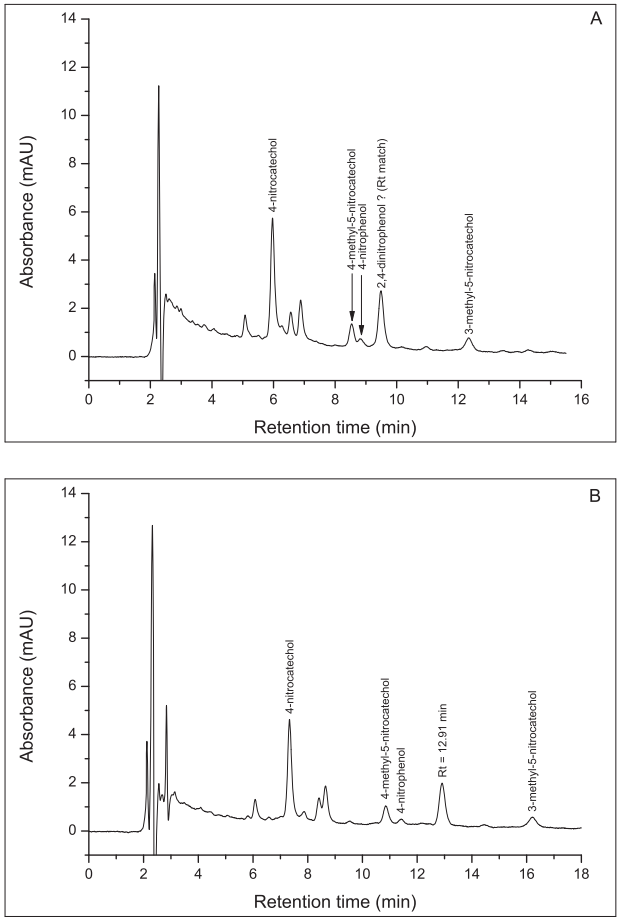

Figure 3. Chromatograms of PM_{10} sample from the city of Maribor, Slovenia (February 2010). Conditions: isocratic elution, methanol/water/200 mM ammonium formate pH 3.0 = 44/36/20 (v/v/v), column T: 32 °C (A) and isocratic elution, methanol/acetonitrile/water/200 mM ammonium formate pH 3.0 = 30/11/39/20 (v/v/v/v), column T: 30 °C (B). For both chromatograms – column: Atlantis T3 (150 x 3.0 mm i.d., 3 µm particle size), flow rate: 0.4 mL min^{-1}, injection volume: 20 µL, detection: UV (345 nm).

phase organic modifier. For that purpose, methanol in the optimized mobile phase (44%) was substituted by another organic solvent giving an equivalent eluotropic strength (34% for acetonitrile, 25% for tetrahydrofuran, and 22% for 2-propanol), while the rest of the mobile phase (up to 100%) was ammonium formate pH 3.0 with a 40 mM final mobile phase concentration. Substituting methanol with acetonitrile (Fig. 4(A) compared to Fig. 2(C)) and tetrahydrofuran gave a substantial change in the selectivity of the separation, while 2-propanol did not result in a substantial change. Further, using different blends of methanol/acetonitrile, methanol/tetrahydrofuran or methanol/2-propanol in the mobile phase, an effort was made to optimize the composition of the ternary mobile phase that would give the best (baseline) separation for the targeted compounds (Figs. 4(B) and 4(C)). The mobile phase containing a blend of methanol and acetonitrile (30/11; v/v) as strong solvent was used for further analysis (Fig. 4(B)). This mobile phase composition gave a satisfactory chromatographic separation and also a lower back pressure compared to the mobile phases containing methanol/tetrahydrofuran or methanol/2-propanol blends.

