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High-performance liquid chromatographic study of the aromatic nitrile metabolism in soil bacteria

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Abstract

Simultaneous HPLC determination of bromoxynil, ioxynil and dichlobenil, three arylnitrile herbicides, and their metabolic products in soil extracts and microbiological media is described. Limits of detection (LODs) ranged from 0.56 to 3.97 ppb. Slight modification of the mobile phase composition allowed determination of 13 other aromatic nitriles. Assay of aromatic nitrile hydratase, amidase or nitrilase activities is possible by the method developed.

Keywords: Nitriles; Bromoxynil; Ioxynil; Dichlobenil

1. Introduction

Herbicidal action of halogenated arylnitriles was discovered in the early seventies [1]. The herbicides bromoxynil (3,5-dibromo-4-hydroxybenzonitrile), its iodine analog ioxynil and dichlobenil (2,6-dichlorobenzonitrile) are the main compounds currently used in many countries. For example, about $2.5 \cdot 10^3$ km^2 in North Dakota were treated with bromoxynil, a contact herbicide used against dicotyledonous weeds immediately after their outgrowth on cereals in 1989 [2]. Several formulations containing bromoxynil, ioxynil and dichlobenil are registered in the Czech Republic (Labuctril 25, Oxytril CN). These compounds are toxic and may persist in the soil or surface water for a long time. Biodegradation is the most important mechanism of removal of herbicides from the soil.

Soil decomposers capable of aromatic nitriles degradation produce two types of enzymes: either nitrilase [3], converting nitriles directly to their corresponding acids, or nitrile hydratase [4], which converts the nitrile group to the amide group. Resulting amides can be subsequently hydrolysed to acids by amidase [5]. New strains of microbial decomposers may differ in their enzymatic equipment. The substrate specificities of the enzymes responsible for arylnitrile herbicide metabolism can also be different. From this viewpoint, a simple and reliable method for the determination of all three currently used arylnitrile herbicides and their possible metabolic products would be useful. As no colour test has been described for amides in the presence of nitriles in biological matrices so far and since the differences in UV spectra between aromatic nitriles and amides are negligible, chromatography is the only method applicable herein.

Thin-layer and high-performance liquid chromatography (HPLC) with a silica-gel stationary phase were originally used in the bromoxynil assay [6]. Reversed-phase HPLC separations were then proposed [7,8]. GC techniques of bromoxynil and

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bromoxynil esters determination were also described [9,10]. In this work, a simultaneous determination of the herbicides and their metabolic products in biological materials (mineral and complex cultivation media, soil extracts) is described. Separation of a mixture of several other nitriles may be carried out with a slight modification of the mobile phase composition. Two soil bacterial strains, *Pseudomonas putida* 13XF and *Agrobacterium radiobacter* 8/4, were studied from the point of view of aromatic nitrile metabolism.

2. Experimental

2.1. Chemicals

Bromoxynil, 3,5-dibromo-4-hydroxybenzoic acid, 2-, 3-, and 4-hydroxybenzonitriles, 1,2-, 1,3- and 1,4-dicyanobenzenes and 4-bromobenzonitrile were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Ioxynil and dichlobenil were from Riedel-de Haën (Seelze, Germany). 3-Cyanopyridine was obtained from Fluka (Fuchs, Switzerland) and indol-3-propionitrile and β-indolylacetonitrile were obtained from Prof. F. Kunc, Institute of Chemical Technology (Prague, Czech Republic). Organic solvents were of HPLC grade from Merck (Darmstadt, Germany) or from Sigma-Aldrich. All other chemicals were of analytical grade from Lachema (Brno, Czech Republic). 3,5-Dibromo-4-hydroxybenzamide and 3,5-diiodo-4-hydroxybenzamide were prepared enzymatically as previously described [4].

2.2. Equipment and mobile phase

An HP 1090 L (Hewlett-Packard, Amstelveen, Netherlands) liquid chromatograph with diode-array detector was used. All measurements were carried out on a reversed-phase column (Hypersil ODS C_{18}, 100×2.1 mm I.D., 5 μm) with a guard column (40×2.1 mm I.D.) from the same manufacturer. Two types of elution were used: (A) for isocratic separation of nitriles, composition of the mobile phase was H_2O–MeOH–AcOH (150:70:1, v/v); (B) gradient elution was used for the separation of bromoxynil, ioxynil, dichlobenil, 3,5-dibromo-4-hydroxybenzoic acid and the corresponding amides. Solvent A was a mixture of H_2O–MeOH–AcOH (59.5:40:0.5, v/v), solvent B was acetonitrile. Linear gradient elution was applied from 0 to 10% B in 5 min. The compounds were detected at 240 nm. All measurements were carried out at 30°C. Flow-rate was 1.0 ml min^{-1}. The hold-up time (t_0) was determined by injection of 0.01 *M* NaCl.

