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Short communication

Use of narrow-bore high-performance liquid chromatography–diode array detection for the analysis of intermediates of the biological degradation of 2,4,6-trinitrotoluene

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Abstract

A single method was developed for the separation and quantitation of hexahydro-1,3,5-trinitro-1,3,5-triazine, 2,4,6-trinitrotoluene (TNT), and most of the known and suspected biodegradation intermediates of TNT by RP-HPLC and diode array detection. The known biodegradation intermediates of TNT analyzed were 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, 2,6-diamino-4-nitrotoluene, 2,4-diamino-6-nitrotoluene, 2,4,6-triaminotoluene, 2,2′,6,6′-tetranitro-4,4′-azoxytoluene, and 4,4′,6,6′-tetranitro-2,2′-azoxytoluene. The suspected biodegradation intermediates of TNT included 1,2,3-benzenetriol (pyrogallol), 1,3,5-benzenetriol (phloroglucinol), 2-methyl-1,3,5-benzenetriol (methyl phloroglucinol) and 4-methylphenol (*p*-cresol). Mobile phases consisting of aqueous buffers adjusted to three different pH values in a gradient with acetonitrile were examined for their efficiency in separating the intermediate compounds and for the minimization of speciation of the ionizable intermediates (e.g. 2,4,6-triaminotoluene). A final aqueous buffer pH of 3.2 was selected to minimize the interference to the separation caused by 2,4,6-triaminotoluene speciation. Solvent consumption was minimized by the use of a narrow-bore column. All of the known reduction products as well as *p*-cresol and methylphloroglucinol were identified in culture supernatants from TNT-degrading cultures while pyrogallol and phloroglucinol were not.

1. Introduction

Nitro-substituted munitions compounds such as 2,4,6-trinitrotoluene (TNT) have been found to contaminate soils [1] and could also contaminate groundwater [2] because of past disposal practices at explosives manufacturing, loading and packing facilities. The toxicological effects of TNT on various organisms are well documented [3–6]; in humans, these effects may result in toxic hepatitis and aplastic anemia [7]. TNT has also been found to be mutagenic by the Ames bacterial assay [8]. Concern over the environmental fate of these compounds has intensified research into safe and economical treatment options like biodegradation. However, the degradation of TNT does not necessarily mean a removal of its deleterious characteristics; the primary biotransformation products of TNT, 2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene (4ADNT), have also been shown to have toxic [9] and mutagenic [10] effects on certain biological species. The toxicity and mutagenicity of secondary and subsequent

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biodegradation intermediates of TNT, such as 2,6-diamino-4-nitrotoluene (DA4NT), 2,4-diamino-6-nitrotoluene (DA6NT) and 2,4,6-triaminotoluene (TAT), have yet to be explored. Therefore, a simple and accurate method for the identification and quantitation of TNT and its biotransformation products is needed so that acceptable mass balances can be established during biodegradation.

A separation step must precede identification and quantitation in the selected method owing to the functional similarities of many of the intermediates. Separation of the intermediates by gas chromatography can result in the coelution of aminodinitrotoluenes (ADNTs) and diaminonitrotoluenes (DANTs) [11]. In addition, some of the analytes are thermally unstable [1], making gas chromatography an unfavorable separation option. Past work has favored reversed-phase high-performance liquid chromatography (RP-HPLC) as the method of choice for the separation of munitions compounds. Most of this work involved isocratic elution [12–14] of compounds with either a water–methanol or a water–methanol–acetonitrile mobile phase. Unfortunately, isocratic elution poses a problem when separating analytes of a wide range of polarities, such as the ones presented in this paper; there is a tradeoff between eluting the less polar tetranitroazoxytoluene (TNAZT) compounds [2,2′,6,6′-tetranitro-4,4′-azoxytoluene (4,4′-Az) and 4,4′,6,6′-tetranitro-2,2′-azoxytoluene (2,2′-Az)] within reasonable time and achieving a separation between the more polar compounds (DA4NT and DA6NT) because significantly different organic fractions in the mobile phase are required for each of these tasks. Kaplan and Kaplan [15] demonstrated this problem when they did isocratic elutions with 20 and 75% methanol to separate DANTs and TNAZTs, respectively. The answer to this problem lies in using gradient elution. Gradient elutions were performed by Kaplan and Kaplan [15] and Schuster and Gratfeld-Huesgen [16] using a water–methanol mobile phase.

