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Use of pressurized liquid extraction (PLE)/gas chromatography–electron capture detection (GC–ECD) for the determination of biodegradation intermediates of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in soils

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Abstract

A rapid, sensitive, and reproducible method was developed for quantitative determination of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and its biodegradation intermediates, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) in soils. RDX, MNX, DNX, or TNX was extracted from soil by pressurized liquid extraction (PLE), followed by cleanup using florisil. Instrumental analysis was performed using gas chromatography with electron capture detection (GC–ECD), which was highly sensitive to the parent explosive and its metabolites. The method detection limits (MDLs) were 0.243, 0.095, 0.138, and 0.057 ng/g for RDX, MNX, DNX, and TNX, respectively. The method gave high recovery (98–102%), good precision (0.22–5.14%), and reproducibility, and proved to be suitable for real world sample analysis.

Keywords: Explosives; RDX; Gas chromatography (GC); Electron capture detection (ECD); Soil analysis; Pressurized liquid extraction (PLE)

1. Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is one of the most important energetic compounds used extensively around the world. Various military and civil activities in certain areas have resulted in the contamination of soil and water with RDX [1]. Under anaerobic conditions, some bacteria can sequentially reduce the N–NO_2 groups on RDX to the corresponding N–NO, ultimately producing (sequentially) hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) [2–4] (Fig. 1). One important characteristic of these bacteria is that they reduce the N–NO_2 groups to N–NO using a type I nitroreductase [2–5]. These biodegradation metabolites of RDX have been observed in laboratory studies [2–5], suggesting that MNX, DNX, and TNX may also be produced via bacterial degradation in the natural environment and co-exist in water and soils where RDX occurs. This hypothesis was strengthened by observations of Beller and Tiemeier [6]. In their study, MNX, DNX, and TNX were found in groundwater samples at the Iowa Army Ammunition Plant with average concentrations of 65, 24, and 39 μg/L, respectively [6].

Recent studies in our laboratory have indicated that MNX and TNX can have adverse effects on earthworms (growth inhibition) and mice (DNA damage) [7,8]. Thus, there are some concerns about the potential effects of these RDX biodegradation intermediates on indigenous organisms, especially at sites requiring some form of remediation due to explosive production activities, field usage, or disposal of munitions. In some instances, these organisms include threatened or endangered species. Hence, evaluation of the potential hazards to terrestrial organisms/wildlife exposed

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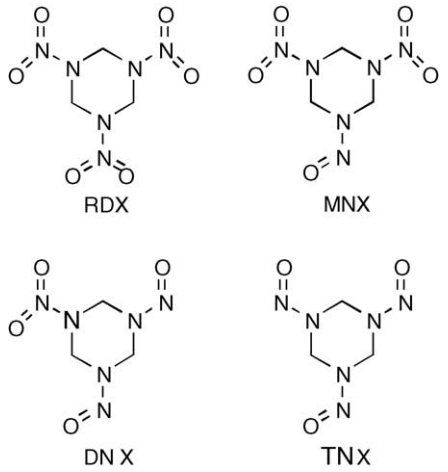

Fig. 1. Structures of RDX and its nitroso-derivatives.

to explosive-contaminated soils is required. Despite numerous analytical methods for RDX determination in various matrices, analytical methods for MNX, DNX, and TNX are limited. To the best of our knowledge, this is the first study describing a method for MNX, DNX, and TNX analysis in a soil matrix.

Because MNX, DNX, and TNX are biodegradation intermediates of RDX, they are likely to occur only at trace levels in soil and water. Thus, sensitive analysis methods as well as efficient sample preparation techniques capable of minimizing matrix effects are required to accurately quantify RDX and its biodegradation intermediates. Recently, Chow et al. reported an HPLC method for the determination of RDX biodegradation intermediates (MNX, DNX, and TNX) in liquid media [9]. Compared with HPLC-UV, GC–ECD is an analytical method that has some advantages in terms of its lower detection limits and improved chromatographic resolution [10–12]. Here, we describe a rapid, sensitive, and reproducible sample preparation and GC–ECD analysis method for determining trace quantities of RDX biodegradation intermediates in soil.

