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Validation of a novel extraction method for studying hexahydro-1,3,5-trinitro-1,3,5 triazine (RDX) biodegradation by ruminal microbiota

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ABSTRACT

A simple, fast liquid–liquid extraction method was developed for studying hexahydro-1,3,5-trinitro-1,3,5 triazine (RDX) biodegradation using small sample volumes. The method was tested *in vitro* with anaerobic incubations of RDX with whole rumen fluid (WRF) and a commercial *Sporanaerobacter acetigenes* strain in methanogenic media for RDX. Additionally, validation experiments were conducted in deionized water in order to show applicability toward various aqueous matrices. Conditions for extraction were as follows: 300 μL of sample were mixed with an equal volume of a 0.34 M ammonium hydroxide solution to reach a basic pH, extracted with a hexane/ethyl acetate 1:1 (*v*/*v*) solution (1 mL) and shaken vigorously for 10 s. The resulting organic phase was transferred, then dried under a constant flow of N_2 and reconstituted with acetonitrile (300 μL) for HPLC–UV and LC–MS/MS analysis. Percent recovery values were obtained (83–101%) in all matrices for RDX. In WRF ($n = 3$ animals), RDX degradation was observed with almost 100% elimination of RDX after 4 h. The five nitroso and ring cleavage metabolites were observed by mass spectrometry. Liquid cultures of *S. acetigenes* did not show significant RDX biodegradation activity. RDX extractions from deionized water samples indicated acceptable recoveries with low variability, suggesting suitability of the method for aqueous matrices. Overall, the new method demonstrated acceptable efficiency and reproducibility across three matrices, providing an advantageous alternative for studies where complex matrices and small volume samples are in use.

1. Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), also known as royal demolition explosive, is a widely used compound in military and commercial explosives. Its extensive application has resulted in environmental accumulation in soil and groundwater, making contaminated areas unsuitable for agriculture or other uses [1]. RDX is toxic to a wide variety of organisms, including animals and plants, and is classified as a group C possible human carcinogen and a priority pollutant [2]. Its toxic effects on humans have been well documented as the direct cause of several conditions such as convulsions, loss of consciousness, vomiting, skin lesions and even death [3].

Traditional methods for removing explosive compounds such as RDX from the soil include extensive excavation and subsequent incineration. These methods are effective, but expensive and logistically inefficient. *Ex situ* bioremediation utilizing anaerobic/aerobic degradation in bioreactors fueled by various microbial consortia is another alternative. Although it is effective in forming strongly bound munitions residues and degrading these compounds, it is quite expensive as compared to *in situ* decontamination [4,5]. Therefore, the high costs of traditional remediation for RDX contaminated soil and groundwater has led to a search for more efficient and cost effective alternatives. Several microorganisms have been studied for their ability to degrade RDX. Recently, strains from the genera *Rhodococcus* were shown to degrade RDX under both anaerobic and microarofilic conditions; however, degradation required conditions involving specific oxygen levels and carbon sources [6].

Transformation routes of RDX are known to include two distinctive pathways: (a) a reduction pathway that is initiated with

Abbreviations: RDX, hexahydro-1,3,5-trinitro-1,3,5 triazine; MNX, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine; DNX, hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine; TNX, hexahydro-1,3,5-trinitroso-1,3,5-triazine; MEDINA, methylene dinitramine; NDAB, 4-nitro-2,4-diazabutanal; WRF, whole ruminal fluid.

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the sequential reduction of nitro groups into the corresponding nitroso derivatives 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-5-nitro-1,3,5-triazine (TNX) and (b) a denitrification pathway characterized by the cleavage of N-NO_2 bonds producing nitrite (NO_2^-), nitrous oxide (N_2O), ammonia (NH_3), formic acid (HCOOH) and formaldehyde (HCHO). Two characteristic intermediates are formed during RDX degradation, namely, methylenedinitramine (MEDINA) and 4-nitro-2,4-diazabutanal (NDAB). These intermediates are characteristic of both biotic and abiotic RDX degradation [7–13].