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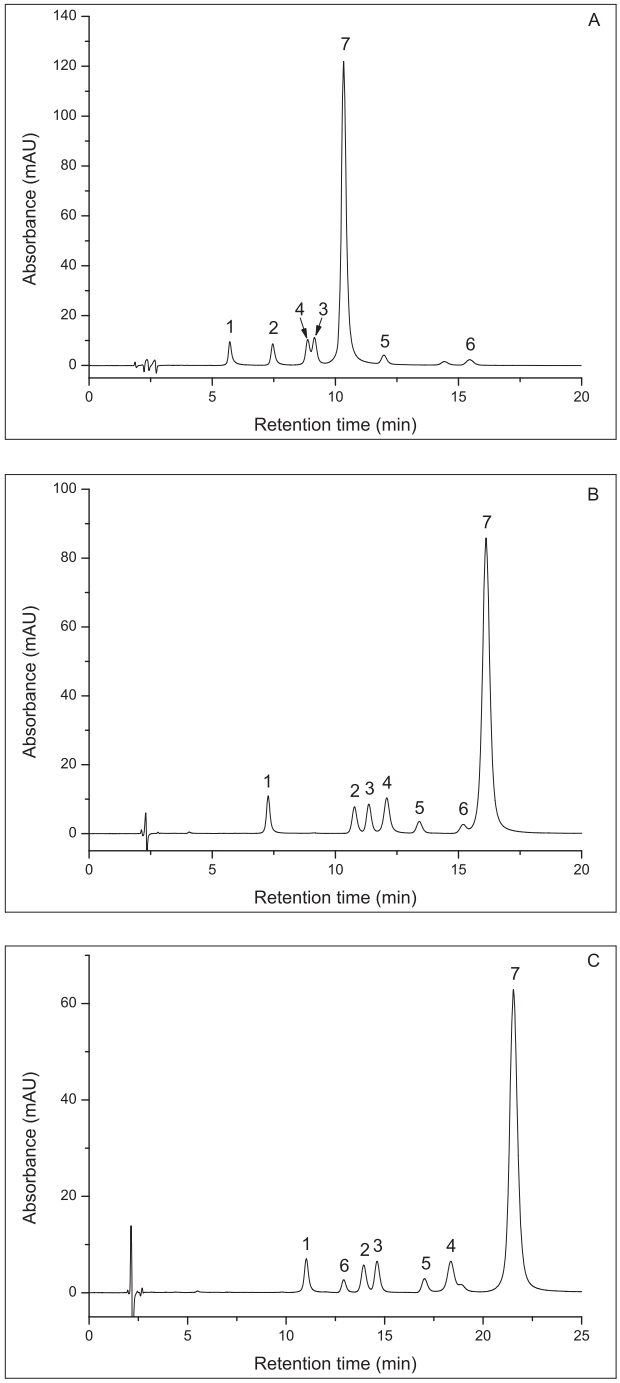

Figure 4. Chromatograms of the same standard mixture as in Fig. 2, obtained under following conditions: acetonitrile/water/200 mM ammonium formate pH 3.0 = 34/46/20 (v/v/v) (A), methanol/acetonitrile/water/200 mM ammonium formate pH 3.0 = 30/11/39/20 (v/v/v/v) (B), methanol/tetrahydrofuran/water/200 mM ammonium formate pH 3.0 = 30/15/35/20 (v/v/v/v) (C). Other chromatographic parameters are the same as in Fig. 2.

Figure 3(B) presents a chromatogram obtained from the same urban PM_{10} sample as in Fig. 3(A); it can be seen that using the ternary mobile phase (methanol/acetonitrile/buffer pH 3.0) gave better separation and specificity for the peaks in the PM_{10} sample chromatogram. The relatively intense peak at RT 9.49 min in Fig. 3(A), which was assigned to 2,4DNP based on its RT, elutes now at 12.91 min, and no longer co-elutes with the dinitrophenol (RT 13.41 min) and is at this stage unknown.

Adaptation of the LC/UV–vis method to LC/(−)ESI-MS

The experiments performed for adapting the LC/UV–vis method outlined above to LC/(−)ESI-MS analysis were made using system 2. For the purpose of LC/(−)ESI-MS analysis, some modifications of the developed LC/UV–vis method were necessary in order to accommodate the chromatographic conditions for (−)ESI-MS detection. The ionic strength of the mobile phase was decreased from 40 mM to 5 mM (keeping the pH at 3.0) to avoid excessive ion suppression in the ion source as well as clogging problems in transfer lines and the ion source. Due to the decrease in the ionic strength of the mobile phase, a lowering of the overall chromatographic resolution occurred. However, a sufficient resolution (>2) is still kept among the methyl nitrocatechol isomers under the new chromatographic conditions.

