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Use of reversed-phase high-performance liquid chromatography–diode array detection for complete separation of 2,4,6-trinitrotoluene metabolites and EPA Method 8330 explosives: influence of temperature and an ion-pair reagent

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Abstract

Explosives such as 2,4,6-trinitrotoluene (TNT), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) are widely distributed environmental contaminants. Complete chromatographic separation is necessary in order to accurately determine and quantify explosives and their degradation products in environmental samples and in (bio)transformation studies. The present study describes a RP-HPLC method with diode array detection using a LC-8 guard column, a Supelcosil LC-8 chromatographic column, and a gradient elution system. This gradient method is capable of baseline separating the most commonly observed explosives and TNT transformation metabolites including 2,4,6-triaminotoluene (TAT) in a single run. The TNT metabolites separated were 2-hydroxylamino-4,6-dinitrotoluene, 4-hydroxylamino-2,6-dinitrotoluene, 2,4-dihydroxylamino-6-nitrotoluene, 4,4′,6,6′-tetranitro-2,2′-azoxytoluene, 2,2′,6,6′-tetranitro-4,4′-azoxytoluene, 4,4′,6,6′-tetranitro-2,2′-azotoluene, 2,2′,6,6′-tetranitro-4,4′-azotoluene, 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, 2,6-diamino-4-nitrotoluene, 2,4-diamino-6-nitrotoluene, and TAT. The same gradient method at a different column temperature can also be used to baseline separate the explosives targeted in the Environmental Protection Agency (EPA) Method 8330 with approximately 22% reduction in total run time and 48% decrease in solvent consumption compared to previously published methods. Good separation was also obtained when all TNT metabolites and EPA Method 8330 compounds (a total of 23 compounds) were analyzed together; only 2,6-DANT and HMX co-eluted in this case. The influence of temperature (35–55 °C) and the use of an ion-pair reagent on the chromatographic resolution and retention were investigated. Temperature was identified as the key parameter for optimal baseline separation. Increased temperature resulted in shorter retention times and better peak resolution especially for the aminoaromatics investigated. The use of an ion-pair reagent (octanesulfonic acid) generally resulted in longer retention times for compounds containing amine functional groups, more baseline noise, and decreased peak resolution.

Keywords: Explosives; Environmental analysis; EPA Method 8330; Temperature selectivity; 2,4,6-Trinitrotoluene; Hydroxylaminodinitrotoluene; Tetranitroazoxytoluene; Tetranitroazotoluene

1. Introduction

The nitroaromatic compound 2,4,6-trinitrotoluene (TNT), has been found to contaminate soils at former and present munitions manufacturing facilities, storage depots, and former sites of explosives use [1,2]. Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) are also contaminants at these locations as they are routinely components of TNT-based explosive mixtures [1,3,4].

At these sites, TNT is often being biologically reduced stepwise to hydroxylaminodinitrotoluenes (HADNTs), aminodinitrotoluenes (ADNTs), diaminonitrotoluenes (DANTs), and 2,4,6-triaminotoluene (TAT) (Fig. 1). The HADNTs, which are intermediates in the transformation of TNT, include 2-hydroxylamino-4,6-dinitrotoluene

