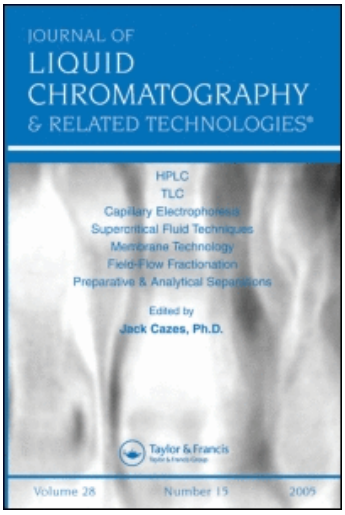

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Determination of Explosives and Some Metabolites of TNT in Biological and Environmental Samples by Liquid Chromatography on a Mixed-Mode C_{18}-anion Column

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DETERMINATION OF EXPLOSIVES AND SOME METABOLITES OF TNT IN BIOLOGICAL AND ENVIRONMENTAL SAMPLES BY LIQUID CHROMATOGRAPHY ON A MIXED-MODE C_{18}-ANION COLUMN

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ABSTRACT

This method employs a ternary gradient generated from (1) a 90:10 water:methanol solution that is 0.015 M in potassium phosphate at pH 5.1; (2) methanol; and (3) acetonitrile to separate fifteen explosives, byproducts, and metabolites of 2,4,6-trinitrotoluene (TNT) on a C_{18}/anion exchange stationary phase. TNT, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), six reduction metabolites and three oxidation metabolites of TNT have been separated and quantitated in a single chromatographic run. Because of the anion exchange character of the stationary phase, the anionic TNT metabolite 2,4,6-trinitrobenzoic acid also may be separated and quantitated using this method. Quantitation limits generally lower than 1 ppm have been established by a systematic certification procedure. The performance of the mixed mode separation method combined with diode array ultraviolet absorbance detection has been demonstrated with

samples derived from both biological (tissues and physiological fluids) and environmental (soil, composts, and leachates) sources.

INTRODUCTION

Remedial action and environmental restoration require fast and efficient methods for determining both explosives and their transformation products or metabolites. Methods that have been utilized for the determination of explosives are quite diverse including gas chromatography,[1] liquid chromatography.[2-6] liquid chromatography-mass spectrometry,[7-9] thin layer chromatography,[10] micellar electrokinetic capillary chromatography,[11] and supercritical fluid chromatography.[12-14] Reverse phase high performance liquid chromatography has emerged as the method most often used. However, the separation and determination problem becomes more complicated when an explosive compound such as 2,4,6-trinitrotoluene (TNT) is exposed to environmental or biological processes which lead to the formation of transformation products or metabolites.[15-17] Characterization of the metabolites resulting from environmental and/or biological action on both TNT[15-23] and dinitrotoluenes[24-30] have shown that nitrotoluenes are susceptible to both oxidation and reduction processes. Some of the known transformation products generated from TNT are listed in Table 1. The reduction process[17] involves attack on the nitro- function with the formation of a hydroxylamino-compound such as 4-hydroxylamino-2,6-dinitrotoluene (4OHA). This hydroxylamino compound, (4OHA), may dimerize to form 2,6,2',6'-tetranitro-4,4'-azoxytoluene (44'AT) or it may be further reduced to 4-amino-2,6-dinitrotoluene (4ADNT). The monoaminodinitro-compound may be further reduced to the corresponding diaminonitro-compound, 2,4-diamino-6-nitrotoluene (24DANT).

