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An improved method for analysis of 2,4,6-trinitrotoluene and its metabolites from compost and contaminated soils

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Abstract

The United States Army Toxic and Hazardous Materials Agency (USATHAMA) Method SMO2, routinely used for the high pressure liquid chromatographic analysis of nitrobenzenes in contaminated soils, was modified to incorporate the use of *para*-nitroaniline (PNA) as an internal standard and also to permit the quantitation of two additional metabolites of 2,4,6-trinitrotoluene (TNT). In our laboratory, these two metabolites, 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT), were found to co-elute with each other, as well as with dinitrotoluenes (DNT), 2,4-DNT and 2,6-DNT, using the original method. The modified method described below is a two-step procedure in which one aliquot of the extract mixture is analyzed directly to quantitate trinitrobenzene (TNB), *meta*-dinitrobenzene (*m*-DNB), and TNT, and a second aliquot is simultaneously treated with trifluoroacetic acid anhydride (TFAA) to convert 2-ADNT and 4-ADNT into derivatives that chromatographically separate cleanly from each other and from DNT. Standard curves for the derivatized compounds are linear over a range of 30–400 ng/25 µl injection. Quantitation of TNT and its metabolites in spiked municipal compost demonstrated intra-assay and inter-assay variabilities of ±15% or less. This two-step analysis for these compounds was found to be successful equally after extraction and dilution with either acetonitrile (ACN) [USATHAMA Method SMO2] or with methylene chloride as in an alternate method (EPA Method 3550). This new derivatization procedure allows for the quantitation of additional metabolites of TNT which is difficult or not possible with Method SMO2.

Introduction

Since World War II, 2,4,6-trinitrotoluene (TNT) has been a widely used explosive. Manufacturing and military wastes containing TNT have been

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accumulating in the environment for decades. The U.S. Army alone has an estimated one million cubic yards of contamined soils at 28 sites across the country [1]. Explosive wastes are known to be toxic to aquatic organisms [2, 3]. Concerns about the health effects upon exposure in humans to TNT arise from evidence which links extensive occupational contact to TNT to an increased incidence of aplastic anemia, liver damage, dermatitis, ocular disorders [4], and gastrointestinal distress [5]. In response to these concerns, there is an increasing initiative supported by military and other government agencies to evaluate the content of existing waste disposal sites and to explore environmentally desirable ways to remediate contaminated soils.

Appropriate analytical methods are essential for these efforts. Quantitation of TNT, its metabolites and degradation products in soils can characterize the wastes at disposal sites. The persistence and fate of intermediate products formed during the decomposition process are of particular interest because there is reason to believe that they may be of toxicological importance [6]. Chemical analysis is important in assessing methods of disposal and degradation of TNT wastes; for example, composting where the microbial decomposition of TNT is accomplished in an organic-rich soil environment. Efficient extraction procedures and chromatographic separation of the analytes must be achieved for quantitation to be possible.

In the course of developing capabilities to analyze TNT contaminated soils in our laboratory, it became evident that, using the HPLC parameters specified in USATHAMA Method SMO2, three TNT degradation products of interest, 2-ADNT, 4-ADNT, and 2,4,-DNT co-eluted. To simultaneously quantitate these compounds, a procedure was developed to convert 2-ADNT and 4-ADNT into trifluoroacetyl derivatives, thereby differentially increasing their retention times and accomplishing chromatographic separation of the compounds from each other and from 2,4-DNT. The derivatization process was appended as a second step to the existing USATHAMA Method SMO2.

Para-nitroaniline (PNA) was used as the internal standard in both steps of the analysis. PNA had a slightly shorter retention time than TNB, *m*-DNB, and TNT in step one of the procedure when these compounds were quantitated. It was derivatized along with 2-ADNT and 4-ADNT in the second step, and its retention time was increased to be intermediate between that of these two compounds.

Municipal compost spiked with the analytes was used to assess the reproducibility and efficiency of the two-step procedure.

Materials and methods

Reagents and supplies

Pure, crystalline standards of 2,4,6-trinitrotoluene, 2-amino-4,6-dinitrotoluene, and 4-amino-2,6-dinitrotoluene were obtained from U.S. Biomedical Research and Development Laboratory, Fort Detrick (Frederick, MD).