A novel method for RDX biodegradation has been reported using the ovine ruminal microbiota and is termed 'phyto-ruminal-bioremediation'. The entire method involves the planting of cool season grasses to take up explosive compounds from soil and then sheep are employed to consume the explosives-laden grass and remediate the compounds in their rumen using their anaerobic microbes. *In vitro* experiments using the ovine rumen microbiota present in WRF have resulted in the degradation of greater than 90% of 40 $\mu g\,mL^{-1}$ RDX following 4 h of incubation [9,10]. Anaerobic conditions can lead to direct attack on the ring structure or consecutive reduction of nitro groups followed by ring cleavage [7,14].

To analyze RDX degradation, a manual solid-phase extraction method is often used [15,16]. The protocol is tedious and requires several time consuming steps including vacuum syphoning of the samples and conditioning of the extraction cartridges. Additionally, the use of extraction cartridges makes it more expensive. Other extraction methods commonly used include adaptations of method 8330A from the Environmental Protection Agency (EPA), which are based on a salting out solvent extraction using acetonitrile and sodium chloride [15]. This method requires large volumes of sample and organic solvents for low RDX concentration (1–50 $\mu g\,L^{-1}$) samples. Direct dilution of samples with acetonitrile is also an option for some samples; the presence of growing bacteria and organic matter typical of ruminal microcosms and bacterial cultures can require sample filtration prior to analysis [15].

Most methods to monitor RDX degradation have required large sample sizes. In order to study the biodegradation of RDX in small samples, a new, simple and rapid method was developed and examined in WRF and a commercial strain of *Sporanaerobacter acetigenes* (a bacterium believed to be capable of degrading RDX). As the majority of the matrices used in RDX biodegradation are aqueous in nature, deionized water was included as a control to evaluate RDX recovery and the coefficient of variability. This new method uses commonly available organic solvents in a small-scale liquid–liquid extraction and thus provides an inexpensive and rapid alternative for monitoring RDX breakdown in several matrices. The novelty of the proposed method lies in the elimination of extraction cartridges and filtration steps. Results show that the method allows very small volume samples to be processed as well as reduces the production of hazardous waste materials. The new method was evaluated using the parameters of quantification and detection limits.

2. Materials and methods

2.1. Chemicals and reagents

A stock solution of hexahydro-1,3,5-trinitro-1,3,5-triazine (1000 $\mu g\,mL^{-1}$ in acetonitrile) was obtained from Chemservice Inc. (West Chester, PA). Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) (99% purity), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) (55% purity + 17% MNX + 23% TNX), hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) (>99% purity), 4-nitro-2,4-diazabutanal (4-NDAB) (98% purity) and methylenedinitramine (MEDINA) (98% purity) were provided by R.J. Spanggord from SRI International (Menlo Park, CA, USA). Experimental concentrations of RDX were prepared by diluting the stock solution directly into the matrices of interest (WRF, methanogenic media and deionized water). Fresh standard solutions of each analyte were prepared by diluting the analyte with acetonitrile in a concentration range of 40–200 $\mu g\,mL^{-1}$. Solutions were stored at −20 °C for later use over a period of 15 days. An ELGA Ultra PureLab (Cary, NC) reverse osmosis water purification system was used to generate Milli-Q (resistance >18.2 MΩ/cm) quality water for all aqueous solutions.Solvents were HPLC/MS grade and were purchased from VWR (Radnor, PA). Reagents were of analytical grade and were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Matrices

A volume of 100 mL of WRF was collected from three one-year-old rams (*Ovis aries*) at a slaughter. Collections were made using a sterile cannula and placed in plastic conical tubes and then placed in a thermos containing warm water for transport to the laboratory. Samples were kept at 39 °C in an anaerobic glove box (Coy Laboratory Products Inc., Grass Lake, MI) for 1 h to acclimatize prior to incubation with RDX. Methanogenic media was prepared according to Rychlik and May, 2000 [18]. Deionized water was obtained from an ELGA Ultra PureLab system (Cary, NC) by reverse osmosis.

