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Short communication

Determination of 4-nitrocatechol in biodegradation samples by gas chromatography–mass spectrometry

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Abstract

4-Nitrocatechol was identified as a product of transformation of 4-nitrophenol by bacterial strain *Corynebacterium* sp.8/3 using direct acetylation of biodegradation samples by acetic anhydride followed by GC–MS analysis. The identity of 4-nitrocatechol, in the form of diacetate, was confirmed by electron-impact spectra and spectra recorded under chemical ionization conditions (positive and negative modes). Negative-ion chemical ionization was used for quantification of 4-nitrocatechol in biodegradation samples in a concentration range of 1–25 mg/l. © 1998 Elsevier Science B.V.

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1. Introduction

Nitroaromatics, including 4-nitrophenol, are present in the environment mostly due to anthropogenic activities. They are widely used in the manufacture of dyes, pesticides, plastics, explosives and solvents. Many of these compounds are toxic to living organisms, and 4-nitrophenol with another three nitrophenols is listed as a priority pollutant by the US Environmental Protection Agency [1] for its adverse effects to the environment [2]. Thus, it is not surprising that a considerable research effort has been devoted to removal of nitroaromatics from the environment [3,4].

The use of microorganisms for degradation of these compounds seems to be the method of choice for remediation of nitroaromatic-contaminated soil, water or sewage [5], but only good knowledge of degradation pathways based on the use of reliable identification techniques enables wide commercial exploitation of bioremediation technologies.

In the case of 4-nitrophenol, bacteria were described which can oxidatively remove the nitro group via various pathways [6–9]. 4-Nitrocatechol (4-NC) was found to be an important intermediate in a degradation pathway of some bacterial strains [7,8]. Therefore, the identification of 4-NC in biodegradation samples indicates a type of a degradation pathway.

New bacterial strains are tested for their capabilities to transform nitroaromatics. The strain *Corynebacteriunl* sp. 8/3 was found to degrade 4-nitrophenol [10], but the degradation pathway is unknown. The identification of 4-NC in analyzed samples is an important step to understanding the degradation scheme.

In this note, the determination of 4-nitrocatechol as a transformation product of 4-nitrophenol, involv-

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ing derivatization of 4-NC with acetic anhydride, liquid–liquid extraction, and gas chromatography–mass spectrometry (GC–MS) detection is described.

2. Experimental

2.1. Samples

Samples of biodegradation products were obtained from Department of Microbiology, Masaryk University, Brno, Czech Republic. Bacterial cells *Corynebacterium* sp. 8/3 were cultivated in 4-nitrophenol water solution (50 mg/l) of pH 6.0 or 7.0 (adjusted with 50 mM Tris-HCl buffer) at a temperature of 26°C, under aerobic conditions and continuous shaking. The duration of the cultivation was 8 days. Samples were taken every 24 h. The volume of each sample was limited to 2 ml so that excessive decreasing of the cultivation solution volume throughout the whole experiment was avoided. Residual cells were removed by centrifugation, and samples of liquid containing degradation products were frozen immediately and analyzed within 1 h. All chemicals used for cell cultivation were analytical grade and purchased from Sigma (St. Louis, MO, USA).

2.2. Derivatization

Direct acetylation of 4-nitrocatechol was performed using a modified derivatization procedure with acetic anhydride, used in determination of phenols [11–14]. Samples were allowed to melt at a laboratory temperature. An aliquot (1 ml) of sample solution was transferred into a glass vial, and 60 mg of sodium hydrogencarbonate was added. After dissolution of $NaHCO_3$, 20 μl of acetic anhydride and subsequently 200 μl of trichloromethane were added. The mixture was shaken until evolving of carbon dioxide was finished. After layer separation, trichloromethane extract was transferred into an 0.2 ml amber glass vial and analyzed by GC–MS. The derivatization procedure of one sample took about 10 min.