Standards of 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) were obtained from the USATHAMA Standard Analytical Reference Materials Repository (Aberdeen, MD). All standards were stored in the dark at 4 °C. *Para*-nitroaniline (Matheson-Coleman, NJ) was used as the internal standard. HPLC grade acetonitrile and methanol (Fisher Scientific) and a Chempure calcium chloride dihydrate (Curtin Matheson) were used in the extractions. Trifluoroacetic acid anhydride was obtained from Aldrich Chemical Company. Derivatization was carried out in 4 ml amber vials with screw-caps (Fisher) holding a Teflon-lined septum (Pierce #12712). A Barnstead Nanopure II 3-cartridge water purification system supplied laboratory grade water. During the extraction/dilution, samples were filtered through a 0.45 μm polytetrafluoroethylene (PTFE) filter (Nalgene #199-2045) using a 10 ml plastic syringe (Becton Dickinson and Co.).

Instrumentation

High pressure liquid chromatography was performed on a Hewlett Packard 1090 HPLC equipped with an autoinjector and linked to a Perkin-Elmer LC-85B ultraviolet spectrophotometric detector. Tracings were recorded with a Houston Instruments OmniScribe chart recorder. Sonication of the sample during extraction was performed with a 375 watt Sonics and Materials Vibra Cell sonicator equipped with a standard half inch horn. Samples were dried under nitrogen and subsequently incubated at 4 °C in a Precision Scientific waterbath. GC/MS confirmation of the structures of the derivatized products was performed using electron impact analyses with a Hewlett Packard 5988 gas chromatography/mass spectrometer.

Chromatographic conditions

The HPLC chromatographic conditions for analysis of the extracts were as described in USATHAMA Method SMO2. The methanol : water mobile phase (50 : 50) was pumped at 1 ml/min; absorbance was measured at a wavelength of 254 nm; the recorder chart speed was 0.2 inch/min. The column was a 25 cm HS-10 C18 reverse phase cartridge column (Perkin-Elmer 0258-0172). Sample injection volume was generally 25 μl. Instrument sensitivity settings ranged from 2 to 16 depending upon the concentration of the material being analyzed.

Preparation of standards and controls

Stock standards at concentrations of 1 mg/ml were prepared in acetonitrile and stored for the duration of the study (3 months). Stock solutions were further diluted weekly to 100 μg/ml with acetonitrile. The standards were stored in test tubes wrapped in aluminum foil to protect solutions from light and were refrigerated at 4 °C.

Working standards were made by dispensing known quantities of the diluted stock solutions into 4 ml amber vials, combining the analytes to make mixed-compound standards over the range of the standard curve (30–400 ng). The internal standard solution (100 μg/ml PNA in acetonitrile) was prepared as

needed. Fifteen micrograms (150 µl) were added to each standard (and sample). A portion of these standards was analyzed directly by HPLC and the rest evaporated to complete dryness and derivatized by procedures to be described later.

A 10-year-old municipal compost was collected in a noncorrosive plastic drum and stored in the dark until used. To spike the compost with TNB, *m*-DNB, TNT, 2,4-DNT, 2-ADNT, and 4-ADNT, 2 g aliquots of the sieved moist compost were placed into 20 ml screw capped glass test tubes, and to each tube 4 ml of methanol and analyte were added. The samples were mixed on a vortex mixer until a slurry was formed. The tubes were then wrapped in foil, and the moisture was evaporated by placing the samples under a stream of nitrogen at 23 °C.

Extraction procedure

Twelve milliliters of acetonitrile were added to the 2 g sample of spiked soil. The sample was vortexed for 1 min, sonicated at 50% duty cycle for 10 min, and allowed to sit overnight (15 h) at room temperature in the dark. It then was centrifuged at 2000 rpm for 10 min. Two and a half milliliters of the acetonitrile supernatant extract was transferred to a tube and mixed with an equal volume of aqueous calcium chloride solution (10 g/l). Fifteen micrograms of PNA (150 µl) were added, the mixture vortexed, and then allowed to equilibrate for 15 min to allow for flocculation and settling of particulates. The solution was drawn into a disposable 10 ml plastic syringe before dispensing through a 0.45 µm filter. The first ml was discarded and then a portion of the remaining filtrate was put into an autoinjector vial for direct analysis (Step 1). The last 2.5 ml was transferred to a 4 ml amber vial and evaporated to complete dryness in a 40 °C waterbath under a stream of nitrogen in preparation for derivatization.

