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Extraction and analysis of trace amounts of cyclonite (RDX) and its nitroso-metabolites in animal liver tissue using gas chromatography with electron capture detection (GC–ECD)

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Abstract

An efficient extraction and cleanup technique, and an instrumental detection method suitable for determination of trace amounts of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and its nitroso-metabolites in animal liver tissue were developed and validated in this paper. The method includes the extraction of explosives from liver tissue samples using accelerated solvent extraction (ASE) followed by cleanup using florisil and styrene-divinyl benzene (SDB) cartridges to remove interfering naturally endogenous compounds. The instrumental analysis was conducted using a capillary column gas chromatograph coupled with an electron capture detector (GC–ECD). High recoveries (58.9–106.8%) of RDX, 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) were achieved at all concentrations studied. RDX, MNX, and TNX gave higher recoveries than DNX at all three tested concentrations (50, 250, 1250 ng/g). Overall recoveries of RDX, MNX, DNX, and TNX from 1 g beef liver samples containing 50, 250, and 1250 ng/g were 80.1, 82.8, 68.9, and 80.4%, respectively. The optimal injection port temperature range was 160–170 °C for analysis of RDX and its nitroso-metabolites. Higher or lower temperatures than 160–170 °C decreased signal amplitudes. RDX was unstable in the liver extraction matrix; as much as 50% of RDX was degraded 10 days after extraction if keeping the liver sample extracts at room temperature. Degradation of RDX to MNX, DNX, or TNX was not detected during the sample storage, extraction, or instrument analysis processes. Other optimized extraction and GC conditions are also discussed.
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Keywords: RDX; Explosive; Liver; ASE; SDB; Gas chromatography; Electron capture detection

1. Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is one of the most widely used explosives in the US and around the world. RDX enters the environment mainly through discharge during manufacturing and military operations [1]. The US army has identified 583 RDX contaminated sites, as well as 88 suspicious RDX contaminated sites [2]. The reported concentration of RDX ranged from 0.044 to 13,900 μg/g in soil samples from various army sites in the US [3]. Moreover, RDX is relatively water soluble as indicated by its logarithmic octanol/water partition coefficient (log K_{ow}) of 0.8; thus, RDX is readily leachable and can migrate rapidly among environmental compartments [2].

Under aerobic and anaerobic conditions, bacteria can degrade RDX to various intermediates and end-products [4,5]. One of the degradation pathways of RDX involves the consequential reduction of RDX to N-nitroso metabolites: 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) [5–8]. Some or all of the N-nitroso metabolites have been detected in laboratory studies under anaerobic conditions [4,5]. These findings indicated that RDX may also undergo degradation and yield N-nitroso metabolites in the natural environment.

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Recently, MNX, DNX, and TNX, as well as RDX have been detected environmentally in ground water at the Iowa Army Ammunition Plant [9]. In that study, relatively high MNX, DNX, and TNX concentrations (up to 430 μg/l) were reported in groundwater samples collected from wells in three different areas of the Iowa Army Ammunition Plant.

RDX is toxic to a wide range of organisms including terrestrial [10], soil dwelling [11], and aquatic organisms [12] due to its cytotoxicity, genotoxicity, neurotoxicity, or possible carcinogenicity [1,2]. The US Environmental Protection Agency (USEPA) has classified RDX as a potential human carcinogen (Class C) [13]. However, the carcinogenic mechanism of RDX remains largely unknown. Since N-nitroso compounds are well-documented genotoxic carcinogens [14–16], concerns are intensified about whether these N-nitroso metabolites of RDX can accumulate in aquatic or territorial organisms and cause direct adverse effects in animals and humans. Recent studies indicated that MNX and TNX were toxic to earthworms and mice by inhibiting growth, causing death, and inducing DNA damage [17,18].

