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Selective Determination of TNT in Soil Extracts by Sequential Injection Spectrophotometry

Roger T. Echols,[†,‡] Marcy M. Christensen,[†,§] Ryan M. Krisko,[†,§] and Joseph H. Aldstadt, III*,[||]

Division of Science and Mathematics, University of Minnesota-Morris, Morris, MN 56267, Department of Chemistry, University of Wisconsin-Milwaukee, 3210 N. Cramer Street, Milwaukee, WI 53211

A rapid sequential injection spectrophotometric method for the selective determination of 2,4,6-trinitrotoluene (TNT) in soil samples is presented. The method is based on a derivatization reaction of TNT with sodium sulfite in a basic acetone medium. The reaction conditions, namely the percentage of acetone used in the reaction, the sample and reagent volumes, the mixing coil volume, and the reaction time, were optimized. The reaction was found to be particularly sensitive to the concentration of acetone; an acetone/water medium of 88% (v/v) acetone was found to be optimal. A study of the response of the method to other explosives demonstrated that the method is selective for TNT. In particular, it was shown that the method had little (<2%) or no response to other secondary explosives such as 2,4- or 2,6-dinitrotoluene (DNT). An average precision of 6.1% RSD was established for five soil samples ($n = 4$). The limit of detection was 0.5 μg mL^{-1} for aqueous standards and 80 μg g^{-1} for ~300 mg soil samples. Analysis time was approximately three minutes per sample. Quantitative results from the determination of TNT in authentic contaminated soil samples compared favorably to those obtained using a standard method. The extension of the method to water samples and the means by which samples can be preconcentrated on-line using solid-phase extraction (SPE) techniques are discussed.

The determination of the most common secondary explosive, 2,4,6-trinitrotoluene (TNT), in environmental samples has been the focus of much research.[1−3] A variety of chromatographic, spectrophotometric, and electrochemical methods to determine TNT in environmental samples have been reported: spectropho-

tometry (colorimetry),[4,5] thin-layer chromatography,[6,7] ion-exchange membranes with colorimetric and fluorescent detection,[8,9] PVC membranes with colorimetric detection,[10] and high-performance liquid chromatography (HPLC).[11−15] HPLC as embodied in U. S. EPA Method 8330 has emerged as the method of choice for laboratory determinations of TNT and other explosives.[3,16,17] Crockett et al.[3] have reported the cost for analyses by the EPA Method 8330 to be $250−$350 per sample for turnaround times of 30 days and $1,000 per sample for turnaround times of 3 days. Thus, there is interest in the development of inexpensive and rapid methods for the determination of TNT, in particular those that can withstand the rigors of field usage for process monitoring.

A promising development in meeting this need was reported recently by Ligler and co-workers[18−21] who have developed a capillary displacement flow immunosensor to determine TNT at trace levels in aqueous media and have demonstrated the statistical equivalency of the method to Method 8330.[22] An alternative approach that uses inexpensive off-the-shelf reagents is based on the chemical reactivity of TNT. Red-colored complexes (so-called Jackson-Meisenheimer anions) result from the reaction of TNT with a number of bases (including sulfite and hydroxide).

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* Corresponding author: (tel.) 414-229-5605; (fax) 414-229-5530; (e-mail) aldstadt@uwm.edu.
† University of Minnesota-Morris.
‡ Current address: Department of Civil and Environmental Engineering, University of Michigan, Ann Arbor, MI 48109; email: rtechols@umich.edu.
§ Current address: Department of Chemistry, University of Kansas, Lawrence, KS 66045.
|| University of Wisconsin-Milwaukee.

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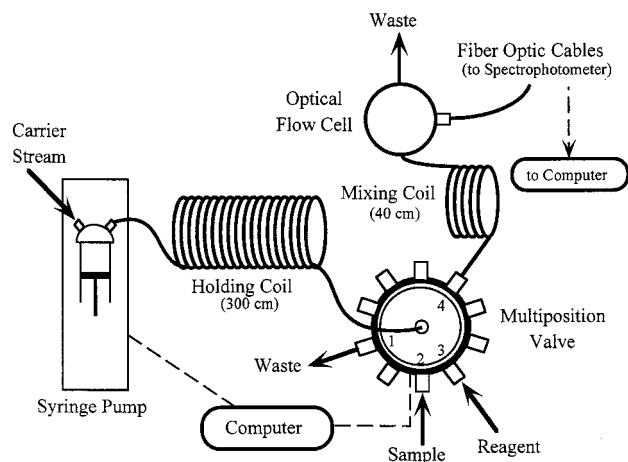
Figure 1. Schematic diagram of the SI system. Flow tubing has an internal diameter of 0.76 mm. All components are mounted on the face plate of a 37 × 32 × 45 cm³ case, which is interfaced to the DPA20 spectrophotometer, interface box, and microcomputer.