TNT is also susceptible to oxidation whereby the methyl group may be attacked to first form trinitrobenzyl alcohol (TNBOH), which can be further oxidized to trinitrobenzoic acid (TNBCOOH). Subsequently, the acid may be decarboxylated to form 1,3,5-trinitrobenzene (TNB).[31] Because the possible transformation products are both acidic and basic with a wide range of polarities it is difficult to develop a single procedure for the separation of all metabolites.[2,22] Compounds representing the range of TNT metabolites have been separated on a C_8 reverse phase column using a series of isocratic elutions with different acetonitrile:water mixtures.[22] In this present work a wide range of explosives and of TNT metabolites, (including the essentially anionic

Table 1

Listing of Abbreviations and Some Chromatographic Characteristics for Explosives and Some Metabolites of 2,4,6-Trinitrotoluene

COMPOUND (Abbreviation)	QL[1]	K[2]	A[3]
Explosives or ByProducts			
2,4,6-Trinitrotoluene (TNT)	0.2	5.36	1.22
Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)	0.4	4.47	1.30
Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)	0.4	6.51	1.25
2,4-Dinitrotoluene (24DNT)	0.4	6.11	1.21
2,6-Dinitrotoluene (26DNT)	0.5	6.08	1.24
1,3-Dinitrobenzene (13DNB)	0.2	4.12	1.23
Reduction Metabolites of TNT			
2-Amino-4,6-dinitrotoluene (2ADNT)	0.2	8.40	1.19
4-Amino-2,6-dinitrotoluene (4ADNT)	0.6	7.80	1.20
2,4-Diamino-6-nitrotoluene (24DANT)	0.7	1.84	1.37
2,6-Diamino-4-nitrotoluene (26DANT)	0.2	1.41	1.40
4-Hydroxyamino-2,6-dinitrotoluene (4OHA)	0.8	12.2	1.39
2,2'6,6'-Tetranitro-4,4'-azoxytoluene (44AT)	2.7	13.3	1.35
Oxidation Metabolites of TNT			
2,4,6-Trinitrobenzyl alcohol (TNBOH)	0.5	2.58	1.23
2,4,6-Trinitrobenzoic acid (TNBCOOH)	0.4	16.9	1.23
1,3,5-Trinitrobenzene (TNB)	0.2	3.18	1.32

[1] Quantitation Limit expressed as ppm concentration of injected sample when a 50 μL aliquot is injected, calculated per Hubaux and Vos (33).

[2]Capacity factor: ratio of retention time for given component to time to elute void volume from the system.

[3]Peak Asymmetry expressed as a ratio of the peak area after the peak maximum to the peak area before the maximum peak height.

metabolite, TNBCOOH) have been separated by a single gradient elution on a C_{18}/anion mixed mode column and determined using diode array UV absorbance detection.

MATERIALS

Analyses were carried out on a Hewlett-Packard Model 1090M Liquid Chromatograph equipped with a DR5 ternary solvent delivery system, a diode array ultraviolet (UV)-visible wavelength detector and the Hewlett-Packard 79994A ChemStation data system. This liquid chromatography system was fitted with an Alltech (Deerfield, IL) RP-C_{18}/Anion Mixed-Mode reverse phase/anion-exchange column (Product No. 72628) that was 150 mm X 4.6 mm. The mixed-mode support was bonded to spherical silica with a pore size of 100 Å and a particle size of 5 μM. The column cartridge was fitted with a matching guard cartridge (10 mm X 4.6 mm, Alltech No. 28013) containing the same mixed-mode packing.

Water and all organic solvents were "Baker Analyzed" HPLC reagent grade obtained from J. T. Baker, Inc. (Phillipsburg, NJ). 2ADNT, 4ADNT, 24DANT, 26DANT, and 13DNB were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). TNT, TNBCOOH, and TNB were purchased from ChemService (West Chester, PA). RDX (Lot No. 1130), HMX (Lot No. 1217), 24DNT (Lot No. 1147), and 26DNT (Lot No. 1148) were Standard Analytical Reference Materials (SARMS) obtained from the U. S. Army Environmental Center SARMS Repository (Aberdeen Proving Ground, MD). TNBOH, 44'AT, and 4OHA were synthesized in this laboratory by established procedures which are described elsewhere.[16] Monobasic and dibasic potassium phosphate were "Baker Analyzed" reagent obtained from J. T. Baker, Inc.

Except for standards prepared in HPLC-grade solvents, all samples were filtered through 0.μm nylon syringe filters that were 13 mm in diameter (Gelman No. 4427, Gelman Sciences, Ann Arbor, MI).

