

Report No. DRXTH-TE-CR-80086

LEVEL II

MIDWEST RESEARCH INSTITUTE

ADA107346

REPORT

IDENTIFICATION OR DEVELOPMENT OF CHEMICAL ANALYSIS
METHODS FOR PLANTS AND ANIMAL TISSUES

DTIC
ELECTE
NOV 1 0 1981

H

FINAL REPORT
January 1981

Contract No. DAAK11-79-C-0110
MRI Project No. 4849-A

DISTRIBUTION STATEMENT A
Approved for public release;
Distribution Unlimited

For

U.S. Army Toxic and Hazardous Materials Agency
DRXTH-TE-A
Aberdeen Proving Ground (EA), Maryland 21010

MIDWEST RESEARCH INSTITUTE 425 VOLKER BOULEVARD, KANSAS CITY, MISSOURI 64110 • 816 753-7600

81 11 10 051

DTIC FILE COPY

The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

MRI - NORTH STAR LABORATORIES 10701 Red Circle Drive, Minnetonka, Minnesota 55343 • 612 933-7880

MRI WASHINGTON, D.C. 20006 - Suite 250, 1750 K Street, N.W. • 202 293-3800

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

19 REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER DRXTH-TE/CR-80086	2. GOVT ACCESSION NO. AD-A106346	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Identification or Development of Chemical Analysis Methods for Plants and Animal Tissues.		5. TYPE OF REPORT & REMARKS COVERED Final Report. 19 Aug 79-24 Aug. 19, 1979 - Oct. 1980
7. AUTHOR(s) D. B. Lakings & O. Gan	6. PERFORMING ORG. REPORT NUMBER MRI Project No. 4849-A	
9. PERFORMING ORGANIZATION NAME AND ADDRESS Midwest Research Institute 425 Volker Boulevard Kansas City, Missouri 64110		8. CONTRACT OR GRANT NUMBER(s) DAAK11-79-C-0110
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Armament Research and Development Command, Aberdeen Proving Ground (Edgewood Area) Maryland 21010		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 12346
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office) U.S. Army Toxic and Hazardous Materials Agency Aberdeen Proving Ground (Edgewood Area) Maryland 21010		12. REPORT DATE January 1981
		13. NUMBER OF PAGES 311
		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Unlimited		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report) DTIC NOV 13 1981 H		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Methods development Trinitrotoluene (TNT) Biological Matrices Pentaerythrite tetranitrate (PETN) Cyclotrimethylenetrinitramine (RDX) High performance liquid chromatography Dinitrotoluene (DNT) (HPLC)		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) HPLC-UV analytical methods have been defined for the quantitative determination of RDX, DNT, and TNT in animal plasma, kidney, muscle/fat, and liver; DNT and TNT in plants stems; and PETN in animal plasma. The HPLC separation and detection of RDX, DNT, and TNT was accomplished on a Spherisorb ODS, 5 µ, 250 x 4.6 mm ID column using 28-30% acetonitrile in water containing 1% acetic acid eluent operated at a flow rate of 1.5 ml/min with a 254 nm detector. Tetryl was also separated and		

DD FORM 1 JAN 73 1473

EDITION OF 1 NOV 68 IS OBSOLETE

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

230350

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

19. Key Words (concluded)

Ultraviolet detection, 254 nm or 215 nm

Animal tissues - plasma, kidney, muscle/fat, liver

Plants - leaves, stems

20. Abstract (concluded)

quantified with this analytical system. PETN required a 215-nm detector for detection and was assayed by HPLC using a Spherisorb ODS column and an eluent of 40% acetonitrile in high-purity water. Each of the analytical systems was evaluated for linearity, precision, and sensitivity using SARM reference solutions of the five munitions. Linearity was obtained over a concentration range of 100 to 2,000 ng/ml for each munition, and the sensitivities of the techniques were 5 ng munition injected on column.

The analytical techniques were employed to evaluate sample preparation techniques for the isolation of the munitions from four animal tissues and two plant matrices. A defined sample preparation technique was validated by preparing and analyzing duplicate samples of the matrix spiked at five different levels with the munitions and matrix blanks on four separate days. The sensitivity of the developed method was to be 100 ng/g for each munition. The sample preparation procedures defined for RDX, DNT, and TNT in animal plasma, kidney, and liver samples were similar and consisted of extracting a weighed sample diluted with a 10% sodium chloride solution containing 1% acetic acid three times with toluene. The toluene extracts were dried, the residue dissolved in 1.0 ml HPLC eluent containing an internal standard, the sample filtered through a 0.45- μ Fluoropore filter, and an aliquot of the prepared sample injected onto the HPLC system. For the muscle/fat and plant stem matrices, a different extraction solvent was necessary to eliminate matrix component interferences in the HPLC determination of RDX, DNT, and TNT. Acetonitrile was employed for extraction of the muscle/fat matrix and 2% isopropanol in hexane was utilized to extract the munition from the plant stems. After extraction, a protocol similar to the one described above was followed to prepare the samples for HPLC injection. During these studies, tetryl was included in all samples; however, no HPLC peaks for tetryl were observed in any of the matrices studied using a variety of sample preparation techniques. Studies on tetryl indicated absorption of the munition to the protein or other macromolecules present in the matrices. Statistical evaluation of the data by the Hubaux and Vos detection limit program gave the following detection limits for RDX, DNT, and TNT in the various matrices: plasma RDX, 146 ng/ml, DNT, 246 ng/ml, TNT, 248 ng/ml; kidney - RDX, 95 ng/g, DNT, 179 ng/g, TNT, 211 ng/g; muscle/fat - RDX, 62 ng/g, DNT, 66 ng/g, TNT, 66 ng/g; liver - RDX, 58 ng/g, DNT, 50 ng/g, TNT, 50 ng/g; and plant stems, DNT, 65 ng/g, TNT, 90 ng/g. The 58 ng/g RDX detection limit in the liver matrix is questionable since the blank liver samples contained a component which co-eluted with RDX and represented 113 ± 18 ng/g RDX. A more realistic RDX detection limit in the liver matrix is 150 ng/g (blank liver value plus two standard deviations). No method was defined for RDX, DNT, TNT, and tetryl in the plant leaves matrix. The procedure defined for the extraction of PETN from animal plasma utilized hexane as the extracting solvent. The detection limit determined for PETN in plasma was 50 ng/ml. The other animal and two plant matrices could not be assayed for PETN using simple extraction techniques because many 215 nm adsorbing matrix components were extracted with each solvent evaluated, and the HPLC analytical system was not able to isolate the PETN from these interferences.

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

PREFACE

This report was prepared at Midwest Research Institute, 425 Volker Boulevard, Kansas City, Missouri 64110, under U.S. Department of the Army Contract No. DAAK11-79-C0110, MRI Project No. 4849-A, entitled "Identification or Development of Chemical Analysis Methods for Plants and Animal Tissues." The research effort was supported by the U.S. Army Toxic and Hazardous Materials Agency. Dr. Leslie Eng, DRXTH-TE-A, was the Project Officer for this program.

This work was conducted in the Analytical Chemistry Department, Dr. James L. Spigarelli, Director. The research effort was directed by Dr. Duane B. Lakings, Program Manager and Senior Bioanalytical Chemist, with assistance from Mr. Owen Gan, Assistant Chemist, and Ms. Mary Valla, Senior Technician. This report was prepared by Dr. Lakings and Mr. Gan.

MIDWEST RESEARCH INSTITUTE

Owen Gan

Owen Gan
Assistant Chemist

Duane B. Lakings

Duane B. Lakings
Program Manager and Senior
Bioanalytical Chemist

Approved:

James L. Spigarelli

James L. Spigarelli, Director
Analytical Chemistry Department

Accession For	
NTIS GRA&I	☒
DTIC TAB	☐
Unannounced	☐
Justification	
By	
Distribution/	
Availability Codes	
or	
or	
A	

TABLE OF CONTENTS

	<u>Page</u>
Summary	1
Conclusions	3
Recommendations	4
I. Introduction.	5
II. Equipment and Materials	5
A. Equipment	5
B. Laboratory Glassware and Equipment.	6
C. Chemicals	6
D. Animal Tissues and Plants	6
E. Reference Stock Solutions	7
F. Calculation of Data	7
III. Analytical (Instrumental) Techniques.	8
A. RDX, DNT, TNT, and Tetryl HPLC Parameters	10
B. PETN HPLC Parameters.	15
IV. Sample Preparation Procedures	17
A. Animal Plasma	17
B. Animal Kidney Samples	29
C. Animal Muscle/Fat Samples	32
D. Animal Liver Samples.	33
E. Plant Leaves.	39
F. Plant Steams.	46
Appendices	
A Raw Data and Calculations for Final Report.	47
B Method Report No. 1	59
C Method Report No. 2	101
D Method Report No. 3	145
E Method Report No. 4	189
F Method Report No. 5	233
G Method Report No. 6	263

RESEARCH AND ANALYSIS DIVISION

LIST OF FIGURES

<u>Number</u>	<u>Title</u>	<u>Page</u>
1	HPLC Separation for TNT, DNT, RDX and Internal Standard on the Spherisorb ODS Column.	13
2	HPLC Determination of RDX, TNT, DNT, and Tetryl SARM Reference Solution Using HPLC System II	14
3	HPLC System I Analysis of Blank Plasma Sample for Method Development Using Acetonitrile Precipitation Technique. .	24
4	HPLC System II Determination of RDX, DNT, and TNT in Plasma Sample Prepared by Acetonitrile Precipitation Technique	25
5	HPLC-UV (215 nm) Method Development for PETN Determination in Kidney Samples	31
6	HPLC Method Development for RDX, DNT, and TNT Determination in Animal Liver	35
7	HPLC Chromatograms of RDX, DNT, and TNT Extracted from Liver with Various Solvents	37
8	HPLC Chromatograms of RDX, DNT, and TNT Extracted from Liver with Various Solvents	38
9	HPLC Chromatograms for PETN Method Development in Liver . .	40
10	HPLC Chromatogram of Blank Leaf Sample (5.0 g) Extracted with Toluene.	42
11	HPLC Chromatogram of 5.0 g Leaves Containing 200 ng/g Each Munition Extracted with Isopropanol in Hexane.	43
12	HPLC Chromatogram of Plant Leaves Containing Munitions and Extracted with Toluene Plus 2% IPA.	44
13	HPLC-UV (215 nm) System IV Analysis of 2% IPA in Hexane Extracted Leaves for Method Development for PETN Determination	45

LIST OF TABLES

<u>Number</u>	<u>Title</u>	<u>Page</u>
Summary Table	Linear Regression Equations and Detection Limits for the HPLC Determination of RDX, DNT, TNT, and PETN in Various Biological Matrices	2
1	HPLC Analytical System Investigated for Munition Compound Determination	9
2	HPLC Linearity of RDX, DNT, and TNT Interim SARM Reference Solution, HPLC System I	11
3	HPLC Precision and Linearity of RDX, DNT, TNT, and Tetryl SARM Reference Solutions, HPLC System II.	16
4	Summary of Sample Preparation Procedure Evaluations for Munitions in Biological Matrices.	18
5	Evaluation of Acetonitrile Precipitation Procedure for the Determination of DNT and TNT in Plasma.	23
6	Tetryl Recovery from Plasma Using Two Sample Preparation Techniques.	28
7	HPLC-UV Determination of RDX, DNT, and TNT in Liver Samples Initial Evaluation of Toluene Extraction Method	34

SUMMARY

The primary objective of this research program for the U.S. Army Toxic and Hazardous Materials Agency was to define simple, quantitative, sensitive analytical methods for the determination of cyclotrimethylenetri-nitramine (RDX), dinitrotoluene (DNT), trinitrotoluene (TNT), 2,4,6-trini-trophenylmethylnitramine (tetryl) and pentaerythrite tetranitrate (PETN) in four animal tissues (plasma, kidney, muscle/fat, and liver) and two plant matrices (leaves and stems). High performance liquid chromatography (HPLC) was selected as the analytical technique for this research project because it had the necessary sensitivity, linearity, precision, accuracy, and separation characteristics for the detection and quantification of the munitions at low levels, i.e., 100 ng/g. An HPLC analytical system was defined which separated RDX, DNT, TNT, and tetryl in a single chromatographic analysis and with an ultraviolet (UV) detector at 254 nm, detected and quantitated a minimum of 5 ng of each munition injected on column. This analytical system consisted of a Spherisorb ODS, 5 μ , 250 x 4.6 mm ID column; a 28-30% aceto-nitrile in 1% acetic acid in water eluent; and a flow rate of 1.5 ml/min. The analytical technique was employed to evaluate sample preparation pro-cedures to isolate the munitions from the various matrices. Simple liquid-liquid or liquid-solid extraction techniques were defined for the determi-nation of RDX, DNT, and TNT in each of the animal tissues and for DNT and TNT in the plant stem matrix. Tetryl could not be analyzed in any of the matrices because it apparently adsorbed to the protein or other macromole-cules present in the matrix. Matrix components which interfered with HPLC elution positions of RDX and DNT prevented the definition of a simple extrac-tion technique for these munitions in the plant leaves matrix. Each of the developed sample preparation techniques gave a linear response to the muni-tion during validation of the method. The methods were validated by prepar-ing and analyzing duplicate samples of each matrix spiked at five different levels and matrix blanks on four separate days. The summary table presents the linear regression equations and correlation coefficients for each muni-tion for the various matrices and gives the detection limit for the munition as determined by the Hubaux and Vos detection limit program.

PETN required a 215-nm UV detector for quantification and a slightly different HPLC eluent and could not be analyzed with the other munitions. The analytical system defined for PETN utilized the same HPLC column and 40% acetonitrile in high-purity water (the use of an acid modi-fied with this system was not possible since the organic acid has absor-bance at 215 nm). The system had the necessary sensitivity and linearity to provide quantitative data at the 100 ng/g level. Studies were conducted to define sample preparation procedures for PETN in the biological matrices. A simple liquid-liquid extraction technique was developed and validated for PETN in plasma. However, simple extraction of the other matrices was not sufficient for PETN determination as a number of components with 215 nm ad-sorption were also extracted and interfered with the HPLC determination of PETN. The summary table gives the linear regression, correlation coefficient, and detection limit for PETN in plasma.

SUMMARY TABLE

LINEAR REGRESSION EQUATIONS AND DETECTION LIMITS FOR THE HPLC DETERMINATION OF RDX, DNT, TNT, AND PETN IN VARIOUS BIOLOGICAL MATRICES

	Linear Regression ^a				Detection Limits ^b (ng/ml or ng/ml)			
	RDX	DNT	TNT	PETN	RDX	DNT	TNT	PETN
Plasma	$y = 0.825x + 14$ 0.990	$y = 0.659x + 6$ 0.982	$y = 0.785x + 16$ 0.988	$y = 0.594x - 0.8$ 0.986	146 ^d	256 ^d	248 ^d	50 ^e
Kidney	$y = 0.973x + 7.1$ 0.999	$y = 0.707x - 3.5$ 0.991	$y = 0.746x - 5.4$ 0.992		95 ^d	179 ^d	211 ^d	
Muscle/Fat	$y = 0.965x + 6.5$ 0.994	$y = 0.781x + 3.7$ 0.990	$y = 0.850x + 1.8$ 0.999		62 ^e	66 ^e	62 ^e	
Liver	$y = 0.904x + 107$ 0.998	$y = 0.640x - 1.9$ 0.995	$y = 0.521x - 6.2$ 0.989		58 ^{e,f}	50 ^e	50 ^e	
Plant Stems		$y = 0.514x + 7.0$ 0.923	$y = 0.449x + 9.8$ 0.957		65 ^e		90 ^e	

- ^a Linear regression - equation determined from the duplicate analysis of matrix samples spiked at five different levels plus matrix blanks on four separate days; 48 data points utilized for each equation.
- ^b Detection limit - statistical evaluation of the data by the Hubaux and Vos detection limit program.
- ^c Correlation coefficients obtained for each linear regression evaluation.
- ^d Detection limits determined from spiking level series of 0, 100, 500, 1,000, 1,500, and 2,000 ng/g using the Hubaux and Vos detection limit program.
- ^e Detection limits determined from spiking level series of 0, 50, 100, 200, 500, and 1,000 ng/g using the Hubaux and Vos detection limit program.
- ^f A liver component co-eluted with RDX and represented 113 ± 18 ng/g RDX in the blank samples. Thus, a 150-ng/g detection limit for RDX in the liver matrix is realistic.

CONCLUSIONS

Quantitative HPLC analytical methods have been defined for the determination of RDX, DNT, and TNT in animal plasma, kidney, muscle/fat, and liver samples; DNT and TNT in plant stems; and PETN in plasma. A simple liquid-liquid or liquid-solid technique was employed to isolate the munitions from the biological matrix followed by reverse phase HPLC to separate the munitions from each other and matrix components; detection and quantification were by UV at 254 nm for RDX, DNT, and TNT and 215 nm for PETN. Each of the developed methods has the necessary linearity, precision, accuracy, and sensitivity to quantitate low levels, i.e., approximately 100 ng/g, of the munitions.

Methods were not defined for tetryl in any matrix due to apparent adsorption of the munition by macromolecules in the matrix. HPLC interferences from the matrix prevented method definition for RDX in plant stems; RDX, DNT, and TNT in plant leaves, and PETN in animal kidney, muscle/fat, and liver and plant leaves and stems. Additional studies are necessary using more elaborate sample preparation procedures such as adsorption or partition column chromatography to define those methods.

RECOMMENDATIONS

Additional studies using more elaborate sample cleanup procedures should be conducted for the isolation of RDX, DNT, and TNT in the plant matrix and PETN in all matrices except plasma. The cleanup procedures recommended for future study include double liquid-liquid extraction using solvents with different polarity, adsorption column chromatography, and/or partition column chromatography. These techniques may provide sufficient cleanup of the biological samples so they can be analyzed by the defined HPLC analytical techniques.

The results and statistical evaluation of the data for the HPLC determination of RDX, DNT, and TNT in plasma and kidney matrices were obtained using a spiking series of 0, 100, 500, 1,000, 1,500, and 2,000 ng/g. For the other matrices, the spiking series was 0, 50, 100, 200, 500, and 1,000 ng/g. The plasma and kidney matrices should be reanalyzed using the second series so that the statistical evaluation of the data by the Hubaux and Vos detection limit program will provide detection limits closer to the values observed during the assay of these samples. The Hubaux and Vos detection limit program uses the standard deviation at the various levels to determine the confidence of analysis at that level. As would be expected, the standard deviation at a level of 2,000 ng/g is substantially higher than at 100 ng/g. By using more low level spiked samples, as is done with the second series, the standard deviation term is lower, giving a more accurate detection limit. Another means of calculating the detection limit is to replace the standard deviation term with a relative standard deviation term. Then, the percent variation at each level, which is usually relatively constant over a concentration range, is employed to define the detection limit.

I. INTRODUCTION

Under Contract No. DAAK11-79-C-0110, entitled "Identification or Development of Chemical Analysis Methods for Plants and Animal Tissues," studies have been conducted to define quantitative analytical methods for the determination of various munition compounds in biological matrices. The munition compounds evaluated included cyclotrimethylenetrinitramine (RDX), dinitrotoluene (DNT), trinitrotoluene (TNT), pentaerythrite tetra-nitrate (PETN), and 2,4,6-trinitrophenylmethylnitramine (tetryl). The biological matrices selected for study were animal plasma, kidney, muscle/fat, and liver and plant leaves and stems.

The analytical methods were to be able to detect and quantitate the munition compounds at the 100 ng/g (parts per billion) level and were to be designed to utilize readily available analytical instrumentation, to be able to analyze a number of samples in a routine manner, and to be capable of providing the final results in a relatively short time. The developed methods may have application in assessing the environmental contamination of these munitions in the plants and animals at munition production facilities or in the immediate area.

This report describes the research effort to develop the analytical methods for munition compounds in biological matrices.

II. EQUIPMENT AND MATERIALS

A. Equipment

The instrumentation employed during the course of the research program consisted of:

1. Isocratic HPLC instrument consisting of a Waters Model 6000A pump, Waters Model U6K injector, Waters Model 440 UV detector (254 nm filter), and a Heath-Schlumberger Model SR-204 strip chart recorder.
2. Isocratic HPLC instrument consisting of a Waters Model 6000A pump, Waters Model U6K injector, Varian Model UV-50 variable wavelength detector (190 to 700 nm), and a Heath-Schlumberger Model SR-204 strip chart recorder.
3. General purpose centrifuge, Dynac, Clay Adams 0101, with 24-place and 8-place heads.
4. A Waring multi-speed blender with a glass container.
5. A Teflon-glass motor driven homogenizer.

B. Laboratory Glassware and Equipment

1. Culture tubes (Pyrex) with Teflon-lined screw caps.
2. Volumetric flasks (100 ml).
3. Volumetric syringes (0-100 μ l, 0-500 μ l, and 0-1,000 μ l).
4. Automatic pipettor (0-5 ml).
5. Filtering apparatus including filter holders, 5-ml disposable syringes, and 0.45 μ Fluoropore filters.
6. Inert gas (nitrogen) drying train with 12 ports.
7. Inert gas (helium) apparatus for degassing HPLC eluents.

C. Chemicals

1. Toluene, hexane, ethyl acetate, isopropanol, and acetonitrile, "Distilled in Glass" grade from Burdick and Jackson. Note: Solvents of lesser quality should not be employed or interfering peaks may be observed on the HPLC chromatograms.
2. Acetic acid, sodium chloride, and ammonium dihydrogen phosphate, ACS grade.
3. High purity water from a Milli-Q water purification system.
4. RDX, DNT, TNT, PETN, and tetryl SARMS from the U.S. Army Toxic and Hazardous Materials Agency.
5. Internal standards - propiophenone, butyrophenone, and valero-phenone, analytical grade.

D. Animal Tissues and Plants

The various animal tissues, plasma, kidney, muscle/fat, and liver, to be studied were obtained from cattle at the time of slaughter at a local (Kansas City, Missouri) slaughter house. The cattle blood was collected in 250 ml centrifuge tubes containing heparin (to prevent clotting), placed in ice, and transported to Midwest Research Institute (MRI). Upon arrival at MRI, the blood was centrifuged and the plasma transferred to culture tubes. The plasma was frozen and stored at -80°C until use in method development. The kidney, muscle/fat, and liver samples obtained at the same time as the blood were placed in freezer bags and transported to MRI in ice chests containing dry ice. Upon arrival at MRI, the tissues were cut into pieces (approximately 2-in. squares), placed in freezer bags, and stored at -80°C until use in method development.

Plant leaves and stems were obtained from a horse pasture. The leaves, including grass, and the stems were placed in freezer bags, transported to MRI in ice chests containing dry ice, and stored at -80°C until use in method development.

E. Reference Stock Solutions

Reference stock solutions of each munition compound and internal standard were prepared and stored at 4°C. The tetryl stock solutions were wrapped in foil to prevent degradation by UV light.

1. Munition Stock Solutions: Approximately 20 mg to the nearest 0.1 mg of munition compound SARM (RDX, DNT, TNT, PETN, and tetryl) were weighed into separate 100-ml volumetric flasks and the weight recorded. Each munition was dissolved in acetonitrile and volume adjusted to 100 ml. The concentration of each munition was 200 µg/ml. A 20-ml aliquot from the RDX, DNT, TNT, and tetryl stocks was quantitatively pipetted into a 100-ml volumetric flask and the volume adjusted to 100 ml with high-purity water. The concentration of RDX, DNT, TNT, and tetryl in this stock solution was 40 µg/ml. A 20-ml aliquot from the PETN stock was quantitatively pipetted into a 100-ml volumetric flask and diluted to volume with high-purity water (PETN concentration - 40 µg/ml). The PETN stock was prepared separately from the other munitions since the analytical technique for PETN was different from the method for RDX, DNT, TNT, and tetryl.

2. Internal Standard Stock Solutions: Approximately 10 mg to the nearest 0.1 mg of each internal standard (IS) (propiophenone, butyrophenone, and valerophenone) were weighed into separate 100-ml volumetric flasks, the weights recorded, and the sample diluted to volume with acetonitrile. The concentration of each IS was 100 µg/ml. A 10-µg/ml stock of each IS was prepared by quantitatively pipetting 10 ml of the 100-µg/ml stock into separate 100-ml volumetric flasks and diluting to volume with acetonitrile.

F. Calculation of Data

The data obtained during this program were calculated using the relative weight response (RWR) to an internal standard method. Reference solutions of the munition compounds were assayed and the RWR of each compound determined by Eq. 1.

$$\text{RWR Cpd/IS} = \frac{\text{Peak Height Cpd}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml Cpd}} \quad (\text{Eq. 1})$$

The RWR value of the reference solution was then employed to calculate the level of the munition in a prepared sample aliquot by:

$$\frac{\text{ng Cpd}}{\text{aliquot}} = \frac{\text{Peak Height Cpd}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{RWR Cpd/IS}} \quad (\text{Eq. 2})$$

If the sample taken for analysis was 1.0 ml or 1.0 g, the ng Cpd/aliquot was equal to ng Cpd/ml or parts per billion (ppb) compound in the sample. If the sample taken was not 1.0 ml or 1.0 g, the ng Cpd/ml was calculated by dividing the ng Cpd/aliquot by the same volume or weight.

The data obtained for the analysis of duplicate samples at five levels on four separate days for each munition were subjected to statistical analysis. The average of the eight data points at each level, the standard deviation, coefficient of variation (relative standard deviation), and percent inaccuracy were determined by the following equations.

$$\text{Average} = \Sigma x/n = \bar{x} \quad (\text{Eq. 3})$$

x = data point; n = number of data points

$$\text{Standard Deviation} = \left(\frac{n \Sigma x^2 - (\Sigma x)^2}{n(n-1)} \right)^{\frac{1}{2}} = \sigma \quad (\text{Eq. 4})$$

$$\text{Coefficient of Variation} = \sigma/\bar{x} \times 100 \quad (\text{Eq. 5})$$

$$\text{Percent Inaccuracy} = \frac{\text{ng/g Cpd Found} - \text{ng/g Cpd Added}}{\text{ng/g Cpd Added}} \times 100 \quad (\text{Eq. 6})$$

The data were also subjected to linear regression analysis and the slope, y-intercept, and correlation coefficient determined for each munition in each matrix. The detection limits for the munitions in a matrix were determined by the Hubaux and Vos detection limit program and were generated at the U.S. Army Toxic and Hazardous Materials Agency.

III. ANALYTICAL (INSTRUMENTAL) TECHNIQUES

The first phase of the research program was to define and validate the analytical techniques to be employed for the determination of RDX, DNT, TNT, PETN, and tetryl in the various biological matrices. Each munition compound was scanned from 350 to 200 nm to determine the wavelength maxima and extinction coefficients. These data indicated that RDX, DNT, TNT, and tetryl had sufficient UV chromophores at 254 nm for detection and quantification by HPLC. However, PETN gave a minimum at 254 nm and a maximum at 215 nm and required a different detection system than the other munitions. Studies were conducted to determine the HPLC conditions necessary to separate RDX, DNT, TNT, and tetryl and to define an internal standard (IS) for calculation purposes. Also, the HPLC conditions necessary to analyze PETN were evaluated. The various HPLC systems evaluated during the project are summarized in Table 1. The development of each of these methods is presented in detail in the following paragraphs.

TABLE 1

HPLC ANALYTICAL SYSTEM INVESTIGATED FOR MUNITION COMPOUND DETERMINATION

<u>System</u>	<u>Analytical Column</u>	<u>Eluent</u>	<u>Munition Compounds</u>	<u>Comments</u>
I	Spherisorb ODS, 5 μ	40% CH ₃ CN in 1% acetic acid	RDX, DNT, TNT	Could not separate TNT and tetryl
II	Spherisorb ODS, 5 μ	20% CH ₃ CN in 0.035 M NH ₄ Ac, pH 7	RDX, DNT, TNT, tetryl	High pump pressure and short column life prevented use for routine analyses
III	Spherisorb ODS, 5 μ	28-30% CH ₃ CN in 1% acetic acid	RDX, DNT, TNT, tetryl	Utilized for method development for several biological matrices
IV	Spherisorb ODS, 5 μ	40% CH ₃ CN in high-purity water	PETN	Utilized for plasma method development

A. RDX, DNT, TNT, and Tetryl HPLC Parameters

Previous studies¹ on these munitions had shown that each could be analyzed on a reverse phase column (Waters μ Bondapak C-18). The separation of the four compounds in a single HPLC system had not been demonstrated. Two reverse phase HPLC columns, μ Bondapak C-18, 10 μ , and Spherisorb ODS, 5 μ , were evaluated using methanol or acetonitrile in 1% acetic acid eluents. The initial studies were conducted using interim SARMs of RDX, DNT, and TNT; no interim SARM for tetryl was available. The results indicated that baseline separation of RDX, DNT, and TNT was possible with either column or organic solvent. The elution position of TNT varied depending on the eluent; with methanol, TNT eluted prior to DNT and with acetonitrile, after DNT. The peak shape was superior with the Spherisorb column, and acetonitrile was expected to be employed in the sample preparation of biological matrices (see Section IV). The Spherisorb column with an acetonitrile eluent was selected (HPLC System I). Two possible IS's were identified, propiophenone (IS-1) and butyrophenone (IS-2). The retention volumes for the three munition compounds and the two IS's on the Spherisorb ODS, 5- μ column with a 40% acetonitrile in 1% acetic acid eluent were: RDX - 8.5 ml, propiophenone - 13.5 ml, DNT - 16.5 ml, TNT - 18 ml, and butyrophenone - 21 ml. A short linearity study indicated that the three munitions gave a linear response from 25 to 10,000 ng/ml and that 3 ng injected on column could be detected and quantitated. The results of the linearity study are summarized in Table 2. The raw data and calculations are given in Tables A-1, A-2, and A-3 of Appendix A. Table 2 gives the relative weight response (RWR) for each munition to both IS's at the various concentration levels studied and presents the linear regression evaluation of the ratios of the peak heights of compound to IS to the ng/ml compound present. Figure 1 presents an HPLC chromatogram of a reference solution containing 50 ng/ml of each munition (2.5 ng of each compound injected on column) on the Spherisorb ODS column with the 40% acetonitrile in 1% acetic acid eluent.

The evaluations described above were conducted using interim SARMs of RDX, DNT, and TNT. After receiving the SARMs for each munition from the U.S. Army Toxic and Hazardous Materials Agency, reference stock solutions as described in Section II.E. were prepared. The HPLC system defined above did not separate TNT and tetryl, and additional studies were conducted to determine the necessary parameters for separation. Changes in both the organic phase concentration and the aqueous phase modifier were evaluated. Separation of the four munition compounds was possible with a 20% acetonitrile in 0.035 M ammonium acetate, pH 7 eluent (HPLC System II). Figure 2 presents a representative HPLC chromatogram for the separation of the four munitions with this system. Butyrophenone (IS-2) was not included with this system because it had a elution time of over 40 min. The linearity of this HPLC system was evaluated for the four compounds by preparing and analyzing duplicate SARM reference solutions at 100, 500, 1,000, 1,500, and 2,000 ng/ml.

¹ Doali, J. O., and Juhasz, J. Chromatog. Sci., 12, 51 (1974); Yinon, J., CRC Critical Reviews in Analytical Chemistry, December 1977; and personal communication from Dan Helton, MRI, on USAMBRDL contracts.

TABLE 2

HPLC LINEARITY OF RDX, DNT, AND TNT INTERIM SARH REFERENCE SOLUTION
HPLC SYSTEM I

Solution No.	RDX			DNT			TNT		
	ng/ml ^a	RWR ^b IS-1	RWR ^c IS-2	ng/ml ^a	RWR ^b IS-1	RWR ^c IS-2	ng/ml ^a	RWR ^b IS-1	RWR ^c IS-2
1-1	10,620	0.565	0.949	10,050	1.15	1.94	10,210	0.788	1.32
1-2	10,620	0.578	0.981	10,050	1.17	1.98	10,210	0.794	1.35
2-1	5,310	0.572	0.977	5,025	1.17	1.99	5,105	0.783	1.34
2-2	5,310	0.555	0.949	5,025	1.15	1.97	5,105	0.775	1.32
3-1	1,062	0.565	0.976	1,005	1.11	1.92	1,021	0.744	1.29
3-2	1,062	0.552	0.943	1,005	1.17	1.99	1,021	0.765	1.31
4-1	531	0.562	0.968	502	1.13	1.94	510	0.779	1.34
4-2	531	0.570	0.985	502	1.15	1.98	510	0.772	1.36
5-1	266	0.552	0.947	251	1.11	1.90	255	0.760	1.30
5-2	266	0.549	0.946	251	1.11	1.92	255	0.752	1.30
6-1	106	0.507	0.956	100	0.99	1.87	102	0.725	1.37
6-2	106	0.497	0.950	100	0.99	1.89	102	0.678	1.30
7-1	53	0.482	0.968	50	0.96	1.92	51	0.751	1.51
7-2	53	0.535	1.08	50	0.98	1.98	51	0.649	1.31
8-1	26	^h — ^h	^h — ^h	25	1.13	1.95	25	0.730	1.26
8-2	26	^h — ^h	^h — ^h	25	1.16	2.01	25	0.969	1.67
Average ^d		0.546	0.970		1.10	1.95		0.763	1.35
SD ^e		± 0.030	± 0.035		± 0.07	± 0.04		± 0.068	± 0.10
RSD ^f		5.5%	3.6%		6.9%	2.0%		8.8%	7.5%

TABLE 2 (concluded)

Correlation
CoefficientLinear Regression^g - IS-1

RDX: $y = 2.27 \times 10^{-3}x - 0.039$ 0.9999
 DNT: $y = 4.61 \times 10^{-3}x - 0.043$ 1.000
 TNT: $y = 3.13 \times 10^{-3}x - 0.053$ 1.000

Linear Regression^g - IS-2

RDX: $y = 1.95 \times 10^{-3}x + 0.0004$ 0.9998
 DNT: $y = 3.97 \times 10^{-3}x + 0.007$ 0.9999
 TNT: $y = 2.70 \times 10^{-3}x + 0.017$ 1.000

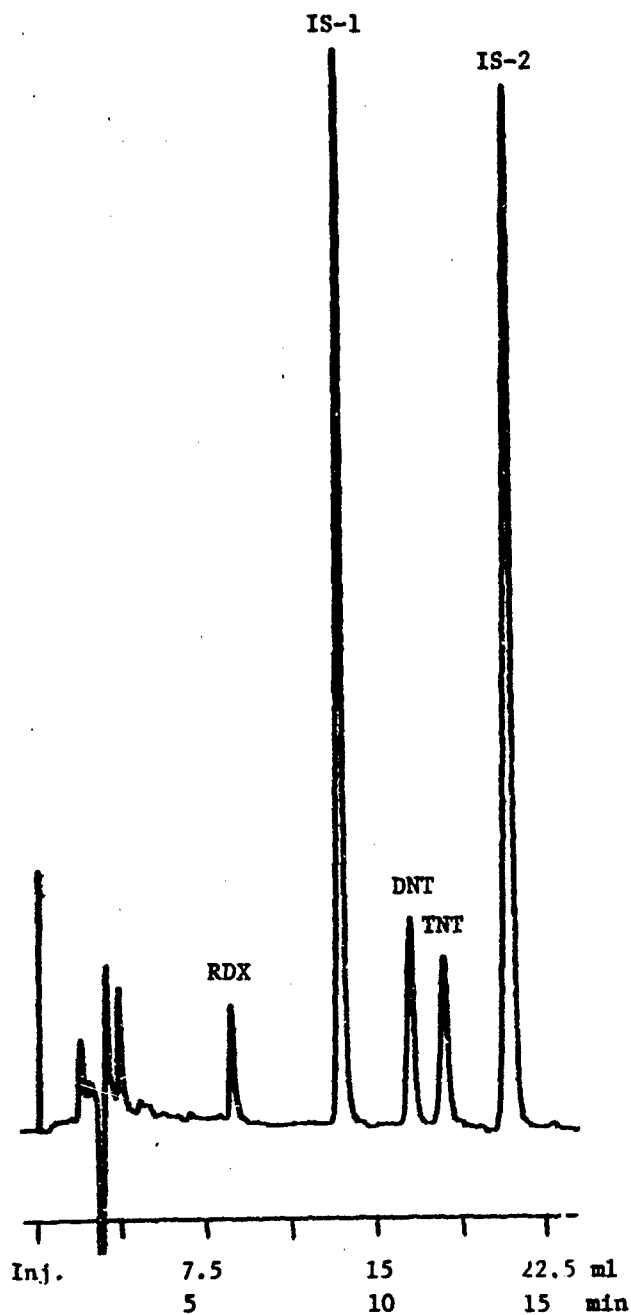
^a ng/ml - Nanograms munition per milliliter solution.^b RWR IS-1 - relative weight response of the munition to IS-1

$$RWR = \frac{\text{Peak Height Cpd}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml Cpd}}$$

^c RWR IS-2 - relative weight response to IS-2.^d Average - average RWR value for all levels.

$$e \text{ SD - standard deviation} = \frac{n\bar{x}^2 - (\sum x)^2}{n(n-1)} \quad h$$

^f RSD - relative standard deviation = SD/Average x 100.^g Linear regression - peak height ratio of munition to IS versus ng/ml munition present.^h Interfering HPLC peak resulted in high peak height for RDX.



HPLC Parameters

Column - Spherisorb ODS, 5 μ
 Eluent - 40% CH₃CN in water containing 1% acetic acid
 Flow Rate - 1.5 ml/min
 Chart Speed - 0.2 in/min
 Detector - UV, 254 nm
 Injection - 50 μ l

Sample Characteristics

<u>Compound</u>	<u>ng Injected</u>
TNT	2.5
DNT	2.5
RDX	2.5
IS-1	12.5
IS-2	25

Retention Indices

<u>Compound</u>	<u>Retention Volume (ml)</u>	<u>Retention Time (min)</u>
TNT	18.0	12.0
DNT	16.5	11.0
RDX	8.5	5.5
IS-1	13.5	9.0
IS-2	21.0	14.0

Figure 1 - HPLC Separation for TNT, DNT, RDX and Internal Standard on the Spherisorb ODS Column

HPLC Parameters

Column: Spherisorb ODS, 5 μ , 250 x 4.6 mm ID
Eluent: 20% acetonitrile in 0.035 M ammonium acetate,
pH 7.0

Flow Rate: 1.5 ml/min
Chart Speed: 0.1 in./min
Detector: UV, 254 nm
Injection: 50 μ l

Sample Characteristics

Compound	Concentration (ng/ml)	Retention Volume (ml)	Retention Time (min)
RDX	500	17	11.2
IS-1	1,000	39	26.2
TNT	500	43	29.0
DNT	500	48.5	32.5
Tetryl	500	52.5	35.0

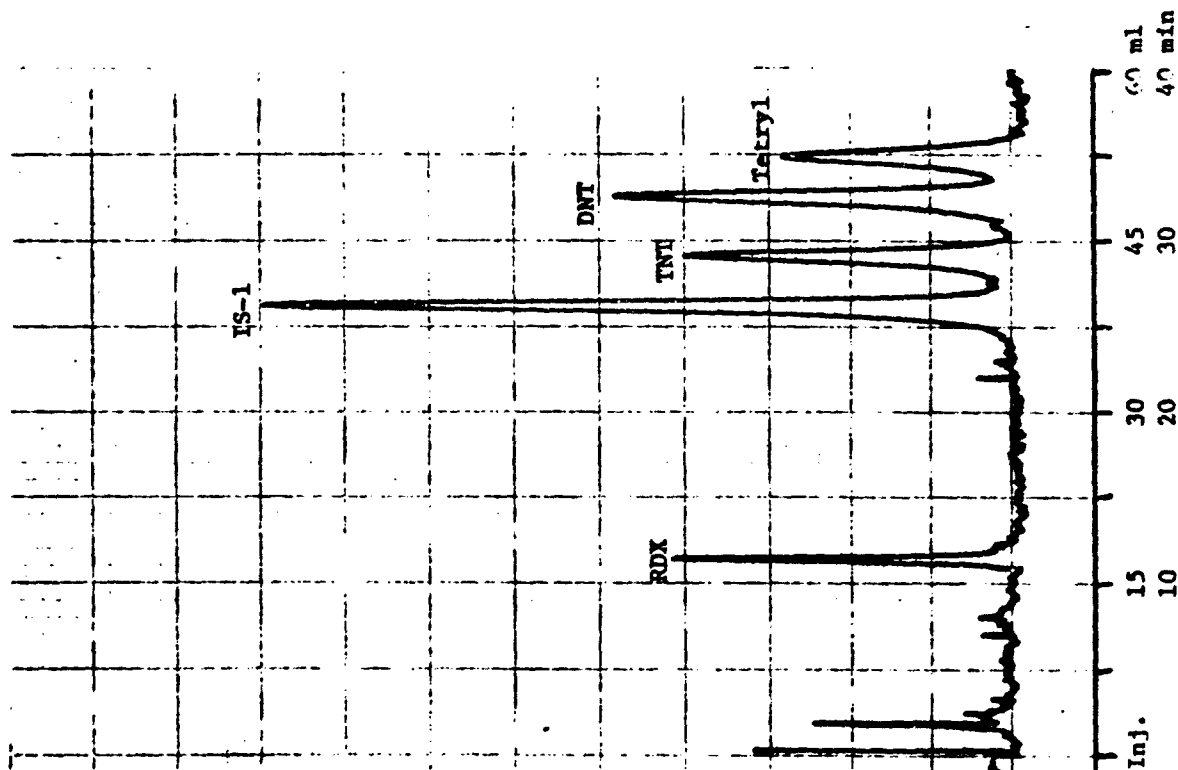


Figure 2 - HPLC Determination of RDX, TNT, DNT, and Tetryl SARM Reference Solution Using HPLC System II

The results are summarized in Table 3. The raw data and calculations are given in Table A-4 of Appendix A. The constant RWR values over the concentration range indicate a linear response. The precision of the analytical technique is shown in the relative standard deviations from the average RWR value for each munition compound.

During the initial sample preparation procedure evaluations for the determination of RDX, DNT, TNT, and tetryl in the various biological matrices, both the HPLC System I and System II were employed. HPLC System I was used with interim SARMs until tetryl was available and found to co-elute with TNT. Then, HPLC System II was defined and utilized for sample preparation procedure studies on plasma, liver, kidney, and muscle/fat tissues. During these evaluations, problems were encountered with the HPLC system. The analytical column appeared to deteriorate (i.e., poor resolution between the various munitions) after about 20 injections of biological matrix extracts, and an increased HPLC pump pressure occurred after a few injections. HPLC System II was not satisfactory for the routine determination of the munitions in biological matrices. Another study was conducted to evaluate the HPLC conditions necessary to resolve the munition compounds. A new Spherisorb ODS column was used and a 1% acetic acid aqueous phase with various acetonitrile levels was studied. Complete separation of the four munitions compounds was obtained with the new column and an eluent of 30% acetonitrile in 1% acetic acid (HPLC System III). The precision and linearity of this system was evaluated and these data are presented in Technical Reports 1, 2, 3, 4, and 6 in Appendices B through E and G for RDX, DNT, and TNT. Tetryl was not included in these data since the determination of tetryl in the biological matrices was not possible (see Section IV). HPLC System III proved to be stable and reproducible and was utilized for each of the methods developed for RDX, DNT, and TNT determination. Slight changes in the retention indices of the munitions occurred with fresh eluent or a change in the analytical column. A 1 to 2% adjustment in the acetonitrile content of the eluent was required to obtain the desired resolution.

B. PETN HPLC Parameters

As mentioned earlier, PETN required a 215-nm detector for detection and quantification. A system similar to the one defined for the other munitions but employing a 215-nm UV detector was desirable to allow the analysis of any of the munitions with only slight modifications. Acetic acid (and other organic acids) have characteristic UV absorption at 215 nm; thus, a system containing only acetonitrile and high-purity water was developed. A relatively long chromatographic time for PETN was necessary since many of the biological compounds that may be present in the prepared sample also absorb at 215 nm and a long retention volume may allow the isolation of PETN from interfering compounds. The HPLC parameters which met these criteria consisted of a Spherisorb ODS, 5- μ column and an eluent of 40% acetonitrile in high-purity water. An internal standard of valerophenone eluted prior to PETN but late enough in the chromatogram to isolate the IS from possible interfering peaks. Precision and linearity data for PETN for this system (HPLC System IV) are presented in Technical Report No. 5 in Appendix F.

TABLE 3

HPLC PRECISION AND LINEARITY OF RDX, DNT, TNT, AND TETRYL
SARM REFERENCE SOLUTIONS
HPLC SYSTEM II

SARM Reference Solution No.	ng/ml Each Munition	Relative Weight Responses ^a			
		RDX	TNT	DNT	Tetryl
1-A	100	1.08	1.00	1.17	0.58
1-B	100	0.98	0.89	1.07	0.63
2-A	500	0.97	0.85	1.05	0.62
2-B	500	0.95	0.86	1.02	0.62
3-A	1,000	0.91	0.85	1.06	0.50
3-B	1,000	0.90	0.88	1.08	0.49
4-A	1,500	0.98	0.89	1.05	0.59
4-B	1,500	0.91	0.91	1.04	0.59
5-A	2,000	0.98	0.90	1.06	0.56
5-B	2,000	0.91	0.87	1.03	0.57
	Average	0.96	0.89	1.06	0.58
	SD ^b	± 0.06	± 0.04	± 0.04	± 0.05
	RSD ^c	5.8%	4.8%	3.8%	8.3%

^a Relative Weight Response = $\frac{\text{Peak Height Cpd}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml Cpd}}$

^b SD = Standard deviation.

^c RDS = Relative standard deviation.

IV. SAMPLE PREPARATION PROCEDURES

The analytical techniques for RDX, DNT, TNT, and tetryl and for PETN, defined in Section III, were employed to evaluate sample preparation procedures for the quantitative determination of the munitions in animal plasma, kidney, muscle/fat, and liver and in plant leaves and stems. The primary objective of the research program was to develop sample preparation procedures which provided quantitative data on the various munitions at the 100-ng/g level. These procedures were to be validated by analyzing duplicate matrix samples spiked at five levels and matrix blanks on four separate days.

Another objective of the program was to provide sample preparation procedures which were relatively simple and routine and had application for a variety of matrices. Plasma was selected as the first matrix to be evaluated. During the evaluations of procedures for plasma level determination of the five munitions, samples of the other matrices were also studied to determine the potential of the plasma technique for kidney, muscle/fat, liver, plant leaves, and plant stems sample preparation. A summary of the sample preparation techniques evaluated for the five munition compounds in the six biological matrices is given in Table 4. These studies are presented in detail in the following sections.

A. Animal Plasma

The assay of organic compounds with intermediate polarities in plasma samples can usually be accomplished by precipitating the plasma protein with an organic solvent such as acetonitrile or ethanol and injecting the supernatant onto a reverse phase HPLC column. The technique is successful because most of the plasma components are fairly polar and can be separated from the compounds of interest. Reverse phase HPLC has the capability of separating compounds with similar chemical and physical properties, i.e., DNT and TNT; the elution order of the technique is based on the polarity of the compounds, the more polar compounds being eluted first. The polar plasma components may be eluted early in the chromatographic analysis followed by the munition compounds.

1. RDX, DNT, TNT, and Tetryl in Plasma: Acetonitrile was selected as the organic solvent for plasma protein precipitation since it was utilized in the HPLC eluent. Initial studies with this technique were conducted using HPLC System I and interim SARMS of RDX, DNT, and TNT. These experiments with this simple technique for sample preparation of plasma were encouraging. Blank plasma samples had little interference at the elution position of the munitions; and acceptable recovery, i.e., greater than 75%, was obtained for plasma containing 10 µg/ml of each munition. However, after the plasma sample had been precipitated with acetonitrile, the resulting solution did not contain a sufficient munition concentration to detect and quantitate the compounds at the 100-ng/ml level. By concentrating the acetonitrile-plasma supernatant and then diluting to a final volume of 1.0 ml, a munition concentration of 100 ng/ml was detectable. This procedure is outlined below.

TABLE 4

SUMMARY OF SAMPLE PREPARATION PROCEDURE EVALUATIONS
FOR MUNITIONS IN BIOLOGICAL MATRICES

<u>Matrix</u>	<u>Munition Compounds Studied</u>	<u>HPLC Analytical System</u>	<u>Sample Preparation Procedure</u>	<u>Comments</u>
Animal Plasma	RDX, DNT, TNT, tetryl	I	CH ₃ CN precipitation	RDX interference, good DNT and TNT recovery, no tetryl recovery
		II	CH ₃ CN precipitation	Partial RDX separation, good DNT and TNT recovery, no tetryl recovery
	Tetryl	II	Extraction with toluene EtOAc, 2% IPA in hexane	Recovery at high levels (200 µg/ml) but below 10 µg/ml no recovery
	RDX, DNT, TNT, tetryl	II	Toluene extraction of a salt-plasma solution	Good recovery of RDX, DNT, TNT; no tetryl. Problems with HPLC system including high pressure and short column life.
		III	Toluene extraction of a salt-plasma solution	Method employed for validation, Appendix B
Animal Kidney	PETN	IV	CH ₃ CN precipitation	HPLC interference from plasma components
			Extraction with toluene EtOAc, CH ₂ Cl ₂ , hexane from a salt-plasma solution	Polar solvent extracted interfering plasma components; hexane extraction employed for validation, Appendix F.
Animal Kidney	RDX, DNT, TNT, tetryl	II	CH ₃ CN precipitation	Interfering kidney components

TABLE 4 (continued)

Matrix	Munition Compounds Studied	HPLC Analytical System	Sample Preparation Procedure	Comments
	PETN	II	Extraction from salt-kidney solution	Interfering HPLC peaks with polar solvents, and poor recovery with nonpolar solvents. HPLC instrument high pressure.
		III	Toluene extraction from salt-kidney solution	Good RDX, DNT, TNT recovery, no tetryl; utilized for method validation, Appendix C.
		IV	CH ₃ CN precipitation	Interfering matrix components.
			Extraction with organic solvents	Polar solvents, toluene EtOAc, extracted interfering matrix components; hexane not capable of extracting PETN; IPA in hexane promising; however, 2% IPA gave only 25% recovery.
Animal Muscle/Fat	RDX, DNT, TNT, tetryl	II	CH ₃ CN precipitation	Good recovery of RDX, DNT, TNT; no tetryl. High HPLC pressure due to matrix components adsorbed on the column.
		II	Extraction of salt-muscle/fat solution with organic solvent	Fat component solubilized in the organic solvent and prevented HPLC determination.
		III	CH ₃ CN precipitation	Stable HPLC system if washed with CH ₃ CN after each sample; employed for method validation, Appendix D.
		IV	CH ₃ CN precipitation	Interfering matrix components
	PETN		Hexane extraction	No PETN recovery; extraction residue not soluble in CH ₃ CN

TABLE 4 (continued)

Matrix	Munition Compounds Studied	HPLC Analytical System	Sample Preparation Procedure	Comments
Animal Liver	RDX, DNT, TNT, tetryl	III	CH ₃ CN precipitation	Interfering matrix components
			EtOAc extraction from salt-liver solution	Interfering matrix components
			Hexane extraction from salt-liver solution	Poor recovery of munitions
			Toluene extraction from salt-liver solution	Good recovery of RDX, DNT, TNT initially; no tetryl; varying recovery during validation for DNT and TNT
			1. pH effect on extraction	Acidic media optimal for extraction; poor chromatography with neutral or basic solutions
			2. Salt concentration required for extraction	At least 10% required for optimal extraction
			3. Other solvents possibilities	No improvement over toluene as extracting solvent
			4. Toluene evaporation and residue reconstitution	No loss during evaporation; vortexing and ultrasonication aid in dissolving munitions from liver extract residue.
			5. Toluene emulsion formation	Improved DNT and TNT recovery indicating importance of complete emulsion formation for optimal extraction; used for method validation, Appendix E.

TABLE 4 (concluded)

<u>Matrix</u>	<u>Munition Compounds Studied</u>	<u>HPLC Analytical System</u>		<u>Sample Preparation Procedure</u>	<u>Comments</u>
Plant Leaves	PETN	IV		2% IPA in hexane extraction	Poor PETN recovery
				Higher polarity solvents for extraction	Poor chromatography with PETN interference from matrix components
	RDX, DNT, TNT, tetryl	III		Polar solvent extraction from salt solution-leaves mixture	Interfering matrix components
				Toluene extraction from salt solution-leaves mixture	RDX, DNT interference; HPLC eluent changes were not able to isolate munitions from interferences
Plant Stems				Nonpolar solvent extraction from salt solution-leaves mixture	Matrix components interference for RDX and DNT; poor recovery.
	PETN	IV		Nonpolar solvent extraction from salt solution-leaves mixture	PETN interference from matrix components with all solvents; hexane, IPA in hexane, CH ₂ Cl ₂ , toluene
	RDX, DNT, TNT, tetryl	III		2% IPA in hexane extraction from salt solution-stem mixture	Low, but consistent, DNT and TNT recovery; RDX interference from matrix; employed for method validation, Appendix G.
	PETN	IV		Hexane or 2% IPA in hexane extraction from salt solution-stem mixture	Poor chromatography with stems matrix interference

Plasma Sample Preparation Using Acetonitrile

1. Pipette 1.0 ml plasma into a Teflon-lined screw cap vial.
2. Add 2.0 ml acetonitrile. Note: When spiking the plasma with the munition compounds, add the proper level of munitions in a small volume of acetonitrile and mix thoroughly. Then, add acetonitrile to make the total acetonitrile volume 2.0 ml.
3. Mix thoroughly on a vortex mixer and allow to stand a minimum of 2 hr at 4°C to completely precipitate the protein.
4. Centrifuge at 1,000 rpm for 10 min.
5. Transfer the supernatant to a properly labeled culture tube.
6. Wash the precipitate with 2.0 ml acetonitrile.
7. Centrifuge and add the supernatant to the first supernatant.
8. Concentrate to about 100 µl on a 40°C hot plate under a stream of nitrogen. Note: Do not allow the sample to evaporate to dryness or the munition compounds may be lost.
9. Add 500 µl acetonitrile containing the internal standards and mix thoroughly.
10. Add 400 µl 1% acetic acid in high-purity water and mix thoroughly. Note: Final volume is approximately 1.0 ml.
11. Filter the prepared sample through a 0.45-µ Fluoropore filter into a properly labeled culture tube.
12. Analyze an aliquot (50-70 µl) by HPLC using HPLC System I.

This technique was evaluated by preparing and analyzing duplicate plasma aliquots spiked with RDX, DNT, and TNT at the following levels: 0, 100, 200, 400, 750, 1,000, and 2,000 ng/ml. The results showed that a plasma component co-eluted with RDX preventing the quantification of this munition. Excellent recovery of DNT and TNT was obtained at each level; these data are summarized in Table 5; the average recovery and standard deviation of DNT and TNT for these plasma samples was 99 ± 4 and 102 ± 5 , respectively. Figures 3 and 4 present representative HPLC chromatograms for a blank plasma and a plasma containing 200 ng/ml RDX, DNT, and TNT using this sample preparation procedure and HPLC System I.

TABLE 5

EVALUATION OF ACETONITRILE PRECIPITATION PROCEDURE FOR THE
DETERMINATION OF DNT AND TNT IN PLASMA

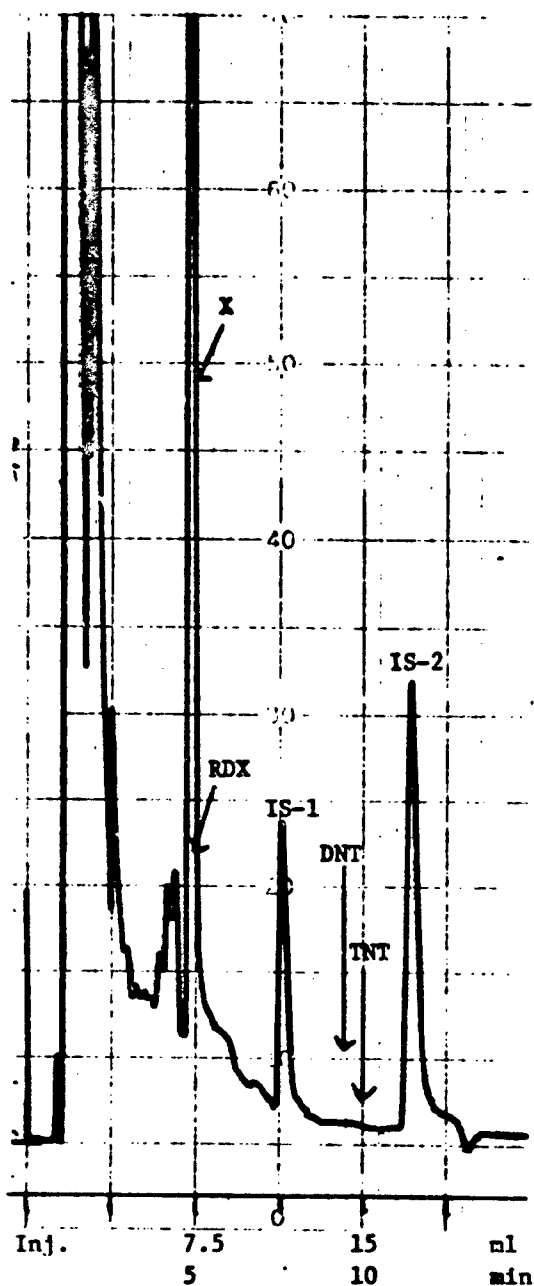
Plasma Solution Number	ml Plasma	ng/ml ^a Munition Added	ng/ml Found ^b		% Recovery ^c	
			DNT	TNT	DNT	TNT
1-A	1.0	0	ND	ND	-	-
1-B	1.0	0	ND	ND	-	-
2-A	1.0	100	94	95	94	95
2-B	1.0	100	94	96	94	96
3-A	1.0	200	194	196	97	98
3-B	1.0	200	195	198	98	99
4-A	1.0	400	386	392	96	98
4-B	1.0	400	401	405	100	101
5-A	1.0	750	717	742	96	99
5-B	1.0	750	728	777	97	104
6-A	1.0	1,000	1,003	1,074	100	107
6-B	1.0	1,000	1,022	1,087	102	109
7-A	1.0	2,000	2,118	2,200	106	110
7-B	1.0	2,000	2,059	2,156	103	108
Average					99	102
Standard Deviation					± 4	± 5

a ng/ml munition added - Nanograms of RDX, DNT, and TNT added to 1.0 ml plasma.

b ng/ml found - Nanograms DNT and TNT found per milliliter plasma, RDX not included due to plasma component interference.

c % Recovery = $\frac{\text{ng/ml Found}}{\text{ng/ml Added}} \times 100$

Note: Detection of RDX was not possible due to an interfering plasma component.



HPLC Conditions

Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 40% acetonitrile in 1%
acetic acid in water
Flow Rate: 1.5 ml/min
Chart Speed: 0.2 in./min
Detector: UV, 254 nm

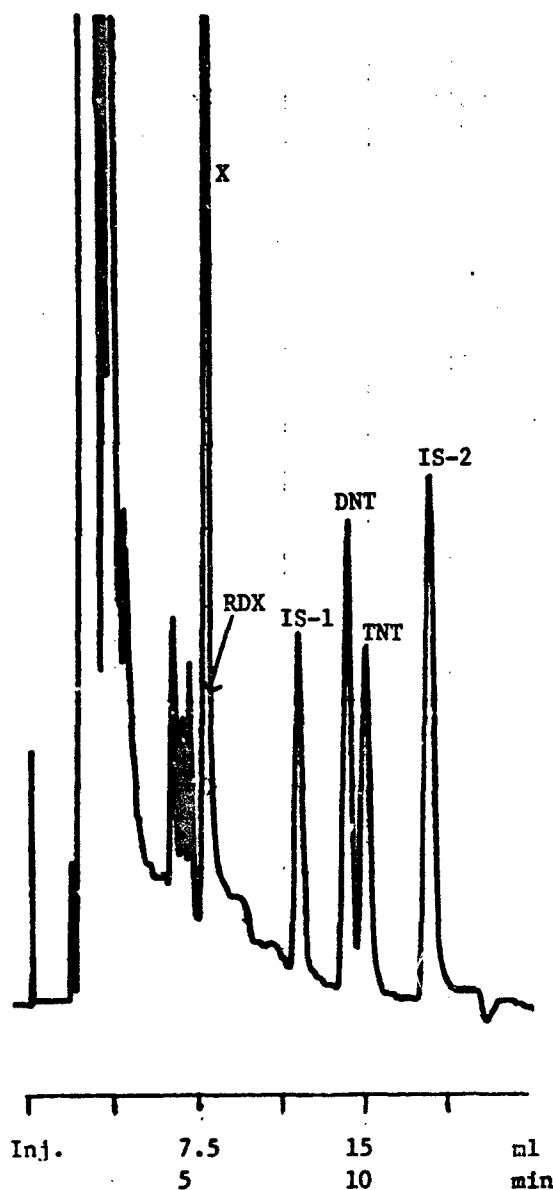
Sample Characteristics

1.0 ml Plasma precipitated with
acetonitrile. Supernatant concen-
trated to about 100 μ l, diluted
with 500 μ l IS stock, and 400 μ l
1% acetic acid in water. Sample
filtered and analyzed.

IS Concentration: 200 ng/ml IS-1
400 ng/ml IS-2

Injection Volume: 50 μ l
Attenuation: 0.005 X

Figure 3 - HPLC System I Analysis of Blank Plasma Sample for Method Development Using Acetonitrile Precipitation Technique. "X" indicates plasma component eluting at the RDX elution position. Arrows show elution positions for RDX, DNT, and TNT.



HPLC Conditions

Column: Spherisorb ODS, 5 μ ,
 250 x 4.6 mm ID
 Eluent: 40% acetonitrile in 1%
 acetic acid in water
 Flow Rate: 1.5 ml/min
 Chart Speed: 0.2 in./min
 Detector: UV, 254 nm

Sample Characteristics

1.0 ml Plasma containing 200 ng/ml
 RDX, DNT, and TNT precipitated with
 acetonitrile. Supernatant concen-
 trated to about 100 μ l, diluted
 with 500 μ l IS stock and 400 μ l
 1% acetic acid in water. Sample
 filtered and analyzed.

IS Concentration: 200 ng/ml IS-1
 400 ng/ml IS-2

Injection Volume: 50 μ l
 Attenuation: 0.005 X

Figure 4 - HPLC System I Determination of RDX, DNT, and TNT in Plasma
 Sample Prepared by Acetonitrile Precipitation Technique. "X"
 indicates plasma component co-eluting with RDX.

During these plasma sample evaluations, the SARM RDX, DNT, TNT, PETN, and tetryl were obtained from the U.S. Army Toxic and Hazardous Materials Agency. The UV characteristics of tetryl indicated that this munition had a sufficient UV chromophore for detection and quantification at 254 nm. PETN cannot be analyzed at 254 nm and required a different UV detector. Tetryl was combined with RDX, DNT, and TNT and method development on the four munitions in plasma conducted. Attempts to separate the four munition compounds using HPLC System I were unsuccessful. Another HPLC System (II) was defined which provided baseline separation of the four munitions. Evaluations with this system and the acetonitrile precipitation procedure were conducted on samples of plasma, kidney, liver, and muscle/fat. Only plasma and muscle/fat samples could be assayed for the munitions, and the plasma component which co-eluted with RDX in earlier studies was still not completely resolved from RDX. Also, during these evaluations, tetryl was not detected in any of the prepared samples even though its elution position was relatively free of interference. Method development studies were undertaken to define a sample preparation which provided a relatively clean chromatogram for each of the tissues being studied and gave a sufficient recovery for the munitions, including tetryl.

Tetryl recovery studies conducted included evaluations of the extraction solvent, addition of salt solutions to the matrix to aid in extraction, and the effects of sample handling during the preparation procedure. The solvents evaluated included acetonitrile, toluene, ethyl acetate, and 2% isopropanol in hexane. When high tetryl levels, i.e., 200 µg/ml, were added to plasma and the plasma protein precipitated with acetonitrile, about 60% of the tetryl was recovered. At levels below 10 µg/ml tetryl, no HPLC peak was observed. Similar data were obtained when the plasma was extracted with the organic solvents. To aid in the extraction, 1.0 ml of 20% sodium chloride was added to 1.0 ml plasma aliquots containing 50 µg/ml tetryl. The plasma aliquots were prepared by precipitation or extraction. However, tetryl recovery was not improved. Tetryl appeared to be binding to the plasma protein, co-precipitated with the protein, or enzymatically altered prior to extraction. To evaluate these possibilities, a number of experiments were conducted. When tetryl was added to a saturated albumin solution and the protein precipitated with acetonitrile, 100% tetryl recovery was obtained indicating the co-precipitation was not occurring. Plasma samples were denatured by heating to 100°C or by the addition of acid and tetryl added. After protein precipitation with acetonitrile or organic solvent extraction of the denatured plasma, no tetryl was detected indicating that denatured plasma protein was still capable of adsorbing tetryl and preventing analysis of the munition. The possibility that tetryl was being lost due to improper sample handling was studied by preparing and analyzing a series of tetryl solution from 2.0 to 200 µg/ml. In one series, 1.0 ml water was used as matrix and in another, 1.0 ml plasma was employed. The two series were handled in identical manner, and quantitative recovery was obtained from the water series while little or no tetryl was detected in the plasma series. Tetryl irreversibly binds to plasma protein and cannot be extracted from this matrix except at very high concentration levels, i.e., 200 µg/ml. Short evaluations with each of the other animal tissue matrices indicated that tetryl cannot be assayed in these matrices. A final experiment for tetryl recovery in plasma was conducted using the two sample preparation

techniques most likely to provide some tetryl recovery. These procedures were the acetonitrile precipitation procedure and a toluene extraction method from a 1.0-ml plasma containing 1.0 ml, 10% sodium chloride solution plus 1% acetic acid. Tetryl levels of 2, 10, 50, 100, and 200 µg/ml were evaluated and the results are presented in Table 6. The data show poor recovery at the high level and no recovery at the low levels for both techniques. These studies on tetryl were conducted using HPLC System II as the analytical technique.

During the above experiments to define tetryl recovery from plasma, studies were also conducted to determine a sample preparation technique for the other munitions in plasma. The acetonitrile precipitation procedure provided excellent data on DNT and TNT in plasma; however, RDX could not be determined with this technique. Liquid-liquid extraction was evaluated as an alternate sample preparation technique. A number of organic solvents were evaluated including toluene, ethyl acetate, methylene chloride, and hexane. The hexane did not extract the munitions and the methylene chloride was not efficiently separated from the aqueous phase. Both toluene and ethyl acetate provided positive results. However, ethyl acetate was too polar and extracted a number of plasma components including the compound which co-eluted with RDX. Toluene gave a relatively clean extract, but the recovery of TNT was low. By adding a sodium chloride solution to the plasma, the recovery of TNT was improved. Also, when the plasma was made slightly acidic, TNT recovery was improved. The organic solvent extracts required evaporation of the solvent prior to HPLC analysis using an aqueous mobile phase. To prevent loss of the munitions during this evaporation step, 0.5 ml of the HPLC aqueous phase was added to the extract and the solvent evaporated at room temperature under a stream of nitrogen. After the solvent had been completely removed, the IS in acetonitrile was added and the final volume adjusted to 1.0 ml. A sample preparation procedure for the toluene extraction of RDX, DNT, and TNT from plasma was defined (see 5. "Procedures for Plasma Sample Determination" in Appendix B). However, prior to evaluating this sample preparation procedure in the five duplicate levels on four separate day protocol, a problem with the HPLC System II occurred. During the evaluations of a method for plasma, studies were also being conducted on the other animal tissue matrices. The HPLC System II was providing the necessary resolution of the munition; but the back pressure on the system was high, i.e., 2,500 psi, and increased substantially after injecting a few samples due to buildup of uneluted matrix components on the column. The increased back pressure was alleviated by washing the column with 100% acetonitrile and removing and cleaning the column frits which had become clogged with column particulates. This caused the column life to be shortened and prevented the routine assay of the munitions in the animal matrices. Another HPLC System (III) was developed which resolved the munitions and provided a stabler chromatographic system (the HPLC Spherisorb column packing deteriorates above pH 8 and is stable between pH's 2 and 6.5; the 1% acetic acid aqueous phase gave an eluent with a 3.2 pH). The toluene extraction sample preparation technique and HPLC System III were employed to validate the method for the determination of RDX, DNT, and TNT in plasma. This method is presented in Appendix B, Technical Report No. 1, Method Development of RDX, DNT, and TNT in Plasma.

TABLE 6

TETRYL RECOVERY FROM PLASMA USING TWO SAMPLE
PREPARATION TECHNIQUES

Sample No.	ml Plasma	Tetryl Added	Peak Heights ^b		$\mu\text{g IS}$	$\mu\text{g Tetryl}^c$	% Recovery ^d
			Tetryl	IS		ml Found	
Acetonitrile Precipitation Technique							
A-1	1.0	2	< 4	93.0	1	ND ^e	-
A-2	1.0	10	< 4	39.0	5	ND	-
A-3	1.0	50	< 4	57.0	25	ND	-
A-4	1.0	100	10.0	99.0	50	6.5	6.5
A-5	1.0	200	48.0	72.0	100	85	43

Toluene Extraction Technique

T-1	1.0	2	< 4	172.0	1	ND	-
T-2	1.0	10	< 4	109.0	5	ND	-
T-3	1.0	50	< 4	214.0	25	ND	-
T-4	1.0	100	10.0	228.0	50	2.8	2.8
T-5	1.0	200	64.0	155.2	100	53	26

Reference Solutions

Standard No.	$\mu\text{g/ml}^a$ Tetryl	Peak Height		$\mu\text{g/ml IS}$	RWR ^f
		Tetryl	IS		
S-1	2	108.0	76.0	1	0.71
S-2	10	71.0	55.0	5	0.65
S-3	50	150.0	100.0	25	0.75
S-4	100	166.0	107.0	50	0.78
S-5	200	126.4	76.8	100	0.82

Average 0.78
SD ± 0.07
RSD 8.4%

a $\mu\text{g/ml Tetryl}$ added - micrograms of tetryl added to 1.0 ml plasma

b Peak Heights - measured peak heights in millimeters of tetryl and IS.

c $\mu\text{g/ml Tetryl}$ found - microgram tetryl detected in 1.0 ml plasma after sample preparation

$$\mu\text{g/ml tetryl found} = \frac{\text{Peak Height tetryl}}{\text{Peak Height IS}} \times \frac{\mu\text{g IS}}{\text{Avg. RWR}}$$

d % Recovery - $\mu\text{g/ml tetryl found} / \mu\text{g/ml tetryl added} \times 100$.

e ND - not detectable, tetryl level below 0.1 $\mu\text{g/ml}$.

f RWR - relative weight response

$$\text{RWR} = \frac{\text{Peak Height tetryl std}}{\text{Peak Height IS}} \times \frac{\mu\text{g IS}}{\mu\text{g tetryl std.}}$$

SD - standard deviation; RSD - relative standard deviation

2. PETN in Plasma: The definition of sample preparation techniques for PETN was initiated after the analytical technique HPLC System IV (Section III.B.) had been defined and validated. Each of the sample preparation procedures studied for RDX, DNT, and TNT determination in plasma was evaluated for PETN in plasma. The acetonitrile precipitation procedure proved unsuccessful because many of the plasma components present after the protein precipitation adsorbed at 215 nm and interfered with PETN detection. The four organic solvents (toluene, ethyl acetate, methylene chloride, and hexane) evaluated earlier were studied for PETN extraction from plasma. Only hexane was able to extract PETN without also extracting PETN interfering plasma components. As with the toluene extraction of RDX, DNT, and TNT from plasma, the addition of an acidic (2% acetic acid) salt (20% sodium chloride) solution was found to provide better recovery of PETN from plasma. The validation of the hexane extraction sample preparation procedure for the HPLC System IV determination of PETN in plasma using five duplicate levels on four separate days is presented in Appendix F, Technical Report No. 5, Method Development for PETN in Plasma.

B. Animal Kidney Samples

1. RDX, DNT, TNT, and Tetryl in Kidney: Earlier studies had shown that the acetonitrile precipitation procedure and the ethyl acetate extraction procedure gave HPLC chromatograms with many extraneous kidney matrix peaks which prevented the detection and quantitation of the munition compounds. The use of less polar organic solvent, i.e., hexane, was also unsuccessful due to low recovery of RDX and TNT. The toluene extraction of a kidney sample diluted 1/1 w/v (weight/volume) with a 10% sodium solution containing 2% acetic acid (the plasma sample preparation method) gave relatively clean chromatograms with acceptable recovery for RDX, DNT, and TNT. As with the plasma matrix, tetryl was not recovered from the kidney matrix at the concentration level required, i.e., 100 ng/g. Two additions to the plasma method for RDX, DNT, and TNT were required to determine these munitions in the kidney samples. The first addition was in the initial sample preparation. The kidney samples required liquefaction to disrupt the cells present. This liquefaction was accomplished by first grinding the kidney sample in a standard blender on "liquefy" speed and then using a Teflon-glass, motor-driven tissue homogenizer to disrupt the cell walls and solubilize the intercellular materials. This technique is required to free the munitions that may be present within the kidney cells. The second addition to the plasma method was in the HPLC procedure. A column wash step with 100% acetonitrile was found to be necessary after each sample injection to elute the nonpolar matrix components. The wash eliminated the pressure build-up problem and also removed the matrix compounds still present in the analytical system.

In addition to these changes in the plasma method, the sample extract drying procedure used for the plasma sample was evaluated and modified. The addition of 0.5 ml water to the toluene extract followed by the room temperature evaporation of the toluene resulted in a large HPLC peak just after the elution position of TNT. The peak was attributed to the

incomplete evaporation of the toluene, and sometimes the component interfered with TNT determination. An evaluation of the drying procedure indicated that if no heat was applied to the samples, complete recovery of the munition compounds was possible even if the samples were taken to dryness. By evaporating all the toluene from the kidney sample extracts and adding a small amount of ethyl acetate to the residue and evaporating, the toluene was almost completely removed from the sample and did not cause problems with TNT determination.

The final sample preparation procedure and analysis technique for RDX, DNT, and TNT was validated by analyzing duplicate kidney samples spiked at five levels and matrix blanks on four separate days. These data and the statistical evaluation of the results are given in Appendix C, Technical Report No. 2, Method Development for the Determination of RDX, DNT, and TNT in Kidney.

2. PETN in Kidney: The earlier studies on PETN indicated that extracted kidney samples contained a substantial number of compounds which adsorbed at 215 nm and interfered with PETN. Also, the hexane extraction procedure employed for plasma did not have sufficient polarity to extract PETN from kidney. When more polar organic solvents i.e., toluene, ethyl acetate, were used, the extract contained many 215 nm adsorbing compounds, and PETN was not observed at 2,000 ng/g. By adding a small amount of isopropanol (IPA) to hexane, the polarity of the extracting solvent was slightly increased. An evaluation of 1, 2, 5, and 10% IPA in hexane as extracting solvent showed that 5 and 10% IPA were too polar and 1% IPA not polar enough. The 2% IPA in hexane showed some promise. The chromatography at the PETN and IS elution positions was relatively clean of interference and some PETN was recovered. However, the recovery was low, i.e., about 25%, and attempts to increase the recovery without affecting the chromatography were unsuccessful. Figure 5 presents representative HPLC-UV (215 nm) chromatograms for PETN extracted from 1.0 g kidney with 2% IPA in hexane. The sample preparation procedure consisted of placing a 1.0-g liquefied kidney sample in a Teflon-lined screw cap vial, adding 2.0 ml 10% sodium chloride solution containing 1% acetic acid, and extracting 3 x 5 ml with 2% IPA in hexane. The extracts were combined, evaporated to dryness at room temperature under a stream of nitrogen, and the residue dissolved in HPLC eluent. After filtering the prepared sample through a 0.45- μ Fluoropore filter, a 100- μ l aliquot was injected onto HPLC System IV.

