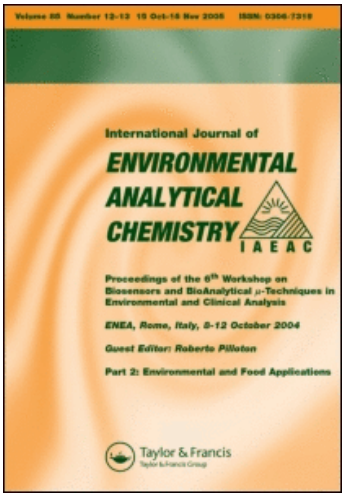

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

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To cite this Article Muir, Derek C. G. and Grift, Norbert P.(1980) 'Determination of Niclosamide (Bayer 2353) in Water and Sediment Samples', International Journal of Environmental Analytical Chemistry, 8: 1, 1 — 14
To link to this Article: DOI: 10.1080/03067318008071875
URL: http://dx.doi.org/10.1080/03067318008071875

Intern. J. Environ. Anal. Chem., 1980. Vol. 8, pp. 1–14
0306-7319/80/0801-0001 $4.50/0

Printed in Great Britain

Determination of Niclosamide (Bayer 2353) in Water and Sediment Samples

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(*Received August 30, 1979*)

A method is described for the determination of niclosamide (2′,5-dichloro-4′-nitrosalicylanilide) in river water and sediment. River water is extracted by shaking with ethyl acetate. Sediment is shaken with methanol : water (4 : 1), the mixture is centrifuged and the methanol is evaporated. The sediment extract is then partitioned with methylene chloride and the extracts are cleaned up on a Florisil column. Niclosamide can be analysed, after methylation with methyl iodide, by gas chromatography with electron-capture or alkali-flame detection, or directly by high pressure liquid chromatography with a UV absorbance (313 nm) detector. Recoveries of niclosamide ranged from 99 to 116% in fortified river water and 73 to 126% in fortified pond sediment samples.

INTRODUCTION

Niclosamide (2′,5-dichloro-4′-nitrosalicylanilide) (Bayer 2353) is used in the form of its 2-aminoethanol salt (Bayluscide, registered trademark of Bayer A.G.[1]) as a molluscicide to control aquatic snails that are the intermediate hosts of organisms which cause schistosomiasis. It is also used in the Canadian Great Lakes region in combination with TFM (3-trifluoro-methyl-4-nitrophenol) to control larvae of the sea lamprey (*Petromyzon marinus*).[2] Recently, we carried out a study on the fate of niclosamide in the aquatic environment to provide advice on the use of the compound for control of leeches in Manitoba lakes. We required an analytical method for the determination of niclosamide in water and sediment.

Several colorimetric methods for the determination of niclosamide in water samples following treatments for snail or lamprey control have been published.[3, 4, 5] A gas chromatographic method was described, recently, in

which the hydrolysis product of niclosamide, 2-chloro-4-nitroaniline, was analysed with an electron-capture detector.[6] In both the colorimetric and gas chromatographic procedures, however, niclosamide is hydrolysed before analysis so that it cannot be determined separately from its hydrolysis products. A procedure for determining niclosamide in its intact form would be of value in degradation studies as well as for monitoring field treatments for snail or lamprey control. In this report, methods for the determination of intact niclosamide in water and sediment samples by gas chromatography (GLC) and by high pressure liquid chromatography (HPLC) are described and compared.

EXPERIMENTAL

Analytical Standards

Niclosamide (99.4%) was obtained from the Pesticide Standards Laboratory, Agriculture Canada, Ottawa. Stock solutions were prepared in methanol (10 mg/100 ml) and diluted for use in working standards.