At this stage, two nitroguaiacols (4- and 5-nitroguaiacol: 4NG and 5NG), two nitrosalicylic acids (3- and 5-nitrosalicylic: 3NSA and 5NSA) and two methyl-nitrophenols (2-methyl-4-nitrophenol and 3-methyl-4-nitrophenol: 2M4NP and 3M4NP) were additionally included in the method. The separation of all analytes was achieved using the optimized mobile phase containing methanol/acetonitrile/aqueous ammonium formate buffer pH 3 = 30/11/59 (v/v/v), with 5 mM salt concentration in the final mobile phase mixture. Other chromatographic parameters were not modified. The mobile phase flow was decreased to 0.2 mL min^{-1} because of the narrower column (2.1 mm i.d.) used for the corresponding LC/MS analysis. LC/MS chromatographic data for the same wintertime PM_{10} sample (previously analyzed with system 1) is shown in Fig. 5. The two most intense peaks in the ambient sample at RTs 7.08 and 16.30 min correspond to 4NC (Fig. 5(B)) and 3M5NC (Fig. 5(C)), respectively. 4M5NC (Fig. 5(C)) and 4NP (Fig. 5(D)) also show intense peaks (RTs 10.74 and 11.08 min, respectively), while 3M6NC (Fig. 5(C)) is present as a minor peak (RT 12.04 min). In addition, the PM_{10} sample from Maribor also revealed the presence of 3NSA and 5NSA (Fig. 5(E); RTs 5.24 and 7.45, respectively), and 3M4NP and 2M4NP (Fig. 5(F); RTs 18.01 and 22.88 min, respectively).

Characterization of methyl nitrocatechols and related tracers using LC/(−)ESI-MS^2

The experiments for obtaining LC/(−)ESI-MS^2 data were carried out with system 2. In this section, we only deal with LC/(−)ESI-MS^2 data for nitro-aromatic compounds that occur in ambient atmospheric aerosol.

Figure 6 presents $[M–H]^-$ product ion MS^2 spectra for 4NP (Fig. 6(A)), 4NC (Fig. 6(B)), 3M5NC (Fig. 6(C)), 3M6NC (Fig. 6(D)), and 4M5NC (Fig. 6(E)). Under the selected CID conditions (i.e. 35% normalized collision energy) the deprotonated molecules of 4NP and 4NC are quite stable and only result in a weak loss of NO, affording an ion at *m/z* 108 and 124, respectively. Furthermore, the precursor ions have a weak satellite ion one unit higher at *m/z* 139 and 155, respectively, which can be explained by the addition of a hydrogen radical, a reaction that is known to occur in the ion trap.[30,31] It is worth noting that the extent of loss of NO (*m/z* 138) is quite

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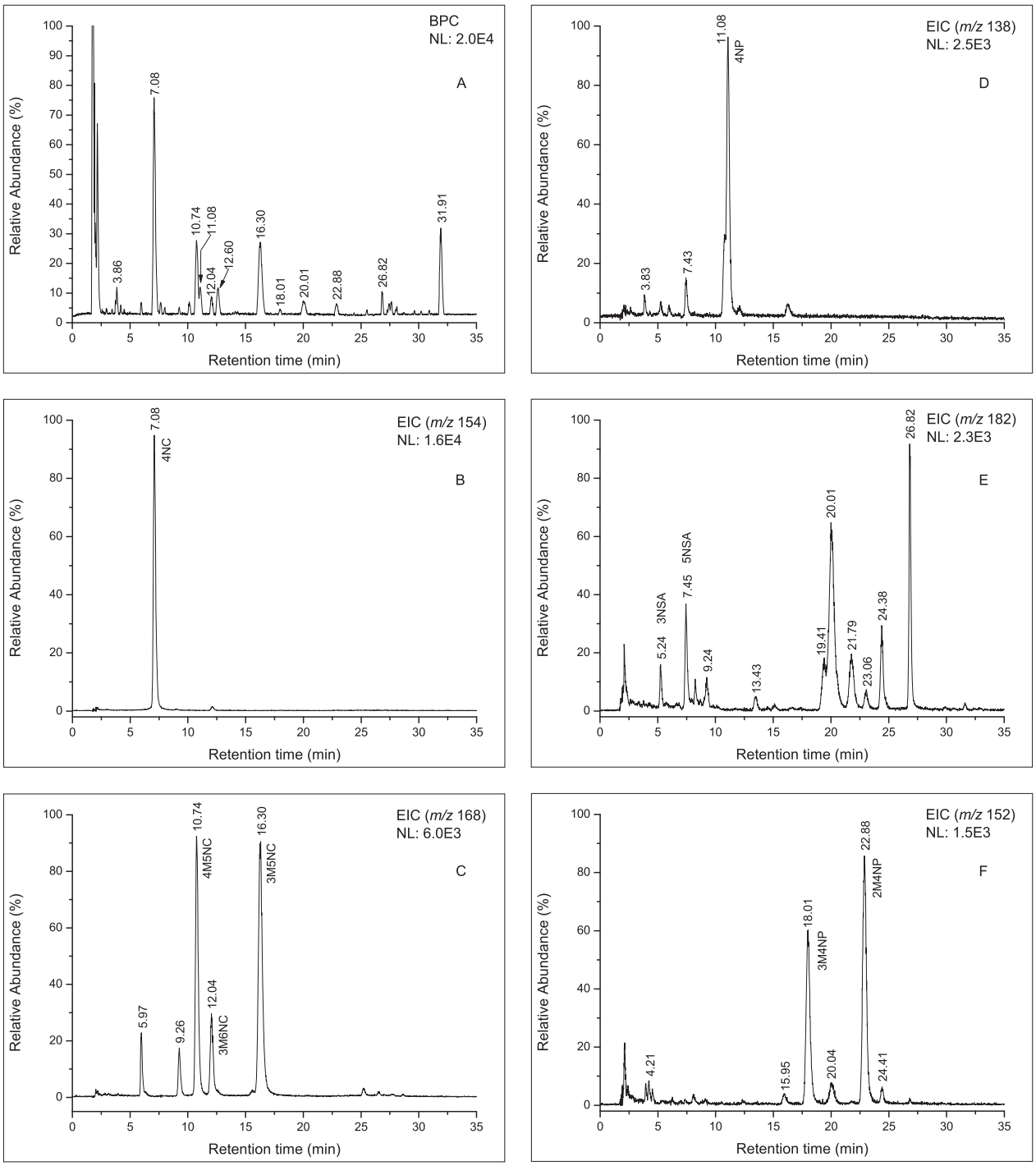