Table 1. Typical Procedure for the SI Method for TNT

device	action
syringe pump	aspirate 2500 μL (100 μL/s) of carrier into holding coil
multiposition valve	step to position one (waste).
syringe pump	dispense 600 μL (50 μL/s) of carrier through holding coil
multiposition valve	step to position two (TNT sample).
syringe pump	aspirate 120 μL (30 μL/s) of sample into holding coil
multiposition valve	step to position three (reagent).
syringe pump	aspirate 90 μL (30 μL/s) of reagent into holding coil
	wait 30 s for reaction to occur
multiposition valve	step to position four (flow cell)
syringe pump	dispense continuously through mixing coil collect absorbance for ~45 s at 470 nm[a]
syringe pump	stop pump

[a] Absorbance data is collected over time by repeated measurements at approximately 0.8-s intervals

In the presence of acetone and a base, Janovsky complexes can form, which also are red-colored and, thus, conveniently monitored in the visible spectrum.[5,23] Colorimetric methods for the qualitative and quantitative determination of TNT have been published over the last 60 years.[24–27] Recent research by Jenkins and co-workers[5,28] using this chemistry has led to the development of a simple quantitative field assay for TNT in soils. TNT is extracted from soil samples with acetone and reacted with sodium sulfite and potassium hydroxide reagents. After the red-colored product forms at equilibrium, the absorbance is measured and compared to the absorbance of a series of standards. A detection limit of 0.72 μg g^{-1} TNT is reported.

The adaptation of the Janovsky reaction to an automated sequential injection (SI) system for the rapid quantitative determination of TNT in soil is described in this paper. The reaction chemistry was optimized, and a study of the selectivity of the method was undertaken. Furthermore, a prototype field-portable instrument was developed for on-site studies of contaminated waters and soils. These results and a comparative study of the SI method and an HPLC method based on EPA Method 8330[16] are presented and discussed.

EXPERIMENTAL SECTION

Apparatus. All research was conducted on a spectrophotometric SI system which consisted of the following components (Figure 1): syringe pump (5 mL, Cavro, Sunnyvale, CA), 300-cm holding coil (0.76-mm ID PTFE flow tubing), multiposition valve (ten-port, Valco, Houston, TX), 35-cm mixing coil (0.76-mm ID flow tubing), flow cell with quartz insert (Global FIA, Gig Harbor, WA), and DPA20 visible spectrophotometer (DSquared Develop-

ment, LaGrande, OR). The DPA20 is a spectrophotometer that has a 2048-element CCD array detector and fiber-optic cables that connect to the flow cell. The components depicted in Figure 1 were mounted on the face plate of a field-portable six-panel instrument chassis (Global FIA) of dimensions 37 × 32 × 45 cm³.

The hardware required to control the SI system consisted of a notebook computer (486, Windows 3.1) and the spectrophotometer, which was interfaced to the syringe pump and the multiposition valve. The software used to control the SI system, Delight 1.4 (DSquared Development), allowed the system to be automatically controlled (see Procedures and Table 1).

A Waters 600E System Controller (Waters, Milford, MA) and Waters 490E Detector (254-nm) were the principle components of the HPLC system used in the quantitative work. A 15-cm C18 HPLC column, an injection volume of 50 μL, and a flow rate of 1.5 mL min^{-1} were used in the analysis.