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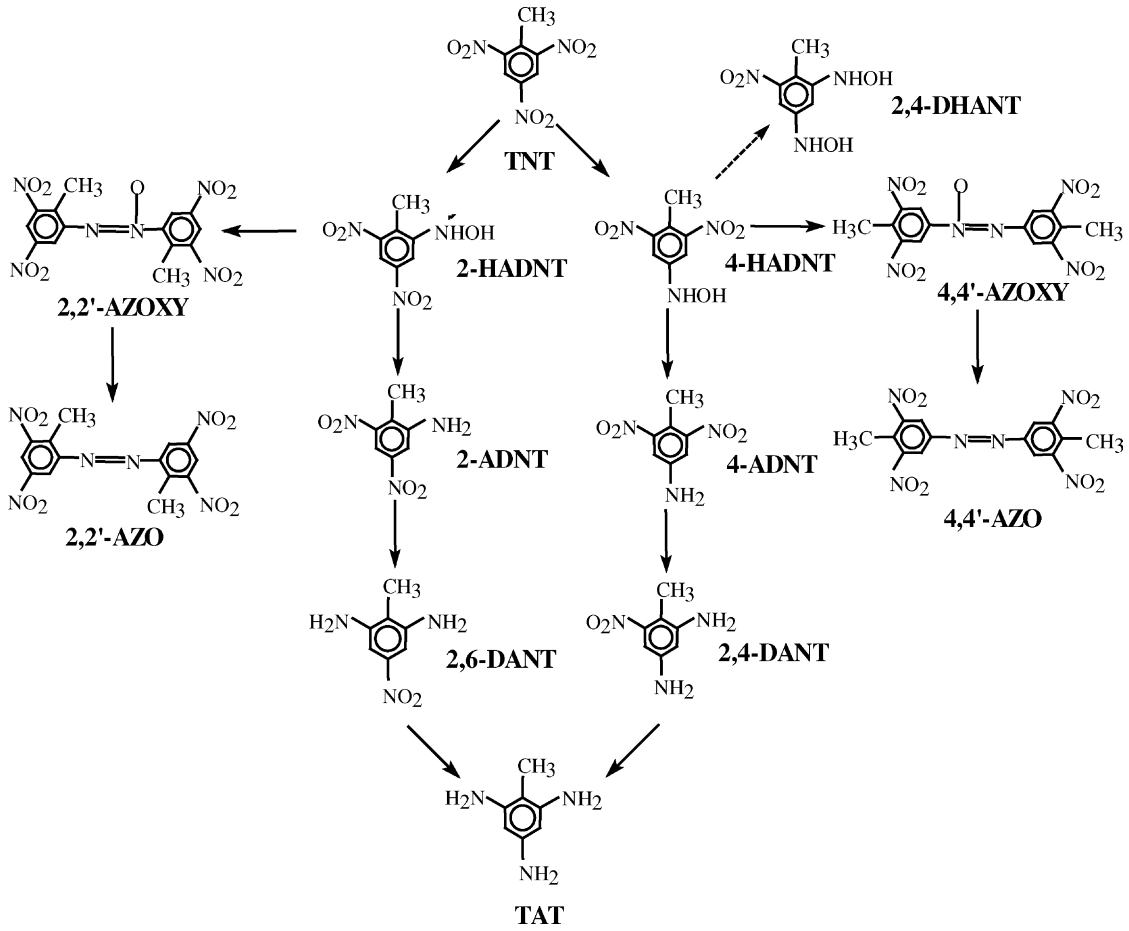

Fig. 1. 2,4,6-Trinitrotoluene (TNT) transformation pathways observed in soil and water.

(2-HADNT), 4-hydroxylamino-2,6-dinitrotoluene (4-HADNT), 2,4-dihydroxylamino-6-nitrotoluene (2,4-DHANT) and (not shown in Fig. 1) 2,6-dihydroxylamino-4-nitrotoluene (2,6-DHANT) [5]. The ADNTs are 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT). The DANTs are 2,4-diamino-6-nitrotoluene (2,4-DANT) and 2,6-diamino-4-nitrotoluene (2,6-DANT) [1,3,6]. The azoxy compounds, such as 4,4′,6,6′-tetranitro-2,2′-azoxytoluene (2,2′-azoxy) and 2,2′,6,6′-tetranitro-4,4′-azoxytoluene (4,4′-azoxy) are believed to be the products of spontaneous abiotic hydroxylamino–nitroso condensation reactions (Fig. 1) [1,5,7].

Environmental concerns stem from the mutagenic, carcinogenic, and toxic effects of nitroaromatic and aminoaromatic compounds. TNT, 2-ADNT, 4-ADNT, 2,4-DANT, 2,6-DANT and TAT have been found to be cytotoxic presumably due to induced oxidative stress [8,9]. TNT, RDX, HMX, and the TNT-derived metabolites all demonstrate mutagenic capability [9] and the EPA classifies TNT and RDX as possible human carcinogens [10].