METHODS

The three eluting solutions for the ternary gradient were (A) 90:10 water:methanol (v/v) solution that was 0.015 M in potassium phosphate, (B)

methanol, and (C) acetonitrile. Eluting solution A was prepared by dissolving 4 grams (294 mmoles) of potassium dihydrogen phosphate in 1800 mL water and then adjusting the pH to 5.1 with approximately 0.8 mL of a 0.5 M stock solution of dipotassium hydrogen phosphate. Subsequently 200 mL of methanol were added causing the apparent pH to rise to 5.4. Other pH and phosphate concentrations in the 90:10 water:methanol eluting solution were prepared by adjusting the molar proportions and amounts of potassium dihydrogen phosphate and dipotassium hydrogen phosphate added to the water before adding the methanol.

Two gradient elution programs, which are summarized in Table 2, were developed for the C_{18}/Anion column. The longer gradient program (method 1, 45 minutes) starts by equilibrating the system with a mixture that contains 72% of the buffered water:methanol solution and 28 % acetonitrile. This equilibration eluent is continued for 1 min after the injection of a 50-µl sample aliquot. From 1 to 5 min a linear gradient reduces the proportion of the buffered water:methanol solution to 68% and increases the acetonitrile to 32%. Isocratic elution conditions are then maintained for the time interval from 5 to 14 min. Between 14 and 20 min a linear gradient reduces the buffered water:methanol solution by 4% per min and increases both methanol and acetonitrile by 2% per min so that at 20 min the eluting solution composition is 44% buffered water:methanol, 12 % methanol and 44% acetonitrile. From 20 to 26 min a linear gradient reduces the buffered water:methanol portion to 2% and increases methanol from 12% to 54% while maintaining the acetonitrile at 44%. Isocratic conditions are maintained from 26 to 33 min. From 33 to 38 min after injection a very steep gradient restores the eluting solution mixture to its initial conditions after which the column is re-equilibrated for 7 min.

The shorter ternary gradient program summarized in Table 2 (method 2) is 25 min in duration. This shorter program will work for sample mixtures that do not contain either TNBCOOH or interfering constituents that are strongly retained by the column.

Environmental solid samples such as soil were prepared by mixing one-gram portions with 4 mL of acetonitrile in a tightly capped vial and extracting for 18 hours in a cooled ultrasonic bath.[31] These extracts were then filtered through a 0.2 µm nylon membrane syringe filter before HPLC analysis. Aqueous leachates of composts were prepared according to the EPA SW-846 method 1312.[32] Organic extracts of tissue were also filtered in the same

Table 2

Ternary Gradient Programs for Elution of Explosives and TNT Metabolites on C_{18}/Anion Column with Flow Rate of 1mL/min

Method 1	% Buffer	% Methanol	%Acetonitrile	Method 2
0 min	72	0	28	0 min
1 min	72	0	28	0 min
5 min	68	0	32	5 min
14 min	68	0	32	11 min
20 min	44	12	44	15 min
26 min	2	54	44	18 min
33 min	2	54	44	21 min
38 min	72	0	28	22 min
45 min	72	0	28	25 min

manner following a preparation procedure described elsewhere[16] that involved tissue homogenization and ultrasonic cell disruption before the solvent extraction.

RESULTS AND DISCUSSION

The separation of a 15-component mixture of explosives and metabolites of TNT following the 45-minute gradient program is shown in Figure 1. To test the influence of the total concentration of phosphate in the 90:10 water:methanol component of this gradient the separation was carried out at three phosphate concentrations, (0.0075 M, 0.015 M and 0.03 M) during which the pH of the aqueous component was held constant at 5.1. The influence of pH was observed by holding the total phosphate concentration at 0.015 M while adjusting the pH of the aqueous component to 4.5, 5.1, 5.3, 5.7, and 6.4. Although the capacity factors for components other than TNBCOOH are not significantly influenced by pH and ionic strength, as illustrated in Figures 2 and 3, there is a major effect on peak asymmetry for some other components. In Figures 4 and 5 the influence of pH and phosphate concentration is shown for all components found to have a peak shape falling outside an asymmetry range between 0.9 and 1.4. The peak asymmetry for all 15 components is listed in Table 1 for pH 5.1 and a total phosphate concentration of 0.015 M. This was