Reagents. All chemicals used in the experimental work were analytical reagent grade or better. A mixture of HPLC-grade acetone and deionized (18 MΩ-cm) water was used as the carrier stream, to prepare reagent solutions and for the dilution of stock solutions. Typically, a mixture of 88% (v/v) acetone/12% (v/v) water was employed (all subsequent percentages expressed as % (v/v)). Acetone and water were degassed with pure helium or neon gas for 15−20 min prior to use in the flow system or in making solutions. The reagent solution was prepared by dissolving 0.2 g of potassium hydroxide and 0.3 g of sodium sulfite in 6 mL of water and adding 44 mL of acetone; the resulting solution contained insoluble salts which were filtered with 0.45-μm glass fiber filter disks (Whatman, Ann Arbor, MI). Standards of the secondary explosives (including TNT) were purchased from Supelco (Bellefonte, PA) as 1000 μg mL^{-1} stock solutions in acetonitrile. Dilutions were made in the acetone/water mixture. Authentic soil samples containing TNT contamination were obtained from Iowa Army Ammunition Plant (Middletown, IA). HPLC-grade methanol and water were used in the HPLC work.

Procedures. Table 1 lists the procedure used for the comparison study of the SI and HPLC methods, and it is typical for the experimental work (with slight changes in volumes or times) described herein. Two details have been excluded from the table

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for the sake of simplicity: (1) The Cavro syringe pump has a control valve that allows the holding coil to be by-passed when loading the pump with carrier solution. (2) A few data points were collected with carrier stream in the detector prior to the reaction to establish a baseline absorbance. The method required approximately 3 min per sample. The complete method was programmed to run automatically and continuously. Absorbance was collected in approximately 0.8-s intervals. Peak height absorbance was used as the system response for the optimization study and the quantitative work.

For the quantitative comparison of the SI and HPLC methods, approximately 0.3 g of soil containing TNT was extracted into 22.0 mL of acetone by shaking for 3 min; 3 mL of water was added after the extraction to make the solution 88% in acetone. The extracts were filtered with 0.45-μm glass fiber filter disks prior to analysis. Dilutions of one part extract to one part 88%/12% acetone/water solution were required to bring the concentrations into the linear working range of the calibration curve (TNT standards from 0 to 18 μg mL^{-1}). The sample absorbance of the analytical blank was measured by replacing the reagent solution with carrier solution. A 50-μL injection volume and a flow rate of 1.5 mL min^{-1} were employed in the HPLC experiments with a mobile phase composition of 50% methanol/50% water.

Experiments to optimize the percentage of acetone used in the reaction, the sample volume, and the reagent volume were conducted by measuring the peak height as a function of the change in the variable of interest. A TNT standard (8.0 μg mL^{-1}) was used in each set of experiments. For the study of the percentage of acetone and water, carrier stream, reagent, and standard solutions with different acetone and water compositions were prepared. For the study of the effect of volume on the peak height absorbance, the volume was changed by reprogramming the method. Sample volume was varied from 80 to 360 μL; reagent volumes were varied from 60 to 160 μL. The optimization of mixing-coil volume and reaction time was conducted by the method of steepest ascent[29] over a range of values deemed practical on the basis of experiment time.

The interference study was conducted by comparing the peak height absorbance of an 8.0 μg mL^{-1} solution of TNT with the peak height absorbances of 8.0 μg mL^{-1} solutions of the other secondary explosives (one solution at a time). The ratio of the absorbance of the potential interfering species to the absorbance of TNT was used as the figure of merit.

Preliminary work was conducted with the SI system using manual control of the pump and valve. Two problems with the SI system required modifications in the method. The formation of bubbles, which interfered with the mixing of sample with reagent, necessitated the degassing of the acetone/water carrier stream and solutions, and the low solubility of reagents in acetone required that the reagent solution be filtered to prevent the flow tubing from becoming blocked. Additionally, the order of sample and reagent solutions in the flow tubing was studied: the TNT sample was aspirated before the reagent solution to reduce the formation of refractive-index peaks.
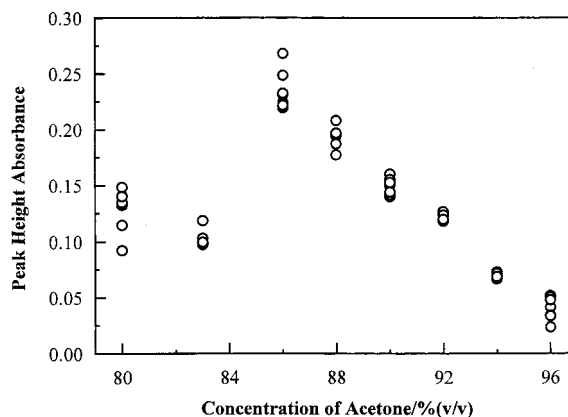
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Figure 2. Optimization of amount of acetone and water in the reaction system. Peak height absorbance of an 8.0 μg mL^{-1} TNT standard for a series of acetone and water compositions. Replicate data points plotted for each acetone/water composition ($n = 6$).