Detection of RDX residues and its nitroso-metabolites in environmental samples is important for exposure evaluation and ecological risk assessment. Some methods have been developed to analyze explosives in water [19–21], soil [22–25], or plant tissues [24,26]. Jenkins et al. developed a standard analytical method for determination of explosives in soil, which is now known as the EPA method 8330 [22]. This method involves 18 h of sonication with acetonitrile followed by precipitation of interfering compounds with calcium chloride prior to high-performance liquid chromatography with ultraviolet detection (HPLC–UV) analysis. The extraction method for soil samples, developed by Jenkins et al. has been adopted by some studies with or without modifications [25–27]. The extraction efficiency, as pointed out by Jenkins et al., depends largely on the efficiency of sonic agitation and the variables of sonic device [22]. As an alternative, accelerated solvent extraction (ASE) was demonstrated to be equivalent to existing extraction methodologies and was adopted by some environmental labs for explosive analysis due to its fast extraction (12–20 min/sample) and high extraction efficiency [23,24]. However, the cleanup techniques described in these methods are limited and may be inadequate when dealing with challenging samples such as animal tissues. Compared with water, soil, and plant samples, animal tissues are more complicated due to the presence of many proteins, lipids, and other compounds. Intensive cleanup during sample preparation is often necessary in order to obtain better resolution and protect expensive instruments. To our knowledge, only one report prepared by Lakings and Gan described an analysis method specific for detection of explosives in animal tissues to date. In Lakings and Gan's study, a HPLC–UV method was used to determine RDX in bovine kidney, muscle/fat, and liver samples. The reported detection limits in their study were 95, 62, and 150 ng/g for bovine kidney, muscle/fat, and liver samples with a recovery range of 87.7–102.9% [1]. GC–ECD is a prevalent instrument in environmental labs. Detection using GC–ECD is advantageous due to its lower detection limits and improved chromatographic resolution [25]. In this study, we described an alternative GC–ECD method for determining tens of nanograms of RDX, MNX, DNX, and TNX in animal tissues. Also, extraction of MNX, DNX, and TNX from animal tissue is discussed for the first time.

2. Experimental

2.1. Reagents and materials

RDX, MNX, DNX, and TNX (Fig. 1) were studied in this experiment. RDX (99.5% pure), at a concentration of 1000 mg/l in acetonitrile, were obtained from Supelco (Bellefonate, PA). Standards for TNX (>99.0% pure), DNX (59% pure), and MNX (99.5% pure) were purchased from SRI International (Menlo Park, CA). Work standards for RDX, MNX, DNX, and TNX were prepared by diluting stock solutions with acetonitrile to desired concentrations. All stock and work solutions were stored at 4 °C.

Styrene-divinylbenzene (SDB) solid phase extraction (SPE) cartridges (500 mg) were obtained from Supelco (Bellefonate, PA). Ultra-pure water (>18 MΩ) was used for all aqueous solutions. Extraction solvent was HPLC-grade acetonitrile (Fisher Scientific, Pittsburg, PA). Na_2SO_4 was purchased from VWR (West Chester, PA).

2.2. Fortification procedure

One whole piece of fresh beef liver, packed in vacuum-sealed plastic bag, was purchased from a local supermarket, and was stored at −20 °C. Prior to use, the beef liver was defrosted, cut into small pieces, and then was thoroughly homogenized using a PowerGen 700D homogenizer (Fisher Scientific, Pittsburg, PA) in an ice bath. Homogenized liver slurry was dispensed into glass test tubes by carefully weighting 1 g (0.98–1.02 g) for each sample. Each sample was spiked to desired concentration with 10–15 μl stock standard containing RDX, MNX, DNX, and TNX. Three levels of spiking were performed: 50, 250, and 1250 ng/g. Spiking solution was absorbed into liver slurry by thoroughly mixing for 1 min using a Genie 2 vortex mixer (Scientific Industries, Bohemix, NY). No solution layer on the surface of the sam-

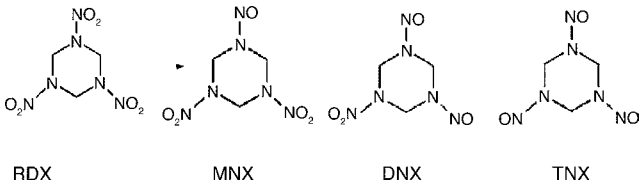

Fig. 1. Structures of RDX, MNX, DNX, and TNX.

ples was observed. Numerous samples were prepared at each spiking level. Blank liver samples were prepared at the same way by spiking 10–15 μl acetonitrile to serve as negative control. Test tubes containing fortified and blank samples were sealed with Para film and stored at −20 °C for no more than 7 days before extraction and analysis.

2.3. Extraction and cleanup procedure

2.3.1. Extraction of RDX, MNX, DNX, and TNX from animal liver tissue by ASE

Prior to ASE extraction, 1 g samples were dehydrated by grinding with 8–10 g of dehydrated Na_2SO_4, which also served as a dispersing agent. All extractions were conducted using a Dionex ASE 200 extractor (Salt Lake City, UT). Sample Na_2SO_4 mixtures were loaded into the 22-ml cells, which were previously fitted with one cellulose filter. Each extraction began with a 5-min preheating, followed by a 5-min static extraction with acetonitrile. The extract was then purged from the system with 1 min nitrogen and was collected in a 60 ml glass collection vial [23]. The sample extract with a volume of 35–40 ml in acetonitrile was evaporated to 1–2 ml using rotary evaporation and stored for subsequent florisil cleanup. The total ASE extraction time was about 17 min per sample.