Derivatization

Two hundred microliters of trifluoroacetic acid anhydride (TFAA) were added to each completely dry sample or standard, and the amber vial was tightly capped and vortexed thoroughly. The sample was incubated in a 40 °C waterbath for 45 min. The derivatizing agent was deactivated immediately when incubation was complete by adding 200 µl of laboratory quality water to the sample and then shaking the vial for a few seconds. The sample was thoroughly mixed and then transferred to a 300 µl vial for the HPLC autoinjector. Derivatized samples in which the TFAA was deactivated with water could be stored overnight in the dark at room temperature without affecting the quality of the analysis.

Results

The trifluoroacetyldinitrotoluenes produced by the derivatization of 2-ADNT and 4-ADNT with trifluoroacetic acid anhydride (TFAA) each have

a molecular weight of 293. The chemical structure of a derivatized 2-ADNT is presented in Fig. 1. The structures of the derivatized products were confirmed by GC/mass spectrometry. Fragmentation patterns for the two compounds are presented in Fig. 2.

Derivatization increased the retention time for each of the three compounds. For 2-ADNT, the increase was from 15.1 to 16.2 min; for 4-ADNT, from 14.1 to 39.3 min; and for *para*-nitroaniline, from 6 to 19 min. Relative retention times of the analytes to the derivatized and underivatized PNA are presented in Table 1.

Figure 3 presents two HPLC chromatograms containing TNB, *m*-DNB, TNT, 2,4-DNT, 2-ADNT, 4-ADNT (A) prior to and (B) following derivatization. The co-elution of 2,4-DNT, 2-ADNT, and 4-ADNT in (A) is apparent and can be compared to the more widely spaced peaks from the derivatized compounds in (B). The shift in the retention time of the internal standard PNA resulting from derivatization can be seen by comparing the two graphs. Two peaks are from the derivatizing compound TFAA, the retention time of the first peak matching that of trifluoroacetic acid.

Standard curves for the derivatized compounds are presented in Fig. 4. The peak height ratio of analyte to derivatized internal standard is plotted versus the concentration of the standard. The plot was linear from 30 to 400 ng per 25 µl injection for each analyte, with HPLC injections of absolute quantities as low as 10 ng being easily detected.

The reproducibility of the extraction/derivatization method for the Step 2 compounds in the procedure is presented in Table 2. The values are the mean calculated concentrations of analytes from three spiked replicates plus or minus one standard deviation. The mean inter-assay and intra-assay variability did not exceed 15%. The calculated values for 2-ADNT and 4-ADNT were 106% and 104% of the target value, respectively, with that for 2,4-DNT being substantially less, at 40%.

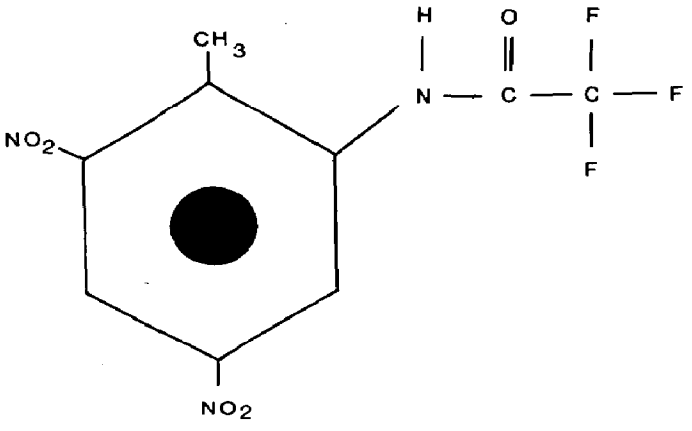

Fig. 1. Chemical structure of the trifluoroacetyl derivative of 2-ADNT. Derivatization was accomplished by reacting 2-ADNT with trifluoroacetic acid anhydride (TFAA) for 30 min at 40 °C.