2. Experimental

2.1. Chemicals and reagents

RDX (purity > 99%) was purchased from Supelco (Bellafonte, PA) as an acetonitrile solution at a concentration of 1 mg/mL. MNX (purity > 99%), DNX (purity 59%), and TNX (purity > 99%) were obtained as solids from SRI International (Menlo Park, CA). HPLC-grade acetonitrile was purchased from Fisher Scientific (Pittsburg, PA). Ultra-pure water (>18 MΩ) was prepared by a Barnstead NANOpure infinity ultrapure water system (Dubuque, Iowa). Anhydrous Na_2SO_4 (99.16% pure, 10–60 mesh) was purchased from VWR (West Chester, PA). Florisil solid phase extraction (SPE) cartridges (bed weight: 500 mg, tube volume: 3 mL) were obtained from Supelco (Bellafonte, PA).

2.2. PLE and soil extract cleanup

One gram of soil (sandy loam, Terry County, TX or silt loam, Harlan County, NE) was individually spiked using 10–20 μL stock solutions containing calculated amounts of RDX, MNX, DNX, or TNX to desired final concentrations of 10, 50, 100, and 500 ng/g. After samples were thoroughly mixed using a small mortar and pestle, 8–10 g of dried Na_2SO_4 (to further dehydrate the soils) was added to each sample. Then, the soil sample–Na_2SO_4 mixture was loaded into a 22-mL cell and extracted using a Dionex Accelerated Solvent Extractor (Model 200, Salt Lake City, UT). Each extraction began with a 5-min preheating step, followed by a 5-min static extraction with acetonitrile. Static extraction was performed at constant temperature and pressure (100 °C and 1500 psi). One extraction cycle was used (total extraction time = 15 min). The extract (15–20 mL) was then purged from the cell and collected into a 60-mL glass vial [13]. The extracts were then reduced to 1–2 mL using rotary evaporation.

Florisil SPE cartridges placed on a 24-port manifold (Supelco, Bellafonte, PA, USA) were used to clean extracts. Before loading samples, florisil cartridges were conditioned with acetonitrile (2 × 5 mL). Samples were then loaded (without vaccumn), and eluates were collected into 10-mL graduated centrifuge tubes. The florisil cartridges were rinsed 3× with acetonitrile (3 × 1 mL). The cleaned sample (4–5 mL) was concentrated to 0.5–1.0 mL under nitrogen using a N-EVAP^{TM}111 nitrogen evaporator (Organomation Associates Inc., Berlin, MA, USA). The final volume was adjusted to 1 mL with clean acetonitrile, filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA) into a GC vial, and stored (4 °C) prior to GC analysis.

2.3. Sample analysis by GC–ECD

An Agilent 6890 series gas chromatograph (GC) was employed to analyze RDX, MNX, DNX, and TNX. The GC was equipped with an autosampler and an electron capture detector (ECD) and was controlled by ChemStation chromatography software (Agilent, Palo Alto, CA, USA). Separation was performed on a capillary HP-5 column (30 m × 0.25 mm × 0.25 μm) (Agilent Company, Wilmington, DE, USA). The GC oven temperature was initially held at 90 °C for 3 min, increased to 200 °C at a rate of 10 °C/min, and then raised to 250 °C at 25 °C/min, and finally held at 250 °C for 5 min. The injector temperature was 170 °C. The detector temperature was 270 °C. The injection volume was 2 μL. The carrier gas was helium (99.999% purity) at a constant flow-rate of 9.2 mL/min. The makeup gas for the ECD detector was argon:methane (95:5) at a combined flow-rate of 60.0 mL/min. The ECD was operated in the constant current mode.

2.4. Standard solution and calibration curve

For each analyte (RDX, MNX, DNX, and TNX), 10 standard concentration levels (1, 2, 5, 10, 20, 50, 100, 200, 500, 1000 ng/mL) were prepared daily, and stored at 4 °C in the dark. Standards were analyzed in order of increasing concentrations. Calibration curves were constructed by plotting concentration of the analyte versus response peak area. These relationships were best fitted to a polynomial regression. Thus, polynomial regression analysis was used to calculate the slope, intercept, and correlation coefficient for the calibration curves.

2.5. Recovery, precision, and method detection limit

Four concentration levels (10, 50, 100, and 500 ng/g) were employed to determine the precision and recovery of the developed method. Each concentration contained five spiked samples (replicates). Recovery was determined by comparing the measured concentration to the nominal concentration. Precision was represented by the relative standard deviation (R.S.D.), which is equal to S.D./mean × 100%. The intra-day precision was determined by repeated injections ($n = 5$) of the same samples on a single day. The inter-day precision was determined by repeated injections of the same samples on five different days. The method detection limit (MDL) for each analyte was calculated by the following formula: MDL = 3.14 × S.D., where S.D. is the standard deviation of the measurements of seven spiked samples, and 3.14 is the student's *t*-value at the 99% confidence level ($t = 3.14$ for $n - 1$ degrees of freedom).

