BASELINE SEPARATION OF 2,4,6-TRINITROTOLUENE (TNT) AND ITS BIOTRANSFORMATION PRODUCTS USING HPLC: PRECAUTIONS FOR ANALYTES LOSS

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Abstract: Analysis of TNT and its metabolites using HPLC is a convenient and efficient method especially for samples from a biological origin. However, it is not always easy to separate the numerous intermediates, especially isomers of reduced TNT, and often be an obstacle for accurate and reliable analytical results. We are reporting a practical baseline separation of TNT and two intermediates, 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene, using isocratic elution of water and methanol on a C-8 column. The developed analytical method is simple but baseline separation of two isomers was possible with high accuracy.

Sample pretreatment was also important for the accurate analysis of TNT and its intermediates. Analyte loss on syringe filters during filtration was tested with nine commercial products including three kinds of PTFE filters, two PVDF filters, a polysulfone, a nylon, and a polycarbonate filter. The loss of TNT was the greatest (>93.9%) on the PVDF filter, followed by polysulfone (>71.8%) and nylon (> 24.2%). The loss of TNT was statistically significant in all filtered samples with a 95% confidence limit. The same experiment was repeated using the supernatant of a TNT degrading aerobic bacterial culture resulting in an analyte loss for all the tested filters. The lowest analyte loss occurred using a PTFE filter, with the highest loss on the polysulfone filter. Analyte loss seems to depend on the filter and the specific analyte interactions such as hydrophobicity. Unaccounted for losses to filters used for sample pretreatment can result in an overestimation of transformation or introduce significant error for materials balance calculations.

Key Words: 2,4,6-trinitrotoluene, baseline separation, HPLC, analyte loss

INTRODUCTION

2,4,6-Trinitrotoluene (TNT) is a representative energetic material that comprises a major portion of the composite explosives with peak TNT production of 2 million pounds in 1985¹. The production and use of TNT resulted in contamination of soil and surface water in the vicinity of several munitions and/or ammunition plants in the U.S. and other countries. As much as 493 g TNT/kg in sediment/subsoil, 484 g TNT/kg in sludge, and 1 g TNT/kg in soil were found at the Louisiana Army Ammunition Plant². In streams that receive wastes from army ammunition plants, TNT was detected up to 60 mg/L³.

Due to the toxicity and mutagenic effects of TNT⁴, bioremediation efforts are being investigated for the elimination of explosives. Biodegradation of explosive compounds is considered as to be a potentially economical and environmentally safe alternative treatment technology. Biological transformation of TNT results in its sequential reduction to nitroso-,
hydroxyl-, and amino- compounds due to the electron withdrawing characteristics of the nitro-groups\(^5\). Abiotic coupling reactions between reduced intermediates complicate the transformation of TNT\(^6\). The major intermediates of TNT transformation are three azoxy-compounds, two isomers of diaminomononitrotoluene, and monoaminodinitrotoluene\(^6\).

High performance liquid chromatography (HPLC) is an effective analytical tool for quantitation of TNT and its intermediates. The analytical conditions are well documented in many reports\(^6\)–\(^8\). A particulate free sample, separation of each intermediate, and an efficient elution scheme are required for an accurate and precise analysis. Preparation of HPLC samples from a bioreactor usually involves a filtration step to remove particulate matters including biomass to prevent interference on the analytical column. However, membrane filters in many disposable syringes are reported to sorb significant quantities of TNT. The degree of sorption varies depending on the properties of membrane material, filtration rate, amount of sample, solubility of analyte, and pore size of the filter\(^6\). With its low solubility and numerous intermediates, use of filtration for TNT sample analysis can be problematic enough that some researchers do not use filtration for the analysis of TNT and its biotransformation intermediates\(^9\). A quantitative study was undertaken in this research to evaluate the loss of analytes on membrane filters, not only for TNT but also its biotransformation products.