Standard solutions for determination of a calibration curve were prepared just before derivatization by diluting fresh 25 mg/l stock solution of 4-nitrocatechol (Fluka, Buchs, Switzerland). Solid ascorbic acid (150–200 mg/l) was added to 4-nitrocatechol solutions to prevent their autooxidation [15].

Trichloromethane used for extraction was spiked with 1,4-dinitrobenzene (20 mg/l) and trinitrotoluene (10 mg/l) as internal standards.

2.3. Instrumentation

All experiments were carried out on mass spectrometer Trio-1000 connected to gas chromatograph GC 8060 (Fisons, Manchester, UK).

Gas chromatograph was equipped with 1.2 m× 0.32 mm deactivated fused-silica precolumn connected to DB-225 fused-silica capillary column (30 m×0.25 mm, film thickness 0.25 μm) supplied by J&W Scientific (Folsom, CA, USA). The carrier gas was helium (99.999%, Linde, Munich, Germany) at a linear velocity of 33 cm/s. On-column injector was used for introduction of samples (0.5 or 1.0 μl) into the column. The temperature program was initiated at 45°C for 2 min, then the temperature was increased to 200°C at 5°C/min and held for 20 min. The temperature of GC–MS interface was 200°C.

Mass spectrometer was operated in scan mode in all experiments. Three ionization modes were exploited for identification of 4-nitrocatechol: classic positive electron impact mode with a range of m/z 35–250, positive-ion chemical ionization with a range of m/z 50–250 and negative-ion chemical ionization (NICI) with a range of m/z 50–250. The electron ionizing energy was 70 eV. The scan time was 0.6 s. The reagent gas for chemical ionization was methane (99.95%, Linde). The temperature of ion source was 200°C for electron impact and NICI, and approximately 100°C for positive-ion chemical ionization.

The quantification of 4-NC was based on total ion-current area of its chromatographic peak.

3. Results and discussion

Direct liquid extraction of 4-nitrocatechol by hexane, dichloromethane and trichloromethane did not give good results even though samples (or standard solutions) were acidified below pH 3.

Therefore, derivatization by acetic anhydride was used to convert 4-nitrocatechol into a less polar derivative. Hexane, dichloromethane and trichloromethane were examined for extraction of 4-NC derivative. The best results were obtained with trichloromethane (five times better extraction efficiency than with hexane, and slightly higher than dichloromethane) which was then used in all subsequent experiments. Because of limited amounts of samples available, small aliquots (1 ml) of samples were used for acetylation. The volume of 200 μl of trichloromethane was found to be sufficient for extraction of 4-NC derivative (no 4-NC diacetate was detected in a second extract with another 200 μl of trichoromethane). Due to the presence of high amounts of 4-nitrophenol and other substances which were subjected to acetylation, the amount of acetic anhydride had to be increased to 20 μl per sample (about 10 times more in comparison with lower amounts sufficient in common applications) to ensure quantitative derivatization of 4-NC.

Because of the possible occurrence of 1,2,4-benzenetriol, another known important degradation intermediate [8], the following preventative measure was taken to improve conditions of identification of this compound. DB5 column was replaced by DB225 column because the coelution of 4-NC diacetate with 1,2,4-benzenetriol triacetate from DB5 column was proved experimentally. 1,2,4-Benzenetriol triacetate was formed under given acetylation conditions when 1,2,4-benzenetriol was added to a sample prior to derivatization, and its retention time on DBS stationary phase was almost identical with that of 4-NC diacetate. The capillary column covered by DB225 stationary phase gave very good separation of 4-NC diacetate from 1,2,4-benzenetriol triacetate, even though the analysis time was extended twice. However, no 1,2,4-benzenetriol was detected in analyzed biodegradation samples.

The identification of 4-nitrocatechol was based on the comparison of mass spectra obtained in all three ionization modes and retention time of the standard compound with those of the suspected compound in samples, because the available NlST library does not contain electron-impact spectra of either 4-NC acetates or 4-nitrocatechol itself.