Methylated niclosamide was synthesized by a scaled-up procedure similar to that of Greenhalgh and Kovacicova:[7] 40 mg niclosamide was dissolved in 10 ml dimethylsulfoxide (DMSO) and added to a flask containing NaH (1 g, hexane washed) suspended in 5 ml hexane. Methyl iodide (10 ml) was added and the mixture was stirred (1 hr, 60°C) under a reflux condenser. Water (10 ml) was then added and the solution was extracted with hexane (2 × 90 ml). The hexane extracts were dried over Na_2SO_4 and evaporated to dryness. The residue was dissolved in ethyl acetate : hexane (1:1) and chromatographed on a column of alumina (activated Woelm basic) (2 cm × 22 mm dia.). The initial 20 ml ethyl acetate : hexane (1 : 1) eluate was discarded and the product was eluted with 25 ml ethyl acetate : hexane (1:1). The product was recrystallized in hexane and gave an overall yield of 65%. Mass spectroscopy (Finnagan Model 1015 direct inlet probe, 70 ev ionization potential) showed a parent ion at m/e 254, and a base peak at m/e 169 corresponding to the loss of the 3-chloro-4-nitroaniline fragment (Figure 1), indicating that two methyl groups were added to the molecule. The melting point of the crystalline product was 167–168.5°C.

Radiolabelled niclosamide (^{14}C-salicyclic acid ring) (Sp. Act. 10 mCi/mM) was obtained from the U.S. Fish and Wildlife Service, La Crosse, Wisconsin. Before use, the ^{14}C-niclosamide was purified by thin-layer chromatography (TLC) on silica gel plates using methanol: chloroform (3:1) as the solvent system.

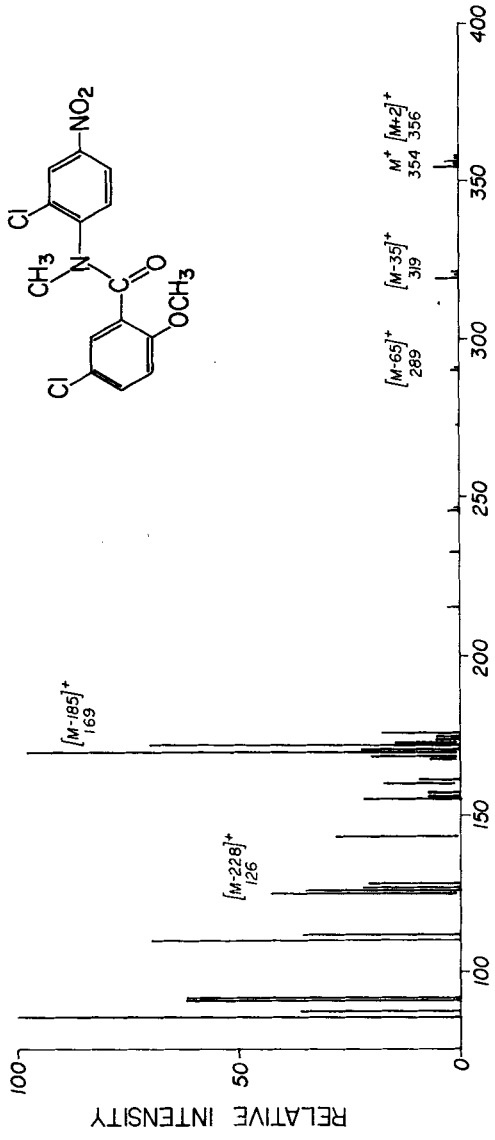

FIGURE 1 Mass spectrum of methylated niclosamide showing major fragments. The structure of the methylated derivative is also shown.

Extraction procedure

(1) Water: River water (55 mg/1 suspended solids) samples (500–1000 ml) were extracted by shaking 3 times with ethyl acetate (200, 100, 100 ml) in a separatory funnel. The ethyl acetate extract was dried over Na_2SO_4, evaporated to dryness and transferred to a test tube using methanol (5 ml). Methanol extracts were assayed directly by HPLC or were evaporated to dryness for derivatization.

(2) Sediment: Excess water was removed from the sediment sample by filtration (Whatman #1 paper). The wet sediment was mixed with a spatula and 25 g were weighed into a centrifuge tube (Sorvall No. 522, stainless steel). A portion (10 g) of the sediment sample was taken for moisture determination (usually about 50% water by weight). The sediment was shaken on a wrist-action shaker with 150 ml methanol : water (4 :1) (1 hr), allowed to stand overnight, and reshaken for 20 min. The mixture was then centrifuged (700 g, 10 min) and the supernatant decanted. The residuum was reextracted with 100 ml methanol: water (4 : 1), and after centrifugation, the combined supernatants were evaporated on a rotary evaporator (45°C) to remove the methanol. The aqueous residue was transferred to a separatory funnel with water to give a volume of 50 ml.