Figure 5. Base peak (BPC) (A) and extracted ion current (EIC) chromatograms (B–F) of PM_{10} sample from city of Maribor, Slovenia (February 2010). Conditions: 0–13 min: isocratic methanol/acetonitrile/8.5 mM ammonium formate pH 3.0 = 30/11/59 (v/v/v), 13–19 min: linear gradient methanol: 30 → 50% and buffer: 59 → 39%, 19–35 min: isocratic methanol/acetonitrile/8.5 mM ammonium formate pH 3.0 = 50/11/39 (v/v/v), column: Atlantis T3 (150 x 2.1 mm i.d., 3 µm particle size), column T: 30 °C, flow rate: 0.2 mL min^{-1}, injection volume: 10 µL, detection: LIT-MS (scan *m/z* 50–500).

different among the three methyl nitrocatechol isomers. The deprotonated form of 4M5NC reveals the most extensive loss, that of 3M5NC the smallest one, and that of 3M6NC a loss that is intermediate between that observed for 4M5NC and 3M5NC. The *m/z* 138 also shows a small satellite ion at *m/z* 139 due to the addition of a hydrogen radical, as already outlined above for deprotonated 4NP and 4NC. In addition, deprotonated 3M6NC (Fig. 6(D)) leads to minor ions at *m/z* 150, due to the loss of a molecule of water (an ortho effect); *m/z* 123, corresponding to the combined loss of NO and a methyl radical, and at *m/z* 94, corresponding to a loss of 74 units that cannot be readily explained. Thus, the three methyl nitrocatechol isomers can be differentiated on the basis of the MS^2 product ion spectra of their deprotonated forms. With regard to the observed NO loss, Levsen *et al.*[32] reported that nitro-aromatic compounds show characteristic

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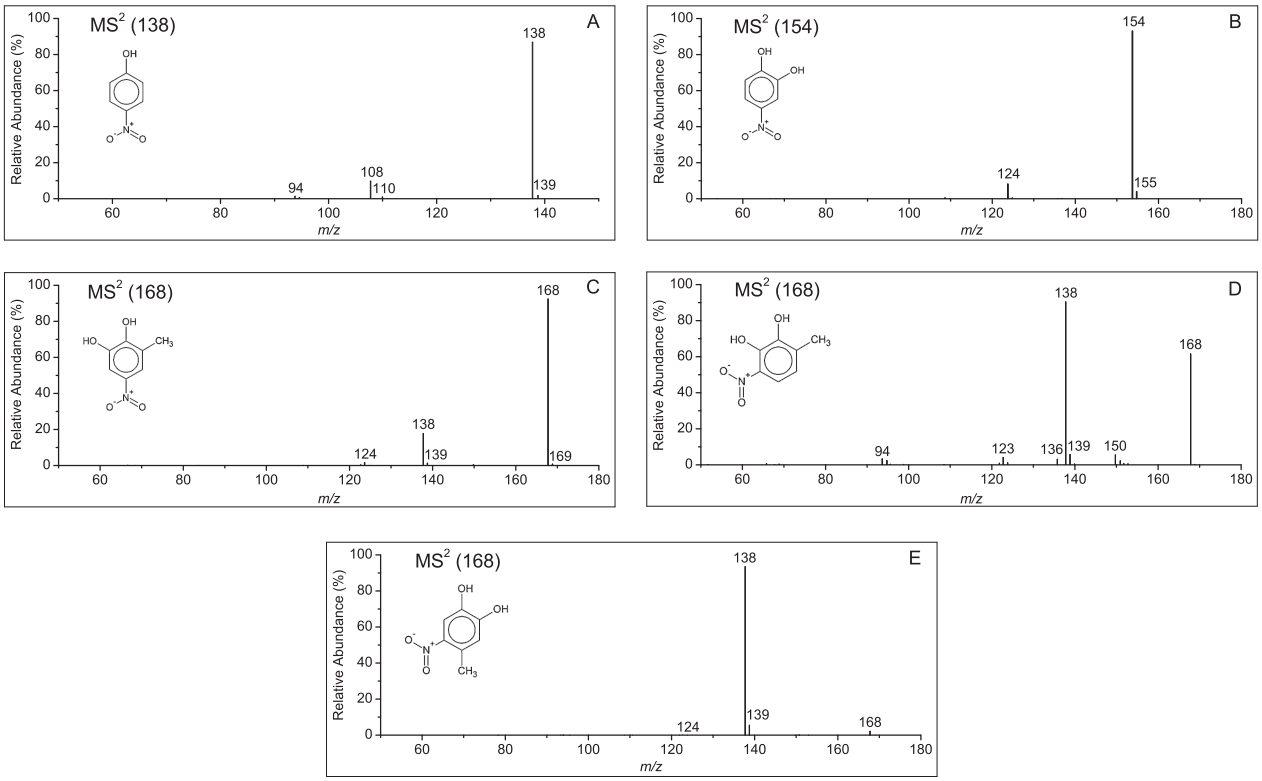