The electron impact mass spectra (Fig. 1A) showed ester fragmentation with a dominating peak of acetyl group (m/z 43), and low-intensity ions in the rest of the spectra. The losses of 42 (CH_2CO) and 30 (NO), typical for aromatic acetates and nitro compounds [16], respectively, were apparent. The low abundant ion with m/z 239 was suspected to be a molecular ion. It corresponds to a formation of 4-nitrocatechol diacetate by the acetylation procedure.

The existence of 4-nitrocatechol diacetate was confirmed by recording mass spectra under chemical ionization conditions. Ion m/z 198 (NC+Ac+H) became one of the main peaks in comparison with the electron-impact spectrum and the protonated molecular ion (m/z 240) was getting more abundant in positive chemical ionization spectra (Fig. 1B). However, experiments in this ionization mode were accompanied by problems with run-to-run response stability. On the other hand, negative-ion chemical ionization mode proved to be more reliable. Although the spectra obtained under NICI conditions are not usually as useful as electron impact spectra for structure elucidation, in this case they are instructive with only three ions corresponding to a molecular ion (m/z 239) and fragment ions with loss of one (m/z 196) or two (m/z 154) acetyl groups (Fig. 1C).

Mass spectra and retention time of the suspected compound in all samples were in good agreement with these characteristics of 4-NC standard, which proved the presence of 4-nitrocatechol in biodegradation samples.

The capability of NICI mass spectrometry for quantification of 4-nitrocatechol was tested. The choice of negative-ion chemical ionization mode was based on two reasons. First, the NICI spectrum of 4-NC is simple and contains high abundant specific ions (m/z 196 and 239) compared to the electron-impact spectrum. This feature can be also advantageous if the selected ion monitoring operation is necessary. Second, due to NICI selectivity for particular classes of compounds [17–19], chromatograms of samples were simplified (Fig. 2). In this case nitrated aromatics were visible almost exclusively. The linear calibration curve was measured by derivatization of freshly prepared standard solutions (1–25 mg/l) which covered the range of 4-NC concentrations in biodegradation samples. The average correlation coefficient of three calibration curves was 0.997. Under given conditions, the detection