Acetone : formic acid (98 : 2)[6] was also investigated as an extraction solvent for sediments containing ^{14}C-niclosamide. In this case, 25 g wet sediment were shaken as described with 150 ml acetone : formic acid (98 : 2) for 1 hr and then centrifuged. The residum was reextracted with 100 ml acetone : formic acid (98 : 2). The combined supernatants were evaporated to remove most of the acetone, and the aqueous extract was diluted to 50 ml with water and adjusted to pH 9.0 with 3 N NH_4OH before partitioning with methylene chloride in order to prevent the extraction of formic acid into the organic phase.

The aqueous phases from both the methanolic and acetone extracts were extracted with methylene chloride (2 × 50 ml). The methylene chloride extracts were dried by passing through a small column of Na_2SO_4 and evaporated just to dryness (40°C, rotary evaporator). The residue was dissolved in ethyl acetate. Extracts containing radiolabelled niclosamide were then assayed directly by liquid scintillation counting (Packard Model 3030) using PCS (Amersham): xylene (2 :1) as scintillation fluid.

Sediment extracts were cleaned up for HPLC analysis or for derivatization, on a Florisil (5% deactivated) column (6 cm × 10 mm i.d., topped with 0.5 cm Na_2SO_4). The residue (dissolved in ethyl acetate) was applied to the column (prewashed with ethyl acetate) and the flask and sides of the chromatographic column were washed with additional ethyl

acetate (2 × 2 ml). The initial ethyl acetate eluate (9.0 ml) was discarded and niclosamide was eluted with methanol (12 ml). The eluate was collected in a test tube (15 ml graduated centrifuge tube) and evaporated to dryness for derivatization or direct HPLC analysis.

Derivatization

The base catalysed methylation procedure described by Lawrence[8] and Greenhalgh and Kovacicova[7] was used with minor modifications. The residue following Florisil clean-up was dissolved in 0.5 ml DMSO and 0.5 ml methyl iodide and about 20 mg NaH (hexane slurry) were added. The test tube was capped and the mixture was shaken gently on a mechanical shaker at room temperature for 1 hr. Water (1 ml) was added slowly to remove excess NaH and the mixture was extracted twice with hexane : ethyl acetate (4 : 1) (2 × 3 ml). The combined extracts were dried over Na_2SO_4 and evaporated to 1 ml for GLC analysis.

Cleanup of the derivatization mixture was necessary for GLC with electron-capture detection. This was accomplished on a small column of alumina (activated Woelm basic) (6 cm × 6 mm i.d. column). The sample (1 ml volume) was transferred to the column and the test tube and sides of the column were washed with hexane : ethyl acetate (1 :1) (2 × 1 ml). The initial 3 ml eluate was discarded and methylated niclosamide was eluted with 5 ml hexane : ethyl acetate (1 : 1) which was evaporated to 1 ml for GLC analysis.

HPLC analysis

A Waters Model 6000A pump and Model 440 absorbance detector were used with a Valco 6-port injection valve. Columns: μBondapak-NH_2 (30 cm × 3.8 mm i.d.) with methanol : water (80 : 20) or acetonitrile : water (60 : 40) as solvent systems (2.0 ml/min); Lichrosorb Si60 (25 cm × 4.2 mm i.d.) with hexane : isopropanol (95 : 5) at 1.5 ml/min. Niclosamide shows a strong UV absorbance in the 300 to 350 nm range (Figure 2) so that 313 nm was chosen as the detection wavelength. Most analyses were carried out at 0.005 absorbance units full scale. The retention times and responses of niclosamide are listed in Table I.