The low recovery of PETN from kidney using 2% IPA in hexane extraction and the poor chromatography obtained with other organic solvents for extraction prevented the development of an analytical method for PETN in kidney at the 100-ng/g level. Additional studies on this matrix are necessary to define a solvent that will quantitatively extract PETN from kidney and to evaluate additional clean-up steps such as adsorption chromatography to remove interfering kidney components from the extract. The time limitation on the present program prevented the necessary studies to determine these parameters.

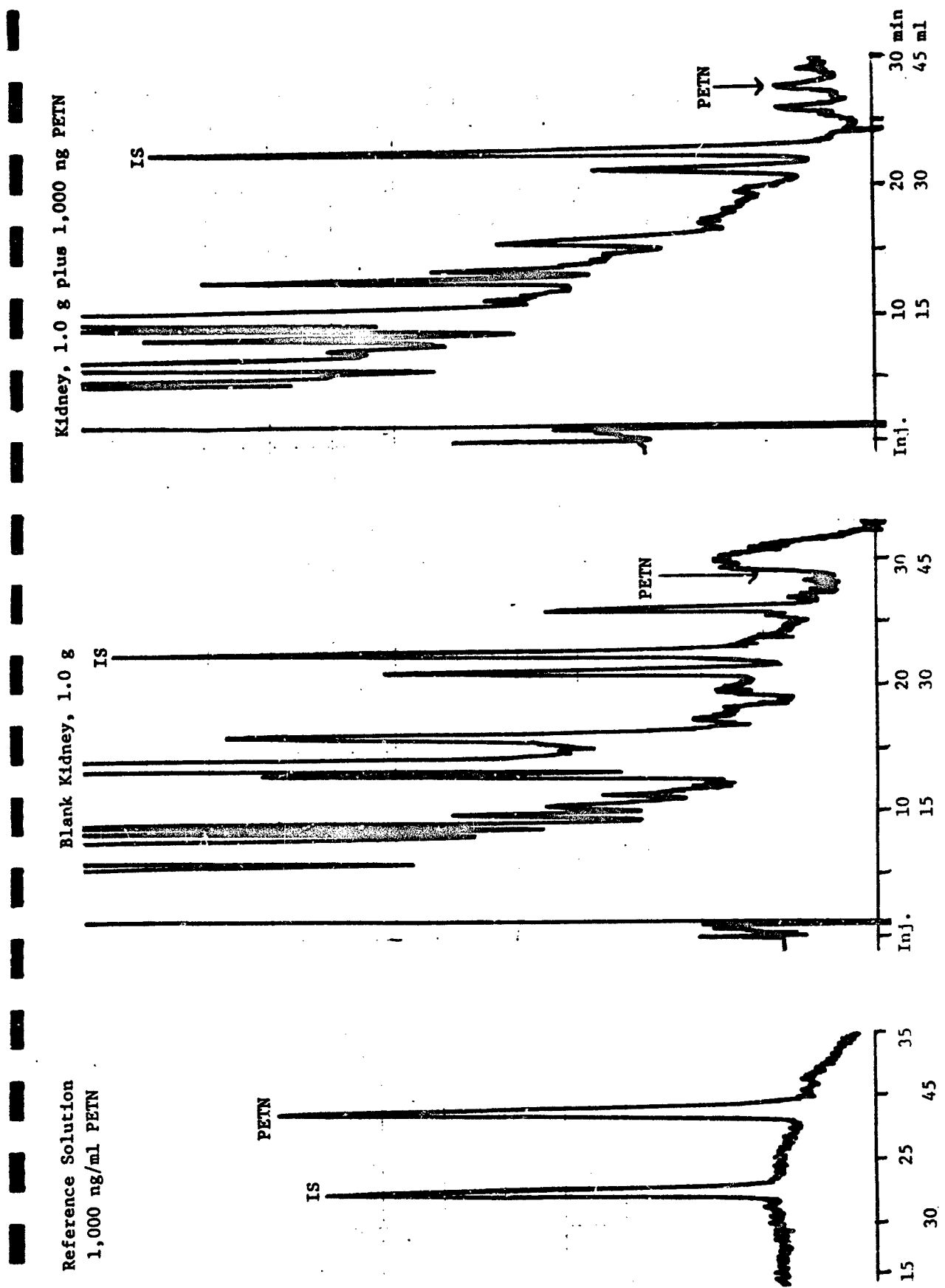


Figure 5 - HPLC-UV (215 nm) Method Development for PETN Determination in Kidney Samples. Sample preparation and HPLC parameters are listed in text. Arrows indicate PETN elution position.

C. Animal Muscle/Fat Samples

1. RDX, DNT, TNT, and Tetryl in Muscle/Fat: Initial evaluation of the plasma method for application to muscle/fat samples indicated that toluene and other nonpolar solvents could not be employed for this matrix because they solubilize the fat component of the matrix. However, acetonitrile, because of its higher polarity, did not dissolve the fat and gave acceptable recovery and chromatography for the munition compounds. Similar to the plasma and kidney matrices, the muscle/fat matrix did not allow recovery of tetryl.

The final sample preparation procedure as outlined in Appendix E, Technical Report No. 3, Method Development for the Determination of RDX, DNT, and TNT in Muscle/Fat Samples was being validated using HPLC System III when the project officer requested that the munition spiking levels be changed from 100, 500, 1,000, 1,500, and 2,000 ng/g to 50, 100, 200, 500, and 1,000 ng/g. This change was requested to aid in the statistical determination of the lower detection limit of the munition by the Hubaux and Vos detection limit program. Since the HPLC system was already operating at close to the highest sensitivity limit, the 1.0-g matrix sample was increased to 2.0 g to provide a similar instrument sensitivity at the newly defined spiking levels. The total procedure, as presented in Appendix D, was validated by analyzing duplicate 2.0-g muscle/fat samples spiked with RDX, DNT, and TNT at 0, 50, 100, 200, 500, and 1,000 ng/g on four separate days.

2. PETN in Muscle/Fat: The evaluations of sample preparation procedures for RDX, DNT, and TNT in the muscle/fat matrix had shown that nonpolar organic solvents, i.e., hexane, could not be employed to extract the munition compound from this matrix. For PETN in muscle/fat, the acetonitrile procedure utilized for the other munitions was evaluated. This procedure proved unsatisfactory in that the HPLC-UV (215 nm) chromatogram contained many extraneous peaks and the recorder pen was off-scale for the first 50 min of the chromatographic run. When the attenuation was increased to provide on-scale peaks, the possible sensitivity for PETN determination was greater than 5 µg/g. Even though hexane solubilized the fat in the matrix, a study was conducted to evaluate this extraction technique. As expected, the dried hexane extract contained a substantial level of fat which was not solubilized when 0.5 ml acetonitrile containing the IS was added. The sample was diluted with water (0.5 ml) to provide a sample compatible with the HPLC eluent, filtered, and analyzed. No PETN was detected even though the matrix had been spiked at 2,000 ng/g; however, the elution position of PETN was relatively free of interfering muscle/fat components. The PETN was most likely still with the undissolved fat. Additional studies are necessary to define methodology to separate the PETN from the fat. Possible techniques for this separation include adsorption or gel permeation column chromatography or a liquid-liquid extraction of the hexane muscle/fat solution using a solvent that can extract the PETN without extracting the fat components present in the hexane solution. The time limitation on the present program prevented evaluation of these sample preparation techniques.

D. Animal Liver Samples

1. RDX, DNT, TNT, and Tetryl in Liver: The initial evaluations of sample preparation procedures for the determination of RDX, DNT, TNT, and tetryl in the animal liver matrix indicated that the toluene extraction technique employed for the plasma and kidney matrices was the most promising. The other techniques evaluated, including acetonitrile, ethyl acetate, and hexane extraction, either extracted too many liver components which interfered with the HPLC determination of the munitions or gave poor recoveries. Studies were initiated to validate the toluene extraction technique for the liver matrix using duplicate samples spiked at five levels on four separate days. Three separate days of liver sample sets were analyzed and the results summarized in Table 7 (the raw data and calculations are given in Appendix A, Tables 5-A, 6-A, and 7-A). At this time, the spiking levels being employed on the program were 0, 100, 500, 1,000, 1,500, and 2,000 ng/ml. The data for Day 1 showed acceptable recovery for RDX, DNT, and TNT (as with the other animal matrices, no tetryl was detected at any of the spiking levels) with good linearity of recovery over the concentration range. However, the Day 2 and Day 3 data for DNT and TNT varied considerably from Day 1 results and were not linear within a day set. Figure 6 presents HPLC chromatograms of 1.0-g liver samples spiked at the 1,000-ng/g level with the munitions from the Day 1 and Day 2 sets. A substantial decrease in the recovery of DNT and TNT in the Day 2 liver sample is apparent. Since the data from Day 1 was acceptable, a systematic error in the sample preparation procedure was considered to be the cause for the variation in the results. Evaluations of the sample preparation procedure were conducted to define the source of error and to eliminate the problem.

The first aspect of the sample preparation procedure evaluated was the effect of the pH of the aqueous phase on the extractability of the munitions from liver. The original protocol had a 1.0-ml 10% sodium chloride solution containing 1% acetic acid added to 1.0 g liver to aid in the extraction; the pH of the aqueous phase under these conditions was approximately 4. To determine if the pH was important, duplicate liver samples containing 1,000 ng/g each munition were prepared and buffered to pH's 2, 4, 6, and 8 with 1.0 ml, 10% sodium chloride plus 0.1 M sodium acetate at the appropriate pH. Each solution was extracted 3 x 3 ml with toluene and analyzed by HPLC as described earlier. The results showed consistent, but low for DNT and TNT, recovery of the munitions for the acidic solutions and very poor chromatography for the basic liver samples. An acidic solution was required for extraction; however, pH was not the apparent cause of the lower recoveries of DNT and TNT.

The rationale for using sodium chloride solutions during extraction was to "salt out" the compounds being extracted. This aspect of the sample preparation procedure was studied by preparing 1,000 ng/g each munition liver samples with the following salt solutions (all contained 1% acid): (1) 0% sodium chloride; (2) 10% sodium chloride (normal procedure, served as control for experiment); (3) 20% sodium chloride; (4) 10% ammonium acetate; (5) 10% sodium dihydrogen phosphate plus phosphoric acid; and (6) 10% sodium sulfate. The results of this study indicated that salt aided in the extraction of the munitions but the salt type or concentrations above 10% were

TABLE 7

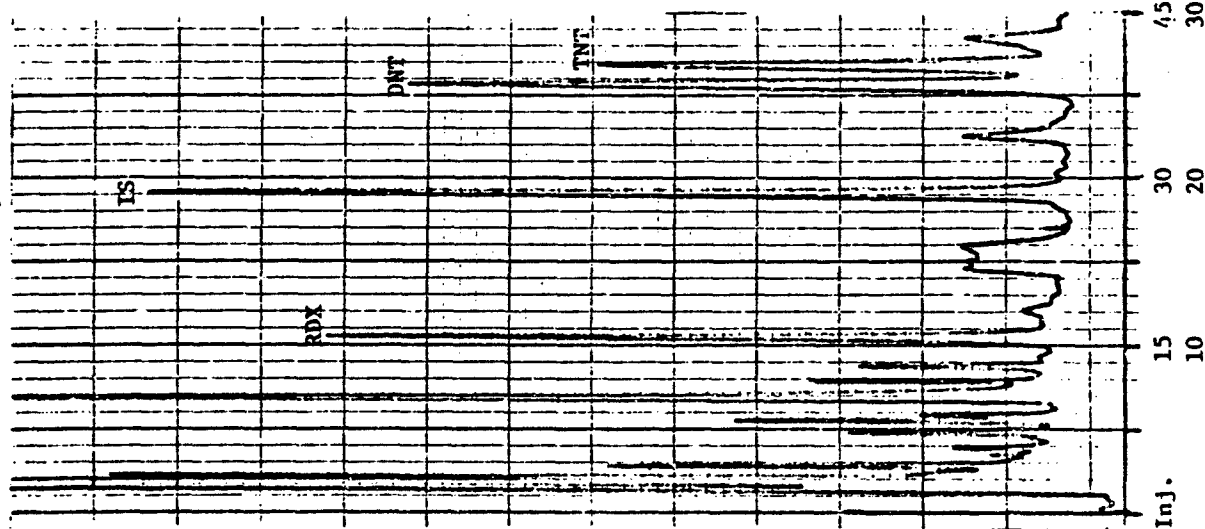
HPLC-UV DETERMINATION OF RDX, DNT, AND TNT IN LIVER SAMPLES
INITIAL EVALUATION OF TOLUENE EXTRACTION METHOD

Sample Description	g Liver	ng/g Munition Added	RDX ng/g Found ^a			DNT ng/g Found ^a			TNT ng/g Found ^a		
			Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
A-0	1.0	0	105	110	106	ND ^b	ND	ND	ND	ND	ND
B-0	1.0	0	66	102	131	ND	ND	ND	ND	ND	ND
A-100	1.0	100	168	164	134	64	41	52	61	46	53
B-100	1.0	100	153	146	233	80	52	70	55	37	59
A-500	1.0	500	538	541	516	328	194	240	275	157	191
B-500	1.0	500	527	588	569	331	274	202	282	148	151
A-1000	1.0	1,000	1,024	1,031	1,020	662	420	549	643	265	384
B-1000	1.0	1,000	1,018	1,064	1,006	752	522	345	637	321	332
A-1500	1.0	1,500	1,540	1,420	1,496	1,021	901	699	991	532	507
B-1500	1.0	1,500	1,494	1,548	1,530	1,029	786	690	892	519	893
A-2000	1.0	2,000	1,901	1,971	1,927	1,322	1,065	930	1,316	664	629
B-2000	1.0	2,000	1,977	1,930	1,964	1,467	1,028	1,076	1,286	687	1,260

^a ng/g Found - nanograms each munition found, determined using the relative weight response to an internal standard method.

^b ND - Not detectable, less than 20 ng/g.

Day 1 Liver Sample



HPLC Conditions

Column: Spherisorb ODS, 5 μ ,
 250 x 4.6 mm ID
 Eluent: 30% acetonitrile in
 1% acetic acid in water
 Flow Rate: 1.5 ml/min
 Chart Speed: 0.1 in./min
 Detector: UV, 254 nm

Sample Characteristics

Day 1 and Day 3 1.0 g liver
 sample containing 1,000 ng/g
 each munition, diluted with
 1 ml 10% NaCl plus 1% acetic
 acid, extracted with 3 x 3 ml
 toluene. Toluene extracts
 evaporated to dryness and
 residue dissolved in 1.0 ml
 HPLC eluent.

IS Concentration: 1,000 ng/ml
 Injection Volume: 70 μ l
 Attenuation: 0.01 X

Day 3 Liver Sample

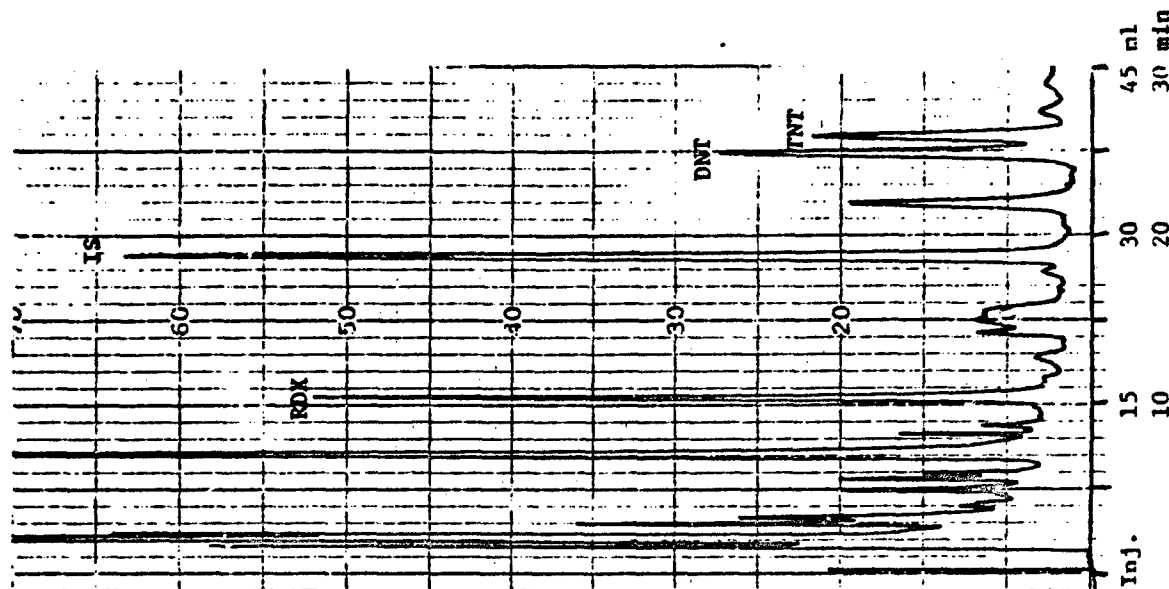


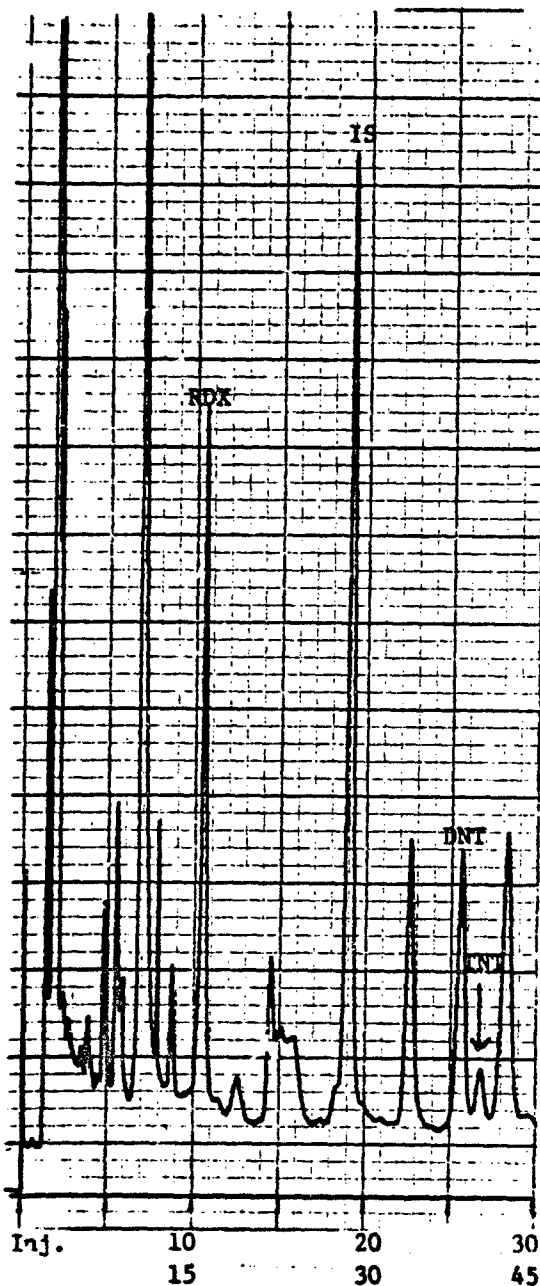
Figure 6 - HPLC Method Development for RDX, DNT, and TNT Determination in Animal Liver. Day 1 and Day 3 1.0 g liver samples containing 1,000 ng/g each munition prepared using the toluene extraction procedure (initial evaluation).

not important. Very low recovery was observed for the 0% salt solution and consistent, but low, recovery was found for the other samples. The addition of 1.0 ml, 10% sodium chloride containing 1% acetic acid aided in the extraction of the munitions from liver; however, the salt solution was not the cause of the systematic error.

Toluene had been employed as the extraction solvent for plasma, kidney, and the Day 1 liver matrices with acceptable recovery of the munitions. The possibility that a slightly more polar solvent was required for uniform extraction of the munitions from liver was explored by evaluating a number of other solvents. The solvents evaluated included toluene (normal method, control); acetonitrile; 2% IPA in toluene; 2%, 3%, 4%, 5%, and 20% IPA in hexane, and 50:45:5 v/v/v toluene:hexane:IPA. Representative HPLC chromatograms for some of these solvent evaluations are shown in Figures 7 and 8. The results indicate that none of these solvents improved the recovery of DNT and TNT without affecting the chromatography. The data did show that a polar solvent, i.e., acetonitrile, ethyl acetate, gave better recovery; however, RDX determination was affected (see Figure 7). Another study was conducted to evaluate the possible use of a double extraction of the liver matrix, first with a polar solvent to obtain acceptable DNT and TNT recovery, and after drying the first extract, with a second less polar solvent such as toluene or 2% IPA in hexane to isolate RDX from the early eluting interference. The polar solvents evaluated were acetonitrile, ethyl acetate, and methanol and the second solvents evaluated were toluene and 2% IPA in hexane. The results showed relatively clean chromatograms for each solvent pair; however, the recoveries of the munition added to the liver were not improved and, in most cases, were lower than with the toluene extraction procedure. The experiments conducted had shown that the inconsistent results obtained for the recovery of DNT and TNT from the liver matrix were not due to the extraction solvent being employed.

The next aspect evaluated was the toluene evaporation and residue reconstitution steps. After drying the toluene from the liver extract, a substantial amount of liver components was present, and this residue was not completely solubilized with 0.5 ml acetonitrile. The poor recoveries of DNT and TNT may have been caused by adsorption of the munitions on these components. Experiments were designed to determine if all the munitions extracted with the toluene were solubilized. These studies utilized vortex mixing and ultrasonication for various times and liver samples spiked with the munitions prior to extraction and after extraction. The results showed quantitative recovery, i.e., greater than 90%, for the munitions from the toluene spiked after extraction while low DNT and TNT recoveries were observed for the livers spiked prior to extraction. While vortexing and ultrasonication aided in solubilizing the residues from the toluene liver extracts, the reconstitution procedure was not the cause of the inconsistent data for DNT and TNT from liver.

1.0 g liver plus 1,000 ng/g each
munition extracted with toluene
(normal procedure)



1.0 g liver plus 1,000 ng/g each
munition extracted with acetonitrile

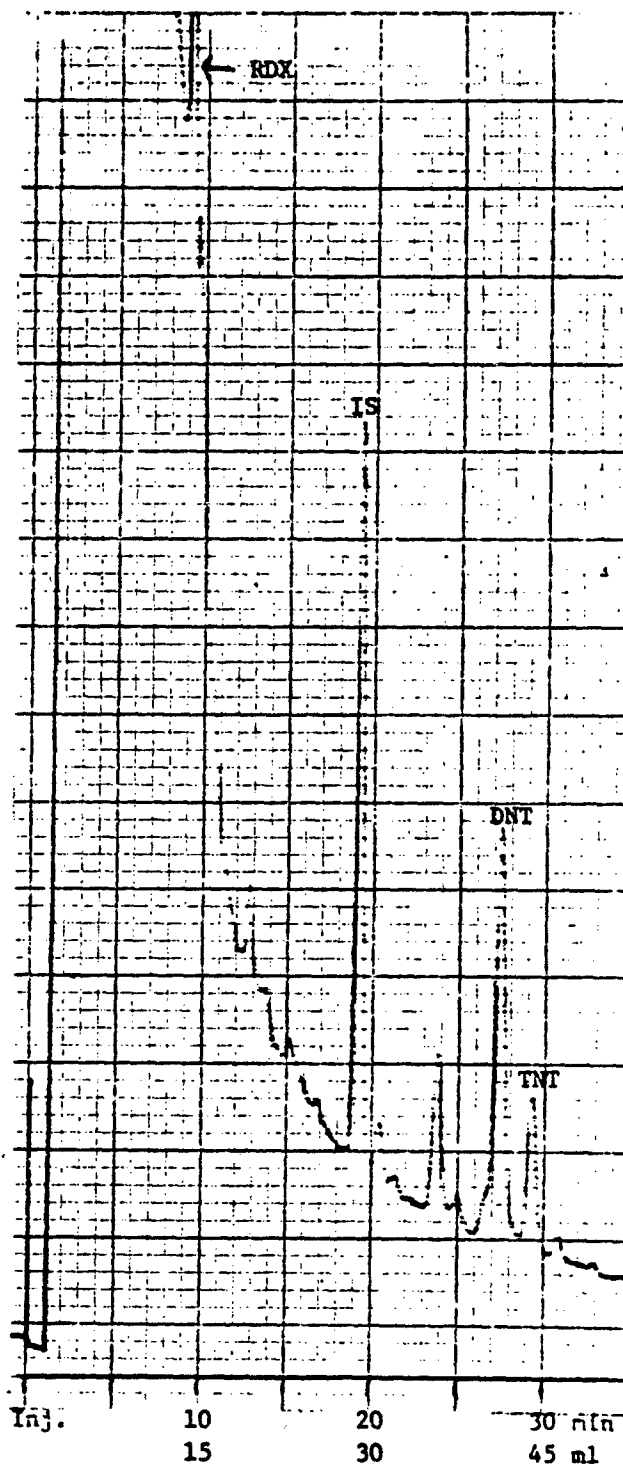


Figure 7 - HPLC Chromatograms of RDX, DNT, and TNT Extracted from Liver with Various Solvents. Difference in elution parameters for the munitions attributed to column change and fresh eluent. HPLC conditions as listed in Figure 6.

1.0 g liver plus 400 ng/g each munition
extracted with 2% IPA in hexane

1.0 g liver plus 400 ng/g each munition
extracted with 2% IPA in toluene

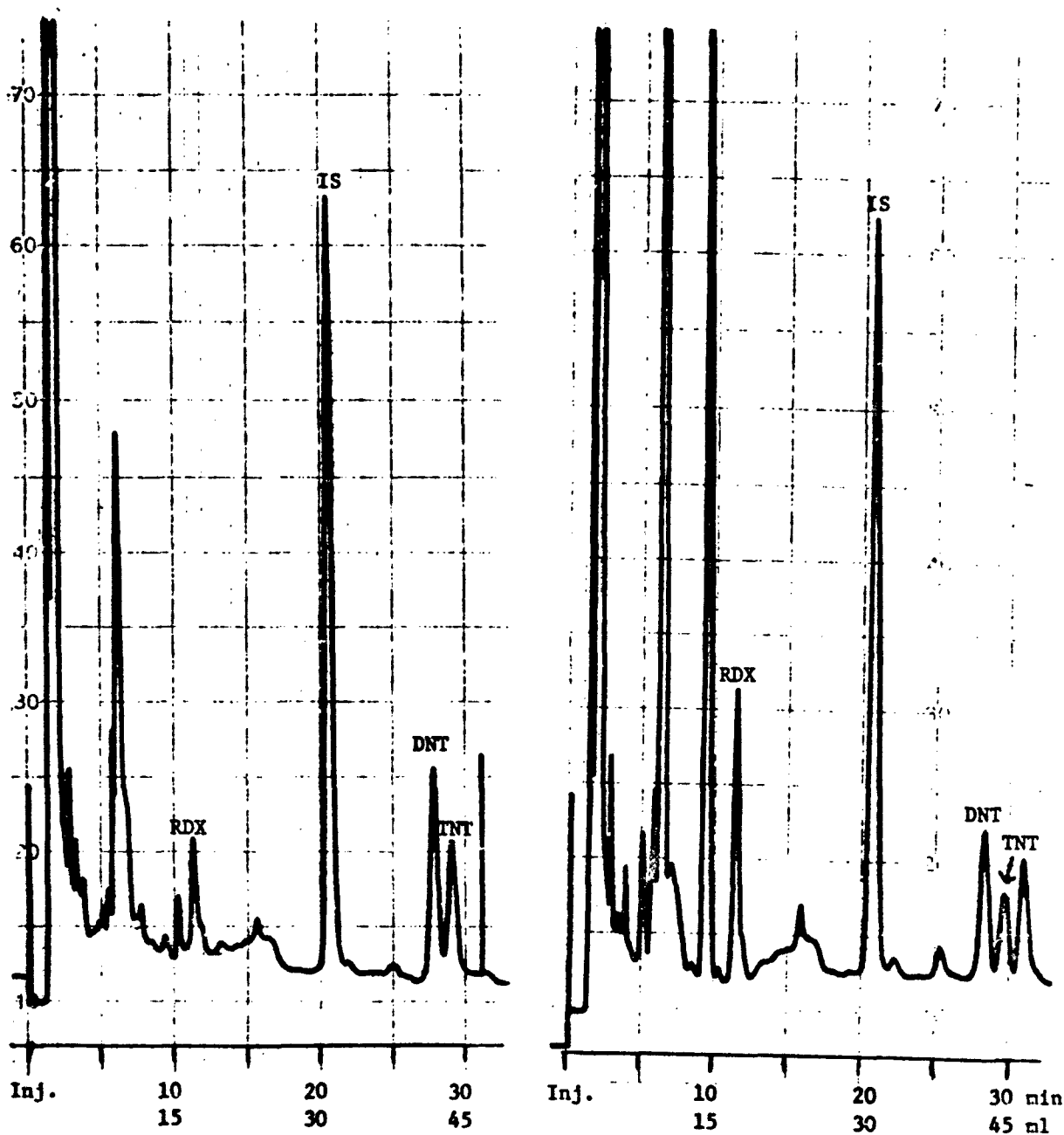


Figure 8 - HPLC Chromatograms of RDX, DNT, and TNT Extracted from Liver with Various Solvents. Differences in elution parameters for the munitions attributed to column change and fresh eluent. HPLC conditions as listed in Figure 6.

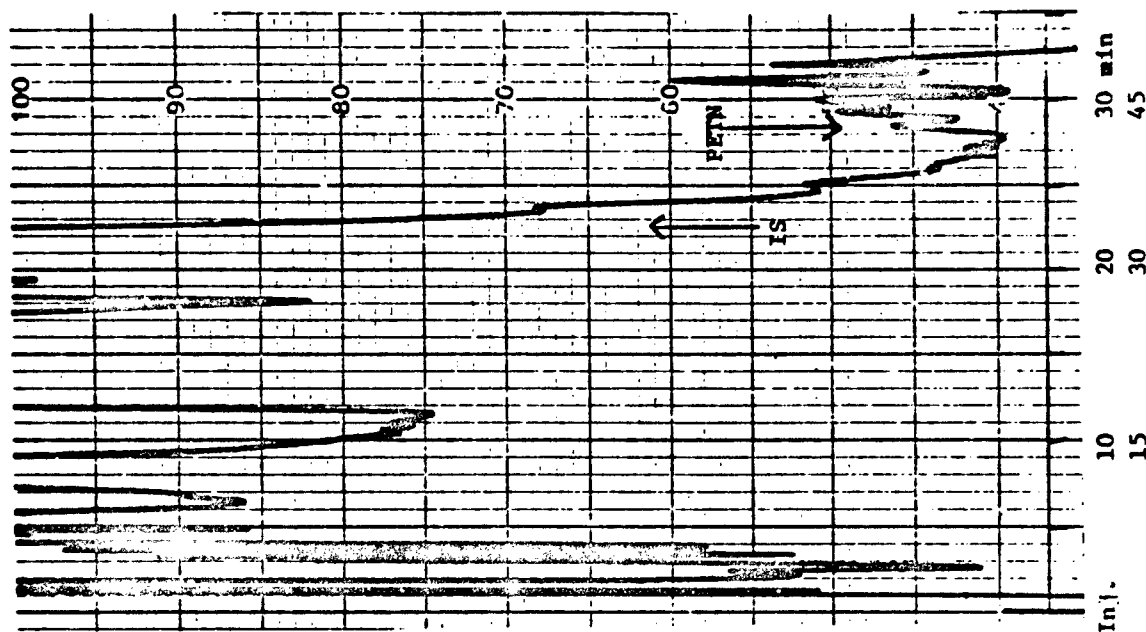
Each of the various parameters of the sample preparation procedure had been evaluated without defining the systematic error which gave varying recoveries for DNT and TNT from liver. Careful review of the entire procedure indicated that the extraction efficiency of the toluene for the liver matrix may not be as high as in the other animal tissues. The formation of extraction emulsions with biological tissues is common and usually undesirable. During the extraction procedure, care had been taken to minimize the emulsion, and this may have prevented optimal extraction of the munitions. When liver samples containing the munitions were mixed with toluene in a manner which guaranteed the formation of a complete emulsion and were then centrifuged to separate the phases, recoveries of DNT and TNT were similar to the Day 1 results presented in Table 5. The emulsion resulted in a longer centrifugation time, i.e., 40-50 min, to separate the phases; however, the extractability of the munitions required the close contact of the toluene and aqueous phases. During these evaluations, the spiking level for the munition in a matrix had been changed. Also, the analytical system had been modified from System II to System III. The final sample preparation procedure and HPLC System III were validated for RDX, DNT, and TNT in liver samples by analyzing duplicate samples spiked at 0, 50, 100, 200, 500, and 1,000 ng/g on four separate days. The method is presented in Appendix E, Technical Report No. 4, Method Development for the Determination of RDX, DNT, and TNT in Animal Liver Samples.

2. PETN in Liver: The results obtained from the earlier studies to define a sample preparation procedure for PETN in kidney samples had shown that 2% IPA in hexane provided some recovery and had acceptable chromatography in the elution region of the IS and PETN. Similar low recovery results, as shown in Figure 9, were obtained for PETN in liver using this extraction technique. When the polarity of the extracting solvent was increased, the chromatography deteriorated and interfering liver components prevented the detection of PETN (also shown in Figure 9). Additional studies are necessary to define a procedure to extract PETN from the liver samples followed by additional clean-up to isolate the PETN from co-extracted liver components.

E. Plant Leaves

1. RDX, DNT, TNT, and Teteryl in Plant Leaves: The knowledge gained during the sample preparation procedure evaluations for RDX, DNT, TNT, and teteryl in animal tissue matrices was applied to defining a protocol for plant leaves. The plant leaves matrix included grass and soft stem plant leaves. Since the availability of this matrix is greater, a larger sample size, i.e., 5 g, was chosen for evaluation. Initial evaluations indicated that polar solvents, i.e., acetonitrile, ethyl acetate, cannot be employed for this matrix as they extracted many plant components. Less polar solvents such as toluene, hexane, and 2% IPA in hexane showed promise and were evaluated further. The animal tissue matrices had utilized a salt solution containing 1% acid to aid in the extraction efficiency. Both the extracting solvent and the aqueous phase parameters were evaluated for plant leaves. Experiments with 0, 10, and 20% sodium chloride containing 1% acetic acid added to 5 g plant leaves both blank and spiked with 200 ng/g each munition and extracted with toluene, hexane, or 2% IPA in hexane were conducted.

1.0 g liver containing 2,000 ng/g PETN
extracted with toluene



1.0 g liver containing 2,000 ng/g PETN
extracted with 2X IPA in hexane

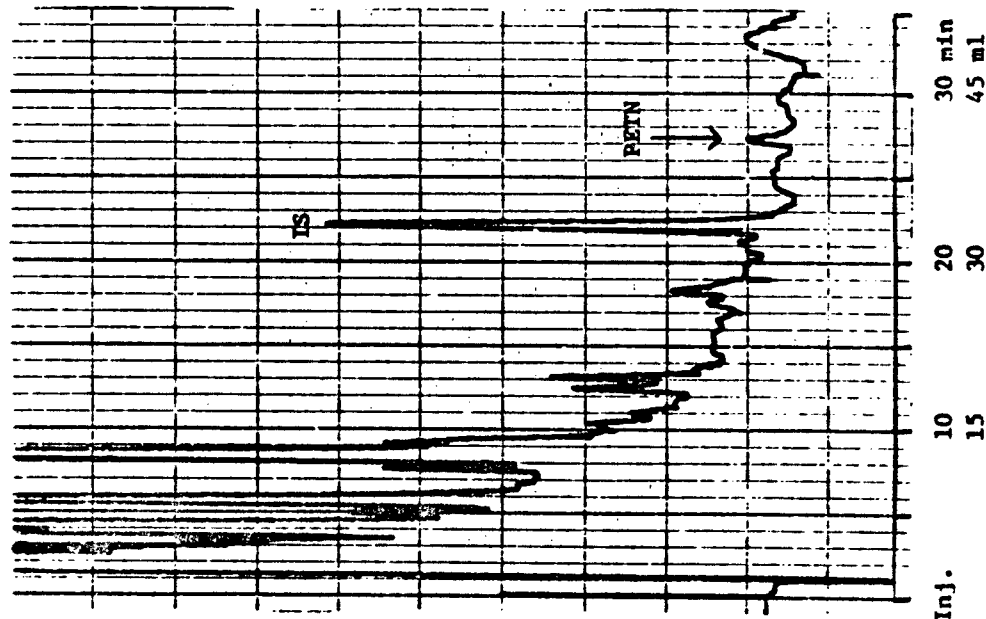
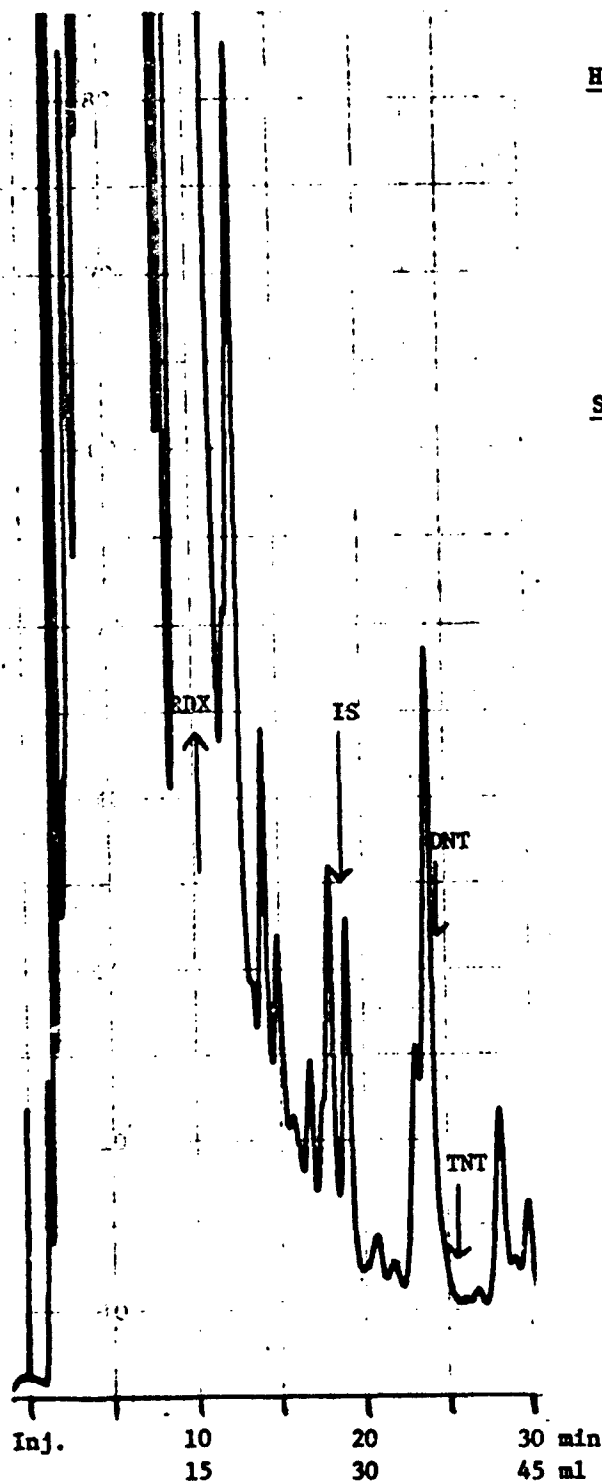


Figure 9 - HPLC Chromatograms for PETN Method Development in Liver.
HPLC conditions as described for Figure 5.

Figure 10 shows the HPLC chromatogram for 10% sodium chloride (5 ml) added to 5 g leaves and extracted with 20 ml toluene. A 10-ml aliquot of the toluene (2.5-g equivalents of plant leaves) was dried, dissolved in HPLC eluent, filtered, and analyzed. The elution positions of the various munitions are indicated by arrows and RDX and DNT have co-eluting leaf compounds. Figure 11 presents an HPLC chromatogram for a 5.0-g leaf sample containing 200 ng/g each munition, diluted with 5 ml 20% sodium chloride plus 1% acetic acid, and extracted with 20 ml 2% IPA in hexane. A 10-ml aliquot of the hexane was taken for analysis. As with the toluene extract, interferences are present at the elution position of RDX and DNT; however, TNT is detected. No tetryl was observed in any plant leaf extracts. Similar chromatograms were obtained for the other conditions.

The plant leaves interferences present in the HPLC chromatograms from toluene and hexane extract prevented the use of HPLC System III for the assay of RDX, DNT, and TNT in this matrix. An HPLC eluent study was undertaken to determine if the munitions could be separated from the interferences. By lowering or raising the percentage of acetonitrile in the eluent, the retention indices of the munitions were changed. RDX was not separated from the large interfering peak with any HPLC eluent evaluated. By lowering the acetonitrile percentage from 30% to 25%, partial separation of DNT and the plant component (X-1 on Figure 11) was achieved. However, under these conditions, TNT and another plant component (X-2 on Figure 11) co-eluted. This HPLC separation is shown in Figure 12. Additional studies are necessary to define a sample preparation procedure to isolate the munitions from plant matrix components.

3. PETN in Plant Leaves: The possible use of a simple extraction technique for the HPLC-UV (215 nm) determination of PETN in plant leaves, was evaluated using a variety of organic solvents. The plant leaves were prepared by grinding in a Waring-type blender, weighing 5-g aliquots into a 50-ml centrifuge tube, and adding 5 ml 10% sodium chloride containing 1% acetic acid. The organic solvents selected for evaluation were hexane, 1% and 2% IPA in hexane, 1/1 (v/v) hexane methylene chloride, methylene chloride, and toluene. These solvents were chosen to provide a slight polarity increase with each solvent, to define a system which extracted PETN and only limited plant leaf material. Each solvent was evaluated by preparing duplicate 5-g leaf samples spiked with 0, 100, and 1,000 ng/g and extracting with 20 ml of solvent. A 10-ml aliquot of the extracting solvent (2.5 g leaves equivalent) was evaporated to dryness, reconstituted with 1.0 ml HPLC eluent containing the IS, and analyzed by HPLC System IV. Figure 13 shows chromatograms for a reference PETN solution and a 5-g leaf sample containing 1,000 ng/g PETN extracted with 2% IPA in hexane. The chromatograms obtained from the other solvents evaluated were similar or worse than that shown in Figure 13. Additional studies are necessary to define a sample preparation procedure to isolate PETN from plant leaves. A more detailed procedure than simple extraction will be required, and the time limitations on the present program prevented the complete evaluation of more elaborate techniques.



HPLC Parameters

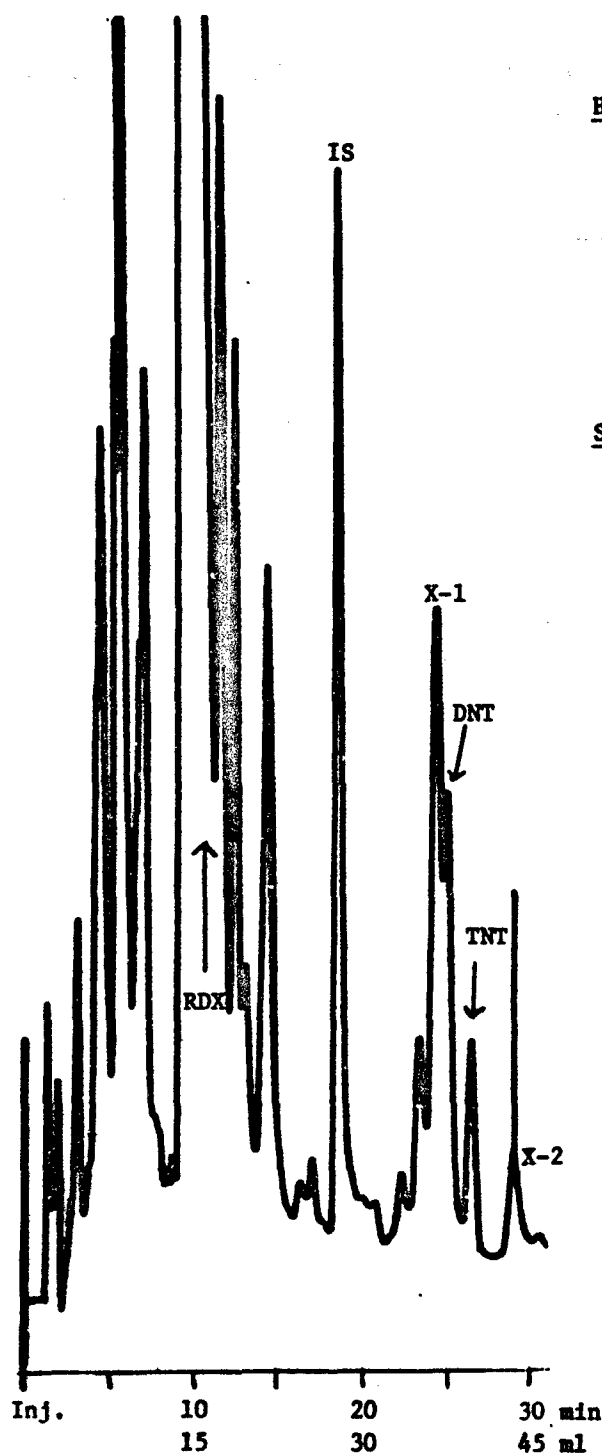
Column: Spherisorb ODS, 5 μ ,
 250 x 4.6 mm ID
 Eluent: 30% acetonitrile in water
 containing 1% acetic acid
 Flow Rate: 1.5 ml/min
 Chart Speed: 0.1 in./min
 Detector: UV, 254 nm

Sample Characteristics

5.0 g Plant leaves plus 5.0 ml 10%
 sodium chloride solution plus 1%
 acetic acid extracted with 20 ml
 toluene. A 10-ml aliquot of toluene
 was evaporated to dryness, recon-
 stituted with 1.0 ml HPLC eluent,
 and injected.

IS Concentration: 0
 Injection Volume: 70 μ l
 Attenuation: 0.01 X

Figure 10 - HPLC Chromatogram of Blank Leaf Sample (5.0 g) Extracted with Toluene.
 Arrows indicate the elution position of the munitions and IS.



HPLC Parameters

Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID

Eluent: 30% acetonitrile in water
containing 1% acetic acid

Flow Rate: 1.5 ml/min

Chart Speed: 0.1 in./min

Detector: UV, 254 nm

Sample Characteristics

5.0 g Leaves containing 200 ng/g
each munition plus 5.0 ml 20%
sodium chloride solution contain-
ing 1% acetic acid extracted with
20 ml 2% IPA in hexane. A 10-ml
aliquot of hexane was evaporated
to dryness, reconstituted in 1.0 ml
HPLC eluent containing the IS, and
injected.

IS Concentration: 1,000 ng/ml

Injection Volume: 70 μ l

Attenuation: 0.01 X

Figure 11 - HPLC Chromatogram of 5.0 g Leaves Containing 200 ng/g Each Munition
Extracted with 2% Isopropanol in Hexane. Munition elution positions indicated
by arrows. "X" denotes leaf components.

HPLC Conditions

Column: Spherisorb ODS, 5 μ , 250 x 4.6 mm ID
Eluent: 25% acetonitrile in water containing
1% acetic acid
Flow Rate: 1.5 ml/min
Chart Speed: 0.1 in./min
Detector: UV, 254 nm

Sample Characteristics

5.0 g Leaves containing 200 ng/g each munition plus
5.0 ml 20% sodium chloride solution containing 1%
acetic acid extracted with 20 ml 2% IPA in hexane.
A 10-ml aliquot of hexane was evaporated to dryness,
reconstituted in 1.0 ml HPLC eluent containing the
IS, and injected.

IS Concentration: 1,000 ng/ml
Injection Volume: 70 μ l
Attenuation: 0.01 X

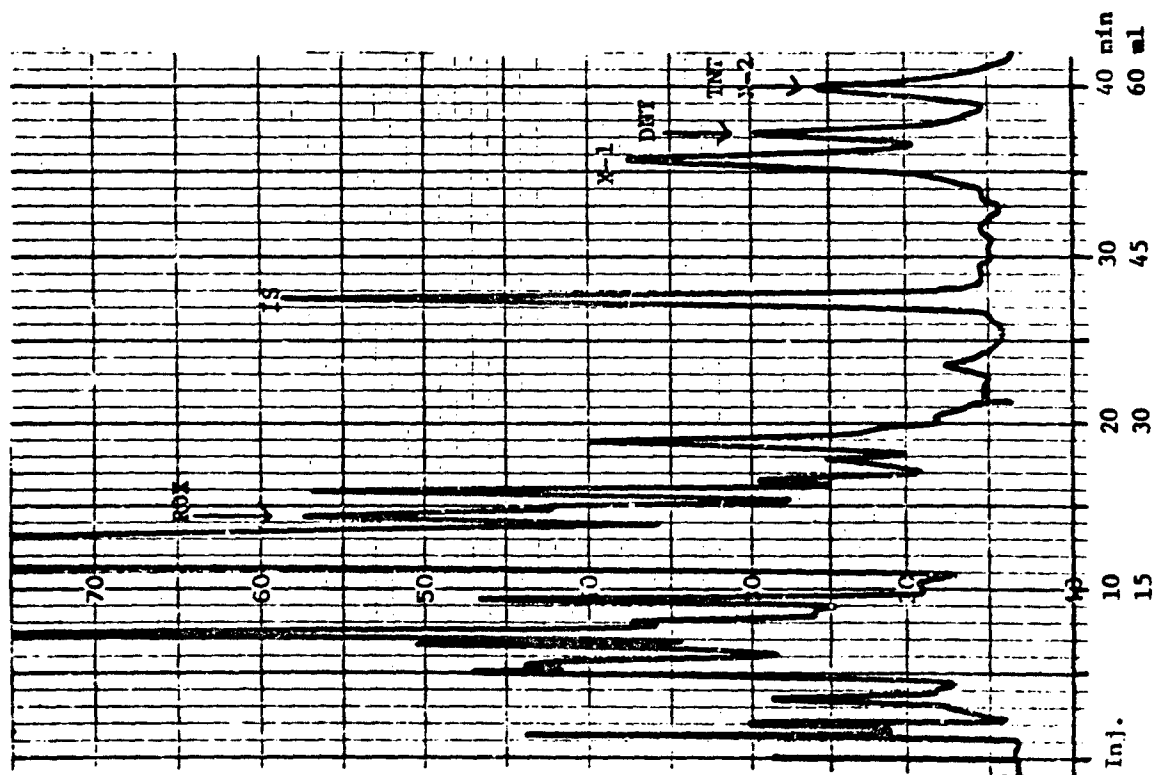
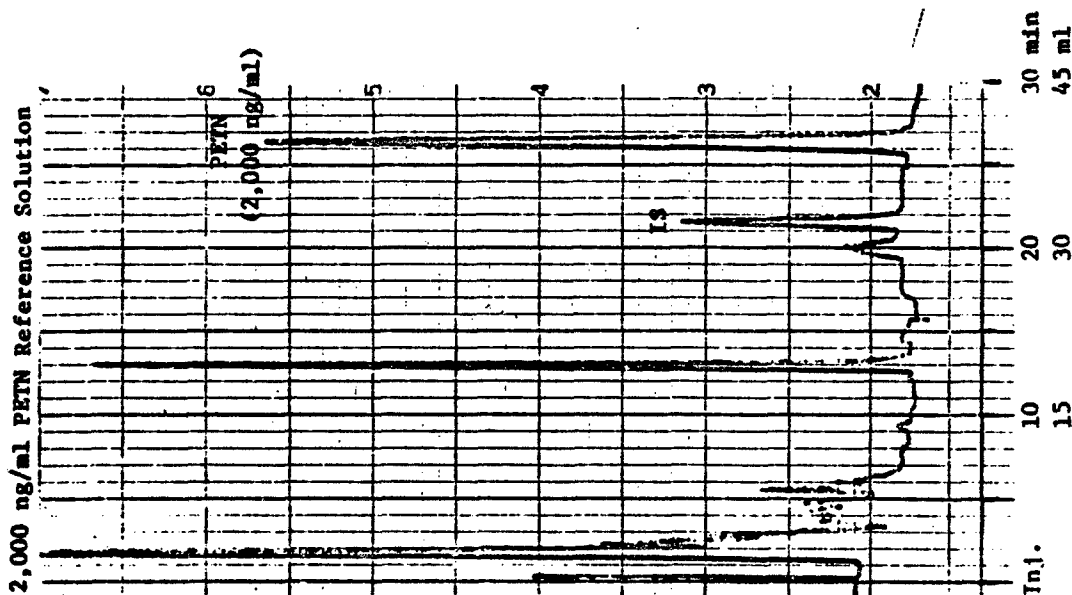


Figure 12 - HPLC Chromatogram of Plant Leaves Containing Munitions and Extracted with Toluene Plus 2% IPA.
"X" denotes leaf components.



5.0 g Leaves plus 1,000 ng/g PETN extracted with 20 ml
2% IPA in hexane; 10-ml extract taken for assay

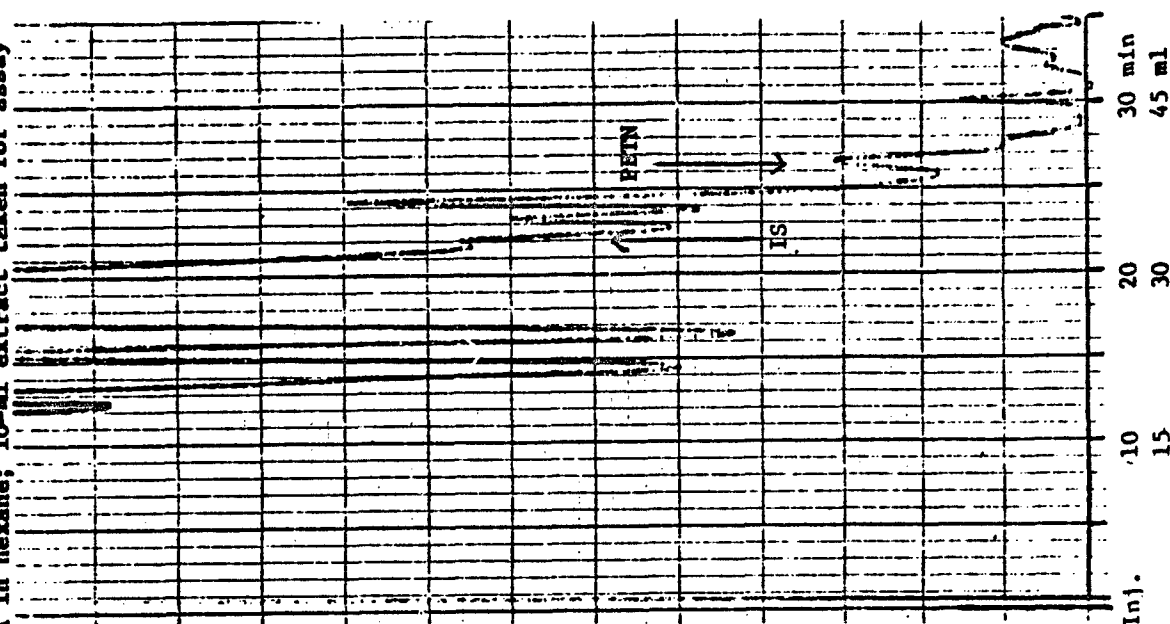


Figure 13 - HPLC-UV (215 nm) System IV Analysis of 2% IPA in Hexane Extracted Leaves for Method Development for PETN Determination. Sample preparation as outlined in text.

F. Plant Stems

1. RDX, DNT, TNT, and Tetryl in Plant Stems: The results obtained during the sample preparation procedure evaluations for RDX, DNT, TNT, and tetryl in plant leaves was applied to studies for these munitions in the plant stems matrix. The plant stems matrix was defined to include plants with soft stems; no grass or hard stem plants, i.e., trees, were included. The plant leaves data had shown the 2% IPA in hexane and toluene were the optimal extracting solvents and these two solvents were evaluated. The leaves matrix component which interfered with DNT was not present in the stems matrix; however, RDX co-eluted with a large peak preventing its determination and no peak was detected at the tetryl elution position. The 2% IPA in hexane solvent gave clearer chromatograms than toluene and was selected for additional study. The nature of the plant stems matrix prevented efficient grinding of the matrix in the blender and an alternate procedure was required to prepare the matrix for extraction. The technique which provided the best grinding of the stems matrix consisted of freezing the stems and using dry ice to maintain a frozen matrix during grinding. The sample was then allowed to thaw and weighed. The single extraction technique described in Section E.2. was employed using 2% IPA in hexane, and DNT and TNT were recovered at about 50%. The sample preparation procedure and HPLC System III were validated for DNT and TNT in the plant stem matrix by analyzing duplicate 5.0-g samples spiked at 0, 50, 100, 200, 500, and 1,000 ng/g each munition on four separate days. This method is presented in Appendix G, Technical Report No. 6, Method Development for the Determination of DNT and TNT in Plant Stems.

2. PETN in Plant Stems: The possibility of determining PETN in the plant stems matrix was evaluated using the same techniques employed for the plant leaves matrix. Only hexane and 2% IPA in hexane were evaluated since the other solvents studied earlier had extracted many interfering compounds from the plant leaves. Unless more promising results were obtained with hexane and 2% IPA in hexane, these solvents were not considered as being able to extract the plant stems without the interferences also being present. The HPLC-UV (215 nm) chromatograms obtained from the hexane and 2% IPA in hexane extraction of PETN from plant stems were similar to the chromatogram presented in Figure 13. Additional studies are necessary to define a procedure to isolate PETN from the co-extracted plant stems components before HPLC-System IV can be utilized to determine PETN in this matrix.

APPENDIX A

RAW DATA AND CALCULATIONS FOR FINAL REPORT

TABLE A-1

HPLC LINEARITY OF RDX INTERIM STANDARD SOLUTION

Solution No.	Injection No.	ng a/ ml RDX	Peak b/ Height RDX	ng a/ ml IS-1	Peak b/ Height IS-1	ng a/ ml IS-2	Peak b/ Height IS-2	PH ^{c/} Ratio		RWR ^{d/}	
								IS-1	IS-2	IS-1	IS-2
1	1	10,620	3,000	5,040	2,520	9,880	2,940	1.19	1.02	0.565	0.949
	2		3,290		2,700		3,120	1.22	1.05	0.578	0.981
2	1	5,310	1,460	5,040	2,420	9,880	2,780	0.603	0.525	0.572	0.977
	2		1,520		2,600		2,980	0.585	0.510	0.555	0.949
3	1	1,062	300	5,040	2,520	9,880	2,860	0.119	0.105	0.565	0.976
	2		300		2,580		2,960	0.116	0.101	0.552	0.943
4	1	531	154	252	130	494	148	1.18	1.04	0.562	0.968
	2		162		135		153	1.20	1.06	0.570	0.985
5	1	266	78	252	134	494	153	0.582	0.510	0.552	0.947
	2		80		138		157	0.580	0.510	0.549	0.946
6	1	106	32	252	150	494	156	0.213	0.205	0.507	0.956
	2		32		153		157	0.209	0.204	0.497	0.950
7	1	53	16	252	158	494	154	0.101	0.104	0.482	0.968
	2		18		160		155	0.113	0.116	0.535	1.08
8	1	26	-	252	152	494	172	-	-	-	-
	2		-		156		177	-	-	-	-

a/ ng/ml RDX, ng/ml IS-1, and ng/ml IS-2 - nanograms per milliliter of RDX, IS-1 (propilphenone) and IS-2 (butyrophene) present in each solution.

b/ Peak Height RDX, IS-1, and IS-2 - measured peak heights of RDX, IS-1, and IS-2 in millimeters at an attenuation of 0.005 O.D.

c/ PH Ratio IS-1 and IS-2 - peak height ratio of RDX to the internal standards.

d/ RWR IS-1 and IS-2 - relative weight response of RDX to IS-1 and to IS-2.

$$RWR = \frac{\text{Peak Height RDX}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml RDX}}$$

TABLE A-2

HPLC LINEARITY OF DNT INTERIM STANDARD SOLUTION

Solution No.	Injection No.	ng a/ ml DNT	Peak b/ Height DNT	ng a/ ml IS-1	Peak b/ Height IS-1	ng a/ ml IS-2	Peak b/ Height IS-2	PH ^{c/} Ratio IS-1	PH ^{c/} Ratio IS-2	d/ RWR IS-1	d/ RWR IS-2
1	1	10,050	5,800	5,040	2,520	9,880	2,940	2.30	1.97	1.15	1.94
	2		6,280		2,700		3,120				
2	1	5,025	2,820	5,040	2,420	9,880	2,780	1.17	1.01	1.17	1.99
	2		2,980		2,600		2,980				
3	1	1,005	560	5,040	2,520	9,880	2,860	0.222	0.196	1.11	1.92
	2		600		2,580		2,960				
4	1	502	292	252	130	494	148	2.25	1.97	1.13	1.94
	2		308		135		153				
5	1	251	148	252	134	494	153	1.10	0.967	1.11	1.90
	2		153		138		157				
6	1	100	59	252	150	494	156	0.393	0.378	0.99	1.87
	2		60		153		157				
7	1	50	30	252	158	494	154	0.190	0.195	0.96	1.92
	2		31		160		155				
8	1	25	17	252	152	494	172	0.112	0.099	1.13	1.95
	2		18		156		177				

a/ ng/ml DNT, ng/ml IS-1, and ng/ml IS-2 - nanograms per milliliter of DNT, IS-1 (propionophenone) and IS-2 (butyropheneone) present in each solution.

b/ Peak Height DNT, IS-1, and IS-2 - measured peak heights of DNT, IS-1, and IS-2 in millimeters at an attenuation of 0.005 O.D.

c/ PH Ratio IS-1 and IS-2 - peak height ratio of DNT to the internal standards.

d/ RWR IS-1 and IS-2 - relative weight response of DNT to IS-1 and to IS-2.

$$RWR = \frac{\text{Peak Height DNT} \times \frac{\text{ng/ml IS}}{\text{ng/ml DNT}}}{\text{Peak Height IS}}$$

TABLE A-3

HPLC LINEARITY OF TNT INTERIM STANDARD SOLUTION

Solution No.	Injection No.	ng a/ ml TNT	Peak b/ Height TNT	ng a/ ml IS-1	Peak b/ Height IS-1	ng a/ ml IS-2	Peak b/ Height IS-2	PH ^{c/} Ratio IS-1	PH ^{c/} Ratio IS-2	RWR ^{d/} IS-2	RWR ^{d/} IS-2
1	1	10,210	4,020	5,040	2,520	9,880	2,940	1.59	1.37	0.788	1.32
	2		4,340		2,700		3,120	1.61	1.39	0.794	1.35
2	1	5,105	1,920	5,040	2,420	9,880	2,780	0.793	0.691	0.783	1.34
	2		2,040		2,600		2,980	0.785	0.685	0.775	1.32
3	1	1,021	380	5,040	2,520	9,880	2,860	0.151	0.133	0.744	1.29
	2		400		2,580		2,960	0.155	0.135	0.765	1.31
4	1	510	205	252	130	494	148	1.57	1.39	0.779	1.34
	2		211		135		153	1.56	1.34	0.772	1.30
5	1	255	103	252	134	494	153	0.769	0.673	0.760	1.30
	2		105		138		157	0.761	0.669	0.752	1.30
6	1	102	44	252	150	494	156	0.293	0.282	0.725	1.37
	2		42		153		157	0.275	0.268	0.678	1.30
7	1	51	24	252	158	494	154	0.152	0.156	0.751	1.51
	2		21		160		155	0.131	0.136	0.649	1.31
8	1	25	11	252	152	494	172	0.072	0.064	0.730	1.26
	2		15		156		177	0.096	0.085	0.969	1.67

a/ ng/ml TNT, ng/ml IS-1, and ng/ml IS-2 - nanograms per milliliter of TNT, IS-1 (propionophenone) and IS-2 (butyrophene) present in each solution.

b/ Peak Height TNT, IS-1, and IS-2 - measured peak heights of TNT, IS-1, and IS-2 in millimeters at an attenuation of 0.005 O.D.

c/ PH Ratio IS-1 and IS-2 - peak height ratio of TNT to the internal standards.

d/ RWR IS-1 and IS-2 - relative weight response of TNT to IS-1 and to IS-2.

$$RWR = \frac{\text{Peak Height TNT} \times \frac{\text{ng/ml IS}}{\text{ng/ml TNT}}}{\text{Peak Height IS}}$$

TABLE A-4

HPLC LINEARITY OF RDX, TNT, DNT, AND TETRYL SARM REFERENCE SOLUTIONS
USING HPLC SYSTEM 2

SARM Reference Solution No.	ng/ml Each Munition	Peak Height (mm)				IS		Relative Weight Response ^a			
		RDX	TNT	DNT	Tetryl	ng/ml	Peak Height	RDX	TNT	DNT	Tetryl
1-A	100	13.0	12.0	14.0	7.0	1,000	120.0	1.08	1.00	1.17	0.58
1-B	100	11.0	10.0	12.0	7.0	1,000	112.0	0.98	0.89	1.07	0.63
2-1	500	59.0	52.0	64.0	38.0	1,000	122.0	0.97	0.85	1.05	0.62
2-B	500	55.0	50.0	59.0	36.0	1,000	116.0	0.95	0.86	1.02	0.62
3-A	1,000	104.0	97.0	121.0	57.0	1,000	114.0	0.91	0.85	1.06	0.50
3-B	1,000	121.0	118.0	145.0	66.0	1,000	134.0	0.90	0.88	1.08	0.49
4-A	1,500	172.0	157.0	184.0	103.0	1,000	117.0	0.98	0.89	1.05	0.59
4-B	1,500	150.0	144.0	172.0	97.0	1,000	110.0	0.91	0.91	1.04	0.59
5-A	2,000	228.0	208.0	246.0	130.0	1,000	116.0	0.98	0.90	1.06	0.59
5-B	2,000	210.0	202.0	240.0	132.0	1,000	116.0	0.91	0.87	1.03	0.57

$$^a \text{ Relative Weight Response} = \frac{\text{Peak Height Cpd}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml Cpd}}$$

TABLE A-5

DETERMINATION OF RDX, DNT, AND TNT IN LIVER SAMPLES

Day 1 - Initial Toluene Extraction Evaluation

Sample Number	ng/g ^a Compound Added	g Liver	Peak Height (mm)			Internal Standard ^b ng/ml	Peak Height	ng/g ^c Detected	
			RDX	DNT	TNT			RDX	TNT
Day 1A-0	0	1.0	12.0	< 2	< 2	1,000	145.0	105	ND
Day 1A-100	100	1.0	19.0	10.0	7.0	1,000	143.0	168	61
Day 1A-500	500	1.0	59.2	51.0	31.4	1,000	142.8	538	275
Day 1A-1000	1,000	1.0	112.0	102.4	73.0	1,000	142.0	1,024	643
Day 1A-1500	1,500	1.0	168.4	158.0	112.6	1,000	142.0	1,540	991
Day 1A-2000	2,000	1.0	209.6	206.4	150.8	1,000	143.2	1,901	1,316
Day 1B-0	0	1.0	8.0	< 2	< 2	1,000	152.6	66	ND
Day 1B-100	100	1.0	18.0	12.0	6.5	1,000	148.8	153	55
Day 1B-500	500	1.0	60.0	53.4	33.4	1,000	148.0	527	282
Day 1B-1000	1,000	1.0	118.0	123.4	76.8	1,000	150.6	1,018	637
Day 1B-1500	1,500	1.0	171.0	166.6	106.0	1,000	148.6	1,494	892
Day 1B-2000	2,000	1.0	220.4	231.6	149.2	1,000	144.8	1,977	1,286

TABLE A-5 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)			Internal Standard ^b		Relative Weight ^c Response		
		RDX	DNT	TNT	ng/ml	Peak Height	RDX	DNT	TNT
Std-Day 1-5	2,000	216.0	306.0	228.0	1,000	142.0	0.76	1.08	0.80
Std-Day 1-4	1,500	158.4	224.8	166.6	1,000	139.2	0.76	1.08	0.80
Std-Day 1-5	2,000	214.8	302.0	224.0	1,000	142.0	0.76	1.06	0.79
Std-Day 1-2	500	53.0	74.0	56.0	1,000	139.0	0.76	1.06	0.81
Std-Day 1-1	100	11.0	16.0	14.0	1,000	138.6	0.79	1.15	0.82
						Average	0.77	1.09	0.80

^a ng/g compound added - nanograms of RDX, DNT, and TNT added per gram of liver sample.

^b Internal standard - compound (propiophenone) added to liver sample after sample preparation for calculation of data.

^c ng/g detected - nanograms of RDX, DNT, and TNT detected in the 1.0 g liver sample.

$$\text{ng compound/g} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}}$$

^d ND - not detectable, less than 20 ng/g.

$$\text{e Relative Weight Response - RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

TABLE A-6

DETERMINATION OF RDX, DNT, AND TNT IN LIVER SAMPLES

Day 2 - Initial Toluene Extraction Evaluation

Sample Number	ng/g ^a Compound Added	g Liver	Peak Height (mm)			Internal Standard ^b		ng/g ^c Detected	
			RDX	DNT	TNT	ng/ml	Peak Height	RDX	DNT
Day 2A-0	0	1.0	24.0	< 2	< 2	1,000	284.4	110	MD ^d
Day 2A-100	100	1.0	35.6	12.4	10.4	1,000	282.0	164	41
Day 2A-500	500	1.0	116.0	58.4	35.0	1,000	278.4	541	194
Day 2A-1000	1,000	1.0	217.6	124.4	58.0	1,000	274.0	1,031	420
Day 2A-1500	1,500	1.0	308.4	274.4	120.0	1,000	282.0	1,420	901
Day 2A-2000	2,000	1.0	443.2	336.0	155.0	1,000	292.0	1,971	1,065
Day 2B-0	0	1.0	22.0	< 2	< 2	1,000	279.0	102	MD
Day 2B-100	100	1.0	32.0	16.0	8.4	1,000	285.0	146	52
Day 2B-500	500	1.0	128.4	84.0	33.6	1,000	283.6	588	274
Day 2B-1000	1,000	1.0	236.0	162.4	74.0	1,000	288.0	1,064	522
Day 2B-1500	1,500	1.0	339.6	242.0	118.4	1,000	285.0	1,548	786
Day 2B-2000	2,000	1.0	422.0	315.2	156.0	1,000	284.0	1,930	1,028

TABLE A-6 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)			Internal Standard ^b		Relative Weight ^c Response		
		RDX	DNT	TNT	ng/ml	Peak Height	RDX	DNT	TNT
Std-Day 2-3	1,000	212.6	300.0	222.0	1,000	276.0	0.77	1.09	0.80
Std-Day 2-2	500	107.0	149.6	111.6	1,000	276.0	0.78	1.08	0.81
Std-Day 2-1	100	22.0	30.4	23.0	1,000	277.0	0.79	1.10	0.83
Std-Day 2-2	500	105.6	148.4	109.6	1,000	278.4	0.76	1.07	0.79
Std-Day 2-5	2,000	428.8	606.0	447.2	1,000	282.0	0.76	1.07	0.79
Std-Day 2-4	1,500	323.6	444.0	322.4	1,000	280.0	0.77	1.06	0.77
Std-Day 2-3	1,000	217.0	304.4	226.0	1,000	280.0	0.77	1.09	0.81
						Average	0.77	1.08	0.80

- a ng/g compound added - nanograms of RDX, DNT, and TNT added per gram of liver sample.
- b Internal standard - compound (propiphenone) added to liver sample after sample preparation for calculation of data.
- c ng/g detected - nanograms of RDX, DNT, and TNT detected in the 1.0 g liver sample.

$$\text{ng compound/g} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}}$$

d ND - not detectable, less than 20 ng/g.

$$\text{e Relative Weight Response - RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

TABLE A-7

DETERMINATION OF RDX, DNT, AND TNT IN LIVER SAMPLES

Day 3 - Initial Toluene Extraction Evaluation

Sample Number	ng/g ^a Compound Added	g Liver	Peak Height (mm)			Internal Standard ^b ng/ml	Peak Height	ng/g ^c Detected		
			RDX	DNT	TNT			RDX	DNT	TNT
Day 3A-0	0	1.0	24.0	< 2	< 2	1,000	289.0	106	ND ^d	ND
Day 3A-100	100	1.0	30.4	16.4	12.4	1,000	291.0	134	52	53
Day 3A-500	500	1.0	115.6	74.4	44.4	1,000	287.0	516	240	191
Day 3A-1000	1,000	1.0	227.0	169.6	89.0	1,000	286.0	1,020	549	384
Day 3A-1500	1,500	1.0	339.6	219.6	119.6	1,000	291.0	1,496	699	507
Day 3A-2000	2,000	1.0	434.0	290.0	147.2	1,000	288.8	1,927	930	629
Day 3B-0	0	1.0	30.0	< 2	< 2	1,000	292.4	131	ND	ND
Day 3B-100	100	1.0	53.6	22.4	14.0	1,000	294.4	233	70	59
Day 3B-500	500	1.0	134.4	66.0	37.0	1,000	303.0	569	202	151
Day 3B-1000	1,000	1.0	227.6	108.0	78.0	1,000	290.0	1,006	345	332
Day 3B-1500	1,500	1.0	342.4	214.0	207.6	1,000	287.0	1,530	690	893
Day 3B-2000	2,000	1.0	447.2	339.2	298.0	1,000	292.0	2,964	1,076	1,260

TABLE A-7 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)			Internal Standard ^b		Relative Weight ^c Response	
		RDX	DNT	TNT	ng/ml	Peak Height	RDX	TNT
Std-Day 3-1	100	23.0	30.4	22.4	1,000	278.0	0.83	1.09
Std-Day 3-5	2,000	428.8	603.2	439.2	1,000	284.0	0.76	1.06
Std-Day 3-4	1,500	336.0	452.4	348.4	1,000	287.6	0.78	1.05
Std-Day 3-5	2,000	438.0	619.2	460.0	1,000	288.8	0.76	1.07
Std-Day 3-2	500	118.0	165.0	129.6	1,000	300.0	0.79	1.10
						Average	0.78	1.08
								0.81

^a ng/g compound added - nanograms of RDX, DNT, and TNT added per gram of liver sample.

^b Internal standard - compound (propiophenone) added to liver sample after sample preparation for calculation of data.