Figure 6. LC/MS^2 product ion spectra of 4-nitrophenol (A), 4-nitrocatechol (B), 3-methyl-5-nitrocatechol (C), 3-methyl-6-nitrocatechol (D), and 4-methyl-5-nitrocatechol (E) (Fig. 5).

$[M–H–NO]^{\bullet-}$ and $[M–H–NO_2]^{\bullet-}$ ions in the product ion mass spectra of their deprotonated molecules $[M–H]^-$, with the loss of NO$^\bullet$ being favored over the loss of $NO_2^\bullet$, owing to the preferential formation of a thermodynamically more stable product ion $ArO^{\bullet-}$ over the less stable product ion $Ar^{\bullet-}$ (in the case of deprotonated Ar-NO_2, where Ar denotes an aromatic moiety). It is noted that in the case of 4NP, 4NC, and the methyl nitrocatechols discussed above, the loss of $NO_2^\bullet$ from the deprotonated molecule was not observed at a normalized collision energy of 35%. The formation of $ArO^{\bullet-}$ corresponds to a rearrangement reaction involving a 1,2-phenyl shift (Ar-N(O)O → Ar-O-NO) followed by the loss of NO$^\bullet$.

It can be seen in the *m/z* 168 extracted ion chromatogram (EIC) (Fig. 5(C)) that in addition to the three methyl nitrocatechol isomers there are other *m/z* 168 compounds eluting earlier at RTs of 5.97 and 9.26 min. The $[M–H]^-$ product ion MS^2 spectra for the latter compounds is given in Figs. 7(A) and 7(B), respectively. The compound eluting at 9.26 min (Fig. 7(B)) could be assigned to 4NG, 5NG or 6-nitroguaiacol (6NG, 2-methoxy-6-nitrophenol), based on the loss of 15 units corresponding to the loss of a methyl radical that is characteristic for an aromatic methoxy group.[9,33] However, subsequent LC/(−)ESI-MS^2 experiments with 4NG and 5NG reference compounds allowed us to rule out 4NG and 5NG, which elute at RTs 11.97 and 10.76 min, respectively. Since 6NG is not available commercially we cannot at this stage unambiguously confirm or reject its presence in our sample. However, if we take into account the possible nitration of guaiacol in *ortho*- or *para*-position with respect to the hydroxyl group, 6NG is a very probable candidate.[33] The deprotonated form of the compound eluting at 5.97 min (Fig. 7(A)) shows an abundant product ion at *m/z* 166 (formal loss of 2 units), likely due to the loss of H_2 and a peak at *m/z* 138 due to the loss of NO. The formation of a prominent product ion $[M–H_2]^-$ in negative ESI has been reported by Attygalle *et al.*[34] for 2,4-dihydroxybenzaldehyde (2,4-DHBA). By using deuterated 2,4-DHBA and high-resolution MS, it was established that the coexistence of a hydroxyl and an aldehyde group in the *ortho*-position on the aromatic ring is a prerequisite for H_2 elimination. Taking this behavior into account the investigated *m/z* 168 compound should have the 2-hydroxybenzaldehyde moiety (salicylaldehyde group). Since the more oxidized form, i.e. salicylic acid, has already been