GLC analysis

The methylated derivative of niclosamide was determined on a Tracor 560 GC equipped with an alkali-flame detector (AFD) (Tracor Model 702) and a ^{63}Ni electron capture detector (ECD) (linearized). A 1.8 m × 4 mm i.d. glass column packed with 5% OV-1 on Chromosorb W-HP and operated

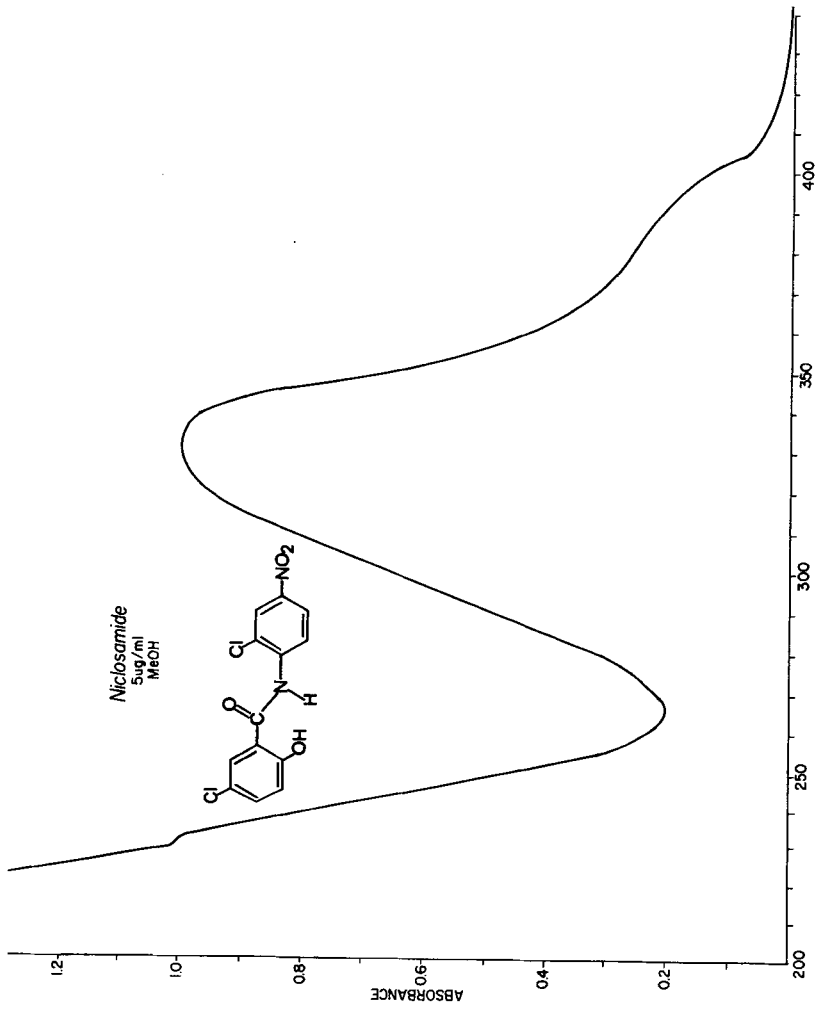

FIGURE 2 UV spectrum of niclosamide (5 μg/ml in MeOH) carried out on a Carey Model 14 spectrophotometer. Structure of niclosamide is also shown.

TABLE I

Responses (A) of niclosamide by HPLC and (B) of methylated niclosamide by GLC.

A. HPLC conditions

Compound	Column	Solvent system	Retention time (min)	Response (ng, $\frac{1}{2}$ f.s.d.[1])
Niclosamide	Lichrosorb Si60 (10μ) 25 cm × 4.2 mm	2-propanol: hexane (5 : 95) 1.5 ml/min	6.5	20
	μBondapak-NH_2 (10μ) 30 cm × 3.8 mm	CH_3CN : water (80 : 20) 2.0 ml/min	12.0	50
	μBondapak-NH_2 (10μ) 30 cm × 3.8 mm	CH_3Oh : water (60 : 40) 2.0 ml/min	10.0	40