^c ng/g detected - nanograms of RDX, DNT, and TNT detected in the 1.0 g liver sample.

$$\text{ng compound/g} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}}$$

^d ND - not detectable, less than 20 ng/g.

$$\text{e Relative Weight Response - RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

APPENDIX B

IDENTIFICATION OR DEVELOPMENT OF CHEMICAL ANALYSIS
METHODS FOR PLANTS AND ANIMAL TISSUES

METHOD REPORT NO. 1

METHOD DEVELOPMENT FOR THE DETERMINATION OF CYCLOTRIMETHYLENE-
TRINITRAMINE (RDX), DINITROTOLUENE (DNT), AND
TRINITROTOLUENE (TNT) IN PLASMA

September 1980

Contract No. DAAK11-79-C-0110
MRI Project No. 4849-A

For

U.S. Army Toxic and Hazardous Materials Agency
Dr. L. Eng, DRKTH-TE-D, Project Officer
Aberdeen Proving Ground (EA), MD 21010

The view, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER Technical Report No. 1	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Method Development for the Determination of Cyclotrimethylenetrinitramine (RDX), Dinitro- toluene (DNT), and Trinitrotoluene (TNT) in Plasma		5. TYPE OF REPORT & PERIOD COVERED Method Report, August 19, 1979 to December 20, 1979
7. AUTHOR(s) D. B. Lakings and O. Gan		6. PERFORMING ORG. REPORT NUMBER MRI Project No. 4849-A
9. PERFORMING ORGANIZATION NAME AND ADDRESS Midwest Research Institute 425 Volker Boulevard Kansas City, MO 64110		8. CONTRACT OR GRANT NUMBER(s) DAAK11-79-C-0110
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Armament Research and Development Command, Aberdeen Proving Ground (Edgewood Area), MD 21020		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		12. REPORT DATE September 1980
		13. NUMBER OF PAGES
		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Unlimited		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Cyclotrimethylenetrinitramine (RDX) High Performance Liquid Chromatography Dinitrotoluene (DNT) Trinitrotoluene (TNT) Plasma Determination		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A high performance liquid chromatographic (HPLC) method for the quantitative determination of cyclotrimethylenetrinitramine (RDX), dinitrotoluene (DNT), and trinitrotoluene (TNT) in plasma has been developed. The analytical system consists of an isocratic HPLC unit with a Spherisorb ODS 5µ, 250 x 4.6 mm ID column, an eluent of 30% acetonitrile in 1% acetic acid in water, and a flow rate of 1.5 ml/min. The compounds, including the internal standard (IS), propiophenone, have the following retention characteristics: RDX - 15 ml, 10 min;		

DD FORM 1473 1 JAN 73 EDITION OF 1 NOV 65 IS OBSOLETE

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE(When Data Entered)

IS - 28.5 ml, 19 min; DNT - 37.5 ml, 25 min; and TNT - 42 ml, 28 min and are detected at 254 nm. Reference solutions of the compounds gave a linear response from 100 ng/ml to 2,000 ng/ml. The plasma samples were prepared by adding 1 ml 10% sodium chloride containing 2% acetic acid to 1 ml plasma and extracting the sample with 3 x 2 ml toluene. The toluene extracts were combined and 0.5 ml water added. The toluene was evaporated at room temperature under a stream of nitrogen gas. The aqueous phase was combined with 0.5 ml acetonitrile containing the IS (1,000 ng/sample), filtered through a 0.45- μ Fluoropore filter and injected onto the HPLC system. The analytical method was evaluated by preparing and analyzing duplicate samples containing 0, 100, 500, 1,000, 1,500, and 2,000 ng/ml of each compound on four succeeding days. Linear regression analysis of the data gave the following equations and correlation coefficients: RDX - $y = 0.825x + 14$, 0.990; DNT - $y = 0.659x + 6$, 0.982; and TNT - $y = 0.785x + 16$, 0.988. The average coefficient of variation and average percent inaccuracy for RDX, DNT, and TNT determination in plasma were 10%, - 12; 12% - 32; and 10% - 19, respectively. A statistical evaluation of the data by the Hubaux and Vos detection limit program gave detection limits of 146 ng/ml for RDX; 256 ng/ml for DNT, and 248 ng/ml for TNT for the HPLC determination of these compounds in plasma samples.

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE(When Data Entered)

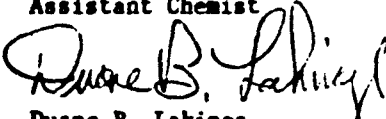
PREFACE

The report was prepared at Midwest Research Institute, 425 Volker Boulevard, Kansas City, Missouri 64110 under U.S. Army Toxic and Hazardous Materials Agency, Contract No. DAAX11-79-C-0110, MRI Project No. 4849-A entitled "Identification or Development of Chemical Analysis Methods for Plants and Animal Tissues." Dr. Leslie Eng, DRXTH-TE-D was the Project Officer for this research effort.

This work was conducted in the Analytical Chemistry Department Dr. James L. Spigarelli, Director. The research effort was directed by Dr. Duane B. Lakings, Program Manager and Senior Chemist, with assistance from Mr. Owen Gan, Assistant Chemist, and Ms. Mary Valla, Senior Technician. This report was prepared by Dr. Lakings and Mr. Gan.

MIDWEST RESEARCH INSTITUTE


Owen Gan
Assistant Chemist


Duane B. Lakings
Program Manager and Senior Chemist

Approved:

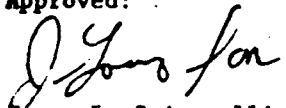

James L. Spigarelli, Director
Analytical Chemistry Department

TABLE OF CONTENTS

	<u>Page</u>
1. Application	1
2. Chemistry	1
3. Apparatus	2
4. Standards	3
5. Procedures for Plasma Sample Determination.	4
6. Calculation	6
7. Statistical Evaluation of Data.	6
Appendix - Raw Data and Calculation for Plasma Method Determination for RDX, DNT, and TNT	24

LIST OF FIGURES

<u>Number</u>	<u>Title</u>	<u>Page</u>
1	HPLC Separation of RDX, DNT, TNT and Tetryl SARMS and Propiophenone (IS)	7
2	Determination of RDX in Plasma	12
3	Determination of DNT in Plasma	13
4	Determination of TNT in Plasma	14
5	Standard Deviation for RDX, DNT, and TNT in Plasma	15
6	Coefficient of Variation for RDX, DNT, and TNT in Plasma .	16
7	Percent Inaccuracy for RDX, DNT, and TNT in Plasma	17
8	HPLC Analysis of Blank Plasma for RDX, DNT, and TNT Method Development	18
9	HPLC Analysis of Plasma Containing 100 ng/ml RDX, DNT, and TNT.	19
10	HPLC Analysis of Plasma Containing 1,000 ng/ml RDX, DNT, and TNT.	20

LIST OF TABLES

<u>Number</u>	<u>Title</u>	<u>Page</u>
1	Linearity and Precision of the HPLC-UV Determination of SARM Reference Solutions of RDX, DNT, and TNT.	8
2	HPLC-UV Determination of RDX in Plasma	9
3	HPLC-UV Determination of DNT in Plasma	10
4	HPLC-UV Determination of TNT in Plasma	11
5	Statistical Evaluation of RDX in Plasma Data by the Hubaux and Vos Detection Limit Program	21
6	Statistical Evaluation of DNT in Plasma Data by the Hubaux and Vos Detection Limit Program	22
7	Statistical Evaluation of TNT in Plasma Data by the Hubaux and Vos Detection Limit Program	23
<u>Appendix</u>		
8	Linearity and Precision of RDX, DNT, and TNT Determination by High Performance Liquid Chromatography.	25
9	Determination of RDX, DNT, and TNT in Plasma Samples . . .	27
10	Determination of RDX, DNT, and TNT in Plasma Samples . . .	29
11	Determination of RDX, DNT, and TNT in Plasma Samples . . .	31
12	Determination of RDX, DNT, and TNT in Plasma Samples . . .	33

Midwest Research Institute
Analytical Chemistry Department
Kansas City, Missouri 64110

for

U.S. Army Armament Research and Development Command
Aberdeen Proving Ground (Edgewood Area)
Maryland 21020

Contract No. DAAK11-79-C-0110

Identification or Development of Chemical Analysis Methods
for Plant and Animal Tissues

METHOD DEVELOPMENT FOR THE DETERMINATION OF CYCLOTRIMETHYLENE-
TRINITROAMINE (RDX), DINITROTOLUENE (DNT), AND
TRINITROTOLUENE (TNT) IN PLASMA

1. APPLICATION: The developed method is for the quantitative determination of RDX, DNT, and TNT in animal plasma using high performance liquid chromatography (HPLC) with ultraviolet (UV) detection at 254 nm.

a. Evaluated Concentration Range: The concentration range of RDX, DNT, and TNT studied in reference solutions and in plasma samples was 100 to 2,000 parts per billion (ppb, ng/ml).

b. Sensitivity: A signal-to-noise ratio of 6 to 1 for RDX (peak height (PH), 25 mm), 9 to 1 for DNT (PH, 30 mm), and 8 to 1 for TNT (PH, 30 mm) was obtained with an injection of 50 µl of a 100 ng/ml solution of each compound (ca. 5 ng each compound on column).

c. Detection Limits: 146 ng/ml for RDX, 256 ng/ml for DNT, and 248 ng/ml for TNT based on the Hubaux and Vos detection limits program.

d. Interferences: No interfering plasma components were found to elute with the same retention volumes as RDX, DNT, or TNT. However, an impurity in the extracting solvent (toluene) eluted 1.5 min prior to RDX.

e. Analysis Rate: The chromatographic time per injection for the plasma determination of RDX, DNT, and TNT was 40 min. With two reference solutions analyzed first and two during the day (160 min total time), a total of eight samples (320 min total time) can be analyzed during an 8-hr day.

2. CHEMISTRY: RDX, DNT, and TNT are munition compounds manufactured at various installations. The possible environmental contamination of these compounds, particularly in plants and animals, is of concern. The determination of plasma levels of RDX, DNT, and TNT in animals may provide

information on the extent and level of contamination at the production facilities and surrounding area. Each of these munitions has a sufficient UV chromophore at 254 nm to allow UV detection and quantification. These munitions are of intermediate polarity and have a limited water solubility. Normally, biological matrices have a large number of components which will interfere with the detection and quantification of low levels of compounds. Reverse phase HPLC is capable of separating compounds with similar chemical and physical properties; the elution order of this technique is based on the polarity of the compounds with the more polar compounds being eluted first. Thus, by extracting the biological matrix, i.e., plasma, with an intermediate polarity solvent and analyzing the extract by HPLC, a simple sample preparation and analysis system may be defined for the determination of RDX, DNT, and TNT in plasma.

3. APPARATUS:

a. Instrumentation: A Waters isocratic liquid chromatographic system consisting of a Model 6000A pump, Model U6K injector, and Model 440 UV detector. A general purpose centrifuge (Dynac, Clay Adams 0101) with a 24-place head.

b. HPLC Parameters:

1. Column: Spherisorb ODS, 5 μ , 250 x 4.6 mm ID.
2. Eluent: 30% acetonitrile in 1% acetic acid in water.
3. Flow rate: 1.5 ml/min.
4. Detector: UV, 254 nm.
5. Internal standard: Propiophenone, 1,000 ng/ml
6. Injection volume: 50 to 100 μ l.
7. Retention volumes and times: RDX, 15 ml, 10 min; DNT, 37.5 ml, 25 min; TNT, 42 ml, 28 min; IS, 28.5 ml, 19 min. NOTE: Slight changes in the retention indices may occur with fresh eluent or a change in columns.

A representative HPLC chromatogram for a SARM reference solution of RDX, DNT, and TNT is shown in Figure 1. Also included on the chromatogram are peaks for an internal standard (propiophenone) and 2,4,6-trinitrophenylmethylnitramine (teteryl).

c. Laboratory Glassware and Equipment:

1. Culture tubes (Pyrex) with Teflon-lined screw caps.
2. Volumetric flasks (100 ml).
3. Volumetric syringes (0-100 μ l, 0-500 μ l, and 0-1,000 μ l).
4. Automatic pipetter (0-5 ml).
5. Filtering apparatus including filter holders, 5-ml disposable syringes, and 0.45 μ Fluoropore filters.
6. Inert gas (nitrogen) drying train with 12 ports.

d. Chemicals:

1. Toluene and acetonitrile, "Distilled in Glass" grade.
2. Acetic acid and sodium chloride, ACS grade.
3. High purity water from a Milli-Q water purification system.
4. RDX, DNT, and TNT SARMs, obtained from the U.S. Army Toxic and Hazardous Materials Agency.
5. Propiophenone (internal standard), analytical grade.

4. STANDARDS:

a. Stock: Weigh approximately 20 mg of RDX, DNT, TNT, and tetryl SARM or interim SARM into separate 100-ml volumetric flasks. Dissolve each compound in acetonitrile and dilute to volume. The concentration of each compound is 200 μ g/ml. Quantitatively pipette 20 ml from each stock above into a 100-ml volumetric flask and dilute to volume with high-purity water. Concentration of each compound is 40 μ g/ml.

b. Working: Pipette 10 ml of the 40 μ g/ml of each compound stock into a 100-ml volumetric flask and dilute to volume with high-purity water. Concentration of each compound is 4 μ g/ml.

Reference solutions were prepared from this stock as follows:

<u>µl Working Stock</u>	<u>µl IS Stock*</u>	<u>µl 10% Acetonitrile in Water</u>	<u>Concentration Each Compound (ng/ml)</u>
500	500	0	2,000
375	500	125	1,500
250	500	250	1,000
125	500	375	500
25	500	475	100
0	500	500	0

* Preparation of IS stock given in "c."

c. Internal Standard Stock: Weigh 10 mg propiophenone into a 100-ml volumetric flask and dilute to volume with acetonitrile (concentration, 100 µg/ml). Quantitatively pipette 10 ml of the 100-µg/ml stock into a 100-ml volumetric flask and dilute to volume with acetonitrile (concentration, 10 µg/ml). A final working solution of 2.0 µg/ml is prepared by pipetting 20 ml of the 10-µg/ml stock into a 100-ml volumetric flask and diluting to volume with acetonitrile.

5. PROCEDURES FOR PLASMA SAMPLE DETERMINATION:

a. Plasma Sample Preparation: The procedure employed to prepare plasma samples for the HPLC-UV determination of RDX, DNT, TNT, and tetraol consisted of:

1. Quantitatively pipette 12 1.0-ml plasma aliquots into culture tubes with Teflon-lined screw caps.
2. Spike two each of the plasma aliquots with the working stock (4 µg/ml) at the following levels: 2,000 ng (500 µl), 1,500 ng (375 µl), 1,000 ng (250 µl), 500 ng (125 µl), and 100 ng (25 µl). The remaining two plasma aliquots serve as plasma blanks. All samples were adjusted to a total volume of 1.5 ml with high-purity water containing 10% acetonitrile.
3. Add 1.0 ml of a 10% sodium chloride solution containing 2% acetic acid to each aliquot.

4. Mix thoroughly on a vortex mixer.
5. Extract the plasma samples with 2 ml toluene ("Distilled in Glass" grade) by vortexing for 30 sec followed by centrifugation at 1,000 rpm for 20 min.
6. Transfer the toluene extracts to properly labeled culture tubes with Teflon-lined screw caps.
7. Repeat the toluene extraction (steps 5 and 6) twice more combining the toluene extracts in the appropriate tubes.
8. Quantitatively pipette 0.5 ml high-purity water into each toluene extract.
9. Evaporate the toluene at room temperature under a stream of nitrogen. NOTE: Continue evaporation until the toluene has been completely removed from culture tube. Do not heat the samples during the evaporation step or loss of RDX, DNT, and TNT may occur.
10. Add 500 µl IS stock (1,000 ng) to each plasma extract and mix thoroughly. NOTE: Final volume of the prepared samples is 1.0 ml.
11. Filter the solutions through 0.45 µ Fluoropore filters into culture tubes.
12. Analyze a 50- to 100-µl aliquot by HPLC.

b. Calibration: The reference solutions described in Section 4.b were prepared and analyzed in quadruplicate. The relative weight response (RWR) (Eq. 1) of each compound was calculated and the average RWR utilized to determine the nanograms of each compound in every reference solution (Eq. 2). The nanograms found were plotted against the nanograms added and a linear regression evaluation of the data was conducted. The slope, intercept, and correlation coefficient for each compound were determined. The data are summarized in Table 1 which include the average value at each level for each compound, the standard deviation, coefficient of variation, and percent inaccuracy. The raw data and calculations are given in Table 8 of the Appendix.

$$RWR = \frac{\text{Peak Height Cpd}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml Cpd}} \quad (\text{Eq. 1})$$

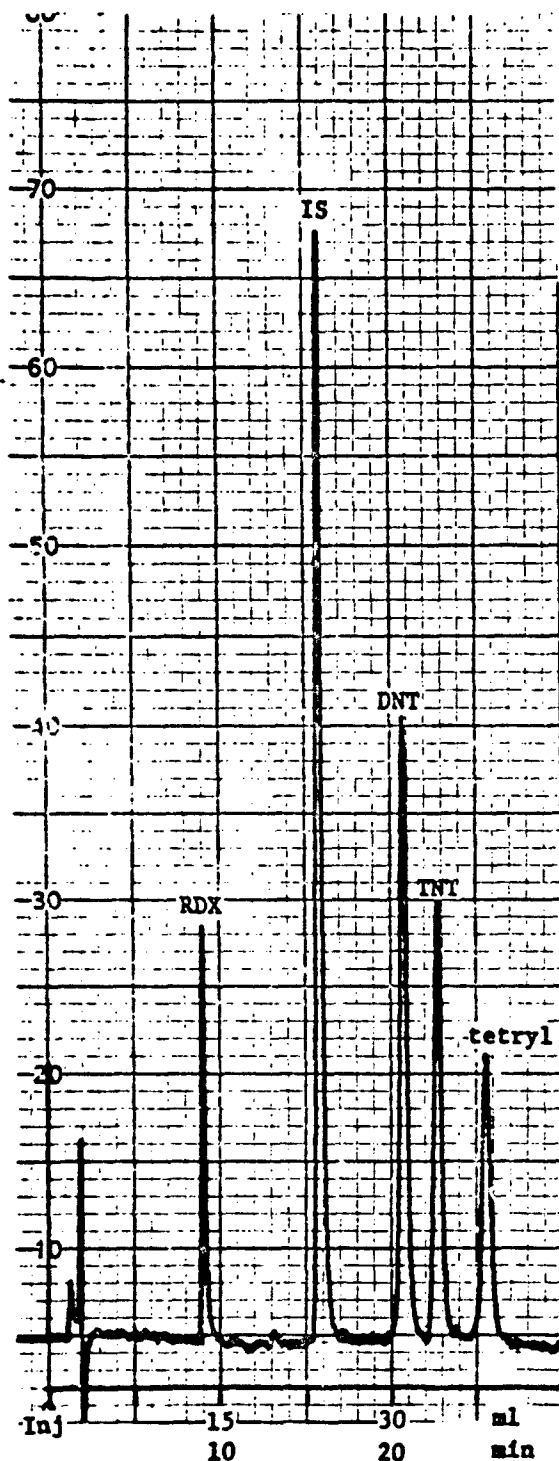
$$\frac{\text{ng}}{\text{ml}} \text{ compound found} = \frac{\text{Peak Height Cpd}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{Avg. RWR}} \quad (\text{Eq. 2})$$

c. Plasma Sample Analysis: The plasma samples prepared as outlined in Section 5.a were injected onto the HPLC system. The peak height of each compound was measured and recorded. Plasma samples were prepared and analyzed on four succeeding days.

6. CALCULATION: The level (nanograms per milliliter) of each compound found in the plasma samples was determined using the relative weight response to an internal standard method. The RWR values for reference solutions (Eq. 1) analyzed with a day set of plasma samples were calculated and the average RWR values for RDX, DNT, and TNT were determined. These values were employed to determine the plasma level of each compound by Eq. 2 where nanograms per milliliter compound represents the level found in the plasma sample. The results for the duplicate determinations of RDX, DNT, and TNT in plasma at five levels on four succeeding days are summarized in Tables 2, 3 and 4, and the average level found, the standard deviation, coefficient of variation, and percent inaccuracy are also included. A linear regression evaluation of the results was made; the slope, intercept, and correlation coefficient are given in the tables. The level of each compound in the plasma was plotted against the amount added and these data are shown in Figures 2 to 4.

Graphic presentations of the standard deviation, coefficient of variation, and the percent inaccuracy for RDX, DNT, and TNT determination in plasma are given in Figures 5, 6, and 7, respectively. Representative HPLC chromatograms are shown for a plasma blank (Figure 8), a 100-ng/ml plasma sample (Figure 9), and a 1,000-ng/ml plasma sample (Figure 10). The raw data and calculations for the plasma sample determinations are given in Tables 9 to 12 in the Appendix.

7. STATISTICAL EVALUATION OF DATA: A statistical evaluation of the data obtained for the determination of RDX, DNT, and TNT in plasma (Tables 2, 3 and 4) by the Hubaux and Vos detection limit program was made at the U.S. Army Toxic and Hazardous Materials Agency. The results of these evaluations are given in Table 5 for RDX, Table 6 for DNT, and Table 7 for TNT. When the 2000 and 1500 ng/ml data points were omitted, the detection limits for RDX, DNT, and TNT in plasma as determined by the program were 145, 256, and 248 ng/ml, respectively. The average nanograms per milliliter found at each level were determined from the linear regression equation for the 48 data points and the nanogram per milliliter added at that level. The standard deviation and percent imprecision (coefficient of variation) at each level were calculated based on this average nanograms per milliliter found. Thus, these values and the values given in Tables 2, 3, and 4 (based on the average of the eight assays at each level) are not comparable. The present inaccuracy term was calculated from the average of the eight data points at each level, and thus agrees with the values in Tables 2, 3 and 4.



HPLC Conditions

Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 30% acetonitrile in
1% acetic in water
Flow Rate: 1.5 ml/min
Chart Speed: 0.1 in./min
Detector: UV, 254 nm

Sample Characteristics

Concentrations: RDX, DNT, TNT,
and tetryl - 500 ng/ml;
IS - 1,000 ng/ml
Injection volume: 70 μ l
Attenuation: 0.01 X

Retention Indices

Compound	Retention Volume (ml)	Retention Time (min)
RDX	13.5	9
IS	22.5	15
DNT	31.5	21
TNT	34.5	23
tetryl	39	26

Figure 1 - HPLC Separation of RDX, DNT, TNT, and Tetryl
SARMS and Propiophenone (IS)

TABLE 1

LINEARITY AND PRECISION OF THE HPLC-UV DETERMINATION OF
SARM REFERENCE SOLUTIONS OF RDX, DNT, AND TNT

Compound	ng/ml Added	ng/ml Detected				Average ^a	Standard Deviation ^b	Coefficient ^c of Variation	Percent ^d Inaccuracy
		A	B	C	D				
RDX	0	ND ^e	ND	ND	ND	-	-	-	-
	100	109	114	105	103	108	± 4.9	4.5	+ 8.0
	500	517	509	499	490	504	± 11.8	2.3	+ 0.8
	1,000	960	950	923	985	955	± 26	2.7	- 4.5
	1,500	1,551	1,547	1,435	1,444	1,494	± 63	4.2	- 0.4
	2,000	2,031	2,069	1,906	1,924	1,983	± 80	4.0	- 0.8
DNT	0	ND	ND	ND	ND	-	-	-	-
	100	113	109	109	100	108	± 5.5	5.1	+ 8.0
	500	499	490	475	483	487	± 10.2	2.1	- 2.6
	1,000	992	1,011	981	1,032	1,004	± 22	2.2	+ 0.4
	1,500	1,500	1,470	1,461	1,439	1,468	± 25	1.7	- 2.1
	2,000	1,968	1,982	1,934	1,901	1,946	± 36	1.9	- 2.7
TNT	0	ND	ND	ND	ND	-	-	-	-
	100	107	112	112	100	108	± 5.7	5.3	+ 8.0
	500	495	479	484	484	486	± 6.8	1.4	- 2.8
	1,000	956	989	967	997	977	± 19	2.0	- 2.3
	1,500	1,498	1,508	1,471	1,432	1,478	± 34	2.3	- 1.5
	2,000	2,011	2,015	1,957	1,879	1,966	± 63	3.2	- 1.7

Linear Regression

RDX: $y = 0.988x + 0.6$

Correlation coefficient - 0.998

DNT: $y = 0.974x + 7.7$

Correlation coefficient - 0.999

TNT: $y = 0.982x + 1.2$

Correlation coefficient - 0.999

- ^a Average = $\sum x/n = \bar{x}$
^b Standard deviation = $(\sum (\bar{x} - x)^2 / (n-1))^{1/2} = \sigma$
^c Coefficient of variation = $\sigma / \bar{x} \times 100$
^d Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\bar{x}} \times 100$
^e ND = Not detectable, less than 20 ng/ml

TABLE 2

HPLC-UV DETERMINATION OF RDX IN PLASMA

Amount Added (ng/ml)	Level Found (ng/ml)								Average ^{a/}	Standard ^{b/} Deviation (ng)	Coefficient ^{c/} of Variation	Percent ^{d/} Inaccuracy
	Day 1		Day 2		Day 3		Day 4					
	A	B	A	B	A	B	A	B				
0	ND ^e	ND	ND	ND	ND	ND	NC	ND	-	-	-	-
100	95	117	113	104	83	71	129	96	101	+ 19	19	+1
500	478	456	403	428	410	385	446	444	431	+ 31	7	-14
1,000	987	839	841	830	814	775	849	870	851	+ 62	7	-15
1,500	1,324	1,345	1,153	1,168	1,259	1,148	1,321	1,299	1,252	+ 83	7	-16
2,000	1,927	1,760	1,576	1,521	1,581	1,337	1,823	1,722	1,658	+ 188	11	-17

Note: Linear regression: $y = 0.825x + 14$

Correlation coefficient: 0.990

^{a/} Average = $\Sigma x/n = \bar{x}$ ^{b/} Standard deviation = $(\Sigma |\bar{x} - x|^2 / (n-1))^{1/2} = \sigma$ ^{c/} Coefficient of variation = $\sigma / \bar{x} \times 100$ ^{d/} Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$ ^{e/} ND = Not detectable, less than 20 ng/ml

TABLE 3

HPLC-UV DETERMINATION OF DNT IN PLASMA

Amount Added (ng/ml)	Level Found (ng/ml)								Standardb/ Deviation (ng)	Coefficientc/ of Variation	Percentd/ Inaccuracy
	Day 1		Day 2		Day 3		Day 4				
	A	B	A	B	A	B	A	B			
0	ND	ND	ND ^e	ND	ND	ND	ND	ND	-	-	-
100	84	62	86	72	79	54	83	75	+ 11	15	-26
500	388	333	333	343	351	321	302	361	+ 26	8	-32
1,000	786	595	669	750	700	505	607	587	+ 94	14	-35
1,500	1,055	1,000	915	1,111	1,052	1,019	956	1,082	+ 65	6	-32
2,000	1,509	1,391	1,533	1,041	1,456	1,041	1,123	1,366	+ 207	16	-35

Note: Linear regression: $y = 0.659x + 6$

Correlation coefficient: 0.982

a/ Average = $\Sigma x/n = \bar{x}$ b/ Standard deviation = $(\Sigma |\bar{x} - x|^2 / (n-1))^{1/2} = \sigma$ c/ Coefficient of variation = $\sigma / \bar{x} \times 100$ d/ Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$

e/ ND = Not detectable, less than 20 ng/ml

TABLE 4

HPLC-UV DETERMINATION OF TNT IN PLASMA

Amount Added (ng/ml)	Level Found (ng/ml)								Average ^a / (ng)	Standard ^b / Deviation (ng)	Coefficient ^c / of Variation	Percent ^d / Inaccuracy
	Day 1		Day 2		Day 3		Day 4					
	A	B	A	B	A	B	A	B				
0	ND ^e	ND	ND	ND	ND	ND	ND	ND	-	-	-	-
100	95	97	96	102	70	76	93	78	88	+ 12	13	-12
500	451	419	412	416	419	404	371	415	413	+ 22	5	-17
1,000	940	840	830	825	793	579	707	710	778	+ 110	14	-22
1,500	1,238	1,202	1,173	1,177	980	1,181	1,223	1,250	1,178	+ 85	7	-21
2,000	1,870	1,748	1,687	1,558	1,600	1,338	1,355	1,507	1,583	+ 185	12	-21

Note: Linear regression: $y = 0.785x + 6$

Correlation coefficient: 0.988

^a/ Average = $\Sigma x/n = \bar{x}$ ^b/ Standard deviation = $(\Sigma |x - \bar{x}|^2 / (n-1))^{1/2} = s$ ^c/ Coefficient of variation = $s/\bar{x} \times 100$ ^d/ Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$ ^e/ ND = Not detectable, less than 20 ng/ml

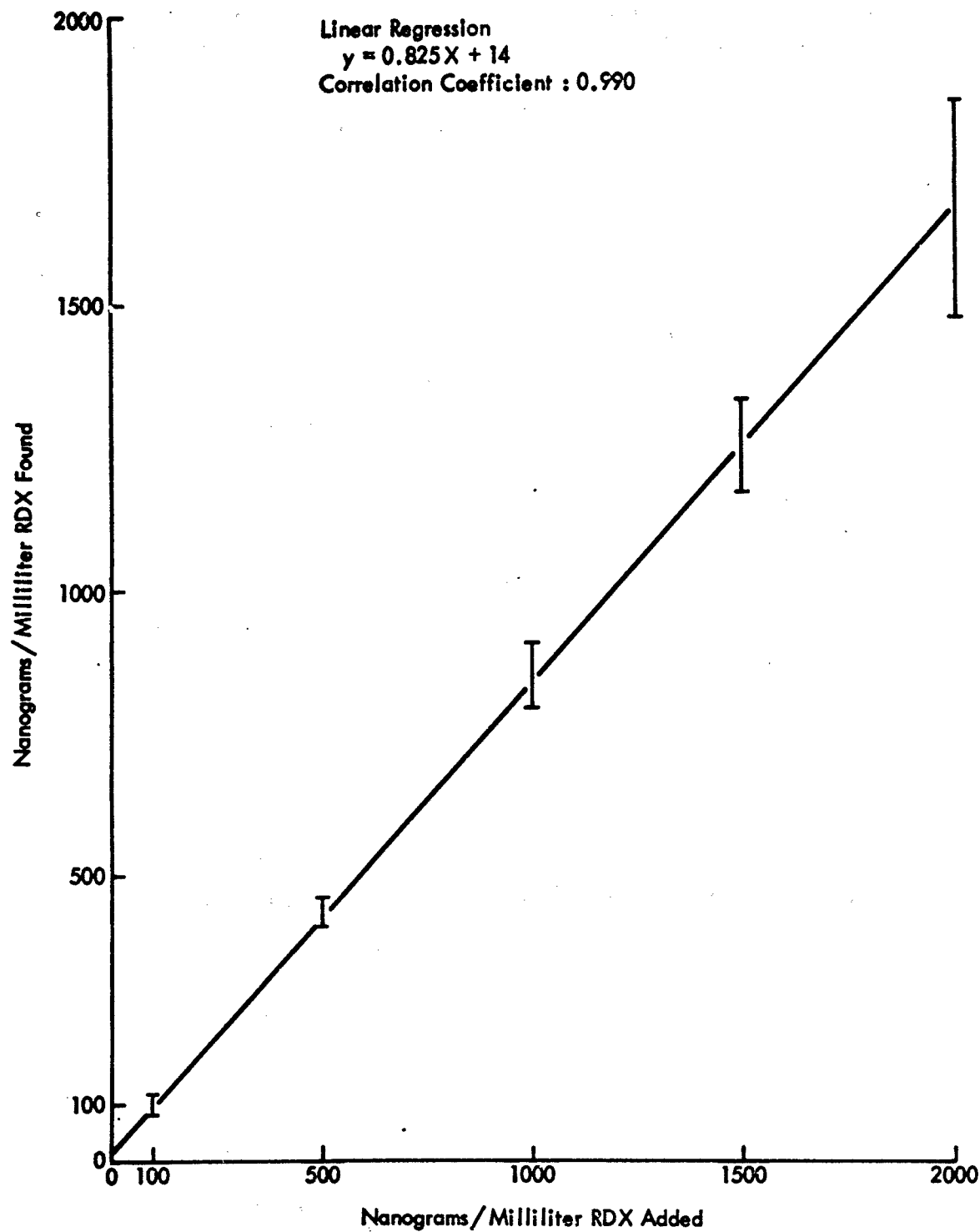


Figure 2 - Determination of RDX in Plasma

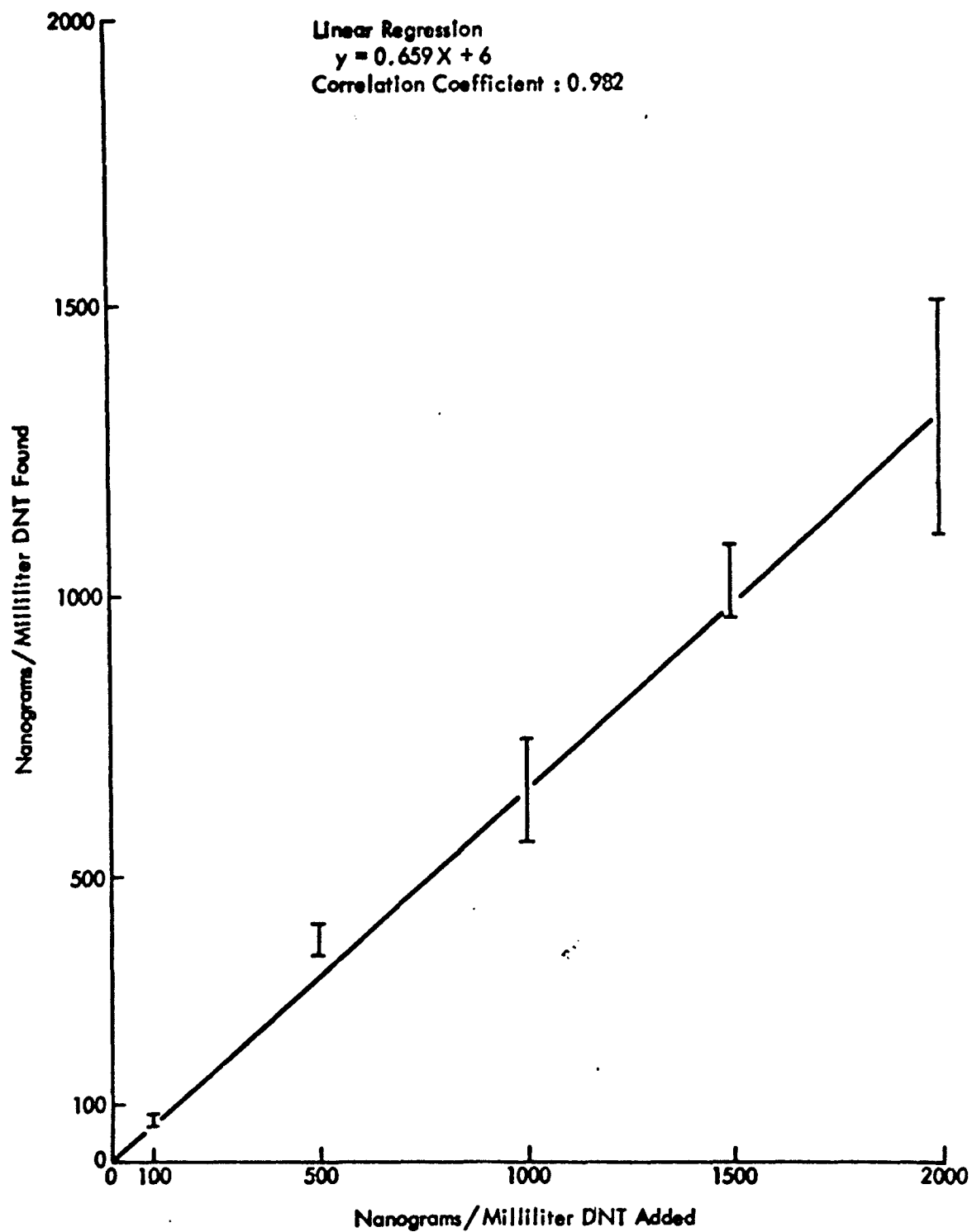


Figure 3 - Determination of DNT in Plasma

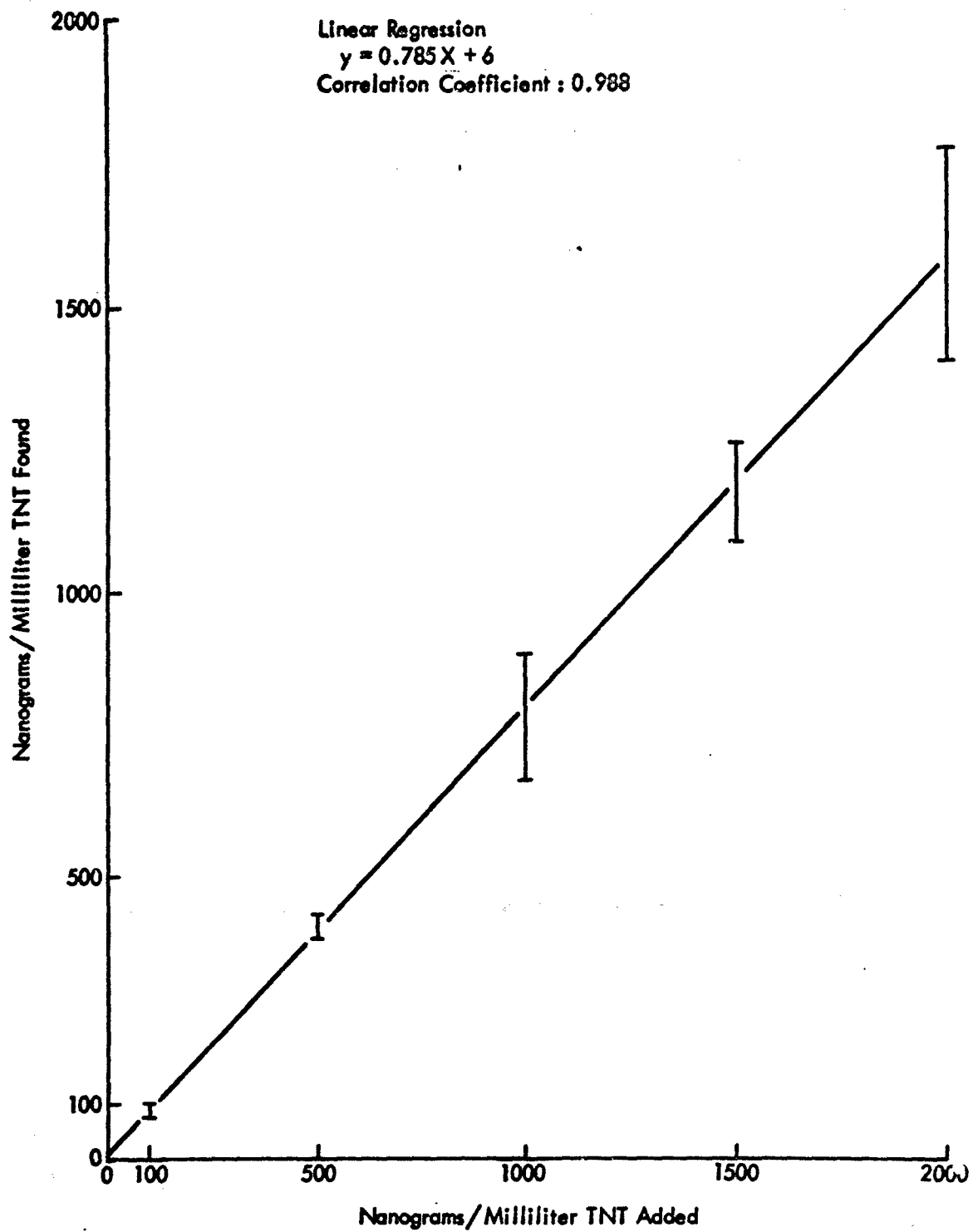


Figure 4 - Determination of TNT in Plasma

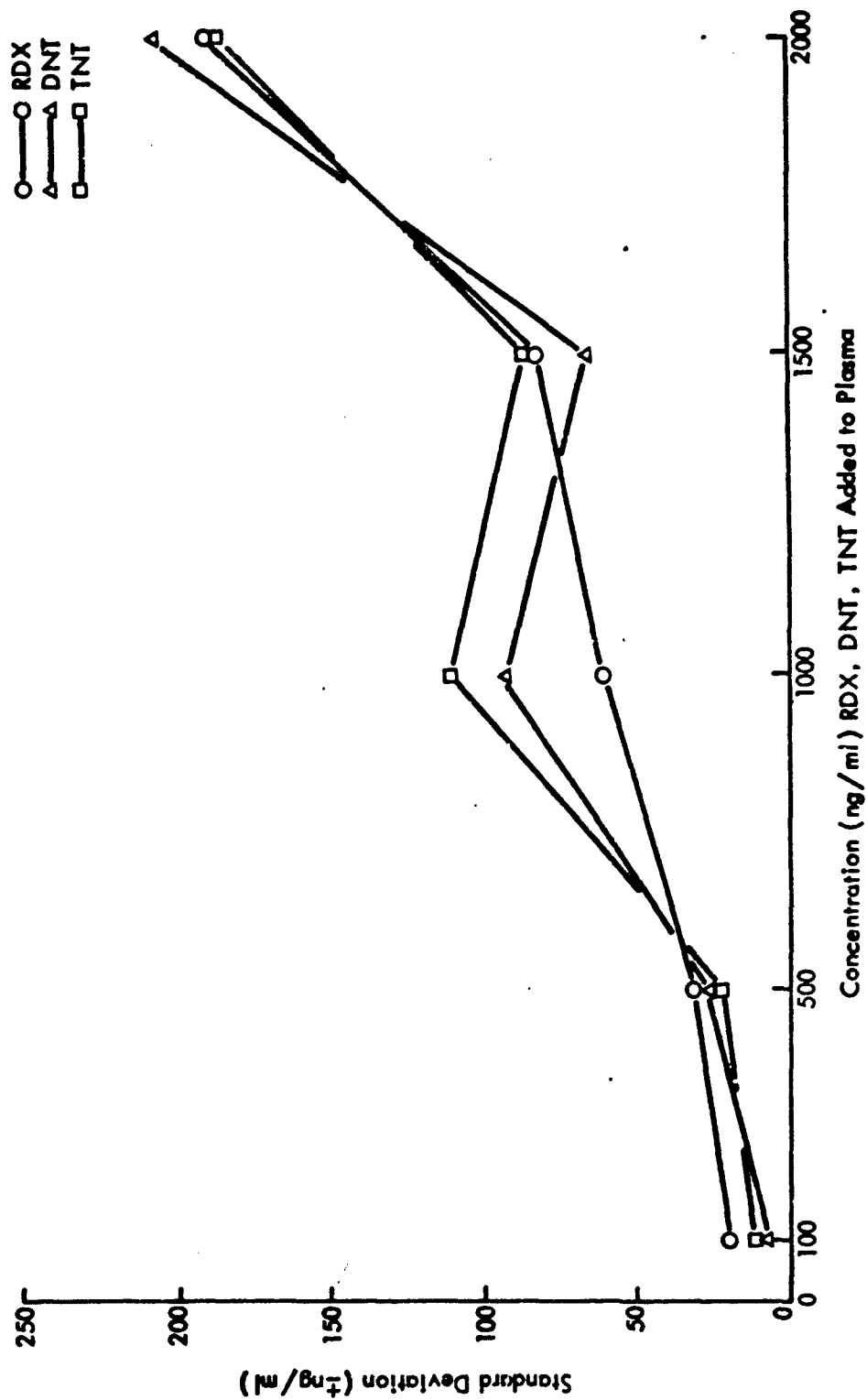


Figure 5 - Standard Deviation for RDX, DNT, TNT Added to Plasma

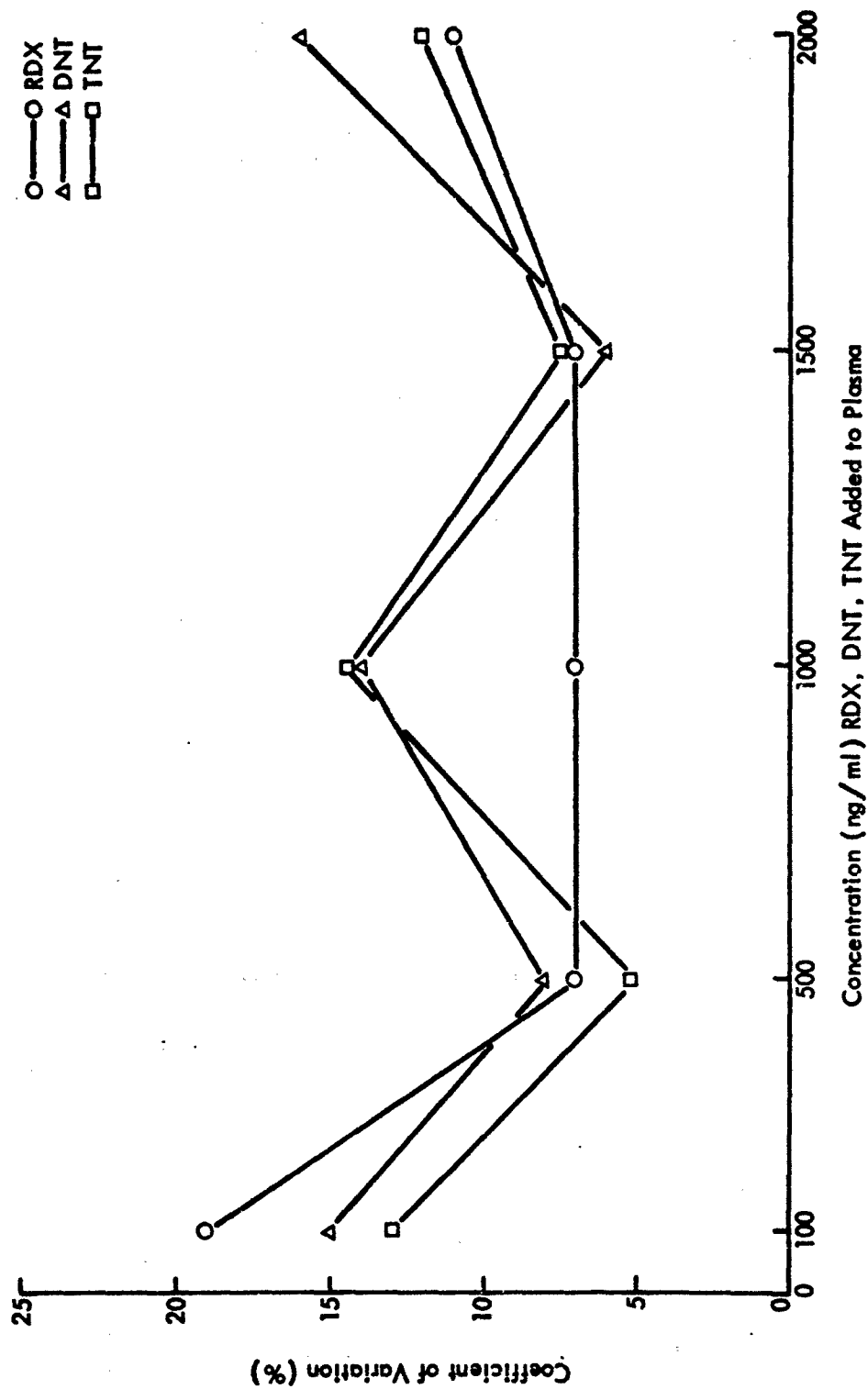


Figure 6 - Coefficient of Variation for RDX, DNT, TNT Added to Plasma

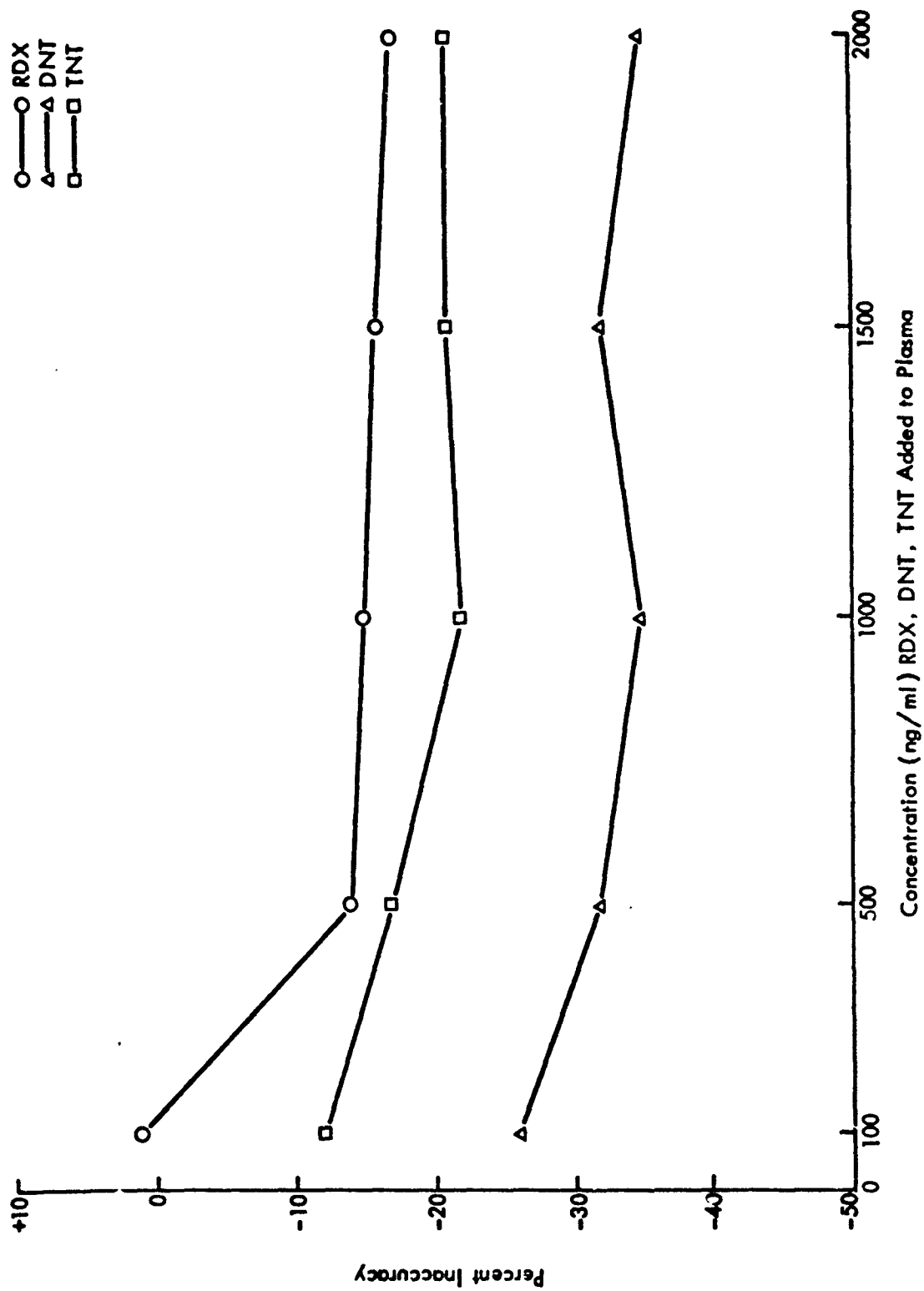
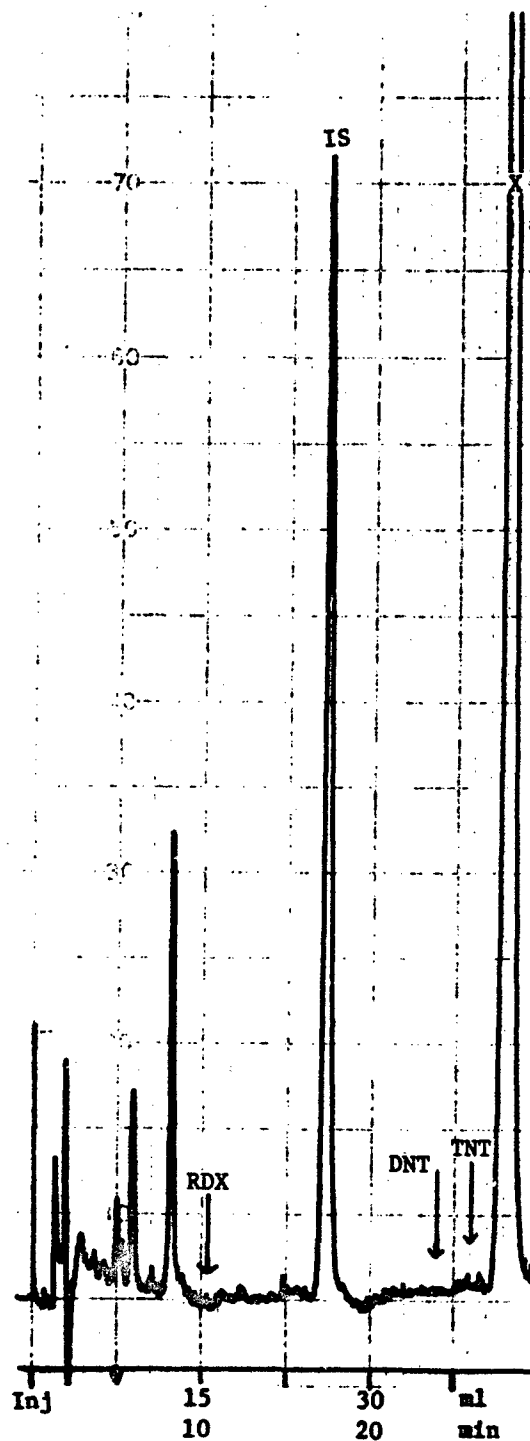


Figure 7 - Percent Inaccuracy for RDX, DNT, TNT Added to Plasma



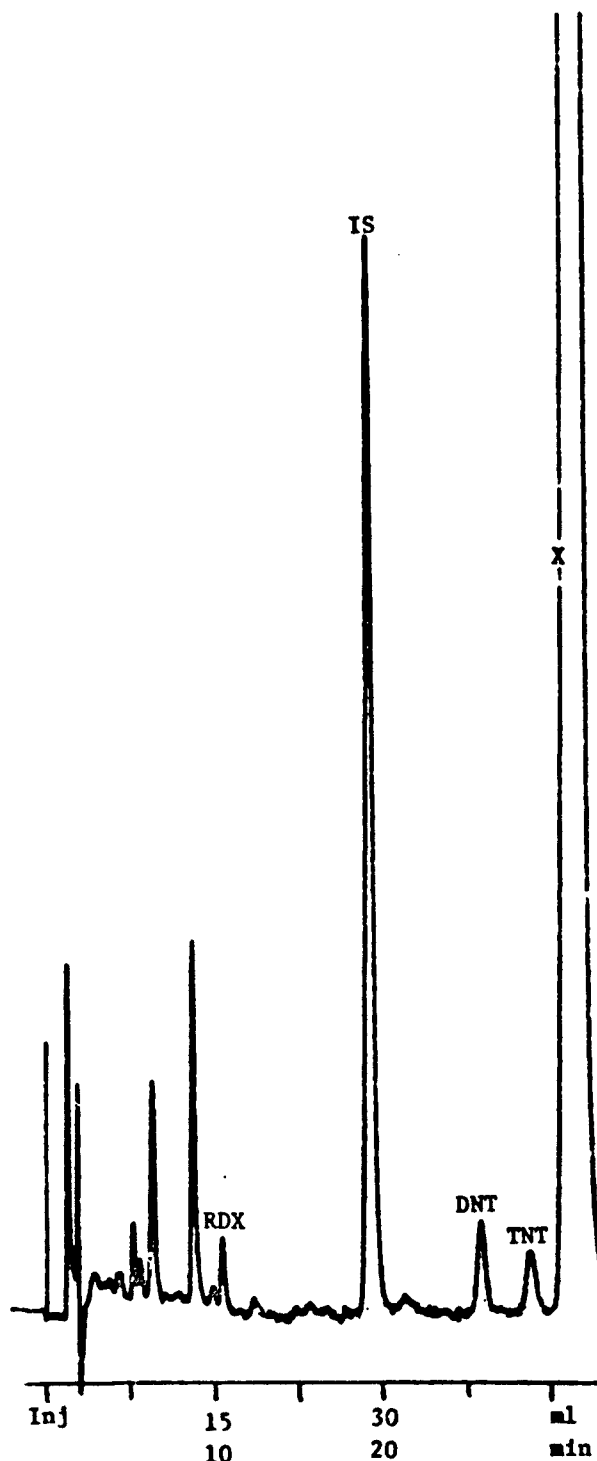
HPLC Conditions

Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 30% acetonitrile in
1% acetic acid in water
Flow rate: 1.5 ml/min
Chart speed: 0.1 in./min
Detector: UV, 254 nm

Sample Characteristics

1.0 ml plasma extracted 3 x 2 ml
with toluene. Toluene evaporated
and sample reconstituted to 1 ml
IS concentration: 1,000 ng/ml
Injection volume: 70 μ l
Attenuation: 0.01 X

Figure 8 - HPLC Analysis of Blank Plasma for RDX, DNT, and TNT
Method Development. "X" indicates toluene contaminant.
Arrows indicate elution position of RDX, DNT, and TNT.



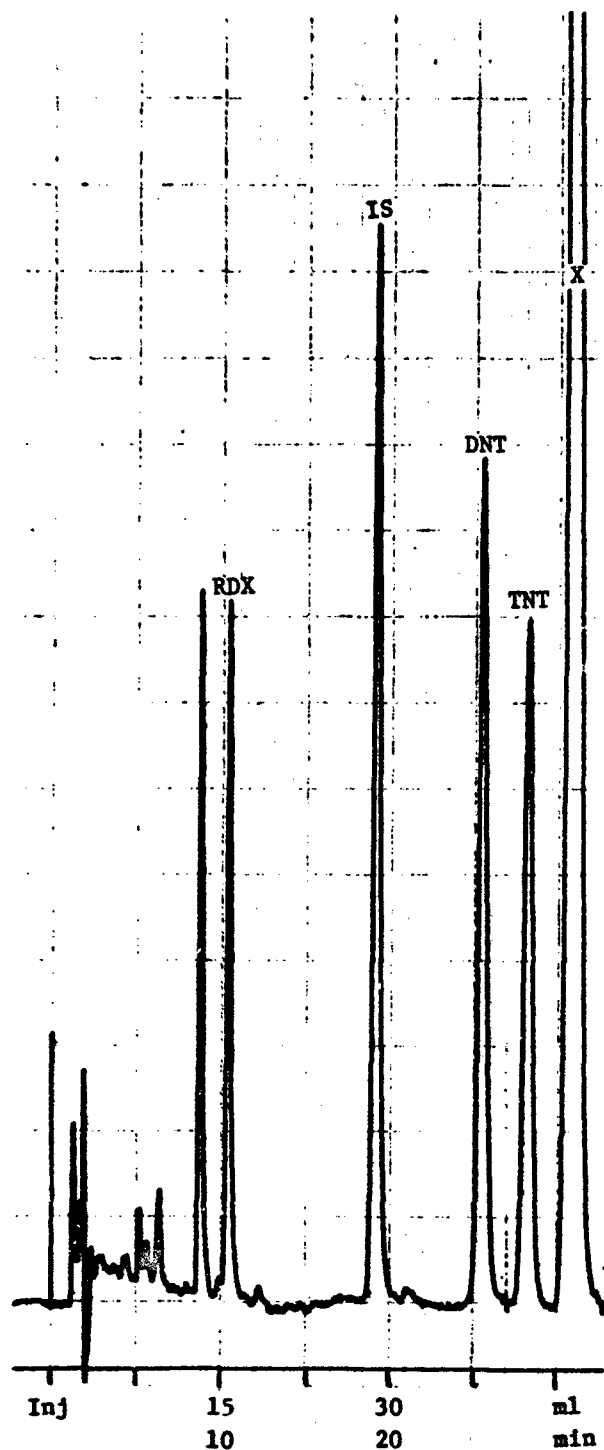
HPLC Conditions

Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 30% acetonitrile in
1% acetic acid in water
Flow rate: 1.5 ml/min
Chart speed: 0.1 in./min
Detector: UV, 254 nm

Sample Characteristics

1.0 ml plasma containing
100 ng/ml RDX, DNT and TNT
extracted 3 x 2 ml with toluene.
Toluene evaporated and sample
reconstituted to 1 ml
IS concentration: 1,000 ng/ml
Injection volume: 70 μ l
Attenuation: 0.01 X

Figure 9 - HPLC Analysis of Plasma Containing 100 ng/ml RDX, DNT, and TNT.
"X" indicates toluene contaminant.



HPLC Conditions

Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 30% acetonitrile in
1% acetic acid in water
Flow rate: 1.5 ml/min
Chart speed: 0.1 in./min
Detector: UV, 254 nm

Sample Characteristics

1.0 ml plasma containing
1,000 ng/ml RDX, DNT, TNT
extracted 3 x 2 ml with toluene.
Toluene evaporated and sample
reconstituted to 1 ml.

IS concentration: 1,000 ng/ml
Injection volume: 70 μ l
Attenuation: 0.01 X

Figure 10 - HPLC Analysis of Plasma Containing 1,000 ng/ml
RDX, DNT, and TNT. "X" indicates toluene contaminant.

TABLE 5

STATISTICAL EVALUATION OF RDX IN PLASMA DATA BY
THE HUBAUX AND VOS DETECTION LIMIT PROGRAM

Number ^a of Data Points	Linear Regression	Correlation Coefficient	Degrees of Freedom	y^c		Detection Limit
				b	Intercept	
48	$y = 0.825x + 14.0$	0.990	46	1.679	161	355
32	$y = 0.844x + 8.2$	0.995	30	1.697	70	145

ng/ml RDX Added	Average ^e ng/ml Found	Standard ^f Deviation	Percent ^g Imprecision	Percent ^h Inaccuracy
100	97	± 7.1	7.0	+ 1.0
500	427	± 11.6	2.7	- 13.8
1,000	839	± 23.3	2.7	- 14.9
1,500	1,252	± 31.5	2.5	- 16.5
2,000	1,664	± 71.2	4.3	- 17.2

^a Number of data points - data points used to calculate linear regression and detection limits;
48 - all data; 40 - 2,000 ng/ml samples omitted; 32 - 2,000 ng/ml and 1,500 ng/ml samples
omitted.

^b t - 2 tail p level (usually 0.1, each confidence band is 0.05 so total p = 0.1).

^c y intercept - intercept on y-axis of upper confidence limit line.

^d Detection limit - x-intercept of y-intercept and lower confidence limit line.

^e Average ng/ml found - average at each level determined from linear regression
equation for 48 points.

^f Standard deviation - determined from average value (e above) and observed values.

^g Percent imprecision - standard deviation divided by average value times 100%.

^h Percent inaccuracy - determined from the average values of the eight observed
values at each level

$$\% \text{ Inaccuracy} = \frac{\text{Average observed values} - \text{level added}}{\text{level added}} \times 100$$

TABLE 6

STATISTICAL EVALUATION OF DNT IN PLASMA DATA BY
THE HUBAUX AND VOS DETECTION LIMIT PROGRAM

Number ^a of Data Points	Linear Regression	Correlation Coefficient	Degrees of Freedom	b ^b	y ^c Intercept	Detection Limit
48	y = 0.659x + 6.3	0.982	46	1.679	168	489
40	y = 0.673x + 0.8	0.991	38	1.686	91	267
32	y = 0.648x + 7.2	0.984	30	1.697	90	256

ng/ml DNT Added	Average ^c ng/ml Found	Standard ^f Deviation	Percent ^g Imprecision	Percent ^h Inaccuracy
100	72	± 4.3	5.8	- 25.6
500	336	± 9.9	2.9	- 31.7
1,000	665	± 35.3	5.4	- 35.0
1,500	995	± 24.7	2.4	- 31.8
2,000	1,324	± 78.2	6.0	- 34.6

a Number of data points - data points used to calculate linear regression and detection limits;
48 - all data; 40 - 2,000 ng/ml samples omitted; 32 - 2,000 ng/ml and 1,500 ng/ml samples
omitted.

b t - 2 tail p level (usually 0.1, each confidence band is 0.05 so total p = 0.1).

c y intercept - intercept on y-axis of upper confidence limit line.

d Detection limit - x-intercept of y-intercept and lower confidence limit line.

e Average ng/ml found - average at each level determined from linear regression
equation for 48 points.

f Standard deviation - determined from average value (e above) and observed values.

g Percent imprecision - standard deviation divided by average value times 100%.

h Percent inaccuracy - determined from the average values of the eight observed
values at each level

$$\% \text{ Inaccuracy} = \frac{\text{Average observed values} - \text{level added}}{\text{level added}} \times 100$$

TABLE 7

**STATISTICAL EVALUATION OF TNT IN PLASMA DATA BY
THE HUBAUX AND VOS DETECTION LIMIT PROGRAM**

Number ^a of Data Points	Linear Regression	Correlation Coefficient	Degrees of Freedom	^b _t	^c Intercept	Detection Limit
48	$y = 0.785x + 6.4$	0.988	46	1.679	163	398
40	$y = 0.779x + 8.7$	0.991	38	1.686	114	271
32	$y = 0.777x + 9.2$	0.985	30	1.697	106	248

ng/ml TNT Added	Average ^e ng/ml Found	Standard ^f Deviation	Percent ^g Imprecision	Percent ^h Inaccuracy
.00	85	± 4.5	5.1	- 11.6
500	399	± 8.3	2.0	- 17.3
1,000	791	± 41.5	5.3	- 22.2
1,500	1,184	± 32.1	2.7	- 21.5
2,000	1,576	± 69.9	4.4	- 20.9

^a Number of data points - data points used to calculate linear regression and detection limits;
48 - all data; 40 - 2,000 ng/ml samples omitted; 32 - 2,000 ng/ml and 1,500 ng/ml samples
omitted.

^b $t - 2$ tail p level (usually 0.1, each confidence band is 0.05 so total $p = 0.1$).

^c y intercept - intercept on y -axis of upper confidence limit line.

^d Detection limit - x -intercept of y -intercept and lower confidence limit line.

^e Average ng/ml found - average at each level determined from linear regression
equation for 48 points.

^f Standard deviation - determined from average value (e above) and observed values.

^g Percent imprecision - standard deviation divided by average value times 100%.

^h Percent inaccuracy - determined from the average values of the eight observed
values at each level

$$\% \text{ Inaccuracy} = \frac{\text{Average observed values} - \text{level added}}{\text{level added}} \times 100$$

APPENDIX

RAW DATA AND CALCULATION FOR PLASMA METHOD DETERMINATION
FOR RDX, DNT, AND TNT

TABLE 8

LINEARITY AND PRECISION OF RDX, DNT, AND TNT DETERMINATION
BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Reference Solution Number	ng ml Compound Added	Peak Height (mm)			Internal Standard		Relative Weight Response			Calculated ng/ml		
		RDX	DNT	TNT	ng ml	Peak Height	RDX	DNT	TNT	RDX	DNT	TNT
A-1	0	< 2	< 2	< 2	1,000	122	-	-	-	ND	ND	ND
A-2	100	12	14	11	1,000	116	1.03	1.21	0.95	109	113	107
A-3	500	58	63	52	1,000	118	0.98	1.07	0.88	517	499	495
A-4	1,000	104	121	97	1,000	114	0.91	1.06	0.85	960	992	956
A-5	1,500	168	183	152	1,000	114	0.98	1.07	0.89	1,551	1,500	1,498
A-6	2,000	220	240	204	1,000	114	0.96	1.05	0.89	2,031	1,968	2,011
B-1	0	< 2	< 2	< 2	1,000	118	-	-	-	ND	ND	ND
B-2	100	13	14	12	1,000	120	1.08	1.17	1.00	114	109	112
B-3	500	59	64	52	1,000	122	0.97	1.05	0.85	509	490	479
B-4	1,000	121	145	118	1,000	134	0.90	1.08	0.88	950	1,011	989
B-5	1,500	172	184	157	1,000	117	0.98	1.05	0.89	1,547	1,470	1,508
B-6	2,000	228	246	208	1,000	116	0.98	1.06	0.90	2,069	1,982	2,015
C-1	0	< 2	< 2	< 2	1,000	120	-	-	-	ND	ND	ND
C-2	100	12	14	12	1,000	120	1.00	1.17	1.00	105	109	112
C-3	500	55	59	50	1,000	116	0.95	1.02	0.86	499	475	484
C-4	1,000	107	128	105	1,000	122	0.88	1.05	0.86	923	981	967
C-5	1,500	150	172	144	1,000	110	0.91	1.04	0.87	1,435	1,461	1,471
C-6	2,000	210	240	202	1,000	116	0.91	1.03	0.87	1,906	1,934	1,957
D-1	0	< 2	< 2	< 2	1,000	119	-	-	-	ND	ND	ND
D-2	100	11	12	10	1,000	112	0.98	1.07	0.89	103	100	100
D-3	500	54	60	50	1,000	116	0.93	1.03	0.86	490	483	484
D-4	1,000	116	137	110	1,000	124	0.94	1.10	0.89	985	1,032	997
D-5	1,500	155	174	144	1,000	113	0.91	1.03	0.85	1,444	1,439	1,432
D-6	2,000	212	236	194	1,000	116	0.91	1.02	0.84	1,924	1,901	1,879

TABLE 8 (concluded)

Relative Weight Response

	<u>Average</u>	<u>Standard Deviation</u>	<u>Relative Standard Deviation</u>
RDX	0.95	± 0.05	5.2%
DNT	1.07	± 0.05	4.9%
TNT	0.89	± 0.04	5.1%

TABLE 9
DETERMINATION OF RDX, DNT, AND TNT IN PLASMA SAMPLES

Sample Number	ng/ml ^a Compound Added	ml Plasma	Peak Height (mm)			Internal ^b Standard		ng/ml ^c Detected		
			RDX	DNT	TNT	ng/ml	Peak Height	RDX	DNT	TNT
Day 1A-0	0	1.0	< 2	< 2	< 2	1,000	238	ND ^d	ND	ND
Day 1A-100	100	1.0	18.3	21.0	17.8	1,000	235	95	84	95
Day 1A-500	500	1.0	88.5	92.8	81.5	1,000	226	478	388	451
Day 1A-1000	1,000	1.0	191	196	177	1,000	236	987	786	940
Day 1A-1500	1,500	1.0	285	293	260	1,000	263	1,324	1,055	1,238
Day 1A-2000	2,000	1.0	427	432	404	1,000	270	1,927	1,509	1,870
Day 1B-0	0	1.0	< 2	< 2	< 2	1,000	232	ND	ND	ND
Day 1B-100	100	1.0	22.3	15.3	18.0	1,000	232	117	62	97
Day 1B-500	500	1.0	87.8	83.0	78.8	1,000	235	456	333	419
Day 1B-1000	1,000	1.0	174	160	170	1,000	253	839	595	840
Day 1B-1500	1,500	1.0	269	259	235	1,000	244	1,345	1,000	1,202
Day 1B-2000	2,000	1.0	366	374	355	1,000	254	1,760	1,391	1,748

TABLE 9 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	Compound Added $\frac{\text{ng}}{\text{ml}}$	Peak Height (mm)			Internal ^b Standard $\frac{\text{ng}}{\text{ml}}$		Relative Weight ^c Response	
		RDX	DNT	TNT	ng	Peak Height	RDX	DNT
Std - Day 1-2	1,000	171	226	172	1,000	214	0.80	1.06
Std - Day 1-1	500	92.3	115	85.0	1,000	218	0.85	1.06
Std - Day 1-4	2,000	366	469	349	1,000	224	0.82	1.05
Std - Day 1-3	1,500	256	346	266	1,000	212	0.81	1.09
						Average	0.82	1.06
							0.80	0.80

23
24^a ng/ml Compound added - nanograms of RDX, DNT, and TNT added to 1.0 ml plasma.^b Internal standard - compound (propiphenone) added to plasma sample after sample preparation for calculation of data.^c ng/ml detected - nanograms of RDX, DNT, and TNT detected in the 1.0 ml plasma sample

$$\text{ng compound/ml} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}}$$

^d ND - not detected, less than 20 ng/ml.^e Relative weight response - RWR

$$\text{RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

TABLE 10
DETERMINATION OF RDX, DNT, AND TNT IN PLASMA SAMPLES

Sample Number	ng/ml ^a Compound Added	ml Plasma	Peak Height (mm)			Internal ^b Standard ng ml	ng/ml ^c Detected		
			RDX	DNT	TNT		RDX	DNT	TNT
Day 2A-0	0	1.0	< 2	< 2	< 2	1,000	ND ^d	ND ^d	ND ^d
Day 2A-100	100	1.0	29.0	34.0	27.6	1,000	113	86	96
Day 2A-500	500	1.0	112	143	129	1,000	403	333	412
Day 2A-1000	1,000	1.0	225	276	250	1,000	841	659	830
Day 2A-1500	1,500	1.0	305	374	350	1,000	1,153	915	1,173
Day 2A-2000	2,000	1.0	427	642	515	1,000	1,576	1,533	1,687
Day 2B-0	0	1.0	< 2	< 2	< 2	1,000	ND	ND	ND
Day 2B-100	100	1.0	28.0	30.0	31.0	1,000	104	72	102
Day 2B-500	500	1.0	123	152	135	1,000	428	343	416
Day 2B-1000	1,000	1.0	220	306	246	1,000	830	750	825
Day 2B-1500	1,500	1.0	311	457	353	1,000	1,168	1,111	1,177
Day 2B-2000	2,000	1.0	405	447	488	1,000	1,457	1,041	1,558

TABLE 10 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	$\frac{\text{ng}}{\text{ml}}$ Compound Added	Peak Height (mm)			Internal ^b Standard $\frac{\text{ng}}{\text{ml}}$	Relative Weight ^c Response	
		RDX	DNT	TNT		RDX	TNT
Std - Day 2-2	500	122	188	135	1,000	0.78	1.20
Std - Day 2-1	100	25.0	39.0	28.6	1,000	0.81	1.27
Std - Day 2-3	1,000	230	365	268	1,000	0.74	1.17
Std - Day 2-4	1,500	359	575	420	1,000	0.74	1.18
Std - Day 2-1	100	31.6	44.0	33.0	1,000	0.96	1.34
Std - Day 2-5	2,000	430	692	496	1,000	0.71	1.14
					Average	0.79	1.22
							0.89

^a ng/ml Compound added - nanograms of RDX, DNT, and TNT added to 1.0 ml plasma.

^b Internal standard - compound (propiphenone) added to plasma sample after sample preparation for calculation of data.

^c ng/ml detected - nanograms of RDX, DNT, and TNT detected in the 1.0 ml plasma sample

$$\text{ng compound/ml} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}}$$

^d ND - not detected, less than 20 ng/ml.

^e Relative weight response - RWR

$$\text{RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

TABLE 11

DETERMINATION OF RDX, DNT, AND TNT IN PLASMA SAMPLES

Sample Number	ng/ml ^a Compound Added	ml Plasma	Peak Height (mm)			Internal ^b Standard ng ml	ng/ml ^c Detected		
			RDX	DNT	TNT		RDX	DNT	TNT
Day 3A-0	0	1.0	< 2	< 2	< 2	1,000	ND ^d	ND	ND
Day 3A-100	100	1.0	21.0	28.4	18.0	1,000	83	79	70
Day 3A-500	500	1.0	106	128	110	1,000	410	351	419
Day 3A-1000	1,000	1.0	208	253	206	1,000	814	700	793
Day 3A-1500	1,500	1.0	322	392	262	1,000	1,259	1,052	980
Day 3A-2000	2,000	1.0	434	565	445	1,000	1,581	1,456	1,600
Day 3B-0	0	1.0	< 2	< 2	< 2	1,000	ND	ND	ND
Day 3B-100	100	1.0	20.0	21.6	21.6	1,000	71	54	76
Day 3B-500	500	1.0	104	122	110	1,000	385	321	404
Day 3B-1000	1,000	1.0	216	198	163	1,000	775	505	579
Day 3B-1500	1,500	1.0	332	416	346	1,000	1,148	1,019	1,181
Day 3B-2000	2,000	1.0	393	432	398	1,000	1,337	1,041	1,338

TABLE 11 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng ml Compound Added	Peak Height (mm)			Internal ^b Standard		Relative Weight ^c Response		
		RDX	DNT	TNT	ng ml	Peak Height	RDX	DNT	TNT
Std - Day 3-1	100	22.0	32.0	24.0	1,000	282	0.78	1.14	0.85
Std - Day 3-2	500	122	172	122	1,000	308	0.79	1.12	0.79
Std - Day 3-2	500	132	186	136	1,000	332	0.80	1.12	0.82
Std - Day 3-4	1,500	362	526	371	1,000	307	0.79	1.14	0.81
Std - Day 3-1	100	24.4	34.0	24.4	1,000	298	0.82	1.14	0.82
Std - Day 3-1	100	24.0	36.0	26.0	1,000	300	0.80	1.20	0.87
Std - Day 3-3	1,000	226	326	236	1,000	297	0.76	1.10	0.80
Std - Day 3-5	2,000	471	692	482	1,000	309	0.76	1.12	0.78
Std - Day 3-5	2,000	537	702	492	1,000	312	0.86	1.12	0.79
						Average	0.80	1.13	0.81

^a ng/ml Compound added - nanograms of RDX, DNT, and TNT added to 1.0 ml plasma.