2.3. RDX biodegradation experiments

Under anaerobic conditions [19] WRF from each of the three animals was supplemented with RDX at 40 $\mu g\,mL^{-1}$. For the experimental procedures, 20 mL of the solution was placed into glass tubes and sealed with butyl rubber stoppers. The solution was incubated at 39 °C on a tray shaker and samples were removed at several time points (10 min, 1 h, 2 h, 2.5 h, 3 h and 4 h) by sterile puncture of the butyl rubber stoppers. The samples were immediately extracted with the liquid–liquid extraction method described below.

Experiments with *S. acetigenes* were performed in methanogenic medium [18]. Three sealed Balch tubes of 10 mL of methanogenic medium in a CO_2:H_2 (90:10) gas mixture were each inoculated with 1 mL of *S. acetigenes* culture (drawn from a 24 h culture in exponential-phase growth). RDX was added to a final concentration of 40 $\mu g\,mL$ to these tubes, as well as to a set of three control tubes. Incubation was performed at 39 °C with shaking for over 96 h. Samples from each tube were drawn at 12 h, 24 h and every 6 h thereafter. The samples were then immediately extracted. Optic density (OD) readings were tracked every 12 h.

2.4. Abiotic degradation control

A deionized water control was used in order to test for abiotic RDX degradation. This control consisted of 4 mL of deionized water at pH 10 containing RDX (40 $\mu g\,mL^{-1}$) and incubated under the same conditions established for the biodegradation assays. Samples (300 μL) were taken at the same time points used for WRF biodegradation experiments (10 min, 1 h, 2 h, 2.5 h, 3 h and 4 h) and extracted using the extraction method described for further analysis by HPLC.

2.5. Sample preparation and extraction

WRF (2 mL) was centrifuged at 10,000 rpm for 5 min. The resulting supernatant (300 μL) was distributed into glass vials and mixed with 300 μL of a 0.34 M ammonium hydroxide solution. Basified samples were transferred to 2 mL screw cap micro-centrifuge tubes, mixed with 1 mL of hexane:ethyl acetate (1:1) solution, and shaken vigorously for 10 s. An 800 μL volume of the organic phase

was transferred to a separate tube; for each sample, this procedure was repeated three times and the organic phase was pooled to a final volume of 2.4 mL of organic phase in a hexane:ethyl acetate (1:1). The pooled extracted organic phase from the samples was dried using a constant N_2 flow and stored at −20 °C until HPLC–UV and LC–MS/MS analysis. Samples from methanogenic media and deionized water were treated identically, as described for WRF.

2.6. Determination of percent recovery

Extraction efficiency was determined by measuring the percentage of recovery from the three different aqueous matrices at 40 μg mL^{-1} (low) and 200 μg mL^{-1} (high) RDX. Comparison of mean recoveries between experimental matrices was determined using the Student's *t*-test after first assessing the equality of variances using Fisher's exact test. All statistical analyses were performed using R software (version 2.15.1). Data are expressed as mean values (M) ± standard deviations (SD) (n = 10, unless otherwise stated).

2.7. HPLC–UV/vis analysis

HPLC–UV/vis analyses were carried out using the method described by Eaton et al., 2011 [17], with minor modifications. In brief, separations were performed using an Ultra carb ODS column (250 mm × 4.6 mm; 5 μm particle size (Phenomenex, Inc., Torrance, CA)). RDX eluted at 10 min under isocratic conditions with water and methanol (55:45, v/v) at 28 °C and a flow rate of 0.8 mL min^{-1}, with a total run time of 30 min. The HPLC system consisted of a Perkin-Elmer (Waltham, MA), Series 200 pump equipped with a Perkin-Elmer Series 200 autosampler and Perkin-Elmer Series 200 UV/VIS detector monitoring at 250 nm. TotalChrom software was used to quantify HPLC data.