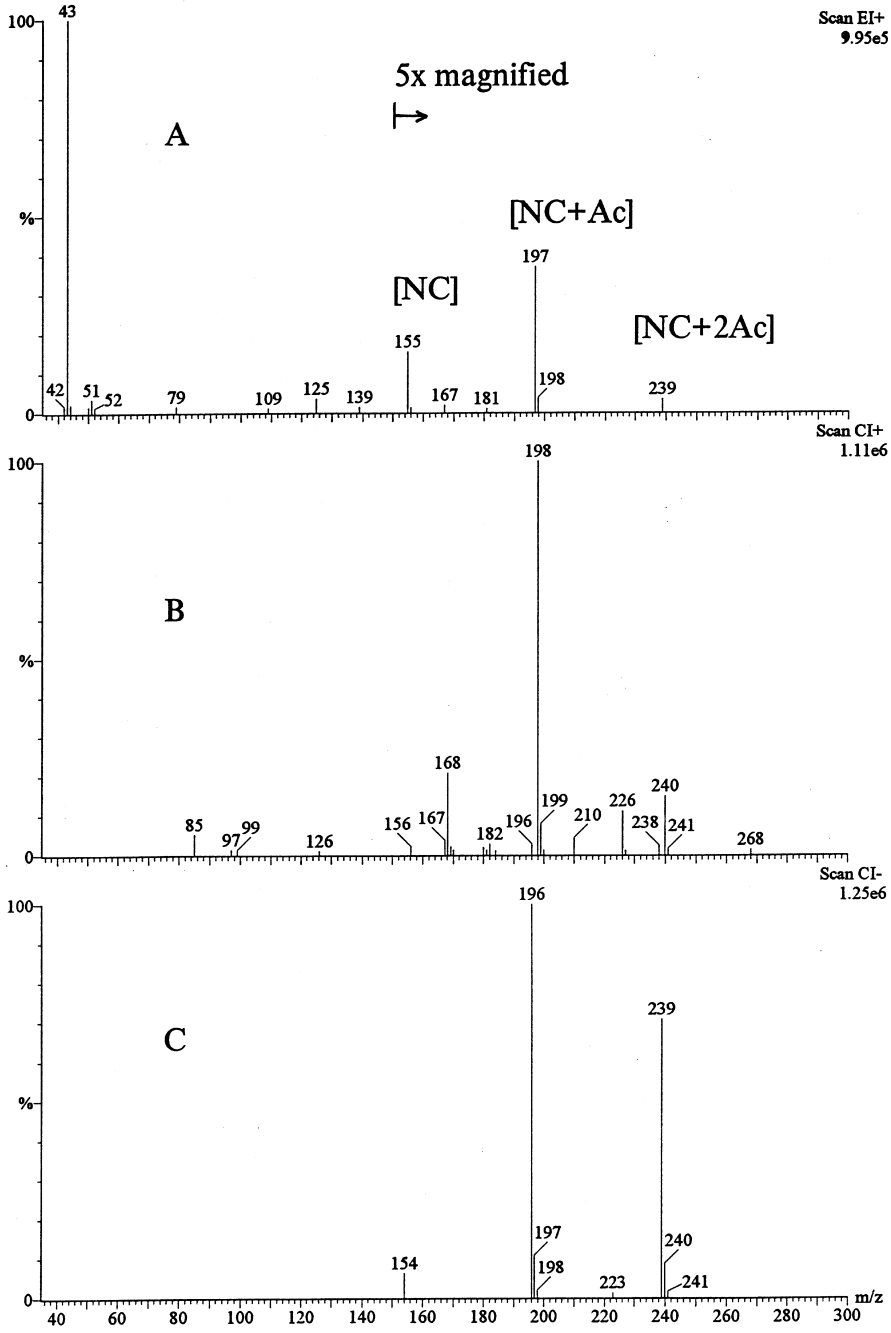

Fig. 1. Mass spectra of 4-nitrocatechol (10 mg/l) obtained under various ionization modes. (A) Electron impact spectrum. Part of the spectrum (m/z>150) is five times magnified because characteristic ions of 4-NC are low abundant. [NC], [NC+Ac] and [NC+2Ac] corresponds to positive fragment ions of 4-NC (m/z 155), 4-NC with one acetyl group (m/z 197) and molecular ion of 4-NC diacetate (m/z 239), respectively. (B) Positive ion chemical ionization spectrum. The protonated molecular ion of 4-NC diacetate (NC+2Ac+H) is more apparent. Ion m/z 198 (NC+Ac+H) is a base peak. (C) Negative ion chemical ionization spectrum. The negative molecular ion of 4-NC diacetate (m/z 239) is along with ion m/z 196 (NC+Ac-H) the most abundant in NICI spectrum. The deprotonated ion of 4-NC (m/z 154) (NC-H) is also visible.