^b Internal standard - compound (propiphenone) added to plasma sample after sample preparation for calculation of data.

^c ng/ml detected - nanograms of RDX, DNT, and TNT detected in the 1.0 ml plasma sample

$$\text{ng compound/ml} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}}$$

^d ND - not detected, less than 20 ng/ml.

^e Relative weight response - RWR

$$\text{RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

TABLE 12

DETERMINATION OF RDX, DNT, AND TNT IN PLASMA SAMPLES

Sample Number	ng/ml ^a Compound Added	ml Plasma	Peak Height (mm)			Internal ^b Standard		ng/ml ^c Detected		
			RDX	DNT	TNT	ng ml	Peak Height	RDX	DNT	TNT
Day 4A-0	0	1.0	< 2	< 2	< 2	1,000	377	ND ^d	ND	ND
Day 4A-100	100	1.0	36.0	34.4	27.0	1,000	364	129	83	93
Day 4A-500	500	1.0	129	130	112	1,000	376	446	302	371
Day 4A-1000	1,000	1.0	253	268	219	1,000	387	849	607	707
Day 4A-1500	1,500	1.0	373	399	358	1,000	366	1,321	956	1,223
Day 4A-2000	2,000	1.0	467	426	107	1,000	333	1,823	1,123	403
Day 4B-0	0	1.0	< 2	< 2	< 2	1,000	379	ND	ND	ND
Day 4B-100	100	1.0	28.0	32.4	23.6	1,000	378	96	75	78
Day 4B-500	500	1.0	122	148	119	1,000	358	444	361	415
Day 4B-1000	1,000	1.0	252	252	214	1,000	376	870	587	710
Day 4B-1500	1,500	1.0	370	456	370	1,000	370	1,299	1,082	1,250
Day 4B-2000	2,000	1.0	580	597	462	1,000	383	1,722	1,366	1,507

TABLE 12 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	Compound Added	ng ml	Peak Height (mm)			Internal ^b Standard ng ml	Relative Weight ^c Response		
			RDX	DNT	TNT		RDX	DNT	TNT
Std - Day 4-1	100		25.0	37.0	25.6	1,000	0.80	1.19	0.82
Std - Day 4-2	500		122	184	130	1,000	0.76	1.14	0.81
Std - Day 4-3	1,000		249	373	262	1,000	0.75	1.13	0.79
Std - Day 4-4	1,500		375	560	393	1,000	0.76	1.14	0.80
Std - Day 4-5	2,000		492	722	500	1,000	0.76	1.12	0.77
Std - Day 4-4	1,000		246	362	252	1,000	0.76	1.12	0.78
Std - Day 4-4	1,000		259	374	264	1,000	0.79	1.14	0.80
Std - Day 4-5	2,000		485	712	493	1,000	0.77	1.13	0.78
Std - Day 4-4	1,000		260	384	274	1,000	0.77	1.13	0.81
Std - Day 4-5	2,000		498	745	522	1,000	0.76	1.14	0.80
Average							0.77	1.14	0.80

^a ng/ml Compound added - nanograms of RDX, DNT, and TNT added to 1.0 ml plasma.

^b Internal standard - compound (propiphenone) added to plasma sample after sample preparation for calculation of data.

^c ng/ml detected - nanograms of RDX, DNT, and TNT detected in the 1.0 ml plasma sample

$$\text{ng compound/ml} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}}$$

^d ND - not detected, less than 20 ng/ml.

^e Relative weight response - RWR

$$\text{RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

APPENDIX C

IDENTIFICATION OR DEVELOPMENT OF CHEMICAL ANALYSIS METHODS
FOR PLANTS AND ANIMAL TISSUE

METHOD REPORT NO. 2

METHOD DEVELOPMENT FOR THE DETERMINATION OF
CYCLOTRIMETHYLENETRINITRAMINE (RDX), DINITROTOLUENE (DNT),
AND TRINITROTOLUENE (TNT) IN KIDNEY

September 1980

Contract No. DAAK11-79-C-0110
MRI Project No. 4849-A

For

U.S. Army Toxic and Hazardous Materials Agency
Dr. L. Eng, DRXTH-TE-D, Project Officer
Aberdeen Proving Ground (EA), Maryland 21010

The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER Technical Report No. 2	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Method Development for the Determination of Cyclotrimethylenetrinitramine (RDX), Dinitro- toluene (DNT), and Trinitrotoluene (TNT) in Kidney		5. TYPE OF REPORT & PERIOD COVERED
7. AUTHOR(s) D. B. Lakings and O. Gan		6. PERFORMING ORG. REPORT NUMBER
9. PERFORMING ORGANIZATION NAME AND ADDRESS Midwest Research Institute 425 Volker Boulevard Kansas City, MO 64110		8. CONTRACT OR GRANT NUMBER(s) DAAK11-79-C-0110
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Armament Research and Development Command, Aberdeen Proving Ground (Edgewood Area), MD 21010		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		12. REPORT DATE September 1980
		13. NUMBER OF PAGES
		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Unlimited		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Cyclotrimethylenetrinitramine (RDX) High Performance Liquid Chromatography Dinitrotoluene (DNT) Trinitrotoluene (TNT) Kidney Level Determination		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A high performance liquid chromatographic (HPLC) method for the quantita- tive determination of cyclotrimethylenetrinitramine (RDX), dinitrotoluene (DNT), and trinitrotoluene (TNT) in kidney has been developed. The analytical system consists of an isocratic HPLC unit with a Spherisorb ODS 5 μ , 250 x 4.6 mm ID column, an eluent of 30% acetonitrile in 1% acetic acid in water, and a flow rate of 1.5 ml/min. The compounds, including the internal standard (IS), pro- piophenone, have the following retention characteristics: RDX, 15 ml, 10 min;		

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE(When Data Entered)

IS, 28.5 ml, 19 min; DNT, 37.5 ml, 25 min; and TNT, 42 ml, 28 min; and are detected and quantified by an ultraviolet detector at 254 nm. Reference solutions of the compounds have a linear response from 100 ng/ml to 2,000 ng/ml. The kidney samples were prepared by adding 1 ml 10% sodium chloride containing 2% acetic acid to 1 g kidney and extracting the sample with 3 x 2 ml toluene. The toluene was evaporated at room temperature under a stream of nitrogen gas. The residue was dissolved with 0.5 ml acetonitrile which contained 1,000 ng IS and the final volume was adjusted to 1 ml with high-purity water. The sample was filtered through a 0.45- μ Fluoropore filter and injected onto the HPLC system. The analytical method was evaluated by preparing and analyzing duplicate samples containing 0, 100, 500, 1,000, 1,500, and 2,000 ng/g of each compound on four succeeding days. Linear regression analysis of the data gave the following equations and correlation coefficients: RDX, $y = 0.973x + 7.1$, 0.999; DNT, $y = 0.707x - 3.5$, 0.991; and TNT, $y = 0.746x - 5.4$, 0.992. The average coefficient of variation and average percent inaccuracy for RDX, DNT, and TNT determination in kidney were 7%, -0.6; 9%, -29; and 11%, -25, respectively. A small peak eluted just prior (approximately 1.5 ml) to RDX and may interfere with the quantitation of RDX at low levels. This peak represented less than 50 ng/g RDX in each of the blank kidney samples analyzed. A statistical evaluation of the data by the Hubaux and Vos detection limit program gave detection limits of 95 ng/g for RDX, 179 ng/g for DNT, and 211 ng/g for TNT for the HPLC determination of these compounds in kidney samples.

UNCLASSIFIED

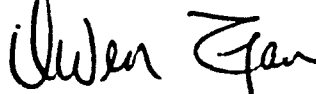
SECURITY CLASSIFICATION OF THIS PAGE(When Data Entered)

PREFACE

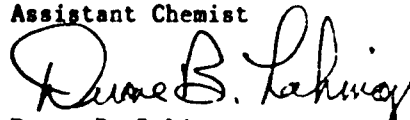
The report was prepared at Midwest Research Institute, 425 Volker Boulevard, Kansas City, Missouri 64110, under U.S. Army Toxic and Hazardous Materials Agency Contract No. DAAK11-79-C-0110, MRI Project No. 4849-A, "Identification or Development of Chemical Analysis Methods for Plants and Animal Tissues." Dr. Leslie Eng, DRXTH-TE-D, was the Project Officer for this research effort.

This work was conducted in the Analytical Chemistry Department Dr. James L. Spigarelli, Director. The research effort was directed by Dr. Duane B. Lakings, Program Manager and Senior Chemist, with assistance from Mr. Owen Gan, Assistant Chemist, and Ms. Mary Valla, Senior Technician. This report was prepared by Dr. Lakings and Mr. Gan.

MIDWEST RESEARCH INSTITUTE

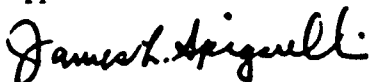


Owen Gan
Assistant Chemist



Duane B. Lakings
Program Manager and Senior Chemist

Approved:



James L. Spigarelli, Director
Analytical Chemistry Department

TABLE OF CONTENTS

	<u>Page</u>
1. Application	1
2. Chemistry	1
3. Apparatus	2
4. Standards	3
5. Procedures for Kidney Sample Determination.	4
6. Calculation	6
7. Statistical Evaluation of Data.	7
Appendix - Method Development for the Determination of RDX, DNT, and TNT in Kidney Samples	25

LIST OF FIGURES

<u>Number</u>	<u>Title</u>	<u>Page</u>
1	HPLC Separation of RDX, DNT, TNT, and Teteryl SARMS and Propiophenone (IS)	8
2	Determination of RDX in Kidney	13
3	Determination of DNT in Kidney	14
4	Determination of TNT in Kidney	15
5	Standard Deviation for RDX, DNT, and TNT in Kidney Samples.	16
6	Coefficient of Variation for RDX, DNT, and TNT in Kidney Samples.	17
7	Percent Inaccuracy for RDX, DNT, and TNT in Kidney Samples.	18
8	HPLC Analysis of Blank Kidney Sample for RDX, DNT, and TNT Method Development	19
9	HPLC Analysis of Kidney Containing 100 ng/g RDX, DNT, and TNT.	20
10	HPLC Analysis of Kidney Containing 1,000 ng/g RDX, DNT, and TNT.	21

LIST OF TABLES

<u>Number</u>	<u>Title</u>	<u>Page</u>
1	Linearity and Precision of the HPLC-UV Determination of SARM Reference Solutions of RDX, DNT, and TNT.	9
2	HPLC-UV Determinations of RDX in Kidney Samples.	10
3	HPLC-UV Determinations of DNT in Kidney Samples.	11
4	HPLC-UV Determinations of TNT in Kidney Samples.	12
5	Statistical Evaluation of RDX in Kidney Data by the Hubaux and Vos Detection Limit Program	22
6	Statistical Evaluation of DNT in Kidney Data by the Hubaux and Vos Detection Limit Program	23
7	Statistical Evaluation of TNT in Kidney Data by the Hubaux and Vos Detection Limit Program	24
Appendix		
8	Linearity and Precision of RDX, DNT, and TNT Determination by High Performance Liquid Chromatography.	26
9	Determination of RDX, DNT, and TNT in Kidney Samples . . .	28
10	Determination of RDX, DNT, and TNT in Kidney Samples . . .	30
11	Determination of RDX, DNT, and TNT in Kidney Samples . . .	32
12	Determination of RDX, DNT, and TNT in Kidney Samples . . .	34

Midwest Research Institute
Analytical Chemistry Department
Kansas City, Missouri 64110

for

U.S. Army Armament Research and Development Command
Aberdeen Proving Ground (Edgewood Area)
Maryland 21020

Contract No. DAAK11-79-C-0110

Identification or Development of Chemical Analysis Methods
for Plant and Animal Tissues

METHOD DEVELOPMENT FOR THE DETERMINATION OF
CYCLOTRIMETHYLENETRINITRAMINE (RDX), DINITROTOLUENE (DNT),
AND TRINITROTOLUENE (TNT) IN KIDNEY

1. APPLICATION: The developed method is for the quantitative determination of RDX, DNT, and TNT in animal kidney samples using high performance liquid chromatography (HPLC) with ultraviolet (UV) detection at 254 nm.

a. Evaluated Concentration Range: The concentration range of RDX, DNT, and TNT studied in reference solutions and in kidney samples was 100 to 2,000 ng/g (parts per billion, ppb).

b. Sensitivity: A signal-to-noise ratio of 6 to 1 for RDX (peak height (PH), 25 mm), 9 to 1 for DNT (PH, 40 mm), and 8 to 1 for TNT (PH, 30 mm) was obtained with an injection of 50 µl of a 100 ng/ml solution of each compound (ca. 5 ng each compound on column).

c. Detection Limits: 95 ng/g RDX, 179 ng/g DNT, and 211 ng/g TNT using the Hubaux and Vos detection limit program.

d. Interferences: No interfering kidney components were found to elute with the same retention volume as DNT or TNT. A small peak eluted just prior to RDX and interfered with the quantification of RDX at low levels. This peak corresponded to less than 50 ng/g RDX.

e. Analysis Rate: The chromatographic time per injection was 40 min. Two reference solutions were analyzed prior to injecting the prepared samples, and two were analyzed during the day (160 min total time). Thus, a total of eight prepared kidney samples (320 min total time) can be analyzed during an 8-hr day.

2. CHEMISTRY: RDX, DNT, and TNT are munition compounds manufactured at various installations. The possible environmental contamination of these compounds, particularly in plants and animals, is of concern.

The determination of the kidney levels of RDX, DNT, and TNT in animals may provide information on the extent and level of contamination at the production facilities and in the surrounding area. Each of these munitions has a sufficient UV chromophore at 254 nm to allow UV detection and quantification. These munitions are of intermediate polarity and have limited water solubility. Normally, biological matrices have a large number of components which will interfere with the detection and quantification of low levels of compounds. Reverse phase HPLC is capable of separating compounds with similar chemical and physical properties; the elution order of the technique is based on the polarity of the compounds, the more polar compounds being eluted first. Thus, by extracting the biological matrix, i.e., kidney, with an intermediate polarity solvent and analyzing the extract by HPLC, a simple sample preparation and analysis system may be defined for the determination of RDX, DNT, and TNT in kidney samples.

3. APPARATUS:

a. Instrumentation: A Waters isocratic liquid chromatographic system consisting of a Model 6000A pump, Model U6K injector, and Model 440 UV detector. A general purpose centrifuge (Dynac, Clay Adams 010i) with a 24-place head.

b. HPLC Parameters:

1. Column: Spherisorb ODS, 5 μ , 250 x 4.6 mm ID.
2. Eluent: 30% acetonitrile in 1% acetic acid in water.
3. Flow rate: 1.5 ml/min.
4. Detector: UV, 254 nm.
5. Internal standard: propiophenone, 1,000 ng/ml.
6. Injection volume: 50 to 100 μ l.
7. Retention volumes and times: RDX, 15 ml, 10 min; DNT, 37.5 ml, 25 min; TNT, 42 ml, 28 min; IS, 28.5 ml, 19 min. NOTE: Slight changes in the retention indices may occur with fresh eluent or a change in columns.

A representative HPLC chromatogram for RDX, DNT, and TNT is shown in Figure 1. Also included on the chromatogram are peaks for an internal standard (propiophenone) and 2,4,5-trinitrophenylmethylnitramine (tetryl).

c. Laboratory Glassware and Equipment:

1. Culture tubes (Pyrex) with Teflon-lined screw caps.
2. Volumetric flasks (100 ml).
3. Volumetric syringes (0-100 μ l, 0-500 μ l, and 0-1,000 μ l).
4. Automatic pipetter (0-5 ml).
5. Six-speed Waring-type blender with glass container.
6. Teflon-glass, motor-driven tissue homogenizer.
7. Filtering apparatus including filter holders, 5-ml disposable syringes, and 0.45 μ Fluoropore filters.
8. Inert gas (nitrogen) drying train with 12 ports.

d. Chemicals:

1. Toluene and acetonitrile, "Distilled in Glass" grade.
2. Acetic acid and sodium chloride, ACS grade.
3. High purity water from a Milli-Q water purification system.
4. RDX, DNT, and TNT SARMs, obtained from the U.S. Army Toxic and Hazardous Materials Agency.
5. Propiophenone (internal standard), analytical grade.

4. STANDARDS:

a. Stock: Weigh approximately 20 mg of TNT, DNT, RDX and tetryl SARM or interim SARM into separate 100-ml volumetric flasks. Dissolve each compound in acetonitrile and dilute to volume. The concentration of each compound is 200 μ g/ml. Quantitatively pipette 20 ml from each stock above into a 100-ml volumetric flask and dilute to volume with distilled water. Concentration of each compound is 40 μ g/ml.

b. Working: Pipette 10 ml of the 40 μ g/ml of each compound stock into a 100-ml volumetric flask and dilute to volume with high purity water. Concentration of each compound is 4 μ g/ml.

Reference solutions were prepared from this stock as follows:

<u>µl Working Stock</u>	<u>µl IS Stock*</u>	<u>µl 10% Acetonitrile in Water</u>	<u>Concentration Each Compound (ng/ml)</u>
500	500	0	2,000
375	500	125	1,500
250	500	250	1,000
125	500	375	500
25	500	475	100
0	500	500	0

* Preparation of IS stock given in "c" below.

c. Internal Standard Stock: Weigh 10 mg propiophenone into a 100-ml volumetric flask and dilute to volume with acetonitrile (concentration, 100 µg/ml). Quantitatively pipette 10 ml of the 100 µg/ml stock into a 100-ml volumetric flask and dilute to volume with acetonitrile (concentration, 10 µg/ml). A final working solution of 2.0 µg/ml is prepared by pipetting 20 ml of the 10 µg/ml stock into a 100-ml volumetric flask and diluting to volume with acetonitrile.

5. PROCEDURES FOR KIDNEY SAMPLE DETERMINATION:

a. Kidney Sample Preparation: The procedure employed to prepare kidney samples for the HPLC-UV determination of RDX, DNT, and TNT consisted of:

1. Place approximately 50 g of kidney into a Waring-type blender and blend for 1 min on speed six (liquify). Note: To completely liquify the kidney sample, the sides of the glass container are scraped with a spatula.
2. Transfer approximately 10 g of the liquified kidney sample to a motor-driven Teflon-glass homogenizer.
3. Homogenize the sample for 30 sec to disrupt the cell walls of the kidney sample. Note: The homogenization step is necessary to solubilize the intercellular compounds prior to the extraction step.
4. Repeat steps 3 and 4 on the remaining liquified kidney samples and combine the homogenized samples.
5. Accurately weigh 12 1.0 g homogenized kidney aliquots into culture tubes with Teflon-lined screw caps.

6. Spike two each of the homogenized kidney aliquots with the working stock (4 µg/ml each RDX, DNT, and TNT) at the following levels: 2,000 ng (500 µl); 1,500 ng (375 µl); 1,000 ng (250 µl); 500 ng (125 µl); and 100 ng (25 µl). The remaining two kidney aliquots serve as kidney sample blanks. All samples are adjusted to a total volume of 1.5 ml with high purity water containing 10% acetonitrile.

7. Add 1.0 ml of a 10% sodium chloride solution containing 2% acetic acid to each aliquot.

8. Mix thoroughly on a vortex mixer.

9. Extract the kidney samples with 2 ml toluene ("Distilled in Glass" grade) by vortexing for 30 sec followed by centrifugation at 1,000 rpm for 20 min. Note: The centrifugation step is required to break the emulsion formed during extraction.

10. Transfer the toluene extracts to properly labeled culture tubes with Teflon-lined screw caps.

11. Repeat the toluene extraction (steps 9 and 10) twice more, combining the toluene extracts in the appropriate tubes.

12. Evaporate the toluene at room temperature under a stream of nitrogen. NOTE: Do not heat the samples during the evaporation step, or loss of RDX, DNT, and TNT may occur. Continue evaporation until toluene has been completely removed from the culture tube.

13. Add about 1.0 ml ethyl acetate to the kidney sample residues and vortex mix for 30 sec.

14. Evaporate the ethyl acetate at room temperature under a stream of nitrogen. NOTE: The ethyl acetate aids in removing the last traces of toluene from the kidney samples.

15. Dissolve the residues in 500 µl acetonitrile containing 1,000 ng IS and mix thoroughly on a vortex mixer.

16. Add 500 µl high-purity water to each extracted kidney sample and mix thoroughly. NOTE: Final volume of the prepared samples is 1.0 ml.

17. Filter the solutions through 0.45-µ Fluoropore filters into culture tubes.

18. Analyze a 50- to 100-µl aliquot of each prepared kidney sample by HPLC.

19. After the elution of the TNT peak, wash the column for 3 min with 100% acetonitrile at 1.5 ml/min to remove any late eluting compounds. NOTE: The acetonitrile wash step is required to prevent possible interference in the chromatographic analysis of the next sample.

20. After the 3-min wash, switch the system back to the eluent. Allow approximately 7 min for equilibration prior to the next injection.

b. Calibration: The reference solutions described in Section 4.b were prepared and analyzed in quadruplicate. The relative weight response (RWR) (Eq. 1) of each compound was calculated and the average RWR utilized to determine the nanograms of each compound in every reference solution (Eq. 2). The nanograms found were plotted against the nanograms added, and a linear regression evaluation of the data was conducted. The slope, intercept, and correlation coefficient for each compound were determined. The data are summarized in Table 1, which includes the average value at each level for each compound, the standard deviation, coefficient of variation (relative standard deviation), and the percent inaccuracy. The raw data and calculations are given in Table 8 of the Appendix.

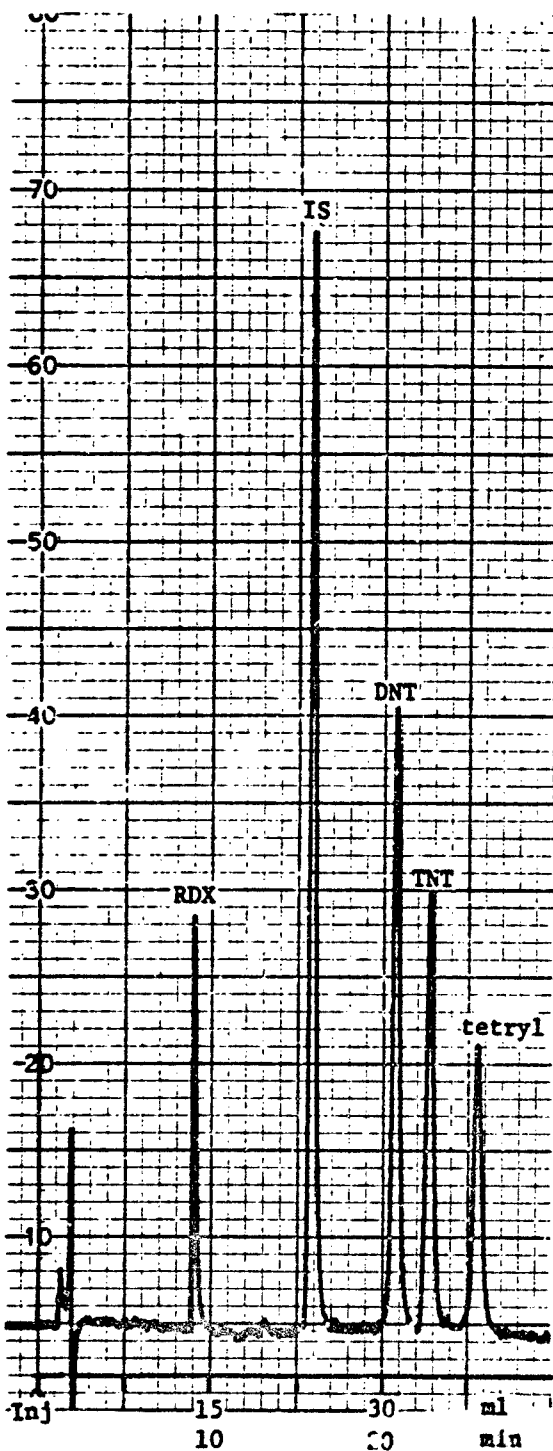
$$RWR = \frac{\text{Peak Height Cpd}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml Cpd}} \quad (\text{Eq. 1})$$

$$\text{ng/ml or ng/g compound} = \frac{\text{Peak Height Cpd}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{Avg. RWR}} \quad (\text{Eq. 2})$$

c. Kidney Sample Analysis: The kidney samples prepared as outlined in Section 5.a were injected onto the HPLC system. The peak height of each compound was measured and recorded. Kidney samples were prepared and analyzed on four succeeding days.

6. CALCULATION: The level (nanograms per gram) of each compound in the kidney samples was determined using the relative weight response to an internal standard method. The RWR values (Eq. 1) for the reference solutions analyzed with a day's set of kidney samples were calculated, and the average values for RDX, DNT, and TNT were determined. These RWR values were employed to calculate the kidney sample level of each compound by Equation 2, where nanograms per gram represents the level found in the kidney sample. The results for the duplicate determinations of RDX, DNT, and TNT in kidney samples at five different levels on four succeeding days are summarized in Tables 2, 3, and 4. The average level found, the standard deviation, coefficient of variation, and percent inaccuracy are also included. A linear regression evaluation of the results was made; the slope, intercept, and correlation coefficient are given in the tables. The level of each compound found in the kidney samples was plotted against the amount added, and these data are shown in Figures 2, 3, and 4. A graphic presentation of the standard deviation, coefficient of variation, and percent inaccuracy for the determination of RDX, DNT, and TNT in kidney samples is given in Figures 5, 6, and 7, respectively. Representative HPLC chromatograms are shown for a kidney sample blank (Figure 8), a 100 ng/g kidney sample (Figure 9), and a 1,000 ng/g kidney sample (Figure 10). The raw data and calculations for the kidney sample determinations are given in Tables 9 to 12 in the Appendix.

7. STATISTICAL EVALUATION OF DATA: A statistical evaluation of the data obtained for the determination of RDX, DNT, and TNT in kidney samples (Tables 2, 3, and 4) by the Hubaux and Vos detection limit program was made at the U.S. Army Toxic and Hazardous Materials Agency. The results of these evaluations are given in Table 5 for RDX, Table 6 for DNT, and Table 7 for TNT. When the 2,000- and 1,500-ng/g data points were omitted, the detection limits for RDX, DNT, and TNT in kidney as determined by the program were 95, 179, and 211 ng/g, respectively. The average nanograms per gram value found at each level was determined from the linear regression equation for the 48 data points and the nanograms per gram added at that level. The standard deviation and percent imprecision (coefficient of variation) at each level were calculated based on this average nanograms per gram value found. Thus, these values and the values given in Tables 2, 3, and 4 (based on the average of the eight assays at each level) are not comparable. The percent inaccuracy term was calculated from the average of the eight data points at each level, and thus agrees with the values in Tables 2, 3, and 4.



HPLC Conditions

Column: Spherisorb ODS, 5 μ ,
 250 x 4.6 mm ID
 Eluent: 30% acetonitrile in
 1% acetic in water
 Flow Rate: 1.5 ml/min
 Chart Speed: 0.1 in./min
 Detector: UV, 254 nm

Sample Characteristics

Concentrations: RDX, DNT, TNT,
 and tetryl - 500 ng/ml;
 IS - 1,000 ng/ml
 Injection volume: 70 μ l
 Attenuation: 0.01 X

Retention Indices

<u>Compound</u>	<u>Retention Volume (ml)</u>	<u>Retention Time (min)</u>
RDX	13.5	9
IS	22.5	15
DNT	31.5	21
TNT	34.5	23
tetryl	39	26

Figure 1 - HPLC Separation of RDX, DNT, TNT, and Tetryl
 SARMS and Propiophenone (IS)

TABLE 1

**LINEARITY AND PRECISION OF THE HPLC-UV DETERMINATION OF
SARM REFERENCE SOLUTIONS OF RDX, DNT, AND TNT**

Compound	ng/ml Added	ng/ml Detected				Average ^a	Standard Deviation ^b	Coefficient ^c of Variation	Percent ^d Inaccuracy
		A	B	C	D				
RDX	0	ND ^e	ND	ND	ND	-	-	-	-
	100	109	114	105	103	108	± 4.9	4.5	+ 8.0
	500	517	509	499	490	504	± 11.8	2.3	+ 0.8
	1,000	960	950	923	985	955	± 26	2.7	- 4.5
	1,500	1,551	1,547	1,435	1,444	1,494	± 63	4.2	- 0.4
	2,000	2,031	2,069	1,906	1,924	1,983	± 80	4.0	- 0.8
DNT	0	ND	ND	ND	ND	-	-	-	-
	100	113	109	109	100	108	± 5.5	5.1	+ 8.0
	500	499	490	475	483	487	± 10.2	2.1	- 2.6
	1,000	992	1,011	981	1,032	1,004	± 22	2.2	+ 0.4
	1,500	1,500	1,470	1,461	1,439	1,468	± 25	1.7	- 2.1
	2,000	1,968	1,982	1,934	1,901	1,946	± 36	1.9	- 2.7
TNT	0	ND	ND	ND	ND	-	-	-	-
	100	107	112	112	100	108	± 5.7	5.3	+ 8.0
	500	495	479	484	484	486	± 6.8	1.4	- 2.8
	1,000	956	989	967	997	977	± 19	2.0	- 2.3
	1,500	1,498	1,508	1,471	1,432	1,478	± 34	2.3	- 1.5
	2,000	2,011	2,015	1,957	1,879	1,966	± 63	3.2	- 1.7

Linear RegressionRDX: $y = 0.988x + 0.6$

Correlation coefficient - 0.998

DNT: $y = 0.974x + 7.7$

Correlation coefficient - 0.999

TNT: $y = 0.982x + 1.2$

Correlation coefficient - 0.999

^a Average = $\Sigma x/n = \bar{x}$ ^b Standard deviation = $(\Sigma |\bar{x} - x|^2 / (n-1))^{1/2} = \sigma$ ^c Coefficient of variation = $\sigma / \bar{x} \times 100$ ^d Percent inaccuracy = $\frac{x - \text{ng added}}{\text{ng added}} \times 100$ ^e ND = Not detectable, less than 20 ng/ml

TABLE 2

HPLC-UV DETERMINATIONS OF RDX IN KIDNEY SAMPLES

Amount Added (ng/g)	Level Found (ng/g)								Average ^a	Standard ^b Deviation	Coefficient ^c of Variation	Percent ^d Inaccuracy
	Day 1		Day 2		Day 3		Day 4					
	A	B	A	B	A	B	A	B				
0	ND ^e	ND	ND	ND	ND	ND	ND	ND	-	-	-	-
100	95	96	124	96	122	115	130	105	103	± 12	11	+3
500	483	501	503	498	511	499	518	532	506	± 15	3	+1
1,000	957	1,027	1,031	900	965	923	963	1,017	973	± 48	5	-3
1,500	1,462	1,525	1,458	1,448	1,471	1,460	1,530	1,496	1,481	± 32	2	-1
2,000	1,868	2,034	1,952	1,985	1,921	1,866	1,975	1,950	1,944	± 58	3	-3

Note: Linear regression: $y = 0.973x + 7.1$
Correlation coefficient: 0.999

a Average = $\Sigma x/n = \bar{x}$

b Standard deviation = $(\Sigma |x - \bar{x}|^2/n-1)^{1/2} = \sigma$

c Coefficient of variation = $\sigma/\bar{x} \times 100$

d Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$

e ND - Not detectable, less than 20 ng/g; HPLC peak eluting 1.5 ml prior to RDX and interfering with the elution position of RDX disregarded in data reduction.

TABLE 3
HPLC-UV DETERMINATIONS OF DNT IN KIDNEY SAMPLES

Amount Added (ng/g)	Level Found (ng/g)								Average ^a	Standard Deviation	Coefficient ^c of Variation	Percent ^d Inaccuracy
	Day 1		Day 2		Day 3		Day 4					
	A	B	A	B	A	B	A	B				
0	ND ^e	ND	ND	ND	ND	ND	ND	ND	-	-	-	-
100	80	55	79	75	84	60	82	73	74	± 11	14	-27
500	387	321	362	301	362	332	365	321	344	± 29	9	-31
1,000	762	635	740	603	735	627	729	772	700	± 67	10	-30
1,500	1,143	1,005	967	890	1,138	1,127	1,043	1,038	1,044	± 90	9	-30
2,000	1,603	1,289	1,495	1,373	1,574	1,214	1,389	1,447	1,423	± 134	9	-29

Note: Linear regression: $y = 0.707x - 3.5$
Correlation coefficient: 0.991

- a Average = $\Sigma x/n = \bar{x}$
b Standard deviation = $(\Sigma |x - \bar{x}|^2 / n - 1)^{1/2} = \sigma$
c Coefficient of variation = $\frac{\sigma}{\bar{x}} \times 100$
d Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$
e ND - Not detectable, less than 20 ng/g.

TABLE 4

HPLC-UV DETERMINATIONS OF TNT IN KIDNEY SAMPLES

Amount Added (ng/g)	Level Found (ng/g)								Average ^a	Standard ^b Deviation	Coefficient ^c of Variation	Percent ^d Inaccuracy
	Day 1		Day 2		Day 3		Day 4					
	A	B	A	B	A	B	A	B				
0	ND ^e	ND	ND	ND	ND	ND	ND	ND	-	-	-	-
100	85	51	81	87	92	68	88	86	80	± 14	17	-20
500	356	300	338	393	358	353	387	359	356	± 29	8	-29
1,000	708	619	728	739	756	634	794	883	733	± 85	12	-27
1,500	1,041	968	1,078	1,159	1,034	1,260	1,134	1,161	1,104	± 92	8	-26
2,000	1,619	1,418	1,407	1,621	1,507	1,283	1,534	1,615	1,501	± 123	8	-25

Note: Linear regression: $y = 0.746x - 5.4$
Correlation coefficient: 0.992

a Average = $\sum x/n = \bar{x}$

b Standard deviation = $(\sum |\bar{x} - x|^2/n-1)^{1/2} = \sigma$

c Coefficient of variation = $\sigma/\bar{x} \times 100$

d Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$

e ND - Not detectable, less than 20 ng/g.

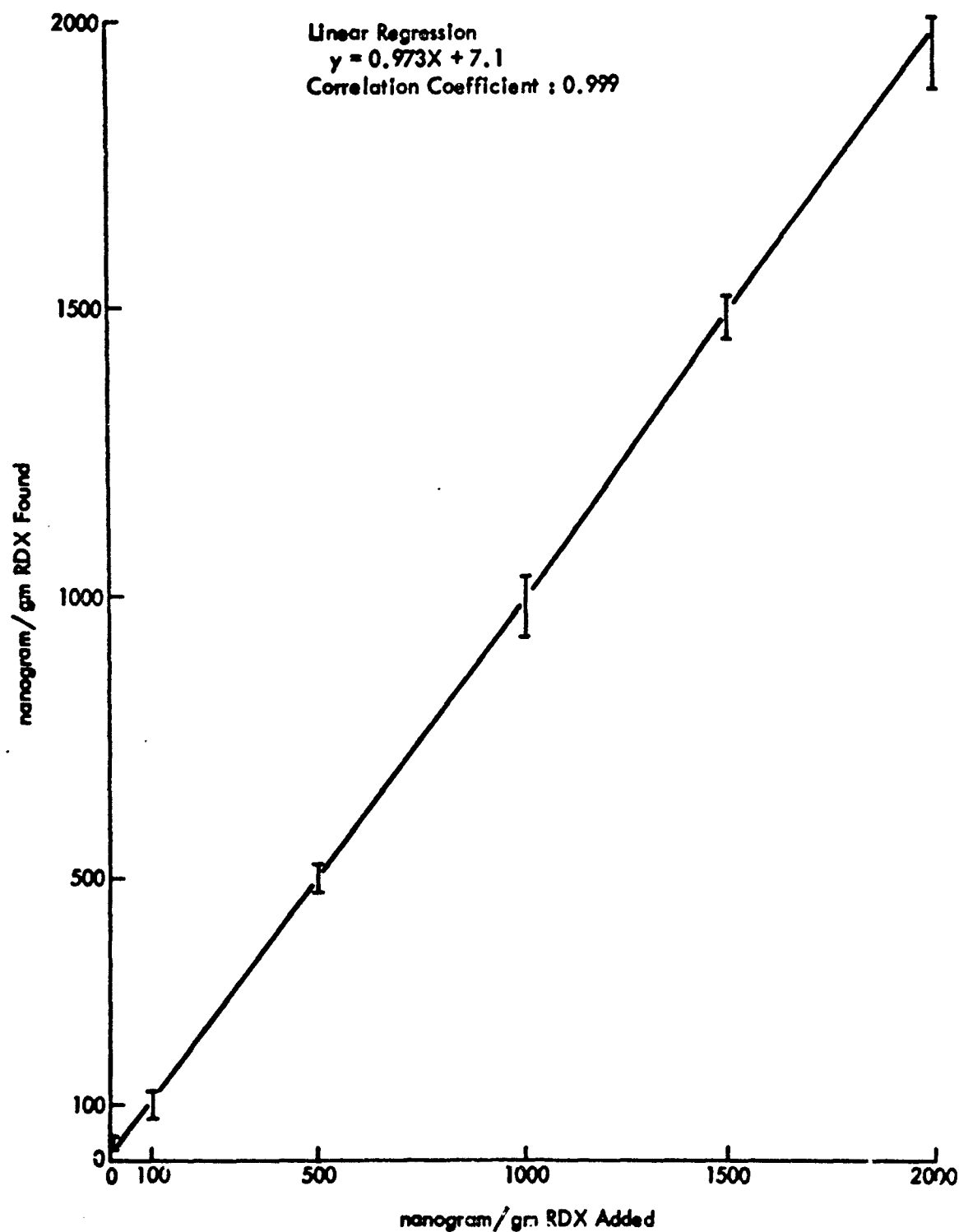


Figure 2 - Determination of RDX in Kidney

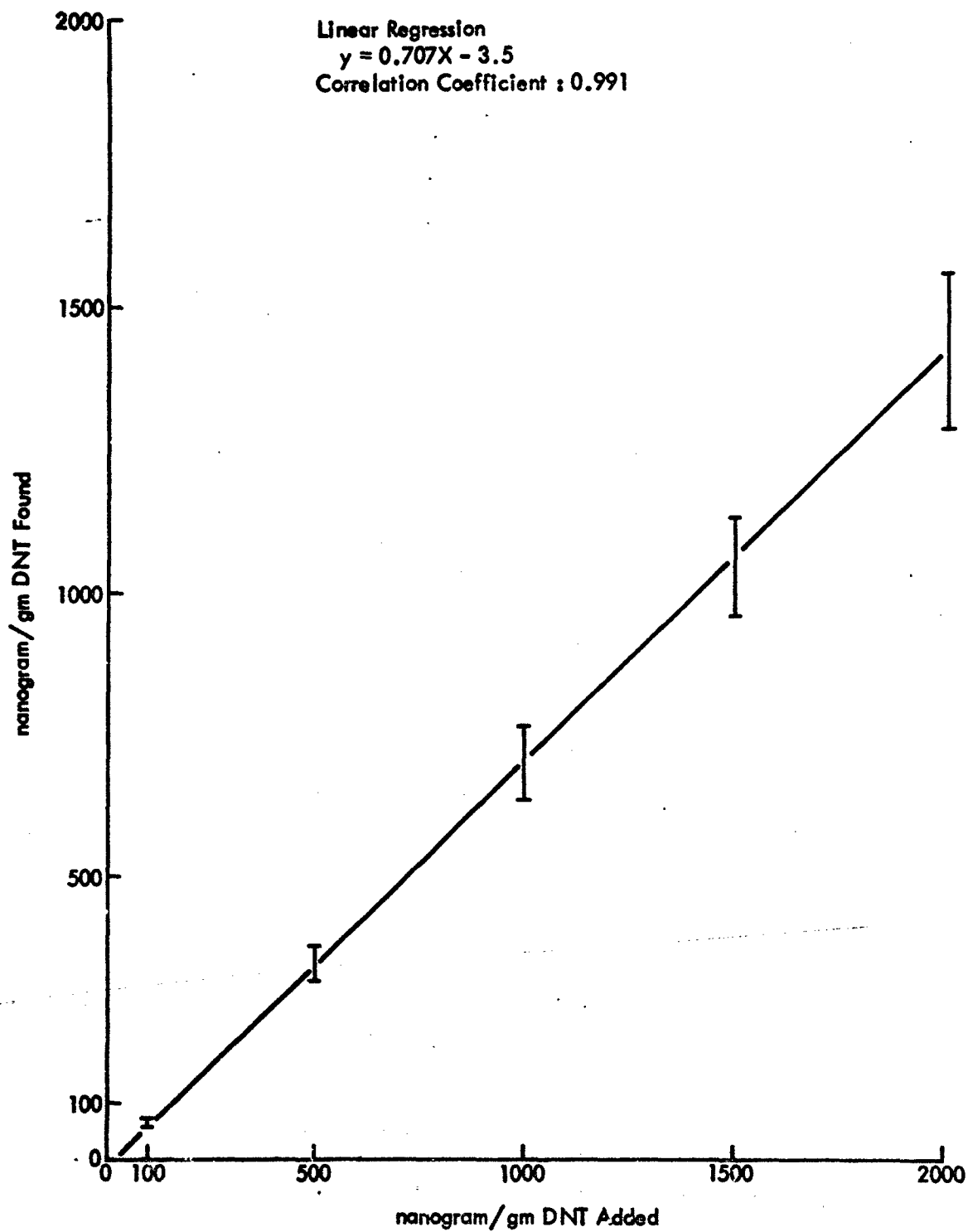


Figure 3 - Determination of DNT in Kidney

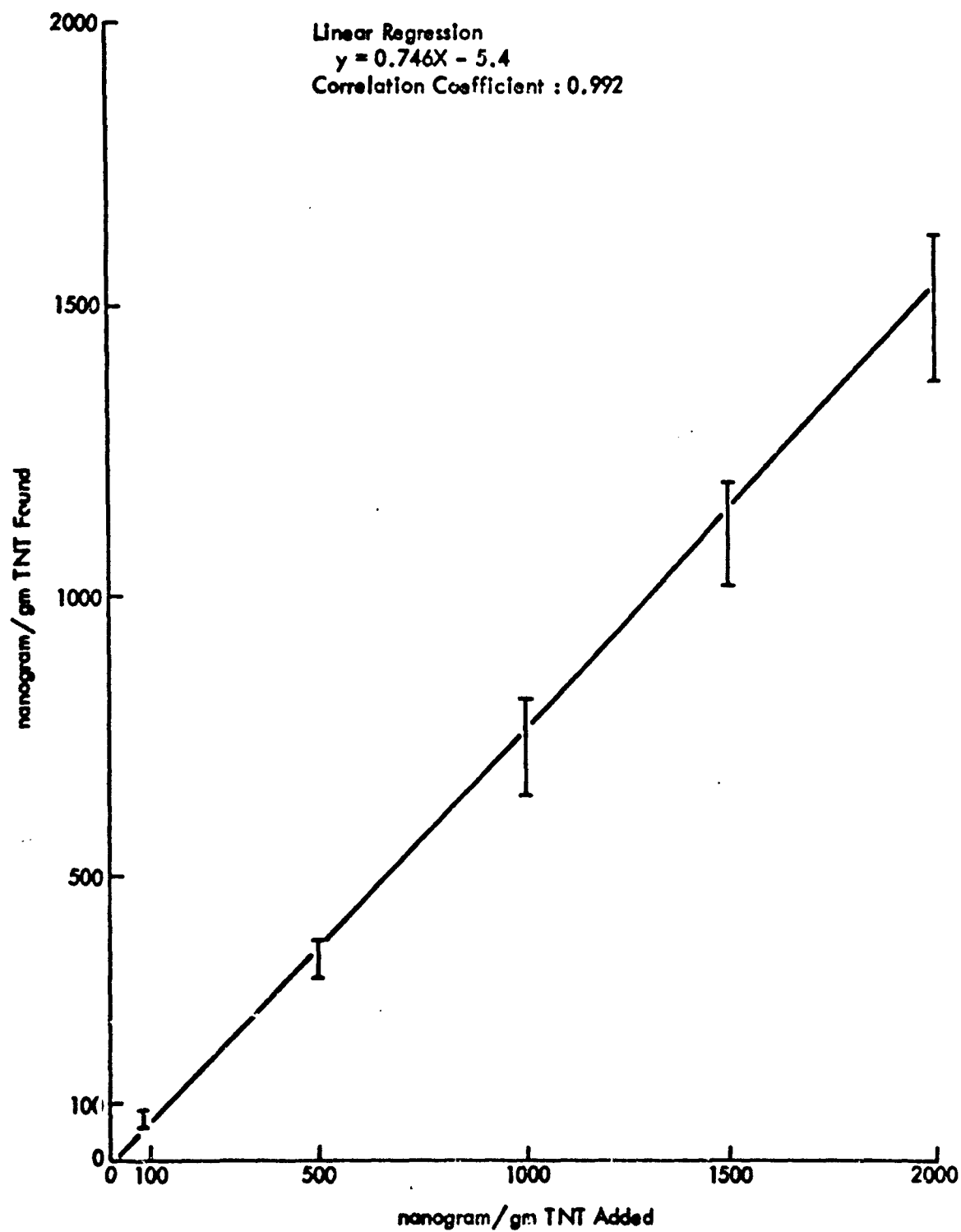


Figure 4 - Determination of TNT in Kidney

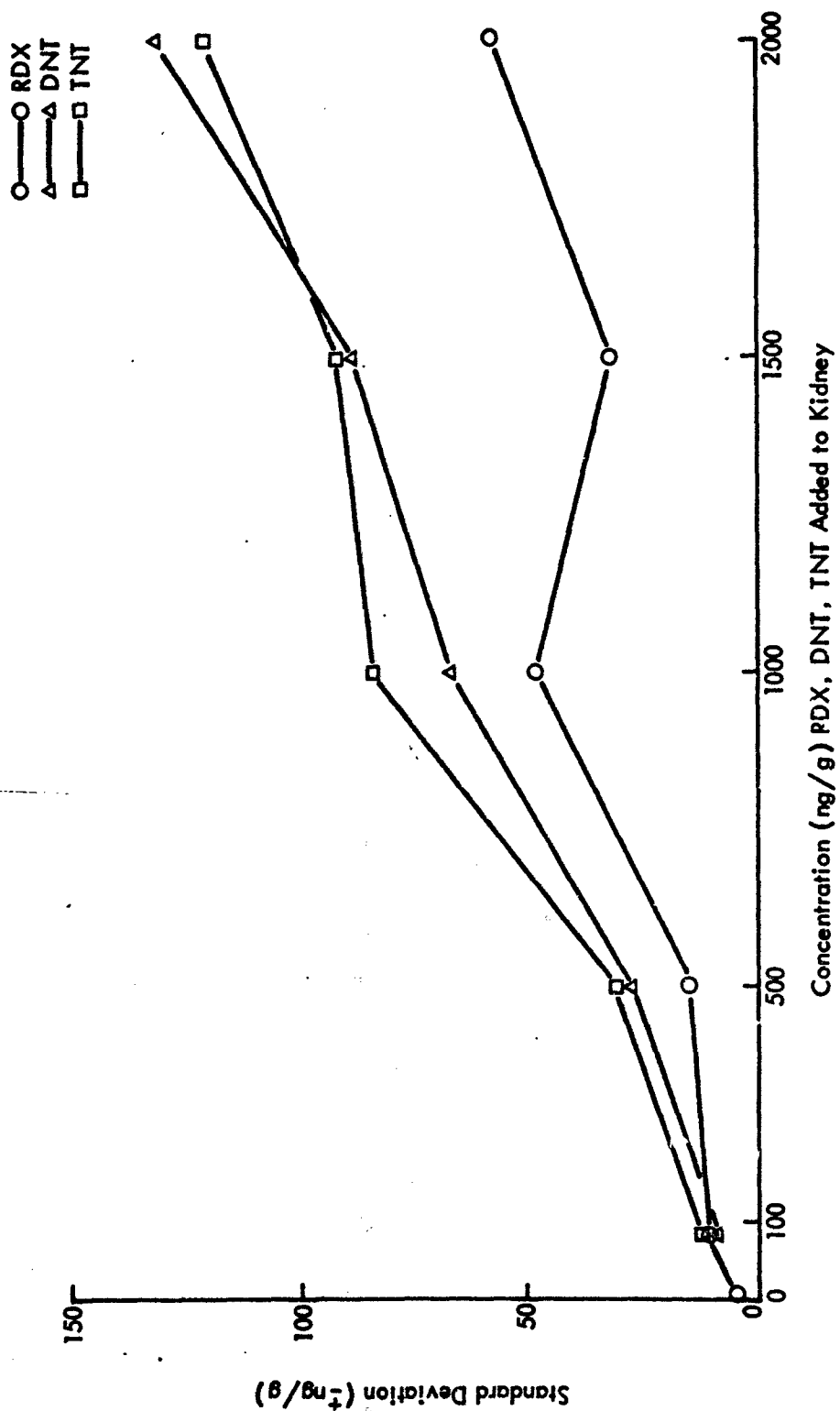


Figure 5 - Standard Deviation for RDX, DNT, and TNT in Kidney Samples

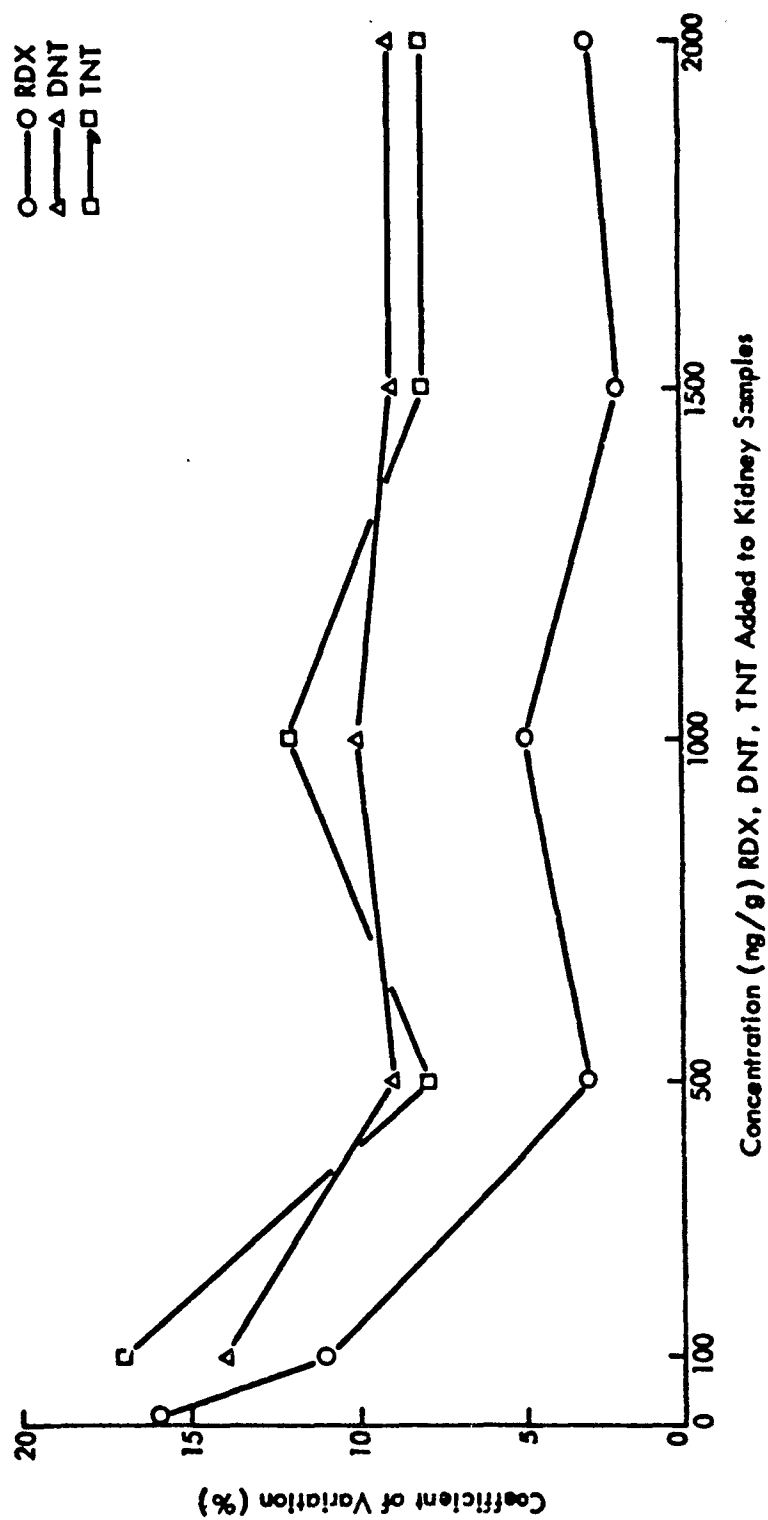


Figure 6 - Coefficient of Variation for RDX, DNT, and TNT in Kidney Samples

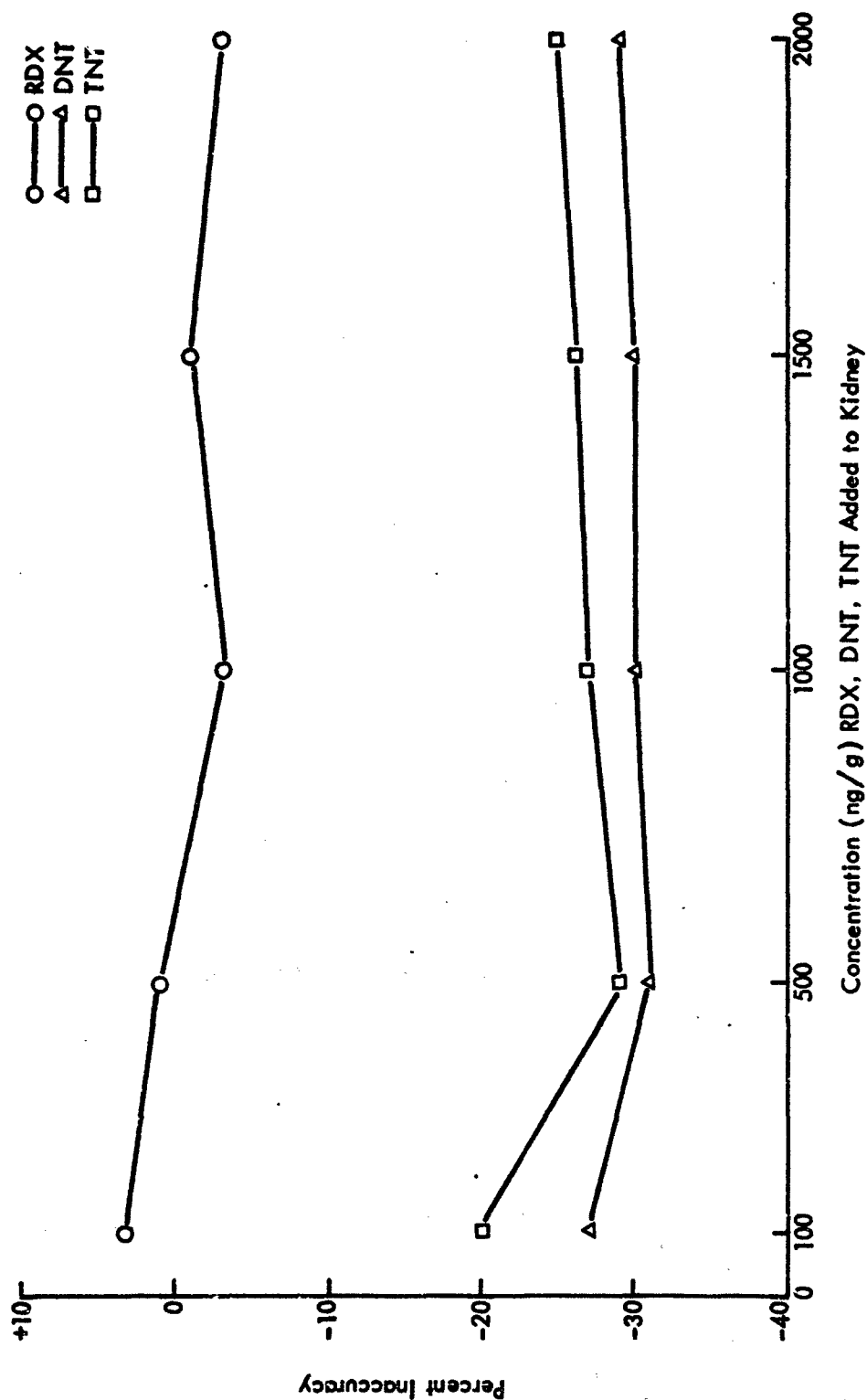


Figure 7 - Percent Inaccuracy for RDX, DNT, TNT Added to Kidney

HPLC Conditions:

Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 30% acetonitrile in
1% acetic acid in water
Flow Rate: 1.5 ml/min
Chart Speed: 0.1 in/min
Detector: UV, 254 nm

Sample Characteristics:

1.0 g kidney extract
3 x 2 ml with toluene.
Toluene evaporated and sample
reconstituted to 1.0 ml.

IS Concentration: 1,000 ng/ml
Injection Volume: 70 μ l
Attenuation: 0.01 X

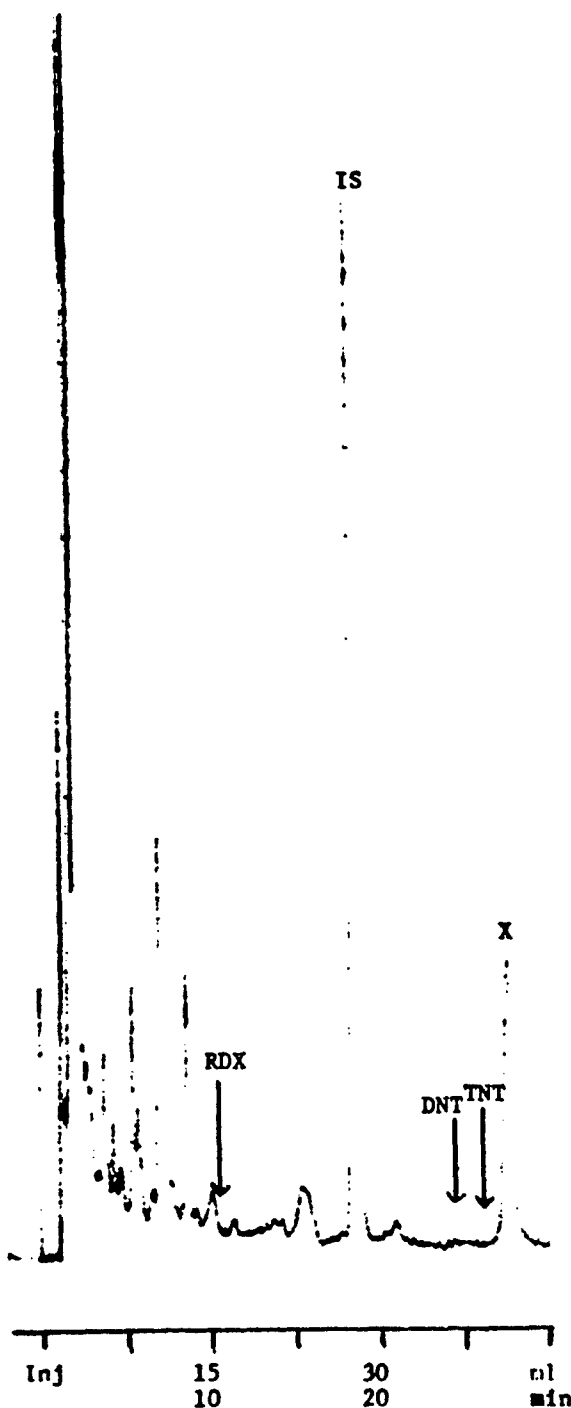
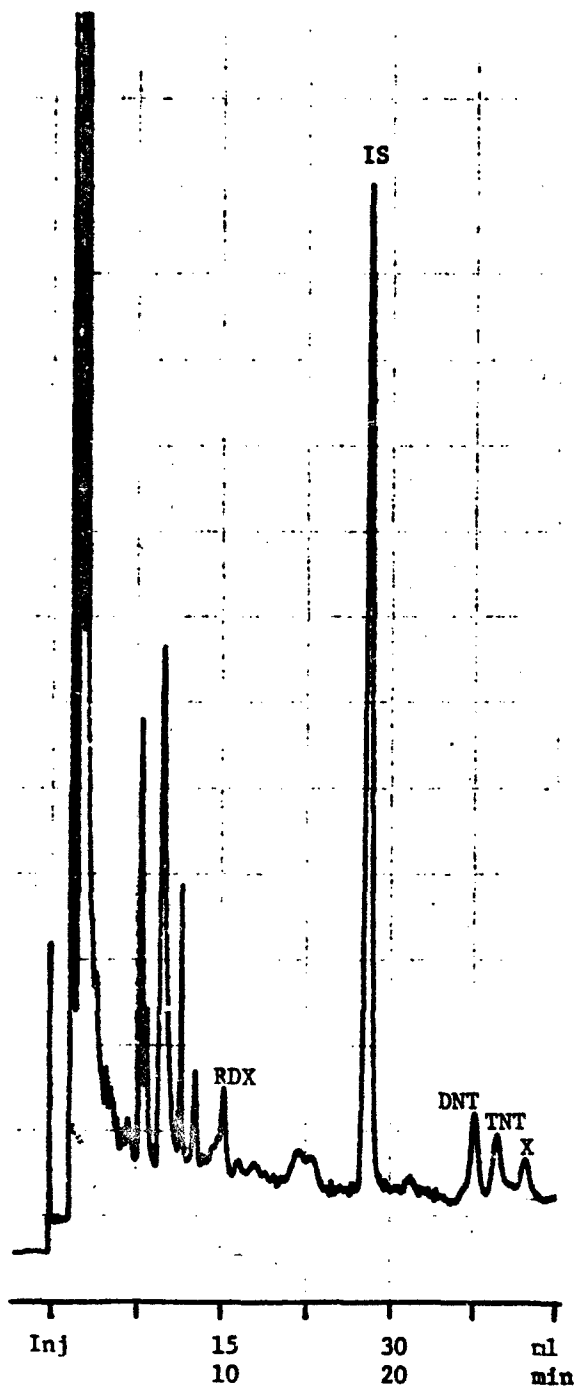


Figure 8 - HPLC Analysis of Blank Kidney Sample for RDX, DNT, and TNT
Method Development. "X" indicates toluene contaminant.
Arrows indicate elution position of RDX, DNT, and TNT.



HPLC Conditions:

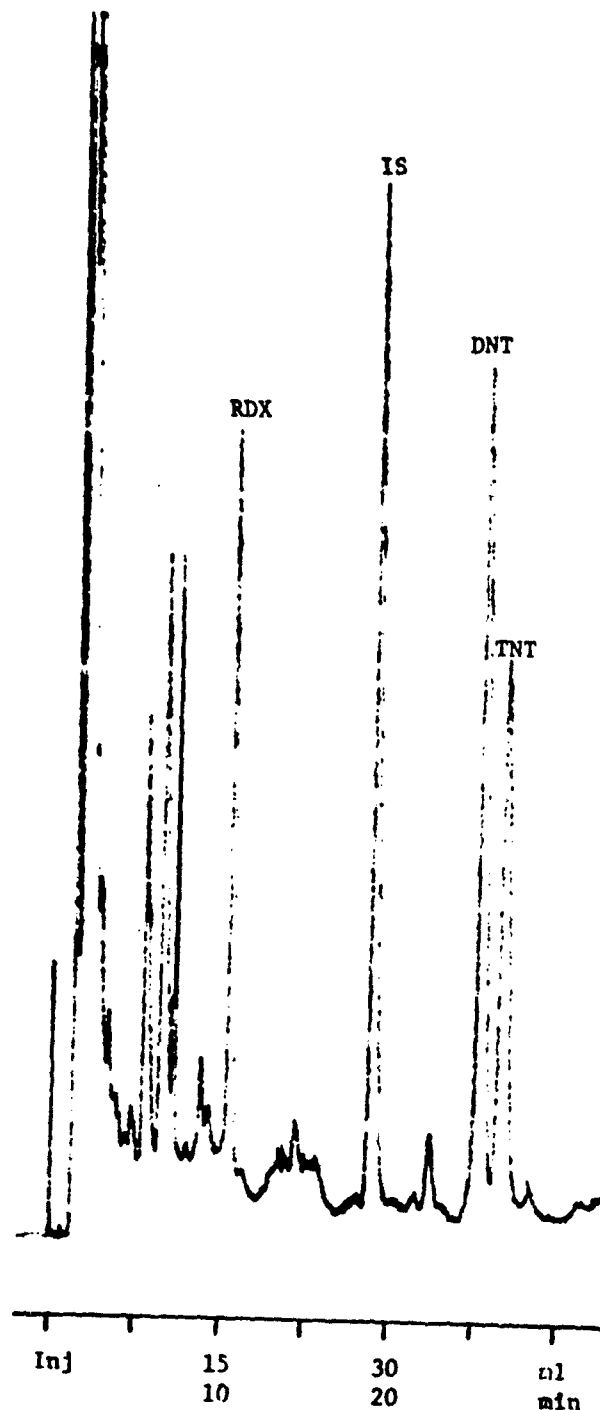
Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 30% acetonitrile in
1% acetic acid in water
Flow Rate: 1.5 ml/min
Chart Speed: 0.1 in/min
Detector: UV, 254 nm

Sample Characteristics:

1.0 g kidney containing
100 ng/g RDX, DNT, and TNT
extracted 3 x 2 ml with toluene.
Toluene evaporated and sample
reconstituted to 1.0 ml.

IS Concentration: 1,000 ng/ml
Injection Volume: 70 μ l
Attenuation: 0.01 X

Figure 9 - HPLC Analysis of Kidney Containing 100 ng/g RDX, DNT, and TNT.
"X" indicates toluene contaminant.



HPLC Conditions:

Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 30% acetonitrile in
1% acetic acid in water
Flow Rate: 1.5 ml/min
Chart Speed: 0.1 in/min
Detector: UV, 254 nm

Sample Characteristics:

1.0 g kidney containing
1,000 ng/g RDX, DNT, and TNT
extracted 3 x 2 ml with
toluene. Toluene evaporated
and sample reconstituted to
1 ml.

IS Concentration: 1,000 ng/ml
Injection: 70 μ l
Attenuation: 0.01 X

Figure 10 - HPLC Analysis of Kidney Containing 1,000 ng/g
RDX, DNT, and TNT

TABLE 5

STATISTICAL EVALUATION OF RDX IN KIDNEY DATA BY
THE HUBAUX AND VOS DETECTION LIMIT PROGRAM

Number ^a of Data Points	Linear Regression	Correlation Coefficient	Degrees of Freedom	t ^b	y ^c Intercept	Detection Limit
48	y = 0.973x + 7.1	0.999	46	1.679	66	120
40	y = 0.982x + 3.7	0.999	38	1.686	51	97
32	y = 0.973x + 1.0	0.998	30	1.697	52	95

ng/g RDX Added	Average ^c ng/g Found	Standard ^f Deviation	Percent ^g Imprecision	Percent ^h Inaccuracy
100	104	± 4.5	4.3	+ 2.6
500	493	± 5.6	1.1	+ 1.1
1,000	978	± 18	1.9	- 2.7
1,500	1,464	± 12	0.8	- 1.2
2,000	1,949	± 21	1.1	- 2.0

^a Number of data points - data points used to calculate linear regression and detection limits;
48 - all data; 40 - 2,000 ng/g samples omitted; 32 - 2,000 ng/g and 1,500 ng/g samples omitted.

^b t - 2 tail p level (usually 0.1, each confidence band is 0.05 so total p = 0.1).

^c y intercept - intercept on y-axis of upper confidence limit line.

^d Detection limit - x-intercept of y-intercept and lower confidence limit line.

^e Average ng/g found - average at each level determined from linear regression equation for 48 points.

^f Standard deviation - determined from average value (e above) and observed values.

^g Percent imprecision - standard deviation divided by average value times 100%.

^h Percent inaccuracy - determined from the average values of the eight observed values at each level

$$\% \text{ Inaccuracy} = \frac{\text{Average observed values} - \text{level added}}{\text{level added}} \times 100$$

TABLE 6

**STATISTICAL EVALUATION OF DNT IN KIDNEY DATA BY
THE HUBAUX AND VOS DETECTION LIMIT PROGRAM**

Number ^a of Data Points	Linear Regression	Correlation Coefficient	Degrees of Freedom	t ^b	y ^c Intercept	Detection ^d Limit
48	y = 0.707x - 3.5	0.991	46	1.679	117	339
40	y = 0.696x + 0.8	0.992	38	1.686	88	248
32	y = 0.698x + 0.4	0.992	30	1.697	63	179

ng/g DNT Added	Average ^e ng/g Found	Standard ^f Deviation	Percent ^g Imprecision	Percent ^h Inaccuracy
100	67	± 4.0	5.4	- 26
500	350	± 11.1	3.2	- 31
1,000	704	± 25	3.6	- 30
1,500	1,057	± 34	3.3	- 30
2,000	1,410	± 51	3.6	- 29

a Number of data points - data points used to calculate linear regression and detection limits;
48 - all data; 40 - 2,000 ng/g samples omitted; 32 - 2,000 ng/g and 1,500 ng/g samples
omitted.

b t - 2 tail p level (usually 0.1, each confidence band is 0.05 so total p = 0.1).

c y intercept - intercept on y-axis of upper confidence limit line.

d Detection limit - x-intercept of y-intercept and lower confidence limit line.

e Average ng/g found - average at each level determined from linear regression

equation for 48 points.

f Standard deviation - determined from average value (e above) and observed values.

g Percent imprecision - standard deviation divided by average value times 100%.

h Percent inaccuracy - determined from the average values of the eight observed
values at each level

$$\% \text{ Inaccuracy} = \frac{\text{Average observed values} - \text{level added}}{\text{level added}} \times 100$$

TABLE 7

**STATISTICAL EVALUATION OF TNT IN KIDNEY DATA BY
THE HUBAUX AND VOS DETECTION LIMIT PROGRAM**

Number ^a of Data Points	Linear Regression	Correlation Coefficient	Degrees of Freedom	t ^b	y ^c Intercept	Detection ^d Limit
48	y = 0.746x - 5.4	0.992	46	1.679	116	323
40	y = 0.734x - 0.8	0.991	38	1.686	96	262
32	y = 0.728x + 0.9	0.989	30	1.697	78	211

ng/g TNT Added	Average ^e ng/g Found	Standard ^f Deviation	Percent ^g Imprecision	Percent ^h Inaccuracy
100	69	± 5.2	6.5	- 20
500	368	± 10.9	3.1	- 29
1,000	741	± 32	4.4	- 27
1,500	1,114	± 35	3.2	- 26
2,000	1,487	± 46	3.1	- 25

^a Number of data points - data points used to calculate linear regression and detection limits;
48 - all data; 40 - 2,000 ng/g samples omitted; 32 - 2,000 ng/g and 1,500 ng/g samples
omitted.

^b t - 2 tail p level (usually 0.1, each confidence band is 0.05 so total p = 0.1).

^c y intercept - intercept on y-axis of upper confidence limit line.

^d Detection limit - x-intercept of y-intercept and lower confidence limit line.

^e Average ng/g found - average at each level determined from linear regression
equation for 48 points.

^f Standard deviation - determined from average value (e above) and observed values.

^g Percent imprecision - standard deviation divided by average value times 100%.

^h Percent inaccuracy - determined from the average values of the eight observed
values at each level

$$\% \text{ Inaccuracy} = \frac{\text{Average observed values} - \text{level added}}{\text{level added}} \times 100$$

APPENDIX

**METHOD DEVELOPMENT FOR THE DETERMINATION
OF RDX, DNT, AND TNT IN KIDNEY SAMPLES**

RAW DATA AND CALCULATIONS

TABLE 8

LINEARITY AND PRECISION OF RDX, DNT, AND TNT DETERMINATION
BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Reference Solution Number	ng/ml Compound Added	Peak Height (mm)		Internal Standard		Relative Weight Response		Calculated ng/ml	
		RDX	TNT	ng ml	Peak Height	RDX	TNT	RDX	TNT
A-1	0	< 2	< 2	1,000	122	-	-	ND	ND
A-2	100	12	14	1,000	116	1.03	1.21	109	113
A-3	500	58	63	1,000	118	0.98	1.07	517	499
A-4	1,000	104	121	1,000	114	0.91	1.06	960	992
A-5	1,500	168	183	1,000	114	0.98	1.07	1,551	1,500
A-6	2,000	220	240	1,000	114	0.96	1.05	2,031	1,968
B-1	0	< 2	< 2	1,000	118	-	-	ND	ND
B-2	100	13	14	1,000	120	1.08	1.17	114	109
B-3	500	59	64	1,000	122	0.97	1.05	509	490
B-4	1,000	121	145	1,000	134	0.90	1.08	950	1,011
B-5	1,500	172	184	1,000	117	0.98	1.05	1,547	1,470
B-6	2,000	228	246	1,000	116	0.98	1.06	2,069	1,982
C-1	0	< 2	< 2	1,000	120	-	-	ND	ND
C-2	100	12	14	1,000	120	1.00	1.17	105	109
C-3	500	55	59	1,000	116	0.95	1.02	499	475
C-4	1,000	107	128	1,000	122	0.88	1.05	923	981
C-5	1,500	150	172	1,000	110	0.91	1.04	1,435	1,461
C-6	2,000	210	240	1,000	116	0.91	1.03	1,906	1,934
D-1	0	< 2	< 2	1,000	119	-	-	ND	ND
D-2	100	11	12	1,000	112	0.98	1.07	103	100
D-3	500	54	60	1,000	116	0.93	1.03	490	433
D-4	1,000	116	137	1,000	124	0.94	1.10	985	1,032
D-5	1,500	155	174	1,000	113	0.91	1.03	1,444	1,439
D-6	2,000	212	236	1,000	116	0.91	1.02	1,924	1,901

TABLE 8 (concluded)

Relative Weight Response

	<u>Average</u>	<u>Standard Deviation</u>	<u>Relative Standard Deviation</u>
RDY	0.95	± 0.05	5.2%
DNT	1.07	± 0.05	4.9%
TNT	0.89	± 0.04	5.1%

TABLE 9

DETERMINATION OF RDX, DNT, AND TNT IN KIDNEY SAMPLES

Sample Number	ng/g ^a Compound Added	g Kidney	Peak Height (mm)			Internal Standard ^b		ng/g ^c Detected	
			RDX	DNT	TNT	ng/ml	Peak Height	RDX	TNT
Day 1A-0	0	1.0	4.0	< 2	< 2	1,000	145	36 ^f	ND
Day 1A-100	100	1.0	11.0	13.0	9.8	1,000	150	95	85
Day 1A-500	500	1.0	55.8	62.8	41.2	1,000	150	483	356
Day 1A-1000	1,000	1.0	112	125	82.8	1,000	152	957	762
Day 1A-1500	1,500	1.0	170	186	121	1,000	151	1,462	1,041
Day 1A-2000	2,000	1.0	218	262	189	1,000	152	1,868	1,619
Day 1B-0	0	1.0	3.0	< 2	< 2	1,000	149	26 ^f	ND
Day 1B-100	100	1.0	11.2	9.0	6.0	1,000	152	96	51
Day 1B-500	500	1.0	58.0	52.2	34.8	1,000	150	501	300
Day 1B-1000	1,000	1.0	121	105	72.8	1,000	153	1,027	619
Day 1B-1500	1,500	1.0	179	166	114	1,000	153	1,525	968
Day 1B-2000	2,000	1.0	238	212	166	1,000	152	2,034	1,418

TABLE 9 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)			Internal Standard ^b		Relative Weight ^c Response		
		RDX	DNT	TNT	ng/ml	Peak Height	RDX	DNT	TNT
Std-Day 1-5	2,000	226	319	230	1,000	148	0.77	1.08	0.78
Std-Day 1-3	1,000	111	155	111	1,000	145	0.77	1.07	0.76
Std-Day 1-5	2,000	229	318	226	1,000	148	0.77	1.08	0.76
Std-Day 1-4	1,500	170	240	168	1,000	145	0.78	1.10	0.77
Std-Day 1-3	1,000	113	161	115	1,000	146	0.77	1.10	0.79
Std-Day 1-2	500	59.5	82.8	59.2	1,000	154	0.77	1.07	0.77
						Average	0.77	1.08	0.77

29
137

^a ng/g compound added - nanograms of RDX, DNT, and TNT added to 1.0 g kidney.