2.8. LC–MS/MS analysis

LC–MS/MS analyses were performed according to Eaton et al., 2012 [19]. Briefly, an ABI/SCIEX 3200 QTRAP LC–MS/MS system (Applied Biosystems, Foster City, CA) using atmospheric pressure chemical ionization in the negative ion mode and multiple reaction monitoring (MRM) was used to detect RDX, MNX, DNX, TNX, MEDINA and 4-NDAB, under a flow rate of 0.65 mL min^{-1} for 35 min using a mobile phase consisting of 0.6 mM ammonium acetate in water (A) and methanol (B). Quantitation of RDX was performed by establishing a calibration curve in Analyst 1.4.2 (Applied Biosystems) using a linear regression from WRF, methanogenic media or deionized water spiked with 40–200 μg mL^{-1} RDX.

3. Results and discussion

3.1. Organic solvents performance

In order to select the organic solvent or the organic solvent mixture to be used for the liquid–liquid extraction, several extractions were selected and their coefficient of recovery was evaluated (Table 1). A mixture of hexane:ethyl acetate (1:1) showed the best recovery for methanogenic media and WRF, 98 and 95%, respectively. This solvent mixture was, therefore, selected for use in the new method and subsequent validation experiments.

3.2. Sample pH

The pH of a sample plays a distinctive role, not only in good extraction of a desired compound but also in better preservation of distinctive intermediates. Other studies have shown that the commonly studied RDX ring cleavage product, MEDINA is more stable in alkaline pH conditions with only a 5% loss after 4 days at pH 12 [20]. It significantly decomposes at pH values between 3 and 8 but shows most stability at pH 1 and 10 [12,14]. Another RDX ring cleavage product formed is 4-NDAB, which is also degraded under extreme alkaline (pH 12) or acidic (pH 3) conditions [20,21].

Table 1
Efficiency of liquid–liquid RDX extraction with the proposed method in three aqueous matrices using different organic solvents, measured by HPLC–UV.

Solvent	Methanogenic media % Recovery[a]	Whole ruminal fluid (WRF) % Recovery[a]
Ethyl acetate	90	54
Hexanes:EtAc, 9:1	36	29
Hexanes:EtAc, 1:1	98	95
Chloroform	87	80

[a] Results expressed as average of three replicates.

In a previous work on RDX biodegradation by WRF samples were diluted (1:1, v/v) using basified water (40 μL of concentrated ammonium hydroxide in 1 mL of deionized water) and generated a good recovery for RDX [19]. The natural physiologic pH of the rumen is slightly acidic, with a pH between 5 and 6 [22,23]. The pH values of evaluated matrices in this work, before extraction, were 6.1, 6.8 and 5.8 for WRF, methanogenic media and deionized water, respectively. The detection of 4-NDAB in WRF samples following a 2 h incubation supports its formation from bacterial-mediated degradation [19]. The findings from this work suggest that a moderate alkaline condition (pH 10.0–10.6) provides good recovery and preservation of labile intermediates such as MEDINA and 4-NDAB [20]. The detection of 4-NDAB in WRF samples following a 2 h incubation in the present study supports its formation from bacterial-mediated degradation [19].

3.3. Method applicability in aqueous matrices of interest

Aqueous matrices (WRF, methanogenic media and deionized water) are commonly used for biodegradation studies. In this study, WRF, methanogenic culture media and deionized water were spiked with two concentrations of RDX. RDX at concentration of 40 μg mL^{-1} was selected as the lower limit because it has proven to be below toxic levels to bacterial systems, RDX has also been used at a concentration of 200 μg mL^{-1} as a nitrogen source [9], this concentration was used as the high level concentration. Both concentrations were used to estimate RDX recovery and potential interferences from the matrices. As shown in Table 2, after using the extraction method proposed in the present work, average recoveries calculated from HPLC–UV data were similar in all matrices with values from 92.0 to 96.3%, 81.0 to 92.0% and 100.0 to 101.0%, for whole rumen fluid, methanogenic media and deionized water, respectively.

For all the studied matrices, LOD's and LOQ's were calculated from the analysis of non-spiked samples (n = 7). Results are summarized in Table 3. The LOQ for WRF was 4.3 μg mL^{-1}, which is acceptable considering the complexity of the matrix and the low sample volumes (300–500 μL) normally used in these experiments. LOQ values of 1.9 and 4.6 μg mL^{-1} were obtained for methanogenic media and deionized water, respectively. These results suggest that the present method is accurate enough for biodegradation studies where sample volume is a limitation.