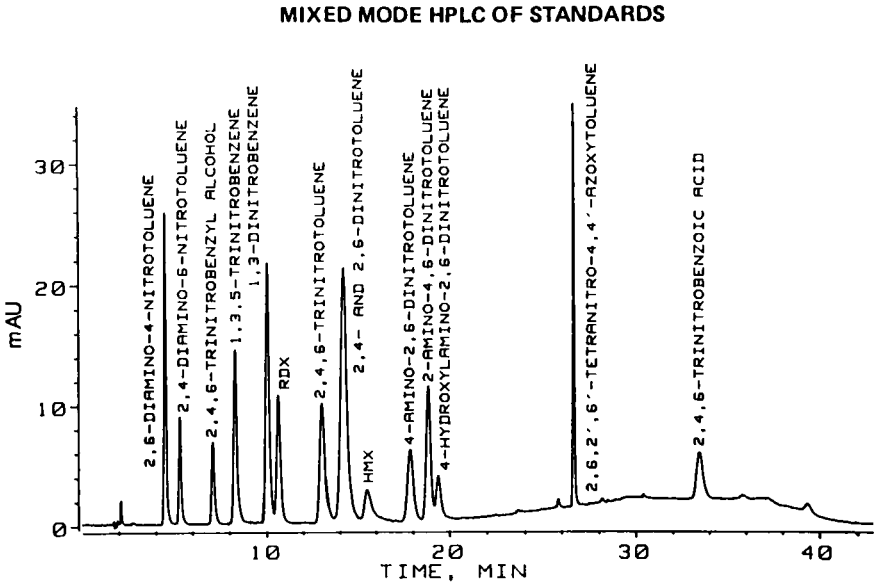

Figure 1. Chromatogram of explosives and TNT metabolites on C18/Anion column using the longer ternary gradient program summarized in Table 1. Chromatogram was monitored at 254 nm. Injection volume was 50 μL and the concentration of most components was 5 ppm.

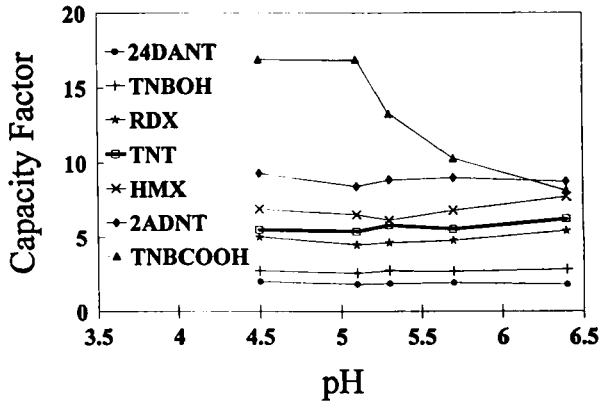

Figure 2. Plots showing the variation in retention with pH at a constant phosphate concentration of 0.015 M. Of all the compounds listed in Table 2 only TNBCOOH showed a significant trend between pH 4.5 and pH 6.4.

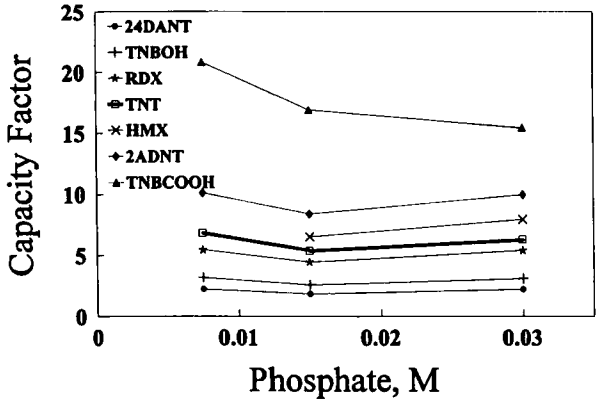

Figure 3. Plots showing the variation in capacity factor with changing total phosphate concentration at pH 5.1. Of all the compounds listed in Table 1 only TNBCOOH showed a significant trend toward lower retention as total phosphate concentration increased from 0.0075 M to 0.03 M.