In order to develop accurate metabolic pathway maps, HPLC methods capable of analyzing and separating all explosives and their transformation products must be available. The reduced metabolites of TNT exhibit varying degrees of polarity and chemical stability making the chromatographic separation extremely challenging. The hydroxylamines are known to be unstable in aqueous solution in the presence of molecular oxygen potentially forming azoxy compounds [5]. Azoxy compounds can undergo further transformation to form 4,4′,6,6′-tetranitro-2,2′-azotoluene (2,2′-azo) and 2,2′,6,6′-tetranitro-4,4′-azotoluene (4,4′-azo) [11]. The formation of HADNTs, azoxy, and azo compounds may be the major cause of poor mass balances obtained in bioremediation systems, where only aminated products are monitored [5,12].

TAT is one of the most unstable reduced TNT metabolites in aqueous solutions. The three amino groups and the methyl group are all activating substituents, which increase the reactivity of the aromatic ring toward electrophiles such as oxygen. Consequently, TAT is highly oxygen sensitive and easily degraded under aerobic conditions [1]. However, even under anaerobic conditions, TAT can participate in oxydimerization and polymerization to form azo and poly-azo compounds [1,13].

Although GC, solid-phase microextraction (SPME)–GC–MS, CE-UV, and TLC have been used, HPLC has remained the major analytical tool for the detection and quantification of nitroaromatic compounds [1,3,7,11,12,14–18]. The most

commonly used method for the analysis of nitroaromatics is HPLC with UV detection due to its widespread availability [3,15], while HPLC combined with MS and electrochemical detection (ED) are also viable but less frequently available methods of detection [13,14,19,20].

The analysis of explosives using HPLC continues to present challenges [3]. Previously published methods have focused on the use of C-18 RP-HPLC [3,5,20,21] and acetonitrile as the organic mobile phase. Acetonitrile is of significant greater health and environmental concern than methanol and therefore methods avoiding the use of acetonitrile are desirable. Many previously published methods had difficulties with the separation of the following isomer pairs: 2- and 4-HADNT, 2- and 4-ADNT, 2,4- and 2,6-DANT, 2,2′- and 4,4′-azoxy [11,12,14,15,21,22]. Consequently, the identification of the polar TAT and the less polar azoxy dimers in TNT degradation studies has often been performed in separate HPLC runs [23].

The goal of the present study was to separate TNT, HADNTs, 2,4-DHANT, ADNTs, DANTs, TAT, azoxy and azo compounds in a single HPLC run and to improve the chromatography of the 14 EPA Method 8330 compounds. The explosives targeted in EPA Method 8330 were included in the present studies due to the fact that they often occur as co-contaminants in environmental samples containing TNT. They could therefore potentially interfere or co-elute with TNT or its reduced metabolites during HPLC analysis.

2. Experimental

2.1. Chemicals and sample preparation

The TNT, ADNT, and DANT standards (1000 μg/ml in acetonitrile, purity >99.0%) were obtained from Supelco (Bellefonte, PA, USA). TAT*3HCl and 4,4′,6,6′-tetranitro-2,2′-azoxytoluene were obtained from Dr. R.J. Spanggord, SRI International, Menlo Park, CA. The chemicals included in EPA Method 8330 (all with a purity >96.8%), 4-hydroxylamino-2,6-dinitrotoluene (purity 96.0%), 2-hydroxylamino-4,6-dinitrotoluene (purity 97.1%), 2,2′,6,6′-tetranitro-4,4′-azoxytoluene (purity 98.8%), 2,2′,6,6′-tetranitro-4,4′-azotoluene (purity 90.7%) and 4,4′,6,6′-tetranitro-2,2′-azotoluene (purity 94.7%) were obtained from AccuStandard (New Haven, CT, USA). 2,4-dihydroxylamino-6-nitrotoluene was synthesized biochemically and kindly provided by Dr. J.B. Hughes, Rice University, Houston, TX [5]. TNT (neat; purity 98.0%) for the degradation studies was obtained from Chem Service (West Chester, PA, USA). HPLC grade methanol (UV cutoff 205 nm) and Optima acetonitrile (UV cutoff 190 nm) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). HPLC grade dibasic sodium phosphate hepta hydrate and enzyme grade monobasic sodium phosphate were obtained from Fisher Scientific and Fisher Biotech, respectively (Fair Lawn, NJ, USA). The ion-pair reagent octanesulfonic acid (sodium salt) was obtained from ACROS (New Jersey, USA). The ion-pair reagent Low-UV PIC B8 was obtained from Waters (Milford, MA, USA). The water used in the preparation of mobile phases and standards was obtained from a Barnstead NANOpure system (resistivity $\geq$ 17.6 MΩ cm).