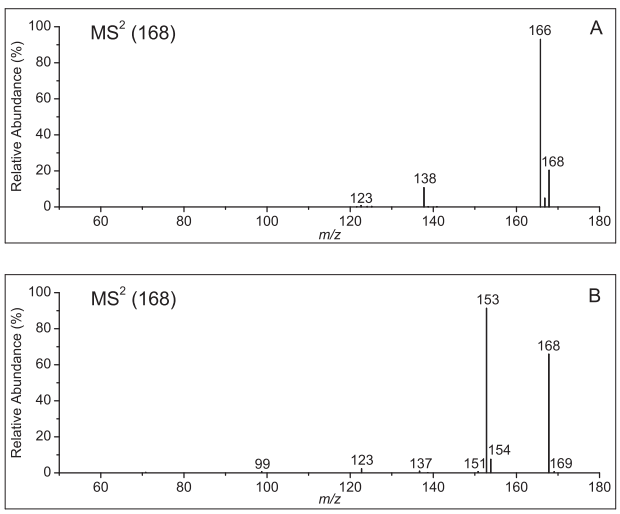

Figure 7. LC/MS^2 product ion spectra of *m/z* 168 compounds at RT = 5.97 min (A) and RT = 9.26 min (B) (Fig. 5).

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reported in the atmosphere,[35,36] this appears a reasonable possibility. However, this nitro-aromatic compound remains only partially characterized.

With respect to the *m/z* 182 compounds, the EIC reveals several peaks in the 4–30 min RT region (Fig. 5(E)). The MS^2 product ion spectra of the three compounds eluting at 5.24, 7.45, and 19.41 min are presented in Figs. 8(A), 8(B) and 8 (C), respectively, while those of the later-eluting compounds are shown in Figs. 8(D) (RT = 20.01 min), 8(E) (RT = 21.79 min), 8(F) (RT = 24.38 min) and 8(G) (RT = 26.82 min). It is worth noting that the *m/z* 182 compounds (i.e. RTs 20.01 and 26.82 min) have a relative abundance that is comparable to those of the methyl nitrocatechols (i.e. RTs 10.74 and 16.30 min); hence, an effort was made to characterize them. The two first-eluting compounds show a fragmentation behavior that is distinctly different from that of the later-eluting *m/z* 182 compounds. The two first-eluting compounds (Figs. 8 (A) and 8(B)) both reveal the loss of 44 units, corresponding to the loss of CO_2 (*m/z* 138) consistent with the presence of a carboxylic group.[37] Subsequent fragmentation of *m/z* 138 leads to the loss of NO resulting in an ion at *m/z* 108 (not shown), and a pattern similar to that observed for the deprotonated form of 4NP (Fig. 6(A)). These (−)ESI-MS^n (n = 2 and 3) data allowed us to conclude that these two compounds contain a carboxyl, a hydroxy and an aromatic nitro group.[32,37,38] This led us to consider nitrosalicylic acid isomers, i.e. 3NSA and 5NSA, which have been reported by van Pinxteren and Herrmann[35] in atmospheric aerosol using capillary electrophoresis/MS. Subsequent LC/(−)ESI-MS^2 experiments with 3NSA and 5NSA reference compounds allowed us to assign the *m/z* 182 compounds eluting at RTs 5.24 and 7.45 min to 3NSA and 5NSA, respectively. The *m/z* 182 compound eluting at RT 19.41 min (Fig. 8(C)) shows a characteristic loss of a methyl radical (*m/z* 167) consistent with the presence of an aromatic methoxy group, as observed for deprotonated nitroguaiacol (Fig. 7(B)). Other losses observed for this *m/z* 182 compound include the loss of NO (*m/z* 152) and the combined loss of NO and a methyl radical (*m/z* 137). This fragmentation behavior allows us to partially characterize this compound as a methyl homolog of 6NG. In this regard Iinuma *et al.*[33] reported an *m/z* 182 compound containing aromatic nitro, methoxy and hydroxyl groups in