In earlier work, only Yinon and Hwang [17], Jenkins and Grant [14] and Schuster and Gratfeld-Huesgen [16] included a post-LC identification/confirmation step. Yinon and Hwang used mass spectral identification, whereas Jenkins and Grant used an LC-CN (cyanopropylmethylsilyl bonded phase) column to confirm the identity of the analytes by an inversion of their retention time sequence. Schuster and Gratfeld-Huesgen used diode array detection (DAD), the same method as the one used in this study, for the UV–visible spectral identification of compounds. This study expands on Schuster and Gratfeld-Huesgen's work with DAD to include most of the known and suspected intermediates of TNT biodegradation.

This paper presents a single method for the separation, identification and quantitation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), TNT, and most of the known and suspected biodegradation intermediates of TNT by RP-HPLC and DAD. The known biodegradation intermediates of TNT analyzed were 2ADNT, 4ADNT, DA4NT, DA6NT, TAT, 4,4′-Az and 2,2′-Az [18,19]. The suspected biodegradation intermediates of TNT included 1,2,3-benzenetriol (pyrogallol), 1,3,5-benzenetriol (phloroglucinol), 2-methyl-1,3,5-benzenetriol (MPG) and 4-methylphenol (*p*-cresol) [20,21]. RDX was included in this study because many munitions contaminated soils are contaminated with appreciable concentrations of RDX as well as TNT [14]. Mobile phases consisting of aqueous buffers adjusted to three different pH values in a gradient with acetonitrile were examined for their efficiency in separating the intermediate compounds and for the minimization of speciation of the ionizable intermediates. Solvent consumption was minimized by the use of a narrow-bore column (2.1 mm I.D.).

2. Experimental

Samples of 2ADNT, 4ADNT, DA6NT, 2,2′-Az and 4,4′-Az were obtained through the generosity of Dr. R.J. Spanggord of SRI International, Menlo Park, CA, USA. Standards for

RDX, pyrogallol, phloroglucinol, MPG and *p*-cresol were acquired from Mr. S. Funk at the University of Idaho, Moscow, ID, USA. TNT and TAT were obtained from Chem Service of Westchester, PA, USA, and DA4NT was obtained from Aldrich, Milwaukee, WI, USA. Stock standards in the 100 mg/l range were prepared. All water used in the preparation of standards and mobile phase was type 1 reagent-grade water obtained from the Barnstead NANOpure II water purification system.

TNT was dried to a constant mass, crushed and made into a 100 mg/l stock standard in water using a method outlined by Ruchhoft and Meckler [22]. Stock standards of DA6NT, *p*-cresol, pyrogallol, phloroglucinol and MPG were made in water. RDX was obtained as a 10000 mg/l stock standard made in methanol. DA4NT, 2ADNT and 4ADNT were made in methanol–water (50:50, v/v). The TNAZT compounds were prepared in acetonitrile–methanol (50:50, v/v), as they were not soluble in the methanol–water mix. Stock standards for TAT were prepared in the aqueous mobile phase with which they were to be analyzed or in methanol or acetonitrile–methanol (50:50) to test TAT's stability in these solvents. For calibration purposes five additional diluted standards were prepared from each stock standard in the same solvent as the given stock standard. Diluted standards showed less than 4% decrease in area over a period of three months and stock standards showed less than 2% decrease over the same period of time.

Two mixed standards were prepared, one in pH 3.2 phosphate buffer and the other in acetonitrile–methanol (50:50). TAT (90 mg/l), phloroglucinol (35 mg/l), pyrogallol (9.4 mg/l) and MPG (24 mg/l) were prepared in pH 3.2 buffer because they showed less peak dispersion in this solvent than in any organic solvents. The second mixture contained 19.8 mg/l DA4NT, 21.3 mg/l DA6NT, 103 mg/l *p*-cresol, 59 mg/l RDX, 29 mg/l 2ADNT, 32 mg/l 4ADNT, 80 mg/l TNT, 30.6 mg/l 2,2′-Az and 37.2 mg/l 4,4′-Az, and was made up in methanol–acetonitrile (50:50).