Incubations of a commercial strain of *S. acetigenes* in methanogenic media culture did not show evidence of RDX biodegradation; however, the extraction method showed acceptable recovery percentages and variability in this complex culture media used for anaerobic bacteria cultures. Deionized water extractions showed the highest percentage of recovery (100–101%) with very low variability. These results suggest that the new method developed is efficient for simple aqueous matrices and a good

Table 2
Extraction efficiency of RDX, using the proposed method, in three aqueous matrices, measured by HPLC–UV.

Concentration (μg/ml)	Water		WRF		Methanogenic Media	
	% Recovery[a]	%CV	% Recovery	%CV	% Recovery	%CV
40	100 ± 0.8	6.9	96.3 ± 1.1	7.7	81 ± 0.6	3.7
200	101 ± 1.7	2.7	92 ± 16.2	29.1	92 ± 5.8	3.7

[a] Results expressed as average ± the standard deviation of $n = 5$ replicates.

alternative for small volume sampling. Further it allows for more replicates per sample and, therefore more statistical robustness.

Different RDX extraction methods are used for ground water, soil and microbial samples analysis. The 8330A method (EPA), is commonly used for RDX extraction, several works have used modified versions of this method to process their samples [24,6]. This method is based in a salting out procedure for low and high concentrations of RDX in aqueous samples and soil samples. However, in the case of aqueous samples it requires a large volume of sample (770 mL) and approximately 175 mL of organic solvent (acetonitrile), this is not only inconvenient with limited amount of sample but it also generates a large amount of hazardous waste. Additionally, it requires certain expertise from the operator to identify when additional filtration steps are required.

Another method cited in previous work is solid phase extraction (SPE) method using Waters Oasis HLB (3 mL/60 mg, 30 μm) cartridges (Milford, MA) following the manufactures instructions [12]. However, this method includes the use of a manifold system, which requires the conditioning of extraction cartridges with methanol (3 mL), deionized water (3 mL) and an additional washing step with methanol/water (5%, v/v) before eluting the sample with methanol. This method also involves a long centrifugation step (10 min) in order to prevent obstruction in the extraction cartridges. Direct dilution of samples before filtration with acetonitrile dilution (1:1) has been used for RDX extraction but it also requires the additional step of sample filtration before dilution.

Based in the experimental design and the specific needs of the current research, it was decided to develop a method that (1) did not require any filtration or the use of extraction cartridges; (2) generated a good recovery for samples volumes below 1 mL (300 μL) and (3) did not generate large amounts of hazardous waste.

The proposed method responds to the above criteria and provides several advantages. First, it eliminates the use of extraction cartridges, which is a very cost effective option for RDX degradation experiments. Further, no filtration is necessary, which increases the effectiveness of the method by reducing the time required to process each sample. Finally, the organic solvents used in the new method have a fast evaporation rate, which reduces the time required for samples to dry before reconstitution.

3.4. HPLC–UV analysis of samples

Calibration curves were established using the square linear regression of peak areas versus amount of analyte injected. Standard solutions of RDX ranged from 40 to 200 μg mL^{-1}. Resulting R^2 values were higher than 0.98, indicating acceptable linearity of the response. Quality parameters such as limits of detection (LOD) and quantification (LOQ) were evaluated for the HPLC–UV system (Table 3). Intra-day reproducibility was 1.5–5.5% for lower concentrations of RDX (40 μg mL^{-1}).

For higher concentrations (200 μg mL^{-1}), intra-day reproducibility values ranged from 16.1 to 39.4% CV, suggesting that in water, higher RDX concentrations lower the reproducibility of the method. However, incubation of ruminal microcosms and other bacterial species with RDX using similar concentrations is toxic to many bacterial species, resulting in inactivation of degradation activity as reported by Eaton et al. [12]. Inter-day variation for the low RDX concentration used (40 μg mL^{-1}) were 2.1, 2.7, and 2.0% for WRF, methanogenic media and deionized water, respectively; for the high RDX concentration (200 μg mL^{-1}), values were 16.1, 0.5 and 0.8%, respectively.