^b Internal standard - compound (propiphenone) added to kidney sample after sample preparation for calculation of data.

^c ng/g detected - nanograms of RDX, DNT, and TNT detected in the 1.0 g kidney sample.

$$\text{ng compound/g} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}}$$

^d ND - not detectable, less than 20 ng/g.

$$\text{Relative Weight Response - RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

^f Not RDX, level calculated from HPLC peak eluting 1.5 ml prior to RDX and disregarded in further data evaluations.

TABLE 10

DETERMINATION OF RDX, DNT, AND TNT IN KIDNEY SAMPLES

Sample Number	ng/g ^a Compound Added	g Kidney	Peak Height (mm)			Internal Standard ^b		ng/g ^c Detected		
			RDX	DNT	TNT	ng/ml	Peak Height	RDX	DNT	TNT
Day 2A-0	0	1.0	5.0	< 2	< 2	1,000	152	43 ^f	ND ^d	ND
Day 2A-100	100	1.0	14.0	12.5	9.5	1,000	147	124	79	81
Day 2A-500	500	1.0	57.2	57.8	40.0	1,000	148	503	362	338
Day 2A-1000	1,000	1.0	115	115	83.0	1,000	144	1,031	740	728
Day 2A-1500	1,500	1.0	169	157	130	1,000	151	1,458	967	1,078
Day 2A-2000	2,000	1.0	223	240	167	1,000	148	1,952	1,495	1,407
Day 2B-0	0	1.0	5.0	< 2	< 2	1,000	149	44 ^f	ND	ND
Day 2B-100	100	1.0	11.0	12.0	10.4	1,000	149	96	75	87
Day 2B-500	500	1.0	57.4	48.6	47.0	1,000	150	498	301	393
Day 2B-1000	1,000	1.0	103	96.8	87.8	1,000	149	900	603	739
Day 2B-1500	1,500	1.0	171	147	142	1,000	153	1,448	890	1,159
Day 2B-2000	2,000	1.0	227	220	192	1,000	148	1,985	1,373	1,621

TABLE 10 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)			Internal Standard ^b		Relative Weight ^c Response		
		RDX	DNT	TNT	ng/ml	Peak Height	RDX	DNT	TNT
Std-Day 2-5	2,000	216	304	226	1,000	146	0.74	1.04	0.78
Std-Day 2-2	500	55.2	76.8	57.8	1,000	144	0.77	1.07	0.80
Std-Day 2-3	1,000	111	158	118	1,000	147	0.76	1.08	0.80
Std-Day 2-5	2,000	222	311	230	1,000	145	0.77	1.07	0.79
Std-Day 2-2	1,000	111	153	115	1,000	145	0.77	1.06	0.80
Std-Day 2-1	100	12.0	16.3	12.0	1,000	148	0.81	1.14	0.81
					Average		0.77	1.08	0.80

^a ng/g compound added - nanograms of RDX, DNT, and TNT added to 1.0 g kidney.

^b Internal standard - compound (propionophenone) added to kidney sample after sample preparation for calculation of data.

^c ng/g detected - nanograms of RDX, DNT, and TNT detected in the 1.0 g kidney sample.

$$\text{ng compound/g} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}}$$

^d ND - not detectable, less than 20 ng/g.

$$\text{Relative Weight Response - RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

^f Not RDX, level calculated from HPLC peak eluting 1.5 ml prior to RDX, and disregarded in further data evaluations.

TABLE 11
DETERMINATION OF RDX, DNT, AND TNT IN KIDNEY SAMPLES

Sample Number	ng/g ^a Compound Added	g Kidney	Peak Height			Internal Standard ^b		ng/g ^c Detected		
			Peak Height (mm)			ng/ml	Peak Height	RDX	DNT	TNT
			RDX	DNT	TNT					
Day 3A-0	0	1.0	4.0	< 2	< 2	1,000	150	35 ^f	ND ^d	ND
Day 3A-100	100	1.0	14.0	13.5	11.0	1,000	151	122	84	92
Day 3A-500	500	1.0	58.5	58.0	42.5	1,000	150	511	362	358
Day 3A-1000	1,000	1.0	109	116	88.5	1,000	148	965	735	756
Day 3A-1500	1,500	1.0	161	174	117	1,000	143	1,471	1,138	1,034
Day 3A-2000	2,000	1.0	214	245	174	1,000	146	1,921	1,574	1,507
Day 3B-0	0	1.0	4.0	< 2	< 2	1,000	156	34 ^f	ND	ND
Day 3B-100	100	1.0	13.0	9.5	8.0	1,000	148	115	60	68
Day 3B-500	500	1.0	56.0	52.0	41.0	1,000	147	499	332	353
Day 3B-1000	1,000	1.0	105	100	75.0	1,000	150	923	627	634
Day 3B-1500	1,500	1.0	160	172	143	1,000	143	1,460	1,127	1,260
Day 3B-2000	2,000	1.0	209	190	149	1,000	147	1,866	1,214	1,283

TABLE 11 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)			Internal Standard ^b		Relative Weight ^c Response	
		RDX	DNT	TNT	ng/ml	Peak Height	RDX	TNT
Std-Day 3-5	2,000	220	302	223	1,000	142	0.78	0.79
Std-Day 3-3	1,000	109	150	111	1,000	142	0.77	0.78
Std-Day 3-2	500	56.0	78.0	58.0	1,000	145	0.78	0.80
Std-Day 3-5	2,000	222	311	229	1,000	148	0.75	0.77
Std-Day 3-4	1,500	166	233	173	1,000	147	0.76	0.79
Std-Day 3-1	100	11.0	16.0	12.0	1,000	147	0.75	0.82
						Average	0.76	0.79

33
141^a ng/g compound added - nanograms of RDX, DNT, and TNT added to 1.0 g kidney.^b Internal standard - compound (propiphenone) added to kidney sample after sample preparation for calculation of data.^c ng/g detected - nanograms of RDX, DNT, and TNT detected in the 1.0 g kidney sample.

$$\text{ng compound/g} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}}$$

^d MD - not detectable, less than 20 ng/g.

$$\text{Relative Weight Response - RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

^f Not RDX, level calculated from HPLC peak eluting 1.5 ml prior to RDX and disregarded in further data evaluations.

TABLE 12

DETERMINATION OF RDX, DNT, AND TNT IN KIDNEY SAMPLES

Sample Number	ng/g ^a Compound Added	g Kidney	Peak Height (mm)			Internal Standard ^b		ng/g ^c Detected		
			RDX	DNT	TNT	ng/ml	Peak Height	RDX	DNT	TNT
Day 4A-0	0	1.0	4.0	< 2	< 2	1,000	151	34 ^f	ND ^d	ND
Day 4A-100	100	1.0	15.0	13.0	10.5	1,000	150	130	82	88
Day 4A-500	500	1.0	60.0	58.2	46.0	1,000	150	518	365	387
Day 4A-1000	1,000	1.0	110	115	93.2	1,000	148	963	729	794
Day 4A-1500	1,500	1.0	170	159	129	1,000	144	1,530	1,043	1,134
Day 4A-2000	2,000	1.0	218	211	174	1,000	143	1,975	1,389	1,534
Day 4B-0	0	1.0	3.0	< 2	< 2	1,000	153	25 ^f	ND	ND
Day 4B-100	100	1.0	12.0	11.6	10.2	1,000	149	105	73	86
Day 4B-500	500	1.0	59.0	49.0	44.0	1,000	144	532	321	359
Day 4B-1000	1,000	1.0	117	122	104	1,000	149	1,017	772	883
Day 4B-1500	1,500	1.0	172	164	137	1,000	149	1,496	1,038	1,161
Day 4B-2000	2,000	1.0	228	232	194	1,000	151	1,950	1,447	1,615

TABLE 12 (concluded)
REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)			Internal Standard ^b		Relative Weight ^c Response	
		RDX	DNT	TNT	ng/ml	Peak Height	RDX	TNT
Std-Day 4-5	2,000	224	320	237	1,000	149	0.75	1.07
Std-Day 4-4	1,500	168	235	174	1,000	148	0.76	1.06
Std-Day 4-2	500	66.8	90.0	67.2	1,000	168	0.80	1.07
Std-Day 4-3	1,000	109	151	109	1,000	143	0.76	1.06
Std-Day 4-2	500	69.4	94.8	68.6	1,000	174	0.80	1.06
Std-Day 4-1	100	11.2	15.4	12.0	1,000	146	0.77	1.06
						Average	0.77	1.06
								0.79

a ng/g compound added - nanograms of RDX, DNT, and TNT added to 1.0 g kidney.

b Internal standard - compound (propionophenone) added to kidney sample after sample preparation for calculation of data.

c ng/g detected - nanograms of RDX, DNT, and TNT detected in the 1.0 g kidney sample.

$$\text{ng compound/g} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}}$$

d ND - not detectable, less than 20 ng/g.

$$\text{e Relative Weight Response - RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

f Not RDX, level calculated from HPLC peak eluting 1.5 ml prior to RDX and disregarded in further data evaluations.

APPENDIX D

IDENTIFICATION OR DEVELOPMENT OF CHEMICAL ANALYSIS
METHODS FOR PLANTS AND ANIMAL TISSUES

METHOD REPORT NO. 3

METHOD DEVELOPMENT FOR THE DETERMINATION OF CYCLOTRIMETHYLENE-
TRINITRAMINE (RDX), DINITROTOLUENE (DNT) AND TRINITROTOLUENE
(TNT) IN MUSCLE/FAT SAMPLES

October 1980

Contract No. DAAK11-79-C-0110
MRI Project No. 4849-A

For

U.S. Army Toxic and Hazardous Materials Agency
Dr. L. Eng, DRXTH-TE-D, Project Officer
Aberdeen Proving Ground (EA), MD 21010

REPRODUCED FROM THE ARMY-NAVY FILES

The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER Technical Report No. 3	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Method Development for the Determination of Cyclotrimethylenetrinitramine (RDX), Dinitrotoluene (DNT), and Trinitrotoluene (TNT) in Muscle/Fat Samples	5. TYPE OF REPORT & PERIOD COVERED Method Report, August 1979 to December 1980	
7. AUTHOR(s) D. B. Lakings and O. Gan	6. PERFORMING ORG. REPORT NUMBER MRI Project No. 4849-A	
9. PERFORMING ORGANIZATION NAME AND ADDRESS Midwest Research Institute 425 Volker Boulevard Kansas City, MO 64110	8. CONTRACT OR GRANT NUMBER(s) DAAK11-79-C-0110	
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Armament Research and Development Command, Aberdeen Proving Ground (Edgewood Area), MD 21010	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS	
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)	12. REPORT DATE October 1980	
	13. NUMBER OF PAGES 44	
	15. SECURITY CLASS. (of this report) Unclassified	
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE	
16. DISTRIBUTION STATEMENT (of this Report) Unlimited		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Cyclotrimethylenetrinitramine (RDX) High Performance Liquid Chromatography Dinitrotoluene (DNT) Trinitrotoluene (TNT) Muscle/Fat Determination		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A high performance liquid chromatographic (HPLC) method for the quantitative determination of cyclotrimethylenetrinitramine (RDX), dinitrotoluene (DNT) and trinitrotoluene (TNT) in muscle/fat samples was developed. The analytical system consists of an isocratic HPLC unit with a Spherisorb ODS, 5 μ , 250 x 4.6 mm ID column, an eluent of 28% acetonitrile in 1% acetic acid in water, and a flow rate of 1.5 ml/min. The compounds, including the internal standard		

DD FORM 1 JAN 75 1473 EDITION OF 1 NOV 65 IS OBSOLETE

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

(IS), propiophenone, have the following retention characteristics: RDX - 18 ml, 12 min; IS - 33 ml, 22 min; DNT - 43.5 ml, 29 min; and TNT - 48 ml, 32 min and are detected at 254 nm. Reference solutions of the compounds gave a linear response from 100 ng/ml to 2,000 ng/ml. The muscle/fat samples were prepared by first homogenizing the matrix to form a uniform sample. A 2.0-g muscle/fat sample was weighed and the munition compounds extracted with first 6 ml and then 3 ml acetonitrile. The acetonitrile extracts were combined and concentrated to about 250 μ l under a stream of nitrogen. A 250- μ l aliquot of acetonitrile containing 1,000 ng IS was added followed by 500 μ l high purity water. The prepared sample was filtered through a 0.45- μ Fluoropore filter and injected onto the HPLC system. The analytical method was evaluated by preparing and analyzing duplicate 2.0-g muscle/fat sample containing 0, 50, 100, 200, 500, and 1,000 ng/g of each sample on four succeeding days. Linear regression analysis of the data gave the following equations and correlation coefficients: RDX, $y = 0.965x + 6.5$, 0.994; DNT, $y = 0.781x + 3.7$, 0.990; and TNT, $y = 0.850x + 1.8$, 0.999. The average coefficient of variation and average percent inaccuracy for RDX, DNT, and TNT determination in muscle/fat samples were 7.5%, +2.9; 8.3%, -19; and 5.9%, -13, respectively. A statistical evaluation of the data by the Hubaux and Vos detection limit program gave detection limits of 62 ng/g for RDX, 66 ng/g for DNT, and 62 ng/g for TNT for the HPLC determination of these compounds in muscle/fat samples.

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

PREFACE

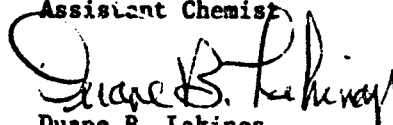
This report was prepared at Midwest Research Institute, 425 Volker Boulevard, Kansas City, Missouri 64110 under U.S. Army Toxic and Hazardous Materials Agency, Contract No. DAAK11-79C-0110, MRI Project No. 4849-A, "Identification or Development of Chemical Analysis Methods for Plants and Animal Tissues." Dr. Leslie Eng, DRXTH-TE-D was the project officer for this research effort.

This work was conducted in the Analytical Chemistry Department, Dr. James L. Spigarelli, Director. The research effort was directed by Dr. Duane B. Lakings, Program Manager and Senior Chemist, with assistance from Mr. Owen Gan, Assistant Chemist, and Ms. Mary Valla, Senior Technician. The report was prepared by Dr. Lakings and Mr. Gan.

MIDWEST RESEARCH INSTITUTE

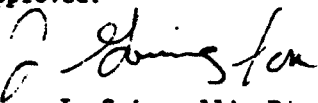


Owen Gan
Assistant Chemist



Duane B. Lakings
Program Manager and Senior Chemist

Approved:



James L. Spigarelli, Director
Analytical Chemistry Department

TABLE OF CONTENTS

	<u>Page</u>
1. Application	1
2. Chemistry.	1
3. Apparatus.	2
4. Standards.	3
5. Procedure for Muscle/Fat Sample.	4
6. Calculation.	6
7. Statistical Evaluation of Data	6
Appendix - Method Development for the Determination of RDX, DNT, and TNT in Muscle/Fat Samples	25

LIST OF FIGURES

<u>Number</u>	<u>Title</u>	<u>Page</u>
1	HPLC Separation of RDX, DNT, TNT, and Tetryl SARMS and Propiophenone (IS)	8
2	Determination of RDX in Muscle/Fat Samples	13
3	Determination of DNT in Muscle/Fat Samples	14
4	Determination of TNT in Muscle/Fat Samples	15
5	Standard Deviation for RDX, DNT, and TNT in Muscle/Fat Samples.	16
6	Coefficient of Variation for RDX, DNT, and TNT in Muscle/Fat Samples	17
7	Percent Inaccuracy for RDX, DNT, and TNT in Muscle/Fat Samples.	18
8	HPLC Analysis of Blank Muscle/Fat Sample for RDX, DNT, and TNT Method Development	19
9	HPLC Analysis of Muscle/Fat Sample Containing 50 ng/g RDX, DNT, and TNT.	20
10	HPLC Analysis of Muscle/Fat Sample Containing 500 ng/g RDX, DNT, and TNT.	21

LIST OF TABLES

<u>Number</u>	<u>Title</u>	<u>Page</u>
1	Linearity and Precision of the HPLC-UV Determination of SARM Reference Solutions of RDX, DNT, and TNT.	9
2	HPLC-UV Determination of RDX in Muscle/Fat Samples	10
3	HPLC-UV Determination of DNT in Muscle/Fat Samples	11
4	HPLC-UV Determination of TNT in Muscle/Fat Samples	12
5	Statistical Evaluation of RDX in Muscle/Fat Sample Data by the Hubaux and Vos Detection Limit Program.	22
6	Statistical Evaluation of DNT in Muscle/Fat Sample Data by the Hubaux and Vos Detection Limit Program.	23
7	Statistical Evaluation of TNT in Muscle/Fat Sample Data by the Hubaux and Vos Detection Limit Program.	24
8	Linearity and Precision of RDX, DNT, and TNT Determination by High Performance Liquid Chromatography.	26
9	Determination of RDX, DNT, and TNT in Muscle/Fat Samples .	28
10	Determination of RDX, DNT, and TNT in Muscle/Fat Samples .	30
11	Determination of RDX, DNT, and TNT in Muscle/Fat Samples .	32
12	Determination of RDX, DNT, and TNT in Muscle/Fat Samples .	34

Midwest Research Institute
Analytical Chemistry Department
Kansas City, Missouri 64110

for

U.S. Army Armament Research and Development Command
Aberdeen Proving Ground (Edgewood Area)
Maryland 21010

Contract No. DAAK11-79-C-0110

Identification or Development of Chemical Analysis Methods
for Plants and Animal Tissues

METHOD DEVELOPMENT FOR THE DETERMINATION OF CYCLOTRIMETHYLENE-
TRINITRAMINE (RDX), DINITROTOLUENE (DNT) AND TRINITROTOLUENE
(TNT) IN MUSCLE/FAT SAMPLES

1. APPLICATION: The developed method is for quantitative determination of RDX, DNT, and TNT in animal muscle/fat tissue samples using high performance liquid chromatography (HPLC) with ultraviolet (UV) detection at 254 nm.

a. Evaluated Concentration Range: The concentration range of RDX, DNT, and TNT studied in reference solutions was 100, 500, 1,000, 1,500, and 2,000 ng/ml and in muscle/fat samples was 50, 100, 200, 500, and 1,000 ng/g (parts per billion, ppb).

b. Sensitivity: A signal-to-noise ratio of 6 to 1 for RDX (peak height (PH) - 25 mm), 9 to 1 for DNT (PH - 40 mm), and 8 to 1 for TNT (PH - 30 mm) was obtained with an injection of 50 µl of a 100 ng/ml solution of each compound (ca. 5 ng each compound on column).

c. Detection Limits: The detection limits in the muscle/fat tissue were 62 ng/g for RDX, 66 ng/g for DNT, and 62 ng/g for TNT using the Hubaux and Vos detection limit program.

d. Interferences: No interfering components from the muscle/fat sample were found to elute with the same retention volumes as RDX, DNT, or TNT. The RDX peak eluted as a shoulder on a large peak(s) and was measured by the tangent method.

e. Analysis Rate: The chromatographic time per injection was 40 min. Two reference solutions were analyzed prior to injecting the prepared muscle/fat samples and two were analyzed during the day (160 min total time). Thus, a total of eight prepared muscle/fat samples (320 min total time) can be analyzed for RDX, DNT, and TNT levels during an 8-hr day.

2. CHEMISTRY: RDX, DNT, and TNT are munition compounds manufactured at various installations. The possible environmental contamination of these compounds, particularly in plants and animals, is of concern. The

determination of muscle/fat levels of RDX, DNT, and TNT in animals may provide information on the extent and level of contamination at the production facilities and in the surrounding area. Each of these munitions has a sufficient UV chromophore at 254 nm to allow UV detection and quantification. These munitions are of intermediate polarity and have limited water solubility. Normally, biological matrices have a large number of components which will interfere with the detection and quantification of low levels of compounds. Reverse phase HPLC is capable of separating compounds with similar chemical and physical properties; the elution order of the technique is based on the polarity of the compounds, the more polar compounds being eluted first. Thus, by extracting the biological matrix, i.e., muscle/fat, with an intermediate polarity solvent and analyzing the extract by HPLC, a simple sample preparation and analysis system may be defined for the determination of RDX, DNT, and TNT in muscle/fat samples.

3. APPARATUS:

a. Instrumentation: A Waters isocratic liquid chromatographic system consisting of a Model 6000A pump, Model U6K injector, and Model 440 UV detector. A general purpose centrifuge (Dynac, Clay Adams 0101) with a 24-place head.

b. HPLC Parameters:

1. Column: Spherisorb ODS, 5 μ , 250 x 4.6 mm ID.
2. Eluent: 28% acetonitrile in 1% acetic acid in water. NOTE: 30% acetonitrile eluent was employed for precision and accuracy evaluations of reference solutions. The 28% acetonitrile eluent was required to obtain better resolution of RDX in the muscle/fat samples.
3. Flow rate: 1.5 ml/min.
4. Detector: UV, 254 nm.
5. Internal standard: Propiophenone, 1,000 ng/ml.
6. Injection volume: 50 to 100 μ l.
7. Retention volumes and times: RDX, 18 ml, 12 min; DNT, 43.5 ml, 29 min; TNT, 48 ml, 32 min; and IS, 33 ml, 22 min in the 28% acetonitrile eluent. NOTE: Slight changes in the retention indices may occur with fresh eluent or a change in column.

A representative HPLC chromatogram for RDX, DNT, and TNT is shown in Figure 1. Also included on the chromatogram are peaks for the internal standard (propiophenone) and 2,4,6-trinitrophenylmethylnitramine (tetryl).

c. Laboratory Glassware and Equipment:

1. Culture tubes (Pyrex) with Teflon-lined screw caps.
2. Volumetric flasks (100 ml).
3. Volumetric syringes (0-100 μ l, 0-500 μ l, and 0-1,000 μ l).
4. Automatic pipetter (0-5 ml).
5. Six speed Waring-type blender with glass container.
6. Teflon-glass, motor-driven tissue homogenizer.
7. Filtering apparatus including filter holders, 5-ml disposable syringes, and 0.45 μ Fluoropore filters.
8. Inert gas (nitrogen) drying train with 12 ports.

d. Chemicals

1. RDX, DNT, and TNT SARMS, obtained from the U.S. Army Toxic and Hazardous Materials Agency.
2. Propiophenone (internal standard), analytical grade.
3. Acetonitrile, "Distilled in Glass" grade; and acetic acid, ACS grade.
4. High purity water from a Milli-Q water purification system.

4. STANDARDS:

a. Stock: Weigh approximately 20 mg of RDX, DNT, TNT, and tetryl SARM or interim SARM into separate 100-ml volumetric flask. Dissolve each compound in acetonitrile and dilute to volume. The concentration of each compound is 200 μ g/ml. Quantitatively pipette 20 ml from each stock above into a 100-ml volumetric flask and dilute to volume with distilled water. Concentration of each compound is 40 μ g/ml.

b. Working: Pipette 10 ml of the 40 μ g/ml each compound stock into a 100-ml volumetric flask and dilute to volume with high purity water. Concentration of each compound is 4 μ g/ml. Reference solutions were prepared from this stock as follows:

<u>µl Working Stock</u>	<u>µl IS Stock*</u>	<u>µl 10% Acetonitrile in Water</u>	<u>Concentration Each Compound (ng/ml)</u>
500	250	250	2,000
375	250	375	1,500
250	250	500	1,000
125	250	625	500
25	250	725	100
0	250	750	0

* Preparation of IS stock given in "c."

These reference solution concentrations were employed for precision and accuracy evaluations of the analytical technique. During the determination of RDX, DNT, and TNT in muscle/fat samples, reference solution-concentrations of 100, 200, 400, 1,000 and 2,000 ng/ml of each compound were used.

c. Internal Standard Stock: Weigh 10 mg propiophenone into a 100-ml volumetric flask and dilute to volume with acetonitrile (concentration, 100 µg/ml). Quantitatively pipette 20 ml of the 100 µg/ml stock into a 100-ml volumetric flask and dilute to volume with acetonitrile (concentration, 20 µg/ml). A final working solution of 4.0 µg/ml is prepared by pipetting 20 ml of the 20 µg/ml stock into a 100-ml volumetric flask and diluting to volume with acetonitrile.

5. PROCEDURE FOR MUSCLE/FAT SAMPLE

a. Muscle/Fat Sample Preparation: The procedure employed to prepare muscle/fat samples for the HPLC-UV determination of RDX, DNT, and TNT consisted of:

1. Place approximately 50 g of the muscle/fat composite tissue into a Waring-type blender and blend for 1 min on speed six (liquify). NOTE: To completely liquify the muscle/fat sample, the sides of the glass containers are scraped with a spatula.

2. Transfer approximately 10 g of the liquified muscle/fat sample to a motor-driven Teflon-glass homogenizer.

3. Homogenize the sample for 30 sec to disrupt the cell walls of the muscle/fat sample. NOTE: The homogenization step is necessary to solubilize the intercellular compounds prior to the extraction step.

4. Repeat steps 3 and 4 on the remaining liquified muscle/fat sample and combine the homogenized samples.

5. Accurately weigh 12 2.0 g homogenized muscle/fat sample aliquots into culture tubes with Teflon-lined screw caps.

6. Spike two each of the homogenized muscle/fat aliquots with the working stock (4 µg/ml each RDX, DNT, and TNT) at the following levels: 2,000 ng (500 µl), 1,000 ng (250 µl), 400 ng (100 µl), 200 ng (50 µl), and 100 ng (25 µl). The remaining two muscle/fat aliquots served as blanks.

7. Adjust the sample volume to 2.5 ml with high purity water containing 10% acetonitrile assuming 2 g muscle/fat sample equals 2 ml.

8. Add 6.0 ml acetonitrile to each aliquot and mix thoroughly on a vortex mixer for at least 2 min.

9. Centrifuge at 1,000 rpm for 20 min.

10. Transfer the acetonitrile layers to properly labeled culture tubes with Teflon-lined screw cap.

11. Repeat the acetonitrile extraction using 3 ml acetonitrile and combine the extracts in the appropriate tubes.

12. Concentrate the acetonitrile to approximately 250 µl at room temperature under a stream of nitrogen. NOTE: Do not heat the samples during the evaporation steps or loss of RDX, DNT, and TNT may occur.

13. Add 250 µl IS stock (1,000 ng) to each extracted muscle/fat sample and mix thoroughly.

14. Add 500 µl high purity water to each sample. NOTE: Final volume of the prepared samples is 1.0 ml.

15. Filter the solutions through a 0.45 µ Fluoropore filter into culture tubes.

16. Analyze a 50- to 100-µl aliquot of each prepared muscle/fat sample by HPLC.

b. Calibration: The reference solutions described in Section 4.b were prepared and analyzed in quadruplicate. The relative weight response (RWR) (Eq. 1) of each compound was calculated and the average RWR utilized to determine the nanograms of each compound in every reference solution (Eq. 2). The nanograms found were plotted against the nanograms added. The slope, intercept, and correlation coefficient for each compound were determined. The data are summarized in Table 1 and include the average value at each level for each compound, the standard deviation, coefficient of variation, and percent inaccuracy. The raw data and calculations are given in Table 8 of the Appendix.

$$RWR = \frac{\text{Peak Height Cpd}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml Cpd}} \quad (\text{Eq. 1})$$

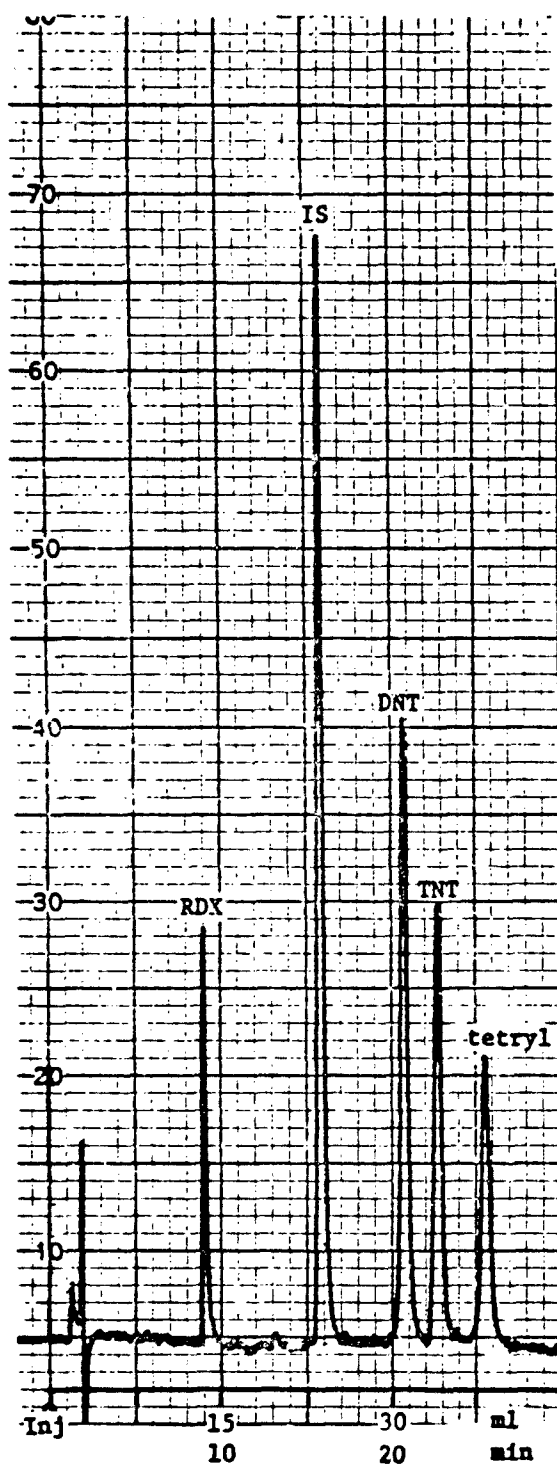
$$\frac{\text{ng}}{\text{ml}} \text{ or ng/2 g compound} = \frac{\text{Peak Height Cpd}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{Avg. RWR}} \quad (\text{Eq. 2})$$

c. Muscle/Fat Sample Analysis: The muscle/fat samples prepared as outlined in Section 5.a were injected onto the HPLC. The peak height of each compound was measured and recorded. Muscle/fat samples were prepared and analyzed on four succeeding days.

6. CALCULATION: The level of each compound in the 2.0 g muscle/fat samples was determined using the relative weight response to an internal standard method. The RWR values (Eq. 1) for the reference solutions analyzed with a day set of muscle/fat samples were calculated and the average values for RDX, DNT, and TNT determined. These RWR values were employed to calculate the level of RDX, DNT, and TNT in the muscle/fat samples (Eq. 2) where the nanograms per milliliter term represents the level found in the 2.0 g sample. The nanograms per gram of each compound were determined by dividing the level found by the sample weight. The results for the duplicate determinations of RDX, DNT, and TNT in muscle/fat samples at five different levels on four succeeding days are summarized in Tables 2, 3, and 4. The average level found, the standard deviation, coefficient of variation, and percent inaccuracy are also included. A linear regression evaluation of the results was made; and the slope, intercept, and correlation coefficient are given in the tables. The level of each compound found in the muscle/fat samples was plotted against the amount added and these data are shown in Figures 2 through 4. The range presented at each level is two standard deviations of the average level found. A graphic presentation of the standard deviation, coefficient of variation, and percent inaccuracy for the determination of RDX, DNT, and TNT in muscle/fat samples is given in Figures 5 through 7, respectively. Representative HPLC chromatograms are shown for a muscle/fat sample blank (Figure 8), a 100 ng/2 g (Figure 9), and a 1,000 ng/2 g (Figure 10) each compound muscle/fat sample. The raw data and calculations for the muscle/fat sample determinations are given in Tables 9 through 12 in the Appendix.

7. STATISTICAL EVALUATION OF DATA: A statistical evaluation of the data obtained for the determination of RDX, DNT, and TNT in muscle/fat samples (Tables 2, 3, and 4) by the Hubaux and Vos detection limit program was made at the U.S. Army Toxic and Hazardous Materials Agency. The results of these evaluations are given in Table 5 for RDX, Table 6 for DNT, and Table 7 for TNT. The data obtained for the blank muscle/fat samples were not included in any of the calculations. Detection limits for RDX and DNT as determined by the program were 62 and 66 ng/g, respectively, when the 1,000 and 500 ng/g data points were omitted. For TNT, the detection limit was 62 ng/g when the 1,000 ng/g data points were omitted. Removal of the 500 ng/g data points from the TNT detection limit calculation resulted in a detection limit below the lowest target concentration, 50 ng/g. The average nanograms per gram value found at each level for each compound was determined from the linear regression for the 40 data points (blank samples omitted) and the nanograms per gram added at that level. The standard deviation and percent imprecision (coefficient of variation) at each level were calculated based on this average nanograms per gram value found. Thus, these values and the

values given in Tables 2, 3, and 4 for these terms (based on the average of the eight assays at each level) are not comparable. The percent inaccuracy term was calculated from the average of the eight data points at each level, and thus agrees closely with the values in Tables 2, 3, and 4.



HPLC Conditions

Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 30% acetonitrile in
1% acetic in water
Flow Rate: 1.5 ml/min
Chart Speed: 0.1 in./min
Detector: UV, 254 nm

Sample Characteristics

Concentrations: RDX, DNT, TNT,
and tetryl - 500 ng/ml;
IS - 1,000 ng/ml
Injection volume: 70 μ l
Attenuation: 0.01 X

Retention Indices

Compound	Retention Volume (ml)	Retention Time (min)
RDX	13.5	9
IS	22.5	15
DNT	31.5	21
TNT	34.5	23
tetryl	39	26

Figure 1 - HPLC Separation of RDX, DNT, TNT, and Tetryl
SARMS and Propiophenone (IS)

TABLE 1

LINEARITY AND PRECISION OF THE HPLC-UV DETERMINATION OF
SARM REFERENCE SOLUTIONS OF RDX, DNT, AND TNT

Compound	ng/ml Added	ng/ml Detected				Average ^a	Standard Deviation ^b	Coefficient ^c of Variation	Percent ^d Inaccuracy
		A	B	C	D				
RDX	0	ND ^e	ND	ND	ND	-	-	-	-
	100	109	114	105	103	108	± 4.9	4.5	+ 8.0
	500	517	509	499	490	504	± 11.8	2.3	+ 0.8
	1,000	960	950	923	985	955	± 26	2.7	- 4.5
	1,500	1,551	1,547	1,435	1,444	1,494	± 63	4.2	- 0.4
	2,000	2,031	2,069	1,906	1,924	1,983	± 80	4.0	- 0.8
DNT	0	ND	ND	ND	ND	-	-	-	-
	100	113	109	109	100	108	± 5.5	5.1	+ 8.0
	500	499	490	475	483	487	± 10.2	2.1	- 2.6
	1,000	992	1,011	981	1,032	1,004	± 22	2.2	+ 0.4
	1,500	1,500	1,470	1,461	1,439	1,468	± 25	1.7	- 2.1
	2,000	1,968	1,982	1,934	1,901	1,946	± 36	1.9	- 2.7
TNT	0	ND	ND	ND	ND	-	-	-	-
	100	107	112	112	100	108	± 5.7	5.3	+ 8.0
	500	495	479	484	484	486	± 6.8	1.4	- 2.8
	1,000	956	989	967	997	977	± 19	2.0	- 2.3
	1,500	1,498	1,508	1,471	1,432	1,478	± 34	2.3	- 1.5
	2,000	2,011	2,015	1,957	1,879	1,966	± 63	3.2	- 1.7

Linear Regression

RDX: $y = 0.988x + 0.6$

Correlation coefficient - 0.998

DNT: $y = 0.974x + 7.7$

Correlation coefficient - 0.999

TNT: $y = 0.982x + 1.2$

Correlation coefficient - 0.999

^a Average = $\Sigma x/n = \bar{x}$ ^b Standard deviation = $(\Sigma |x - \bar{x}|^2 / (n-1))^{1/2} = s$ ^c Coefficient of variation = $s/\bar{x} \times 100$ ^d Percent inaccuracy = $\frac{x - \text{ng added}}{\text{ng added}} \times 100$ ^e ND = Not detectable, less than 20 ng/ml

TABLE 2

HPLC-UV DETERMINATION OF RDX IN MUSCLE/FAT SAMPLES

Amount Added (ng/g)	Level Found (ng/g)										Standard Deviation ^b	Coefficient ^c of Variation	Percent ^d Inaccuracy
	Day 1		Day 2		Day 3		Day 4		Average ^a				
	A	B	A	B	A	B	A	B					
0	ND ^e	ND	ND	ND	ND	ND	ND	ND	-	-	-	-	
50	52	55	58	55	58	55	54	56	56	± 2.2	3.9	+ 12	
100	112	108	104	98	114	114	105	110	108	± 5.6	5.2	+ 8.0	
200	206	130	209	208	210	206	204	209	198	± 27	14	- 1.0	
500	524	526	480	468	482	512	508	453	494	± 27	5.5	- 1.2	
1,000	1,017	906	958	961	1,014	1,024	1,076	798	969	± 86	8.9	- 3.1	

Note: Linear regression: $y = 0.965x + 6.5$
Correlation coefficient: 0.994

a Average = $\Sigma x/n = \bar{x}$

b Standard deviation = $(\Sigma |\bar{x} - x|^2 / n - 1)^{1/2} = \sigma$

c Coefficient of variation = $\sigma / \bar{x} \times 100$

d Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$

e ND - Not detectable, less than 10 ng/g.

TABLE 3
HPLC-UV DETERMINATION OF DNT IN MUSCLE/FAT SAMPLES

Amount Added (ng/g)	Level Found (ng/g)										Average ^a	Standard Deviation ^b	Coefficient ^c of Variation	Percent ^d Inaccuracy
	Day 1		Day 2		Day 3		Day 4							
	A	B	A	B	A	B	A	B	A	B				
0	ND ^e	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-	-	
50	42	46	48	46	42	46	44	42	44	± 2.3	5.3	- 12	- 12	
100	84	84	89	80	84	82	82	80	83	± 2.9	3.5	- 17	- 17	
200	132	108	156	176	164	170	162	168	154	± 23	15	- 23	- 23	
500	420	386	404	384	375	422	424	368	400	± 20	4.9	- 20	- 20	
1,000	709	796	826	862	826	833	846	566	783	± 99	13	- 22	- 22	

Note: Linear regression: $y = 0.781x + 3.7$
Correlation coefficient: 0.990

- a Average = $\Sigma x/n = \bar{x}$
b Standard deviation = $(\Sigma |\bar{x} - x|^2/n-1)^{1/2} = \sigma$
c Coefficient of variation = $\sigma/\bar{x} \times 100$
d Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$
e ND - Not detectable, less than 10 ng/g.

TABLE 4

HPLC-UV DETERMINATION OF TNT IN MUSCLE/FAT SAMPLES

Amount Added (ng/g)	Level Found (ng/g)										Average ^a	Standard Deviation ^b	Coefficient ^c of Variation	Percent ^d Inaccuracy
	Day 1		Day 2		Day 3		Day 4							
	A	B	A	B	A	B	A	B						
0	ND ^e	ND	ND	ND	ND	ND	ND	ND	-	-	-	-	-	
50	46	48	50	48	42	44	44	42	46	± 3.0	6.5	-	8.0	
100	80	84	94	90	86	87	82	88	87	± 3.7	4.2	-	13	
200	154	146	199	178	170	170	160	172	169	± 16	9.6	-	16	
500	409	434	420	437	394	462	428	474	432	± 26	6.1	-	14	
1,000	816	824	872	832	838	862	881	873	850	± 25	3.0	-	15	

Note: Linear regression: $y = 0.850x + 1.8$
Correlation coefficient: 0.999

a Average = $\Sigma x/n = \bar{x}$

b Standard deviation = $(\Sigma |\bar{x} - x|^2/n-1)^{1/2} = \sigma$

c Coefficient of variation = $\sigma/\bar{x} \times 100$

d Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$

e. ND - Not detectable, less than 10 ng/g.

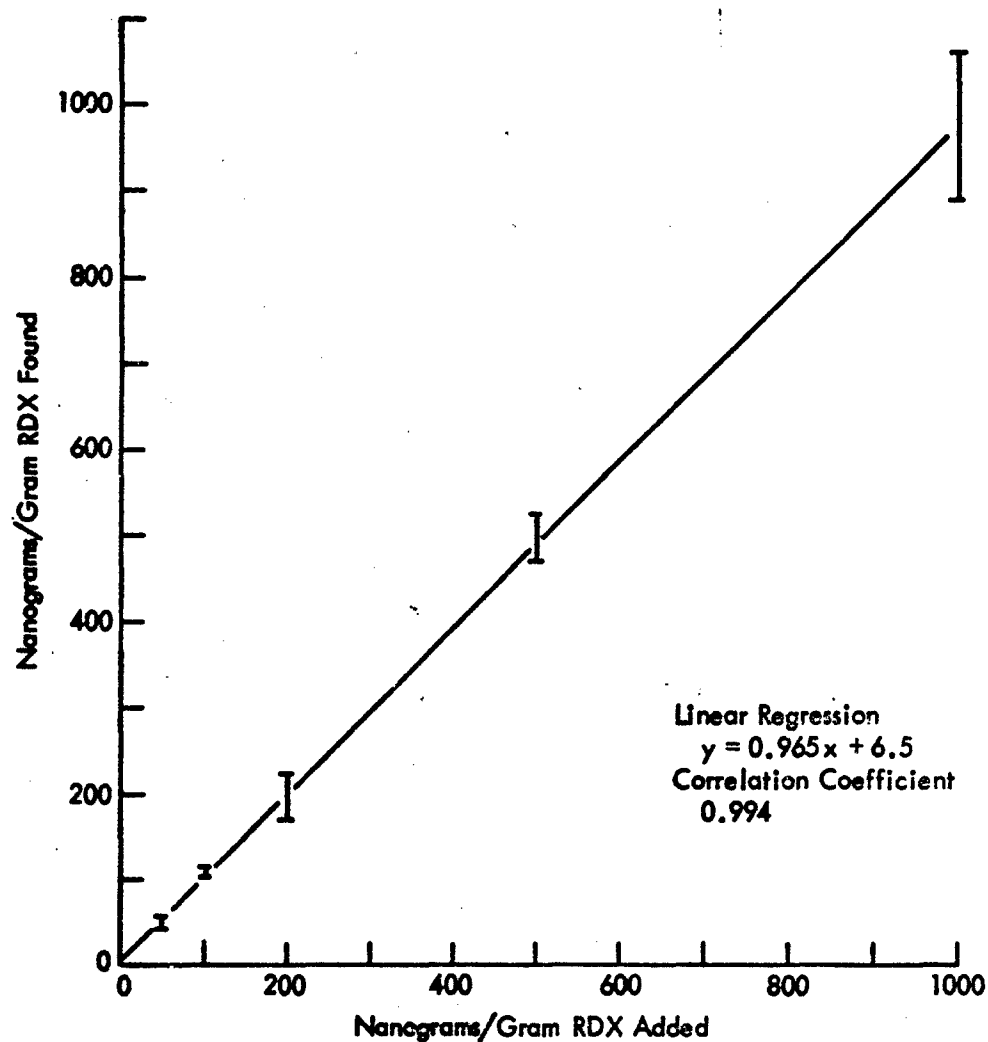


Figure 2 - Determination of RDX in Muscle/Fat Samples

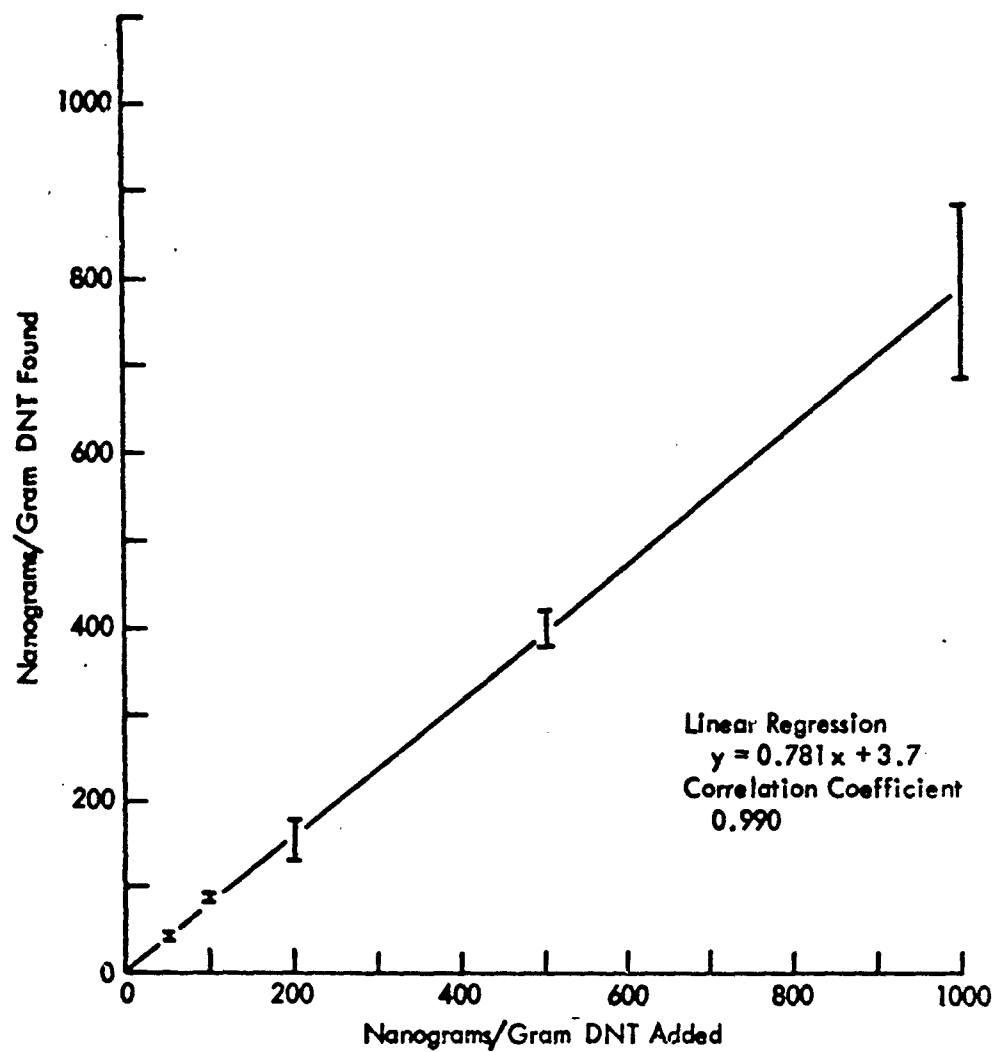


Figure 3 - Determination of DNT in Muscle/Fat Samples

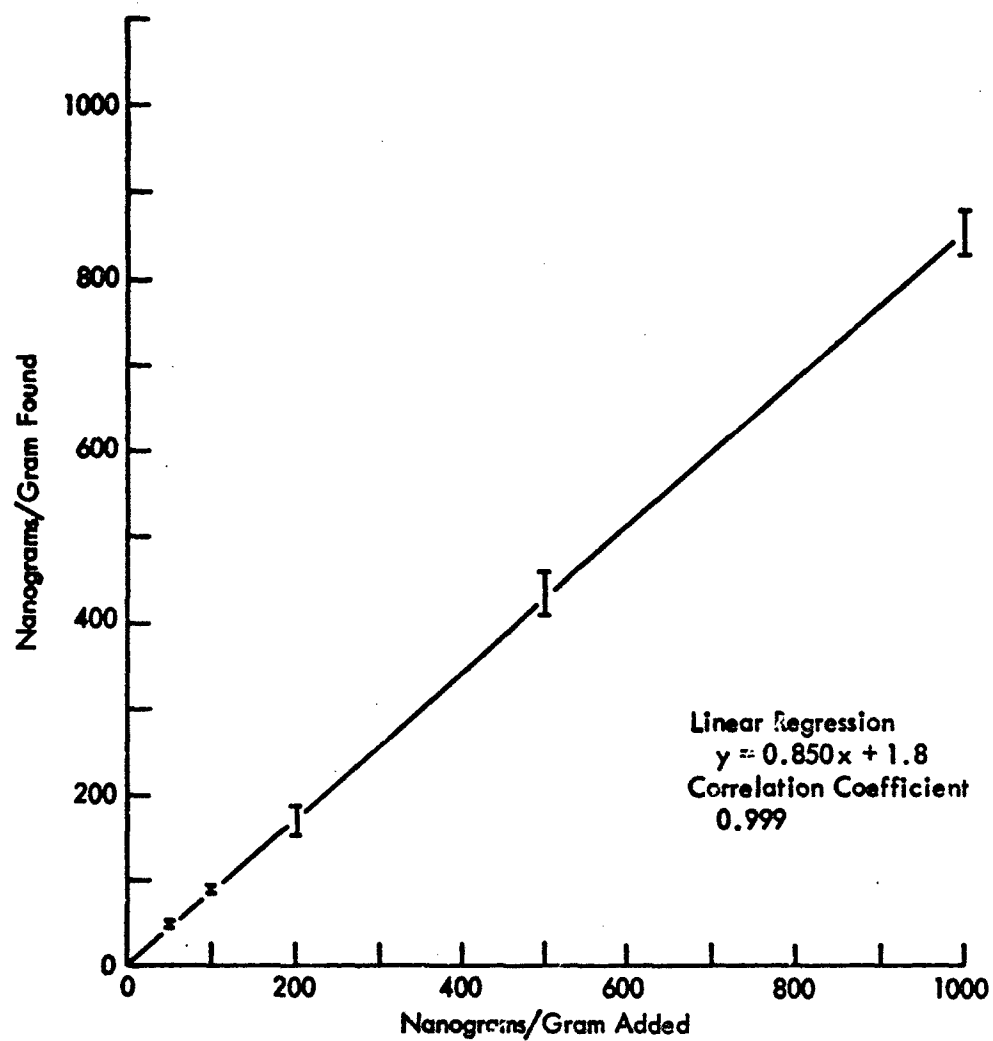


Figure 4 - Determination of TNT in Muscle/Fat Samples

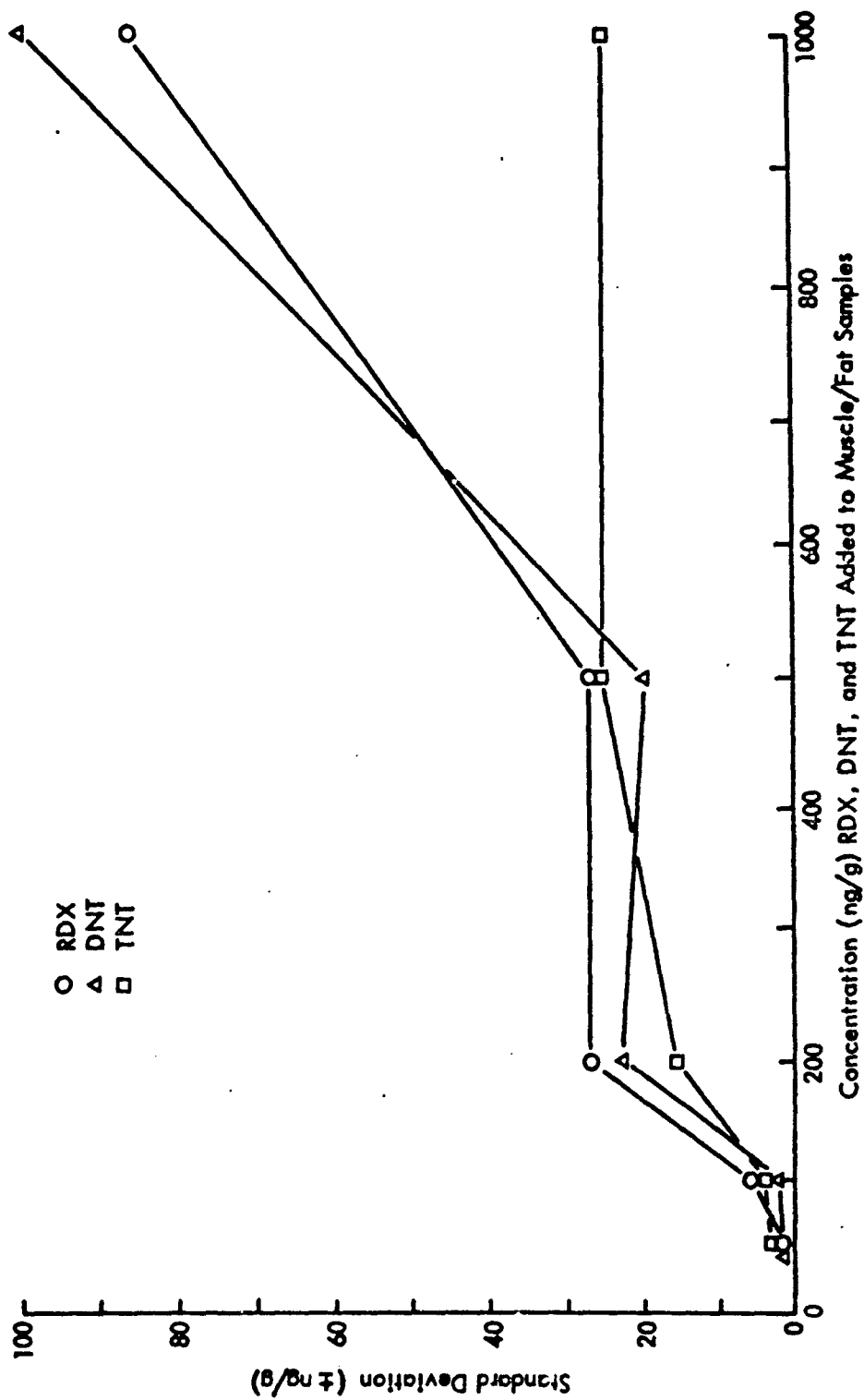


Figure 5 - Standard Deviation for RDX, DNT, and TNT in Muscle/Fat Samples

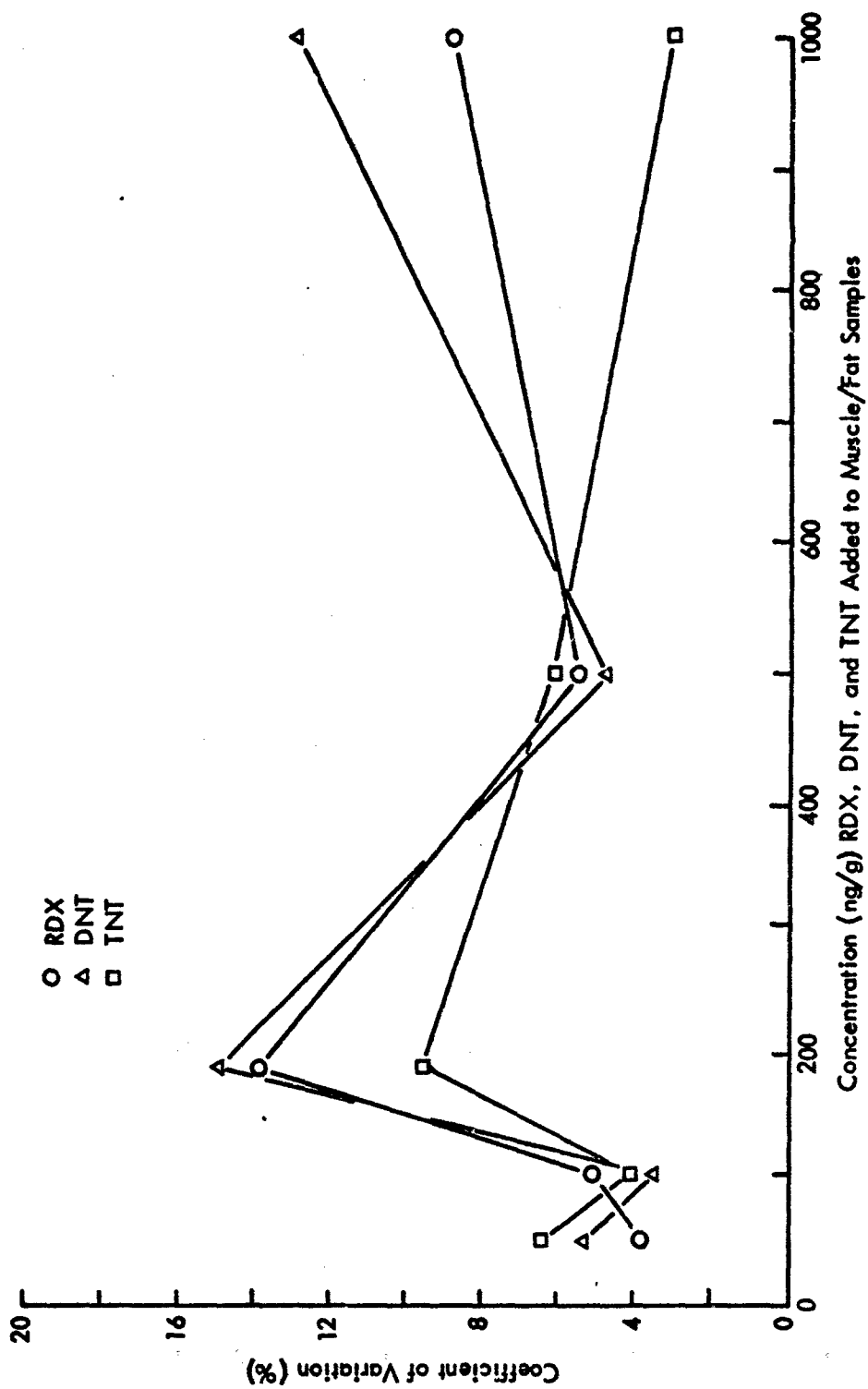


Figure 6 - Coefficient of Variation for RDX, DNT, and TNT in Muscle/Fat Samples

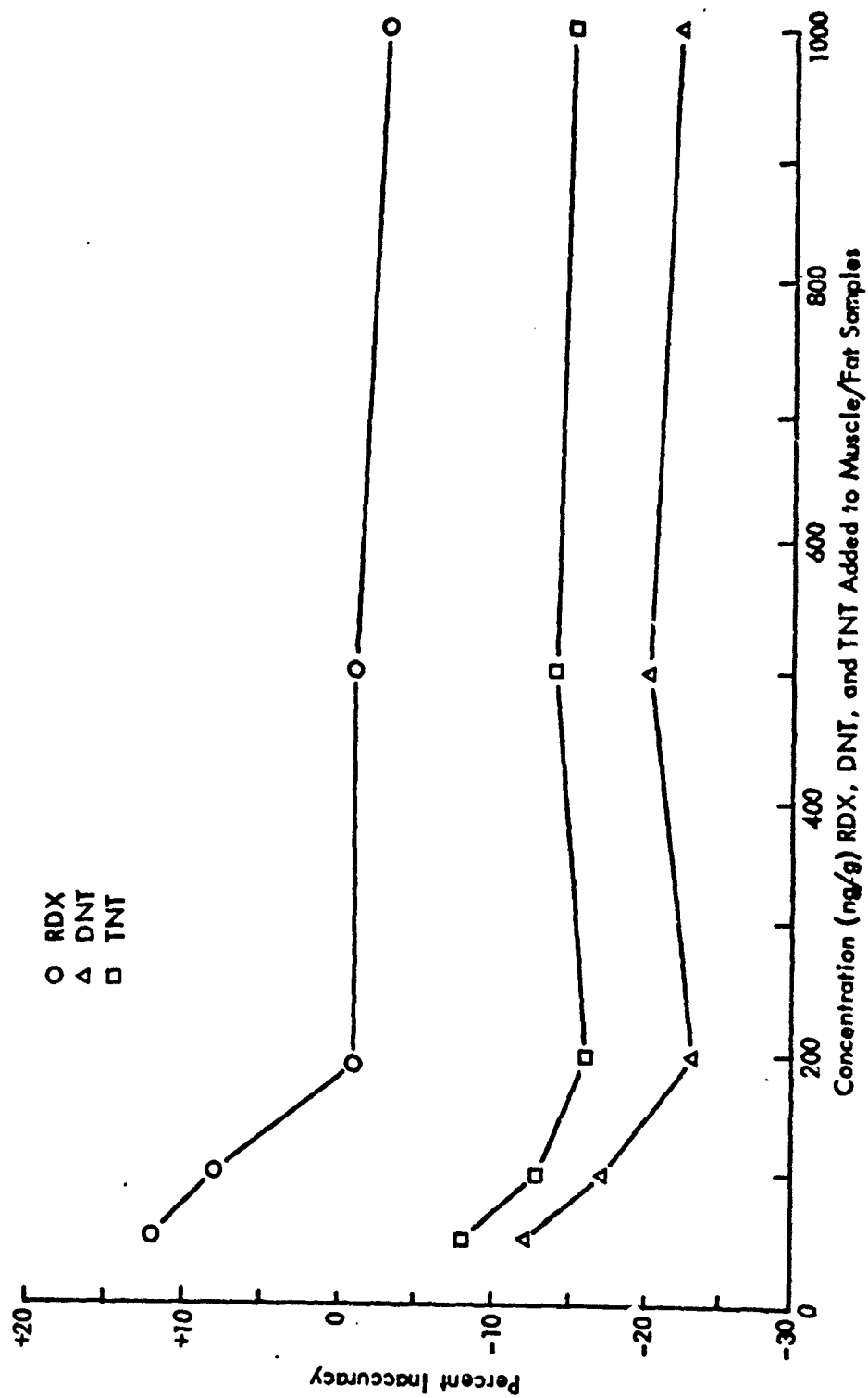
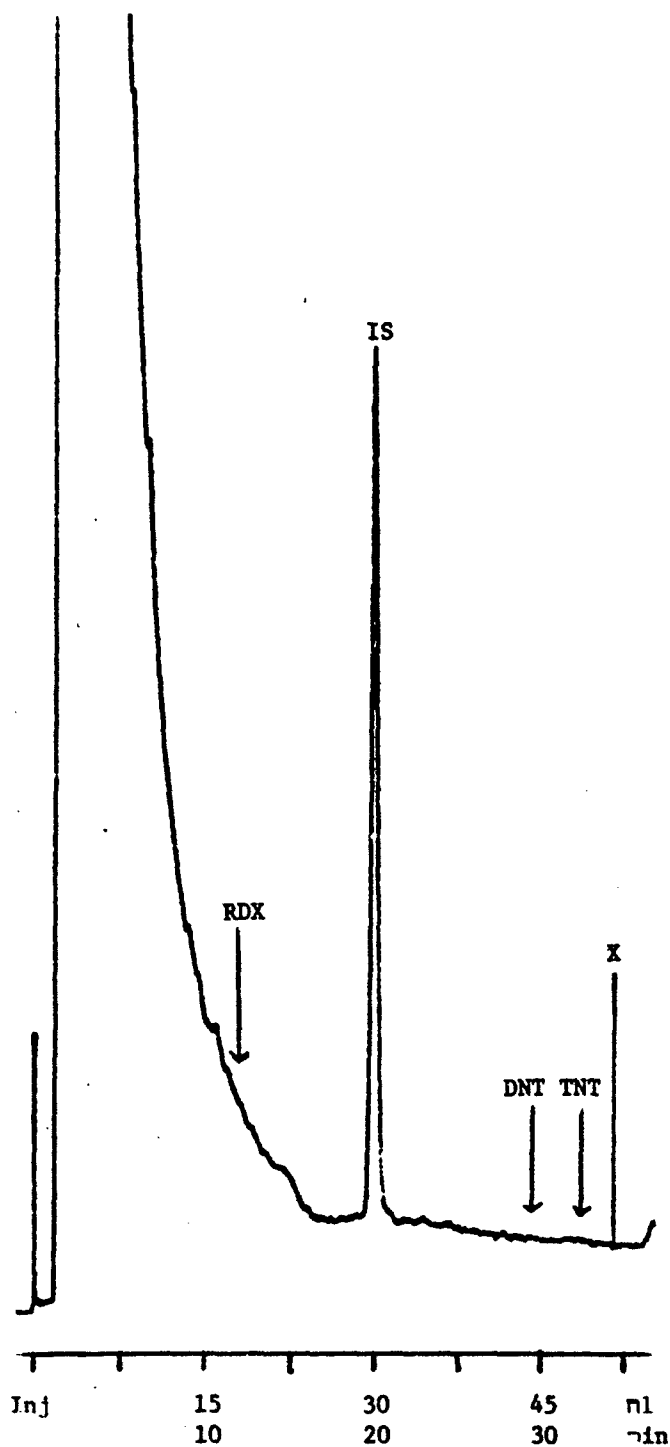


Figure 7 - Percent Inaccuracy for RDX, DNT, and TNT in Muscle/Fat Samples



HPLC Conditions:

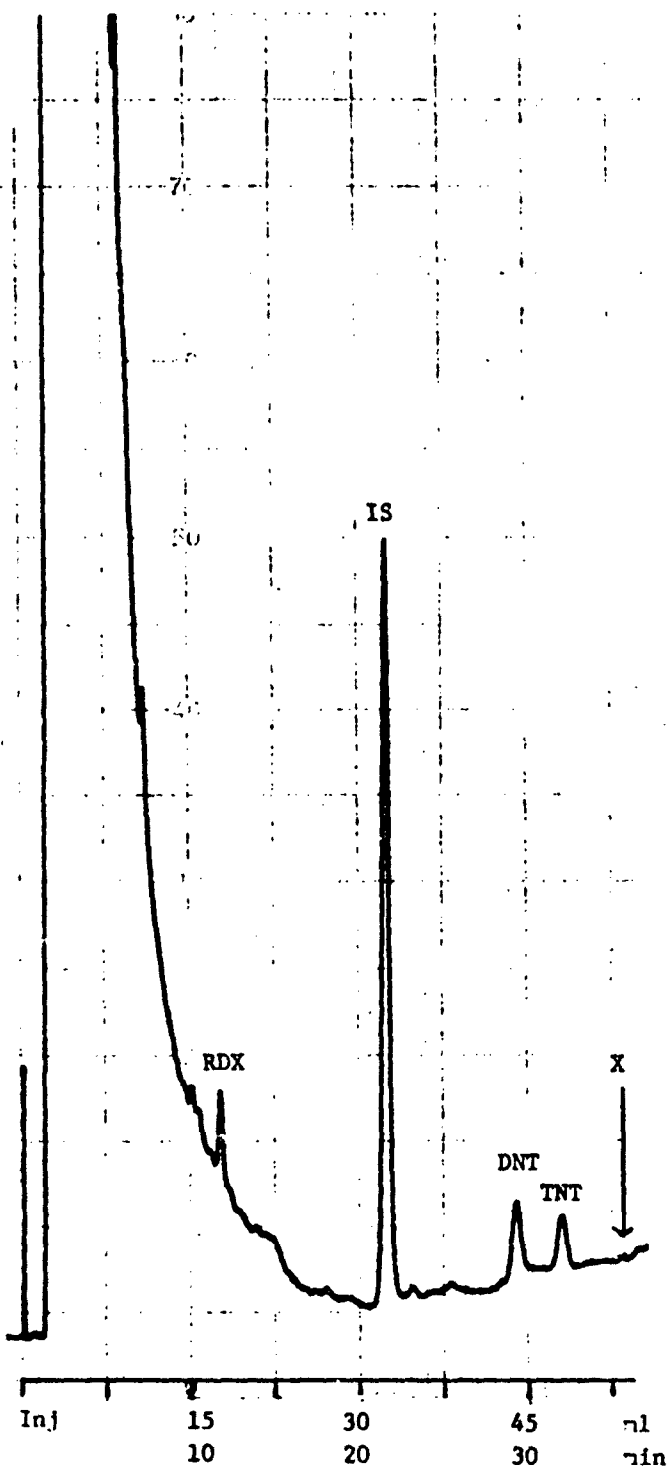
Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 28% acetonitrile in
1% acetic acid in water
Flow Rate: 1.5 ml/min
Chart Speed: 0.1 in/min
Detector: UV, 254 nm

Sample Characteristics:

2.0 g muscle/fat extracted
with 6 ml and then 3 ml
acetonitrile. Acetonitrile
concentrated to about 250 μ l
and then diluted to 1.0 ml
with IS stock and water.

IS Concentration: 1,000 ng/ml
Injection Volume: 70 μ l
Attenuation: 0.01 X

Figure 8 - HPLC Analysis of Blank Muscle/Fat Sample for RDX, DNT, and TNT
Method Development. "X" indicates eluent change to 100% acetonitrile.
Arrows indicate elution position of RDX, DNT, and TNT.



HPLC Conditions:

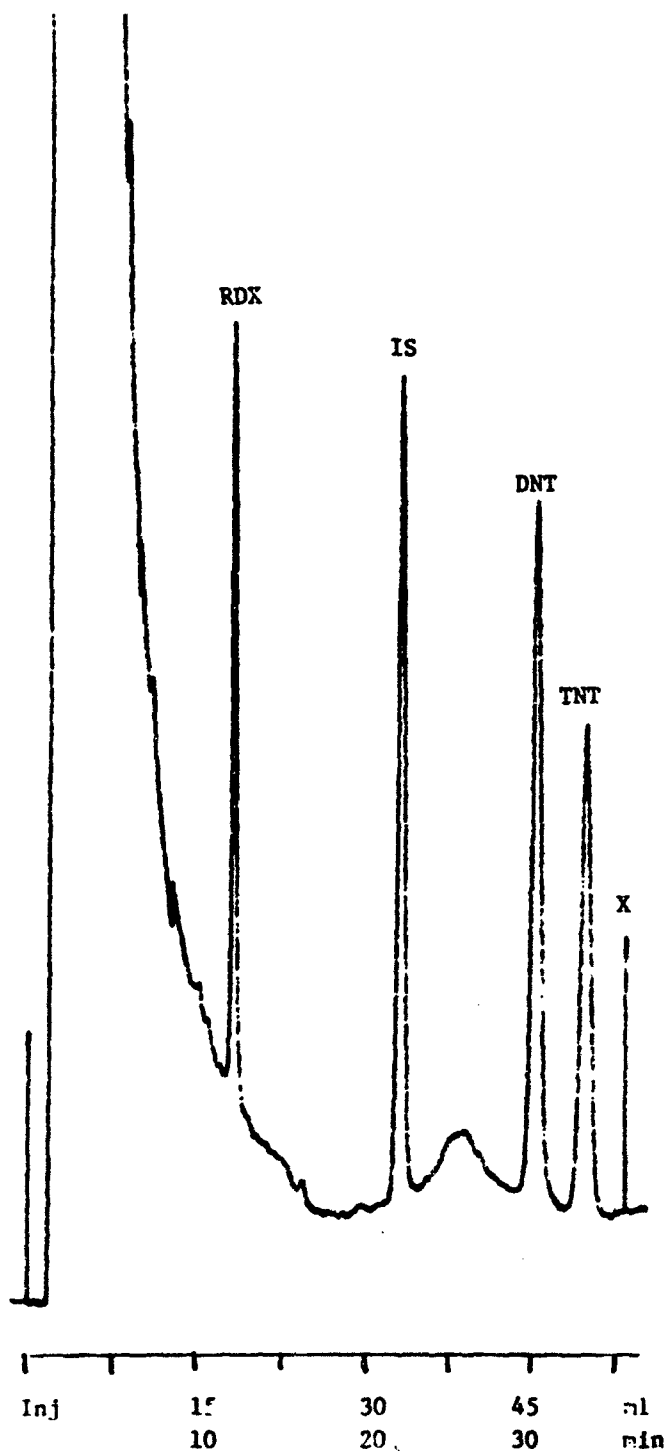
Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 28% acetonitrile in
1% acetic acid in water
Flow Rate: 1.5 ml/min
Chart Speed: 0.1 in/min
Detector: UV, 254 nm

Sample Characteristics:

2.0 g muscle/fat containing
50 ng/g RDX, DNT, and TNT
extracted with 6 ml and then
3 ml acetonitrile. Acetonitrile
concentrated to about 250 μ l
and then diluted to 1.0 ml
with IS stock and water.

IS Concentration: 1,000 ng/ml
Injection Volume: 70 μ l
Attenuation: 0.01 X

Figure 9 - HPLC Analysis of Muscle/Fat Sample Containing 50 ng/g RDX, DNT, and TNT.
"X" indicates eluent change to 100% acetonitrile.



HPLC Conditions:

Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 28% acetonitrile in
1% acetic acid in water
Flow Rate: 1.5 ml/min
Chart Speed: 0.1 in/min
Detector: UV, 254 nm

Sample Characteristics

2.0 g muscle/fat containing
500 ng/g RDX, DNT, and TNT
extracted with 6 ml and then
3 ml acetonitrile. Acetonitrile
concentrated to about 250 μ l
and then diluted to 1.0 ml
with IS stock and water.

IS Concentration: 1,000 ng/ml
Injection Volume: 70 μ l
Attenuation: 0.01 X

Figure 10 - HPLC Analysis of Muscle/Fat Sample Containing 500 ng/g RDX, DNT, and TNT.
"X" indicates eluent change to 100% acetonitrile.

TABLE 5

**STATISTICAL EVALUATION OF RDX IN MUSCLE/FAT SAMPLE DATA BY
THE HUBAUX AND VOS DETECTION LIMIT PROGRAM**

Number ^a of Data Points	Linear Regression	Correlation Coefficient	Degrees of Freedom	b	y ^c Intercept	Detection ^d Limit
40	y = 0.962x + 9.2	0.993	38	1.686	80	146
32	y = 0.971x + 7.6	0.994	30	1.697	41	69
24	y = 0.939x + 10.9	0.967	22	1.717	41	62

ng/g RDX Added	Average ^e ng/g Found	Standard ^f Deviation	Percent ^g Imprecision	Percent ^h Inaccuracy
50	57	± 0.8	1.5	+ 12
100	105	± 2.1	1.9	+ 8.1
200	202	± 10	5.2	- 1.1
500	490	± 10	2.1	- 1.2
1,000	971	± 33	3.4	- 3.1

^a Number of data points - data points used to calculate linear regression and detection limits; the blank samples analyzed were not included in these calculations; 40 - all data;

^b 32 - 1,000 ng/g samples omitted; 24 - 1,000 ng/g and 500 ng/g samples omitted.

^c t - 2 tail p level (usually 0.1, each confidence band is 0.05 so total p = 0.1).

^d y intercept - intercept on y-axis of upper confidence limit line.

^e Detection limit - x-intercept of y-intercept and lower confidence limit line.

^f Average ng/g found - average at each level determined from linear regression equation for 40 points.

^g Standard deviation - determined from average value (e above) and observed value.

^h Percent imprecision - standard deviation divided by average value times 100%.