Samples of the supernatant from TNT biodegradation studies were centrifuged at 14 000 rpm for approximately 15 min (16 000 *g*) and were stored and analyzed under the same conditions as the standards.

Acetonitrile was selected as the organic component of the mobile phase after observing that the TNAZT compounds were not completely soluble in methanol. The acetonitrile used in the mobile phase and the preparation of standards was Fisher Scientific HPLC-grade acetonitrile (UV cutoff 190 nm). Mallinckrodt HPLC-grade methanol was also used in standard preparation. Phosphate-buffered aqueous mobile phase components at pH 3.2 and pH 4.0 were prepared by first adding 50 μl of concentrated phosphoric acid/l water and then adding K_2HPO_4 to adjust to the desired pH. The approximate molarity of these buffers was 11 m*M*. Type 1 reagent-grade water, without any pH change, was used as the third aqueous phase component. The pH of the reagent-grade water was found to be 6.4. All aqueous mobile phase components were vacuum filtered through a 0.2-μm Gelman Sciences (Ann Arbor, MI, USA) membrane filter.

The LC analyses were performed with a Hewlett-Packard HP 1090 Series II/M liquid chromatograph with a DR 5 ternary solvent-delivery system, variable-volume auto-injector, temperature controlled autosampler (TCAS), thermostatically controlled column compartment and a built-in DAD system. Hewlett-Packard ^{3D}Chemstation was used for instrument control and data analysis. The column used for the separation was an Alltech Alltima RP-HPLC C_{18} column that contained 5-μm particles. Column dimensions were 250 mm × 2.1 mm I.D. An Alltech direct-connect refillable guard column packed with the same material was used to protect the analytical column.

Temperatures for the TCAS and the column compartment were maintained at 8 and 45°C, respectively, during analyses. The auto-injector was set to inject 10 μl of each sample. DAD was used for dual-wavelength detection at 210 and 254 nm. Peaks were scanned from 200 to 600 nm for compound characterization. The mobile

phase flow-rate was set at 0.4 ml/min. The initial method employed a 2-min equilibration at 10% acetonitrile, followed by a linear gradient from 2 to 20 min varying acetonitrile from 10 to 100%. The initial method was used to study pH effects on individual compounds. This method was then optimized so that maximum separation could be achieved between the analytes. The final optimized method varied acetonitrile in the following way: 10 to 55% from 0 to 14 min, 55 to 100% from 14 to 18 min, held at 100% from 18 to 19 min and 100 back to 10% from 19 to 22 min.

