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Crucial problem in rapid spectrophotometric determination of 2,4,6-trinitotoluene and its breakthrough method

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Abstract

A rapid spectrophotometric determination for 2,4,6-trinitrotoluene (TNT) is significant because this method is suitable for simultaneous analyses of the numerous samples. We found one problem that TNT reduction products interfere with the TNT detection in this assay, and we overcame this problem by heating the samples at 95℃, resulting in the production of compounds that did not interfere.

Key words: accuracy of quantification, hydroxylamino-dinitrotoluene, rapid spectrometric determination, tetranitro-azoxytoluene, 2,4,6-trinitrotoluene

The highly energetic chemical 2,4,6-trinitrotoluene (TNT) is a nitroaromatic explosive that is released into soil and water ecosystems mainly by many military activities (Comfort et al., 1995). The persistence of TNT and its metabolites are of environmental concern because they may be cytotoxic and genotoxic to many living organisms (Bennett, 1994; Rieger and Knackmuss, 1995; Letzel et al., 2003). Therefore, constructing the effective remediation technology for TNT as early as possible will be required; discoveries of bacteria possessing a high performance for TNT degradation will be necessary for constructing the complete TNT-degradation systems. Also, decontaminating rapidly such TNT will be essential for leveraging the bacteria possessing a high performance of TNT biotransformations and/or for adding many cells in the TNT-polluted sites. There has been much interest in the bioremediation of explosive-polluted water and soil (Nay et al, 1974); both aerobic and anaerobic TNT transformation by bacteria have been reported (Haidour and Ramos, 1996; Martin et al., 1997; Esteve-Nunez and Ramos, 1998; Kalafut et al., 1998). The primary intermediates of TNT transformation by bacteria are hydroxylamino-dinitrotoluenes, amino-dinitrotoluenes, diamino-nitrotoluenes, and tetranitroazoxyltoluenes.

Biological transformation of TNT is usually determined by tracking the loss of the parent TNT and the production of metabolites by HPLC analysis (Ahmad and Roberts, 1995); however, this method may not be suitable for the timely analysis of multiple samples encountered in TNT degradation studies such as screening for TNT-metabolizing bacteria and

enzyme studies and so on. For this issue, a simple spectrophotometric assay, in which TNT can be rapidly detected, is applicable for such many samples and it was developed by Oh et al. (2000), resulting in allowing the high throughput screening. This assay is an excellent method because TNT can be briefly determined by measuring significant absorbance at 447 nm of TNT solution in highly alkaline conditions; however, in this method, we found one crucial problem that a rapid spectrophotometric determination for TNT was not applicable to the TNT-metabolizing samples (Fig. 1), reacted with many cells (about 2.5×10^8 cells/mL) of *Pseudomonas* sp. strain TM15, which was isolated from TNT-contaminated soils in the Yamada Green Zone, Kitakyushu, Japan (Maeda et al., 2003). Cell suspensions of this strain (resuspended in 50 mM sterilized phosphate buffer (pH 7.0) after grown until the late logarithmic phase in LB medium containing TNT (0.1 g/L) at 30℃) were mixed into 100 mL M8 minimal medium (Abrill et al., 1989) containing TNT (100 mg/L) and acetate (10 mM) as nitrogen and carbon sources, and then the mixture was incubated at 30℃ in the dark with shaking (120 rpm). When reacted with many cells, we found the difference in the results of measurement value for TNT between HPLC analysis (detection at 254 nm with a Shimadzu SPD-10AVP UV-VIS detector) on an Inertsil ODS-2 column (GL Sciences Inc., Japan) with acetonitrile-distilled water (40: 60) as the mobile phase and spectrophotometric determination in which one measures significant absorbance at 447 nm generated by mixing 240 μL of 1 M NaOH and distilled water (1 mL) into 0.5 mL of the sample (removing the cells by

centrifugation at 5000×g for 10 min and by filtration with a membrane filter (0.2 μm, Toyo Roshi Kaisha, Ltd., Japan)), with a UV / VIS Spectrophotometer V-530 (Jasco Co. Ltd., Japan) as shown in Fig. 1, although no problems in few cells (about 5.0×10^6 cells/mL). Also, we demonstrated that high concentrations of TNT reduction products, 2-hydroxylamino-4,6-dinitrotoluene (2HADNT) and 4-hydroxylamino-2,6-dinitrotoluene (4HADNT), were detected from the culture medium including many cells in comparison with that including few cells (data not shown) and then suggested that 2HADNT and 4HADNT may be responsible for this problem because these chemicals exhibit significant absorbance at 447 nm as well as TNT in the highly alkaline solution (Fig. 2), although less effects than TNT does.

2HADNT and 4HADNT were spontaneously converted into 4,4',6,6'-tetranitro-2,2'-azoxytoluene (2,2'AzT), 4,2',6,6'-tetranitro-2,4'-azoxytoluene (2,4'AzT), 2,2',6,6'-tetranitro-4,4'-azoxytoluene (4,4'AzT) in agreement with the results of previously studies (Haidour and Ramos, 1996; Maeda et al., 2006; Pereira et al., 1979; Spanggord et al., 1995). These tetranitroazoxytoluenes display no absorbance at 447 nm in highly alkaline solutions (Fig. 2). Also, we found that 2HADNT and 4HADNT were rapidly decreased by treating at 95℃ with a Dry Thermo Unit DTU-1B (TIETECH Co. Ltd., Japan) in comparison with that at room temperature, judging from the results of stability tests of 2HADNT and 4HADNTby HPLC analyses (data not shown). Heat treatment at 95℃ was

the best ways to promote these reaction from 2HADNT and 4HADNT to 2,2'AzT, 2,4'AzT, and 4,4'AzT at short times.

Using our knowledge, the TNT-metabolizing samples were treated for 3 min at 95℃, and then TNT concentrations were analyzed by HPLC and spectrophotometric determination in these samples. As shown in Fig. 3, the accuracy of quantification for TNT in spectrophotometric determination was recovered to the same level as HPLC analyses. TNT was slightly decreased by heat treatment of TNT-metabolizing samples (Fig. 3) although TNT dissolved in distilled water was intact in initial terms (data not shown). These results indicate that some enzymes leaked from TM15 cells may catalyze TNT. We overcame one crucial problem that 2HADNT and 4HADNT interfered with the determination of TNT, by converting the other compounds that did not interfere. Our technique may be applicable for the TNT detection in TNT-metabolizing and/or various environmental samples including many amounts of 2HADNT and 4HADNT, which are interfering substances in a rapid spectrophotometric determination method.

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