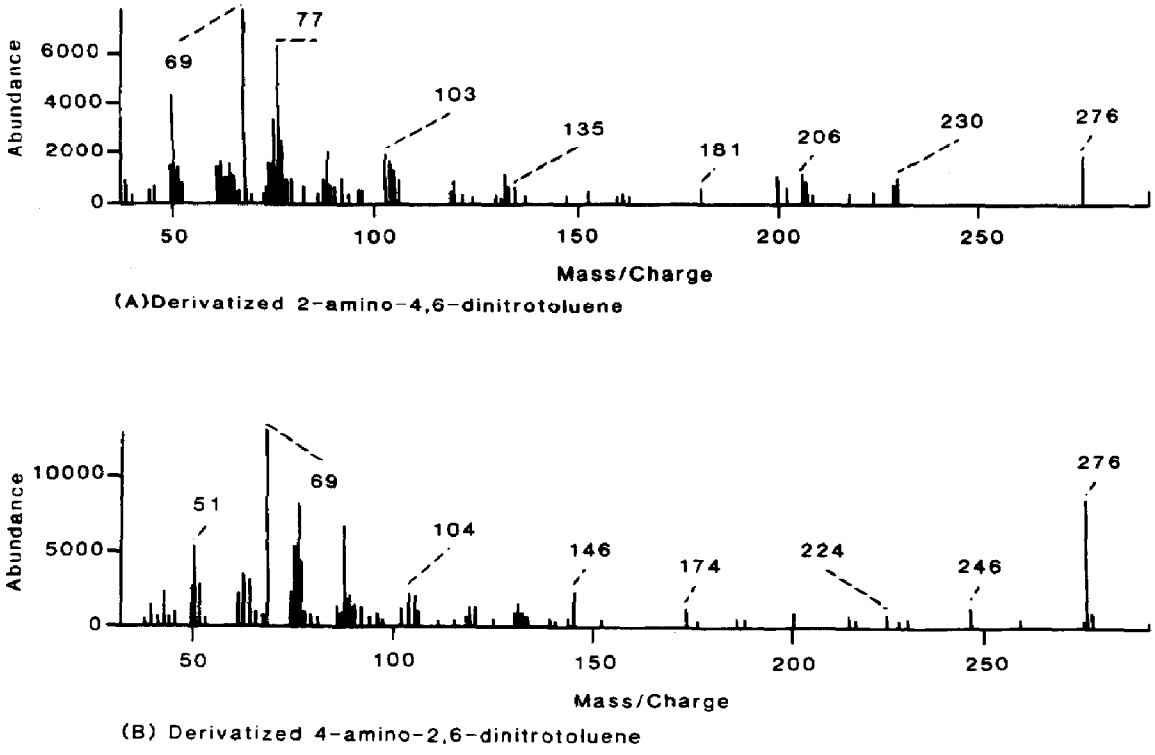

Fig. 2. GC/MS fragmentation patterns for derivatized 2-ADNT and 4-ADNT.

TABLE 1

Chromatography retention times relative to *para*-nitroaniline (PNA)

Compound[a]	Relative retention times[b]	
	Underivatized sample	Derivatized sample
TNB	1.23	0.39
m-DNB	1.65	0.52
TNT	1.90	0.60
2,4-DNT	2.52	0.80
2-ADNT	2.52	0.85
4-ADNT	2.35	2.07

[a] TNB (trinitrobenzene), *m*-DNB (*m*-dinitrobenzene), TNT (trinitrotoluene), 2,4-DNT (2,4-dinitrotoluene), 2-ADNT (2-aminodinitrotoluene), and 4-ADNT (4-aminodinitrotoluene) were chromatographed on a C-18 reverse phase column using methanol:H_2O (50:50).

[b] Relative retention times of TNT and related compounds to underivatized and derivatized PNA are based on elution of underivatized PNA at 6.0 min and derivatized PNA at 19.0 min.