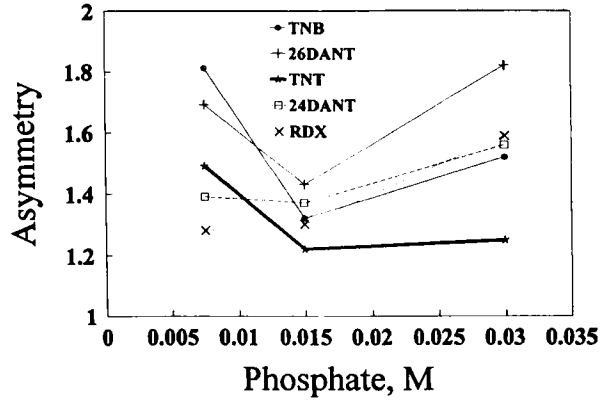

Figure 4. Plots showing the effect of total phosphate concentration on peak asymmetry. Only those compounds with peak asymmetry exceeding 1.4 at one or more of the phosphate concentrations tested are shown and compared to TNT.

the only set of conditions tested in which all components showed a peak asymmetry within the range of 1 to 1.4.

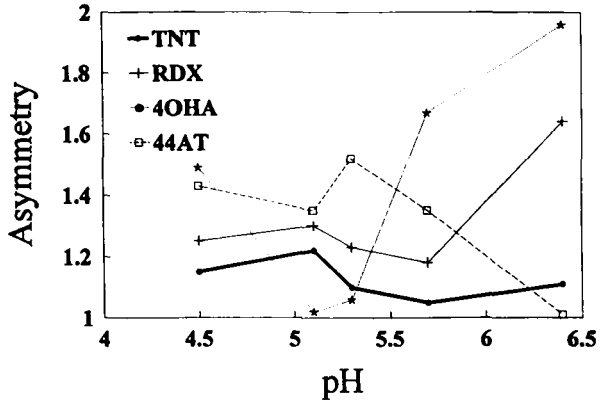

Figure 5. Plots showing the effect of pH on peak asymmetry. Only those compounds with peak asymmetry exceeding 1.4 at one or more of the pH levels tested are shown and compared to TNT.

The anion exchange character of the mixed-mode column would not appear to be a major factor in the retention of any of the tested components except TNBCOOH, for which significant changes in retention were observed for variations in both pH and total phosphate concentration . The mixed-mode column capacity for TNBCOOH was observed to be maximal at lower values of pH and lower total phosphate concentrations. Increasing phosphate concentrations at a given pH should decrease retention by the anion exchange sites on the column because of the increasing amount of anions (phosphate) available in the eluent to displace the anionic TNBCOOH. This same mass action effect of anions in the eluent also explains the decrease in retention with increasing pH at a constant total phosphate concentration. TNBCOOH is a relatively strong acid that should be almost completely ionized in very dilute solutions above pH 4.5. Therefore the fraction of TNBCOOH in the anionic form does not change significantly as pH increases above 4.5. On the other hand, at pH 4.5 the phosphate should be present almost exclusively in the singly ionized dihydrogen phosphate form. Between pH 4.5 and 6.5 the fraction of phosphate present in the doubly ionized hydrogen phosphate form increases by nearly two orders of magnitude.

In short, the total anionic eluting strength of a given total phosphate concentration increases substantially over the pH range tested. This assertion

that the anion exchange characteristics of the mixed mode column are principally responsible for the retention of the TNBCOOH is also supported by observations in other work. Yinon and Hwang[22] separated various groups of TNT metabolites including TNBCOOH using a series of isocratic elutions (acetonitrile:water eluents) on a C_8 column. In these reverse phase separations the TNBCOOH eluted very near the solvent front and always ahead of TNT, TNT metabolites (including 24DANT, 26DANT, 2ADNT, 4ADNT, 4OHA, and TNBOH), and even solvents such as acetone and methylene chloride.