B. GLC conditions

Compound	Column	Retention time (min)	Response (ng, $\frac{1}{2}$ f.s.d.[1]) ECD	AFD
Methylated-niclosamide	5% OV-1 (1.8 m × 6 mm)	4.8	0.2	10.0

[1]One-half full scale recorder deflection

at 260°C was used with both detectors. Carrier gas (N_2, ECD; He, AFD) flow rate was 40 ml/min for both detectors. Detector and inlet oven temperatures (°C) were 350 and 250, respectively, for the ECD and 275 and 250 for the AFD. The AFD had air and hydrogen flows (ml/min) of 120 and 4.0, respectively. The response of methylated niclosamide with each detector is given in Table I. Peak heights of methylated niclosamide in fortified sample extracts were compared with those of working standard solutions of methylated niclosamide synthesized as described previously.

Recovery studies

(1) Methylation efficiency was examined by reacting microgram quantities of niclosamide as described previously and comparing responses with that of the methylated niclosamide standard.

(2) Water: Known quantities (0.1, 1.0 and 10.0 μg) of niclosamide (methanol solution) were added to river water (1000 ml) and the water was shaken and allowed to stand 30 minutes. The water was extracted as described previously and analysed by GLC-ECD. The river water had total suspended solids of 55 mg/l and pH 7.6.

(3) Sediments: Known volumes of niclosamide stock solutions (0.01–0.10 ml) were added to a pond sediment (25 g wet weight). The sediment was mixed and allowed to stand 1 hr before extraction. Extracts were either methylated and analysed by GLC-AFD or were analysed directly by HPLC. The pond sediment contained 58% clay, 37% silt, 6.0% organic matter, and had a pH 7.8.

(4) Extraction of ^{14}C-niclosamide from sediment: Incubation flasks similar to those described by Simsiman and Chesters[9] containing 15 g pond sediment (66% clay, 31% silt, pH 7.7, 4.5% organic matter) and 150 ml pond water were connected to air (aerobic) or nitrogen (anaerobic) lines and equilibrated for 7 days at 25°C (gas flow rates 1–2 ml/min). The flasks were held under Gro-lux fluorescent lamps (16 hr daylight; 8 hr dark); anaerobic flasks were covered with aluminum foil. Anaerobic sediments were amended with cellulose powder (1%). An aqueous solution containing niclosamide was added to give 1 ppm concentrations in the water and 100,000 DPM per flask. The aerobic and anaerobic treatments were carried out in triplicate. After 30 days the sediment and water were separated by filtration (Whatman .#1) and the moisture content of the sediment sample was determined. The sediment was divided in half and extracted with methanol-water or acetone-formic acid as described. Portions of the unextracted sediment and the extracted sediment (0.5 g samples) were mixed with Solkafloc and oxidized on a Packard Model 306 oxidizer. Aliquots of the methylene chloride and aqueous phases were also oxidized. The oxidized samples were counted on a Packard Model 3030 liquid scintillation counter (10 min per sample) using an internal channels ratio method for quench correction.

RESULTS AND DISCUSSION

The efficiency of methylation of various quantities of niclosamide using methyl iodide is shown in Table II. Small quantities (0.3 μg) showed reduction in methylation efficiency. Continuous agitation of the reaction mixture was found to be important for consistent results. The derivatives were stable on storage for more than 6 months (4°C). The GLC response of methylated niclosamide using EC detection was about 50-fold greater than that with the AFD (Table I). No other derivatives were investigated though it is likely that the electron capture response of niclosamide could be increased further by use of perfluoroacylation reagents.

The recovery of niclosamide from fortified river water using ethyl acetate as the extraction solvent and GLC-ECD is shown in Table III. Recoveries were high though the relative standard deviation was high at

TABLE II

Methylation efficiency of niclosamide.[1]

Quantity (μg)	Recovery[2] (%)	Rel. Std. Dev.%
0.30	86.5 (4)	15.8
0.60	100.3 (4)	5.3
2.40	110.9 (6)	8.4
12.0	108.0 (3)	9.3

[1]Calculated by using the GLC response of methylated niclosamide standard.
[2]Replicates in parentheses.

TABLE III

Recovery of niclosamide from fortified river water.