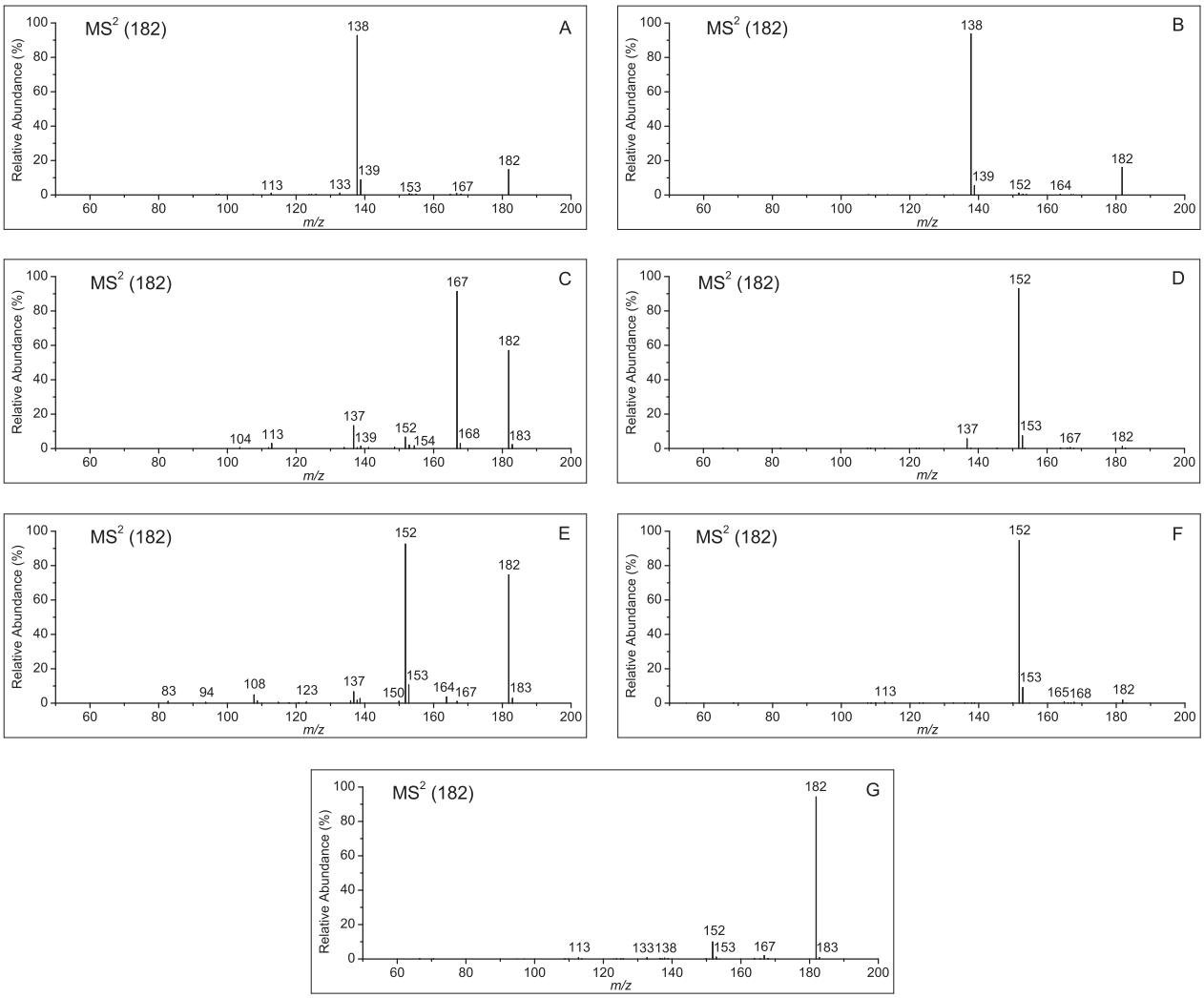

Figure 8. LC/MS^2 product ion spectra of *m/z* 182 compounds at RT = 5.24 min (A), RT = 7.45 min (B), RT = 19.41 min (C), RT = 20.01 min (D), RT = 21.79 min (E), RT = 24.38 min (F), and RT = 26.82 min (G) (Fig. 5).

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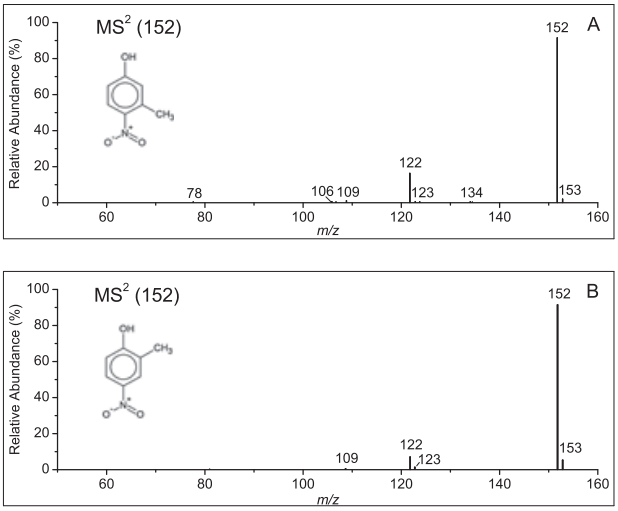