RESULTS AND DISCUSSION

Optimization of the Sequential Injection Method. Of the numerous physical and chemical variables involved in the SI method for TNT, five were chosen for optimization: (1) percentage of acetone and water, (2) volume of sample, (3) volume of reagent, (4) length of the mixing coil (between the valve and the detector), and (5) reaction time. The order in which the sample and reagent volumes were aspirated was determined on the basis of refractive index; the flow rate was deemed to be of little consequence if the volumes and tubing lengths were optimized simultaneously; and the concentration of reagents was a factor controlled by the amount of acetone.

The most important variable of the SI method for TNT was the percentage of acetone used in the reaction (including the solution in which all standards and samples were prepared). At a high percent composition of acetone (Figure 2), the solubility of the reagents is low (the reagent concentrations limit the amount of derivatized TNT formed); at low percent compositions of acetone, the molar absorptivity of the complex is small. The highest absorbance of the TNT standard occurred at 86% acetone/14% water, but a higher percentage of acetone (88%) was chosen for subsequent work because of better precision (2.5% versus 7.6% RSD, respectively). This amount was lower than that used by Jenkins and Walsh[5] (97%/3%) as a consequence of the nature of the SI experiment. In the off-line method, mixing of the TNT and the reagents occurs by manual shaking; in the SI method, mixing of the TNT and the reagents occurs by laminar flow, which requires a higher concentration of the reagents and, consequently, a higher percentage of water.

Results from studies of the change in peak height absorbance as a function of sample volume and reagent volume are shown in Figure 3. The exponential trend for the variation in signal with sample volume is well-established for other flow injection-based methods: The signal rises to a maximum, which is the point at which the sample plug is not diluted by the carrier stream as it reaches the detector (i.e., the dispersion [D] approaches 1.0). The sensitivity of the reaction could be increased slightly with a larger volume, but a sample volume of 120 μL was used to maintain short reaction times and increase throughput. The response relative to reagent volume is unchanged; a sufficient excess of reagent was present at all volumes even though we estimate that ~80% of the
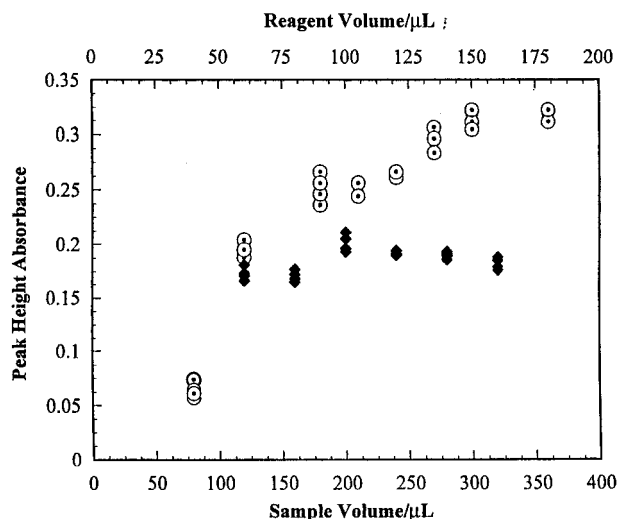
Figure 3. Optimization of the sample volume and reagent volume. Sample volume (⊙) was varied between 80 and 360 μL. Reagent volume (◆) was varied between 60 and 160 μL. Replicate data points plotted for each volume.
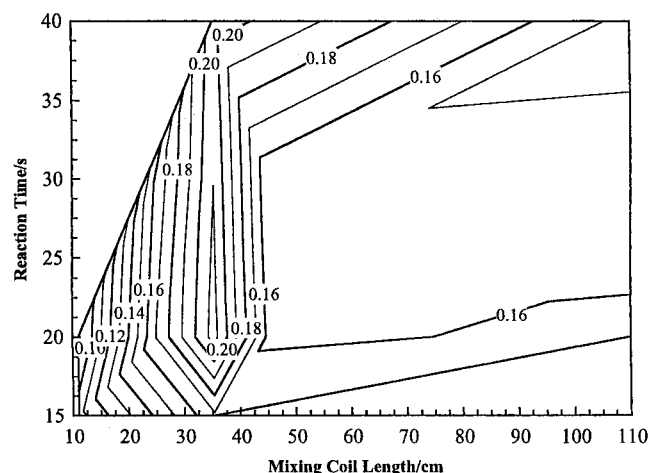