2.3. Sample preparation

Bacterial strains were cultivated either in mineral medium [4] or in the Luria–Bertani medium [11] at 28°C. Flasks (250 ml) containing 70 ml of the medium supplemented with 50 ppm of tested herbicide were inoculated with 1 ml of bacterial suspension ($1 \cdot 10^6$ cells ml^{-1}). All samples were centrifuged at 10 000 *g* for 5 min before analysis.

3. Results and discussion

3.1. Sample preparation

Soil extracts, as well as fermentation broths, contain mainly inorganic ions which are on a C_{18} column eluted at the start and do not interfere with compounds of interest. In some cases, organic compounds present in microbiological media (e.g. yeast extract, pepton) may contaminate the LC column. The effect of this contamination results in the broadening of the peaks with a consequent decrease in their height. However, similar problems may be detected after more than 400 analyses. The use of a guard column and periodical flushing with 250 ml of 5% acetonitrile in water after every 200 analyses, preserved the column in good efficiency with standard peak-height/area ratios. Thus, the sample preparation could be reduced merely to the separation of solid soil or cell particles by centrifugation. Under these conditions no other steps, e.g. pH-adjustment, are necessary.

3.2. Performance of the method

The method allowed the simultaneous detection of bromoxynil, ioxynil, dichlobenil and their corre-

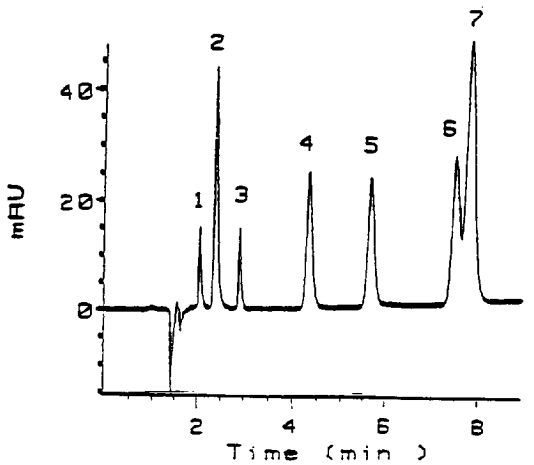

Fig. 1. Chromatogram of 2,6-dichlorobenzamide (1), 3,5-dibromo-4-hydroxybenzamide (2), 3,5-diiodo-4-hydroxybenzamide (3), 3,5-dibromo-4-hydroxybenzoic acid (4), 3,5-dibromo-4-hydroxybenzonitrile (bromoxynil) (5), 3,5-diiodo-4-hydroxybenzonitrile (ioxynil) (6) and 2,6-dichlorobenzonitrile (dichlobenil) (7); 50 ppm each. Column and eluent composition as reported in the text. Detection at 240 nm.

sponding amides. Fig. 1 shows a typical chromatogram with addition of 3,5-dibromo-4-hydroxybenzoic acid. The use of a gradient elution allowed good separation of amides from the herbicides. Ioxynil and dichlobenil co-elute under the conditions described. The presence of nitrile hydratase activity is better confirmed by formation of the amide peak(s) than by decrease of the nitrile peak(s). The compounds were detected using a DAD detector at 240 nm.

Linear calibration graphs were constructed for all compounds in the various concentration ranges of interest. The equations representing the five sets of experimental data indicated good linearity as verified by the r values. Table 1 gives the LODs for all the compounds recorded with a S/N of 3 and the parameters of calibration curves. All values were calculated from chromatograms obtained at 240 nm.

The precision and linearity were validated by analysing in triplicate 10 calibration standards, containing 0–100 mg/l of herbicides, over 3 days. The reproducibility of the peak–area ratios over the calibration range, expressed as relative standard deviations, ranged from 0.88 to 3.22%.