Figure 9. LC/MS^2 product ion spectra of *m/z* 152 compounds: 3-methyl-4-nitrophenol at RT = 18.01 min (A) and 2-methyl-4-nitrophenol at RT = 22.88 min (B) (Fig. 5).

aerosol obtained by combustion of pine wood with green needles. On a basis of its MS^2 fragmentation patterns they attributed this *m/z* 182 compound to 4-methyl-6-nitroguaiacol (2-methoxy-4-methyl-6-nitrophenol).

Concerning the *m/z* 182 compounds eluting between 20 and 27 min (5 peaks), all the *m/z* 182 precursor ions reveal the loss of NO (*m/z* 152), consistent with the presence of an aromatic nitro group. These compounds were partially characterized as methyl homologs of the methyl nitrocatechols, for which six positional isomers are possible. However, without reference compounds no unambiguous attributions of the dimethyl nitrocatechols can be made. It can be seen from the mass spectral data for two of the five isomers (Figs. 8(D) and 8(F)) that the stability of the *m/z* 182 precursor ion upon CID is similar, i.e. the relative abundance of *m/z* 152 corresponding to the loss of NO is quite comparable.

A last set of compounds that could be characterized in the ambient PM_{10} sample from Maribor were *m/z* 152 compounds. These compounds were assigned to isomeric methyl nitrophenols, i.e. 3-methyl-4-nitrophenol (3M4NP: RT 18.01 min) and 2-methyl-4-nitrophenol (2M4NP; RT 22.88 min), based on comparison of chromatographic and mass spectral data with reference compounds (Figs. 9(A) and 9(B)). These nitro-aromatic compounds have been reported in rainwater by Ganranoo *et al.*[15] and in BBA produced by pine wood combustion.[13] Both isomers reveal a weak ion at *m/z* 122 due to the loss of NO, while 3M4NP shows an additional weak ion at *m/z* 134 due to the loss of water; the latter can be explained by an ortho effect.

CONCLUSIONS

A new LC/MS method based on reversed-phase chromatography and negative ion electrospray ionization mass spectrometric detection has been systematically developed for the separation and characterization of methyl nitrocatechols and related nitro-aromatic compounds, including 4-nitrophenol, 4-nitrocatechol, 4- and 5-nitroguaiacols, 3- and 5-nitrosalicylic acids, and 2- and 3-methyl-4-nitrophenols in atmospheric aerosols. The ambiguity concerning the identification of 3-methyl-5-nitrocatechol and 3-methyl-6-nitrocatechol has been clarified on the basis of the structural characterization using NMR for the 3-methyl-5-nitrocatechol synthesized in pure form. The proposed LC/MS method has the advantage compared to a previously published one[9] in that it results in a better separation and specificity for the targeted analytes. For example, a better separation is obtained for the MW 169 compounds (*m/z* 168). In addition, a satisfactory separation is achieved for homologous MW 183 nitro-aromatic compounds (*m/z* 182), some of which are also quite abundant in ambient aerosol. Comparison of LC/(−)ESI-MS^2 data of available reference compounds and compounds observed in ambient aerosol allowed us to unambiguously confirm the presence of 4-nitrophenol, 2-methyl-4-nitrophenol, 3-methyl-4-nitrophenol, 4-nitrocatechol, 3-nitrosalicylic acid, 5-nitrosalicylic acid, 3-methyl-5-nitrocatechol, 3-methyl-6-nitrocatechol, and 4-methyl-5-nitrocatechol. Furthermore, interpretation of LC/(−)ESI-MS^2 data for other nitro-aromatic compounds present in ambient aerosol led to the complete or partial characterization of methyl 6-nitroguaiacol and dimethyl nitrocatechols, which could serve as additional useful SOA tracers. The characterization of these tracers is a prerequisite to develop in a further stage a quantitative method and apply it to BBA. This should enable us to compare the concentrations of the SOA tracers with those of the well-established primary BB tracer, levoglucosan.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Acknowledgements

This work was supported by the Slovenian Research Agency (Contract Nos. P1-0034-0104 and BI-BE/11-12-F-012). The authors would like to thank mag. Tanja Bolte from the Environmental Agency of Republic of Slovenia for providing the PM_{10} samples. We thank also Aleksandar Gaćeša from the Slovenian NMR Center (National Institute of Chemistry, Ljubljana) for the help during the NMR analysis and for useful discussions.

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