2.3.2. Cleanup of ASE extracts

Prior to GC–ECD analysis, each liver sample extract was processed through a florisil cartridge and then a SDB cartridge to remove the interfering compounds and pigments. Initial cleanup of the sample extracts using florisil cartridges (0.8 g) on a 24-port manifold (Supelco, Bellefonate, PA) was performed using the following protocol. First, florisil cartridges were conditioned with 2× 5 ml acetonitrile. Then, the sample extracts were passed through florisil cartridges without using any vacuum. Florisil cartridges were subsequently rinsed three times with a small amount of acetonitrile (3× 1 ml) and the sample eluents in acetonitrile were collected. Previous studies have shown that sample extracts still contained many interfering compounds that would interfere with GC analysis and may cause instrument contamination (Fig. 2a). Therefore, an extra cleanup step using SDB cartridges was employed as follows: the extract (<5 ml) acquired from the florisil cleanup step was diluted in 100 ml water containing 10% NaCl. The SDB cartridges were conditioned using hexane 4 ml, acetone 4 ml, acetonitrile 4 ml, and 10 ml water. Samples were passed through the conditioned SDB cartridges at 2 ml/min under low-pressure vacuum. After washing with 5 ml water, the SDB sorbent bed was dried under vacuum for 20 min. Analytes of interests were eluted with 5 ml acetonitrile and collected, and then the eluent was evaporated under a gentle stream of nitrogen and concentrated to a final volume of 1.5 ml.

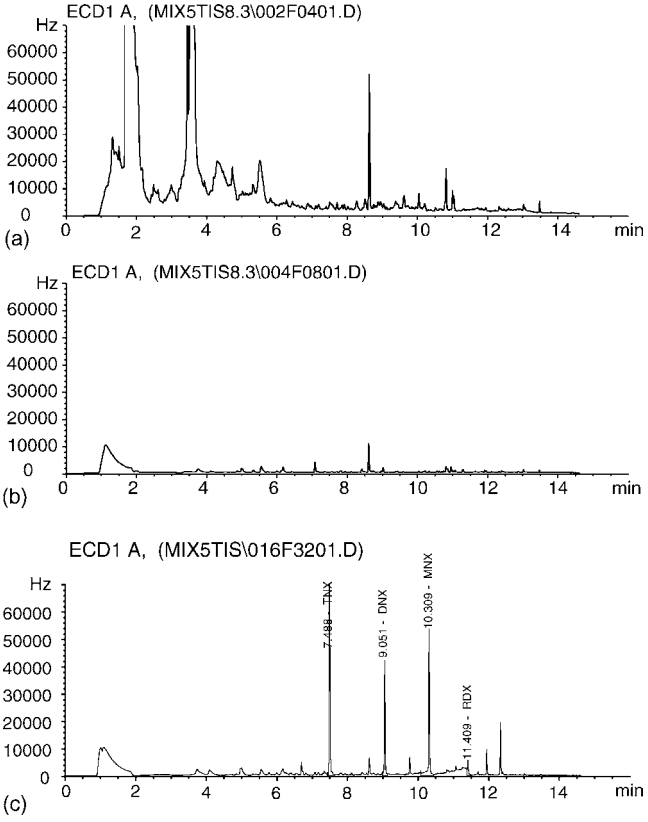

Fig. 2. Representative chromatograms of (a) blank tissue sample cleaned up by a florisil cartridge only, (b) blank tissue sample cleaned up by florisil and SDB columns, and (c) tissue sample containing RDX, TNX, MNX, and DNX.

Final extracts were then filtered through 0.2 μm PTFE membranes and stored in 2-ml vials for GC analysis.

2.4. Instrumental analysis

The stock solution of standards for RDX, MNX, DNX, and TNX (10 μg/ml) were prepared in acetonitrile and stored at 4 °C. Working standards were prepared in acetonitrile, at a concentration range of 1–1000 μg/l. At least eight standards were prepared for calibration. Standard solutions were stored in the dark at 4 °C.

Standards and sample extracts were analyzed using an HP 6890 series GC–ECD (Agilent, Palo Alto, CA). Splitless injections (2 μl) were made by autoinjector. Separations were performed in a 30 m × 0.25 mm (i.d.) × 0.25 μm film thickness HP-5 column (HP company, Wilmington, DE). Helium carrier gas was maintained at a constant flow of 80 cm/s. The temperature program began at 90 °C, held for 2 min, increased to 130 °C at a rate of 25 °C/min, and increased to 200 °C at a rate of 10 °C/min, and then increased to 250 °C at a rate of 25 °C/min. Injector and detector temperatures were 170 and 270 °C, respectively. The ECD was operated in the constant current mode and argon methane served as make-up gas for the detector. The septum and injector liner were replaced after every 50 injections. Extra run time was needed

when analyzing tissue samples to allow the late-eluting interferences to exit the column before the next injection.