Figure 4. Optimization of mixing coil length and reaction time. Surface contour plot of peak height absorbance versus mixing coil length and reaction time. Peak height absorbances are noted on the plot.

reagent is not soluble in the carrier. A reagent volume of 90 μL was used for most of the experimental work. The contour plot (Figure 4) shows the variation of signal as a function of mixing coil volume and reaction time—factors that interact at constant flow rate. A mixing-coil length of 40 cm and a reaction time of 30 s were chosen for subsequent experiments. The lack of dependence of peak height on the reaction time (range of maximum values from 20 to 30 s) indicates that mixing of the reagents via laminar flow is crucial in promoting the reaction.

Jenkins and Walsh[5] reported a molar absorptivity of 2.7×10^4 L mol^{-1} cm^{-1} for the TNT complex at 462 nm. A similar molar absorptivity was obtained for the SI experiment using optimal experimental conditions. Molar absorptivity was typically between 1.0×10^4 and 1.2×10^4 L mol^{-1} cm^{-1} when the measured absorbance for the TNT complex was corrected for the dispersion in the SI system (D $\approx$ 1.9). Because of dispersion, an inherent feature of FI-based methods, the SI method for TNT is slightly less sensitive than the corresponding off-line method, even though the chemistry is the same.

Table 2. Results from the Study of the Selectivity of the SI Method for TNT

secondary explosive	relative absorbance (%)[a]
TNT	100
4-nitrotoluene	2.06
1,3-dinitrobenzene	1.80
nNitrobenzene	0.90
2,4-dinitrotoluene	0.00
2,6-dinitrotoluene	0.00
2-nitrotoluene	0.00
3-nitrotoluene	0.00
RDX[a]	0.00
HMX[b]	0.00

[a] Relative absorbance is the ratio of peak height absorbance of an 8 μg mL^{-1} solution of secondary explosive to the peak height absorbance of an 8 μg mL^{-1} solution of TNT. [b] RDX is 1,3,5-trinitro-1,3,5-triaza-cyclohexane. [c] HMX is 1,3,5,7-tetranitro-1,3,5,7-tetrazacyclo-octane.

Study of Selectivity of Method. Selectivity is an important feature of methods for TNT because of other explosive contaminants that are present along with TNT in environmental waters and soils. In particular, effective bioremediation of TNT waste sites requires effective discrimination of the degradation product DNT to determine the efficiency of the process. As a consequence of the nature of chromatographic experiments, the HPLC method for TNT[16] is inherently selective and if the concentration of several explosives is desired, it is the method of choice. The interference study performed in this research measured the response of the species of interest relative to TNT. Results are displayed in Table 2. The peak height absorbances for the nine species selected for the study are small, the largest having a signal 2% of that of the TNT standard. For these experiments, a signal of approximately 0.01 absorbance was observed in the form of a refractive-index signal, rather than a well-defined SI peak. Existence of the refractive-index peaks was confirmed with a series of blank solutions which had a mean peak height absorbance of 0.013 at 470 nm. All peak heights, including those for TNT, were corrected for the contribution of the refractive-index peaks.