Although there are numerous TNT intermediates potentially generated from the biodegradation of TNT, the aminodinitrotoluene was reported as major intermediates of TNT in frequency and in quantity\(^6\)–\(^11\). Separation of these two isomers can be achieved using reversed phase liquid chromatography (RPLC). However, baseline separation of these isomers is challenging due to the similarity of chemical properties\(^6\). Zou et al. (1994) developed a baseline separation method using a silica column in normal phase liquid chromatography (NPLC). Two isomers of aminonitrotoluenes and diaminonitrotoluenes were separated in NPLC\(^2\). However, co-elution of TNT and azoxy-compounds as well as the unnecessary use of solvent may be prevented if baseline separation of these isomers can be achieved in RPLC.

The two objectives of this study were: 1) to evaluate a quantitative estimation of analyte loss on disposable membrane filters; and 2) to develop a baseline separation technique for the aminodinitrotoluene isomers in RPLC. Experiments were conducted with pure TNT samples and intermediates samples that contained TNT and its transformation products. Results showed that loss of analytes, including TNT transformation products, were statistically significant for all tested disposal membrane filters. Among them, loss of TNT on a PVDF filter was greater than 94%. With a reversed-phased C-8 column and water/methanol elution, each of the dinitrotoluenes isomers and aminonitrotoluenes, TNT, and three azoxy-compounds were separated. In addition, baseline separation of aminodinitrotoluenes was achieved with an isocratic elution in a reversed-phase C-8 column.

**MATERIALS AND METHODS**

TNT (purity > 99.0%) was purchased from Chem. Service (West Chester, PA). 2,6-diamino-4-nitrotoluene was purchased from Aldrich Chemical Co. (Milwaukee, WI). 4-amino-2,6-dinitrotoluene (4amDNT), 2-amino-4,6-dinitrotoluene (2amDNT), 4,4'6,6'-tetrinitro-2,2'-azoxytoluene (2,2'Azo), and 2,2'6,6'-tetrinitro-4,4'-azoxytoluene (4,4'Azo) were provided by Dr. Ronald Spanggord (SRI International, CA). Standard solutions of TNT, 4amDNT, and 2amDNT were purchased from PolyScience (Niles, IL). All chemicals used for the preparation of buffer solution and other nutrients were A.C.S. grade. In all experiments, the conductivity of deionized water was greater than 17.2 MOhms-cm.

Glassware was carefully prepared to prevent possible contamination. Glassware was first
washed with soap, rinsed with acid and deionized water, pyrolyzed overnight at 450°C and cooled to room temperature. Glassware was again rinsed with filtered deionized water and dried in a particle-free laminar flow chamber. All samples were capped with a teflonlined septum and stored in a freezer unless analyzed immediately. Disposable membrane filters were either purchased or provided by each manufacturer. The material and pore size of tested disposable membrane filters is listed in Table 1.

To test the loss of TNT on membrane filters, three TNT samples (1X, 10X, and 20X strength) were prepared with deionized water (the TNT samples). Samples of TNT biotransformation products were obtained from a 1L batch reactor containing nutrients, glucose (1.0 g/L), and TNT (75 mg/L). The reactor pH was maintained at 7.0 with 50 mM phosphate buffer. Initial biomass concentration was estimated to be 540 mg/L (dry mass). Cultures were harvested at 48 hours by centrifuging at 7500 g for 30 minutes. The supernatant (the reactor sample) was used to test the loss of analytes, TNT and its transformation products, after the filtration.

Two mLs of each TNT sample were passed through five different disposable syringe filters from Alltech (Table 1) using disposable B-D plastic syringes. During filtration, a 0.1 ml air pocket was generated between the rubber seal of syringe plunger and the liquid sample in a syringe to prevent possible sorption on the rubber seal. The tested filters were neither equilibrated with samples nor treated with any solvent. The time of contact between the tested filters and the sample was very short, i.e., less than a few second. The bioreactor sample was filtered using the same procedure, but it was tested for all filters shown in Table 1. All filtered samples were analyzed randomly to reduce systematic errors. Unfiltered samples for both the TNT samples and the bioreactor samples were also analyzed and compared as controls. Each sample was analyzed more than four times.