Each analysis sequence began with injections of at least three calibration standards that spanned the needed calibration range. Continuing calibration standards were run following every 10 samples, and blanks were analyzed at intervals of no more than 5 samples. If the response of the continuing calibration standards had changed by 15% or more, then a new standard curve was developed. This frequency of standard analysis ensured that analyte and detector stability were maintained during instrumental analysis.

2.5. Validation design

Method validation was performed with beef liver that had been fortified with TNX. Test procedures were evaluated by fortification of at least 12 samples at each of nine concentrations (Table 2). The recoveries of TNX at each concentration were evaluated on different days. Two persons alternated procedural responsibilities on the different analysis days. This provided different teams for statistical analysis of the effects caused by possible personal operation differences on the method.

Table 1
Percent recoveries of RDX and its nitro-metabolites in animal liver tissues

Statistics	Compound			
	RDX	MNX	DNX	TNX
Level: 50 ng/g				
Average[a]	69.7$^{a,\alpha}$	70.5$^{a,\alpha}$	58.9$^{b,\alpha}$	70.1$^{a,\alpha}$
Minimum[a]	54.5	53.1	37.2	55.8
Maximum[a]	83.4	87.0	87.3	93.3
S.D.[a]	12.3	14.9	25.1	15.1
n	11	11	11	11
Level: 250 ng/g				
Average	78.2$^{a,\beta}$	86.7$^{a,c,\beta}$	73.5$^{a,b,\beta}$	84.2$^{a,c,\alpha,\gamma}$
Minimum	55.3	68.1	50.1	61.5
Maximum	95.0	101.3	85.2	99.7
S.D.	17.0	12.9	12.2	12.1
n	11	11	11	11
Level: 1250 ng/g				
Average	92.3$^{a,\beta}$	88.5$^{a,\beta}$	70.7$^{b,\beta}$	88.1$^{a,\beta}$
Minimum	72.8	72.2	53.4	60.7
Maximum	109.6	106.4	87.8	105.8
S.D.	11.8	15.1	17.1	11.6
n	11	11	11	11
Overall				
Average	80.1[a]	82.8[a]	68.9[b]	80.4[a]
S.D.	13.7	14.3	18.1	12.9
n	33	33	33	33

a, b, c, d—recovery comparisons were made between different compounds at the same concentration level. Data with similar average recoveries ($p > 0.05$) were marked with the same superscript letter; data with significantly different average recoveries ($p < 0.05$) were marked with different superscript letters. α, β, γ—recovery comparisons were made between the concentration levels of the same compound. Data with similar average recoveries ($p > 0.05$) were marked with the same superscript letter; data with significantly different average recoveries ($p < 0.05$) were marked with different superscript letters.

2.6. Stability of RDX in liver and extracted matrix

To determine the possible RDX metabolism occurring during storage, extractions, or analysis processes, we also determined the recovery of RDX without the presence of other analytes. For this test, 12 liver samples, spiked with RDX only, were randomly split across two days ($n = 12$; 6 for day 1, 6 for day 2). This check was performed at the spike level of 1000 ng/g and was conducted according to the same extraction, cleanup, and GC analysis protocols described above. RDX concentrations were measured on different days to study the degradation of RDX in liver sample extracts.

2.7. Statistics analysis

All validation data were processed by statistical analysis using the standard statistical software (SigmaPlot, Version 8.0, SPSS, Chicago, IL, USA). A signific level of $\alpha = 0.05$ was used in all comparative statistics. The plot of the calibration curves containing the 95% confident interval was conducted using computer software R program (R, Version 1.9, R Development Core Team).

3. Results

3.1. Calibration curves and detection limits of GC–ECD

Since the lineary range for ECD response to analytes was very narrow [28], a quadratic model, which provided excellent correlation coefficients (>0.997) for all analytes in the concentration range of 1–1000 ng/g, was used in this study to determine the concentrations of TNX, DNX, MNX, and RDX in liver (Fig. 3). The method detection limits (MDLs) of analytes were calculated according to EPA guidelines [28]. The detection limits were derived by calculating the product of the standard deviation of the seven replicates and the Student's value for a 99% confidence level ($t = 3.14$ for $n = 7$). The MDLs for TNX, DNX, MNX, and RDX in liver samples were 20.8, 34.6, 20.6, and 17.0 ng/g, respectively.

3.2. Extraction, cleaning up, separation, and determination of analyte

Animal tissues are more complicated matrixes than water, soil, and plant tissue because they contain many proteins, lipids, and other endogenous compounds. Consequently, animal tissue samples require intensive cleanup before they can be appropriately analyzed by gas chromatography (GC) or other precision instruments. The GC analysis of only florisil-treated extracts showed many interfering peaks and raised baselines (Fig. 2a). Florisil-treated extracts subsequently processed using SDB cartridges resulted in improved chromatographic baselines and elimination of many peaks of endogenous compounds (Fig. 2b).