The lack of a signal from the reaction of the explosives with the reagents is not surprising for many of the species used in the study. For example, RDX and HMX are not known to react in the same manner as TNT.[5] Other species — 1,3-dinitrobenzene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene — react slowly with potassium hydroxide and sodium sulfite in acetone media to form colored products that absorb in the 550−570 nm region. Jenkins and Walsh[5] were able to apply the chemistry used for their TNT method to the determination of 2,4-dinitrotoluene (DNT) using a reaction time of 30 min. No absorbance was observed in the SI method for TNT because of the short reaction time that is afforded by the method. Thus, the inherent kinetic discrimination of the SI method provides additional selectivity to the method (the selectivity of the SI method in addition to the selectivity of the chemical reaction). By comparison, a similar interference (cross-reactivity) study on two TNT antibodies was conducted by Ligler and co-workers using the flow immunosensor method.[21] For the first antibody, the interference signals relative to TNT were in the 15−20% range. For a second antibody, the interference signals relative to TNT were lower: the relative cross-reactivity signal

Table 3. Comparison of the SI Method ($n = 4$) for TNT Determination to the LC Method ($n = 2$) Using Five Authentic Samples (Concentrations Are in μg/g)

sample	av concn (SI)[a]	s (SI)[a]	RSD (SI) (%)[a]	av concn (LC)	s (LC)	RSD (LC) (%)	t[b]
1	2425	76.45	3.15	2635	6	0.24	2.24
2	2268	261.6	11.5	2526	35	1.37	0.80
3	2145	162.6	7.58	2145	59	2.74	0.00
4	2223	140.3	6.31	2420	51	2.11	1.13
5	2094	43.81	2.09	2365	69	2.90	3.75

[a] SI peak height data is corrected for background absorbance. [b] The critical value of t at the 95% confidence level is 2.78 (4 degrees of freedom for the pooled variances).

for DNT was about 5% and the relative signals for 2-nitrotoluene, HMX, and RDX were less than 1%.

Application to Authentic Samples. Using authentic TNT-contaminated soil samples (i.e., unfortified), a quantitative comparison of the SI method for the determination for TNT was made with the HPLC method for TNT. This comparison was conducted without the use of the 18-hour extraction procedure that is specified in Method 8330[16] to ensure that the simple "shake and shoot" extraction procedure was the same for both methods and because the 3-min extraction is used commonly for TNT field methods.[3]

The TNT concentration in five soil samples was determined by each method (Table 3). The calibration equations and coefficients of determination (r^2) for the linear calibration models were $y = -3.38_0 \times 10^{-3} + 1.768 \times 10^{-2}x$ ($r^2 = 0.998$) for the SI experiment (absorbance vs concentration in μg/mL) and $y = -5.89_2 \times 10^4 + 2.01_8 \times 10^5 x$ ($r^2 = 0.996$) for the HPLC experiment (peak area vs concentration in μg/mL). The average relative standard deviations (RSD) were low for both the SIA and HPLC methods (6.1% [$n = 4$] and 1.9% [$n = 2$], respectively). The results indicated no statistical difference (Student's t-test, pooled variances) between the two methods at the 95% confidence level for four of the five samples. If the HPLC method is assumed to be 'correct', the average percent error for the five samples by SIA is 7.6%, which is similar to or better than the accuracy reported for other TNT field methods.[5,22]

The manner in which the background absorbance of the soil was handled in this work was similar to that used by Jenkins,[28] in which the absorbance of soil in acetone was measured and then used to correct the absorbance of the derivatized TNT. Small background peaks were recorded for each soil extract, and the peak height was measured. While this method is time-consuming, it is necessary because the background absorbance was up to 10 percent of the analyte peak heights. With the SI method it is easy to program the instrument to perform these additional injections automatically. One way to minimize the background is to move the analytical wavelength to 540 nm,[28] a region at which the background absorbance is minimal; this technique was not applied to the SI method, but could also be employed to improve the ratio of the analyte absorbance to the background absorbance.

A series of repeat injections for one of the soil samples is shown in Figure 5 along with background peaks. The concentration of the solution being analyzed was approximately 15 μg mL^{-1} after the extraction (0.3 g of soil diluted by a factor of 50). The precision
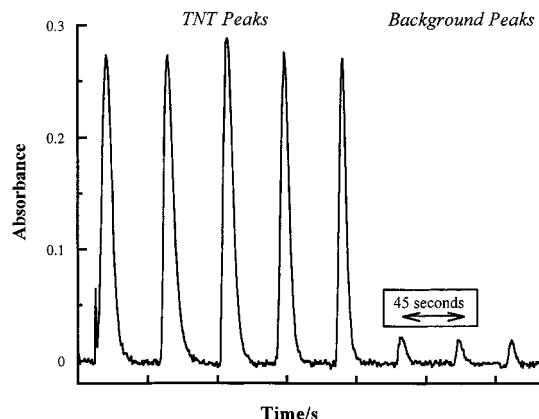