Fortification level, μg/l	Recovery[1] %	Rel. Std. Dev. %
0.10	99.2	20.3
1.0	116.2	4.0
10.0	99.5	13.7

[1]Average of 3 replicates. Determined by electron capture GLC.

low fortification levels. Luhning *et al.*[6] have noted that niclosamide decomposes rapidly in water, especially in alkaline water. A short equilibration time after fortification of the river water was chosen to avoid this problem but the rapid decomposition may account, in part, for some of the variation in recovery efficiency.

In other work we found no significant differences between recoveries of niclosamide from water adjusted to pH 4.0 or to pH 7.0 using ethyl acetate for extraction. Hexane can also be used as an extraction solvent if the aqueous phase is acidified[6] (pH 2–3). The lack of any effect of pH on recovery efficiency with ethyl acetate or chloroform[5] may be due to the intramolecular hydrogen-bonding which niclosamide can undergo, like other salicylic acid derivatives, thus reducing the polarity of the phenolic group. Niclosamide was also found to be extracted from water by a strong anion exchange resin (AG-1 × 4, 100–200 mesh) (acetate form) and eluted quantitatively with methanol : acetic acid (9 : 1). This has been reported previously[10] using AG-1 × 8 (hydroxide form). However, the acetic acid was difficult to remove for subsequent steps in the analysis and this approach was abandoned. Macroreticular resins (XAD 2 and 4) were also investigated for the recovery of niclosamide from fortified river water but quantitative results were not observed.

Figure 3 shows a typical chromatogram of a fortified river water extract. Relatively little interference from co-extractive materials was encountered (after methylation and cleanup on alumina) in the 0.1 to 10 ppb range using the ECD. The AFD (without alumina cleanup) and HPLC separation and detection at 313 nm (non methylated niclosamide) also gave excellent results with water extracts but lacked the sensitivity of the ECD in the sub-ppb range.

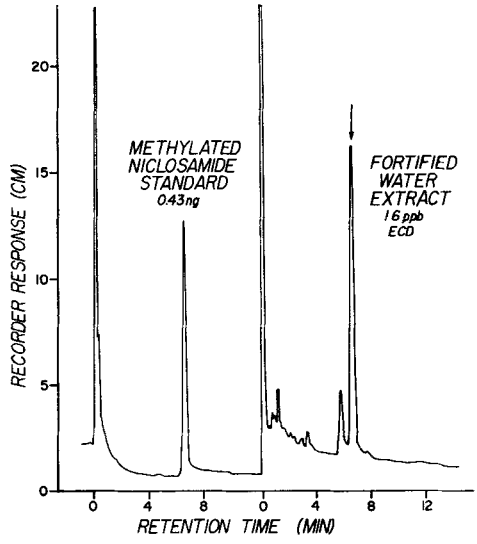

FIGURE 3 Gas chromatograms of a fortified river water extract and an analytical standard containing methylated niclosamide using an electron capture (63 Ni) detector. Oven temperature, 240°C; attenuation × 20.

Recoveries of the niclosamide from a fortified clay pond sediment are shown in Table IV. These results were obtained with GLC-AFD or by direct analysis using HPLC. There was a considerable lack of precision in the results in which methylation was used compared to those in which the Florisil column eluates were analysed directly by HPLC. In some sediment extracts, coextractive materials appeared to reduce methylation efficiency. EC detection was initially investigated for the analysis of cleaned up sediment extracts (Figure 4). Much less interference was encountered when the same sample extracts were analysed with the AFD (Figure 5) or by HPLC (Figure 6) and fewer steps were required in the analysis. All further work with sediment extracts was carried out with the AFD or by HPLC. HPLC chromatograms (Figure 6) indicate that

TABLE IV

Recovery of niclosamide from fortified sediment using GLC or HPLC detection

Fortification level (μg/g, dry wt)	Recovery[1] %	Rel. Std. Dev. %
A. GLC-AFD		
0.05	84.6	18.9
0.12	99.2	15.3
0.24	126.2	24.2
0.60	73.6	24.5
B. HPLC		
0.24	81.8	10.9
0.48	79.9	9.6

[1]Average of 3 replicates.