Table 3
Limit of quantification (LOQ) and limit of detection (LOD) of RDX extraction in three aqueous matrices by HPLC–UV.

	Water	WRF	Methanogenic media
LOQ, μg mL^{-1}	4.6	4.3	1.9
LOD, μg mL^{-1}	1.4	2.7	0.6

3.5. Method applicability to RDX degradation by ovine ruminal microbiota

The incubation of RDX with ovine whole rumen fluid (WRF) from three different animals resulted in the biodegradation of RDX in all of the incubations, as shown in Fig. 1. Degradation rates in samples of 40 μg mL^{-1} were different among the animals when analyzed by HPLC–UV; they were 100%, 86% and 75% for animals 1, 2 and 3, respectively. When similar experiments were carried out with WRF inactivated by autoclaving, no degradation was observed, suggesting that the degradation activity is inherent in the ovine ruminal microbiota. The different efficiency in degrading RDX by the

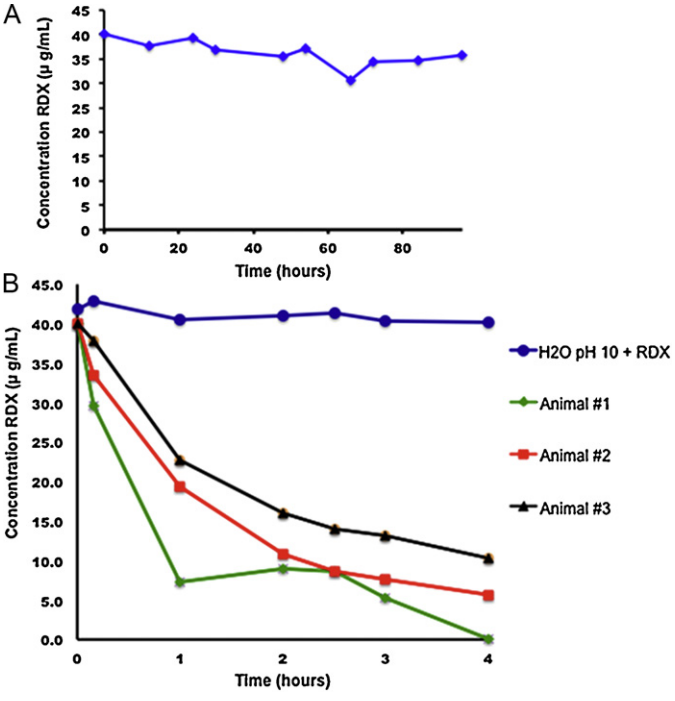

Fig. 1. Degradation of RDX under anaerobic conditions by *S. acetigenes* in methanogenic culture media with 3 replicates per time point (A) and by WRF (B) using an initial RDX concentration of 40 μg mL^{-1}. RDX was quantitated using LC–MS/MS MRM analysis.