3.3. Separation of other nitriles

Chromatographic separation of 4-bromobenzonitrile, 1,2-dicyanobenzene, 1,3-dicyanobenzene and 1,4-dicyanobenzene under the gradient conditions described above is also possible. For the separation of benzonitrile, 2-hydroxybenzonitrile, 3-hydroxybenzonitrile, 4-hydroxybenzonitrile, 3-cyanopyridine, indol-3-propionitrile, β-indolylacetonitrile, 2,6-difluorobenzonitrile and 2-aminobenzonitrile, isocratic elution with modified mobile phase gave better results. Calibration data and LODs are given in Table 1. Chromatogram of benzonitrile, 4-hydroxybenzonitrile, 2-aminobenzamide, 2,6-difluorobenzonitrile, 1,4-dicyanobenzene and 3-cyanopyridine is shown in Fig. 2.

3.4. The use of HPLC in the investigation of aromatic nitrile metabolism

As previously mentioned, nitriles are converted to corresponding amides or acids by a variety of soil microorganisms. Formation of acids from either nitriles (by nitrilase, EC 3.5.5.1) or amides (by amidase, EC 3.5.1.4) is accompanied with a release of ammonium, that may be simply determined. In the nitrile hydratase (EC 4.2.1.84) assay the enzyme activity can be calculated only from the changes in the substrate or product concentration. The method described above allowed simultaneous determination of three often used arylnitrile model herbicides and their corresponding amides. 3,5-Dibromo-4-hydroxybenzamide had the lowest LOD among metabolites. The use of bromoxynil as substrate allowed to confirm or exclude the nitrile hydratase activity after about 1 h of incubation of the tested strain. Chromatographic analysis together with the sample preparation lasted about 15 min.

The use of HPLC in the study of aliphatic nitrile metabolism has been discussed recently [12]. HPLC-based assays of several other enzyme activities were also reviewed [13].

4. Conclusion

The HPLC method described here was developed for screening purposes of the degradative abilities of

Table 1
Values of k and parameters of calibration curve ($y=ax+b$, r)

Compound	Conditions	k	a	b	r	LOD
Benzonitrile	A	4.429	1.268	-4.691	0.9999	23.97[a]
2-Hydroxybenzonitrile	A	2.569	4.776	-13.746	0.9998	6.18[b]
3-Hydroxybenzonitrile	A	2.557	7.545	3.873	0.9997	3.61
4-Hydroxybenzonitrile	A	2.157	48.817	-38.80	0.9999	0.57
3-Cyanopyridine	A	1.240	4.924	-8.455	0.9999	5.79[c]
Indol-3-propionitrile	A	6.208	3.776	-17.346	0.9999	8.18[d]
β-Indolylacetonitril	A	4.594	3.759	-12.396	0.9999	7.94[e]
2,6-Difluorobenzonitrile	A	6.293	1.579	-4.127	0.9999	18.53[f]
2-Aminobenzonitrile	A	2.766	19.374	-98.85	0.9997	1.63
Benzamide	A	1.337	12.886	-21.782	0.9999	2.23
2-Aminobenzamide	A	1.778	15.802	-25.491	0.9999	1.81
3,5-Dibromo-4-hydroxybenzonitrile	B	2.244	12.841	-38.655	0.9999	2.63
3,5-Diiodo-4-hydroxybenzonitrile	B	3.212	9.720	-38.982	0.9997	3.59
2,6-Dichlorobenzonitrile	B	3.324	8.214	-16.418	0.9999	3.97
1,2-Dicyanobenzene	B	0.837	28.605	-60.873	0.9999	1.16
1,3-Dicyanobenzene	B	0.754	4.233	11.4	0.9993	6.85[g]
1,4-Dicyanobenzene	B	0.797	59.682	-153.109	0.9997	0.56
4-Bromobenzonitrile	B	2.592	43.027	-69.036	0.9999	0.77
3,5-Dibromo-4-hydroxybenzamide	B	0.700	8.615	-9.702	0.9999	3.72

LODs were calculated for 240 nm.
[a] 0.97 at 230 nm.
[b] 1.44 at 230 nm.
[c] 1.24 at 220 nm.
[d] 0.56 at 220 nm.
[e] 0.59 at 220 nm.
[f] 0.87 at 230 nm.
[g] 0.53 at 230 nm.