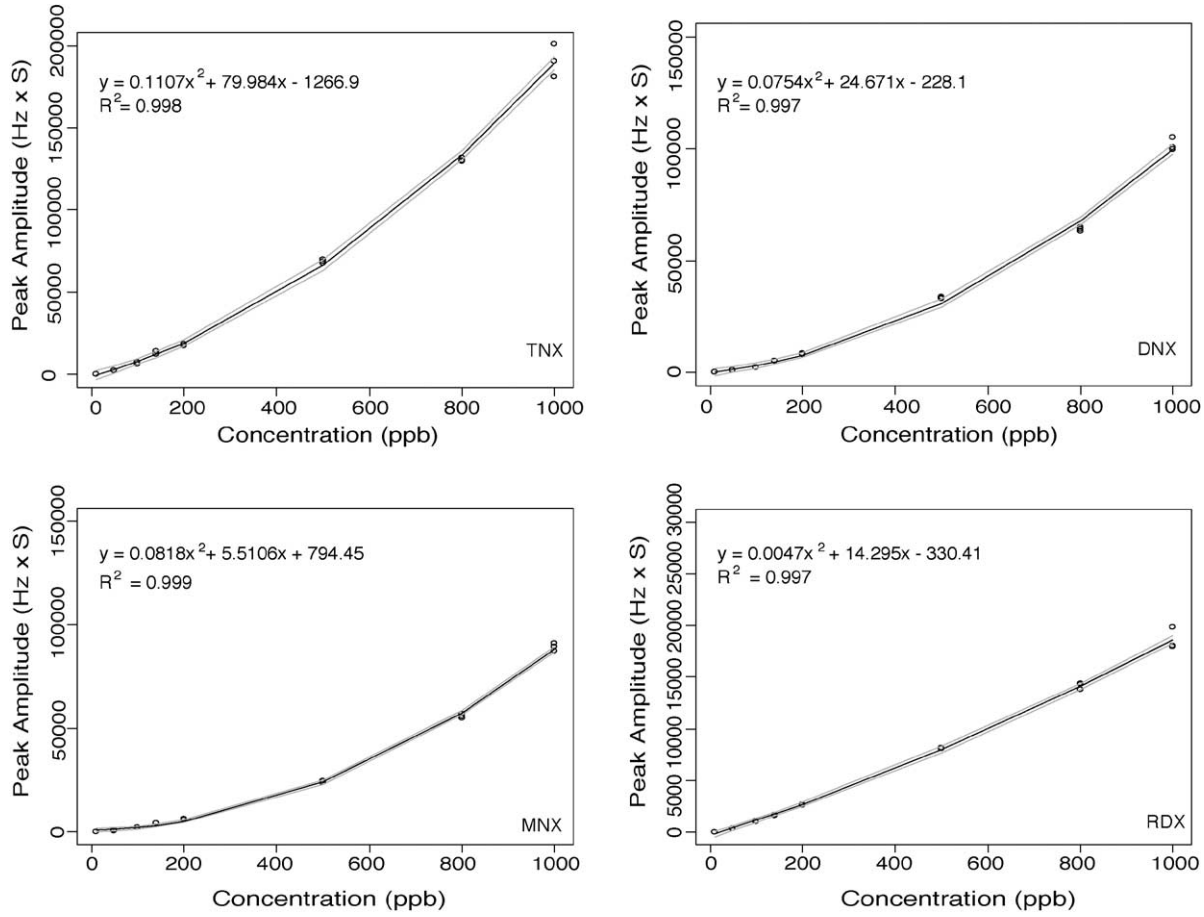

Fig. 3. Calibration curves obtained for TNX, DNX, MNX, and RDX. Black lines stand for the calibration curves ($n = 3$). The gray lines stand for the 95% confidence interval.

RDX, TNX, DNX, and MNX were well separated from each other and from endogenous compounds of liver tissue during a reasonable time. The retention times of TNX, DNX, MNX, and RDX, were 7.48, 9.05, 10.31, and 11.48 min, respectively (Fig. 2c). The chromatogram of blank liver extracts showed that blank samples did not give interfering peaks that had the same retention times as TNX, DNX, MNX, and RDX (Fig. 2b).