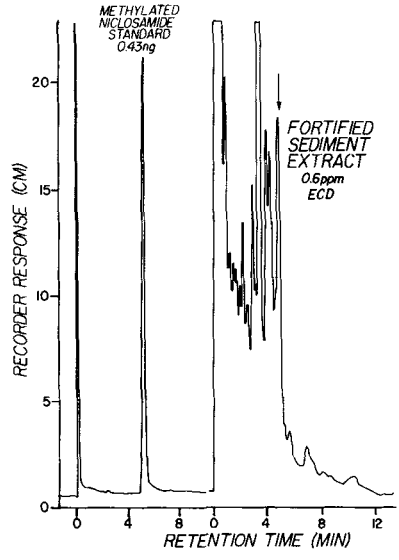

FIGURE 4 Gas chromatograms of a fortified pond sediment extract and an analytical standard containing methylated niclosamide using electron-capture detection. Oven temperature, 260°C, attenuation × 20.

niclosamide could be separated from all coextractives, following Florisil cleanup, using a methanol-water solvent system. We found that methylene chloride extracts could be analysed without Florisil column cleanup but this procedure would be likely to shorten the life of the chromatographic column. Some changes in the retention time of niclosamide were noted with the μBondapak-NH_2 column during a normal operating day.

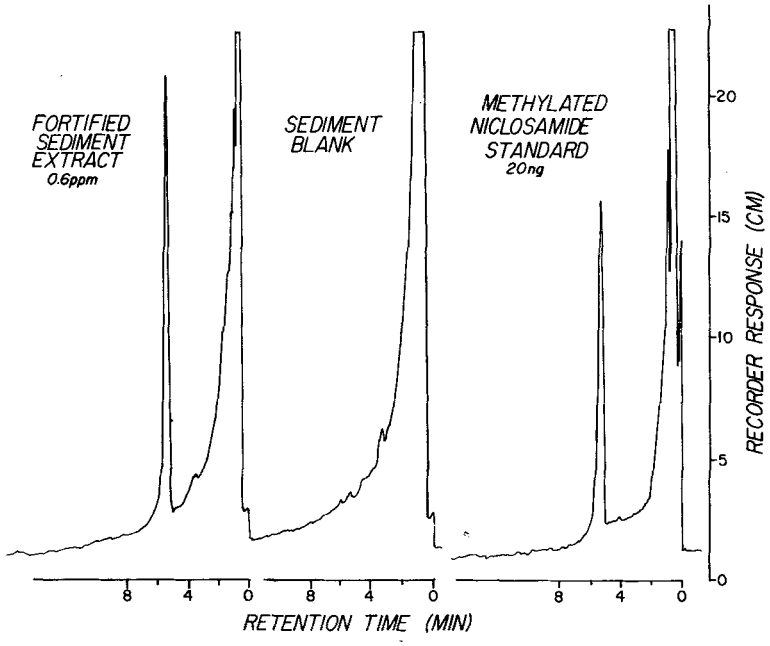

FIGURE 5 Gas chromatograms of a fortified sediment extract (0.6 ppm) blank sediment sample and analytical standard containing methylated niclosamide using an alkali-flame detector. Oven temperature, 250°C; attenuation × 4.

Continuous sparging of the solvent with helium (5–10 ml/min) reduced this variation. A Lichrosorb Si60 column (hexane : isopropanol (95 : 5) solvent system) also appeared to be useful for the analysis of niclosamide in water extracts and may provide a means for partial confirmation of peak identity. We did not investigate this column for sediment extracts, however. It was concluded that HPLC analysis offered the best procedure for determination of niclosamide in sediment extracts.