Quantitation limits have been determined by the approach described by Hubaux and Vos[33] and are listed in Table 1 as sample concentrations when a 50 μL sample aliquot is injected. Quantitation was performed at 254 nm. The quantitation limits are generally 0.2 - 0.8 ppm. This 45-minute gradient method has also been certified for extracts and leachates of soils and composts using the U. S. Army Environmental Center's Quality Assurance Program Precertification and Certification Procedure.[34]

The performance of the mixed mode separation coupled with diode array UV absorbance detection is illustrated by its extensive application to the determination of explosives and TNT metabolites in soils contaminated with explosives, composts of those soils, aqueous leachates of the soils and composts,[35-36] and in tissues from animals living in highly contaminated sites[16] In the soils, TNT, HMX and RDX were the main contaminants detected. During composting, the rapid biotransformation of TNT led to the early appearance of its reduction metabolites. The 2A46DNT and 4A26DNT were observed in both the fresh composts and their leachates. The 24DA6NT and 26DA4NT grew in as the monoaminonitrotoluenes decreased in abundance during the course of composting, and then decreased as composting continued. Except for an azoxy dimer (44'AT) in leachates prepared from soils in the early stages of composting, no other transformation products or reduction/oxidation metabolites of TNT (or HMX and RDX) were observed.

Figure 6 shows the chromatograms (using the shorter gradient program) for the leachates and acetonitrile extract of a soil after composting for 5 days. HMX, RDX, TNT, and its metabolites are clearly separated and free of detectable interferences. The azoxy dimer, 44'AT, was of particular interest because it has only very rarely been observed. It was observed only in the leachate, suggesting that the 18-hr tumbling in water at room temperature promoted further reactions of TNT metabolites present in the soil compost. The dimer was identified by its retention time and UV spectrum (Figure 7A) in