ⁱ Percent inaccuracy - determined from the average values of the eight observed values at each level

$$\% \text{ Inaccuracy} = \frac{\text{Average observed values} - \text{level added}}{\text{level added}} \times 100$$

TABLE 6

**STATISTICAL EVALUATION OF DNT IN MUSCLE/FAT SAMPLE DATA BY
THE HUBAUX AND VOS DETECTION LIMIT PROGRAM**

Number ^a of Data Points	Linear Regression	Correlation Coefficient	Degrees of Freedom	b t	y ^c Intercept	Detection ^d Limit
40	y = 0.781x + 4.3	0.988	38	1.686	82	197
32	y = 0.793x + 2.2	0.994	30	1.697	29	67
24	y = 0.731x + 8.8	0.964	22	1.717	33	66

ng/g DNT Added	Average ^e ng/g Found	Standard ^f Deviation	Percent ^g Imprecision	Percent ^h Inaccuracy
50	43	± 0.9	2.0	- 11
100	82	± 1.1	1.3	- 17
200	161	± 8.7	5.6	- 23
500	395	± 7.4	1.9	- 20
1,000	785	± 38	4.8	- 22

a Number of data points - data points used to calculate linear regression and detection limits; the blank samples analyzed were not included in these calculations; 40 - all data;

b 32 - 1,000 ng/g samples omitted; 24 - 1,000 ng/g and 500 ng/g samples omitted.

c t - 2 tail p level (usually 0.1, each confidence band is 0.05 so total p = 0.1).

d y intercept - intercept on y-axis of upper confidence limit line.

e Detection limit - x-intercept of y-intercept and lower confidence limit line.

f Average ng/g found - average at each level determined from linear regression equation for 40 points.

g Standard deviation - determined from average value (e above) and observed value.

h Percent imprecision - standard deviation divided by average value times 100%.

i Percent inaccuracy - determined from the average values of the eight observed values at each level

$$\% \text{ Inaccuracy} = \frac{\text{Average observed values} - \text{level added}}{\text{level added}} \times 100$$

TABLE 7

STATISTICAL EVALUATION OF TNT IN MUSCLE/FAT SAMPLE DATA BY
THE HUBAUX AND VOS DETECTION LIMIT PROGRAM

Number ^a of Data Points	Linear Regression	Correlation Coefficient	Degrees of Freedom	b	y ^c Intercept	Detection ^d Limit
40	$y = 0.849x + 2.5$	0.998	38	1.686	33	71
32	$y = 0.861x - 0.5$	0.995	30	1.697	27	62
24	$y = 0.820x + 4.9$	0.985	22	1.717	23	43

ng/g TNT Added	Average ^e ng/g Found	Standard ^f Deviation	Percent ^g Imprecision	Percent ^h Inaccuracy
50	45	± 1.1	2.5	- 9.0
100	87	± 1.4	1.6	- 13
200	172	± 6.1	3.6	- 16
500	427	± 9.9	2.3	- 14
1,000	852	± 9.5	1.1	- 15

^a Number of data points - data points used to calculate linear regression and detection limits; the blank samples analyzed were not included in these calculations; 40 - all data;

^b 24 - 1,000 ng/g samples omitted; 24 - 1,000 ng/g and 500 ng/g samples omitted.

^c t - 2 tail p level (usually 0.1, each confidence band is 0.05 so total p = 0.1).

^d y intercept - intercept on y-axis of upper confidence limit line.

^e Detection limit - x-intercept of y-intercept and lower confidence limit line.

^f Average ng/g found - average at each level determined from linear regression equation for 40 points.

^g Standard deviation - determined from average value (e above) and observed value.

^h Percent imprecision - standard deviation divided by average value times 100%.

ⁱ Percent inaccuracy - determined from the average values of the eight observed values at each level

$$\% \text{ Inaccuracy} = \frac{\text{Average observed values} - \text{level added}}{\text{level added}} \times 100$$

APPENDIX

**METHOD DEVELOPMENT FOR THE DETERMINATION OF
RDX, DNT, AND TNT IN MUSCLE/FAT SAMPLES**

RAW DATA AND CALCULATIONS

TABLE 8

LINEARITY AND PRECISION OF RDX, DNT, AND TNT DETERMINATION
BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Reference Solution Number	ng/ml Compound Added	Peak Height (mm)			Internal Standard ng ml	Peak Height	Relative Weight Response			Calculated ng/ml		
							RDX	DNT	TNT	RDX	DNT	TNT
		RDX	DNT	TNT								
A-1	0	< 2	< 2	< 2	1,000	122	-	-	-	ND	ND	ND
A-2	100	12	14	11	1,000	116	1.03	1.21	0.95	109	113	107
A-3	500	58	63	52	1,000	118	0.98	1.07	0.88	517	499	495
A-4	1,000	104	121	97	1,000	114	0.91	1.06	0.85	960	992	956
A-5	1,500	168	183	152	1,000	114	0.98	1.07	0.89	1,551	1,500	1,498
A-6	2,000	220	240	204	1,000	114	0.96	1.05	0.89	2,031	1,968	2,011
B-1	0	< 2	< 2	< 2	1,000	118	-	-	-	ND	ND	ND
B-2	100	13	14	12	1,000	120	1.08	1.17	1.00	114	109	112
B-3	500	59	64	52	1,000	122	0.97	1.05	0.85	509	490	479
B-4	1,000	121	145	118	1,000	134	0.90	1.08	0.88	950	1,011	989
B-5	1,500	172	184	157	1,000	117	0.98	1.05	0.89	1,547	1,470	1,508
B-6	2,000	228	246	208	1,000	116	0.93	1.06	0.90	2,069	1,982	2,015
C-1	0	< 2	< 2	< 2	1,000	120	-	-	-	ND	ND	ND
C-2	100	12	14	12	1,000	120	1.00	1.17	1.00	105	109	112
C-3	500	55	59	50	1,000	116	0.95	1.02	0.86	499	475	484
C-4	1,000	107	128	105	1,000	122	0.88	1.05	0.86	923	981	967
C-5	1,500	150	172	144	1,000	110	0.91	1.04	0.87	1,435	1,461	1,471
C-6	2,000	210	240	202	1,000	116	0.91	1.03	0.87	1,906	1,934	1,957
D-1	0	< 2	< 2	< 2	1,000	119	-	-	-	ND	ND	ND
D-2	100	11	12	10	1,000	112	0.98	1.07	0.89	103	100	100
D-3	500	54	60	50	1,000	116	0.93	1.03	0.86	490	483	484
D-4	1,000	116	137	110	1,000	124	0.94	1.10	0.89	985	1,032	997
D-5	1,500	155	174	144	1,000	113	0.91	1.03	0.85	1,444	1,439	1,432
D-6	2,000	212	236	194	1,000	116	0.91	1.02	0.84	1,924	1,901	1,879

TABLE 8 (concluded)

Relative Weight Response

	<u>Average</u>	<u>Standard Deviation</u>	<u>Relative Standard Deviation</u>
RDX	0.95	± 0.05	5.2%
DNT	1.07	± 0.05	4.9%
TNT	0.89	± 0.04	5.1%

TABLE 9

DETERMINATION OF RDX, DNT, AND TNT IN MUSCLE/FAT SAMPLES

DAY 1 SAMPLES

Sample Number	ng/g ^a Compound Added	g Muscle/Fat	Peak Height (mm)			Internal ^b Standard		ng/g ^c Detected		
			RDX	DNT	TNT	ng/ml	Peak Height	RDX	DNT	TNT
Day 1A-0	0	2.0	< 2	< 2	< 2	1,000	101	ND ^d	ND	ND
Day 1A-100	50	2.0	11.0	10.2	7.8	1,000	116	52	42	46
Day 1A-200	100	2.0	24.0	20.4	15.0	1,000	116	112	84	88
Day 1A-400	200	2.0	45.0	32.6	26.8	1,000	119	206	132	154
Day 1A-1000	500	2.0	109	99.0	67.6	1,000	113	524	524	409
Day 1A-2000	1,000	2.0	234	184	149	1,000	125	1,017	709	816
Day 1B-0	0	2.0	< 2	< 2	< 2	1,000	114	ND	ND	ND
Day 1B-100	50	2.0	12.6	12.0	8.6	1,000	124	55	46	48
Day 1B-200	100	2.0	24.0	21.0	14.6	1,000	120	108	84	84
Day 1B-400	200	2.0	27.0	25.2	24.0	1,000	113	130	108	146
Day 1B-1000	500	2.0	116	96.4	76.0	1,000	120	526	386	434
Day 1B-2000	1,000	2.0	180	178	130	1,000	108	906	796	824

TABLE 9 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)			Internal ^b Standard		Relative Weight ^c Response	
		RDX	DNT	TNT	ng/ml	Peak Height	RDX	TNT
Std - Day 1-4	1,000	124	139	96.0	1,000	135	0.92	1.03
Std - Day 1-1	100	13.0	14.0	10.2	1,000	135	0.96	1.04
Std - Day 1-5	2,000	237	269	182	1,000	131	0.91	1.03
Std - Day 1-2	200	24.6	28.0	19.8	1,000	133	0.92	1.05
Std - Day 1-3	400	50.0	58.0	40.0	1,000	138	0.91	1.05
Average							0.92	1.04
							0.73	0.73

29
181

^a ng/g Compound added - nanograms of RDX, DNT, and TNT added per gram of muscle/fat sample.
^b Internal standard - compound (propionophenone) added to muscle/fat sample after sample preparation for calculation of data.

^c ng/g detected - nanograms of RDX, DNT, and TNT detected in the muscle/fat sample

$$\text{ng compound/g} = \frac{\text{Peak Height Compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}} \times \frac{1}{\text{Sample Weight (g)}}$$

^d ND - not detected, less than 10 ng/g.

^e Relative weight response - RWR

$$\text{RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

TABLE 10

DETERMINATION OF RDX, DNT, AND TNT IN MUSCLE/FAT SAMPLES

DAY 2 SAMPLES

Sample Number	ng/g ^a Compound Added	g Muscle/Fat	Peak Height (mm)			Internal ^b Standard		ng/g ^c Detected		
			RDX	DNT	TNT	ng/ml	Peak Height	RDX	DNT	TNT
Day 2A-0	0	2.0	< 2	< 2	< 2	1,000	130	ND ^d	ND	ND
Day 2A-100	50	2.0	13.0	11.5	8.2	1,000	114	58	48	50
Day 2A-200	100	2.0	24.2	22.2	15.8	1,000	119	104	89	94
Day 2A-400	200	2.0	49.0	39.2	33.8	1,000	120	209	156	199
Day 2A-1000	500	2.0	115	104	73.0	1,000	122	480	404	420
Day 2A-2000	1,000	2.0	207	192	137	1,000	110	958	826	872
Day 2B-0	0	2.0	< 2	< 2	< 2	1,000	122	ND	ND	ND
Day 2B-100	50	2.0	14.8	12.5	8.8	1,000	131	58	46	48
Day 2B-200	100	2.0	26.0	22.8	17.2	1,000	135	98	80	90
Day 2B-400	200	2.0	44.0	40.0	27.2	1,000	108	208	176	178
Day 2B-1000	500	2.0	133	117	90.0	1,000	145	468	384	437
Day 2B-2000	1,000	2.0	226	217	159	1,000	120	961	862	832

TABLE 10 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)		Internal ^b Standard		Relative Weight ^c Response	
		RDX	DNT	ng/ml	Peak Height	RDX	TNT
Std - Day 2-4	1,000	120	138	1,000	134	0.90	1.03
Std - Day 2-2	200	25.8	30.8	1,000	144	0.90	1.07
Std - Day 2-5	2,000	254	300	1,000	144	0.88	1.04
Std - Day 2-2	200	26.6	30.0	1,000	145	0.92	1.04
Std - Day 2-1	100	14.0	15.0	1,000	142	0.99	1.06
					Average	0.91	1.05
							0.66

^a ng/g Compound added - nanograms of RDX, DNT, and TNT added per gram of muscle/fat sample.

^b Internal standard - compound (propiphenone) added to muscle/fat sample after sample preparation for calculation of data.

^c ng/g detected - nanograms of RDX, DNT, and TNT detected in the muscle/fat sample

$$\text{ng compound/g} = \frac{\text{Peak Height Compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}} \times \frac{1}{\text{Sample Weight (g)}}$$

^d ND - not detected, less than 10 ng/g.

^e Relative weight response - RWR

$$\text{RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

TABLE 11

DETERMINATION OF RDX, DNT, AND TNT IN MUSCLE/FAT SAMPLES

DAY 3 SAMPLES

Sample Number	ng/g ^a Compound Added	g Muscle/Fat	Peak Height (mm)			Internal ^b Standard		ng/g ^c Detected	
			RDX	DNT	TNT	ng/ml	Peak Height	RDX	TNT
Day 3A-0	0	2.0	< 2	< 2	< 2	1,000	96.2	ND ^d	ND
Day 3A-100	50	2.0	12.2	10.5	7.2	1,000	115	58	42
Day 3A-200	100	2.0	27.2	23.8	17.0	1,000	132	114	84
Day 3A-400	200	2.0	47.2	43.2	31.5	1,000	124	210	164
Day 3A-1000	500	2.0	113	104	76.2	1,000	129	482	375
Day 3A-2000	1,000	2.0	229	219	156	1,000	124	1,014	826
Day 3B-0	0	2.0	< 2	< 2	< 2	1,000	114	ND	ND
Day 3B-100	50	2.0	13.0	12.8	8.6	1,000	129	55	46
Day 3B-200	100	2.0	27.0	22.6	16.8	1,000	129	114	82
Day 3B-400	200	2.0	47.0	45.8	32.0	1,000	126	206	170
Day 3B-1000	500	2.0	121	117	90.0	1,000	130	512	422
Day 3B-2000	1,000	2.0	226.	216	157	1,000	121	1,024	833

TABLE 11 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)			Internal ^b Standard		Relative Weight ^c Response		
		RDX	DNT	TNT	ng/ml	Peak Height	RDX	DNT	TNT
Std - Day 3-4	1,000	124	147	104	1,000	142	0.87	1.04	0.73
Std - Day 3-3	400	51.2	61.5	43.2	1,000	142	0.90	1.08	0.76
Std - Day 3-1	100	13.0	14.2	10.0	1,000	129	1.01	1.10	0.78
Std - Day 3-5	2,000	255	305	213	1,000	143	0.89	1.07	0.75
Std - Day 3-3	400	53.0	64.4	45.2	1,000	149	0.89	1.08	0.76
Average							0.91	1.07	0.75

^a ng/g Compound added - nanograms of RDX, DNT, and TNT added per gram of muscle/fat sample.
^b Internal standard - compound (propiphenone) added to muscle/fat sample after sample preparation for calculation of data.

^c ng/g detected - nanograms of RDX, DNT, and TNT detected in the muscle/fat sample

$$\text{ng compound/g} = \frac{\text{Peak Height Compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}} \times \frac{1}{\text{Sample Weight (g)}}$$

^d ND - not detected, less than 10 ng/g.

^e Relative weight response - RWR

$$\text{RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

TABLE 12

DETERMINATION OF RDX, DNT, AND TNT IN MUSCLE/FAT SAMPLES

DAY 4 SAMPLES

Sample Number	ng/g ^a Compound Added	g Muscle/Fat	Peak Height (mm)			Internal ^b Standard		ng/g ^c Detected		
			RDX	DNT	TNT	ng/ml	Peak Height	RDX	DNT	TNT
Day 4A-0	0	2.0	< 2	< 2	< 2	1,000	132	ND ^d	ND	ND
Day 4A-100	50	2.0	12.8	12.8	8.5	1,000	134	54	44	44
Day 4A-200	100	2.0	25.5	24.0	16.5	1,000	138	105	82	82
Day 4A-400	200	2.0	50.5	49.0	33.0	1,000	141	204	162	160
Day 4A-1000	500	2.0	125	127	87.5	1,000	140	508	424	428
Day 4A-2000	1,000	2.0	234	224	159	1,000	124	1,076	846	881
Day 4B-0	0	2.0	< 2	< 2	< 2	1,000	134	ND	ND	ND
Day 4B-100	50	2.0	14.0	12.8	8.5	1,000	141	56	42	42
Day 4B-200	100	2.0	29.8	26.5	19.8	1,000	154	110	80	88
Day 4B-400	200	2.0	48.2	47.2	33.0	1,000	131	209	168	172
Day 4B-1000	500	2.0	128	133	111	1,000	161	453	388	474
Day 4B-2000	1,000	2.0	275	238	250	1,000	196	798	566	873

TABLE 12 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)		Internal ^b Standard		Relative Weight ^c Response	
		RDX	TNT	ng/ml	Peak Height	RDX	TNT
Std - Day 4-5	2,000	251	306	1,000	144	0.87	1.06
Std - Day 4-3	400	51.2	69.8	1,000	146	0.88	1.04
Std - Day 4-1	100	13.5	16.8	1,000	146	0.92	1.15
Std - Day 4-5	2,000	252	302	1,000	143	0.88	1.06
Std - Day 4-4	1,000	125	155	1,000	146	0.86	1.06
Std - Day 4-4	1,000	127	156	1,000	146	0.86	1.06
Average						0.88	1.07
							0.73

^a ng/g Compound added - nanograms of RDX, DNT, and TNT added per gram of muscle/fat sample.

^b Internal standard - compound (propiphenone) added to muscle/fat sample after sample preparation for calculation of data.

^c ng/g detected - nanograms of RDX, DNT, and TNT detected in the muscle/fat sample

$$\text{ng compound/g} = \frac{\text{Peak Height Compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}} \times \frac{1}{\text{Sample Weight (g)}}$$

^d ND - not detected, less than 10 ng/g.

^e Relative weight response - RWR

$$\text{RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

APPENDIX E

IDENTIFICATION OR DEVELOPMENT OF CHEMICAL ANALYSIS METHODS
FOR PLANTS AND ANIMAL TISSUE

METHOD REPORT NO. 4

METHOD DEVELOPMENT FOR THE DETERMINATION OF
CYCLOTRIMETHYLENETRINITRAMINE (RDX), DINITROTOLUENE (DNT),
AND TRINITROTOLUENE (TNT) IN ANIMAL LIVER SAMPLES

October 1980

Contract No. DAAK11-79-C-0110
MRI Project No. 4849-A

For

U.S. Army Toxic and Hazardous Materials Agency
Dr. L. Eng, DRXTH-TE-A, Project Officer
Aberdeen Proving Ground (EA), Maryland 21010

The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER Technical Report No. 4	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Method Development for the Determination of Cyclotrimethylenetrinitramine (RDX), Dinitrotoluene (DNT), and Trinitrotoluene (TNT) in Animal Liver Samples		5. TYPE OF REPORT & PERIOD COVERED Method Report August 1979 to December 1980
7. AUTHOR(s) D. B. Lakings and O. Gan		6. PERFORMING ORG. REPORT NUMBER MRI Project No. 4849-A
9. PERFORMING ORGANIZATION NAME AND ADDRESS Midwest Research Institute 425 Volker Boulevard Kansas City, MO 64110		8. CONTRACT OR GRANT NUMBER(s) DAAK11-79-C-0110
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Armament Research and Development Command, Aberdeen Proving Ground (Edgewood Area), MD 21010		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS October 1980
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		12. REPORT DATE October 1980
		13. NUMBER OF PAGES 44
		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Unlimited		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Cyclotrimethylenetrinitramine (RDX) High Performance Liquid Chromatography Dinitrotoluene (DNT) Trinitrotoluene (TNT) Liver Level Determination		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A high performance liquid chromatographic (HPLC) method for the quantitative determination of cyclotrimethylenetrinitramine (RDX), dinitrotoluene (DNT) and trinitrotoluene (TNT) in animal liver samples has been developed. The analytical system consists of an isocratic HPLC unit with a Spherisorb ODS, 5 μ , 250 x 4.6 mm ID column, an eluent of 30% acetonitrile in 1% acetic acid in water, and a flow rate of 1.5 ml/min. The compounds, including the internal standard (IS), propiophenone, have the following retention characteristics:		

DD FORM 1 JAN 73 1473

EDITION OF 1 NOV 65 IS OBSOLETE

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE(When Data Entered)

Abstract (concluded)

RDX, 15 ml, 10 min; IS - 28.5 ml, 19 min; DNT - 37.5 ml, 25 min; and TNT - 39 ml, 26 min (slight changes in the retention indices occurs with a fresh eluent or a change of columns) and are detected at 254 nm. Reference solutions of the munition compounds from 100 to 2,00 ng/ml gave a linear response and an HPLC peak was detected and quantifiable when 3 ng of analyte were injected on column. The animal liver samples were prepared by first homogenizing the matrix to obtain a uniform sample. Then, a 1.0-g liver sample was weighed, mixed with 1.0 ml 10% sodium chloride containing 1% acetic acid, and extracted with 3 x 4 ml toluene. The toluene extract(s) were combined and evaporated to dryness at room temperature under a stream of nitrogen. The residue was dissolved in 500 μ l acetonitrile containing the IS (1,000 ng/ml) and the final volume adjusted to 1.0 ml with high purity water. After filtering the prepared sample through a 0.45 μ Fluoropore filter, an aliquot was analyzed by HPLC. The analytical method was evaluated by preparing and analyzing duplicate liver samples containing 0, 50, 100, 200, 500 and 1,000 ng/g of each munition compound on four separate days. Linear regression of the data gave the following equations and correlation coefficients: RDX, $y = 0.904x + 107$, 0.988; DNT, $y = 0.640x - 1.9$, 0.995; and TNT, $y = 0.521x - 6.2$, 0.989, respectively. The average coefficient of variation and average percent inaccuracy for RDX, DNT, and TNT in liver samples were 16% + 74; 11% - 36; and 15% - 51, respectively. A liver component(s) had the same retention indices as RDX and interfered with the quantification of RDX. This HPLC peak(s) represented an average of 113 ng/g RDX in the eight blank liver samples analyzed. No interference was observed at the elution positions of DNT and TNT. A statistical evaluation of the data by the Hubaux and Vox detection limits program gave detection limits of 58 ng/g for RDX, 50 ng/g for DNT, and 50 ng/g for TNT for the HPLC determination of these compounds in animal liver samples. However, the 58 ng/g detection limit for RDX is unrealistic because the blank liver samples contained a component at the RDX elution position which represented 113 ± 18 ng/g RDX. Thus, a more representative detection limit for RDX in the liver matrix is 150 ng/g.

UNCLASSIFIED

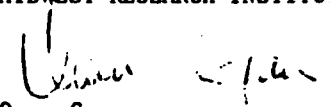
SECURITY CLASSIFICATION OF THIS PAGE(When Data Entered)

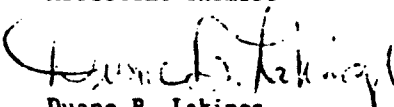
PREFACE

The report was prepared at Midwest Research Institute, 425 Volker Boulevard, Kansas City, Missouri 64110, under U.S. Army Toxic and Hazardous Materials Agency Contract No. DAAK11-79-C-0110, MRI Project No. 4849-A, "Identification or Development of Chemical Analysis Methods for Plants and Animal Tissues." Dr. Leslie Eng, DRXTH-TE-A, was the Project Officer for this research effort.

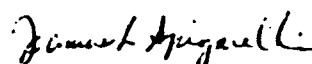
This work was conducted in the Analytical Chemistry Department Dr. James L. Spigarelli, Director. The research effort was directed by Dr. Duane B. Lakings, Program Manager and Senior Chemist, with assistance from Mr. Owen Gan, Assistant Chemist, and Ms. Mary Valla, Senior Technician. This report was prepared by Dr. Lakings and Mr. Gan.

MIDWEST RESEARCH INSTITUTE


Owen Gan
Assistant Chemist


Duane B. Lakings
Program Manager and Senior Chemist

Approved:



James L. Spigarelli, Director
Analytical Chemistry Department

TABLE OF CONTENTS

	<u>Page</u>
1. Application	1
2. Chemistry	2
3. Apparatus	2
4. Standards	3
5. Procedures for Liver Sample Determination	4
6. Calculation	6
7. Statistical Evaluation of Data.	6
Appendix - Method Development for the Determination of RDX, DNT, and TNT in Liver Samples.	25

LIST OF FIGURES

<u>Number</u>	<u>Title</u>	<u>Page</u>
1	HPLC Separation of RDX, DNT, TNT, and Teteryl SARMS and Propiophenone (IS)	8
2	Determination of RDX in Animal Liver Samples	13
3	Determination of DNT in Animal Liver Samples	14
4	Determination of TNT in Animal Liver Samples	15
5	Standard Deviation for RDX, DNT, and TNT in Animal Liver Samples.	16
6	Coefficient of Variation for RDX, DNT, and TNT in Animal Liver Samples.	17
7	Percent Inaccuracy for RDX, DNT, and TNT in Animal Liver Samples.	18
8	HPLC Analysis of Blank Liver Sample for RDX, DNT, and TNT Method Development	19
9	HPLC Analysis of Animal Liver Sample Containing 100 ng/g RDX, DNT, and TNT.	20
10	HPLC Analysis of Animal Liver Sample Containing 500 ng/g RDX, DNT, and TNT	21

LIST OF TABLES

<u>Number</u>	<u>Title</u>	<u>Page</u>
1	Linearity and Precision of the HPLC-UV Determination of SARM Reference Solutions of RDX, DNT, and TNT.	9
2	HPLC-UV Determinations of RDX in Liver Samples	10
3	HPLC-UV Determinations of DNT in Liver Samples	11
4	HPLC-UV Determinations of TNT in Liver Samples	12
5	Statistical Evaluation of RDX in Liver Sample Data by the Hubaux and Vos Detection Limit Program	22
6	Statistical Evaluation of DNT in Liver Sample Data by the Hubaux and Vos Detection Limit Program.	23
7	Statistical Evaluation of TNT in Liver Sample Data by the Hubaux and Vos Detection Limit Program.	24
Appendix		
8	Linearity and Precision of RDX, DNT, and TNT Determination by High Performance Liquid Chromatography.	26
9	Determination of RDX, DNT, and TNT in Liver Samples. . . .	28
10	Determination of RDX, DNT, and TNT in Liver Samples. . . .	30
11	Determination of RDX, DNT, and TNT in Liver Samples. . . .	32
12	Determination of RDX, DNT, and TNT in Liver Samples. . . .	34

Midwest Research Institute
Analytical Chemistry Department
Kansas City, Missouri 64110

for

U.S. Army Armament Research and Development Command
Aberdeen Proving Ground (Edgewood Area)
Maryland 21010

Contract No. DAAK11-79-C-0110

Identification or Development of Chemical Analysis Methods
for Plant and Animal Tissues

METHOD DEVELOPMENT FOR THE DETERMINATION OF
CYCLOTRIMETHYLENETRINITRAMINE (RDX), DINITROTOLUENE (DNT),
AND TRINITROTOLUENE (TNT) IN ANIMAL LIVER SAMPLES

1. **APPLICATION:** The developed method is for the quantitative determination of RDX, DNT, and TNT in animal liver samples using high performance liquid chromatography (HPLC) with ultraviolet (UV) detection at 254 nm.

a. **Evaluated Concentration Range:** The concentration range of RDX, DNT, and TNT studied in reference solutions and in liver samples was 50 to 1,000 ng/g (parts per billion, ppb).

b. **Sensitivity:** A signal-to-noise ratio of 6 to 1 for RDX (peak height (PH), 25 mm), 9 to 1 for DNT (PH, 40 mm), and 8 to 1 for TNT (PH, 30 mm) was obtained with an injection of 50 μ l of a 100 ng/ml solution of each compound (ca. 5 ng each compound on column).

c. **Detection Limits:** 58 ng/g RDX, 50 ng/g DNT, and 50 ng/g TNT using the Hubaux and Vos detection limit program. However, the blank liver samples had a component which co-eluted with RDX. For the eight blank liver samples analyzed, this component represented an average RDX concentration of 113 ng/g with a standard deviation of ± 18 ng/g. Thus, a more representative detection limit for RDX in the liver matrix is 150 ng/g (the average blank value plus two standard deviations).

d. **Interferences:** No interfering liver components were found to elute with the same retention volume as DNT or TNT. A peak eluted with RDX and interfered with the quantification of RDX at low levels. This peak corresponded to greater than 100 ng/g RDX.

e. **Analysis Rate:** The chromatographic time per injection was 40 min. Two reference solutions were analyzed prior to injecting the prepared samples, and two were analyzed during the day (160 min total time). Thus, a total of eight prepared liver samples (320 min total time) can be analyzed during an 8-hr day.

2. CHEMISTRY: RDX (CAS Reg. No. 121-82-4), DNT (CAS Reg. No. 121-14-2), and TNT (CAS Reg. No. 118-96-7) are of intermediate polarity and have limited water solubility. They have good solubility in polar (methanol, acetonitrile) and intermediate polarity (toluene, ethyl acetate) organic solvents. The UV spectrum of these munitions indicates that each has a sufficient UV chromophore at 254 nm to allow UV detection and quantitation.

3. APPARATUS:

a. Instrumentation: A Waters isocratic liquid chromatographic system consisting of a Model 6000A pump, Model U6K injector, and Model 440 UV detector. A general purpose centrifuge (Dynac, Clay Adams 0101) with a 24-place head.

b. HPLC Parameters:

1. Column: Spherisorb ODS, 5 μ , 250 x 4.6 mm ID.
2. Eluent: 28% acetonitrile in 1% acetic acid in water.
3. Flow rate: 1.5 ml/min.
4. Detector: UV, 254 nm.
5. Internal standard: propiophenone, 500 ng/ml.
6. Injection volume: 50 to 100 μ l.
7. Retention volumes and times: RDX, 15 ml, 10 min; DNT, 37.5 ml, 25 min; TNT, 42 ml, 28 min; IS, 28.5 ml, 19 min. NOTE: Slight changes in the retention indices may occur with fresh eluent or a change in columns.

A representative HPLC chromatogram for RDX, DNT, and TNT is shown in Figure 1. Also included on the chromatogram are peaks for an internal standard (propiophenone) and 2,4,5-trinitrophenylmethylnitramine (tetryl) (CAS Reg. No. 479-45-8).

c. Laboratory Glassware and Equipment:

1. Culture tubes (Pyrex) with Teflon-lined screw caps.
2. Volumetric flasks (100 ml).
3. Volumetric syringes (0-100 μ l, 0-500 μ l, and 0-1,000 μ l).
4. Automatic pipetter (0-5 ml).
5. Six-speed Waring-type blender with glass container.

6. Teflon-glass, motor-driven tissue homogenizer.
7. Filtering apparatus including filter holders, 5-ml disposable syringes, and 0.45 μ Fluoropore filters.
8. Inert gas (nitrogen) drying train with 12 ports.

d. Chemicals:

1. Toluene and acetonitrile, "Distilled in Glass" grade.
2. Acetic acid and sodium chloride, ACS grade.
3. High purity water from a Milli-Q water purification system.
4. RDX, DNT, and TNT SARMs, obtained from the U.S. Army Toxic and Hazardous Materials Agency.
5. Propiophenone (internal standard), analytical grade.

4. STANDARDS:

a. Stock: Weigh approximately 20 mg of TNT, DNT, RDX and tetryl SARM or interim SARM into separate 100-ml volumetric flasks. Dissolve each compound in acetonitrile and dilute to volume. The concentration of each compound is 200 μ g/ml. Quantitatively pipette 20 ml from each stock above into a 100-ml volumetric flask and dilute to volume with distilled water. Concentration of each compound is 40 μ g/ml.

b. Working: Pipette 10 ml of the 40 μ g/ml of each compound stock into a 100-ml volumetric flask and dilute to volume with high purity water. Concentration of each compound is 4 μ g/ml; this working stock was employed in the precision and accuracy evaluations of reference solutions. Another working stock of 2 μ g/ml was prepared by diluting 5 ml of the 40 μ g/ml each compound to 100 ml; this stock was utilized for adding the RDX, DNT, TNT, and tetryl to the liver samples.

Reference solutions were prepared from this stock as follows:

<u>μl Working Stock</u>	<u>μl IS Stock*</u>	<u>μl 10% Acetonitrile in Water</u>	<u>Concentration Each Compound (ng/ml)</u>
500	500	0	2,000
375	500	125	1,500
250	500	250	1,000
125	500	375	500
25	500	475	100
0	500	500	0

* Preparation of IS stock given in "c" below.

c. Internal Standard Stock: Weigh 10 mg propiophenone into a 100-ml volumetric flask and dilute to volume with acetonitrile (concentration, 100 µg/ml). Quantitatively pipette 10 ml of the 100 µg/ml stock into a 100-ml volumetric flask and dilute to volume with acetonitrile (concentration, 10 µg/ml). A final working solution of 2.0 µg/ml is prepared by pipetting 20 ml of the 10 µg/ml stock into a 100-ml volumetric flask and diluting to volume with acetonitrile.

5. PROCEDURES FOR LIVER SAMPLE DETERMINATION:

a. Liver Sample Preparation: The procedure employed to prepare liver samples for the HPLC-UV determination of RDX, DNT, and TNT consisted of:

1. Place approximately 50 g of liver into a Waring-type blender and blend for 1 min on speed six (liquefy). Note: To completely liquefy the liver sample, the sides of the glass container are scraped with a spatula.

2. Transfer approximately 10 g of the liquefied liver sample to a motor-driven Teflon-glass homogenizer.

3. Homogenize the sample for 30 sec to disrupt the cell walls of the liver sample. Note: The homogenization step is necessary to solubilize the intercellular compounds prior to the extraction step.

4. Repeat steps 3 and 4 on the remaining liquefied liver samples and combine the homogenized samples.

5. Accurately weigh twelve 1.0 g homogenized liver aliquots into culture tubes with Teflon-lined screw caps.

6. Spike two each of the homogenized liver aliquots with the working stock (2 µg/ml each RDX, DNT, and TNT) at the following levels: 1,000 ng (500 µl); 500 ng (250 µl); 200 ng (100 µl); 100 ng (50 µl); and 50 ng (25 µl). The remaining two liver aliquots serve as liver sample blanks. All samples are adjusted to a total volume of 1.5 ml with high purity water containing 10% acetonitrile.

7. Add 1.0 ml of a 10% sodium chloride solution containing 1% acetic acid to each aliquot.

8. Mix thoroughly on a vortex mixer.

9. Extract the liver samples with 4 ml toluene ("Distilled in Glass" grade) by vortexing for at least 2 min. Note: To obtain optimal extraction, the toluene and aqueous liver phase must be thoroughly mixed; if separation of the phases occurs after the 2-min vortexing, mixing has not been complete and additional vortexing is necessary.

10. Centrifuge the extraction mixture at 1,000 rpm for 20 min to break the emulsion. Note: If 20-min centrifugation does not produce two distinct layers, continue centrifuging for an additional 20 min or until two layers form.

11. Transfer the toluene extracts to properly labeled culture tubes with Teflon-lined screw caps.

12. Repeat the toluene extraction (steps 9, 10, and 11) twice more, combining the toluene extracts in the appropriate tubes.

13. Evaporate the toluene at room temperature under a stream of nitrogen. NOTE: Do not heat the samples during the evaporation step, or loss of RDX, DNT, and TNT may occur. Continue evaporation until toluene has been completely removed from the culture tube.

14. Add about 1.0 ml ethyl acetate to each liver sample residue and vortex mix for 30 sec.

15. Evaporate the ethyl acetate at room temperature under a stream of nitrogen. NOTE: The ethyl acetate aids in removing the last traces of toluene from the liver samples.

16. Dissolve the residues in 250 μ l acetonitrile and mix thoroughly by vortexing and ultrasonication. Note: The residue must be completely suspended in the acetonitrile or the analytes will not solubilize. Ultrasonication aids in this process by breaking the residue into smaller particulates.

17. Add 250 μ l of the 2 μ g/ml IS stock (500 ng) to each extracted liver sample and mix thoroughly by vortexing and ultrasonication.

18. Add 500 μ l high-purity water to each extracted liver sample and mix thoroughly. NOTE: Final volume of the prepared samples is 1.0 ml.

19. Filter the solutions through 0.45- μ Fluoropore filters into culture tubes. Note: If the filtrate is not clear, refilter through another 0.45- μ filter. The filtration step is necessary to remove particulate material from the sample and thus prolong the usefulness of the analytical column.

20. Analyze a 50- to 100- μ l aliquot of each prepared liver sample by HPLC.

21. After the elution of the TNT peak, wash the column for 3 min with 100% acetonitrile at 1.5 ml/min to remove any late eluting compounds. NOTE: The acetonitrile wash step is required to prevent possible interference in the chromatographic analysis of the next sample.

22. After the 3-min wash, switch the system back to the eluent. Allow approximately 7 min for equilibration prior to the next injection.

b. Calibration: The reference solutions described in Section 4.b were prepared and analyzed in quadruplicate. The relative weight response (RWR) (Eq. 1) of each compound was calculated and the average RWR utilized to determine the concentration (ng/ml) of each compound in every reference solution (Eq. 2). The concentrations found were plotted against the concentrations added, and a linear regression evaluation of the data was conducted. The slope, intercept, and correlation coefficient for each compound were determined. The data are summarized in Table 1, which includes the average value at each level for each compound, the standard deviation, coefficient of variation (relative standard deviation), and the percent inaccuracy. The raw data and calculations are given in Table 8 of the Appendix.

$$RWR = \frac{\text{Peak Height Cpd}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml Cpd}} \quad (\text{Eq. 1})$$

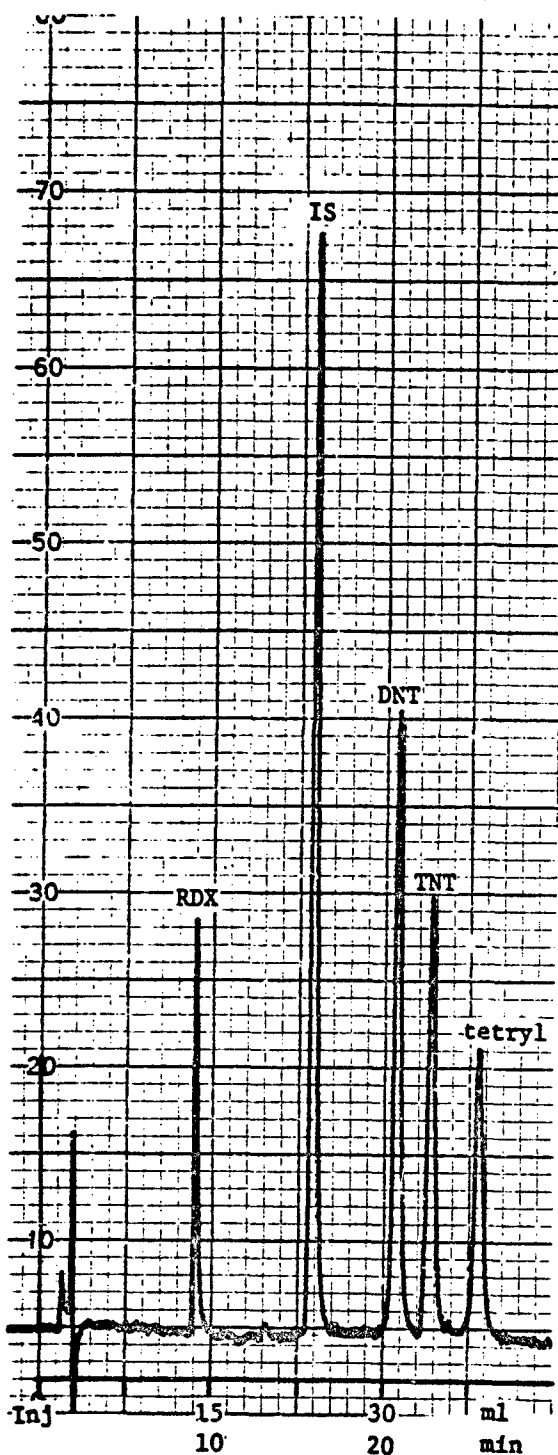
$$\text{ng/ml or ng/g compound} = \frac{\text{Peak Height Cpd}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{Avg. RWR}} \quad (\text{Eq. 2})$$

c. Liver Sample Analysis: The liver samples prepared as outlined in Section 5.a were injected onto the HPLC system. The peak height of each compound was measured and recorded. Liver samples were prepared and analyzed on four separate days.

6. CALCULATION: The concentration (nanograms per gram) of each compound in the liver samples was determined using the relative weight response to an internal standard method. The RWR values (Eq. 1) for the reference solutions analyzed with a day's set of liver samples were calculated, and the average values for RDX, DNT, and TNT were determined. These RWR values were employed to calculate the liver sample level of each compound by Equation 2, where nanograms per gram represents the concentration found in the liver sample. The results for the duplicate determinations of RDX, DNT, and TNT in liver samples at five different levels on four succeeding days are summarized in Tables 2, 3, and 4. The average concentration found, the standard deviation, coefficient of variation, and percent inaccuracy are also included. A linear regression evaluation of the results was made; the slope, intercept, and correlation coefficient are given in the tables. The concentration of each compound found in the liver samples was plotted against the amount added, and these data are shown in Figures 2, 3, and 4. A graphic presentation of the standard deviation, coefficient of variation, and percent inaccuracy for the determination of RDX, DNT, and TNT in the liver samples is given in Figures 5, 6, and 7, respectively. Representative HPLC chromatograms are shown for a liver sample blank (Figure 8), a 100 ng/g liver sample (Figure 9), and a 500 ng/g liver sample (Figure 10). The raw data and calculations for the liver sample determinations are given in Tables 9 to 12 in the Appendix.

7. STATISTICAL EVALUATION OF DATA: A statistical evaluation of the data obtained for the determination of RDX, DNT, and TNT in liver samples (Tables 2, 3, and 4) by the Hubaux and Vos detection limit program was made at the U.S. Army Toxic and Hazardous Materials Agency. The results of these

evaluations are given in Table 5 for RDX, Table 6 for DNT, and Table 7 for TNT. The detection limits, as determined by the program, were 58 ng/g for RDX when the 1,000- and 500-ng/g data points were omitted, 50 ng/g for DNT using all the data points, and 50 ng/g for TNT when the 1,000-ng/g data points were omitted. The average nanograms per gram value found at each fortification level was determined from the linear regression equation for the 48 data points and the amount of analyte added at that level. The standard deviation and percent imprecision (coefficient of variation) at each level were calculated based on this average (nanograms per gram) value found. Thus, these values and the values given in Tables 2, 3, and 4 (based on the average of the eight assays at each level) are not comparable. The percent inaccuracy term was calculated from the average of the eight data points at each level, and thus agrees with the values in Tables 2, 3, and 4.



HPLC Conditions

Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 30% acetonitrile in
1% acetic in water
Flow Rate: 1.5 ml/min
Chart Speed: 0.1 in./min
Detector: UV, 254 nm

Sample Characteristics

Concentrations: RDX, DNT, TNT,
and tetryl - 500 ng/ml;
IS - 1,000 ng/ml
Injection volume: 70 μ l
Attenuation: 0.01 X

Retention Indices

<u>Compound</u>	<u>Retention Volume (ml)</u>	<u>Retention Time (min)</u>
RDX	13.5	9
IS	22.5	15
DNT	31.5	21
TNT	34.5	23
tetryl	39	26

Figure 1 - HPLC Separation of RDX, DNT, TNT, and Tetryl
SARMS and Propiophenone (IS)

TABLE 1

LINEARITY AND PRECISION OF THE HPLC-UV DETERMINATION OF
SARM REFERENCE SOLUTIONS OF RDX, DNT, AND TNT

Compound	ng/ml Added	ng/ml Detected				Average ^a	Standard ^b Deviation	Coefficient ^c of Variation	Percent ^d Inaccuracy
		A	B	C	D				
RDX	0	ND ^e	ND	ND	ND	-	-	-	-
	100	109	114	105	103	108	± 4.9	4.5	+ 8.0
	500	517	509	499	490	504	± 11.8	2.3	+ 0.8
	1,000	960	950	923	985	955	± 26	2.7	- 4.5
	1,500	1,551	1,547	1,435	1,444	1,494	± 63	4.2	- 0.4
	2,000	2,031	2,069	1,906	1,924	1,983	± 80	4.0	- 0.8
DNT	0	ND	ND	ND	ND	-	-	-	-
	100	113	109	109	100	108	± 5.5	5.1	+ 8.0
	500	499	490	475	483	487	± 10.2	2.1	- 2.6
	1,000	992	1,011	981	1,032	1,004	± 22	2.2	+ 0.4
	1,500	1,500	1,470	1,461	1,439	1,468	± 25	1.7	- 2.1
	2,000	1,968	1,982	1,934	1,901	1,946	± 36	1.9	- 2.7
TNT	0	ND	ND	ND	ND	-	-	-	-
	100	107	112	112	100	108	± 5.7	5.3	+ 8.0
	500	495	479	484	484	486	± 6.8	1.4	- 2.8
	1,000	956	989	967	997	977	± 19	2.0	- 2.3
	1,500	1,498	1,508	1,471	1,432	1,478	± 34	2.3	- 1.5
	2,000	2,011	2,015	1,957	1,879	1,966	± 63	3.2	- 1.7

Linear Regression

RDX: $y = 0.988x + 0.6$

Correlation coefficient - 0.998

DNT: $y = 0.974x + 7.7$

Correlation coefficient - 0.999

TNT: $y = 0.982x + 1.2$

Correlation coefficient - 0.959

$$a \text{ Average} = \sum x / n = \bar{x}$$

$$b \text{ Standard deviation} = \left(\frac{n \sum x^2 - (\sum x)^2}{n(n-1)} \right)^{1/2} = \sigma$$

$$c \text{ Coefficient of variation} = \sigma / \bar{x} \times 100$$

$$d \text{ Percent inaccuracy} = \frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$$

e ND = Not detectable, less than 20 ng/ml

TABLE 2
HPLC-UV DETERMINATION OF RDX IN LIVER SAMPLES

Amount Added (ng/g)	Level Found (ng/g)								Average ^a	Standard ^b Deviation	Coefficient ^c of Variation	Percent ^d Inaccuracy
	Day 1		Day 2		Day 3		Day 4					
	A	B	A	B	A	B	A	B				
0	115	122	124	121	75	104	132	108	113	± 18	16	-
50	133	158	148	181	109	136	203	186	157	± 31	20	+ 214
100	147	214	198	232	172	150	227	273	202	± 44	22	+ 102
200	277	304	277	344	234	233	259	348	285	± 45	16	+ 43
500	520	569	565	617	496	367	564	601	537	± 79	15	+ 7
1,000	906	1,042	1,073	1,102	1,012	936	1,029	1,072	1,022	± 69	7	+ 2

Note: Linear regression: $y = 0.904x + 107$
Correlation coefficient: 0.988

a Average = $\Sigma x/n = \bar{x}$

b Standard deviation = $\left(\frac{n \Sigma x^2 - (\Sigma x)^2}{n(n-1)} \right)^{1/2} = \sigma$

c Coefficient of variation = $\sigma/\bar{x} \times 100$

d Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$

e Not detectable, less than 20 ng/g.

TABLE 3
HPLC-UV DETERMINATION OF DNT IN LIVER SAMPLES

Amount Added (ng/g)	Level Found (ng/g)								Average ^a	Standard Deviation ^b	Coefficient ^c of Variation	Percent ^d Inaccuracy
	Day 1		Day 2		Day 3		Day 4					
	A	B	A	B	A	B	A	B				
0	ND ^e	ND	ND	ND	ND	ND	ND	ND	-	-	-	-
50	29	27	39	30	37	38	39	41	35	± 5	16	- 30
100	54	54	56	59	68	66	73	78	64	± 9	14	- 36
200	105	125	114	100	121	134	138	122	120	± 13	11	- 40
500	308	302	288	277	333	322	325	342	312	± 22	7	- 38
1,000	592	685	590	571	707	696	629	661	641	± 53	8	- 36

Note: Linear regression: $y = 0.640x - 1.9$
Correlation coefficient: 0.995

a Average = $\sum x/n = \bar{x}$

b Standard deviation = $\left(\frac{n\sum x^2 - (\sum x)^2}{n(n-1)} \right)^{1/2} = \sigma$

c Coefficient of variation = $\sigma/\bar{x} \times 100$

d Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$

e Not detectable, less than 20 ng/g.

TABLE 4

HPLC-UV DETERMINATION OF TNT IN LIVER SAMPLES

Amount Added (ng/g)	Level Found (ng/g)								Average ^a	Standard ^b Deviation	Coefficient ^c of Variation	Percent ^d Inaccuracy
	Day 1		Day 2		Day 3		Day 4					
	A	B	A	B	A	B	A	B				
0	ND ^e	ND	ND	ND	ND	ND	ND	ND	-	-	-	-
50	21	20	31	22	25	26	30	35	26	± 5	21	- 48
100	37	39	47	48	40	47	55	62	47	± 8	18	- 53
200	77	95	83	75	85	99	113	92	90	± 13	14	- 55
500	241	227	219	199	254	235	273	281	241	± 27	11	- 52
1,000	504	568	499	487	624	489	571	442	523	± 59	11	- 48

Note: Linear regression: $y = 0.521x - 6.2$
Correlation coefficient: 0.989

a Average = $\sum x/n = \bar{x}$

b Standard deviation = $\left(\frac{n\sum x^2 - (\sum x)^2}{n(n-1)} \right)^{1/2} = \sigma$

c Coefficient of variation = $\sigma/\bar{x} \times 100$

d Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$

e Not detectable, less than 20 ng/g.

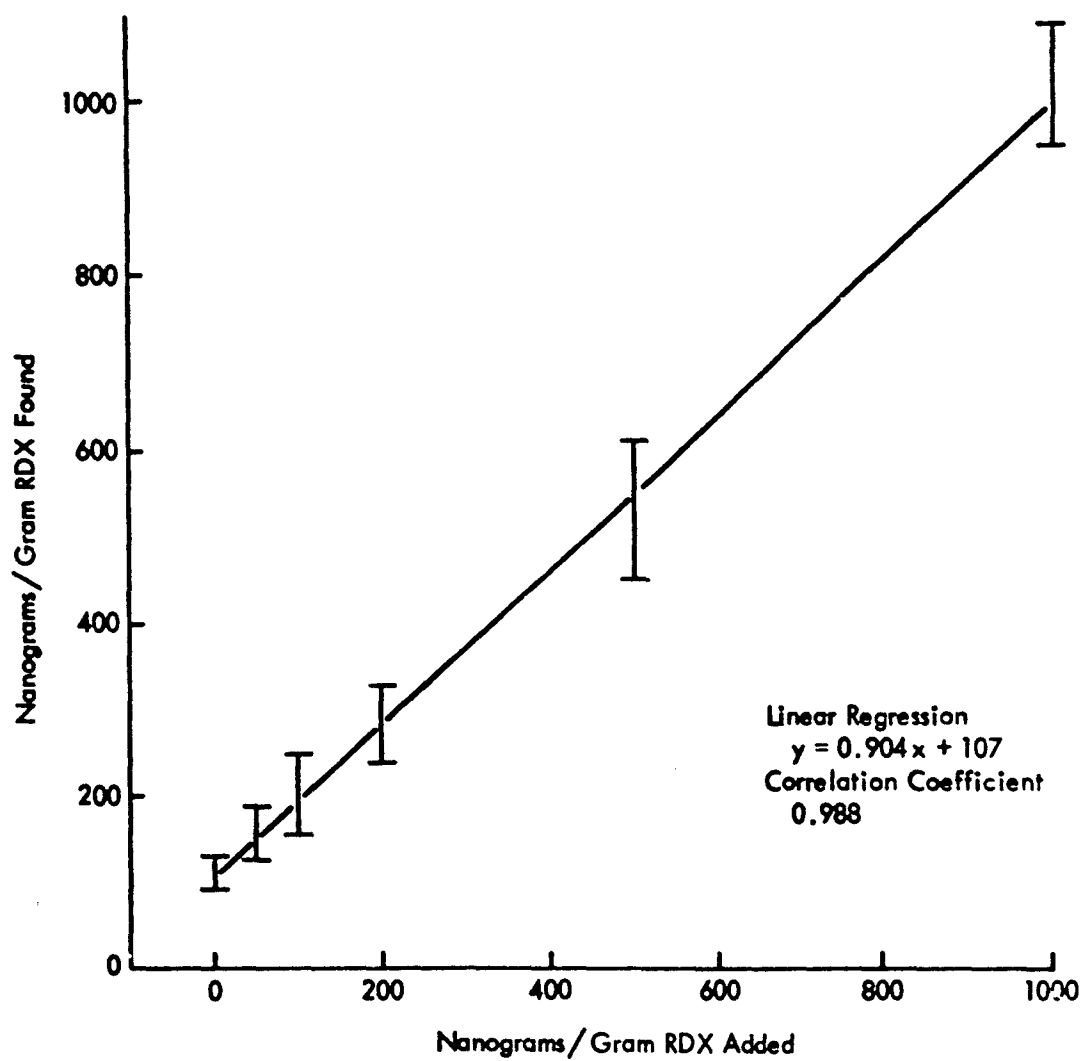


Figure 2 - Determination of RDX in Animal Liver Samples

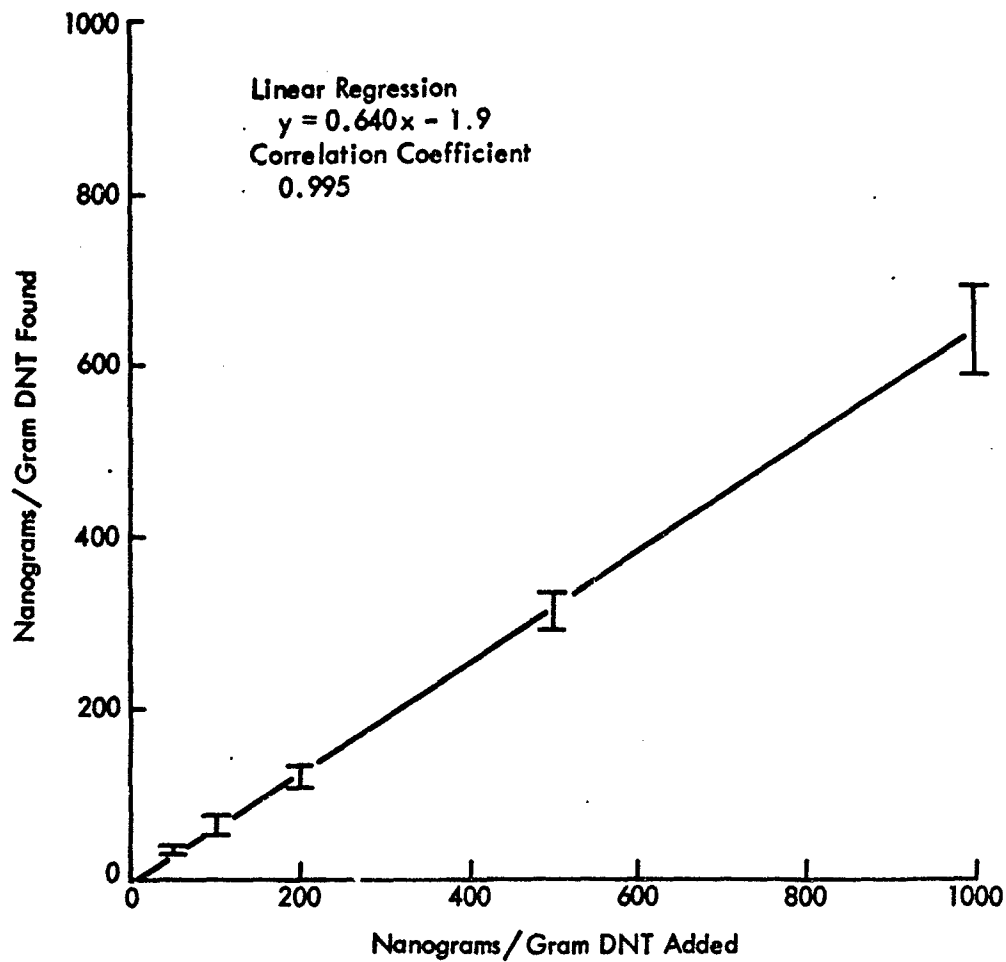


Figure 3 - Determination of DNT in Animal Liver Samples

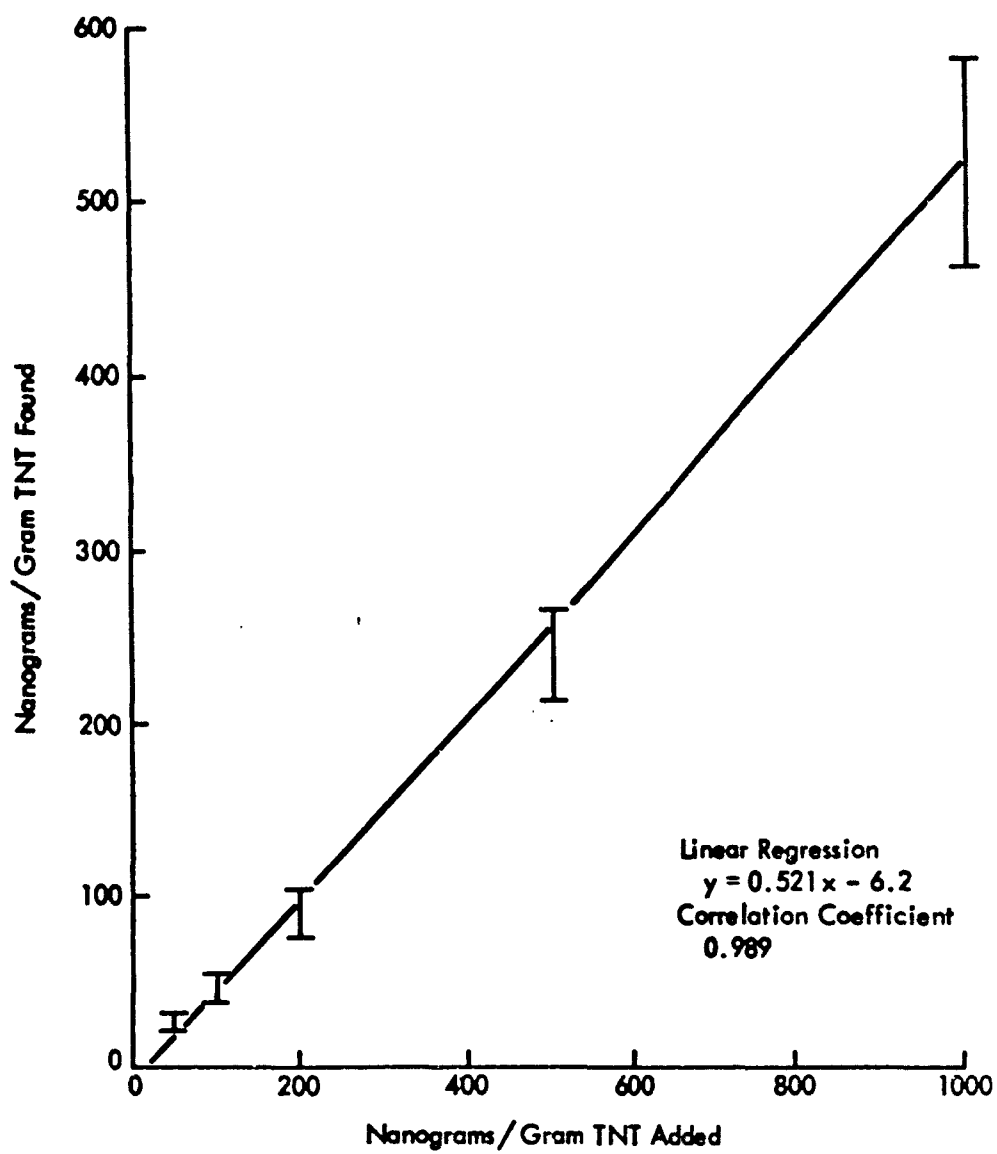


Figure 4 - Determination of TNT in Animal Liver Samples

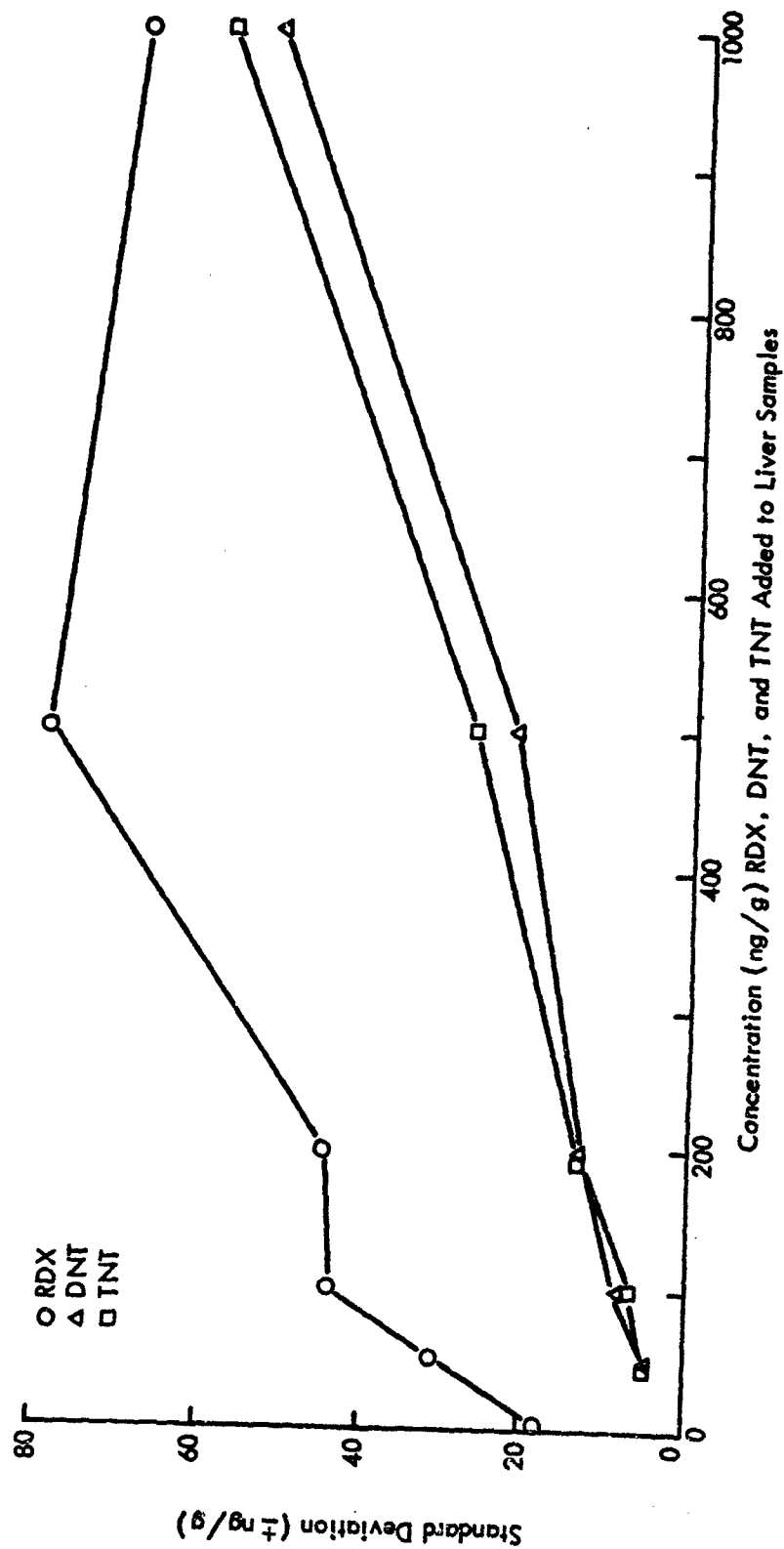


Figure 5 - Standard Deviation for RDX, DNT, and TNT in Animal Liver Samples

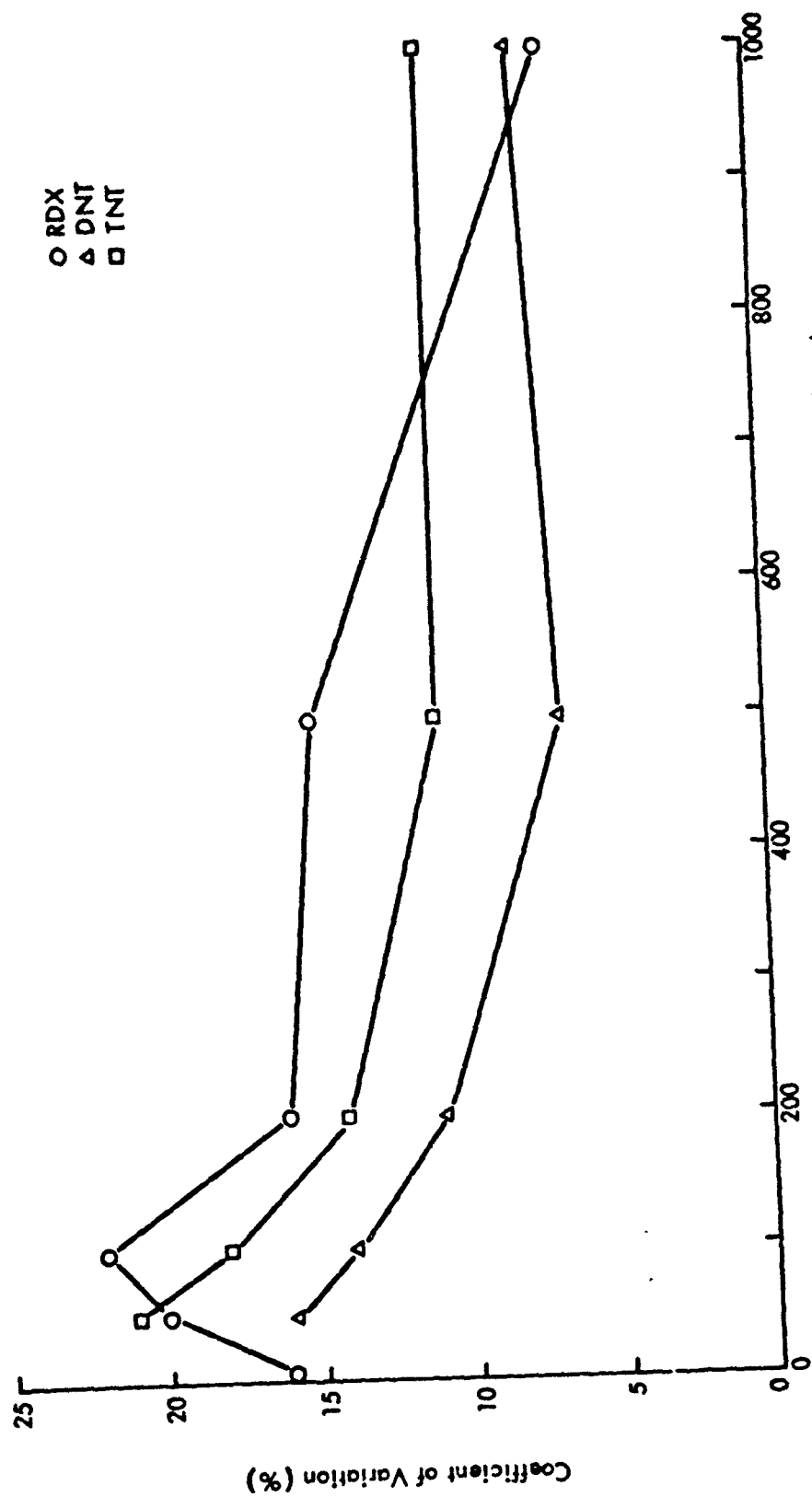


Figure 6 - Coefficient of Variation for RDX, DNT, and TNT in Animal Liver Samples

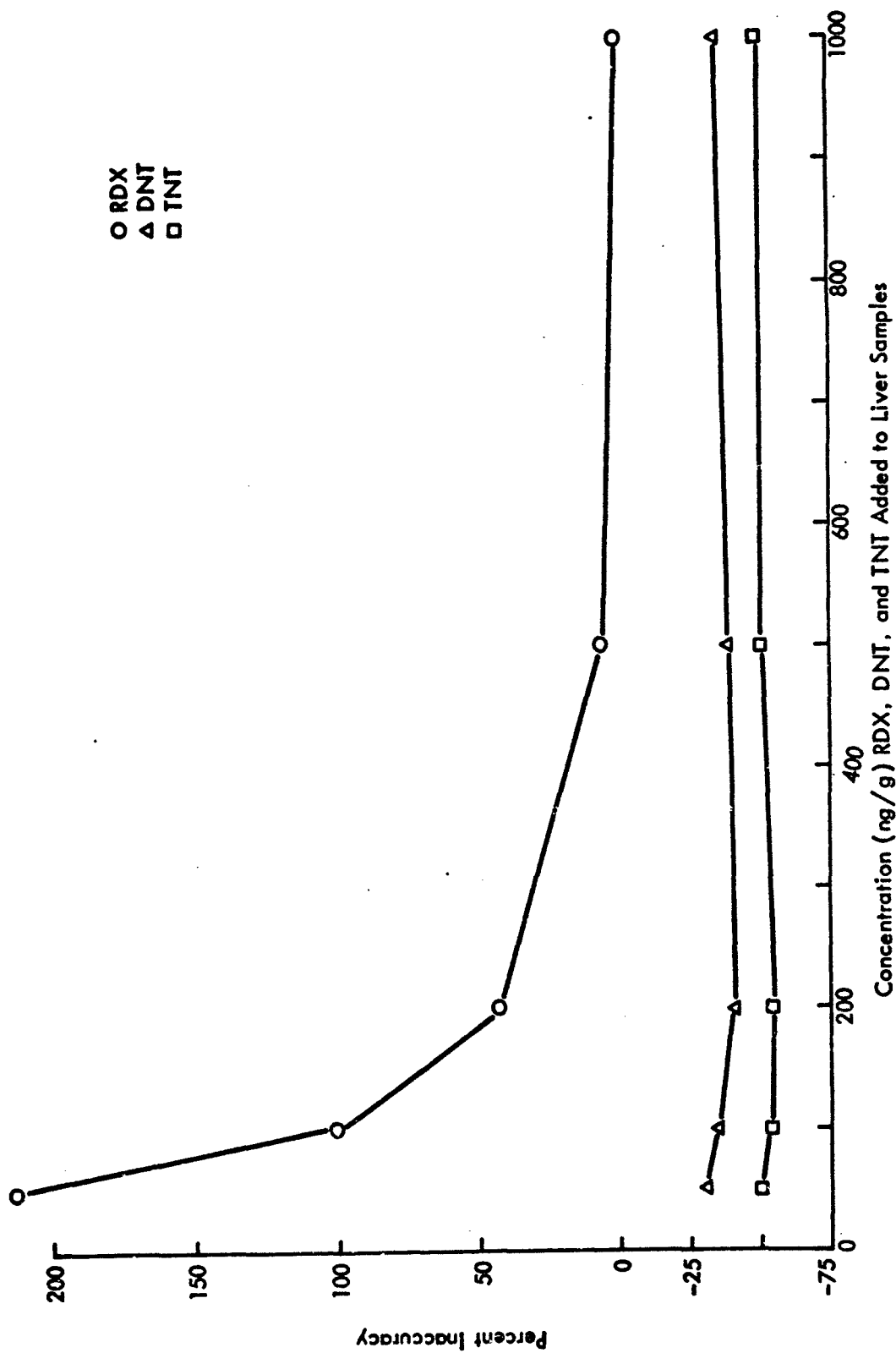
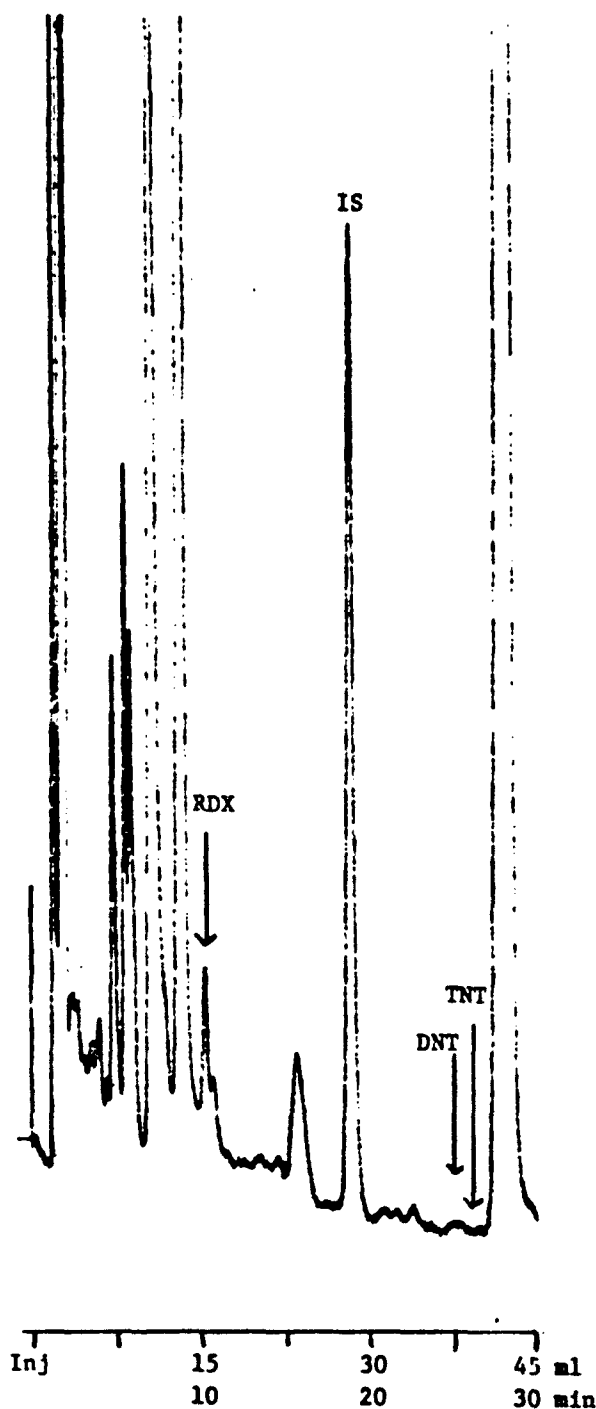


Figure 7 - Percent Inaccuracy for RDX, DNT, and TNT in Animal Liver Samples



HPLC Conditions:

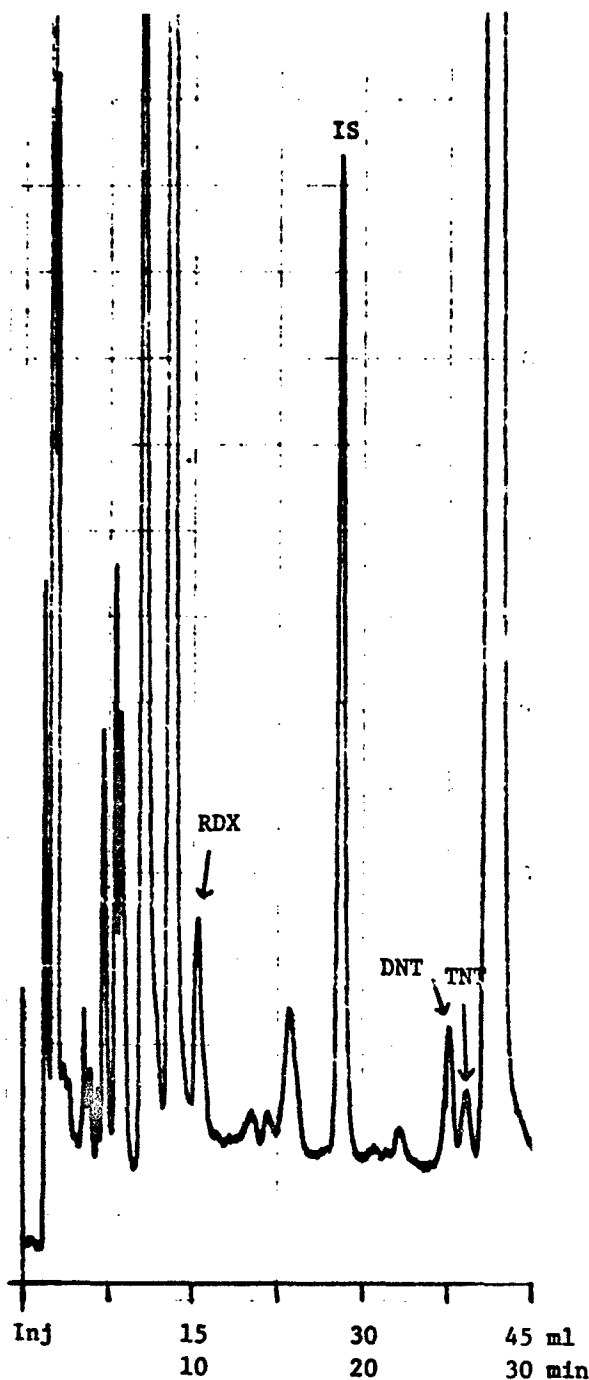
Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 28% acetonitrile in
1% acetic acid
Flow Rate: 1.5 ml/min
Chart Speed: 0.1 in/min
Detector: UV, 254 nm

Sample Characteristics:

1.0 g liver extracted 3 x 4 ml
with toluene. Extract evaporated to
dryness and residue reconstituted
with 1.0 ml HPLC eluent.