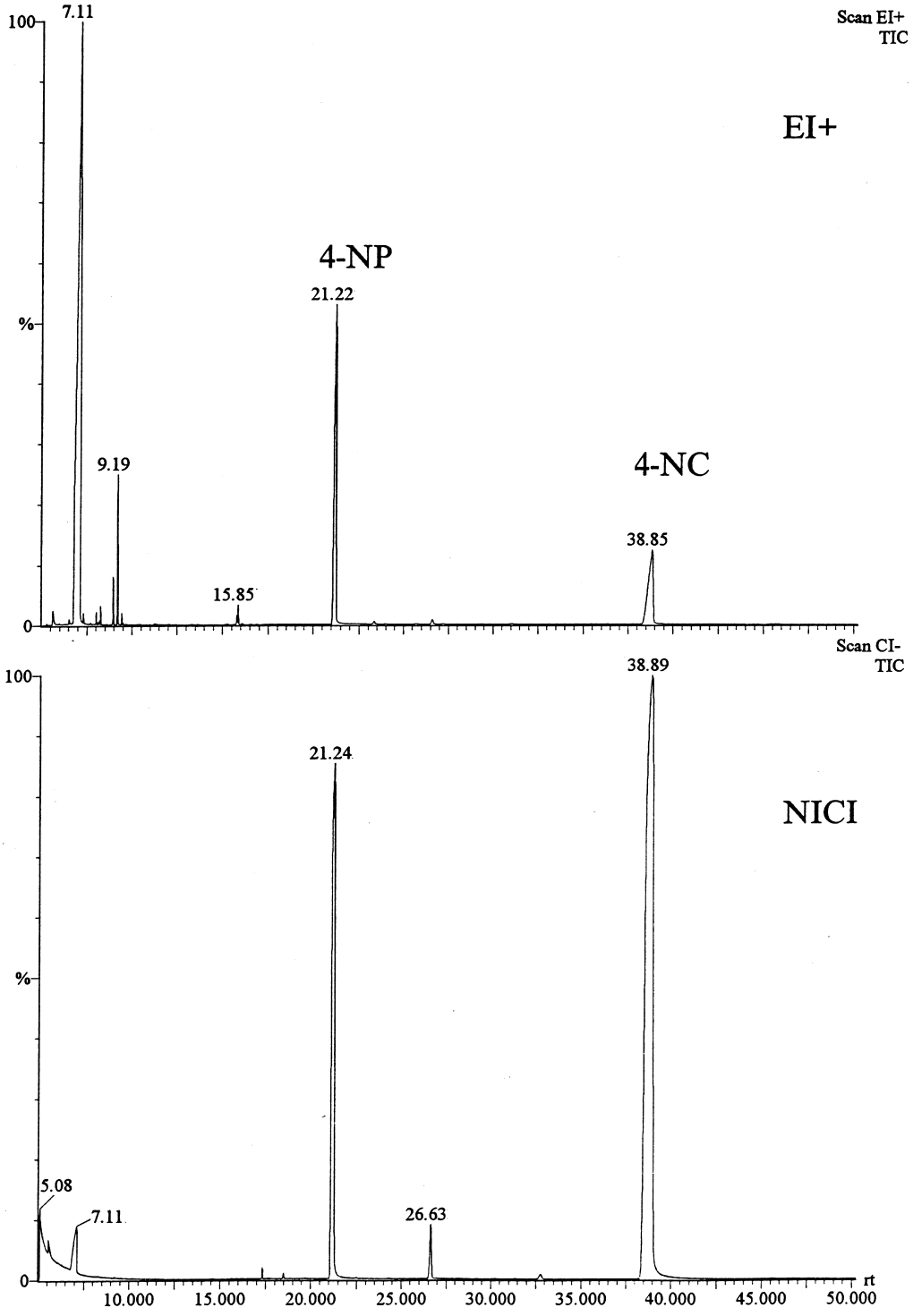

Fig. 2. Chromatograms of degradation sample. Typical total ion current chromatograms of a degradation sample (after 144 h cultivation) taken under electron impact and negative-ion chemical ionization mode conditions. The substance with retention time (rt) 26.63 min (in NICI) is suspected to be another degradation product.

limit was 90 μg/l (calculated from three times the baseline noise and for injection volume of 1 μl). The relative standard deviation of five consecutive analyses (including acetylation) was 5.9% at the 4-NC concentration of 5.14 mg/l. The concentrations of 4-nitrocatechol found in biodegradation samples were in the range of 1.5–9.8 mg/l.

4. Conclusion

Direct acetylation was successfully used for derivatization of 4-nitrocatechol. The identity 4-nitrocatechol formed in the process of biodegradation of 4-nitrophenol was proved using GC–MS with various modes of ionization. 4-Nitrocatechol was identified in all analyzed samples. The negative-ion chemical ionization mode was used for quantification of 4-NC in biodegradation samples with good results.

Acknowledgements

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