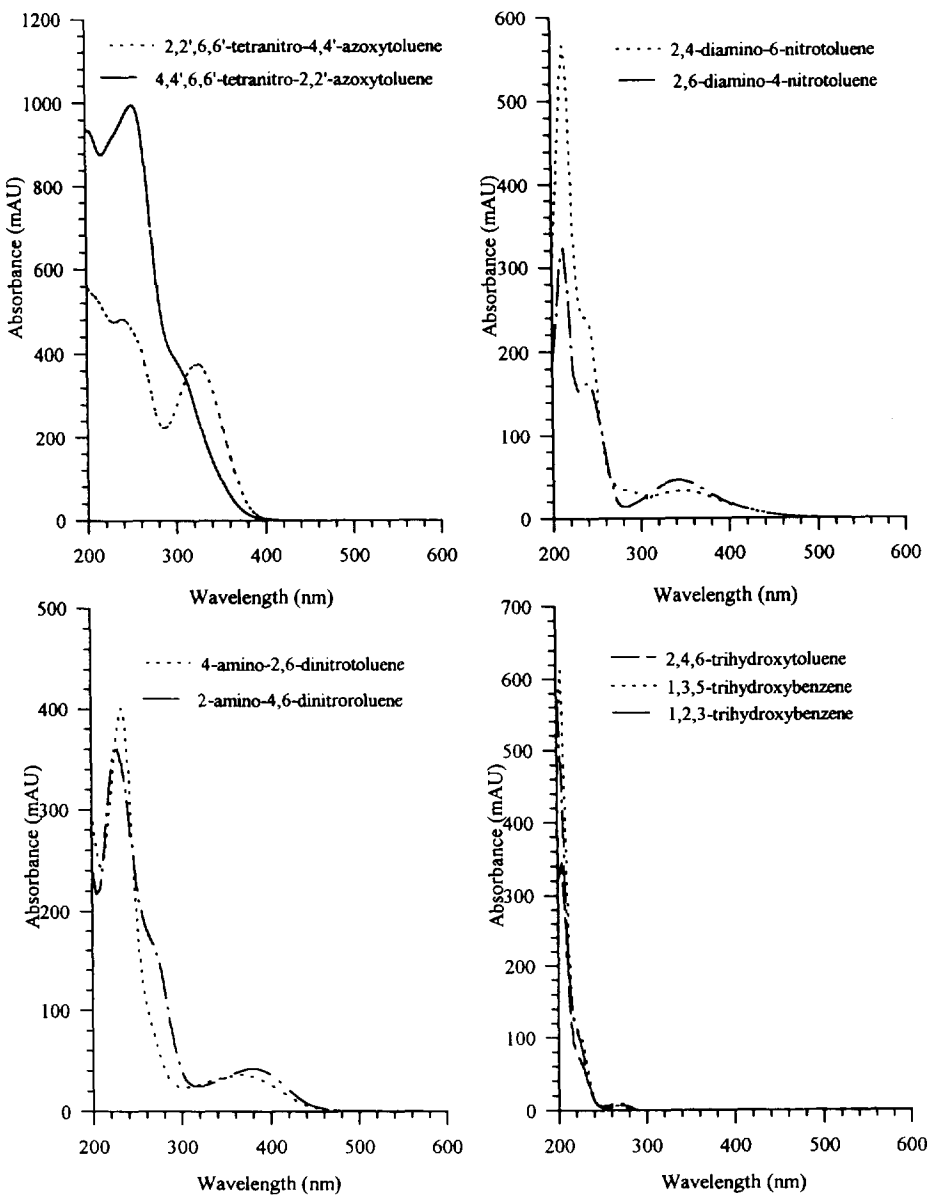

Fig. 1. The UV–Vis spectra of nine of the intermediates or suspected intermediates of TNT degradation. Spectra were obtained by scanning from 210 to 600 nm using the diode array detector.

3. Results

3.1. Effects of aqueous phase pH

No significant changes in retention times or response factors were observed with a variation in aqueous phase pH. There was no change in compound spectra, either; spectral matches of greater than 990 (on a scale of 1000) were obtained for compounds eluted at different pH values. The spectra obtained for most of the analytes are shown in Fig. 1.

The most significant effect of pH was seen on the stability of TAT. At pH 6.4, TAT eluted as five different peaks, causing an interference to the separation of the early eluting alcohols. The number of TAT peaks was reduced to four with a pH 4.0 aqueous phase. A drastic change occurred at pH 3.2 when TAT eluted as only two peaks with greater than 84% of the area in a single, possibly solvent, peak at a retention time of 1.55 min. TAT standards made in methanol and acetonitrile–methanol (50:50) and analyzed at pH 3.2 showed further improvements. TAT in methanol eluted as two peaks with greater than 85% of the area in a single peak. TAT in acetonitrile–methanol (50:50) eluted as a single identifiable peak. Based on these results pH 3.2 aqueous phase was selected for the final optimization of the separation.

3.2. Optimization of separation

The final gradient method selected varied acetonitrile in the following way: 10 to 55% from 0 to 14 min, 55 to 100% from 14 to 18 min, held at 100% from 18 to 19 min and 100 back to 10% from 19 to 22 min. The total analysis time was 22 min with 3 min of re-equilibration allowed between samples. The retention times, response factors and detection limits obtained for the analytes with this gradient are shown in Table 1. Changing the initial composition of the mobile phase to less than 10% acetonitrile resulted in the binding of the TNAZT compounds to the column and lengthening the retention times to extremes, making the method impractical for these compounds. Since the detection of these compounds is very important to the mass balance of TNT metabolism, it was decided that the at least 10% acetonitrile was necessary.