IS Concentration: 500 ng/ml
Injection Volume: 70 μ l
Attenuation: 0.005 X

Figure 8 - HPLC Analysis of Blank Liver Samples for RDX, DNT, and TNT
Method Development. "X" indicates a toluene contaminant.
Arrows indicate the elution position of RDX, DNT, and TNT.



HPLC Conditions:

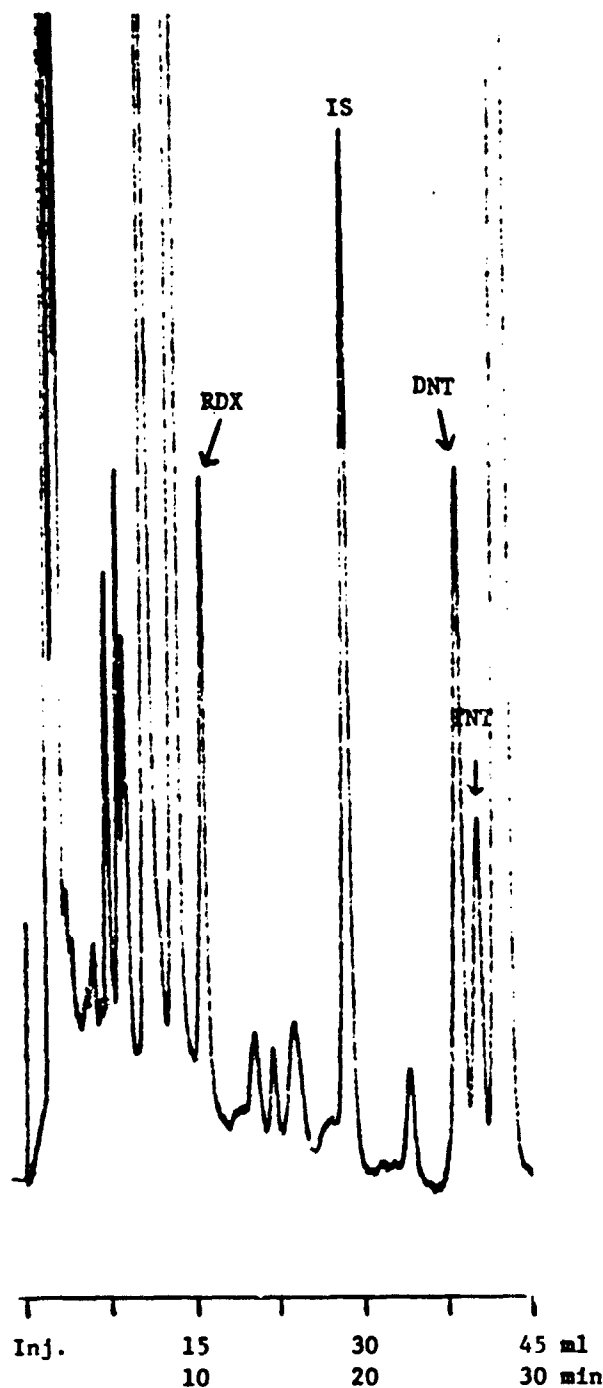
Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 28% acetonitrile in
1% acetic acid
Flow Rate: 1.5 ml/min
Chart Speed: 0.1 in/min
Detector: UV, 254 nm

Sample Characteristics:

1.0 g liver containing 100 ng/g
RDX, DNT, and TNT extracted 3 x 4 ml
with toluene. Extract evaporated to
dryness and residue reconstituted in
1.0 ml HPLC eluent.

IS Concentration: 500 ng/ml
Injection Volume: 70 μ l
Attenuation: 0.005 X

Figure 9 - HPLC Analysis of Animal Liver Sample Containing 100 ng/g RDX, DNT, and TNT. "X" indicates toluene contaminants. Arrows show HPLC peaks for RDX (and co-eluting compound), DNT, and TNT.



HPLC Conditions:

Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 28% acetonitrile in
1% acetic acid
Flow Rate: 1.5 ml/min
Chart Speed: 0.1 in/min
Detector: UV, 254 nm

Sample Characteristics:

1.0 g liver containing 500 ng/g
RDX, DNT, and TNT extracted 3 x 4 ml
with toluene. Extract evaporated to
dryness and residue reconstituted
with 1.0 ml HPLC eluent.

IS Concentration: 500 ng/ml
Injection Volume: 70 μ l
Attenuation: 0.005 X

Figure 10 - HPLC Analysis of Animal Liver Sample Containing 500 ng/g RDX, DNT, and TNT. "X" indicates toluene contaminant. Arrows show HPLC peaks for RDX (and co-eluting compound), DNT, and TNT.

TABLE 5

STATISTICAL EVALUATION OF RDX IN LIVER SAMPLE DATA BY
THE HUBAUX AND VOS DETECTION LIMIT PROGRAM

Number ^a of Data Points	Linear Regression	Correlation Coefficient	Degrees of Freedom	b _t	y ^c Intercept	Detection Limit
48	y = 0.904x + 107	0.988	46	1.679	141	75
40	y = 0.847x + 115	0.958	38	1.686	147	74
32	y = 0.859x + 114	0.883	30	1.697	140	58

ng/g RDX Added	Average ^e ng/g Found	Standard ^f Deviation	Percent ^g Imprecision	Percent ^h Inaccuracy
0	107	± 6.7	5.9	-
50	152	± 12	7.6	+ 214
100	197	± 17	8.2	+ 102
200	288	± 17	5.9	+ 42
500	559	± 30	5.6	+ 7
1,000	1,011	± 26	2.5	+ 2

^a Number of data points - data points used to calculate linear regression and detection limits; the blank samples analyzed included in these calculations; 48 all data; 40 - 1,000 ng/g samples omitted; 32-1,000 ng/g and 500 ng/g samples omitted.

^b t - 2 tail p level (usually 0.1, each confidence band is 0.05 so total p = 0.1).

^c y intercept - intercept on y-axis of upper confidence and lower confidence limit line.

^d Detection limit - x-intercept of y-intercept and lower confidence limit line.

^e Average ng/ml found - average at each level determined from linear regression equation for 48 points.

^f Standard deviation - determined from average value (e above) and observed values.

^g Percent imprecision - standard deviation divided by average value times 100%.

^h Percent inaccuracy - determined from the average values of the eight observed values at each level

$$\% \text{ Inaccuracy} = \frac{\text{Average observed values} - \text{level added}}{\text{level added}} \times 100$$

TABLE 6

STATISTICAL EVALUATION OF DNT IN LIVER SAMPLE DATA BY
THE HUBAUX AND VOS DETECTION LIMIT PROGRAM

Number ^a of Data Points	Linear Regression	Correlation Coefficient	Degrees of Freedom	t_b	y^c Intercept	Detection Limit
48	$y = 0.640x - 1.9$	0.995	46	1.679	14	50
ng/g DNT Added	Average ^e ng/g Found	Standard ^f Deviation	Percent ^g Imprecision	Percent ^h Inaccuracy		
50	30	± 2.0	5.9	- 30		
100	62	± 3.5	5.4	- 37		
200	126	± 5.0	4.2	- 40		
500	318	± 8.5	2.7	- 38		
1,000	638	± 20	3.1	- 36		

^a Number of data points - data points used to calculate linear regression and detection limits;
the blank samples analyzed included in these calculations.

^b t - 2 tail p level (usually 0.1, each confidence band is 0.05 so total $p = 0.1$).

^c y intercept - intercept on y -axis of upper confidence limit line.

^d Detection limit - x -intercept of y -intercept and lower confidence limit line.

^e Average ng/ml found - average at each level determined from linear regression equation for 48 points.

^f Standard deviation - determined from average value (e above) and observed values.

^g Percent imprecision - standard deviation divided by average value times 100%.

^h Percent inaccuracy - determined from the average values of the eight observed values at each level

$$\% \text{ Inaccuracy} = \frac{\text{Average observed values} - \text{level added}}{\text{level added}} \times 100$$

TABLE 7

STATISTICAL EVALUATION OF TNT LIVER SAMPLE DATA BY
THE HUBAUX AND VOS DETECTION LIMIT PROGRAM

Number ^a of Data Points	Linear Regression	Correlation Coefficient	Degrees of Freedom	t ^b	y ^c Intercept	Detection Limit
48	y = 0.521x - 6.2	0.989	46	1.679	12	70
40	y = 0.480x - 0.8	0.987	38	1.686	9	40

ng/g TNT Added	Average ^e ng/g Found	Standard ^f Deviation	Percent ^g Imprecision	Percent ^h Inaccuracy
50	20	± 2.0	7.7	- 48
100	46	± 3.2	6.8	- 53
200	98	± 4.8	5.3	- 55
500	254	± 10	4.3	- 52
1,000	515	± 22	4.3	- 48

a Number of data points - data points used to calculate linear regression and detection limits; the blank samples analyzed included in these calculations; 48 all data; 40 - 1,000 ng/g samples omitted.

b t - 2 tail p level (usually 0.1, each confidence band is 0.05 so total p = 0.1).

c y intercept - intercept on y-axis of upper confidence limit line.

d Detection limit - x-intercept of y-intercept and lower confidence limit line.

e Average ng/ml found - average at each level determined from linear regression equation for 48 points.

f Standard deviation - determined from average value (e above) and observed values.

g Percent imprecision - standard deviation divided by average value times 100%.

h Percent inaccuracy - determined from the average values of the eight observed values at each level

$$\% \text{ Inaccuracy} = \frac{\text{Average observed values} - \text{level added}}{\text{level added}} \times 100$$

APPENDIX

**METHOD DEVELOPMENT FOR THE DETERMINATION
OF RDX, DNT, AND TNT IN LIVER SAMPLES**

RAW DATA AND CALCULATIONS

TABLE 8

LINEARITY AND PRECISION OF RDX, DNT, AND TNT DETERMINATION
BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Reference Solution Number	ng/ml Compound Added	Peak Height (mm)			Internal Standard		Relative Weight Response			Calculated ng/ml	
		RDX	DNT	TNT	ng ml	Peak Height	RDX	DNT	TNT	RDX	DNT
A-1	0	< 2	< 2	< 2	1,000	122	-	-	-	ND	ND
A-2	100	12	14	11	1,000	116	1.03	1.21	0.95	109	113
A-3	500	58	63	52	1,000	118	0.98	1.07	0.88	517	499
A-4	1,000	104	121	97	1,000	114	0.91	1.06	0.85	960	992
A-5	1,500	168	183	152	1,000	114	0.98	1.07	0.89	1,551	1,500
A-6	2,000	220	240	204	1,000	114	0.96	1.05	0.89	2,031	1,968
B-1	0	< 2	< 2	< 2	1,000	118	-	-	-	ND	ND
B-2	100	13	14	12	1,000	120	1.08	1.17	1.00	114	109
B-3	500	59	64	52	1,000	122	0.97	1.05	0.85	509	490
B-4	1,000	121	145	118	1,000	134	0.90	1.08	0.88	950	1,011
B-5	1,500	172	184	157	1,000	117	0.98	1.05	0.89	1,547	1,470
B-6	2,000	228	246	208	1,000	116	0.98	1.06	0.90	2,069	1,982
C-1	0	< 2	< 2	< 2	1,000	120	-	-	-	ND	ND
C-2	100	12	14	12	1,000	120	1.00	1.17	1.00	105	109
C-3	500	55	59	50	1,000	116	0.95	1.02	0.86	499	475
C-4	1,000	107	128	105	1,000	122	0.88	1.05	0.86	923	981
C-5	1,500	150	172	144	1,000	110	0.91	1.04	0.87	1,435	1,461
C-6	2,000	210	240	202	1,000	116	0.91	1.03	0.87	1,900	1,934
D-1	0	< 2	< 2	< 2	1,000	119	-	-	-	ND	ND
D-2	100	11	12	10	1,000	112	0.98	1.07	0.89	103	100
D-3	500	54	60	50	1,000	116	0.93	1.03	0.86	490	483
D-4	1,000	116	137	110	1,000	124	0.94	1.10	0.89	985	1,032
D-5	1,500	155	174	144	1,000	113	0.91	1.03	0.85	1,444	1,439
D-6	2,000	212	236	194	1,000	116	0.91	1.02	0.84	1,924	1,901
										1,879	

TABLE 8 (concluded)

Relative Weight Response

	<u>Average</u>	<u>Standard Deviation</u>	<u>Relative Standard Deviation</u>
RDX	0.95	± 0.05	5.2%
DNT	1.07	± 0.05	4.9%
TNT	0.89	± 0.04	5.1%

TABLE 9
DETERMINATION OF RDX, DNT, AND TNT IN LIVER SAMPLES

DAY 1										
Sample Number	ng/g ^a Compound Added	g Liver	Peak Height (mm)			Internal Standard ^b		ng/g ^c Detected		
			RDX	DNT	TNT	ng/ml	Peak Height	RDX	DNT	TNT
Day 1A-0	0	1.0	27.0	< 4	< 4	500	147.6	115	ND ^d	ND
Day 1A-50	50	1.0	31.0	9.2	5.0	500	146.8	133	29	21
Day 1A-100	100	1.0	35.2	17.2	9.0	500	151.0	147	54	37
Day 1A-200	200	1.0	65.2	33.2	18.2	500	148.4	277	105	77
Day 1A-500	500	1.0	121.0	96.0	56.2	500	146.6	520	308	241
Day 1A-1000	1,000	1.0	226.0	198.0	126.0	500	157.2	906	592	504
Day 1B-0	0	1.0	27.0	< 4	< 4	500	140.0	122	ND	ND
Day 1B-50	50	1.0	36.0	8.2	4.6	500	143.4	158	27	20
Day 1B-100	100	1.0	50.0	17.0	9.2	500	147.0	214	54	139
Day 1B-200	200	1.0	69.0	38.0	21.6	500	143.0	304	125	95
Day 1B-500	500	1.0	132.4	94.2	53.0	500	146.6	569	302	227
Day 1B-1000	1,000	1.0	238.0	210.0	130.0	500	144.0	1,042	685	568

TABLE 9 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)			Internal Standard ^b Peak Height		Relative Weight ^c Response	
		RDX	DNT	TNT	ng/ml	Height	RDX	TNT
Std-Day 1-5	1,000	224.0	300.0	225.2	500	144.0	0.778	1.042
Std-Day 1-1	50	13.0	17.0	13.0	500	159.0	0.818	1.069
Std-Day 1-5	1,000	227.0	312.8	230.0	500	144.0	0.788	1.086
Std-Day 1-4	500	119.6	160.4	118.0	500	151.4	0.790	1.059
Average							0.793	1.064
Standard Deviation							± 0.017	± 0.019

^a ng/g compound added - nanograms of RDX, DNT, and TNT added per gram of liver sample.

^b Internal standard - compound (propionophenone) added to liver sample after sample preparation for calculation of data.

^c ng/g detected - nanograms of RDX, DNT, and TNT detected in the liver sample.

$$\text{ng compound/g} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}}$$

^d ND - not detectable, less than 20 ng/g.

$$\text{e Relative Weight Response - RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

TABLE 10
DETERMINATION OF RDX, DNT, AND TNT IN LIVER SAMPLES

DAY 2									
Sample Number	ng/g ^a Compound Added	g Liver	Peak Height (mm)			Internal Standard ^b		ng/g ^c Detected	
			RDX	DNT	TNT	ng/ml	Peak Height	RDX	DNT
Day 2A-0	0	1.0	28.8	< 4	< 4	500	145.0	124	ND ^d
Day 2A-50	50	1.0	34.0	12.0	7.0	500	144.0	148	31
Day 2A-100	100	1.0	45.6	17.4	10.6	500	144.0	198	47
Day 2A-200	200	1.0	65.0	36.0	19.0	500	147.0	277	83
Day 2A-500	500	1.0	130.0	89.0	49.4	500	144.2	565	219
Day 2A-1000	1,000	1.0	250.0	184.8	114.0	500	146.0	1,073	499
Day 2B-0	0	1.0	27.0	< 4	< 4	500	139.6	121	ND
Day 2B-50	50	1.0	41.2	9.2	5.0	500	142.5	181	22
Day 2B-100	100	1.0	53.0	18.0	10.8	500	143.0	232	48
Day 2B-200	200	1.0	79.0	31.0	17.0	500	144.0	344	75
Day 2B-500	500	1.0	142.0	85.5	45.0	500	144.2	617	199
Day 2B-1000	1,000	1.0	256.0	178.4	111.0	500	145.6	1,102	487

TABLE 10 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)			Internal Standard ^b		Relative Weight ^c Response	
		RDX	DNT	TNT	ng/ml	Peak Height	RDX	TNT
Std-Day 2-5	1,000	228.0	306.0	222.0	500	144.0	0.792	1.063
Std-Day 2-4	500	121.0	162.2	119.6	500	154.0	0.876	1.053
Std-Day 2-3	200	46.0	60.4	44.4	500	144.2	0.798	1.047
Std-Day 2-5	50	13.0	18.0	13.0	500	158.0	0.822	1.138
Std-Day 2-4	500	124.0	165.8	121.5	500	158.2	0.794	1.062
Average							0.798	1.072
Standard Deviation							± 0.014	± 0.037
								± 0.022

^a ng/g compound added - nanograms of RDX, DNT, and TNT added per gram of liver sample.

^b Internal standard - compound (propionophenone) added to liver sample after sample preparation for calculation of data.

^c ng/g detected - nanograms of RDX, DNT, and TNT detected in the liver sample.

$$\text{ng compound/g} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}}$$

^d ND - not detectable, less than 20 ng/g.

$$\text{Relative Weight Response - RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

TABLE 11
DETERMINATION OF RDX, DNT, AND TNT IN LIVER SAMPLES

Sample Number	ng/g ^a Compound Added	g Liver	DAY 3			Internal Standard ^b			ng/g ^c Detected	
			Peak Height (mm)			ng/ml	Peak Height	TNT	RDX	DNT
			RDX	DNT	TNT					
Day 3A-0	0	1.0	17.0	< 4	< 4	500	144.4		75	ND ^d
Day 3A-50	50	1.0	25.0	11.6	5.6	500	145.0		109	37
Day 3A-100	100	1.0	39.0	21.2	9.0	500	144.0		172	68
Day 3A-200	200	1.0	53.0	37.6	19.0	500	143.4		234	121
Day 3A-500	500	1.0	112.0	103.0	57.0	500	143.0		496	333
Day 3A-1000	1,000	1.0	230.0	220.4	141.0	500	144.0		1,012	707
Day 3B-0	0	1.0	24.0	< 4	< 4	500	145.6		104	ND
Day 3B-50	50	1.0	31.2	11.8	6.0	500	145.2		136	38
Day 3B-100	100	1.0	35.0	20.8	11.0	500	147.8		150	66
Day 3B-200	200	1.0	52.0	41.0	22.0	500	141.6		233	134
Day 3B-500	500	1.0	88.0	106.0	56.0	500	152.0		367	322
Day 3B-1000	1,000	1.0	208.0	212.0	108.0	500	140.8		936	696

TABLE 11 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)			Internal Standard ^b		Relative Weight ^c Response	
		RDX	DNT	TNT	ng/ml	Peak Height	RDX	DNT
Std-Day 3-5	1,000	223.0	305.6	219.0	500	144.4	0.772	1.058
Std-Day 3-3	200	45.0	64.0	46.4	500	147.0	0.765	1.088
Std-Day 3-4	500	113.0	154.2	113.0	500	142.2	0.795	1.084
Std-Day 3-4	500	120.0	160.0	116.0	500	145.8	0.823	1.097
Average							0.789	1.082
Standard Deviation ±							0.026	± 0.017
								± 0.018

^a ng/g compound added - nanograms of RDX, DNT, and TNT added per gram of liver sample.

^b Internal standard - compound (propionophenone) added to liver sample after sample preparation for calculation of data.

^c ng/g detected - nanograms of RDX, DNT, and TNT detected in the liver sample.

$$\text{ng compound/g} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}}$$

^d ND - not detectable, less than 20 ng/g.

$$\text{Relative Weight Response - RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

TABLE 12

DETERMINATION OF RDX, DNT, AND TNT IN LIVER SAMPLES

Sample Number	ng/g ^a Compound Added	g Liver	Peak Height			Internal Standard ^b		ng/g ^c Detected		
			(mm)			ng/ml	Peak Height	RDX	DNT	TNT
			RDX	DNT	TNT					
Day 4A-0	0	1.0	30.0	< 4	< 4	500	142.6	132	ND ^d	ND
Day 4A-50	50	1.0	47.2	12.6	7.0	500	145.6	203	39	30
Day 4A-100	100	1.0	53.6	23.6	13.0	500	148.0	227	73	55
Day 4A-200	200	1.0	59.0	44.0	26.0	500	143.0	259	138	113
Day 4A-500	500	1.0	129.2	103.8	63.0	500	143.6	564	325	273
Day 4A-1000	1,000	1.0	230.0	196.0	128.4	500	140.0	1,029	629	571
Day 4B-0	0	1.0	27.0	< 4	< 4	500	156.0	108	ND	ND
Day 4B-50	50	1.0	45.0	13.8	8.6	500	151.6	186	41	35
Day 4B-100	100	1.0	67.0	26.6	15.4	500	153.8	273	78	62
Day 4B-200	200	1.0	80.0	39.0	21.2	500	144.0	348	122	92
Day 4B-500	500	1.0	140.0	111.2	66.0	500	146.0	601	342	281
Day 4B-1000	1,000	1.0	236.0	203.2	98.0	500	138.0	1,072	661	442

TABLE 12 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)			Internal Standard ^b		Relative Weight ^c Response		
		RDX	DNT	TNT	ng/ml	Peak Height	RDX	DNT	TNT
Std-Day 4-5	1,000	224.0	308.0	222.0	500	141.2	0.793	1.091	0.786
Std-Day 4-3	200	46.2	68.0	49.0	500	143.8	0.803	1.182	0.852
Std-Day 4-5	1,000	230.0	316.0	230.0	500	146.8	0.783	1.076	0.783
Std-Day 4-2	100	23.4	31.8	22.8	500	144.0	0.813	1.104	0.792
Average							0.798	1.113	0.803
Standard Deviation							± 0.013 ± 0.047 ± 0.033		

^a ng/g compound added - nanograms of RDX, DNT, and TNT added per gram of liver sample.

^b Internal standard - compound (propiophenone) added to liver sample after sample preparation for calculation of data.

^c ng/g detected - nanograms of RDX, DNT, and TNT detected in the liver sample.

$$\text{ng compound/g} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}}$$

^d ND - not detectable, less than 20 ng/g.

$$\text{Relative Weight Response - RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

APPENDIX F

IDENTIFICATION OR DEVELOPMENT OF CHEMICAL ANALYSIS
METHODS FOR PLANTS AND ANIMAL TISSUES

METHOD REPORT NO. 5

METHOD DEVELOPMENT FOR THE DETERMINATION OF PENTAERYTHRITOL
TETRANITRATE (PETN) IN PLASMA

October 1980

Contract No. DAAK11-79-C-0110
MRI Project No. 4849-A

For

U S. Army Toxic and Hazardous Materials Agency
Dr. L. Eng, DRXTH-TE-A, Project Officer
Aberdeen Proving Ground (EA), MD 21010

The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER Technical Report No. 5	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Method Development for the Determination of Pentaerythritol Tetranitrate (PETN) in Plasma		5. TYPE OF REPORT & PERIOD COVERED Method Report, August 1979 to December 1980
7. AUTHOR(s) D. B. Lakings and O. Gan		6. PERFORMING ORG. REPORT NUMBER MRI Project No. 4849-A
9. PERFORMING ORGANIZATION NAME AND ADDRESS Midwest Research Institute 425 Volker Boulevard Kansas City, MO 64110		8. CONTRACT OR GRANT NUMBER(s) DAAK11-79-C-0110
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Armament Research and Development Command, Aberdeen Proving Ground (Edgewood Area), MD 21010		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		12. REPORT DATE October 1980
		13. NUMBER OF PAGES 30
		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Unlimited		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Pentaerythritol Tetranitrate (PETN) Plasma Determination High Performance Liquid Chromatography (HPLC) UV, 215 nm Detection		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A high performance liquid chromatographic (HPLC) method using ultraviolet (UV) detection at 215 nm has been developed for the quantitative determination of pentaerythritol tetranitrate (PETN) in animal plasma samples. The analytical system consists of an isocratic HPLC unit with a Spherisorb ODS, 5 μ , 250 x 4.6 mm ID column, an eluent of 40% acetonitrile in high purity water, and a flow rate of 1.5 ml/min. The PETN and internal standard (IS), valerophenone, are detected and quantitated at 215 nm using a variable wavelength UV detector.		

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

20. (continued)

The compounds have the following retention indices: PETN, 42 ml, 28 min, and IS, 34.5 ml, 23 min. Reference solutions of PETN have a linear response from 50 ng/ml to 1,000 ng/ml; the linear regression equation and correlation coefficient for the reference solutions were $y = 0.986x + 2.3$, 0.9997. The plasma samples were prepared by adding 2 ml 20% sodium chloride containing 1% acetic acid to 2.0 ml plasma and extracting the mixture with 3 x 5 ml hexane. The hexane was evaporated at room temperature under a stream of nitrogen. The residue was dissolved in 500 μ l acetonitrile containing 500 ng IS and the final volume adjusted to 1.0 ml with high purity water. After filtering the prepared sample through a 0.45 μ Fluoropore filter, a 100- μ l aliquot was assayed by HPLC-UV (215 nm). The analytical method was evaluated by preparing and analyzing duplicate plasma samples containing 0, 50, 100, 200, 500, and 1,000 ng/ml PETN on four separate days. Linear regression analysis of the data gave the following equation and correlation coefficient: $y = 0.594x - 0.8$, 0.986. The average coefficient of variation and average percent inaccuracy for PETN plasma determination at the five levels were 14% and -42, respectively. No plasma components were found which interfered with PETN determination. A statistical evaluation of the data by the Hubaux and Vos detection limit program gave a detection limit of 50 ng/ml for PETN determination in plasma samples.

UNCLASSIFIED

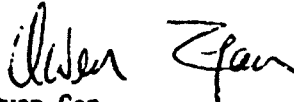
SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

PREFACE

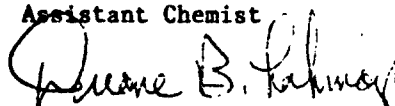
This report was prepared at Midwest Research Institute, 425 Volker Boulevard, Kansas City, Missouri 64110 under U.S. Army Toxic and Hazardous Materials Agency, Contract No. DAAK11-79C-0110, MRI Project No. 4849-A, "Identification or Development of Chemical Analysis Methods for Plants and Animal Tissues." Dr. Leslie Eng, DRXTH-TE-A was the project officer for this research effort.

This work was conducted in the Analytical Chemistry Department, Dr. James L. Spigarelli, Director. The research effort was directed by Dr. Duane B. Lakings, Program Manager and Senior Chemist, with assistance from Mr. Owen Gan, Assistant Chemist, and Ms. Mary Valla, Senior Technician. The report was prepared by Dr. Lakings and Mr. Gan.

MIDWEST RESEARCH INSTITUTE

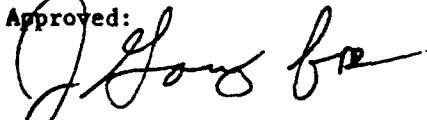


Owen Gan
Assistant Chemist



Duane B. Lakings
Program Manager and Senior Chemist

Approved:



James L. Spigarelli, Director
Analytical Chemistry Department

TABLE OF CONTENTS

	<u>Page</u>
1. Application	1
2. Chemistry	1
3. Apparatus	2
4. Standards	3
5. Procedure for Plasma Sample Determinations	4
6. Calculation.	6
7. Statistical Evaluation of Data	6
Appendix - Method Development for the Determination of PETN in Plasma Samples.	16

LIST OF FIGURES

<u>Number</u>	<u>Title</u>	<u>Page</u>
1	HPLC-UV (215 nm) Separation of PETN SARM and Valerophenone (IS)	7
2	Determination of PETN in Plasma.	10
3	Standard Deviation, Coefficient of Variation, and Percent Inaccuracy for PETN in Plasma Samples.	11
4	HPLC Analysis of Blank Plasma Sample for PETN Method Development.	12
5	HPLC Analysis of Plasma Containing 100 ng/ml PETN.	13
6	HPLC Analysis of Plasma Containing 500 ng/ml PETN.	14

LIST OF TABLES

<u>Number</u>	<u>Title</u>	<u>Page</u>
1	Linearity and Precision of the HPLC-UV (215 nm) Determination of SARM Reference Solutions of PETN.	8
2	HPLC-UV (215 nm) Determination of PETN in Plasma	9
3	Statistical Evaluation of PETN in Plasma Data by the Hubaux and Vos Detection Limit Program	15
Appendix 4	Linearity and Precision of PETN Determination by High Performance Liquid Chromatography.	17
5	Determination of PETN in Plasma Samples - Day 1.	18
6	Determination of PETN in Plasma Samples - Day 2.	19
7	Determination of PETN in Plasma Samples - Day 3.	20
8	Determination of PETN in Plasma Samples - Day 4.	21

Midwest Research Institute
Analytical Chemistry Department
Kansas City, Missouri 64110

for

U.S. Army Armament Research and Development Command
Aberdeen Proving Ground (Edgewood Area)
Maryland 21010

Contract No. DAAK11-79-C-0110

Identification or Development of Chemical Analysis Methods
for Plant and Animal Tissues

METHOD DEVELOPMENT FOR THE DETERMINATION OF PENTAERYTHRITOL
TETRANITRATE (PETN) IN PLASMA

1. APPLICATION: The developed method is for the quantitative determination of PETN in animal plasma samples using high performance liquid chromatography (HPLC) with ultraviolet (UV) detection at 215 nm.

a. Evaluated Concentration Range: The concentration range of PETN studied in plasma samples and reference solutions corresponded to 50 to 1,000 ng/ml (parts per billion, ppb).

b. Sensitivity: A signal-to-noise ratio of 3 to 1 for PETN was obtained with an injection of 100 μ l of a 50 ng/ml reference solution (ca. 5 ng PETN on column). The 100- μ l injection of a 50 ng/ml PETN solution gave a PETN peak height of 6 mm.

c. Detection Limits: The detection limit for PETN in plasma was determined to be 50 ng/ml using the Hubaux and Vos detection limit program.

d. Interferences: No interfering plasma components were found to elute with the same retention volumes as PETN or the IS.

e. Analysis Rate: The chromatographic time per injection was 35 min. Two reference solutions were analyzed prior to injecting the prepared samples and two were analyzed during the day (140 min total time). Thus, a total of nine prepared plasma samples (315 min) can be analyzed during an 8-hr day.

2. CHEMISTRY: PETN (CAS. Reg. No. 4792-15-8) has limited solubility in water and polar organic solvents; however, it has good solubility in intermediate polarity (acetone, benzene) and nonpolar (hexane) solvents. The UV spectrum of PETN shows an absorption maximum at 215 nm with little absorbance at 254 nm.

3. APPARATUS:

a. Instrumentation: The isocratic HPLC instrument utilized during this study consisted of a Waters Model 6000A pump, Waters Model U6K injector, and a Varian Model Vista UV-50 variable wavelength detector. During extraction, samples were centrifuged in a general purpose centrifuge (Dynac, Clay Adams 0101) with a 24-place head.

b. HPLC Parameters:

1. Column: Spherisorb ODS, 5 μ , 250 x 4.6 mm ID.
2. Eluent: 40% acetonitrile in high purity water.
Note: The eluent must be helium degassed since oxygen interferes at 215 nm; also, the use of acetic acid or other modifiers is not recommended since they absorb at 215 nm.
3. Flow rate: 1.5 ml/min.
4. Detector: UV, 215 nm.
5. Internal standard: Valerophenone, 500 ng/ml.
6. Injection volume: 100 μ l.
7. Retention volumes and times: PETN, 42 ml and 28 min;
IS, 34.5 ml and 23 min. Note: Slight changes in the retention indices may occur with fresh eluent or a change in columns.

A representative HPLC-UV (215 nm) chromatogram for PETN and the IS is shown in Figure 1.

c. Laboratory Glassware and Equipment:

1. Culture tubes (Pyrex) with Teflon-lined screw caps.
2. Volumetric flasks (100 ml).
3. Volumetric syringes (0-100 μ l, 0-500 μ l, and 0-1,000 μ l).
4. Automatic pipetter (0-5 ml).
5. Filtering apparatus including filter holder, 5-ml disposable syringes, and 0.45 μ Fluoropore filters.
6. Inert gas (nitrogen) drying train with 12 ports.
7. Inert gas (helium) degassing train.

d. Chemicals:

1. Acetonitrile and hexane, "Distilled in Glass" grade.
Note: Organic solvents of lesser grade should not be used; they may have trace contaminants which will interfere with PETN determination.
2. Acetic acid and sodium chloride, ACS grade.
3. High purity water from a Milli-Q water purification system. Note: Water of lesser quality may contain trace organic impurities which may interfere with PETN determination.
4. PETN SARM, obtained from the U.S. Army Toxic and Hazardous Materials Agency.
5. Valerophenone (internal standard), analytical grade.

4. STANDARDS:

a. Stock: Weigh approximately 20 mg PETN SARM or interim SARM into a 100-ml volumetric flask and record weight. Dissolve the PETN in acetonitrile and dilute to volume (concentration of PETN is 200 µg/ml). Quantitatively pipette 20 ml of the 200 µg/ml stock into a 100-ml volumetric flask and dilute to volume with high purity water (concentration of PETN is 40 µg/ml).

b. Working: Pipette 10 ml of the 40 µg/ml stock into a 100-ml volumetric flask and dilute to volume with 10% acetonitrile in high purity water. The concentration of PETN is 4 µg/ml.

Reference solutions of PETN were prepared from this stock as follows:

<u>µl Working Stock</u>	<u>µl IS Stock*</u>	<u>µl 10% Acetonitrile in Water</u>	<u>Concentration PETN (ng/ml)</u>
500	500	1,000	1,000
250	500	1,250	500
100	500	1,400	200
50	500	1,450	100
25	500	1,475	50
0	500	1,500	0

* Preparation of IS stock given in "c" below.

c. Internal Standard Stock: Weigh 10 mg valerophenone into a 100-ml volumetric flask and dilute to volume with acetonitrile (concentration, 100 µg/ml). Quantitatively pipette 10 ml of the 100 µg/ml stock into a 100-ml volumetric flask and dilute to volume with acetonitrile (concentration, 10 µg/ml). A final working IS solution of 1,000 ng/ml is prepared by pipetting 10 ml of the 10 µg/ml stock into a 100-ml volumetric flask and diluting to volume with acetonitrile.

5. PROCEDURE FOR PLASMA SAMPLE DETERMINATIONS:

a. Plasma Sample Preparation: The procedure employed to prepare plasma samples for the HPLC-UV (215 nm) determination of PETN consisted of:

1. Quantitatively pipette twelve (12) 2.0 ml plasma aliquots into culture tubes with Teflon-lined screw caps.
2. Spike two each of the plasma aliquots with the PETN working stock (4 µg/ml) at the following levels: 2,000 ng (500 µl), 1,000 ng (250 µl), 400 ng (100 µl), 200 ng (50 µl), and 100 ng (25 µl). The remaining two plasma aliquots serve as plasma sample blanks. Adjust all samples to a final volume of 2.5 ml with high purity water containing 10% acetonitrile.
3. Add 2 ml of a 20% sodium chloride solution containing 1% acetic acid to each aliquot.
4. Mix thoroughly on a vortex mixer.
5. Extract the plasma samples with 5 ml hexane ("Distilled in Glass" grade) by hand mixing about 15 times. Note: Extensive mixing or vortexing may result in an emulsion which is difficult to break.
6. Centrifuge the samples at 1,000 rpm for 20 min.
7. Transfer the hexane extracts to properly labeled culture tube with Teflon-lined screw caps.
8. Repeat the hexane extraction (steps 5 and 6) twice more, combining the hexane extracts in the appropriate tubes.
9. Evaporate the hexane at room temperature under a stream of nitrogen. Note: Do not heat the extracts during the evaporation step or loss of PETN may occur. Continue evaporation until the hexane has been completely removed from the culture tube.

10. Dissolve the residue in 500 µl acetonitrile containing 500 ng IS (IS working stock solution) and mix thoroughly by vortexing.
11. Add 500 µl high purity water to each extracted plasma sample and mix thoroughly. Note: Final volume of the prepared samples is 1.0 ml.
12. Filter the prepared samples through 0.45 µ Fluoropore filters into culture tubes. Note: The filtration step removes undissolved particulate matter from the samples and thus prolongs the life of the HPLC analytical column.
13. Analyze a 100-µl aliquot of each prepared plasma sample by HPLC.
14. After the elution of the PETN, inject 200 µl acetonitrile onto the system to remove any late eluting compounds. Note: The acetonitrile wash step is required to prevent possible interference in the chromatographic analysis of the next sample.

b. Calibration: The reference solutions described in Section 4.b were prepared and analyzed in quadruplicate. The relative weight response (Eq. 1) of PETN was calculated for each solution and the average RWR utilized to determine the nanograms per milliliter of PETN in every solution (Eq. 2). The nanograms per milliliter found were plotted against the nanograms per milliliter added, and a linear regression evaluation of the data was conducted. The slope, intercept, and correlation coefficient were determined. The data are summarized in Table 1, which also presents the average value at each PETN level, the standard deviation, coefficient of variation (relative standard deviation), and percent inaccuracy. The raw data and calculations are given in Table 4 of the Appendix.

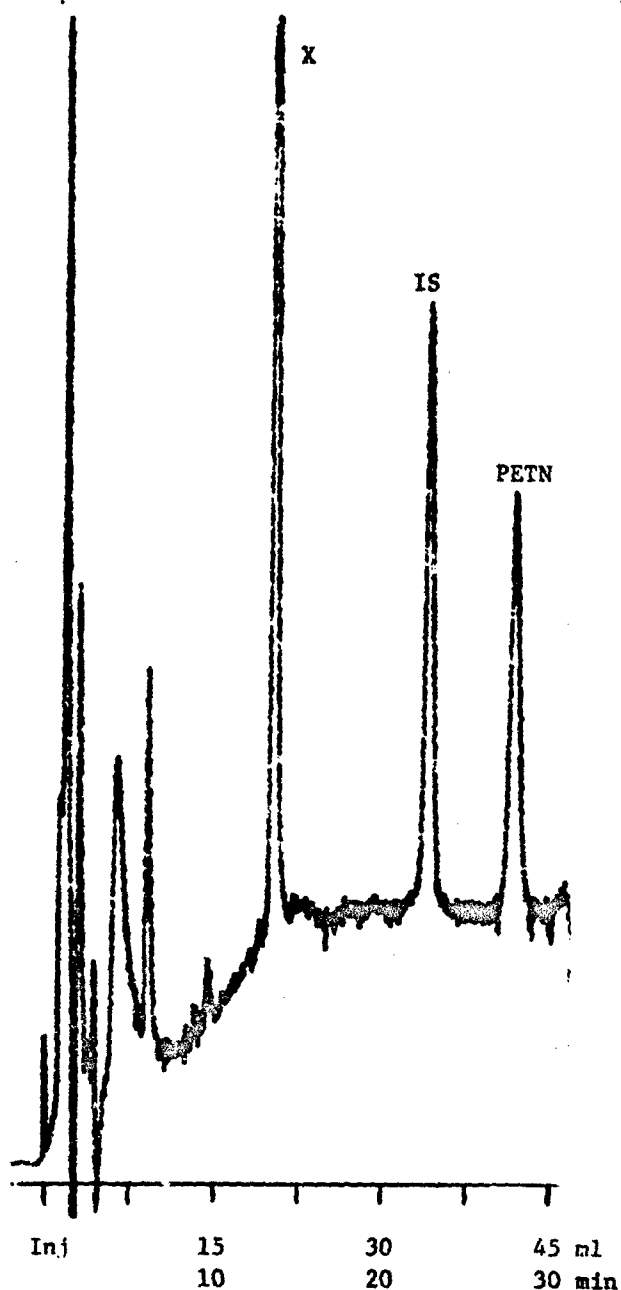
$$RWR = \frac{\text{Peak Height PETN}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml PETN}} \quad (\text{Eq. 1})$$

$$\text{ng/ml or } \frac{\text{ng}}{\text{plasma aliquot}} = \frac{\text{Peak Height PETN}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{Avg. RWR}} \quad (\text{Eq. 2})$$

c. Plasma Sample Analysis: The plasma samples prepared as outlined in Section 5.a were injected onto the HPLC system. The peak heights of PETN and the IS were measured and recorded. Plasma samples were prepared and analyzed on four separate days.

6. CALCULATION: The nanograms PETN per milliliter of plasma in the prepared samples was determined using the relative weight response to an internal standard method. The RWR values (Eq. 1) for PETN reference solutions analyzed with a day's set of plasma samples were calculated and averaged. The nanograms PETN per plasma aliquot was determined by Equation 2 and the nanograms per milliliter PETN calculated by dividing the nanograms per aliquot by the plasma sample volume (2.0 ml). The results for the duplicate determinations of PETN in plasma samples at five different levels on four separate days are summarized in Table 2. The average level found, the standard deviation, coefficient of variation, and percent inaccuracy are also included. A linear regression evaluation of the data was made; the slope, intercept, and correlation coefficient are given in the table. Figure 2 presents the plot of the nanograms per milliliter PETN found against the nanograms per milliliter added; the range shown at each level represents two standard deviations from the average value at that level. A graphic presentation of the standard deviation, coefficient of variation, and percent inaccuracy for the determination of PETN in plasma is given in Figure 3. Representative HPLC-UV (215 nm) chromatograms are shown for a plasma sample blank (Figure 4), a 100 ng PETN/ml plasma sample (Figure 5), and a 500 ng PETN/ml plasma sample (Figure 6). The raw data and calculations for the plasma sample PETN determinations are given in Tables 5 to 8 in the Appendix.

7. STATISTICAL EVALUATION OF DATA: A statistical evaluation of the data obtained for the determination of PETN in plasma samples (Table 2) by the Hubaux and Vox detection limit program was made at the U.S. Army Toxic and Hazardous Materials Agency. The results of this evaluation are given in Table 3. The detection limit for PETN in plasma as determined by the program was 50 ng/ml. The linear regression equations presented in Table 3 were generated using all the plasma samples, the blank plasma samples and the plasma with PETN added. The average nanograms per milliliter value at each level was calculated from the 48-point linear regression equation and the nanograms per milliliter added at that level. The standard deviation and percent imprecision (coefficient of variation) at each level were calculated based on this average nanograms per milliliter value found. Thus, these values and the values given in Table 2 (based on the average of the eight assays at each level) are not comparable. The percent inaccuracy terms in Tables 2 and 3 were calculated from the average of the eight data points at each level and thus agree closely.



HPLC Conditions

Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 40% acetonitrile in
high purity water
Flow Rate: 1.5 ml/min
Detector: UV, 215 nm

Reference Solution Characteristics

Concentrations: PETN - 500 ng/ml;
IS - 500 ng/ml
Injection Volume: 100 μ l
Attenuation: 0.005X

Retention Indices

<u>Compound</u>	<u>Retention Volume (ml)</u>	<u>Retention Time (min)</u>
IS	34.5	23
PETN	42	28

Figure 1 - HPLC-UV (215 nm) Separation of PETN SARM and Valerophenone (IS).
"X" indicates a contaminant in the PETN reference solution stock.

TABLE 1

LINEARITY AND PRECISION OF THE HPLC-UV (215 nm) DETERMINATION
OF SARH REFERENCE SOLUTIONS OF PETN

Compound	ng/ml Added	ng/ml Detected			Average ^a	Standard ^b Deviation	Coefficient ^c of Variation	Percent ^d Inaccuracy
		A	B	C				
PETN	0	ND ^e	ND	ND	-	-	-	-
	50	47	49	49	49	± 1.6	3.3	-2.0
	100	101	104	104	103	± 1.5	1.5	+3.0
	200	196	200	205	199	± 4.9	2.4	-0.5
	500	512	502	483	502	± 14	2.7	+0.4
	1,000	964	997	999	985	± 16	1.7	-1.5

Linear Regression

PETN $y = 0.986x + 2.3$

Correlation Coefficient = 0.9997

^a Average = $\sum x/n = \bar{x}$

^b Standard deviation = $\left(\frac{n\sum x^2 - (\sum x)^2}{n(n-1)} \right)^{1/2} = \sigma$

^c Coefficient of variation = $\sigma/\bar{x} \times 100$

^d Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$

^e ND = Not detectable, less than 25 ng/ml

TABLE 2
HPLC-UV (215 nm) DETERMINATION OF PETN IN PLASMA

Amount PETN Added (ng/ml)	Level Found (ng/ml)						Average ^a	Standard ^b Deviation (ng/ml)	Coefficient ^c of Variation	Percent ^d Inaccuracy
	Day 1		Day 2		Day 3					
	A	B	A	B	A	B				
0	ND ^e	ND	ND	ND	ND	ND	-	-	-	-
50	26	31	31	23	23	27	28	± 5.0	18	-44
100	63	72	58	51	53	51	60	± 8.4	14	-40
200	102	134	120	94	105	118	115	± 13	11	-43
500	279	337	294	228	269	324	298	± 38	13	-40
1,000	524	674	523	516	531	625	593	± 82	14	-41

Linear Regression
 $y = 0.594x - 0.8$

Correlation Coefficient - 0.986

a Average = $\sum x/n = \bar{x}$

b Standard deviation = $\left(\frac{n\sum x^2 - (\sum x)^2}{n(n-1)} \right)^{1/2} = \sigma$

c Coefficient of variation = $\sigma/\bar{x} \times 100$

d Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$

e ND = Not detectable, less than 25 ng/ml

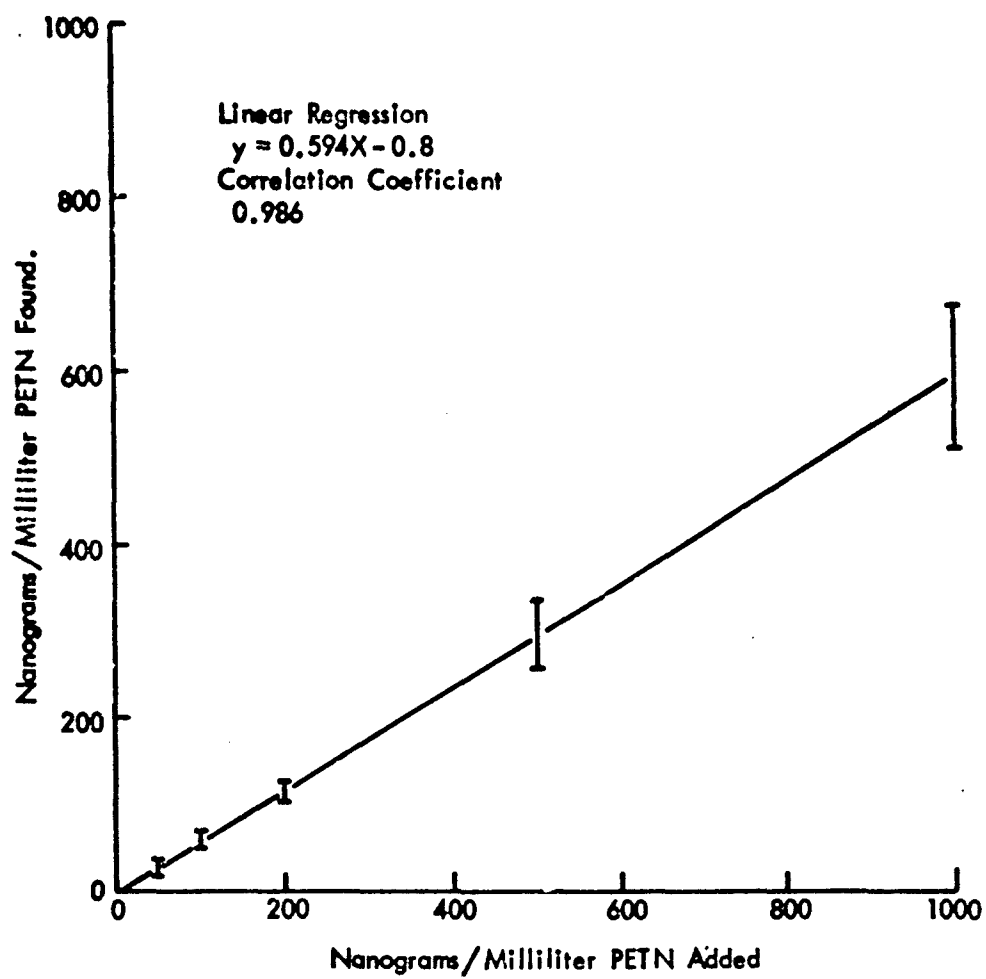


Figure 2 - Determination of PETN in Plasma

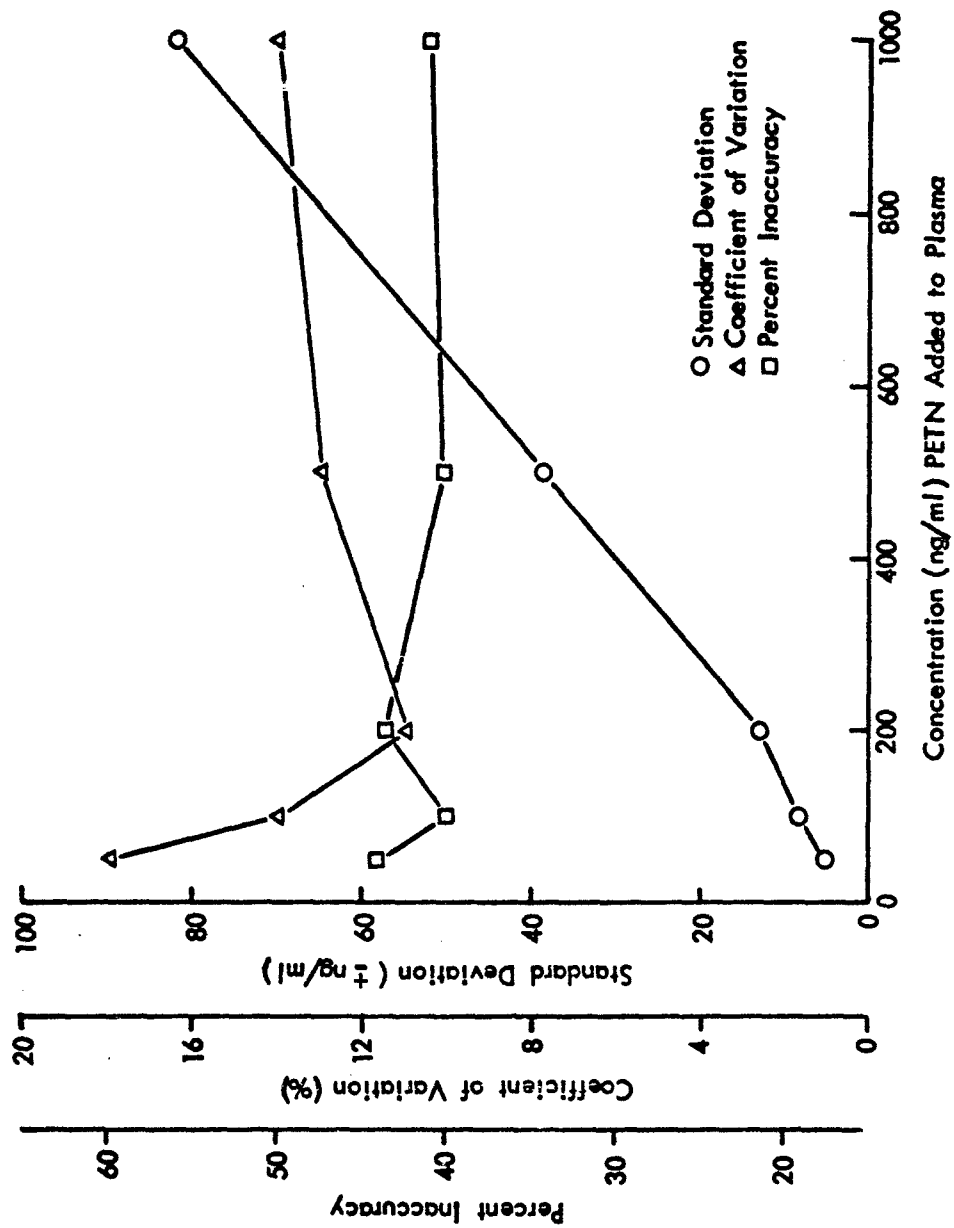
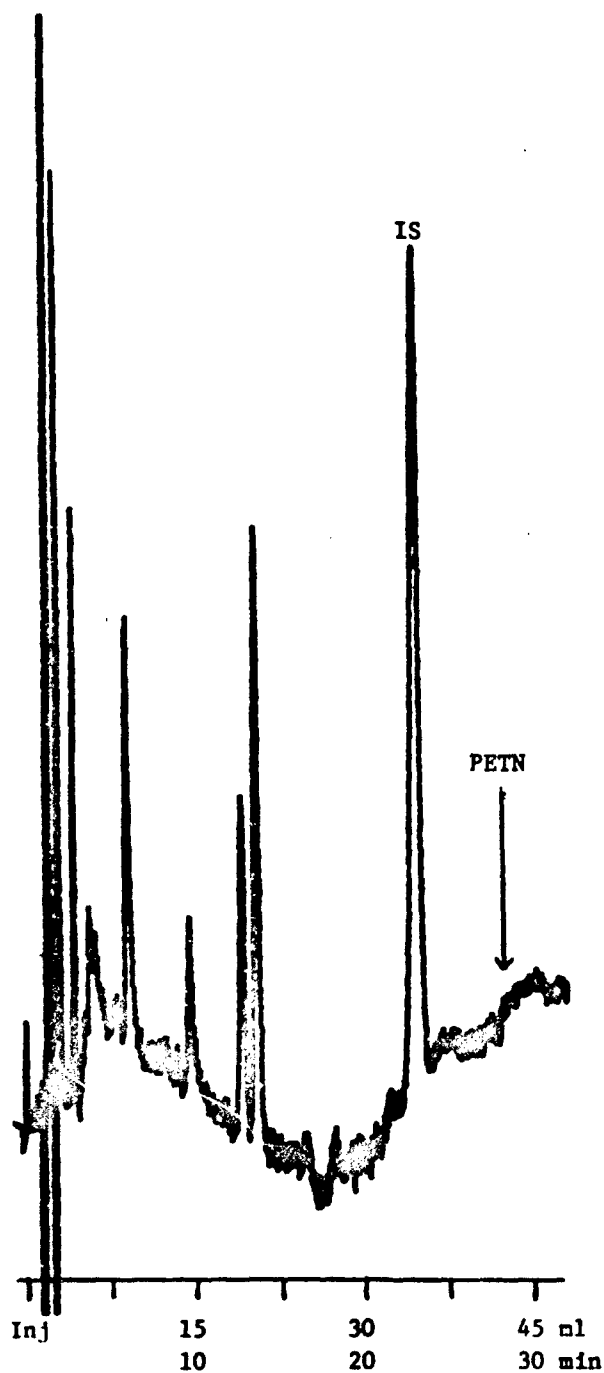


Figure 3 - Standard Deviation, Coefficient of Variation, and Percent Inaccuracy for PETN in Plasma Samples



HPLC Conditions

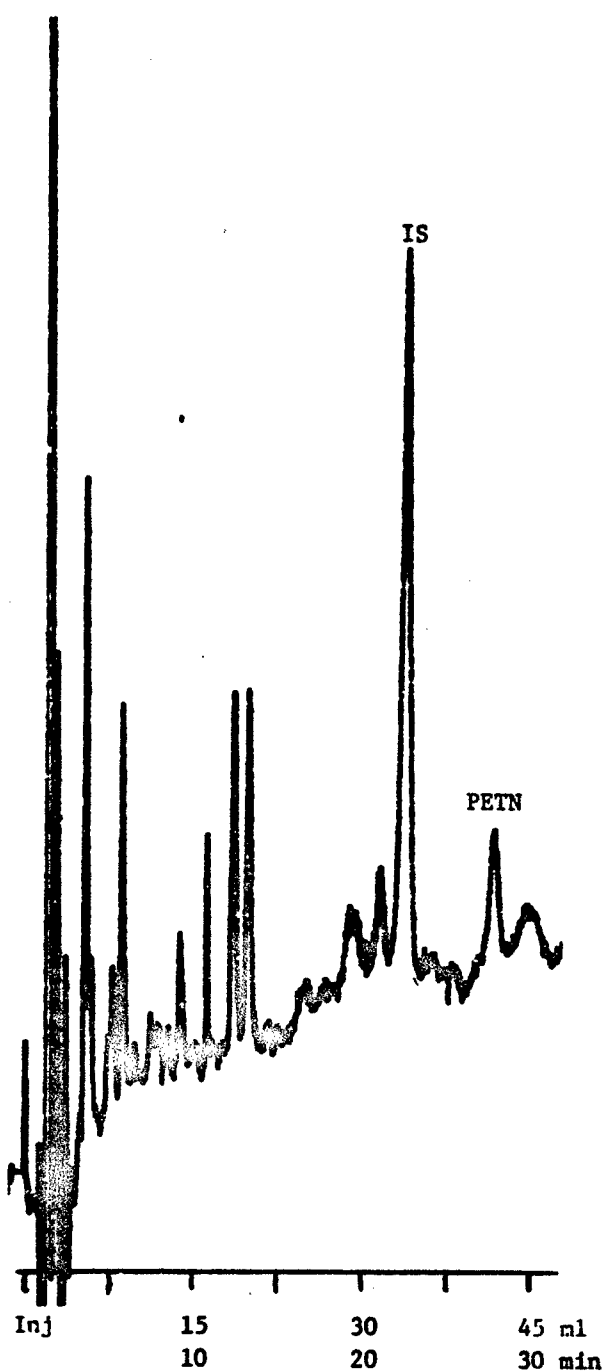
Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 40% acetonitrile in
high purity water
Flow Rate: 1.5 ml/min
Detector: UV, 215 nm

Sample Characteristics

2.0 ml plasma extracted with
3 x 5 ml hexane. Hexane evaporated
and sample reconstituted to 1.0 ml.

IS Concentration: 500 ng/ml
Injection Volume: 100 μ l
Attenuation: 0.005X

Figure 4 - HPLC Analysis of Blank Plasma Sample for PETN Method Development. Arrow indicates PETN elution position.



HPLC Conditions

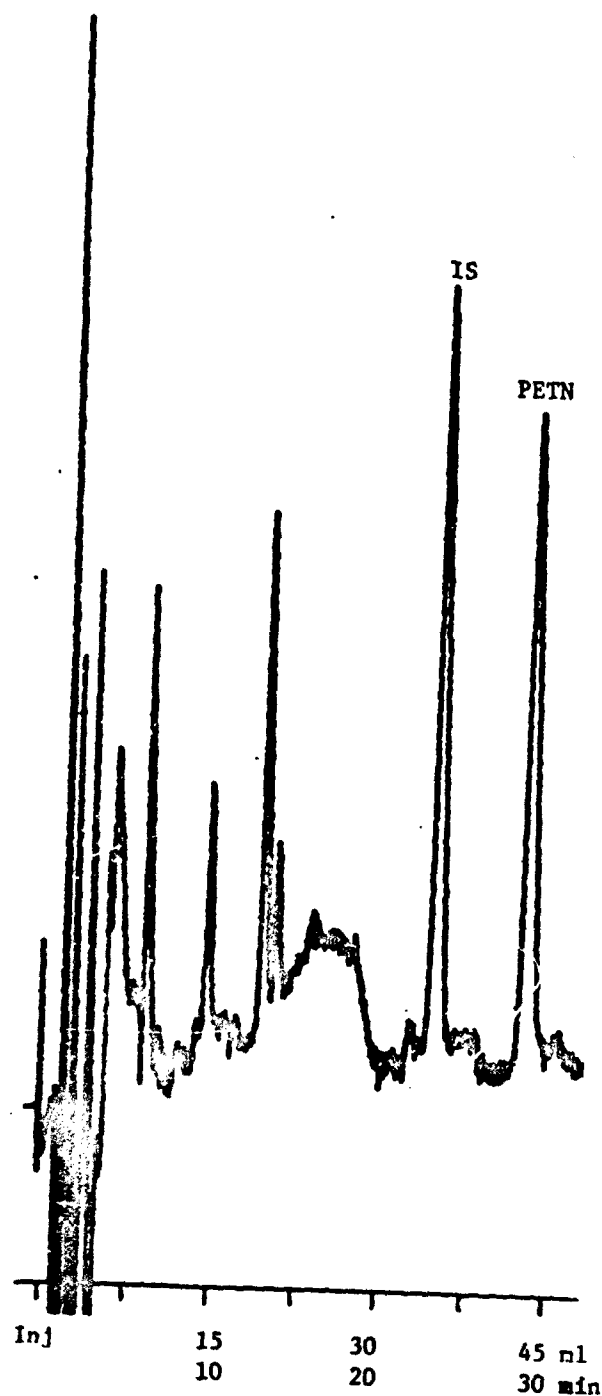
Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 40% acetonitrile in
high purity water
Flow Rate: 1.5 ml/min
Detector: UV, 215 nm

Sample Characteristics

2.0 ml plasma containing 100 ng/ml
PETN extracted 3 x 5 ml with hexane.
Hexane evaporated and sample recon-
stituted to 1.0 ml.

IS Concentration: 500 ng/ml
Injection Volume: 100 μ l
Attenuation: 0.005X

Figure 5 - HPLC Analysis of Plasma Containing 100 ng/ml PETN



HPLC Conditions

Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 40% acetonitrile in
high purity water
Flow Rate: 1.5 ml/min
Detector: UV, 215 nm

Sample Characteristics

2.0 ml plasma containing 500 ng/ml
PETN extracted 3 x 5 ml with hexane.
Hexane evaporated and sample reconstituted to 1.0 ml.

IS Concentration: 500 ng/ml
Injection Volume: 100 μ l
Attenuation: 0.005X

Figure 6 - HPLC Analysis of Plasma Containing 500 ng/ml PETN

TABLE 3

STATISTICAL EVALUATION OF PETN IN PLASMA DATA BY THE HUBAUX AND VOS
DETECTION LIMIT PROGRAM

Number of Data Points	Linear Regression	Correlation Coefficient	Degrees of Freedom	t _b	y ^c Intercept	Detection Limit ^d
48	y = 0.594x - 0.77	0.986	46	1.679	23	80
40	y = 0.597x - 1.06	0.987	38	1.686	12	42

ng/ml PETN Added	Average ^e ng/ml Found	Standard ^f Deviation	Percent ^g Imprecision	Percent ^h Inaccuracy
50	29	± 1.8	6.6	-43
100	59	± 3.2	5.2	-40
200	118	± 5.0	4.4	-43
500	296	± 14	4.9	-40
1,000	593	± 31	5.2	-41

a Number of data points - data points used to calculate linear regression and detection limits; 48 - all data;
40 - 1,000 ng/ml samples omitted.

b t - 2-tail p level (usually 0.1, each confidence band is 0.05, so total p = 0.1).

c y intercept - intercept on y-axis of upper confidence limit line.

d Detection limit - x-intercept of y-intercept and lower confidence limit line.

e Average ng/ml found - average at each level determined from linear regression equation for 48 points.

f Standard deviation - determined from average value ("e" above) and observed values.

g Percent imprecision - standard deviation divided by average value times 100%.

h Percent inaccuracy - determined from the average values of the eight observed values at each level.

$$\% \text{ Inaccuracy} = \frac{\text{Average observed values} - \text{level added}}{\text{level added}} \times 100$$

APPENDIX

METHOD DEVELOPMENT FOR THE DETERMINATION OF PETN
IN PLASMA SAMPLES

RAW DATA AND CALCULATIONS

TABLE 4

LINEARITY AND PRECISION OF PETN DETERMINATION BY HIGH PERFORMANCE
LIQUID CHROMATOGRAPHY

Reference Solution Number	$\frac{\text{ng}}{\text{ml}}$ ^a PETN Added	Peak Height (mm) ^b		$\frac{\text{ng}}{\text{ml}}$ ^a IS	Relative ^c Weight Response	Calculated ^d ng/ml PETN
		PETN	IS			
A-1	0	< 3	89.0	500	-	ND ^e
A-2	50	6.0	93.6	500	0.641	47
A-3	100	12.0	88.0	500	0.682	101
A-4	200	23.0	87.0	500	0.661	196
A-5	500	58.8	85.0	500	0.692	512
A-6	1,000	89.0	116.0	500	0.652	964
B-1	0	< 3	90.0	500	-	ND
B-2	50	6.0	90.0	500	0.667	49
B-3	100	12.4	88.4	500	0.701	104
B-4	200	25.4	94.0	500	0.676	200
B-5	500	60.0	88.4	500	0.679	502
B-6	1,000	88.0	118.6	500	0.674	997
C-1	0	< 3	90.0	500	-	ND
C-2	50	6.0	90.0	500	0.667	49
C-3	100	12.6	90.0	500	0.700	104
C-4	200	25.0	90.0	500	0.694	205
C-5	500	56.4	86.4	500	0.653	483
C-6	1,000	118.0	87.4	500	0.675	999
D-1	0	< 3	89.0	500	-	ND
D-2	50	6.0	87.0	500	0.690	51
D-3	100	12.5	89.0	500	0.702	104
D-4	200	26.0	99.0	500	0.657	194
D-5	500	59.8	86.4	500	0.692	512
D-6	1,000	118.0	89.0	500	0.663	981

Average 0.676

Standard Deviation ± 0.018

Relative Standard Deviation 2.7%

a ng/ml PETN and ng/ml IS - concentration of PETN and IS in nanograms per milliliter in each reference solution.

b Peak Height (mm) - peak height of PETN and IS measured in millimeters.

c Relative Weight Response (RWR)

$$\text{RWR} = \frac{\text{Peak Height PETN}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml PETN}}$$

d Calculated ng/ml PETN - level of PETN calculated to be in the reference solution using the average RWR value for all solutions analyzed.

e ND - not detectable, less than 25 ng/ml.

TABLE 5

DETERMINATION OF PETN IN PLASMA SAMPLES - DAY 1

Sample Number	Sample Volume (ml)	ng ^a PETN Added	Peak Height ^b (mm)		ng ^c IS	ng/ml ^d PETN Detected
			PETN	IS		
Day-1 A-1	2.0	0	< 3	110.0	500	ND ^e
Day-1 A-50	2.0	100	8.0	116.0	500	26
Day-1 A-100	2.0	200	21.0	125.0	500	63
Day-1 A-200	2.0	400	35.4	130.6	500	102
Day-1 A-500	2.0	1,000	93.0	125.0	500	279
Day-1 A-1000	2.0	2,000	186.0	133.2	500	524
Day-1 B-0	2.0	0	< 3	105.0	500	ND
Day-1 B-50	2.0	100	9.6	115.0	500	31
Day-1 B-100	2.0	200	20.0	105.0	500	72
Day-1 B-200	2.0	400	40.0	112.0	500	134
Day-1 B-500	2.0	1,000	98.6	109.8	500	337
Day-1 B-1000	2.0	2,000	187.2	104.2	500	674

Reference Solutions - Day 1

Reference Solution Number	ng/ml ^a PETN	Peak Height (mm) ^b		ng/ml ^c IS	Relative Weight Response ^f
		PETN	IS		
Std-Day-1-5	1,000	118.8	85.6	500	0.694
Std-Day-1-2	100	13.0	91.0	500	0.714
Std-Day-1-4	500	60.0	90.0	500	0.667
Std-Day-1-3	200	26.4	100.0	500	0.660
Std-Day-1-1	50	5.2	87.4	500	0.595

Average 0.666
Standard Deviation ±0.045

- a ng or ng/ml PETN Added - nanograms of PETN added to 2.0 ml plasma or present in reference solution.
b Peak Height (mm) - peak height in millimeters of PETN and IS in plasma samples and reference solutions.
c ng IS - nanograms of the internal standard added to the samples and reference solutions for calculation.
d ng/ml PETN Detected - nanograms of PETN detected per milliliter of plasma.

$$\text{ng/ml PETN} = \frac{\text{Peak Height PETN}}{\text{Peak Height IS}} \times \frac{\text{ng IS}}{\text{Avg. RWR}} \times \frac{1}{2.0 \text{ ml plasma}}$$

- e ND - not detectable, less than 15 ng PETN per milliliter plasma.
f Relative Weight Response (RWR)

$$\text{RWR} = \frac{\text{Peak Height PETN Std}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml PETN}}$$

TABLE 6

DETERMINATION OF PETN IN PLASMA SAMPLES - DAY 2

Sample Number	Sample Volume (ml)	ng ^a PETN Added	Peak Height ^b (mm)		ng ^c IS	ng/ml ^d PETN Detected
			PETN	IS		
Day-2 A-0	2.0	0	< 3	124.0	500	ND ^e
Day-2 A-50	2.0	100	10.0	120.0	500	31
Day-2 A-100	2.0	200	18.0	115.4	500	58
Day-2 A-200	2.0	400	35.0	108.0	500	120
Day-2 A-500	2.0	1,000	96.0	121.2	500	294
Day-2 A-1000	2.0	2,000	176.0	125.0	500	523
Day-2 B-0	2.0	0	< 3	124.0	500	ND
Day-2 B-50	2.0	100	7.4	119.4	500	23
Day-2 B-100	2.0	200	17.0	122.8	500	51
Day-2 B-200	2.0	400	32.0	126.0	500	94
Day-2 B-500	2.0	1,000	76.0	124.0	500	228
Day-2 B-1000	2.0	2,000	170.0	122.4	500	516

Reference Solutions - Day 2

Reference Solution Number	ng/ml ^a PETN	Peak Height (mm) ^b		ng/ml ^c IS	Relative Weight Response ^f
		PETN	IS		
Std-Day-2-4	500	55.8	84.8	500	0.658
Std-Day-2-5	1,000	116.0	83.2	500	0.697
Std-Day-2-3	200	24.2	88.0	500	0.683
Std-Day-2-3	200	22.8	88.6	500	0.643
Std-Day-2-5	1,000	116.0	85.0	500	0.682

Average 0.673

Standard Deviation ± 0.022

a ng or ng/ml PETN Added - nanograms of PETN added to 2.0 ml plasma or present in reference solution.

b Peak Height (mm) - peak height in millimeters of PETN and IS in plasma samples and reference solutions.

c ng IS - nanograms of the internal standard added to the samples and reference solutions for calculation.

d ng/ml PETN Detected - nanograms of PETN detected per milliliter of plasma.

$$\text{ng/ml PETN} = \frac{\text{Peak Height PETN}}{\text{Peak Height IS}} \times \frac{\text{ng IS}}{\text{Avg. RWR}} \times \frac{1}{2.0 \text{ ml plasma}}$$

e ND - not detectable, less than 15 ng PETN per milliliter plasma.

f Relative Weight Response (RWR)

$$\text{RWR} = \frac{\text{Peak Height PETN Std}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml PETN}}$$

TABLE 7

DETERMINATION OF PETN IN PLASMA SAMPLES - DAY 3

Sample Number	Sample Volume (ml)	ng ^a PETN Added	Peak Height ^b (mm)		ng ^c IS	ng/ml ^d PETN Detected
			PETN	IS		
Day-3 A-0	2.0	0	< 3	122.0	500	ND ^e
Day-3 A-50	2.0	100	8.2	133.8	500	23
Day-3 A-100	2.0	200	17.0	122.6	500	53
Day-3 A-200	2.0	400	36.0	130.0	500	105
Day-3 A-500	2.0	1,000	86.8	123.0	500	269
Day-3 A-1000	2.0	2,000	182.8	131.0	500	531
Day-3 B-0	2.0	0	< 3	116.0	500	ND
Day-3 B-50	2.0	100	8.0	114.8	500	27
Day-3 B-100	2.0	200	14.2	105.2	500	51
Day-3 B-200	2.0	400	36.0	116.0	500	118
Day-3 B-500	2.0	1,000	93.8	110.0	500	324
Day-3 B-1000	2.0	2,000	189.2	115.2	500	625

Reference Solutions - Day 3

Reference Solution Number	ng/ml ^a PETN	Peak Height (mm) ^b		ng/ml ^c IS	Relative ^f Weight Response
		PETN	IS		
Std-Day-3-5	1,000	113.0	85.0	500	0.665
Std-Day-3-4	500	60.5	88.8	500	0.681
Std-Day-3-3	200	22.0	82.5	500	0.667
Std-Day-3-4	500	51.2	83.5	500	0.613

Average 0.657
Standard Deviation ±0.030

a ng or ng/ml PETN Added - nanograms of PETN added to 2.0 ml plasma or present in reference solution.

b Peak Height (mm) - peak height in millimeters of PETN and IS in plasma samples and reference solutions.

c ng IS - nanograms of the internal standard added to the samples and reference solutions for calculation.

d ng/ml PETN Detected - nanograms of PETN detected per milliliter of plasma.

$$\text{ng/ml PETN} = \frac{\text{Peak Height PETN}}{\text{Peak Height IS}} \times \frac{\text{ng IS}}{\text{Avg. RWR}} \times \frac{1}{2.0 \text{ ml plasma}}$$

e ND - not detectable, less than 15 ng PETN per milliliter plasma.

f Relative Weight Response (RWR)

$$\text{RWR} = \frac{\text{Peak Height PETN Std}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml PETN}}$$

TABLE 8

DETERMINATION OF PETN IN PLASMA SAMPLES - DAY 4

Sample Number	Sample Volume (ml)	ng ^a PETN Added	Peak Height ^b (mm)		ng ^c IS	ng/ml ^d PETN Detected
			PETN	IS		
Day-4 A-0	2.0	0	< 3	134.0	500	ND ^e
Day-4 A-50	2.0	100	8.8	131.0	500	28
Day-4 A-100	2.0	200	22.0	132.0	500	70
Day-4 A-200	2.0	400	39.5	134.0	500	124
Day-4 A-500	2.0	1,000	106.0	131.8	500	337
Day-4 A-1000	2.0	2,000	195.2	131.5	500	623
Day-4 B-0	2.0	0	< 3	130.0	500	ND
Day-4 B-50	2.0	100	11.0	123.0	500	38
Day-4 B-100	2.0	200	16.0	103.4	500	65
Day-4 B-200	2.0	400	38.8	134.0	500	121
Day-4 B-500	2.0	1,000	99.4	130.6	500	319
Day-4 B-1000	2.0	2,000	221.0	127.0	500	730

Reference Solutions - Day 4

Reference Solution Number	ng/ml ^a PETN	Peak Height (mm) ^b		ng/ml ^c IS	Relative Weight Response ^f
		PETN	IS		
Std-Day-4-5	1,000	106.0	94.5	500	0.561
Std-Day-4-2	100	12.0	101.8	500	0.589
Std-Day-4-2	100	13.0	102.0	500	0.637

Average 0.596
Standard Deviation ±0.039

- a ng or ng/ml PETN Added - nanograms of PETN added to 2.0 ml plasma or present in reference solution.
b Peak Height (mm) - peak height in millimeters of PETN and IS in plasma samples and reference solutions.
c ng IS - nanograms of the internal standard added to the samples and reference solutions for calculation.
d ng/ml PETN Detected - nanograms of PETN detected per milliliter of plasma.

$$\text{ng/ml PETN} = \frac{\text{Peak Height PETN}}{\text{Peak Height IS}} \times \frac{\text{ng IS}}{\text{Avg. RWR}} \times \frac{1}{2.0 \text{ ml plasma}}$$

- e ND - not detectable, less than 15 ng PETN per milliliter plasma.
f Relative Weight Response (RWR)

$$\text{RWR} = \frac{\text{Peak Height PETN Std}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml PETN}}$$

APPENDIX G

IDENTIFICATION OR DEVELOPMENT OF CHEMICAL ANALYSIS
METHODS FOR PLANTS AND ANIMAL TISSUES

METHOD REPORT NO. 6

METHOD DEVELOPMENT FOR THE DETERMINATION OF DINITROTOLUENE (DNT)
AND TRINITROTOLUENE (TNT) IN PLANT STEMS

November 1980

Contract No. DAAK11-79-C-0110
MRI Project No. 4849-A

For

U.S. Army Toxic and Hazardous Materials Agency
Dr. L. Eng, DRXTH-TE-A, Project Officer
Aberdeen Proving Ground (EA), MD 21010

RECEIVED AND INDEXED FILED

The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
Technical Report No. 6		
4. TITLE (and Subtitle) Method Development for the Determination of Dinitrotoluene (DNT) and Trinitrotoluene (TNT) in Plant Stems		5. TYPE OF REPORT & PERIOD COVERED Method Report, August 19, 1979 to December 20, 1979
7. AUTHOR(s) D. B. Lakings and O. Gan		6. PERFORMING ORG. REPORT NUMBER MRI Project No. 4849-A
8. PERFORMING ORGANIZATION NAME AND ADDRESS Midwest Research Institute 425 Volker Boulevard Kansas City, MO 64110		9. CONTRACT OR GRANT NUMBER(s) DAAK11-79-C-0110
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Armament Research and Development Command, Aberdeen Proving Ground (Edgewood Area), MD 21010		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		12. REPORT DATE November 1980
		13. NUMBER OF PAGES 41
		15. SECURITY CLASS. (of this report) Unclassified
		16a. DECLASSIFICATION/DOWNGRADING SCHEDULE
14. DISTRIBUTION STATEMENT (of this Report) Unlimited		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) High Performance Liquid Chromatography Dinitrotoluene (DNT) Trinitrotoluene (TNT) Plant Stem Determination		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A high performance liquid chromatographic (HPLC) method for the quantitative determination of dinitrotoluene (DNT) and trinitrotoluene (TNT) in plant stems has been developed. The analytical system consists of an isocratic HPLC unit with a Spherisorb ODS 5 μ , 250 x 4.6 mm ID column, an eluent of 30% acetonitrile in 1% acetic acid in water, and a flow rate of 1.5 ml/min. The compounds, including the internal standard (IS), propiophenone, have the following retention characteristics: IS - 24 ml, 16 min; DNT - 31.5 ml, 21 min; and		

DD FORM 1 JAN 79 1073 EDITION OF 1 NOV 68 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

TNT - 34.5 ml, 23 min and are detected at 254 nm. Reference solutions of the compounds gave a linear response from 100 ng/ml to 2,000 ng/ml. The plant stem matrix was prepared by adding 2-g sodium chloride to 5-g grounded stems in 10-ml water and extracting the sample with 20-ml hexane containing 2% isopropanol. The hexane extract (10 ml) was transferred to a culture tube and evaporated to dryness at room temperature under a stream of nitrogen gas. A 500- μ l aliquot of acetonitrile containing 1,000 ng IS was added followed by 500- μ l high-purity water. The prepared sample was filtered through a 0.45 μ Fluoropore filter and injected onto the HPLC system. The analytical method was evaluated by preparing and analyzing duplicate 5.0-g plant stems samples containing 0, 50, 100, 200, 500 and 1,000 ng/g of each munition on four succeeding days. Linear regression analysis of the data gave the following equations and correlation coefficients: DNT - $y = 0.514x + 7$, 0.923 and TNT - $y = 0.449x + 10$, 0.957. The average coefficient of variation and average percent inaccuracy for DNT and TNT determination in plant stems were 38%, -43 and 33%, -48, respectively. A statistical evaluation of the data by Hubbaux and Vos detection limit program gave detection limits of 65 ng/g for DNT and 90 ng/g for TNT for HPLC determination of these compounds in stem samples.