2.6. Stability of analytes in acetonitrile extracts of soil samples

The stability of the analytes in soil sample extracts under three storage temperatures (4 °C, −20 °C, and room temperature ∼20 °C) was also studied. Soil sample extracts spiked at concentrations of 50 and 100 ng/mL for all analytes were analyzed at different times during a period of 2 days after extraction.

2.7. Analysis of real samples

Real soil samples were obtained from an ongoing earthworm exposure study involving MNX and TNX in order to further test the developed method. Five grams of soil was sampled and stored at −20 °C. Sample extraction and analysis followed the same protocol described above.

3. Results and discussion

We found acetonitrile to be a suitable extraction solvent for RDX biodegradation intermediates in soil. While other solvents provided good recovery, acetonitrile gave us the option of analyzing the extracts by either HPLC or GC. For these particular explosive metabolites, we chose GC analysis.

No peaks interfering with the compounds of interest were observed within the respective retention windows in the blank soil samples (Fig. 2a). This indicated that an efficient cleanup method was used in this study. Under the chromatographic conditions described in the Section 2, RDX and its biodegradation intermediates (MNX, DNX, and TNX) were completely separated, and the response of GC–ECD was good for each analyte (Fig. 2b and d). At lower concentrations (1–20 ng/g), GC–ECD response was linear with excellent correlation coefficients (>0.999) for each analyte. For a wider concentration range (1–1000 ng/g), a quadratic model ($y = ax^2 + bx + c$) provided a better overall fit for the concentration versus ECD response calibration graphs with $r^2 > 0.999$. This result was similar to results observed by Walsh and Ranney [9] for nitroaromatic, nitramine, and nitrate ester explosives.

Under the chromatographic conditions described in our study, a sharp peak corresponding to each analyte was clear on each chromatogram and the respective retention times were stable. The retention times were 8.262, 10.070, 11.378, and 12.595 min for TNX, DNX, MNX, and RDX respectively. The method detection limits (MDLs) varied for the analytes and ranged from 0.057 to 0.243 ng/g (Table 1). TNX and MNX had lower method detection limits (0.057 and 0.095 ng/g, respectively).

High recoveries of the analytes of interest (range = 98–102%) were achieved at all the concentrations studied (Table 2). The reproducibility of the analytical method was evaluated by determining precision at four different concentrations. The intra-day precision represented as relative standard deviation (R.S.D.) was 0.22–4.51% and the inter-day R.S.D. was 0.54–5.14% (Table 2). We considered both recovery and precision for these types of analyses in a soil matrix to be acceptable.

The stability of RDX, MNX, DNX, and TNX in acetonitrile extracts was investigated under three storage temperatures: 4 °C, −20 °C, and room temperature (∼20 °C). Concentrations of RDX, MNX, DNX, and TNX were analyzed at different times during a 2-day period after extraction. Our results indicated that RDX, MNX, DNX, and TNX were stable in acetonitrile extracts for 48 h at all three storage tem-

Table 1
Retention times and method detection limits (MDLs) for RDX and its nitroso-derivatives in soil (mean ± S.D.)

Analyte	Retention time (min)	Method detection limit[a] (ng/g)
RDX	12.60 ± 0.01	0.243
MNX	11.38 ± 0.01	0.095
DNX	10.07 ± 0.01	0.138
TNX	8.26 ± 0.01	0.057

[a] Method detection limits were calculated using the equation, MDL = 3.14 × S.D., where S.D. is the standard deviation of the measurements of seven spiked samples, and 3.14 is the student's *t*-value at the 99% confidence level ($t = 3.14$ for $n - 1$ degrees of freedom).