The efficiency of extraction of the methanol-water and acetone-formic acid systems was compared by extracting sediment that had been incubated with ^{14}C-niclosamide in a sediment-water system. The results of the extractions (Table V) indicated that neither system could extract the radioactivity efficiently. Most of the radioactivity (71–85%) remained in the sediment residuum after extraction and centrifugation and was determined by oxidative combustion and liquid scintillation counting. Recoveries were slightly higher using the acetone-formic acid solvent system but the methanol-water extracts were somewhat easier to clean up for TLC or HPLC analysis. The acetone-formic acid extracts caused

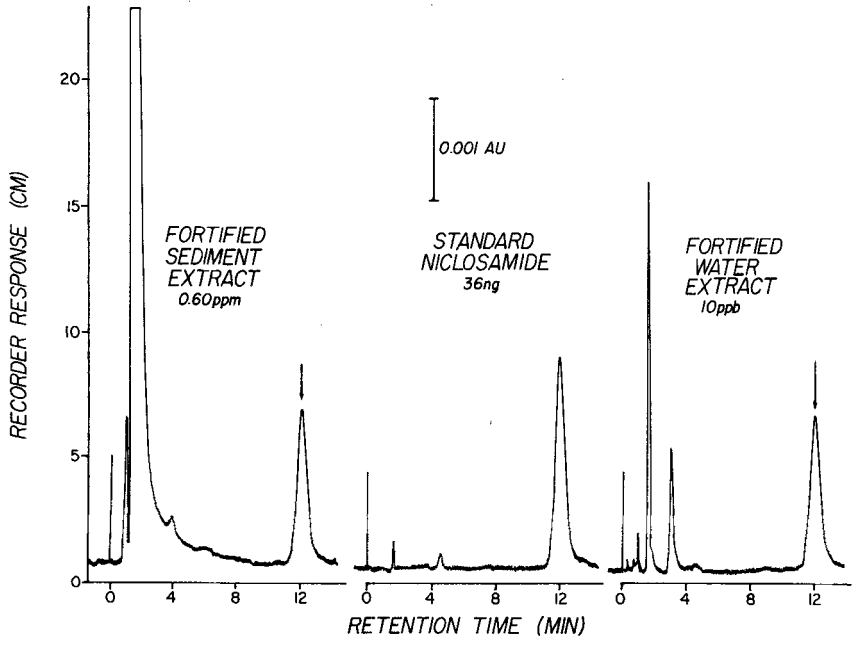

FIGURE 6 Liquid chromatograms of a fortified sediment (0.60 ppm) extract, analytical standard and fortified water extract (10 ppb) on a μBondapak-NH_2 column. Sensitivity, 0.005 A.U.F.S.; methanol: water (80 : 20), 2.0 ml/min.

TABLE V

Extraction of ^{14}C-niclosamide from aerobic and anaerobic sediments

Condition	Percent of total radioactivity in each phase[1]		
	CH_2Cl_2	Aqueous	Residuum
A. Methanol-water extraction[2]			
aerobic	12.7±7.0	1.0±0.4	84.8± 7.3
anaerobic	16.9±5.7	3.7±2.5	71.2±17.4
B. Acetone-formic acid extraction[2]			
aerobic	19.5± 7.0	5.0±0.8	75.4±13.4
anaerobic	20.8±23.7	2.9±2.0	79.9±15.1

[1]Total radioactivity by oxidative combustion of untreated sediment.
[2]Average of 3 replicates ± standard deviation.

considerable color quenching when assayed directly by scintillation counting and aliquots of the extracts were oxidized (Packard Model 306 oxidizer) before counting to overcome the problem. Slightly more radioactivity was extracted from anaerobic than aerobically incubated sediment. A number of phenylcarbamate pesticides have been observed to degrade

more slowly under anaerobic or flooded soil conditions.[11] Autoradiography of sample extracts separated by TLC revealed that none of the radioactivity was in the form of niclosamide. Most of the radioactivity had similar R_f to 5-chlorosalicyclic acid but this observation remains to be confirmed by more detailed study. The recovery study served to illustrate the rapid degradation of niclosamide in sediments. Work is underway to identify niclosamide degradation products in sediment and to determine the nature of the bound residues.

Acknowledgement

The authors would like to thank G. Malis and M. Pitze for their competent technical assistance. We are also indebted to the U.S. Fish and Wildlife Service (La Crosse, Wis) for providing ^{14}C-niclosamide.

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