Decreasing the slope of the gradient caused increased interference between the early-eluting compounds and it did not improve separation between the non-baseline-separated pairs. Baseline separation could not be achieved be-

Table 1
Summary of chromatographic characteristics for the compounds studied

Compound	Retention time (min)	Response factor (mAU s mg^{-1} l^{-1})[a]	Detection limit (ng/injection)[b]	Signal-to-noise ratio at detection limit
Phloroglucinol	2.15	145.3 ± 1.6	10	22
Pyrogallol	2.88	110.3 ± 5.5	10	14
MPG	3.13	129.3 ± 2.3	10	26
DA4NT	6.11	144.2 ± 1.0	10	35
DA6NT	6.68	152.6 ± 9.8	10	33
p-Cresol	10.3	35 ± 1.8	10	114
RDX	10.4	53.2 ± 1.5	5	61
2ADNT	13.3	52.3 ± 0.5	10	26
4ADNT	13.4	56.4 ± 1.1	10	27
TNT	14.7	51.7 ± 0.9	1.1	2.7
2,2'-Az	18.2	54.1	0.66	5.6
4,4'-Az	18.5	52.1	0.58	0.6

[a] Results are average and 1 standard deviation ($n = 4$).
[b] Results indicate the lowest standard tested, detection of smaller amounts of material may be possible, but was not tested.

tween *p*-cresol and RDX and between 2ADNT and 4ADNT. However, separations were sufficient to allow spectral identification and integration of the different peaks. Increasing the slope of the gradient reduced the separation between the ADNTs and between *p*-cresol and RDX. Chromatograms at 210 and 254 nm are shown in Fig. 2. As it can be seen from Fig. 2, all of the