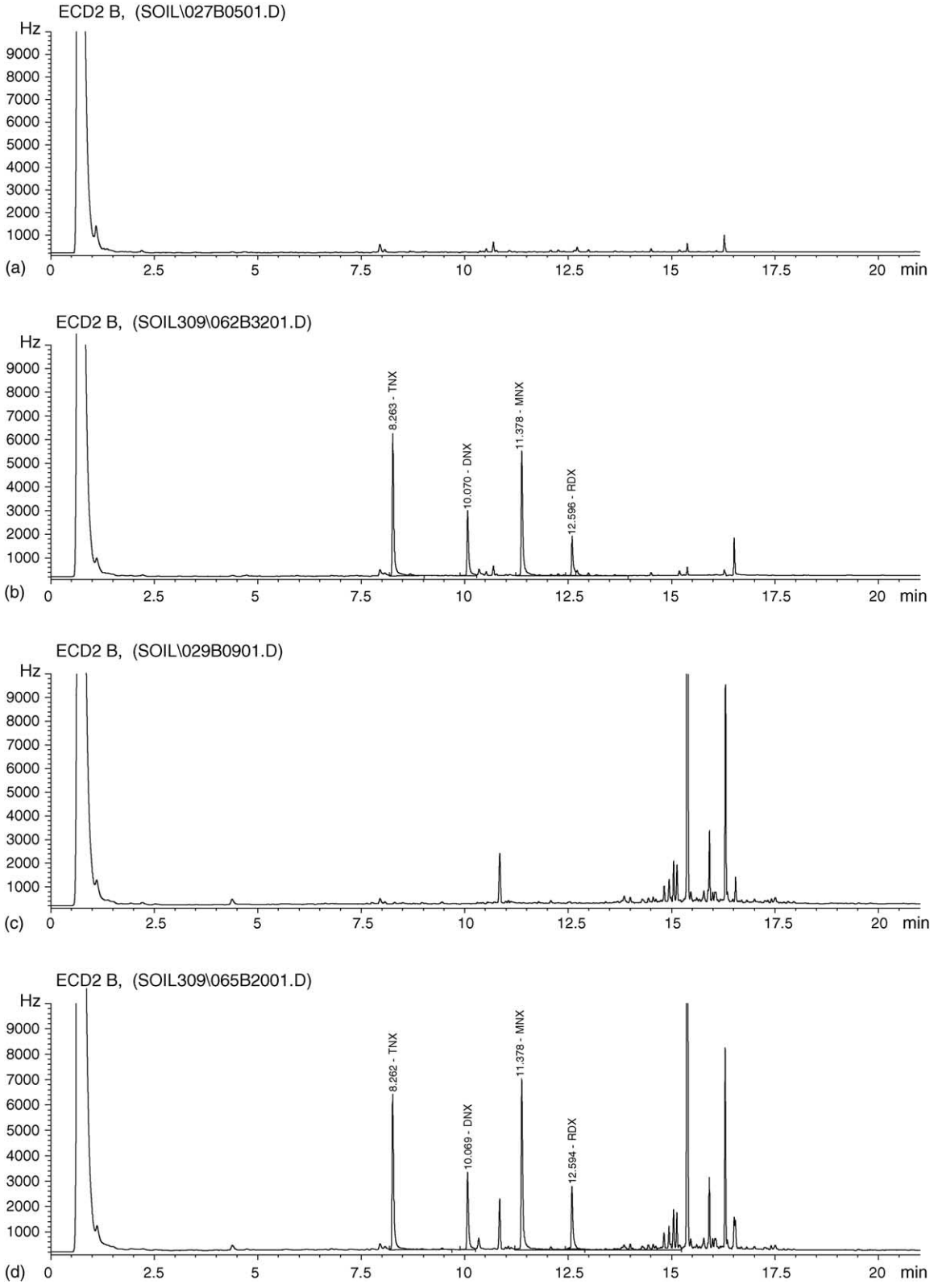

Fig. 2. Representative chromatograms of a blank sample of: (a) sandy loam soil and (c) silt loam soil; (b) sandy loam soil sample and (d) silt loam soil sample spiked with 5 ng/g RDX, MNX, DNX, and TNX.

Table 2
Intra-day and inter-day recovery, precision of RDX, and its nitroso-derivatives in soil[a]