3.3. Recovery study

High recoveries of TNX, DNX, MNX, and RDX (58.9–106.8%) were achieved at all concentrations studied (Table 1). When RDX was the only analyte in liver, recovery was $106.8 \pm 7.9\%$ ($n = 12$). No detectable degradation of RDX to MNX, DNX, or TNX was observed during the storage, extraction, or analysis processes. When tissues con-

Table 2
TNX percent recovery (mean ± S.D.) obtained during method development for animal tissues

TNX concentration (ng/g)	*N*	Day I		Day II		Overall*
		Team I	Team II	Team I	Team II	
1250	30	85.5 ± 12.0	87.1 ± 3.1	86.0 ± 8.8	87.1 ± 6.4	86.7 ± 6.7[a]
1000	12	82.5 ± 12.5	85.0 ± 3.4	77.9 ± 3.3	84.0 ± 1.0	83.3 ± 4.8[a,b,c]
500	12	85.0 ± 11.0	81.5 ± 3.8	84.7 ± 5.7	88.3 ± 2.0	84.9 ± 5.1[a,b]
250	33	81.5 ± 5.8	83.1 ± 3.0	83.2 ± 8.4	82.7 ± 6.5	82.7 ± 6.5[b,c,d]
200	12	73.5 ± 1.0	82.9 ± 5.7	80.2 ± 5.2	79.3 ± 5.8	79.7 ± 5.1[c,d]
100	12	77.8 ± 4.6	81.2 ± 6.9	74.5 ± 11.9	78.7 ± 5.4	78.7 ± 5.4[d]
50	54	66.2 ± 7.0	70.2 ± 8.0	68.1 ± 6.7	68.7 ± 7.3	68.7 ± 7.3[e]
20	12	155.5 ± 32.0	200.1 ± 27.1	186.3 ± 18.8	127.1 ± 46.4	146.7 ± 26.7
0	16	ND**	ND	ND	ND	Undefined

* Data with no significant different average recoveries ($p > 0.05$) were marked with the same superscript letter; data with significant different average recoveries ($p < 0.05$) were marked with different superscript letters.
** Not detected.

taining multiple analytes were extracted, two-way ANOVA indicated that recoveries significantly differed among chemicals and among concentrations. In this experiment, a paired *t*-test was used to compare analyte recoveries within the same spiked level. Our results showed that RDX, MNX, and TNX gave significantly higher recoveries than DNX at concentrations of 250 and 1250 ng/g. At the concentration of 50 ng/g, RDX and MNX gave significantly higher recoveries than DNX. Although recoveries of RDX and DNX were not distinctly different ($p = 0.052$), the *p*-value approached the critical value ($p = 0.05$).

An independent variable *t*-test was used to compare the recoveries of each compound at the different concentrations. Recoveries at concentrations of 250 and 1250 ng/g were significantly higher than recoveries at 50 ng/g for TNX, DNX, and MNX. There were no significant recovery differences for TNX, DNX, and MNX between 250 and 1250 ng/g levels ($p > 0.05$). In the case of RDX, there was no significant difference between 50 and 250 ng/g, but the recovery of 1250 ng/g was significantly higher than 250 ng/g. And, there was no significant recovery difference ($p = 0.084$) between the samples spiked only with RDX ($106.8 \pm 7.9\%$, $n = 12$) and the samples spiked with RDX in mixture ($92.3 \pm 11.8\%$, $n = 11$). Table 3 shows the precision and accuracy of recoveries for RDX, MNX, DNX, and TNX in beef liver.

3.4. Validation test of developed method

We validated the developed method using 175 TNX samples at nine concentrations. The results showed good recoveries that were concentration dependent ($p < 0.0001$) (Table 3). The recovery increased as the TNX concentration increased [Eq. (1)]. The regression line excluded data from 20 ng/g level, since the recoveries are too high (>145%). This set of data was at or below the MDL and were thus excluded from the regression analysis.

$$Y = 0.17 \times X + 0.53 \quad (p = 0.005) \tag{1}$$

where Y is the arcsin (% recovery) and X is the log (TNX, ng/g).

The method showed good intra- and inter-assay precision and accuracy. Two-way ANOVA showed that there was no significant recovery difference between teams ($p = 0.150$) on a given day or within a team on different days ($p = 0.965$) for the nine tested concentrations of TNX (Table 3).

Table 3
Experimental results of precision and accuracy for determination of RDX, MNX, DNX, and TNX in animal liver

Compounds	Recovery (%) (mean ± S.D.)	Precision[a] (% R.S.D.)	Accuracy[b] (%)
RDX	92.3 ± 11.8	12.78	92.3
MNX	88.5 ± 15.1	17.06	88.5
DNX	70.7 ± 17.1	24.19	70.7
TNX	88.1 ± 11.6	13.17	88.1

[a] Precision was measured using % relative standard deviation (% R.S.D.). % R.S.D. = (standard deviation/mean) × 100%.
[b] % Accuracy = % mean concentration measured/ concentration spiked.

3.5. Stability of RDX in liver and extracted matrix

RDX was unstable in the extracted matrix solution at room temperature. After 10 days of extraction, about 50% RDX in sample extracts was degraded. No MNX, DNX, or TNX was detected from 1000 ng/g RDX-spiked sample extracts during the 10-day period after extraction, indicating that RDX was degraded to unidentified products.