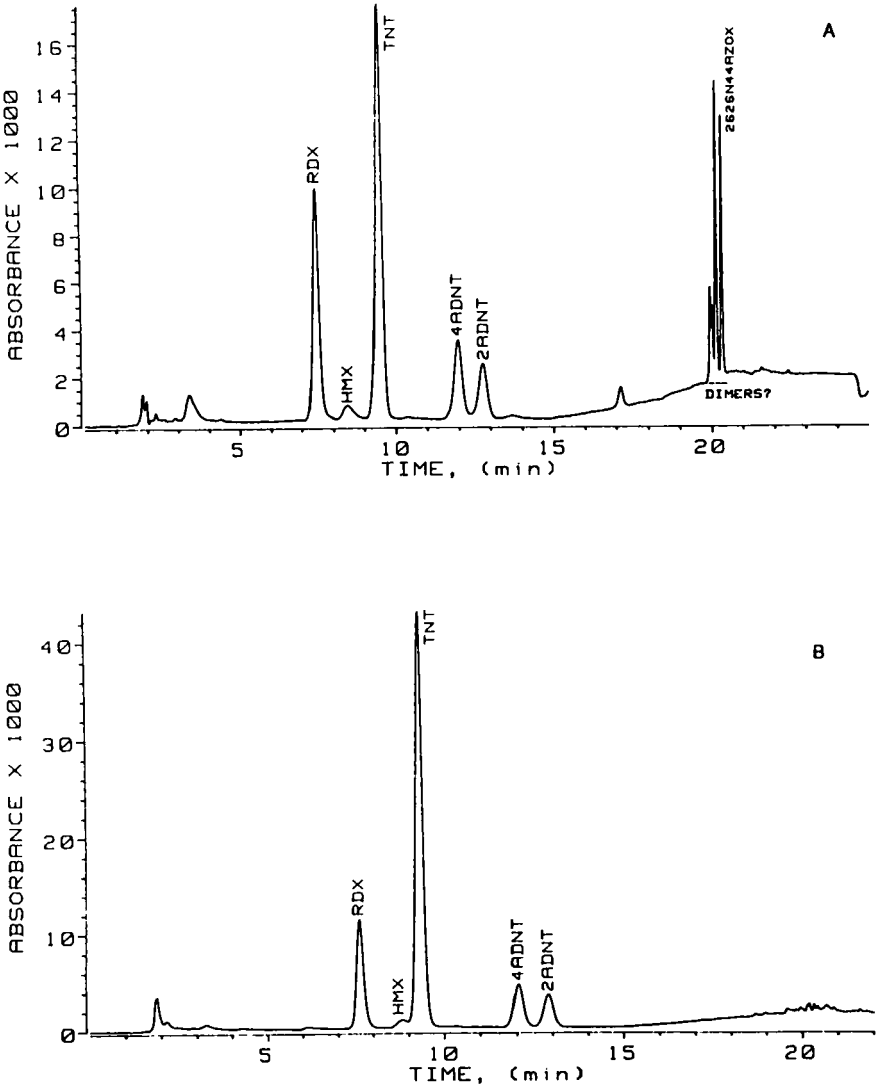

Figure 6. Chromatograms of explosives and TNT metabolites in soil compost leachate (A) and acetonitrile extract (B) on C_{18}/Anion column using the shorter ternary gradient program (method 2 in Table 2).

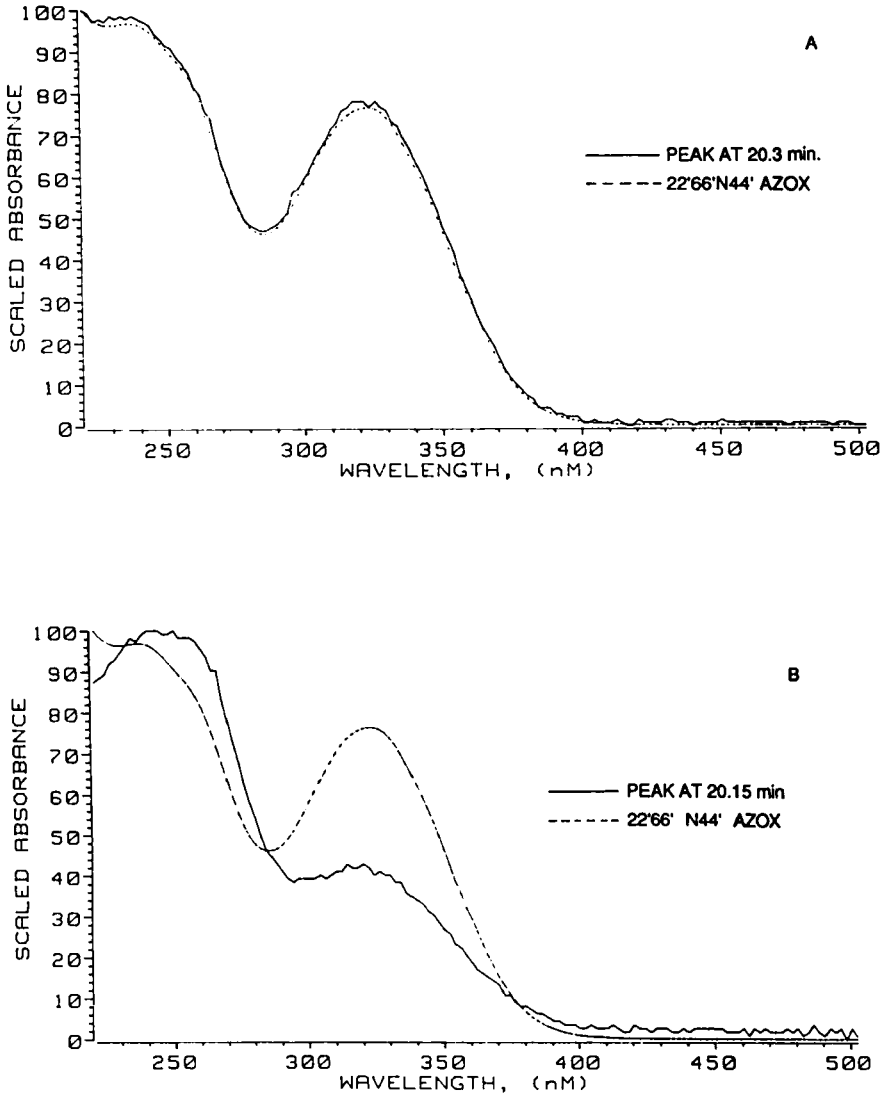

Figure 7. Comparison of the spectrum of authentic 44'AT with HPLC peaks eluting at (A) 20.30 min and (B) 20.15 min. in the chromatogram of the leachate from the soil compost (Figure 6).