Compound	Concentration added (ng/g)	Intra-day			Inter-day		
		Concentration measured (ng/g)	Recovery (%)	Precision (%)	Concentration measured (ng/g)	Recovery (%)	Precision (%)
RDX	10	9.79 ± 0.44	97.92 ± 4.42	4.51	10.04 ± 0.23	100.41 ± 2.32	2.31
	50	49.85 ± 0.82	99.70 ± 1.64	1.64	50.39 ± 1.50	100.79 ± 3.01	2.99
	100	101.42 ± 1.70	101.42 ± 1.70	1.67	101.61 ± 3.33	101.61 ± 3.32	3.27
	500	502.99 ± 3.20	100.60 ± 0.64	0.64	497.90 ± 5.72	99.58 ± 1.14	1.15
MNX	10	10.05 ± 0.12	100.46 ± 1.20	1.20	10.05 ± 0.49	100.45 ± 4.89	4.86
	50	50.13 ± 0.52	100.26 ± 1.04	1.03	50.38 ± 0.91	100.77 ± 1.83	1.81
	100	100.54 ± 0.78	100.54 ± 0.78	0.78	99.99 ± 1.27	99.99 ± 1.27	1.27
	500	503.02 ± 3.75	100.60 ± 0.75	0.75	500.04 ± 3.96	100.01 ± 0.79	0.79
DNX	10	10.00 ± 0.31	100.00 ± 3.06	3.06	9.93 ± 0.51	99.33 ± 5.11	5.14
	50	50.17 ± 0.86	100.34 ± 1.71	1.71	50.34 ± 2.32	100.67 ± 4.63	4.60
	100	100.69 ± 1.98	100.69 ± 1.98	1.96	99.64 ± 4.06	99.64 ± 4.06	4.08
	500	502.71 ± 4.77	100.54 ± 0.95	0.95	495.45 ± 6.08	99.10 ± 1.22	1.23
TNX	10	10.00 ± 0.08	99.99 ± 0.80	0.80	10.15 ± 0.31	101.49 ± 3.11	3.06
	50	50.10 ± 0.11	100.20 ± 0.22	0.22	50.46 ± 1.16	100.93 ± 2.33	2.31
	100	101.37 ± 0.53	101.37 ± 0.53	0.52	101.64 ± 1.55	101.64 ± 1.55	1.53
	500	500.41 ± 2.14	100.08 ± 0.43	0.43	502.79 ± 2.70	100.56 ± 0.54	0.54

The intra-day precision was determined by repeated injections ($n = 5$) of the same samples on a single day. The inter-day precision was determined by repeated injections of the same samples on five different days. Recovery (%) = the measured concentration/the nominal concentration × 100%. The precision (%) = S.D./mean × 100%.

[a] Mean ± S.D., $n = 5$.

peratures. There were no significant concentration changes for samples analyzed immediately after extraction and samples analyzed 2 days after extraction (data not shown). This indicated that no special storage condition is necessary if conducting GC–ECD analysis within 2 days after sample extraction.

The extraction and analysis method was employed in the analysis of soil samples from an earthworm exposure study [7] in which TNX and MNX were spiked into two soil types (sandy loam and silt loam). Uptake of these compounds into earthworms was monitored over time (60 d). We used the extraction and analysis method described here to monitor concentrations of MNX and TNX in the soils during the experimental period. Fig. 3 shows the results of that successful effort. These results further support our contention that the method is suitable for quantifying RDX and its biodegradation intermediates as well as monitoring biodegradation processes of RDX in soils.

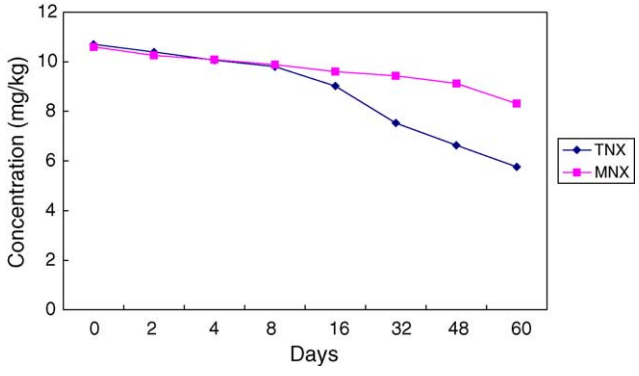

Fig. 3. MNX and TNX concentrations in soil samples from an earthworm exposure study conducted over 60 days.

4. Conclusions

This research resulted in the development of a simple and reproducible sample preparation technique and GC–ECD analysis method for RDX and its biodegradation intermediates in soil samples. PLE followed by florisil SPE cleanup produces both an efficient extraction and adequate cleanup for these samples. High recoveries (98–102%) were achieved at all four tested concentrations for the four analytes (RDX, MNX, DNX, and TNX). Good precision (0.22–5.14%) also demonstrated the high reproducibility of this method. GC–ECD was highly sensitive for RDX and its biodegradation intermediates as indicated by the low MDLs of 0.243, 0.095, 0.138, and 0.057 ng/g for RDX, MNX, DNX, and TNX, respectively. This method is now employed in our laboratory for routine analysis of soil samples.

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