The TNT metabolite mixture was prepared in a glove box in the absence of oxygen (90% N_2, 5% CO_2, 5% H_2) and concentrated by rapid evaporation of acetonitrile. TAT standards have been found to easily degrade in H_2O solutions [24]. However, degradation of the oxygen and pH sensitive TAT standards was prevented when prepared in 0.025 M phosphate buffer (pH 7) as follows. The phosphate buffer was boiled after preparation and cooled down on ice under a constant purge of nitrogen to keep the solution free of oxygen. The oxygen free buffer solution was transferred into the anaerobic glove box and aliquots were added to 15 ml vials containing defined amounts of TAT*3HCl powder. The vials were capped with polytetrafluoroethylene (PTFE) coated butyl rubber septa and crimp-sealed. The headspace was finally replaced with pure nitrogen. For analysis, aliquots of these solutions were transferred to HPLC vials in the glove box and analyzed immediately. The TAT concentration in the stock solutions was constant for at least 10 days (data not shown).

2.2. HPLC

2.2.1. Apparatus

HPLC analyses were performed using a Hewlett-Packard 1090 Liquid Chromatograph equipped with an original autosampler and diode array detector. The Agilent ChemStation software (Rev. A.08.01 [783]) was utilized for instrument control, data acquisition, and analysis. Although multiple Supelcosil octyl (C-8) 150 mm × 4.6 mm (5 μm particle size) columns were used during the method development, all data published herein were obtained using the same column. Guard columns used for the method development included Supelcosil LC-CN, LC-ABZ+, and LC-8 (20 mm × 4.6 mm; 5 μm), but only the LC-8 guard column was used for the final data collection. The injection volume was 10 μl, with an initial syringe draw speed of 83 μl/min.

2.2.2. Column temperature, diode array detection, and mobile phases

The column temperature was controlled using the block heater built into the HPLC system (circulating heated air around the column). Stainless steel tubing (35 cm long, 0.17 mm i.d.) was placed upstream of the column inside the oven compartment to ensure that the inlet solvent was acclimatized in order to minimize potential instrument-to-instrument variation [25,26]. Oven temperatures from 35 to 55 °C were investigated.

Chromatograms were extracted at absorbencies of 220, 230, 254, 360, and 370 nm. Peaks were scanned from 200 to 600 nm to obtain spectrochromatograms for compound characterization.

Two mobile phases were utilized to establish the gradient system. The organic mobile phase was HPLC-grade methanol. The aqueous mobile phase consisted of either 0.025 M sodium phosphate buffer (pH 7) with or without 0.1% or 0.5% w/w 1-octanesulfonic acid (ion-pair reagent), or of a phosphate buffer (0.0144 M Na_2HPO_4) amended with Low-UV PIC B8 resulting in a 0.1% ion-pair reagent solution with a pH of 7. No significant differences were observed for the two different aqueous ion-pair reagent mobile phases. Since the use of the Low-UV PIC B8 ion-pair reagent resulted in significant time savings, all results presented herein were acquired using this phosphate buffer solution. Mobile phases were kept oxygen free by purging them with helium for at least 30 min prior to the first sample run and continuously throughout analyses.

2.2.3. Gradient

The flow rate of the mobile phase was 1 ml/min. The mobile phase initially consisted of 99% phosphate buffer (with or without 0.1% ion-pair reagent) and 1% methanol. By utilizing the Agilent ChemStation "narrow gradient range" option, the gradient was changed to 30% methanol over 2 min, then to 43% methanol over the next 13 min, finally increased to 100% methanol over 12.5 min, and held constant for 0.5 min. The solvent ratio was returned to the initial conditions over 1 min and held for an additional 5 min before injection of the next sample. The total run time including conditioning time was 34 min.