A HPLC system installed Rheodyne injector with a fixed loop (20 µL) was used for the analysis. Reversed-phase Supelcosil LC-18 or LC-8 column was the main analytical column. The flow rate was 1.3 mL/min and the absorbance was monitored using a UV/VIS detector at the wavelength of 254 nm under the following eluent programming: 50/50 of water/methanol for 17 minutes; methanol ratio was increased to 25/75 of water/methanol over 2 minutes; 25/75 of water/methanol for 10 minutes and returned to 50/50 over 2 minutes followed by 5 minutes of equilibration time. The concentration of TNT and resolved intermediates were quantified based on peak area. During the analysis of TNT and its intermediates, theoretical plate number for TNT was greater than 7,500 throughout the analysis.

### Table 1. Physical properties of tested disposable membrane filters

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Material</th>
<th>Pore size (µm)</th>
<th>Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alltech</td>
<td>Nylon</td>
<td>0.20</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Polysulfone</td>
<td>0.22</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>PTFE 1</td>
<td>0.20</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>PTFE 2</td>
<td>0.20</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>PVDF 1</td>
<td>0.22</td>
<td>25</td>
</tr>
<tr>
<td>Cole-Parmer</td>
<td>PTFE 3</td>
<td>0.20</td>
<td>13</td>
</tr>
<tr>
<td>Poretics</td>
<td>Polycarbonate</td>
<td>0.20</td>
<td>25</td>
</tr>
<tr>
<td>Waters</td>
<td>Nylon</td>
<td>0.20</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>PVDF 2</td>
<td>0.20</td>
<td>13</td>
</tr>
</tbody>
</table>

**RESULTS AND DISCUSSION**

The concentration of TNT in the TNT samples were evaluated to be 3.8, 39.2, and 71.0 mg/L as determined by HPLC analysis (Table 2). Loss of TNT on any of the filters was the greatest for the PVDF filter, followed by polysulfone, nylon, and the two PTFE filters. The PVDF syringe filter adsorbed more than 94% of the initial TNT concentration in all the TNT samples. The loss of TNT at higher concentrations was also significant with
Table 2. Summary of TNT sample analysis for the comparison of TNT losses on syringe filter

| Tested Filter | 1X sample | | | 10X sample | | | 20X sample | | |
|--------------|------------|----------------|----------------|------------|----------------|----------------|------------|----------------|----------------|----------------|
|              | Conc. a    | s b           | n c           | Conc.       | s             | n             | Conc.       | s             | n             | Conc.       | s             | n             | |
| PTFE1        | 3.2        | 0.3           | 7             | 33.5        | 2.8           | 5             | 71.2        | 3.2           | 4             |
| PTFE2        | 3.2        | 0.8           | 4             | 36.5        | 3.1           | 5             | 70.1        | 6.3           | 4             |
| Polysulfone  | 0.5        | 0.5           | 4             | 10.6        | 1.1           | 5             | 20.0        | 13.0          | 4             |
| PVDF         | 0.1        | 0.1           | 4             | 1.1         | 0.5           | 5             | 4.3         | 3.9           | 4             |
| Nylon        | 2.6        | 0.2           | 4             | 27.0        | 2.5           | 5             | 53.8        | 10.0          | 4             |
| Control      | 3.8        | 0.1           | 4             | 39.2        | 1.5           | 5             | 71.0        | 5.1           | 4             |

a TNT concentration (mg/L), b Standard deviation (mg/L), c Number of samples

Figure 1. The concentrations of TNT after filtration with disposable syringe filters. (Error bar represents standard deviation).

the polysulfone and nylon filtered samples. The percent TNT loss after filtration through the polysulfone filter was 86.8, 73.0, and 71.8 %, for 1X, 10X, and 20X samples, respectively. For the nylon filter, the percent TNT loss was 31.6, 31.1, and 24.2%, respectively. This is consistent with the previous study 9, in which the loss of TNT on a Nalgene filter (nylon) was approximately 20% for three different concentrations of TNT samples. Jenkins et al. (1987) suggested that this loss is due to partitioning rather than sorption because sorption would produce greater loss at lower analyte concentration. The quantified TNT concentration after filtration was compared with that of the control sample (Figure 1).