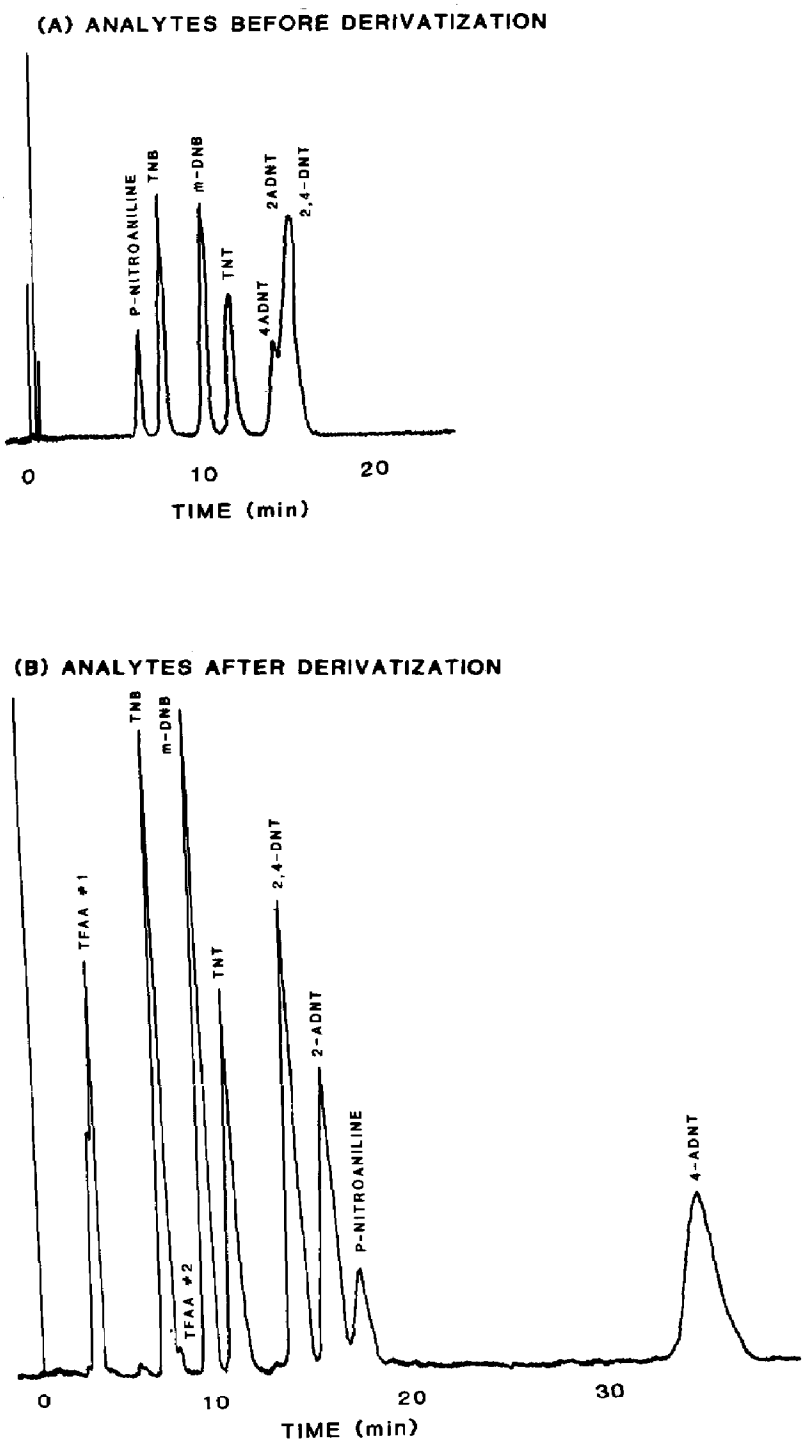

Fig. 3 HPLC chromatograms of the six analytes prior to (A) and following (B) derivatization with TFAA.