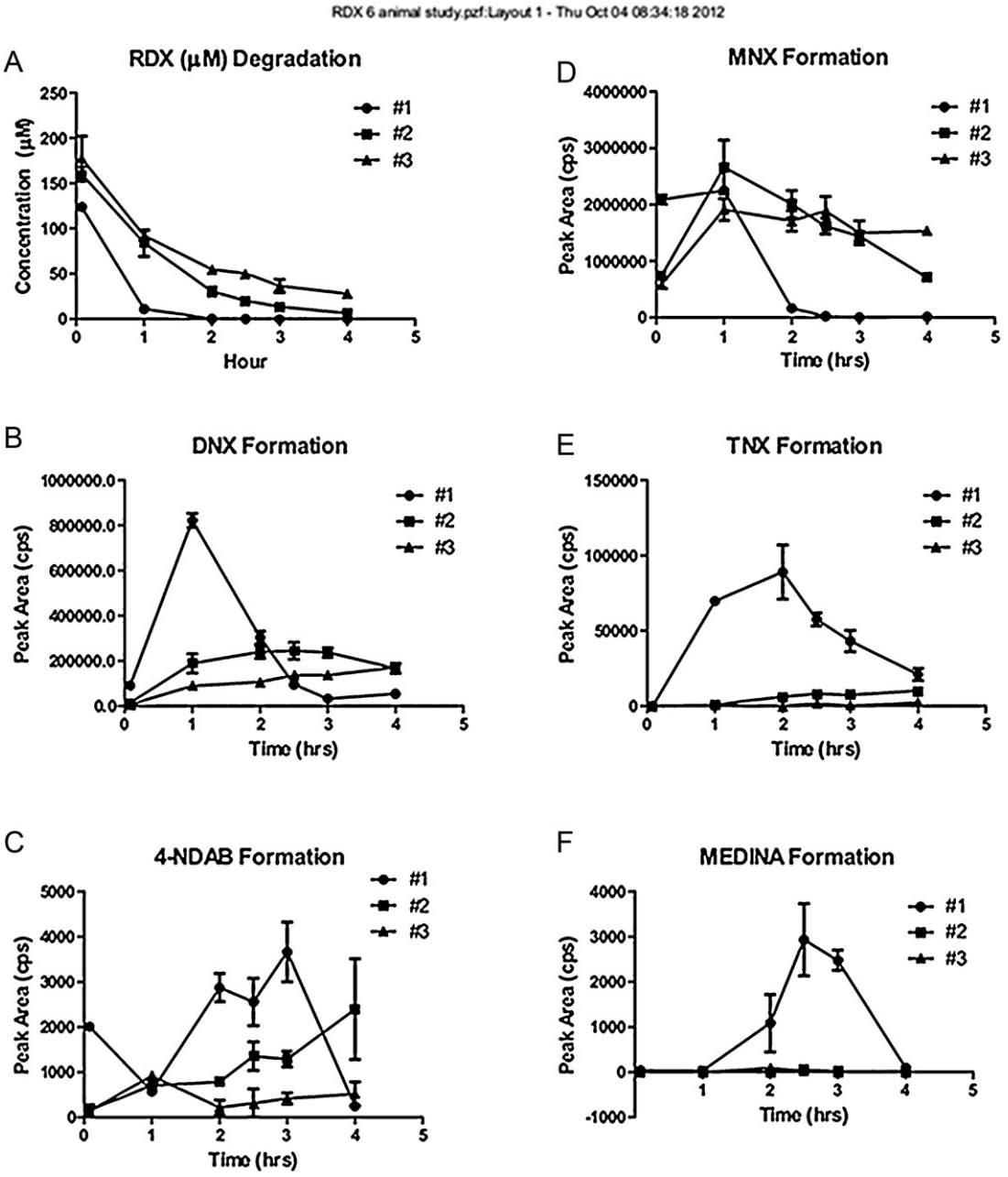

Fig. 2. Measurement of RDX, extracted with our proposed method, in order to measure biodegradation and production of nitroso metabolites in ovine WRF, using 2 replicates per time point, by LC–MS/MS.

different animals can be attributed to the uniqueness of the microbiota in each individual.

RDX degradation was not detected in abiotic controls incubated under the same conditions, indicating that degradation was achieved by ruminal microbes and that pH and culture conditions did not result in RDX break down (Fig. 1). Within 10 min, the reduction products MNX, DNX and TNX were visible (Fig. 2). Also, 4-NDAB was detected at 10 min in all samples.

Ring cleavage products MEDINA and 4-NDAB were detected for the first time in WRF. MEDINA was consistently present in animal #1 (Fig. 2F) but, by the 4 h time point, was not detected in any of the samples, suggesting that MEDINA is most likely an intermediate in the breakdown of RDX in ovine rumen fluid as previously postulated in Eaton et al. [9]. Intermediate formation seems to be very heterogeneous among the studied animals (Fig. 2), suggesting that each animal possess a characteristic and unique microbiota, which generates different degradation rates. The fact that the present method was able to detect and quantify RDX and almost all of its documented metabolites (transformation products) allows us to safely conclude that the method is competitive with other reported method(s) and thus can be used to monitor fate of RDX and its products in the environment.

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50-1265-6-076. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the US Department of Agriculture.

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