4. Discussion

4.1. RDX and its N-nitroso metabolites in the environment

MNX, DNX, and TNX, as anaerobic metabolites of RDX, are well documented in laboratory studies [4,5]. Under anaerobic conditions, some bacteria can sequentially reduce the $N\text{-}NO_2$ groups of RDX to the corresponding N-NO groups and yield MNX, DNX, and TNX. Recent detection of relatively high concentrations of MNX, DNX, TNX, in groundwater surrounding the Iowa Army Ammunition Plant has prompted concerns about the bioavailability and toxicity of these N-nitroso metabolites of RDX to indigenous organisms [9]. Furthermore, the study of the toxicity of these N-nitroso metabolites of RDX may help to better elucidate the potential carcinogenic mechanism of RDX. However, specific analytical methods suitable to detect RDX and its N-nitroso metabolites in animal tissue are limited. Hence, it is important to develop sensitive and reliable analytical methods for determining MNX, DNX, TNX, and RDX in complicated samples, such as animal tissue samples.

4.2. Extraction and cleaning up of MNX, DNX, TNX, and RDX from animal tissue samples

Animal tissues contain significant amounts of proteins, lipids, and other endogenous compounds, most of which interfere with instrumental analysis by contaminating the injector port and GC column, or coeluting with analytes of interests. Initially, we attempted to adopt the extraction and HPLC–UV methods described in previous studies to determine the concentrations of RDX and its nitroso-metabolites in animal tissues. However, the resulting chromatograms contained high backgrounds due to numerous interfering materials present in tissue samples (figure not shown). To improve the chromatogram and to protect the expensive analytical columns and instruments from contamination, we developed a novel three-step extraction and cleanup technique for sample preparation. First, RDX, MNX, DNX, and TNX were extracted from liver samples by accelerated solvent extraction with 100% acetonitrile. High temperature (100 °C)

and high pressure (1500 psi) employed in ASE made the extraction efficient and allowed for high recovery of analytes of interest. Moreover, extracted proteins were precipitated in 100% acetonitrile extracts; thus, these proteins could be easily removed by filtration or centrifugation. Following ASE extraction, we employed two cleanup steps, using florisil and SDB cartridges, to remove a majority of biological macromolecules and pigments that may cause injection port contamination, column fouling, inconsistent retention time, and poor peak shapes. After these three steps, good chromatograms were produced (Fig. 2), and the concentrations of RDX, MNX, DNX, and TNX could be reliably determined using GC–ECD. Initially, C18 cartridges were used instead of SDB cartridges; however, they gave poor recoveries of desired analytes, partially because the analyte is too polar to use C18. C18 cartridges are optimal when analytes of interest have $\log K_{ow}$ ranges of 1–3 [31]. Since the $\log K_{ow}$ of RDX is 0.8, RDX and its N-nitroso metabolites would be more efficiently extracted by more polar SDB sorbent cartridges.

4.3. Instrumental analysis of RDX, MNX, DNX, and TNX from animal tissue samples

HPLC was employed in some analytical methods for determining RDX from water, soil, and plant samples [23,24]. There are a few difficulties associated with HPLC analysis such as relatively poor sensitivity and resolution capacity. When cleanup is inadequate, interfering macromolecules appear as large and broad peaks that would likely obscure the relatively small peaks of trace amounts of analytes in animal tissue. Although analysis using HPLC may be practical after adequate sample cleanup, an alternative GC–ECD method was developed in this study. The detection limit ranged from 17.0 to 34.6 ng/g achieved in this study is superior to the detection limit of 150 ng/g reported in a previous study that used HPLC–UV method [1].

The injection port temperature was important to guarantee both full volatilization of the sample and minimized thermal degradation of the analytes. In our study, 160–170 °C gave the best peak amplitudes for all the analytes (Fig. 4). Higher or lower injection port temperatures than 160–170 °C significantly decreased the peak amplitude of RDX, MNX, DNX, and TNX (Fig. 3) in our system. At higher temperatures, analytes may decompose in the GC injection port, as indicated by a decrease in peak amplitude. Almost no signal was observed when the inlet temperature was at 250 °C. This result was similar to previous studies [29,30].