Significant losses of TNT were observed for several of the filters tested. It is evident that greater than 73% of TNT was lost when the PVDF and polysulfone syringe filters were used. For the two types of PTFE filters, a statistical testing was necessary to evaluate the significance of TNT loss. Using a t-test, the loss of TNT was significant for all filtrates of nylon, polysulfone, and PVDF at 95% confidence level. Also, one out of three TNT samples showed statistical significance for the loss of TNT with two kinds of PTFE filters at 95% confidence level.

The reactor samples were used instead of novel TNT intermediates to quantify analyte loss during filtration because (1) some of the TNT intermediates are not commercially available, (2) nitroso-, and hydroxyl-compounds are unstable, (3) the effect of sample matrix is not documented, and (4) several unidentified but consistent metabolites of TNT are observed during biotransformation studies. Biological transformation resulted in the formation of aminodinitrotoluene isomers and small amounts of dianinonitrotoluene (Figure 2). Other than these compounds, unknown peaks with retention times of 4.7 to 14.6 were also observed (Figure 2). Biologically transformed samples were passed through test syringe filters using the same procedure described previously. The filtered reactor samples along with unfiltered control samples were randomly analyzed by HPLC. The results of analysis are presented as
percent recovery of each peak compared to that of the control sample (Figure 3).

Statistical testing (t' test) showed that there was a significant difference in analyte concentration at 95% confidence levels for all filtered samples. However, the percent analyte loss varied depending on the type of filter material and the properties of intermediates. As was observed with the TNT samples, PVDF1 showed the greatest removal (> 99%) of TNT, the peak eluting at 9.7, and 14.6 minutes. However, the compounds eluting at 2.4 to 7.5 minutes, more polar compounds, showed higher recovery (85.4 ~ 105%) suggesting that sorption occurs only with the late eluting non-polar compounds. In contrast to the PVDF1 syringe filter, the PVDF2 (13 mm ID) filter sorbed less severe analyte loss. More than 90% of the analytes were recovered in samples filtered with the PVDF2. The PVDF2 had a pore size of 0.20 nm suggesting that membrane material is not the only factor that governs analyte loss. As the diameter of PVDF2 filter is 13 mm, the surface area of the filter is one fourth of the PVDF1 filter. Assuming that the contact time to be equal, the analyte loss due to sorption will depend on the filter area. Therefore, an appropriate surface area (or filtration area) must be considered for the selection of syringe filter, too.

PTFE, PVDF2, nylon, and polycarbonate filters resulted in less removal of TNT (13.0 ±

Figure 2. HPLC chromatogram of the bioreactor supernatant at 48 hours prior to filtration. The sample was used to test the analytes loss on filter material (Peak ID: 8. 2,6-diamino-4-nitrotoluene; 9. 2,4-diamino-6-nitrotoluene; 17. 2,4,6-trinitrotoluene; 21. 2-amino-4,6-dinitrotoluene; 22. 4-amino-2,6-dinitrotoluene.

Figure 3. The percent recovery of TNT and each intermediate of the bioreactor sample after filtration with a syringe filter (Error bar represents the coefficient of variation, Number of analysis= 4).
Table 3. Retention time and detection limit for TNT and intermediates

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time(min)</th>
<th>Detection Limit(mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,6-diamino-4-nitrotoluene</td>
<td>2.4</td>
<td>0.024</td>
</tr>
<tr>
<td>2,4-diamino-6-nitrotoluene</td>
<td>2.5</td>
<td>0.022</td>
</tr>
<tr>
<td>2,4,6-trinitrotoluene</td>
<td>13.3</td>
<td>0.030</td>
</tr>
<tr>
<td>2-amino-4,6-dinitrotoluene</td>
<td>15.8</td>
<td>0.038</td>
</tr>
<tr>
<td>2-amino-4,6-dinitrotoluene</td>
<td>16.2</td>
<td>0.052</td>
</tr>
</tbody>
</table>