Figure 5. Representative peaks for determination of TNT in authentic soil samples. Concentration of TNT in extract is approximately 15 μg/L. The three small peaks on the right are sample "blanks," which measure the background absorbance of the soil extract. Precision of the peak height absorbance of the five TNT peaks is 3% RSD.

of the peak heights of the first five peaks is 3% RSD, which is typical for SI experiments. The limit of detection for the SI method was approximately 0.5 μg mL^{-1} (for TNT in solution), which corresponds to approximately 80 μg g^{-1} for 0.3-g soil samples. With an increase in sample size, the detection limit in soil can be decreased by a factor of 10.

One advantage of the SI method for TNT is the reduced volumes of sample, reagents, and carrier solution that are consumed per determination. Less than 1.5 mL of waste are produced per injection, 120 μL of which is from the TNT sample. The batch screening method for TNT[28] specifies the determination to be performed on a 25-mL aliquot from a 100-mL extraction; direct comparison of the methods is difficult because the extraction and analysis steps are combined in the field method (the extraction step is not included in the 1.5-mL total for the SI experiment). The waste volumes generated for the determination of TNT by HPLC can vary. For the flow rates used in this work and a retention time of 10 min, the total volume of waste per injection is estimated at 15 mL. The sample injection volume for HPLC is typically 50–100 μL. Thus, the SI method reduces waste by 10-fold or more in comparison with other methods.

Application to Aqueous Samples. The fact that the TNT derivatization reaction must be run in a highly concentrated acetone medium (88%) complicates the analysis of aqueous samples. The TNT in soil samples that is extracted into acetone can be analyzed directly without any additional sample treatment. Water samples cannot be directly aspirated into the SI system in the same manner. Not only would there not be the right concentration of acetone at the center of the TNT peak, but refractive-index peaks would form from the mixing of (nearly) pure acetone and water. One solution to this problem is to dilute the water samples until the correct ratio of acetone and water is attained, resulting in a dilution factor of more than seven. A better method is to use solid-phase extraction (SPE)[30,31] to load the TNT

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(31) *Test Methods for Evaluating Solid Wastes (SW-846), Method 3535A: Solid-Phase Extraction*; U. S. Environmental Protection Agency, Office of Solid Waste and Emergency Response: Washington, D. C., 1997.

onto a C18 or other SPE column and displace it with an eluent that has the proper amount of acetone and water. Conducting the determination with SPE columns provides the proper reaction medium and provides a means by which the TNT can be preconcentrated. In preliminary experiments to investigate the feasibility of this approach, the analyte was preconcentrated by a factor of 50 with a C18 SPE column (off-line).

Additional investigative experiments demonstrated that SPE methodology can be applied "on-line" to maintain the automation of the SI method. A more complicated SI manifold consisting of an additional two-position valve (six-port) and pump (peristaltic) is required for the complete automation of this procedure. One form of such a SI system was demonstrated to work reasonably well. A six-port valve with a SPE column in the "load" position was placed between the holding coil and the multiposition valve. A water sample containing 0.16 μg mL^{-1} TNT was loaded onto the column and eluted with the 88% acetone/12% water carrier stream. A preconcentration factor of 100 was achieved. On the basis of the detection limit of TNT standard solutions of 0.5 μg mL^{-1} these preliminary results indicate that the detection limit for aqueous samples is 5.0 μg L^{-1} (ppb).

Future Work. In addition to improved analytical figures of merit, the prototype field-portable (at-line or in-line) process monitor for TNT improves sample throughput, decreases secondary waste, and reduces labor costs through automation. Future research will include a study of the applicability of the SI method for determining the concentration of TNT in aqueous samples and will include an optimization of the means by which the preconcentration is achieved. On-site testing of the field-portable instrument for a range of TNT concentrations is also an area for future work. The study of interfering species will be extended to include other TNT metabolites, which would provide more data about the selectivity of the method and the applicability of it to, for example, effective bioremediation process control. Miniaturization of the monitor by replacement of the multichannel spectrophotometer with a photodiode-based colorimeter (with a light-emitting diode (LED) source) is also a focus of further work.