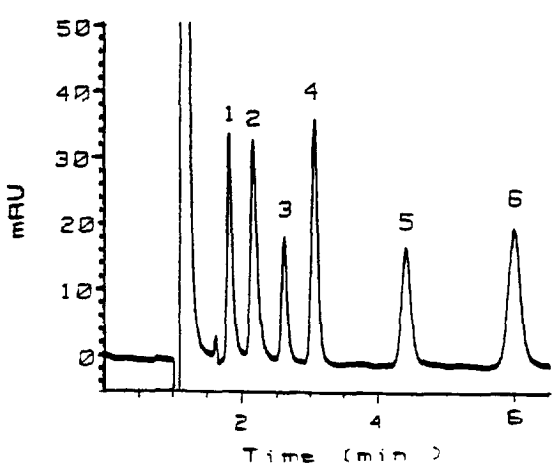

Fig. 2. Chromatogram of benzonitrile (1), 4-hydroxybenzonitrile (2), 3-hydroxybenzonitrile (3), 2,6-difluorobenzonitrile (4), 1,4-dicyanobenzene (5) and 3-cyanopyridine (6); 25 ppm each. Column and eluent composition as reported in the text. Detection at 240 nm.

soil microorganisms. It allows us to distinguish between two metabolic pathways. Information on the substrate specificity among three main arylnitrile herbicides may be also obtained from one chromatographic run.

Two new soil microbial decomposers of aromatic herbicide bromoxynil were isolated in our laboratory. While strain *Ps. putida* 13XF, similarly to a recently described strain of *Agrobacterium* ssp. [14], possesses both nitrile hydratase and amidase activities [15], the latter enzyme in *A. radiobacter* 8/4 has not been found [4]. Chromatograms of fermentation broths containing 50 ppm of bromoxynil after 24 h incubation with *Ps. putida* 13XF and *A. radiobacter* 8/4 are shown. While *Ps. putida* 13XF transformed almost all bromoxynil (Fig. 3) into the 3,5-dibromo-4-hydroxybenzamide (by nitril hydratase) and then subsequently into the 3,5-dibromo-4-hydroxybenzoic acid (by amidase), the presence of the latter compound in the case of *A. radiobacter* 8/4 was not

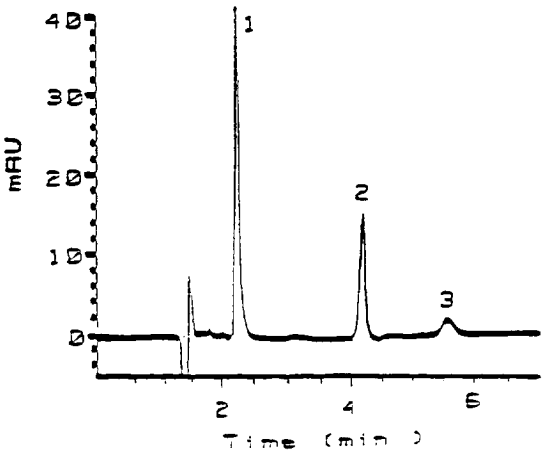

Fig. 3. Chromatogram of fermentation broth containing bromoxynil after 24 h incubation with *Ps. putida* 13XF strain. Metabolites were eluted in the order 3,5-dibromo-4-hydroxybenzamide (1), 3,5-dibromo-4-hydroxybenzoic acid (2) and 3,5-dibromo-4-hydroxybenzonitrile (bromoxynil) (3). For the chromatographic conditions see text. Detection at 240 nm.

detected (Fig. 4). Depending on the activity of the enzymes, amide peaks may be reliably detected after 1–2 h incubation of the tested microbial strain with the substrate.

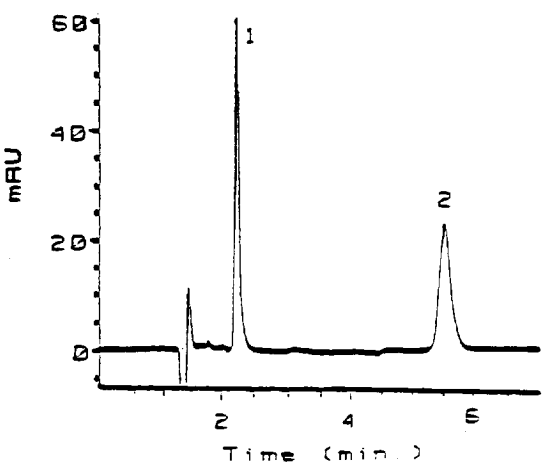

Fig. 4. Chromatogram of fermentation broth containing bromoxynil after 24 h incubation with *A. radiobacter* 8/4 strain. 3,5-Dibromo-4-hydroxybenzamide (1) is present as the only metabolite of 3,5-dibromo-4-hydroxybenzonitrile (bromoxynil) (2). For the chromatographic conditions see text. Detection at 240 nm.

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