*: instrument detection limit

5.0%) compared to PVDF1 and polysulfone filters (>92.0 ± 0.5%). Overall, the analyte loss increased as peak retention time increased, or nonpolar analytes tended to be retained on the filter more than polar analytes. Further, there is an indication that some of the TNT intermediates had a greater affinity for a specific type of filter material. For example, the polycarbonate filter removed more than 99% of 2-amino-4,6-dinitrotoluene (Figure 3). The PVDF1 filter completely removed peaks eluting at 9.7 and 15.3 minutes. The polysulfone filter removed significant quantities of compounds eluting after 9.7 minutes. The same results, although the degree of analyte loss was less severe, were observed with samples treated with nylon filters and PVDF2 filters. The HPLC chromatograms showed that either a large peak was spread into many small peaks over the retention time or it completely disappeared from the chromatogram. These results suggest the following and necessitates pre-test and techniques before analysis: i) use of syringe filter may induce analytical errors in TNT quantitation, ii) other than TNT, loss of analyte is intermediate specific.

Jenkins et al. (1987) recommended pretesting filter materials and the use of an organic solvent such as methanol to avoid chemical leach and sorption. Mayer recommended careful examination of hydrophobicity of a filter that depends on the materials inherent nature and pore size. Other sample cleaning methods for sample cleanup include solid phase extraction methods and the salting-out solvent extraction method. For samples from bioreactors, high-speed centrifugation can be a simple and cost effective alternative. In this case, a guard column and monitoring of back-pressure of the HPLC system is highly recommended. The guard column will protect the analytical column from biomass and the changes of back-pressure will serve as an indication of column clogging.

Baseline Separation of Aminodinitrotoluenes

The separation of two aminodinitrotoluene congeners is challenging in reversed phase liquid chromatography due to the chemical similarity of these compounds. A RP C-18 column was used to separate these compounds without success. However, baseline separation of these two compounds was achieved with RP C-8 column with methanol (43%) and water (57%) at a flow rate of 1.5 mL/min (Figure 4). The methanol mix falls within the optimum range (50 ± 5%) reported by Kaplan and Kaplan (1982). The concentration of each analyte was 9.48 mg-TNT/L, 0.76 mg-4amDNT/L, and 1.79 mg-2amDNT/L. The theoretical plate height was greater than 8100 for TNT and the resolution of two amino congeners was 1.3 according to the following equation.

$$R_s = \frac{2(t_{R_1} - t_{R_2})}{(w_1 - w_2)}$$

where,

- $t_{R_1}$ and $t_{R_2}$ = retention time of peak 1 and 2, respectively (min)
- $R_s$ = resolution of two peaks
- $w_1$ and $w_2$ = peak width of peak 1 and 2, respectively (min)

This $R_s$ value is greater than the theoretical definition of baseline resolution, 1.0, and is close to 1.5, the value of baseline resolution as a practical definition. Kaplan and Kaplan (1982) reported the baseline separation of two
some are less polar than the parent compound increasing the retention time. For purposes of analysis, TNT and its intermediates can be grouped into three categories: (1) the polar intermediates eluting less than 10 minutes including, 2,4-diamino-6-nitrotoluene and 2,6-diamino-4-nitrotoluene; (2) other compounds eluting around TNT (from 10 to 18 minutes) including 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, and hydroxylamino compounds; and (3) the late eluting azoxy-compounds (around 25 minutes). The elution profile used in this study is an unusual combination of a gradient and isocratic scheme. However, it was successful in separating all three groups of intermediates. When necessary, 2amDNT and 4amDNT were accurately analyzed with an isocratic elution flow scheme reported in this study.

The filtration of samples to protect columns and instrument components resulted in the loss of analytes to the filter material. Results showed that statistically significant losses of TNT and its biotransformation products occurred on all tested filter materials although the degree and mechanism was different between analytes. The degree of analyte loss due to adsorption increased with increasing analyte hydrophobicity. For some filter material, partitioning may be an important mechanism for analyte loss. In addition, there is a filter-analyte specific removal of intermediates. Therefore, it is recommended that any filter should be tested using an intermediate sample in an analyzing matrix before routine analysis, especially for samples from bioreactors.

**SUMMARY**

The analysis of TNT and its biotransformation products pose several potential problems. First, the biological and chemical transformation pathways generate many intermediates with a broad range of chemical characteristics. Second, TNT transformation produces isomers that are difficult to separate and

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