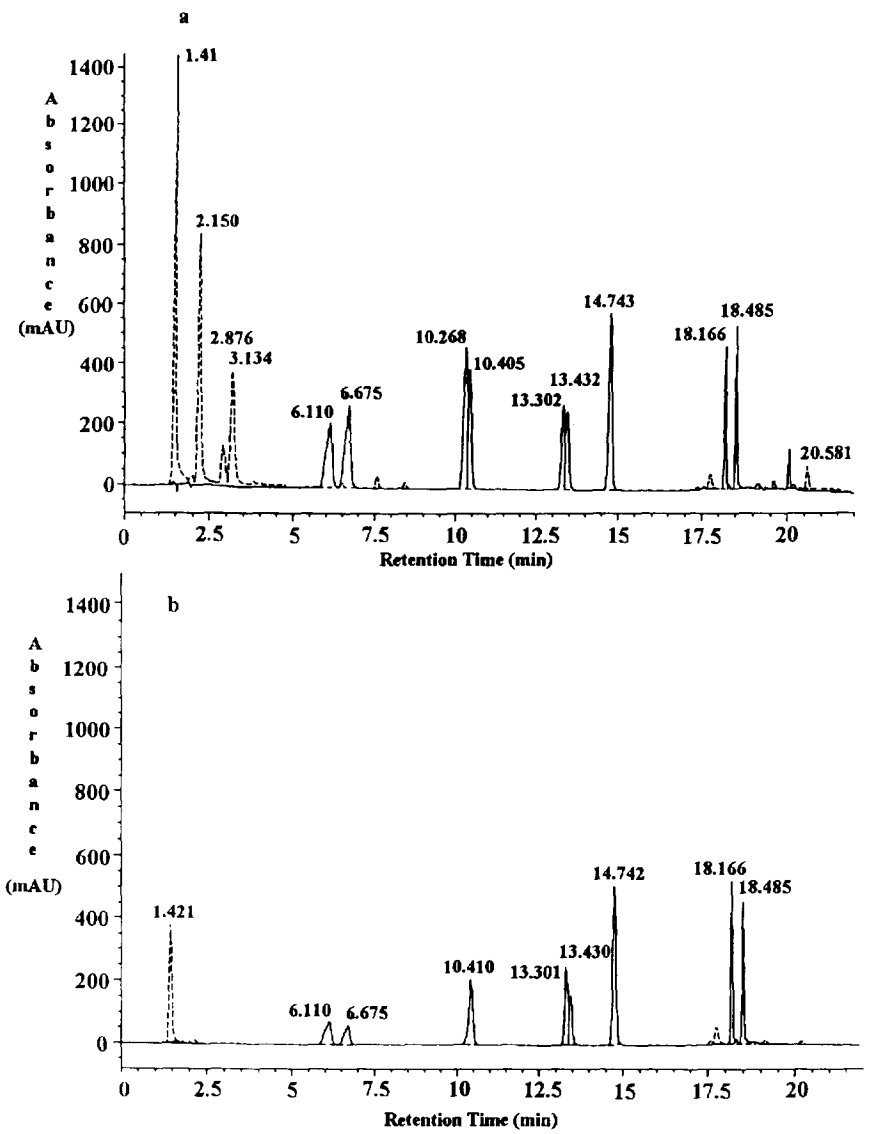

Fig. 2. The chromatographs of compound separation for the mixed standards containing all of the test compounds from signals saved at (a) 210 and (b) 254 nm. Dotted lines, standards of TAT (1.4 min, 90 mg/l), phloroglucinol (2.15 min, 35 mg/l), pyrogallol (2.88 min, 9.4 mg/l) and methylphloroglucinol (3.13 min, 24 mg/l) made up in pH 3.2 buffer. Solid lines, standards of DA4NT (6.11 min, 19.8 mg/l), DA6NT (6.68 min, 21.3 mg/l), *p*-cresol (10.27 min, 103 mg/l), RDX (10.4 min, 59 mg/l), 2ADNT (13.3 min, 29 mg/l), 4ADNT (13.4 min, 32 mg/l), TNT (14.74 min, 80 mg/l) 2,2′-Az (18.17 min, 30.6 mg/l) and 4,4′-Az (18.48 min, 37.2 mg/l) made up in methanol–acetonitrile (50:50).

suspected biodegradation intermediates of TNT showed no absorption in the 250–254 nm wavelength range.

Fig. 3 shows a chromatograph from a sample of a culture spiked with TNT. This culture has produced 4ADNT and DA6NT almost exclusively over the two other amino or diamino isomers. Low concentrations of *p*-cresol and MPG were detected and identified by comparing the spectra and retention times to the authentic standards. This culture was not inoculated under strictly anaerobic conditions but was allowed to create its own anaerobic conditions by the use of glucose by aerobic bacteria present in the culture. In such cases some of the TNAZT compounds are produced but do not remain in the culture for long. The presence of MPG and *p*-cresol indicates that the methyl group of the TNT molecule stays on the ring in its reduced form at least until *p*-cresol. There have been no indications of the presence of phloroglucinol or pyrogallol in our cultures. The peak at 1.9 min is seen occasionally in aqueous cultures spiked with TNT. This compound is as yet unidentified and may be methylresorcinol, resulting from the removal of one hydroxy group from the