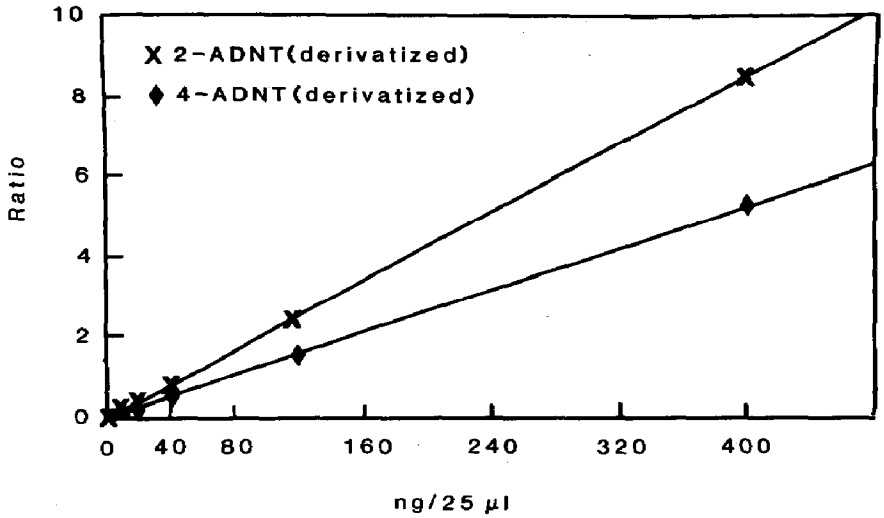

Fig. 4 Standard curves for derivatized 2-ADNT and 4-ADNT. Values on the *y*-axis represent ratios of peak height for either 2-ADNT or 4-ADNT to that obtained with 15 µg of *p*-nitroaniline as the internal standard. For both compounds, the standard curve is linear to at least 400 ng/25 µl.

TABLE 2

Intra-assay and inter-assay variability for Step 2 compounds[a]

Analyte	Intra-assay variability (Day 1)	Intra-assay variability (Day 2)	Intra-assay variability (Day 3)	Inter-assay variability
2,4-DNT	6.3±0.8	8.9±1.8	6.5±1.0	7.2±1.4
2-ADNT	18.5±0.4	21.8±4.2	18.4±4.2	19.6±1.9
4-ADNT	17.4±1.5	18.2±1.2	22.0±4.9	19.2±2.0

[a] Variability in the analysis for the Step 2 metabolites in compost. Values are expressed as mean calculated micrograms analyte per gram of soil ± one standard deviation. Each value represents the mean of three individual values. Compost was spiked at a level of 18 µg/g.

Discussion

Significant contamination of soils by TNT and related munitions has been reported, particularly at United States military installations [1]. Because of the reported toxicity of TNT, biodegradation processes are being employed to facilitate conversion of the parent compound to less toxic metabolites. In order to monitor the extent of soil contamination and evaluate the extent of biodegradation, effective analytical techniques must be available. The most commonly used method for the analysis of TNT and its metabolites is USATHAMA Method SMO2, a high pressure liquid chromatographic technique employing extraction/dilution and ultimate detection with UV spectrophotometry [7–9].

Whereas this method is effective, it may not permit the measurement of the two aminodinitrotoluene compounds, which are significant metabolites of TNT and which have been shown to co-elute with each other, as well as with 2,4-DNT. The method developed above involves a chemical derivatization of the amino metabolites of TNT to chromatographically separate the compounds and thus enables their quantitation to levels as low as 10 ng.

Secondly, this method employs the use of an internal standard, *p*-nitroaniline, which was selected because of its ability to be derivatized and because of its chemical similarity to nitrotoluenes. The use of an internal standard is of great value because it provides a control during the sample transfer, derivatization, and subsequent chromatography and is critical in the preparation of the daily standard curve.

This new two-step approach permits the measurement of TNT compounds (trinitrobenzene, *m*-dinitrobenzene, trinitrotoluene, combined dinitrotoluene (2,4-DNT and 2,6-DNT), 2-amino-4,6-dinitrotoluene, and 4-amino-2,6-dinitrotoluene using a single aliquot of sample. The precision and accuracy for this analytical method are well within the standards which would be expected to be required to maintain good quality assurance.

Finally, the method is convenient, can be incorporated into existing TNT-testing programs with a minimum of additional work, and may be used for testing soils, waters, and even biological specimens. In addition, this method may be used to monitor the content of contaminated soils undergoing bioremediation employing microorganisms.

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