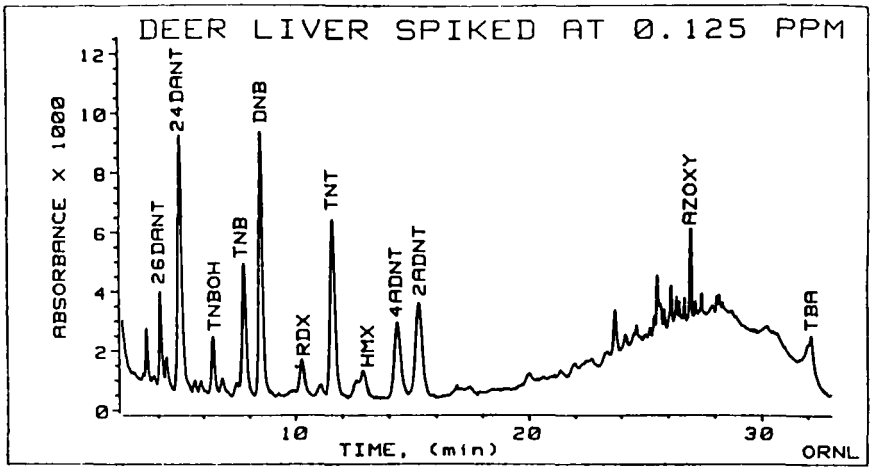

Figure 8. Chromatogram of explosives and metabolites extracted from a deer liver sample which had been spiked with authentic standards at a concentration of 0.125 ppm each. The longer ternary gradient program (method 1 in Table 2) was used.

the leachate. The peak eluting immediately before 44'AT at ca. 21.7 min is suspected to also be an azoxydimer because its spectrum was almost identical to that of 44'AT but shifted a few nm (Figure 7B). The other two peaks in the quartet could be azoxydimers; a total of four isomers (two symmetrical and two unsymmetrical isomers) appears possible in the microbial metabolism of TNT.

TNBCOOH has not yet been observed in any sample extract, most likely because this compound is readily decarboxylated in solvents such as ethanol and dioxane where the carboxylate ion is not as strongly solvated as it is in aqueous solution.[37] In this study TNBCOOH standards in water solution stored at 4°C were observed to be stable for many months, whereas TNBCOOH began to disappear from a methanol solution within a few hours and from an acetonitrile solution within 30 min. It appears likely that common sample preparation methods in which explosives and metabolites are extracted from aqueous-based biological and environmental samples into organic solvents would show increasingly diminished amounts of TNBCOOH as the preparation and storage time before analysis increased.

The utility of the method for analysis of animal tissue extracts is

illustrated by the chromatogram in Figure 8. The chromatogram shows the separation of an extract from a deer liver tissue sample spiked with TNT and its metabolites at 0.125 ppm. Although the levels are near the limit of detection, the peaks are clearly visible and identifiable. In this study, neither TNT nor its soluble free metabolites were detected in any sample at a limit of 0.2 ppm.[16] It was suspected that the metabolites were mainly highly polar, water-soluble forms which eluted in the solvent peak, and also conjugates which would not be detected without performing hydrolysis before the extraction.

CONCLUSIONS

The mixed mode combination of anion exchange and reverse phase mechanisms achieves a highly efficient separation of explosives and also both reduction and oxidation metabolites of TNT in a single chromatographic run. Coupling this separation mechanism with diode array UV absorbance detection allows the confident analysis of extracts and leachates of soils and extracts of animal tissues.

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