4.4. Metabolism of RDX and its potential effect on determination of RDX from animal liver samples

In order to rule out the possibility that RDX might undergo sequential reduction during the sample storage, extraction, or instrumental analysis processes, RDX was spiked (spike level: 1000 ng/g), extracted, cleaned, and analyzed using this method. Results of immediate analysis after extraction showed that recovery of RDX was 106.8 ± 7.9% ($n = 12$, spike level: 1000 ng/g) and no MNX, DNX, or TNX was detected. The results indicated that the sample preparation and analysis processes did not cause RDX to degrade to MNX,

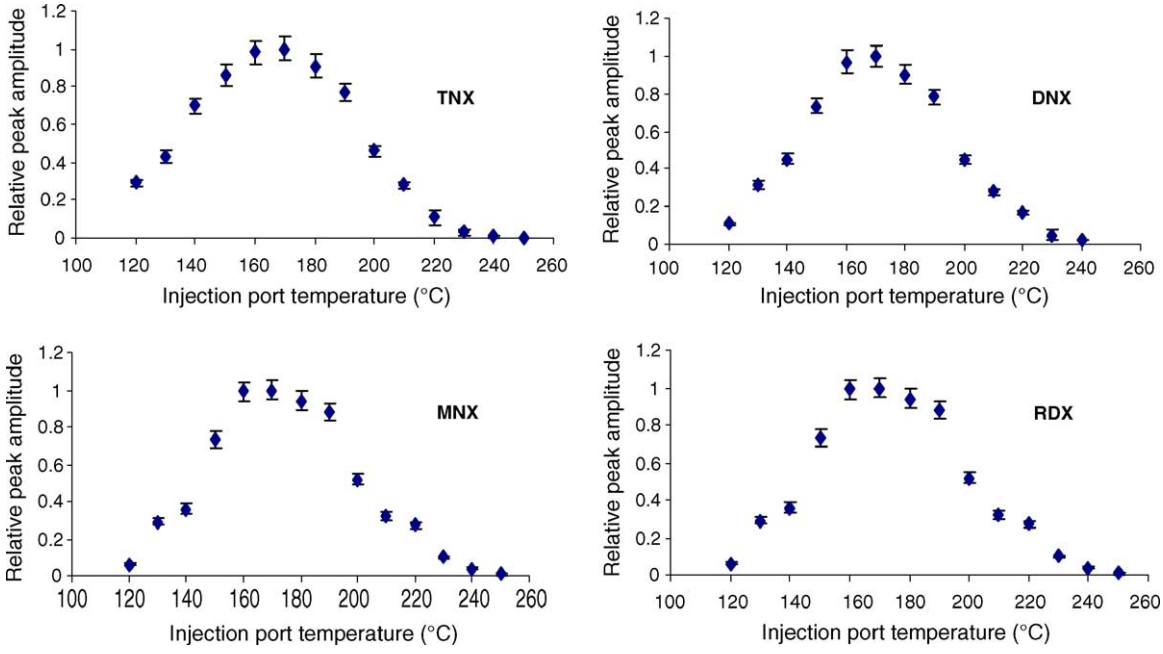

Fig. 4. The peak amplitudes of analytes vs. the injector port temperatures. Y values ($n = 3$ for each temperature point) were calculated as the ratio of signal values at certain temperature to the maximum signal value (obtained at the range of 160–170 °C) of the same compound. Error bars indicated standard deviation for triplicate injection samples.

DNX, or TNX. The stability of RDX in sample extracts matrix was also studied. Sample extracts were analyzed at different times in the following 10 days. The results indicated that RDX was degraded to 50% after 10 days when the sample extracts were stored at room temperature. In contrast, the rate of RDX degradation slow down when sample extracts were stored at 4 °C in refrigerator (data not shown). Again, no MNX, DNX, or TNX were detected in sample extracts 10 days after extraction. The results indicated that RDX was unstable in the extracted matrix solution and may degrade to unknown compounds quickly at room temperature or slowly at low temperature.

Recovery of RDX was $106.8 \pm 7.9\%$ ($n = 12$, spike level: 1000 ng/g) when RDX was the only compound in the liver. It was higher than the recovery of RDX ($92.4 \pm 11.8\%$) spiked at 1250 ng/g along with MNX, DNX, and TNX. Although the difference was insignificant ($p = 0.084$), the p-value approached the critical value ($p = 0.05$). RDX might exhibit a higher tendency to degrade to some unknown compounds when MNX, DNX, and TNX were also present in samples. However, this hypothesis needs further testing.

The significant influences of the analyte concentrations on recoveries may also be due to matrix-catalyzed analyte degradation to unknown compounds during the storage, extraction or instrumental analysis processes. The liver is an important detoxification organ containing many enzymes capable of biotransforming toxicants to other compounds [4]. These degraded products might appear as several unidentified pronounced peaks in chromatogram (Fig. 2). However, the nature of these unidentified peaks is unclear.

5. Conclusion

A method for efficient extraction and cleanup of RDX, MNX, DNX, and TNX in animal tissue, followed by detection using GC–ECD has been established. The method provided good reproducibility and accuracy, and is suitable for studies of explosives uptake by organisms. The method can be used to determine the residues of RDX and its N-nitroso metabolites in animal tissues for forensic interests, exposure evaluation, and ecological risk assessment. This extraction and GC–ECD method may be useful for the analysis of other explosives and their transformation products in animal tissues. This study represents the first reported analysis of MNX, DNX, and TNX in animal tissues.

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