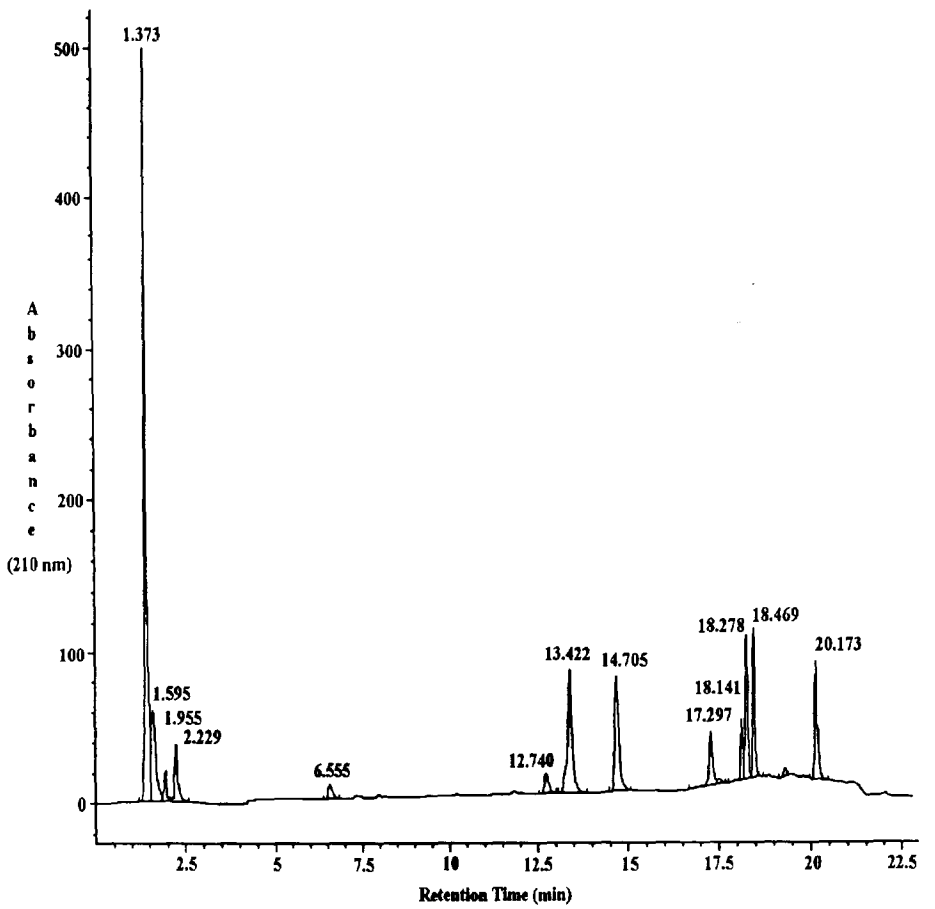

Fig. 3. A randomly selected chromatograph from analysis of the aqueous phase of TNT-degrading cultures. The retention times of some of the peaks have shifted due to column age but DAD analyses of the peak spectra confirm the identifications of methylphloroglucinol (2.229 min), DA6NT (6.555 min), 4ADNT (13.442 min), TNT (14.705 min), 2,2′-Az (18.141 min) and 4,4′-Az (18.469 min).

methylphloroglucinol or it may be an intermediate of *p*-cresol degradation.

4. Conclusions and discussion

The analysis of the intermediates of the biological degradation of TNT was accomplished using narrow bore HPLC with diode array detection. The aqueous phase of pH 3.2 was selected as the most suitable for the separation of the given compounds. This pH allowed the minimization of TAT speciation without causing any adverse changes in the chromatographic properties of the other analytes. Reduction of pH to 3.0 may further decrease the speciation of TAT. However, the operation a silica-based reversed-phase column below pH 3.0 is not recommended, as it can cause the cleavage of silanol groups connecting the alkyl chains to the silica particles. There are, however, several resin-based (e.g. styrene–divinylbenzene) reversed-phase columns available that have shown a wider range of pH tolerance than traditional silica-based reversed-phase columns. The low pH requirements also suggests that samples from TNT biodegradation studies should be acidified before analysis. The decrease in speciation of TAT is important in order to prevent its interference with the elution and quantitation of other compounds. TAT is very unstable and is not detected easily in biological samples due to its extreme sensitivity to oxygen. The sampling procedure used here, exposes the sample to oxygen while centrifuging, and so attempts made to quantitate TAT are done as specific experiments designed with minimized oxygen contact. The procedure developed here was for routine everyday analyses with TAT out of the way, and not for TAT analyses.

A detection wavelength of 250–254 nm is unsuitable for the detection of some of the suspected intermediates of TNT biodegradation. A wavelength in the range of 210–220 nm is more suitable for detection of these compounds. This also precludes the use of methanol as the organic component of the mobile phase, as methanol absorbs in this wavelength range. Prior to this work, only Yinon and Hwang [17] and Harvey et al. [23] have used acetonitrile as the sole organic phase.

Baseline separation of ADNTs could not be achieved using the method presented. In past literature this has only been accomplished by Walsh and Jenkins [24], who used an LC-CN column in series with an RP C_{18} column. Therefore, it is strongly suspected that baseline separation of the ADNTs cannot be achieved without the alteration of the stationary phase to one that is more selective for the ADNTs.

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