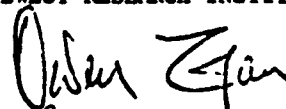
UNCLASSIFIED

PREFACE

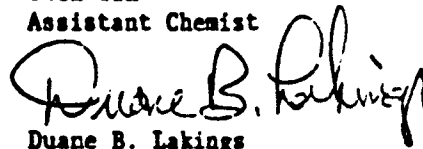
This report was prepared at Midwest Research Institute, 425 Volker Boulevard, Kansas City, Missouri 64110, under U.S. Army Toxic and Hazardous Materials Agency, Contract No. DAAK11-79-C-0110, MRI Project No. 4849-A, entitled "Identification or Development of Chemical Analysis Methods for Plants and Animal Tissues." Dr. Leslie Eng, DRXTH-TE-A, was the project officer for this research effort.

This work was conducted in the Analytical Chemistry Department, Dr. James L. Spigarelli, Director. The research effort was directed by Dr. Duane B. Lakings, Program Manager and Senior Chemist, with assistance from Mr. Owen Gan, Assistant Chemist, and Ms. Mary Valla, Senior Technician. This report was prepared by Dr. Lakings and Mr. Gan.

MIDWEST RESEARCH INSTITUTE

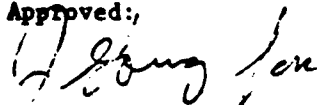


Owen Gan
Assistant Chemist



Duane B. Lakings
Program Manager and Senior Chemist

Approved:



James L. Spigarelli, Director
Analytical Chemistry Department

TABLE OF CONTENTS

	<u>Page</u>
1. Application	1
2. Chemistry	2
3. Apparatus	2
4. Standards	3
5. Procedures for Plant Stems Sample Determination	4
6. Calculation	6
7. Statistical Evaluation of Data.	6
Appendix - Method Development for the Determination of DNT and TNT in Plant Stems.	22

LIST OF FIGURES

<u>Number</u>	<u>Title</u>	<u>Page</u>
1	HPLC Separation of RDX, DNT, TNT, and Tetryl SARMS and Propiophenone (IS)	8
2	Determination of DNT in Plant Stems.	12
3	Determination of TNT in Plant Stems.	13
4	Standard Deviation for DNT and TNT in Plant Stem Samples .	14
5	Coefficient of Variation for DNT and TNT in Plant Stem Samples	15
6	Percent Inaccuracy for DNT and TNT in Plant Stem Samples .	16
7	HPLC Analysis of Blank Plant Stems	17
8	HPLC Analysis of Plant Stems Containing 50 ng/g of RDX, DNT and TNT.	18
9	HPLC Analysis of Plant Stems Containing 500 ng/g RDX, DNT, TNT, and Tetryl	19

LIST OF TABLES

<u>Number</u>	<u>Title</u>	<u>Page</u>
1	Linearity and Precision of the HPLC-UV Determination of SARM Reference Solutions of RDX, DNT, and TNT.	9
2	HPLC-UV Determination of DNT in Plant Stems Samples. . . .	10
3	HPLC-UV Determination of TNT in Plant Stems Samples. . . .	11
4	Statistical Evaluation of DNT in Plant Stems Data by the Hubaux and Vos Detection Limit Program	20
5	Statistical Evaluation of TNT in Plant Stems Data by the Hubaux and Vos Detection Limit Program	21
Appendix 6	Linearity and Precision of RDX, DNT, and TNT Determination by High Performance Liquid Chromatography.	23
7	Determination of DNT and TNT in Plant Stems Samples, Day 1 Samples.	25
8	Determination of DNT and TNT in Plant Stems Samples, Day 2 Samples.	27
9	Determination of DNT and TNT in Plant Stems Samples, Day 3 Samples.	29
10	Determination of DNT and TNT in Plant Stems Samples, Day 4 Samples.	31

Midwest Research Institute
Analytical Chemistry Department
Kansas City, Missouri 64110

for

U.S. Army Armament Research and Development Command
Aberdeen Proving Ground (Edgewood Area)
Maryland 21010

Contract No. DAAK11-79-C-0110

Identification or Development of Chemical Analysis Methods
for Plants and Animal Tissues

METHOD DEVELOPMENT FOR THE DETERMINATION OF DINITROTOLUENE (DNT)
AND TRINITROTOLUENE (TNT) IN PLANT STEMS

1. APPLICATION: The developed method is for quantitative determination of DNT and TNT in plant stems samples using high performance liquid chromatography (HPLC) with ultraviolet (UV) detection at 254 nm.

a. Evaluated Concentration Range: The concentration range of DNT and TNT studied in reference solutions was 100, 500, 1,000, 1,500, and 2,000 ng/ml and in plant stems samples was 50, 100, 200, 500, and 1,000 ng/g (parts per billion, ppb).

b. Sensitivity: A signal-to-noise ratio of 9 to 1 for DNT (PH - 40 mm) and 8 to 1 for TNT (PH - 30 mm) was obtained with an injection of 50 µl of a 100 ng/ml solution of each compound (ca. 5 ng each compound on column).

c. Detection Limits: The detection limits in the plant stem matrix were 65 ng/g for DNT and 90 ng/g for TNT using the Hubaux and Vox detection limit program.

d. Interferences: Small plant component peaks were observed at the elution position of DNT and TNT in the blank plant stems sample and represented about 5 ng/g each for DNT and TNT. Two other munitions, cyclo-trimethylenetrinitramine (RDX), CAS Reg. No. 121-82-4, and 2,4,6-trinitro-phenylmethylnitramine (tetryl), CAS Reg. No. 479-45-8, were also included in these evaluations. RDX co-eluted with a large plant stem component and thus could not be determined. The HPLC elution position of tetryl was relatively free from plant stem component interferences, and in some samples a peak at the elution position of tetryl was observed. However, the recovery of the peak (tetryl) was low and inconsistent from sample to sample, thus preventing the determination of tetryl.

e. Analysis Rate: The chromatographic time per injection was 40 min. Two reference solutions were analyzed prior to injecting the prepared samples, and two were analyzed during the day (160 min total time). Thus, a total of eight prepared plant stems samples (320 min total time) can be analyzed during an 8-hr day.

2. CHEMISTRY: DNT (CAS Reg. No. 121-14-2) and TNT (CAS Reg. No. 118-96-7) have limited solubility in water and nonpolar organic solvents; however, they have good solubility in intermediate polarity and polar solvents. Each of these munitions has a sufficient UV chromophore at 254 nm to allow UV detection and quantification at the required levels.

3. APPARATUS:

a. Instrumentation: A Waters isocratic liquid chromatographic system consisting of a Model 6000A pump, Model U6K injector, and Model 440 UV detector. A general purpose centrifuge (Dynac, Clay Adams 0101) with a 24-place head.

b. HPLC Parameters:

1. Column: Spherisorb ODS, 5 μ , 250 x 4.6 mm ID.
2. Eluent: 30% acetonitrile in 1% acetic acid in water.
3. Flow rate: 1.5 ml/min.
4. Detector: UV, 254 nm.
5. Internal standard: Propiophenone, 1,000 ng/ml.
6. Injection volume: 50 to 100 μ l.
7. Retention volumes and times: DNT, 31.5 ml, 21 min; TNT, 34.5 ml, 23 min; and IS, 24 ml, 16 min in the 30% acetonitrile eluent. NOTE: Slight changes in the retention indices may occur with fresh eluent or a change in column.

A representative HPLC chromatogram for DNT and TNT is shown in Figure 1. Also included on the chromatogram are peaks for the internal standard (propiophenone), RDX, and tetryl.

c. Laboratory Glassware and Equipment:

1. Culture tubes (Pyrex) with Teflon-lined screw caps.
2. Centrifuge tubes (Oak Ridge type, polypropylene, Nalgene 3119, capacity 50 ml) with screw caps.
3. Volumetric flasks (100 ml).
4. Volumetric syringes (0-100 μ l, 0-500 μ l, and 0-1,000 μ l).
5. Automatic pipetter (0-5 ml).

6. Six-speed Waring-type blender with glass container.
7. Teflon-glass, motor-driven tissue homogenizer.
8. Filtering apparatus including filter holders, 5-ml disposable syringes, and 0.45 μ Fluoropore filters.
9. Inert gas (nitrogen) drying train with 12 ports.
10. Ultrasonic cleaner (50/60 Hz type).

d. Chemicals:

1. DNT and TNT SARMS, obtained from the U.S. Army Toxic and Hazardous Materials Agency.
2. Propiophenone (internal standard), analytical grade.
3. Acetonitrile, hexane, and isopropanol (IPA), "Distilled in Glass" grade. The extracting solvent was 2/98 v/v isopropanol:hexane.
4. Acetic acid and sodium chloride, ACS grade.
5. High purity water from a Milli-Q water purification system.
6. Dry ice.

4. STANDARDS:

a. Stock: Weigh approximately 20 mg of RDX, DNT, TNT, and tetryl SARM or interim SARM into separate 100-ml volumetric flasks. Dissolve each compound in acetonitrile and dilute to volume. The concentration of each compound is 200 μ g/ml. Quantitatively pipette 20 ml from each stock above into a 100-ml volumetric flask and dilute to volume with distilled water. Concentration of each compound is 40 μ g/ml.

b. Working: Pipette 10 ml of the 40 μ g/ml each compound stock into a 100-ml volumetric flask and dilute to volume with high purity water. Concentration of each compound is 4 μ g/ml. Reference solutions were prepared from this stock as follows:

<u>μl Working Stock</u>	<u>μl IS Stock*</u>	<u>μl 10% Acetonitrile in Water</u>	<u>Concentration Each Compound (ng/ml)</u>
500	500	0	2,000
375	500	125	1,500
250	500	250	1,000
		3	
		273	

<u>µl Working Stock</u>	<u>µl IS Stock*</u>	<u>µl 10% Acetonitrile in Water</u>	<u>Concentration Each Compound (ng/ml)</u>
125	500	375	500
25	500	475	100
0	500	500	0

* Preparation of IS stock given in "c."

c. Internal Standard Stock: Weigh 10 mg propiophenone into a 100-ml volumetric flask and dilute to volume with acetonitrile (concentration, 100 µg/ml). Quantitatively pipette 10 ml of the 100 µg/ml stock into a 100-ml volumetric flask and dilute to volume with acetonitrile (concentration, 10 µg/ml). A final working solution of 2.0 µg/ml is prepared by pipetting 20 ml of the 10 µg/ml stock into a 100-ml volumetric flask and diluting to volume with acetonitrile.

5. PROCEDURES FOR PLANT STEMS SAMPLE DETERMINATION:

a. Plant Stems Sample Preparation: The procedure employed to prepare stems samples for the HPLC-UV determination of DNT and TNT consisted of:

1. Place approximately 50 g of green plant stems (precut into $\frac{1}{2}$ in.) into a Waring-type blender and blend for 1 min on speed six (liquify). To prevent the pieces of stems from sticking to the side of the blender walls, place small chunks of dry ice into the blender prior to blending.
NOTE: The side of the blender should be scraped with a spatula.
2. Pipette duplicate aliquots of the working stock (4 µg/ml each RDX, DNT, TNT, and tetryl) into the polypropylene centrifuge tubes at the following levels: 5,000 ng (1.25 ml); 2,500 ng (0.625 ml); 1,000 ng (0.25 ml); 500 ng (0.125 ml); and 250 ng (0.0625 ml). Also, prepare two tubes to serve as plant stem blanks. All tubes are adjusted to a total volume of 10 ml with high purity water.
3. Accurately weigh 5.0 g homogenized plant stems into each of the 12 polypropylene centrifuge tubes (with different levels of compound). Cap the tubes and mix thoroughly with hand shaking.

4. Weigh 2 g sodium chloride each into the tubes and again mix thoroughly with hand and vortex mixer.
5. Extract the stems with 20 ml hexane (2% IPA) ("distilled in glass" grade) by vortexing and hand mixing for 30 sec followed by centrifugation at 1,000 rpm for 10 min.
6. Transfer 10 ml of the hexane (2% IPA) extracts to properly labeled culture tubes with Teflon-lined screw caps.
7. Evaporate the hexane (2% IPA) at room temperature under a stream of nitrogen. NOTE: Do not heat the samples during the evaporation step, or loss of DNT and TNT may occur. Continue evaporation until hexane-IPA has been completely removed from the culture tube.
8. Dissolve the residues in 500 μ l acetonitrile containing 1,000 ng IS, i.e., internal standard working solution, mix thoroughly on a vortex mixer, and then place in ultrasonicator for approximately 5 min.
9. Add 500 μ l high purity water to each extracted stems sample and mix thoroughly on a vortex mixer. NOTE: Final volume of the prepared samples is 1.0 ml.
10. Filter the solutions through 0.45 μ Fluorepore filters into culture tubes.
11. Analyze a 50- to 100- μ l aliquot of each prepared stem sample by HPLC.
12. After the elution of TNT peak, wash the column for 3 min with 100% acetonitrile at 1.5 ml/min to remove any late-eluting compounds. NOTE: The acetonitrile wash step is required to prevent possible interference in the chromatographic analysis of the next sample injection.
13. After the 3-min wash, switch the system back to the eluent. Allow approximately 7 min for equilibration prior to the next injection.

b. Calibration: The reference solutions described in Section 4.b were prepared and analyzed in quadruplicate. The relative weight response (RWR) (Eq. 1) of each compound was calculated and the average RWR utilized to determine the nanograms of each compound in every reference solution (Eq. 2). The nanograms found were plotted against the nanograms added.

The slope, intercept, and correlation coefficient for each compound were determined. The data are summarized in Table 1 and include the average value at each level for each compound, the standard deviation, coefficient of variation, and percent inaccuracy. The raw data and calculations are given in Table 6 of the Appendix.

$$RWR = \frac{\text{Peak Height Cpd}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml Cpd}} \quad (\text{Eq. 1})$$

$$\frac{\text{ng}}{\text{ml}} \text{ or ng/5 g compound} = \frac{\text{Peak Height Cpd}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{Avg. RWR}} \quad (\text{Eq. 2})$$

c. Plant Stems Sample Analysis: The plant stems samples prepared as outlined in Section 5.a were injected onto the HPLC. The peak height of each compound was measured and recorded. Plant stems samples were prepared and analyzed on four succeeding days.

6. CALCULATION: The level of each compound in the 5.0 g plant stems samples was determined using the relative weight response to an internal standard method. The RWR values (Eq. 1) for the reference solutions analyzed with a day set of plant stems samples were calculated and the average values for DNT and TNT determined. These RWR values were employed to calculate the level of DNT and TNT in the plant stems samples (Eq. 2) where the nanograms per milliliter term represents the level found in the 5.0 g sample. The nanograms per gram of each compound were determined by dividing the level found by the sample weight. The results for the duplicate determinations of DNT and TNT in plant stems samples at five different levels on four separate days are summarized in Tables 2 and 3. The average level found, the standard deviation, coefficient of variation, and percent inaccuracy are also included. A linear regression evaluation of the results was made; and the slope, intercept, and correlation coefficient are given in the tables. The level of each compound found in the plant stems samples was plotted against the amount added, and these data are shown in Figures 2 and 3. The range presented at each level is two standard deviations of the average level found. A graphic presentation of the standard deviation, coefficient of variation, and percent inaccuracy for the determination of DNT and TNT in plant stems samples is given in Figures 4 through 6, respectively. Representative HPLC chromatograms are shown for a plant stems sample blank (Figure 7), a 50 ng/g (Figure 8), and a 500 ng/g (Figure 9) each compound plant stems sample. The raw data and calculations for the plant stems sample determinations are given in Tables 7 and 10 in the Appendix.

7. STATISTICAL EVALUATION OF DATA: A statistical evaluation of the data obtained for the determination of DNT and TNT in plant stems samples (Tables 4 and 5) by the Hubaux and Vos detection limit program was made

at the U.S. Army Toxic and Hazardous Materials Agency. The results of these evaluations are given in Table 4 for DNT and Table 5 for TNT. When the 1,000- and 500-ng/g data points were omitted, the detection limit for DNT in plant stems as determined by the program was 65 ng/g. For TNT, the detection limit was 90 ng/g when the 1,000-ng/g data points were omitted. Removal of the 500-ng/g data points from the TNT detection limit calculation resulted in a detection limit below the lowest target concentration, 50 ng/g. The average nanograms per gram value found at each level for each compound was determined from the linear regression for the 48 data points and the nanograms per gram added at that level. The standard deviation and percent imprecision (coefficient of variation) at each level were calculated based on this average nanograms per gram value found. Thus, these values and the values given in Tables 2 and 3 for these terms (based on the average of the eight assays at each level) are not comparable. The percent inaccuracy term was calculated from the average of the eight data points at each level, and thus agrees closely with the values in Tables 2 and 3.

HPLC Conditions

Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 30% acetonitrile in
1% acetic in water
Flow Rate: 1.5 ml/min
Chart Speed: 0.1 in./min
Detector: UV, 254 nm

Sample Characteristics

Concentrations: RDX, DNT, TNT,
and tetryl - 500 ng/ml;
IS - 1,000 ng/ml
Injection volume: 70 μ l
Attenuation: 0.01 X

Retention Indices

Compound	Retention Volume (ml)	Retention Time (min)
RDX	13.5	9
IS	22.5	15
DNT	31.5	21
TNT	34.5	23
tetryl	39	26

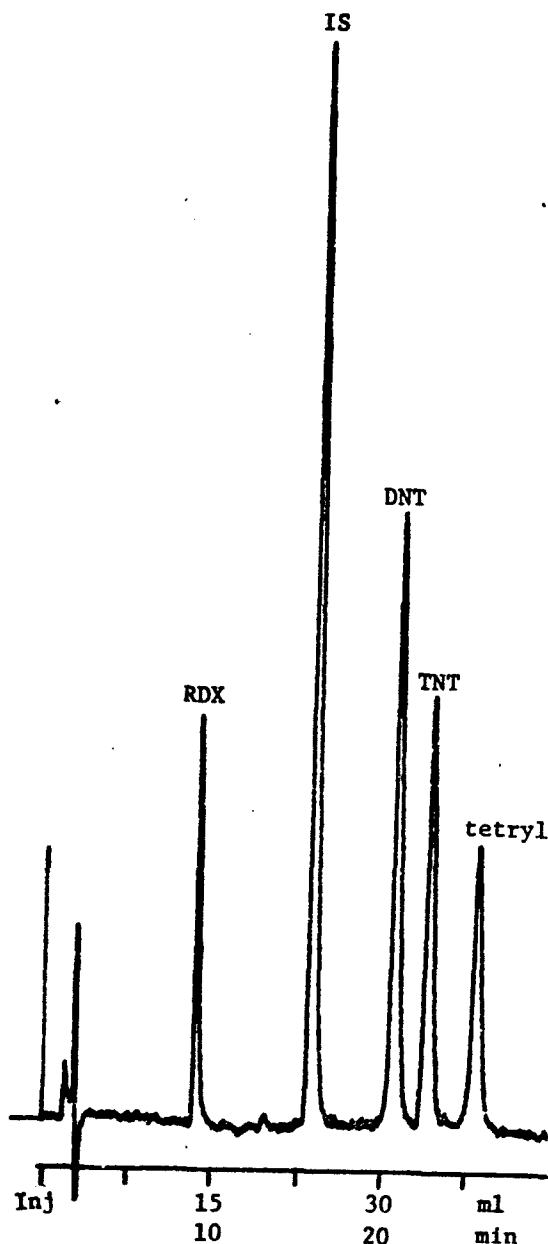


Figure 1 - HPLC Separation of RDX, DNT, TNT, and Tetryl
SARMS and Propiophenone (IS)

TABLE 1
LINEARITY AND PRECISION OF THE HPLC-UV DETERMINATION OF
SARM REFERENCE SOLUTIONS OF RDX, DNT, AND TNT

Compound	ng/ml Added	ng/ml Detected				Average ^a	Standard ^b Deviation	Coefficient ^c of Variation	Percent ^d Inaccuracy
		A	B	C	D				
RDX	0	ND ^e	ND	ND	ND	-	-	-	-
	100	109	114	105	103	108	± 4.9	4.5	+ 8.0
	500	517	509	499	490	504	± 11.8	2.3	+ 0.8
	1,000	960	950	923	985	955	± 26	2.7	- 4.5
	1,500	1,551	1,547	1,435	1,444	1,494	± 63	4.2	- 0.4
	2,000	2,031	2,069	1,906	1,924	1,983	± 80	4.0	- 0.8
DNT	0	ND	ND	ND	ND	-	-	-	-
	100	113	109	109	100	108	± 5.5	5.1	+ 8.0
	500	499	490	475	483	487	± 10.2	2.1	- 2.6
	1,000	992	1,011	981	1,032	1,004	± 22	2.2	+ 0.4
	1,500	1,500	1,470	1,461	1,439	1,468	± 25	1.7	- 2.1
	2,000	1,968	1,982	1,934	1,901	1,946	± 36	1.9	- 2.7
TNT	0	ND	ND	ND	ND	-	-	-	-
	100	107	112	112	100	108	± 5.7	5.3	+ 8.0
	500	495	479	484	484	486	± 6.8	1.4	- 2.8
	1,000	956	989	967	997	977	± 19	2.0	- 2.3
	1,500	1,498	1,508	1,471	1,432	1,478	± 34	2.3	- 1.5
	2,000	2,011	2,015	1,957	1,879	1,966	± 63	3.2	- 1.7

Linear Regression

RDX: $y = 0.988x + 0.6$

Correlation coefficient - 0.998

DNT: $y = 0.974x + 7.7$

Correlation coefficient - 0.999

TNT: $y = 0.982x + 1.2$

Correlation coefficient - 0.999

- a Average = $\sum x/n = \bar{x}$
b Standard deviation = $(\sum (\bar{x} - x)^2 / (n-1))^{1/2} = \sigma$
c Coefficient of variation = $\sigma / \bar{x} \times 100$
d Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$
e ND = Not detectable, less than 20 ng/ml

TABLE 2
HPLC-UV DETERMINATION OF DNT IN PLANT STEMS SAMPLES

Amount Added (ng/g)	Level Found (ng/g)								Average ^a	Standard Deviation	Coefficient ^c of Variation	Percent ^d Inaccuracy
	Day 1		Day 2		Day 3		Day 4					
	A	B	A	B	A	B	A	B				
0	2.2	ND ^e	5	5	5	4	4	4	3.4	± 1.7	49	-
50	20	22	49	47	32	25	25	24	31	± 11	37	-38
100	32	46	91	90	49	42	56	57	58	± 22	37	-42
200	44	89	186	169	112	102	115	91	114	± 45	40	-43
500	159	175	367	439	274	271	235	243	270	± 94	35	-45
1,000	333	412	707	814	479	416	435	544	518	± 164	32	-48

Note: Linear regression: $y = 0.514x + 7$
Correlation coefficient: 0.923

a Average = $\Sigma x/n = \bar{x}$

b Standard deviation = $\left(\frac{n \Sigma x^2 - (\Sigma x)^2}{n(n-1)} \right)^{1/2} = \sigma$

c Coefficient of variation = $\sigma/\bar{x} \times 100$

d Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$

e ND - Not detectable, less than 2 ng/g.

TABLE 3
HPLC-UV DETERMINATION OF TNT IN PLANT STEMS SAMPLES

Amount Added (ng/g)	Level Found (ng/g)								Average ^a	Standard Deviation	Coefficient ^c of Variation	Percent ^d Inaccuracy
	Day 1		Day 2		Day 3		Day 4					
	A	B	A	B	A	B	A	B				
0	11	ND ^e	6	7	3	6	3	3	4.9	± 3.4	69	-
50	24	28	32	31	44	32	18	21	29	± 8	28	-42
100	37	41	62	63	75	48	67	42	54	± 14	26	-46
200	70	78	127	120	120	97	140	116	108	± 24	23	-46
500	136	154	301	322	244	240	245	253	237	± 64	27	-52
1,000	311	356	597	593	462	454	401	472	456	± 102	22	-54

Note: Linear regression: $y = 0.449x + 10$
Correlation coefficient: 0.957

a Average = $\Sigma x/n = \bar{x}$

b Standard deviation = $\left(\frac{n \Sigma x^2 - (\Sigma x)^2}{n(n-1)} \right)^{1/2} = \sigma$

c Coefficient of variation = $\sigma/\bar{x} \times 100$

d Percent inaccuracy = $\frac{\bar{x} - \text{ng added}}{\text{ng added}} \times 100$

e ND - Not detectable, less than 2 ng/g.

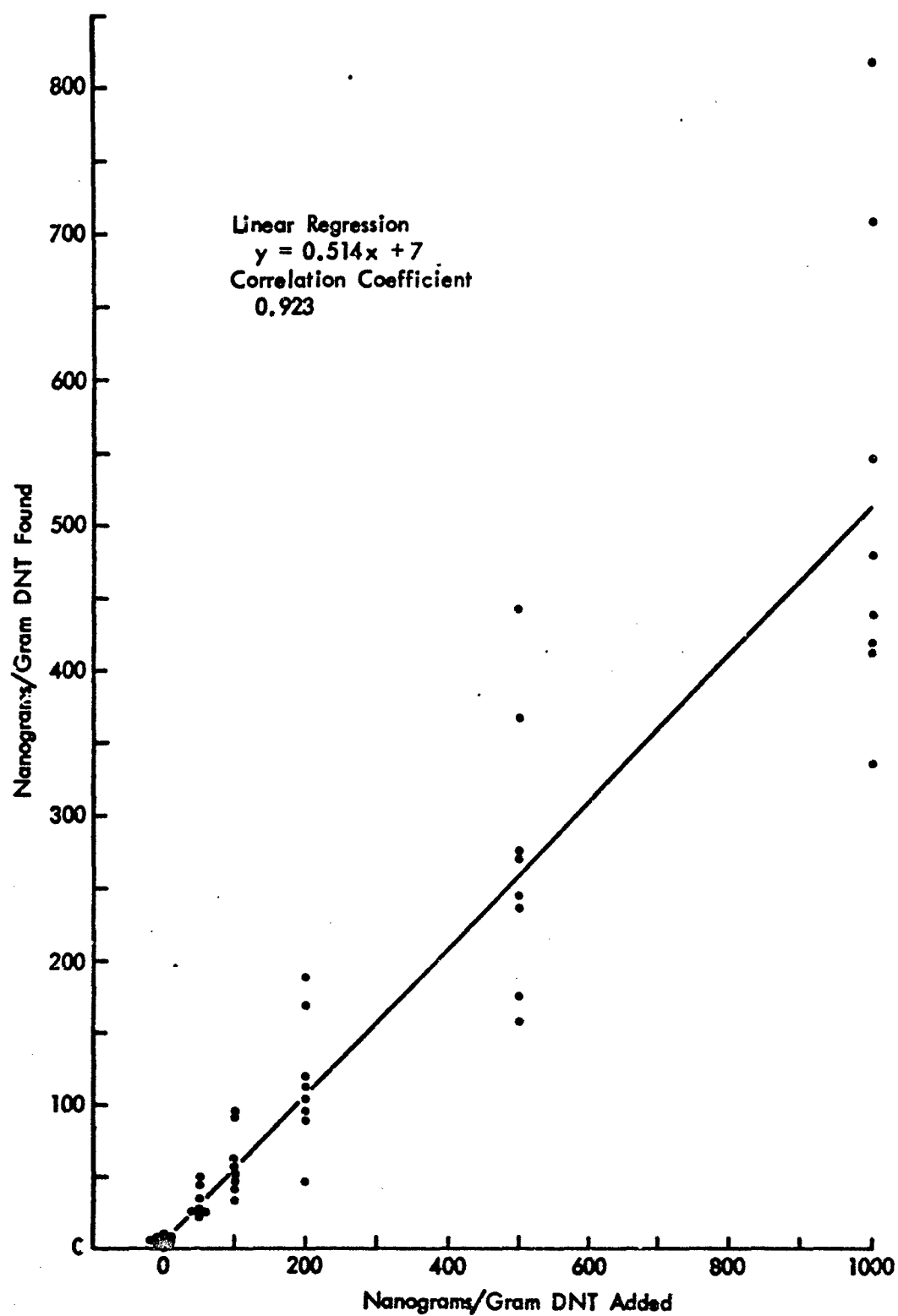


Figure 2 - Determination of DNT in Plant Stems

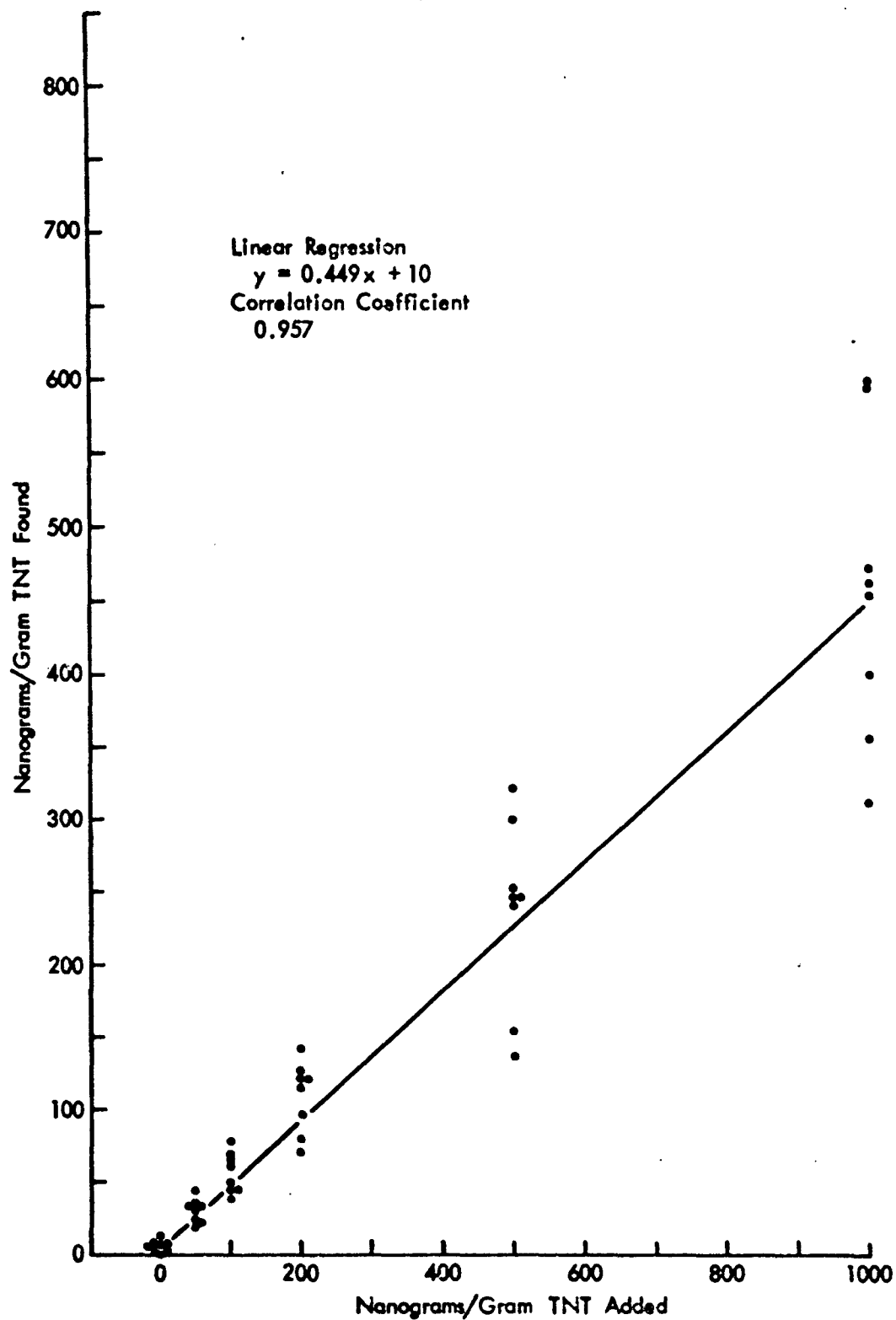


Figure 3 - Determination of TNT in Plant Stems

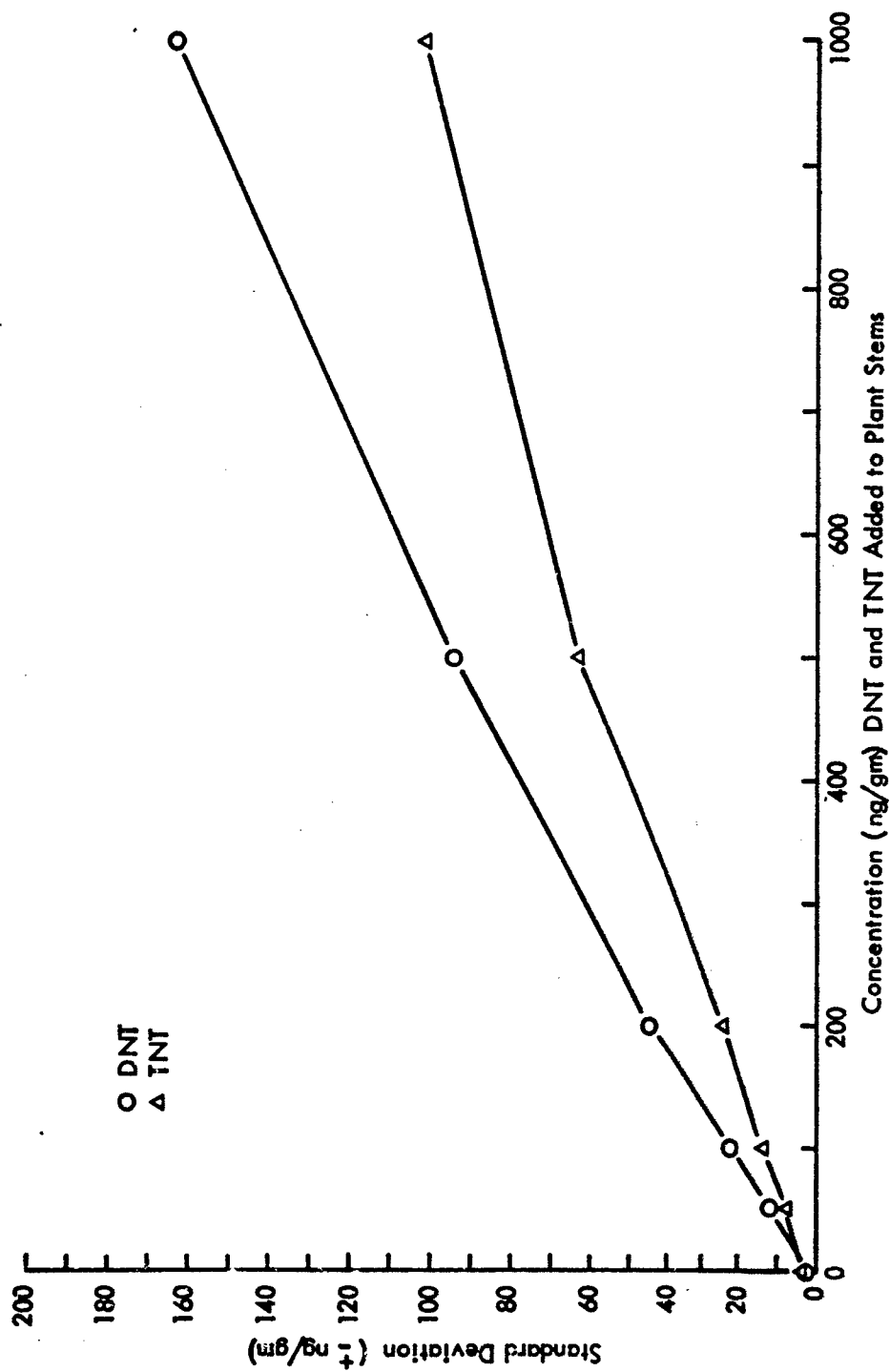


Figure 4 - Standard Deviation for DNT and TNT in Plant Stem Samples

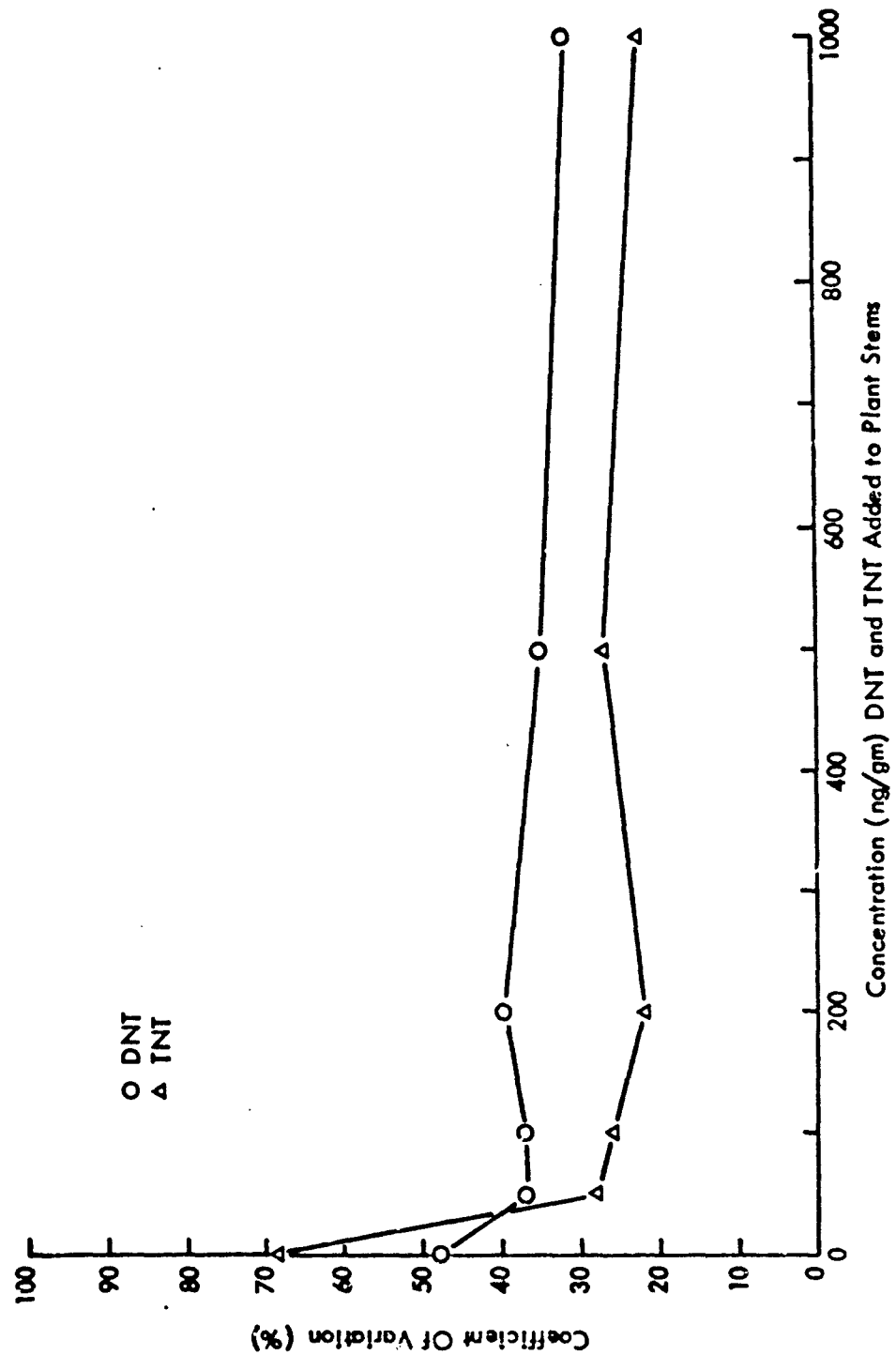


Figure 5 - Coefficient of Variation for DNT and TNT in Plant Stem Samples

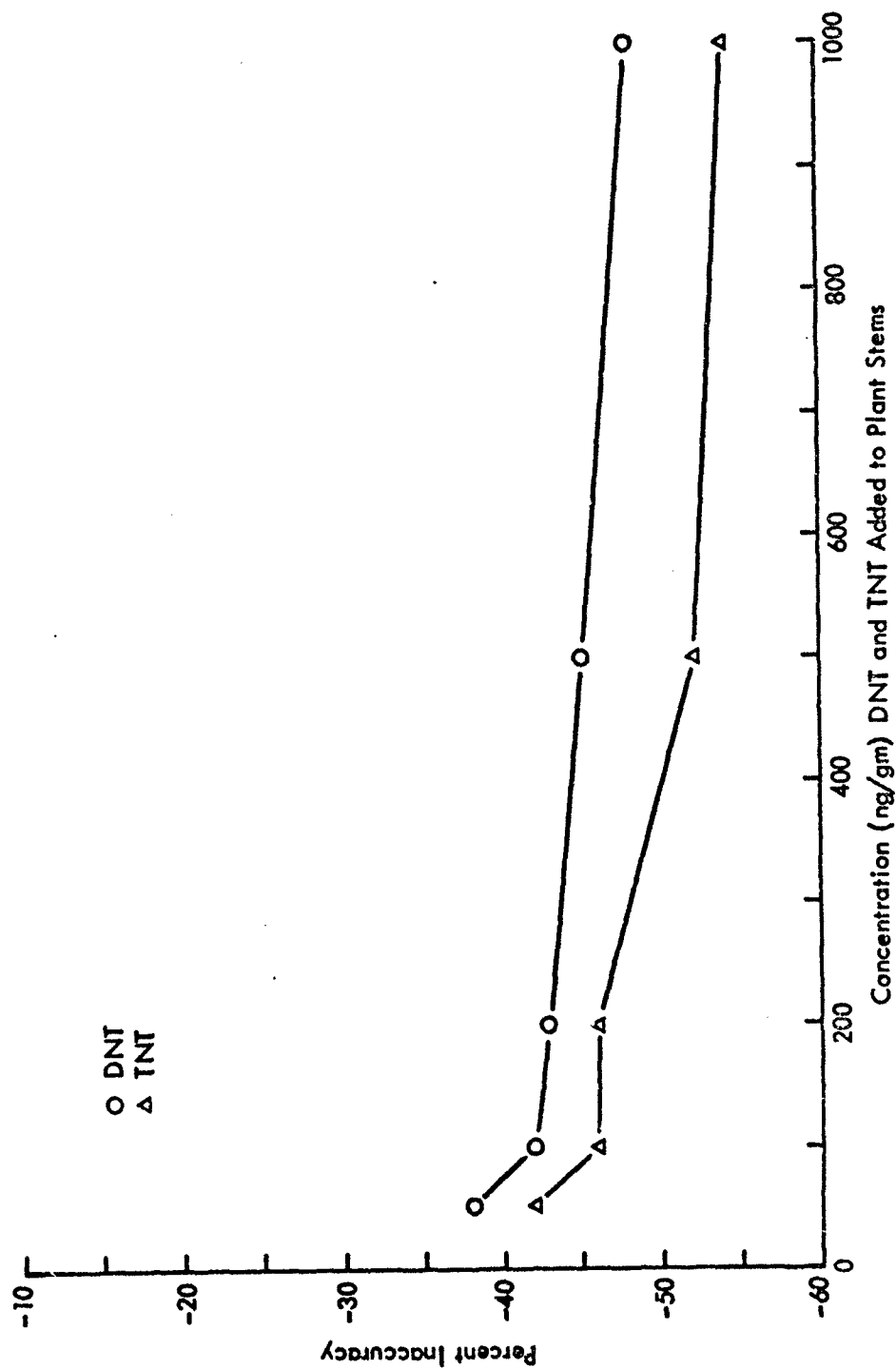
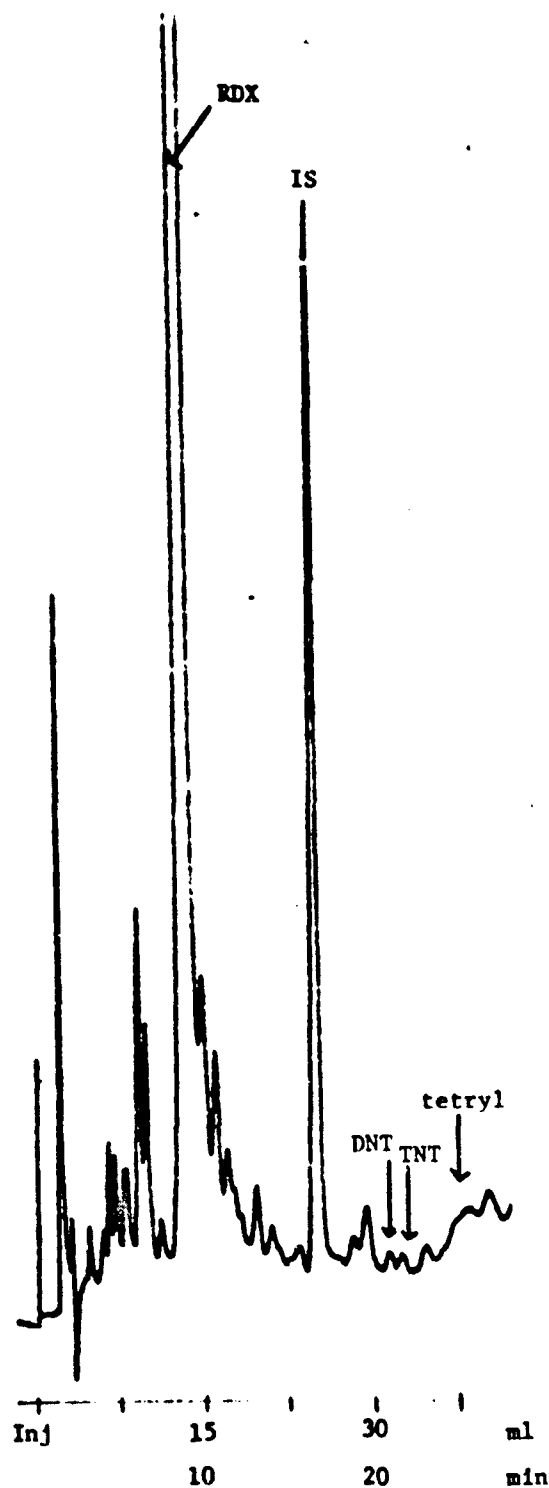


Figure 6 - Percent Inaccuracy for DNT and TNT in Plant Stem Samples



HPLC Conditions:

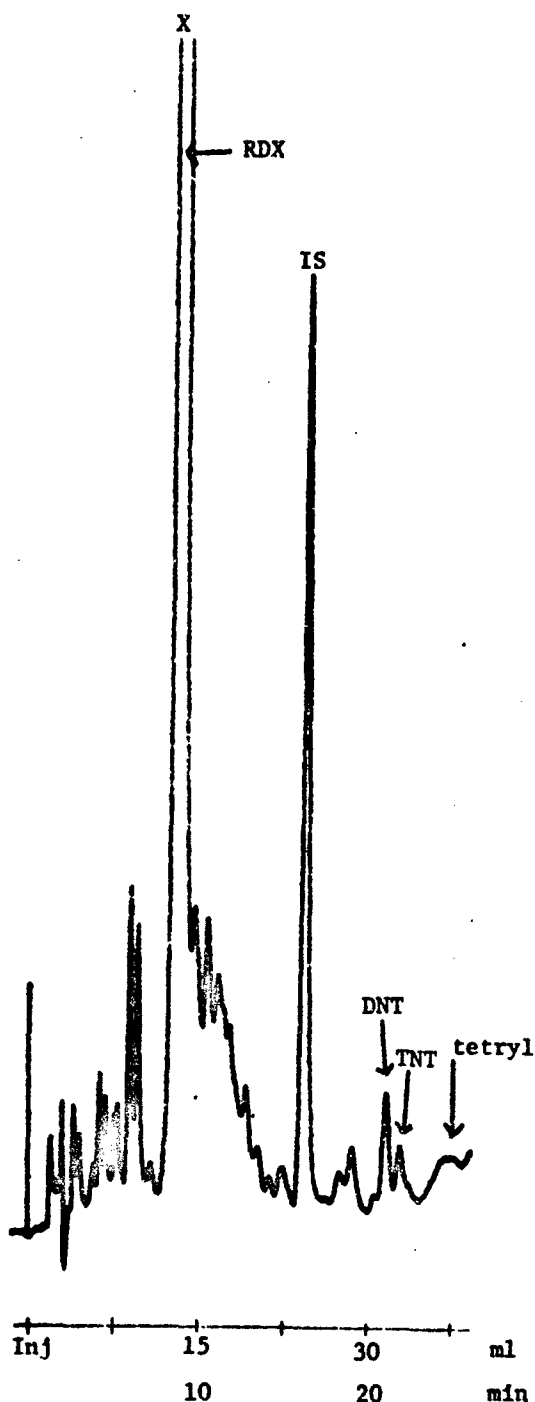
Column: Spherisorb ODS, 5 μ ,
250 x 4.6 mm ID
Eluent: 30% acetonitrile in
1% acetic acid in water
Flow Rate: 1.5 ml/min
Chart Speed: 0.1 in./min
Detector: UV, 254 nm

Sample Characteristics:

5.0 g plant stem extracted with
20 ml hexane (2% IPA). Extract
evaporated, and residue recon-
stituted with 500 μ l acetonitrile
and 500 μ l water.

IS Concentration: 1,000 ng/ml
Injection Volume: 70 μ l
Attenuation: 0.01 X

Figure 7 - HPLC Analysis of Blank Plant Stems. Sample for DNT and TNT Method Development. Arrows indicate elution positions for RDX, DNT, TNT, and tetryl.



HPLC Conditions:

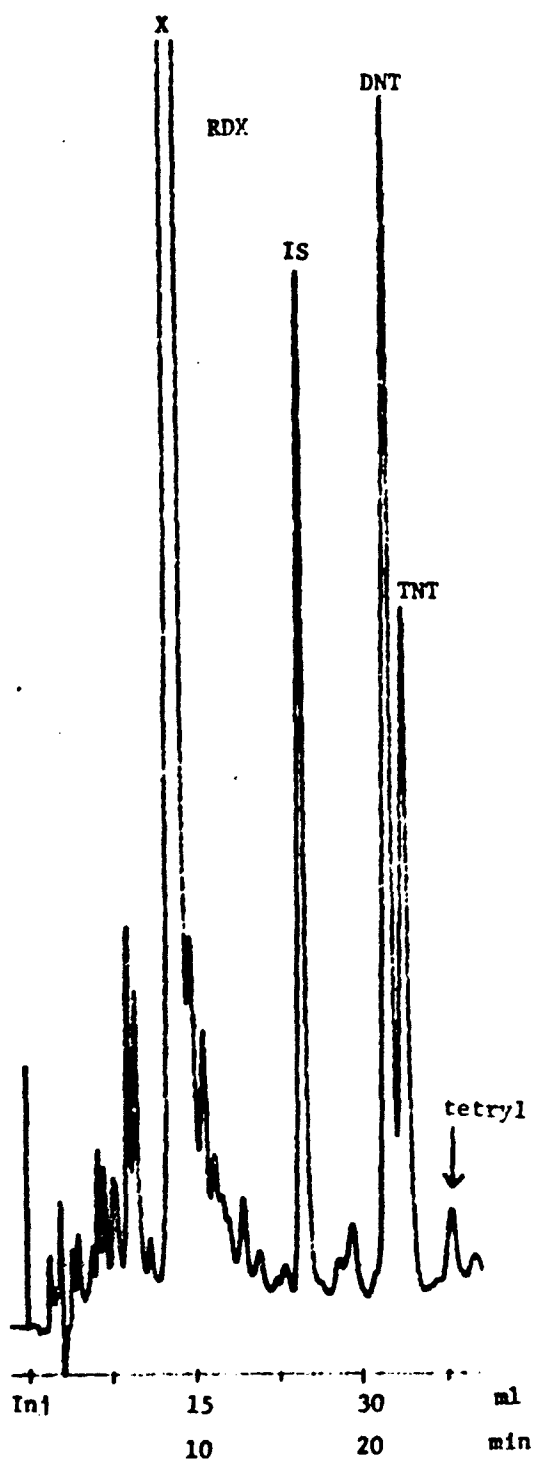
Column: Spherisorb ODS, 5 μ ,
 250 x 4.6 mm ID
 Eluent: 30% acetonitrile in
 1% acetic acid in water
 Flow Rate: 1.5 ml/min
 Chart Speed: 0.1 in./min
 Detector: UV, 254 nm

Sample Characteristics:

5.0 g plant stem containing 50 ng/g
 each munition extracted with 20 ml
 hexane (2% IPA). Extract evapor-
 ated, and residue reconstituted
 with 500 μ l acetonitrile and 500 μ l
 water.

IS Concentration: 1,000 ng/ml
 Injection Volume: 70 μ l
 Attenuation: 0.01 X

Figure 8 - HPLC Analysis of Plant Stems Containing 50 ng/g
 of RDX, DNT and TNT. "X" denotes plant component. Munition
 elution positions indicated by arrows.



HPLC Conditions:

Column: Spherisorb ODS, 5 μ ,
 250 x 4.6 mm ID
 Eluent: 30% acetonitrile in
 1% acetic acid in water
 Flow Rate: 1.5 ml/min
 Chart Speed: 0.1 in./min
 Detector: UV, 254 nm

Sample Characteristics:

5.0 g plant stem containing 500
 ng/g each munition extracted
 with 20 ml hexane (2% IPA).
 Extract evaporated, and residue
 reconstituted with 500 μ l aceto-
 nitrile and 500 μ l water.

IS Concentration: 1,000 ng/ml
 Injection Volume: 70 μ l
 Attenuation: 0.01 X

Figure 9 - HPLC Analysis of Plant Stems Containing 500 ng/g
 RDX, DNT, TNT, and Tetryl. "X" indicates elution position
 plant component interfering with RDX.

TABLE 4

**STATISTICAL EVALUATION OF DNT IN PLANT STEMS DATA BY
THE HUBAUX AND VOS DETECTION LIMIT PROGRAM**

Number ^a of Data points	Linear Regression	Correlation Coefficient	Degrees of Freedom	b	y ^c Intercept	Detection ^d Limit
48	y = 0.514 x + 7.0	0.923	46	1.679	59	196
40	y = 0.534 x + 4.5	0.904	38	1.686	37	117
32	y = 0.550 x + 3.3	0.860	30	1.697	22	65

ng/g DNT Added	Average ^e n/g/ Found	Standard ^f Deviation	Percent ^g Imprecision	Percent ^h Inaccuracy
0	7	± 0.7	18	-
50	33	± 4.3	14	- 39
100	58	± 8.2	14	- 42
200	110	± 17	15	- 43
500	264	± 35	13	- 46
1,000	521	± 62	12	- 48

a Number of data points - data points used to calculate linear regression and detection limits;

48 - all data; 40 - 1,000 ng/g samples omitted; 32 - 1,000 ng/g and 500 ng/g samples omitted.

b t - 2 tail p level (usually 0.1, each confidence band is 0.05 so total p = 0.1).

c y intercept - intercept on y-axis of upper confidence limit line.

d Detection limit - x-intercept of y-intercept and lower confidence limit line.

e Average ng/g found - average at each level determined from linear regression equation for 48 points.

f Standard deviation - determined from average value (e above) and observed values.

g Percent imprecision - standard deviation divided by average value times 100%.

h Percent inaccuracy - determined from the average values of the eight observed values at each level

$$\% \text{ Inaccuracy} = \frac{\text{Average observed values} - \text{level added}}{\text{level added}} \times 100$$

TABLE 5

**STATISTICAL EVALUATION OF TNT IN PLANT STEMS DATA BY
THE HUBAUX AND VOS DETECTION LIMIT PROGRAM**

Number ^a of Data points	Linear Regression	Correlation Coefficient	Degrees of Freedom	b	y ^c Intercept	Detection ^d Limit
48	y = 0.449 x +9.8	0.957	46	1.679	43	144
40	y = 0.464 x +7.8	0.940	38	1.686	29	90
32	y = 0.520 x +3.6	0.941	32	1.697	14	40

ng/g TNT Added	Average ^e n/g/ Found	Standard ^f Deviation	Percent ^g Imprecision	Percent ^h Inaccuracy
0	10	± 1.3	26	-
50	32	± 3.1	11	- 43
100	55	± 5.3	9.8	- 46
200	100	± 9.3	8.5	- 46
500	235	± 24	10	- 53
1,000	459	± 39	8.5	- 54

^a Number of data points - data points used to calculate linear regression and detection limits;
48 - all data; 40 - 1,000 ng/g samples omitted; 32 - 1,000 ng/g and 500 ng/g samples omitted.

^b t - 2 tail p level (usually 0.1, each confidence band is 0.05 so total p = 0.1).

^c y intercept - intercept on y-axis of upper confidence limit line.

^d Detection limit - x-intercept of y-intercept and lower confidence limit line.

^e Average ng/g found - average at each level determined from linear regression equation for 48 points.

^f Standard deviation - determined from average value (e above) and observed values.

^g Percent imprecision - standard deviation divided by average value times 100%.

^h Percent inaccuracy - determined from the average values of the eight observed values at each level

$$\% \text{ Inaccuracy} = \frac{\text{Average observed values} - \text{level added}}{\text{level added}} \times 100$$

APPENDIX

METHOD DEVELOPMENT FOR THE DETERMINATION
OF DNT AND TNT IN PLANT STEMS

RAW DATA AND CALCULATIONS

TABLE 6

LINEARITY AND PRECISION OF RDX, DNT, AND TNT DETERMINATION
BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Reference Solution Number	ng/ml Compound Added	Peak Height (mm)			Internal Standard ng ml	Relative Weight Response			Calculated ng/ml		
		RDX	DNT	TNT		RDX	DNT	TNT	RDX	DNT	TNT
A-1	0	< 2	< 2	< 2	1,000	-	-	-	ND	ND	ND
A-2	100	12	14	11	1,000	1.03	1.21	0.95	109	113	107
A-3	500	58	63	52	1,000	0.98	1.07	0.88	517	499	495
A-4	1,000	104	121	97	1,000	0.91	1.06	0.85	960	992	956
A-5	1,500	168	183	152	1,000	0.98	1.07	0.89	1,551	1,500	1,498
A-6	2,000	220	240	204	1,000	0.96	1.05	0.89	2,031	1,968	2,011
B-1	0	< 2	< 2	< 2	1,000	-	-	-	ND	ND	ND
B-2	100	13	14	12	1,000	1.08	1.17	1.00	114	109	112
B-3	500	59	64	52	1,000	0.97	1.05	0.85	509	490	479
B-4	1,000	121	145	118	1,000	0.90	1.08	0.88	950	1,011	989
B-5	1,500	172	184	157	1,000	0.98	1.05	0.89	1,547	1,470	1,508
B-6	2,000	228	246	208	1,000	0.98	1.06	0.90	2,069	1,982	2,015
C-1	0	< 2	< 2	< 2	1,000	-	-	-	ND	ND	ND
C-2	100	12	14	12	1,000	1.00	1.17	1.00	105	109	112
C-3	500	55	59	50	1,000	0.95	1.02	0.86	499	475	484
C-4	1,000	107	128	105	1,000	0.88	1.05	0.86	923	981	967
C-5	1,500	150	172	144	1,000	0.91	1.04	0.87	1,435	1,461	1,471
C-6	2,000	210	240	202	1,000	0.91	1.03	0.87	1,906	1,934	1,957
D-1	0	< 2	< 2	< 2	1,000	-	-	-	ND	ND	ND
D-2	100	11	12	10	1,000	0.98	1.07	0.89	103	100	100
D-3	500	54	60	50	1,000	0.93	1.03	0.86	490	483	484
D-4	1,000	116	137	110	1,000	0.94	1.10	0.89	985	1,032	997
D-5	1,500	155	174	144	1,000	0.91	1.03	0.85	1,444	1,439	1,432
D-6	2,000	212	236	194	1,000	0.91	1.02	0.84	1,924	1,901	1,879

TABLE 6 (concluded)

Relative Weight Response

	<u>Average</u>	<u>Standard Deviation</u>	<u>Relative Standard Deviation</u>
RDX	0.95	± 0.05	5.2%
DNT	1.07	± 0.05	4.9%
TNT	0.89	± 0.04	5.1%

TABLE 7

DETERMINATION OF DNT AND TNT IN PLANT STEMS SAMPLES

DAY 1 SAMPLES

Sample Number	ng/g ^a Compound Added	g Plant Stem	Peak Height (mm)		Internal ^b Standard		ng/g ^c Detected	
			DNT	TNT	ng/ml	Peak Height	DNT	TNT
Day 1A-0	0	5.0	1.0	3.8	1,000	165.5	2.2	11.1
Day 1A-250	50	5.0	8.8	8.0	1,000	164.0	20	24
Day 1A-500	100	5.0	14.5	12.5	1,000	163.5	32	37
Day 1A-1000	200	5.0	20.0	23.8	1,000	164.8	44	70
Day 1A-2500	500	5.0	71.5	46.0	1,000	163.5	159	136
Day 1A-5000	1,000	5.0	149.2	105.2	1,000	162.8	333	311
Day 1B-0	0	5.0	< 1.0	< 1.0	1,000	166.8	ND ^d	ND
Day 1B-250	50	5.0	10.0	9.8	1,000	165.8	22	28
Day 1B-500	100	5.0	20.8	14.0	1,000	166.0	46	41
Day 1B-1000	200	5.0	39.8	26.2	1,000	163.0	89	78
Day 1B-2500	500	5.0	77.0	51.0	1,000	159.8	175	154
Day 1B-5000	1,000	5.0	184.8	120.5	1,000	163.2	412	356

TABLE 7 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)		Standard ng/ml	Peak Height	Relative Weight Response	
		DNT	TNT			DNT	TNT
Std - Day 1-2	200	32.0	24.0	1,000	150.5	1.06	0.80
Std - Day 1-1	100	16.2	12.0	1,000	151.8	1.07	0.79
Std - Day 1-4	500	82.5	64.5	1,000	148.5	1.11	0.87
Std - Day 1-1	100	16.0	12.0	1,000	147.5	1.08	0.81
				Average		1.08	0.82
				Standard Deviation		± 0.022	± 0.036

26
296

^a ng/g compound added - nanograms of DNT and TNT added per gram of plant stems sample.

^b Internal standard - compound (propionophenone) added to plant stems sample after sample preparation for calculation of data.

^c ng/g detected - nanograms of DNT and TNT detected in the plant stems sample.

$$\text{ng compound/g} = \frac{\text{Peak Height Compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}} \times \frac{1}{\text{Sample Weight (g)}}$$

^d ND - not detected, less than 2 ng/g.

^e Relative weight response - RWR.

$$\text{RWR} = \frac{\text{Peak Height Compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

TABLE 8

DETERMINATION OF DNT AND TNT IN PLANT STEMS SAMPLES

DAY 2 SAMPLES

Sample Number	ng/g ^a Compound Added	g Plant Stem	Peak Height (mm)		Internal ^b Standard		ng/g ^c Detected	
			DNT	TNT	ng/ml	Peak Height	DNT	TNT
Day 1A-0	0	5.0	2.0	2.0	1,000	156.6	5	6
Day 1A-250	50	5.0	20.8	11.0	1,000	161.0	49	32
Day 1A-500	100	5.0	37.2	20.0	1,000	153.0	91	62
Day 1A-1000	200	5.0	74.0	39.8	1,000	149.0	186	127
Day 1A-2500	500	5.0	152.0	98.2	1,000	155.2	367	301
Day 1A-5000	1,000	5.0	295.6	196.0	1,000	156.4	707	597
Day 1B-0	0	5.0	2.0	2.0	1,000	143.0	5	7
Day 1B-250	50	5.0	17.4	9.0	1,000	139.0	47	31
Day 1B-500	100	5.0	43.0	23.6	1,000	178.0	90	63
Day 1B-1000	200	5.0	72.0	40.0	1,000	159.2	169	120
Day 1B-2500	500	5.0	176.0	101.6	1,000	150.0	439	322
Day 1B-5000	1,000	5.0	340.4	194.8	1,000	156.4	814	593

TABLE 8 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)		Internal ^b Standard		Relative Weight ^c Response	
		DNT	TNT	ng/ml	Peak Height	DNT	TNT
Std - Day 2-3	500	69.2	53.8	1,000	128.2	1.08	0.84
Std - Day 2-2	200	27.8	21.2	1,000	129.2	1.08	0.82
Std - Day 2-5	2,000	311.0	240.0	1,000	144.4	1.08	0.83
Std - Day 2-2	200	29.0	23.4	1,000	137.0	1.06	0.85
				Average		1.07	0.84
				Standard Deviation		± 0.010	± 0.014

28
298

- ^a ng/g Compound added - nanograms of DNT and TNT added per gram of plant stems sample.
^b Internal standard - compound (propiphenone) added to plant stems sample after sample preparation for calculation of data.
^c ng/g detected - nanograms of DNT and TNT detected in the plant stems sample

$$\text{ng compound/g} = \frac{\text{Peak Height Compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}} \times \frac{1}{\text{Sample Weight (g)}}$$

^d ND - not detected, less than 2 ng/g.

^e Relative weight response - RWR

$$\text{RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

TABLE 9

DETERMINATION OF DNT AND TNT IN PLANT STEMS SAMPLES

DAY 3 SAMPLES

Sample Number	ng/g ^a Compound Added	g Plant Stem	Peak Height (mm)		Internal ^b Standard		ng/g ^c Detected	
			DNT	TNT	ng/ml	Peak Height	DNT	TNT
Day 1A-0	0	5.0	2.5	1.0	1,000	163.0	5	3
Day 1A-250	50	5.0	14.6	15.4	1,000	159.6	32	44
Day 1A-500	100	5.0	22.0	26.0	1,000	159.0	49	75
Day 1A-1000	200	5.0	51.6	42.4	1,000	163.0	112	120
Day 1A-2500	500	5.0	124.0	85.0	1,000	160.0	274	244
Day 1A-5000	1,000	5.0	210.0	156.0	1,000	155.2	479	462
Day 1B-0	0	5.0	2.0	2.0	1,000	157.0	4	6
Day 1B-250	50	5.0	10.2	10.0	1,000	144.0	25	32
Day 1B-500	100	5.0	17.0	15.0	1,000	144.0	42	48
Day 1B-1000	200	5.0	48.0	35.0	1,000	166.0	102	97
Day 1B-2500	500	5.0	124.8	85.0	1,000	162.8	271	240
Day 1B-5000	1,000	5.0	187.6	157.6	1,000	159.6	416	454

TABLE 9 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)		Internal ^b Standard		Relative Weight ^c Response	
		DNT	TNT	ng/ml	Peak Height	DNT	TNT
Std - Day 3-5	1,000	146.0	116.0	1,000	134.0	1.09	0.86
Std - Day 3-1	100	16.6	13.0	1,000	142.0	1.17	0.91
Std - Day 3-3	500	86.0	64.0	1,000	152.0	1.13	0.84
Std - Day 3-2	200	35.0	26.0	1,000	152.0	1.15	0.86
Average						1.13	0.87
Standard Deviation						± 0.034	± 0.032

30

300

^a ng/g Compound added - nanograms of DNT and TNT added per gram of plant stems sample.
^b Internal standard - compound (propiphenone) added to plant stems sample after sample preparation for calculation of data.

^c ng/g detected - nanograms of DNT and TNT detected in the plant stems sample

$$\text{ng compound/g} = \frac{\text{Peak Height Compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}} \times \frac{1}{\text{Sample Weight (g)}}$$

^d ND - not detected, less than 2 ng/g.

^e Relative weight response - RWR

$$\text{RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

TABLE 10
DETERMINATION OF DNT AND TNT IN PLANT STEMS SAMPLES

DAY 4 SAMPLES

Sample Number	ng/g ^a Compound Added	g Plant Stem	Peak Height (mm)		Internal ^b Standard		ng/g ^c Detected	
			DNT	TNT	ng/ml	Peak Height	DNT	TNT
Day 1A-0	0							
Day 1A-250	50	5.0	2.0	1.0	1,000	166.4	4	3
Day 1A-500	100	5.0	11.8	6.4	1,000	165.0	25	18
Day 1A-1000	200	5.0	27.2	24.0	1,000	169.4	56	67
Day 1A-2500	500	5.0	53.0	47.6	1,000	160.4	115	140
Day 1A-5000	1,000	5.0	109.0	84.0	1,000	161.6	235	245
			210.0	142.8	1,000	168.0	435	401
Day 1B-0	0							
Day 1B-250	50	5.0	2.0	1.0	1,000	158.0	4	3
Day 1B-500	100	5.0	11.0	7.0	1,000	161.0	24	21
Day 1B-1000	200	5.0	29.6	16.0	1,000	181.4	57	42
Day 1B-2500	500	5.0	42.4	40.0	1,000	162.4	91	116
Day 1B-5000	1,000	5.0	117.0	90.0	1,000	167.8	243	253
			264.0	169.2	1,000	168.8	544	472

TABLE 10 (concluded)

REFERENCE SOLUTIONS

Reference Solution Number	ng/ml ^a Compound Added	Peak Height (mm)		Internal ^b Standard		Relative Weight ^c Response	
		DNT	TNT	ng/ml	Peak Height	DNT	TNT
Std - Day 4-5	1,000	178.0	130.0	1,000	159.0	1.12	0.82
Std - Day 4-1	100	18.0	13.4	1,000	154.0	1.17	0.87
Std - Day 4-2	200	35.6	26.0	1,000	151.4	1.18	0.86
Std - Day 4-3	500	94.2	70.0	1,000	165.4	1.14	0.85
				Average		1.15	0.85
				Standard Deviation		± 0.026	± 0.022

^a ng/g Compound added - nanograms of DNT and TNT added per gram of plant stems sample.

^b Internal standard - compound (propiphenone) added to plant stems sample after sample preparation for calculation of data.

^c ng/g detected - nanograms of DNT and TNT detected in the plant stems sample

$$\text{ng compound/g} = \frac{\text{Peak Height Compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{average RWR compound}} \times \frac{1}{\text{Sample Weight (g)}}$$

^d ND - not detected, less than 2 ng/g.

^e Relative weight response - RWR

$$\text{RWR} = \frac{\text{Peak Height compound}}{\text{Peak Height IS}} \times \frac{\text{ng/ml IS}}{\text{ng/ml compound}}$$

DISTRIBUTION LIST

<u>Distribution</u>	<u>No. of Copies</u>
DCASMA, Kansas City Room 201, Noland Plaza Office Building 3675 South Noland Road Independence, MO 64055	1
Defense Logistics Studies Information Exchange U.S. Army Logistics Management Center Fort Lee, VA 23801	2
Defense Technical Information Center Cameron Station Alexandria, VA 22314	12
Commander U.S. Army Toxic and Hazardous Materials Agency Attn: DRXTH-TE-A	4
Attn: DRXTH-ES Aberdeen Proving Ground, MD 21010	2
Commander U.S. Army Environmental Hygiene Agency Attn: HSE-AA-L Aberdeen Proving Ground, MD 21010	1
Commander-Director Chemical Systems Laboratory Attn: DRDAR-CLB-R Aberdeen Proving Ground, MD 21010	1
Dr. David H. Rosenblat U.S. Army Medical Bioengineering Research and Development Laboratories Fort Detrick Frederick, MD 21701	1
Cold Regions Research and Engineering Laboratories Box 282 Lyme Road Hanover, NH 03755	1