2.3. Detection and peak performance parameters

Performance assessment was based on calibration standards of authentic compounds and multipoint standard calibration curves. All compounds investigated had a linear detection range of at least 1–100 mg/l and were easily detected in the range of 5–10 ng per injection. Standard solutions with a concentration of 10–25 mg/l were used to generate the chromatograms with the exception of the azo compounds, which had a concentration of up to 40 mg/l.

Retention times (t_r), retention time factors (k'), and the chromatographic resolution (R_s) were calculated for all compounds. k' was calculated using a void time which was determined from the column parameters supplied ($k' = (t_r - (V_m/F))/(V_m/F)$), where V_m is the column void volume (L^3) and F the flow rate (L^3/t). The porosity of the column packing material was 0.71 based on information from Supelco (Bellefonte, PA, USA). R_s and number of theoretical plates (N) were calculated using the half-width method.

3. Results and discussion

3.1. Analysis of TNT metabolites

Separation of TNT and its reduced metabolites has been problematic due to the co-elution of the isomers 2-ADNT and 4-ADNT, difficulties with the quantification of ionizable intermediates such as TAT due to speciation, and poor retention [21]. Octanesulfonic acid has been used as an ion-pair reagent to increase the retention time of the highly polar TAT [13].

The development of a gradient elution method for the separation of the most commonly reported TNT metabolites was accomplished after extensive investigation of various types of chromatographic columns (including both C-8 and C-18 packing materials), guard columns, and mobile phases (at various pH values). This method was then optimized further by the choice of temperature and addition of an ion-pair reagent.

UV-Spectra of all investigated TNT metabolites were compared to published spectra [21,24] for positive identification, except for the UV-absorption spectra of 4,4′,6,6′-tetranitro-2,2′-azotoluene and 2,2′,6,6′-tetranitro-4,4′-azotoluene, which are to our best knowledge shown here for the first time (Fig. 2). The highest UV absorption was obtained at a wavelength of 230 nm for the majority of TNT metabolites. However, TAT, DANTs, 4,4′-azoxy, and 4,4′-azo had a higher absorption at 220 nm and 2,2′-azoxy and 2,2′-azo a slightly increased absorption at 254 nm (Figs. 2 and 3a) [21,24]. Most chromatograms shown herein are given at 254 nm unless otherwise noted, since it is the wavelength achievable on most HPLC-UV systems and the detector wavelength suggested by the EPA [27].

3.1.1. Influence of temperature and ion-pair reagent

The impact of temperature, on the separation of TNT metabolites in the absence of an ion-pair reagent is shown in Fig. 3a and b and in the presence of ion-pair reagent in Fig. 4. Although a temperature increase from 35 to 55 °C decreased the overall separation time by less than one minute, the retention time of compounds such as the

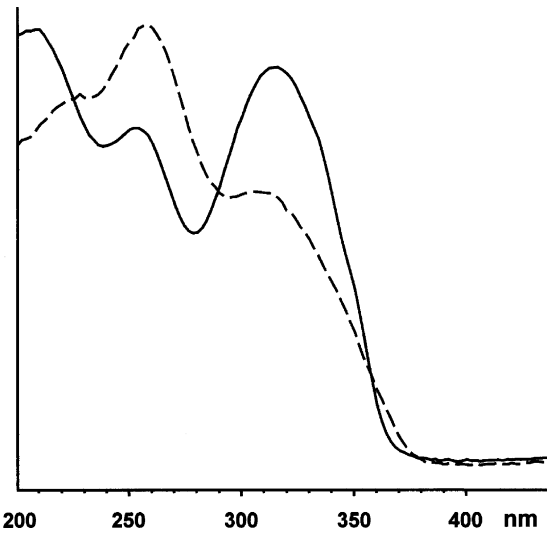

Fig. 2. UV-Spectra of 4,4′,6,6′-tetranitro-2,2′-azotoluene (dashed line) and 2,2′,6,6′-tetranitro-4,4′-azotoluene (solid line).

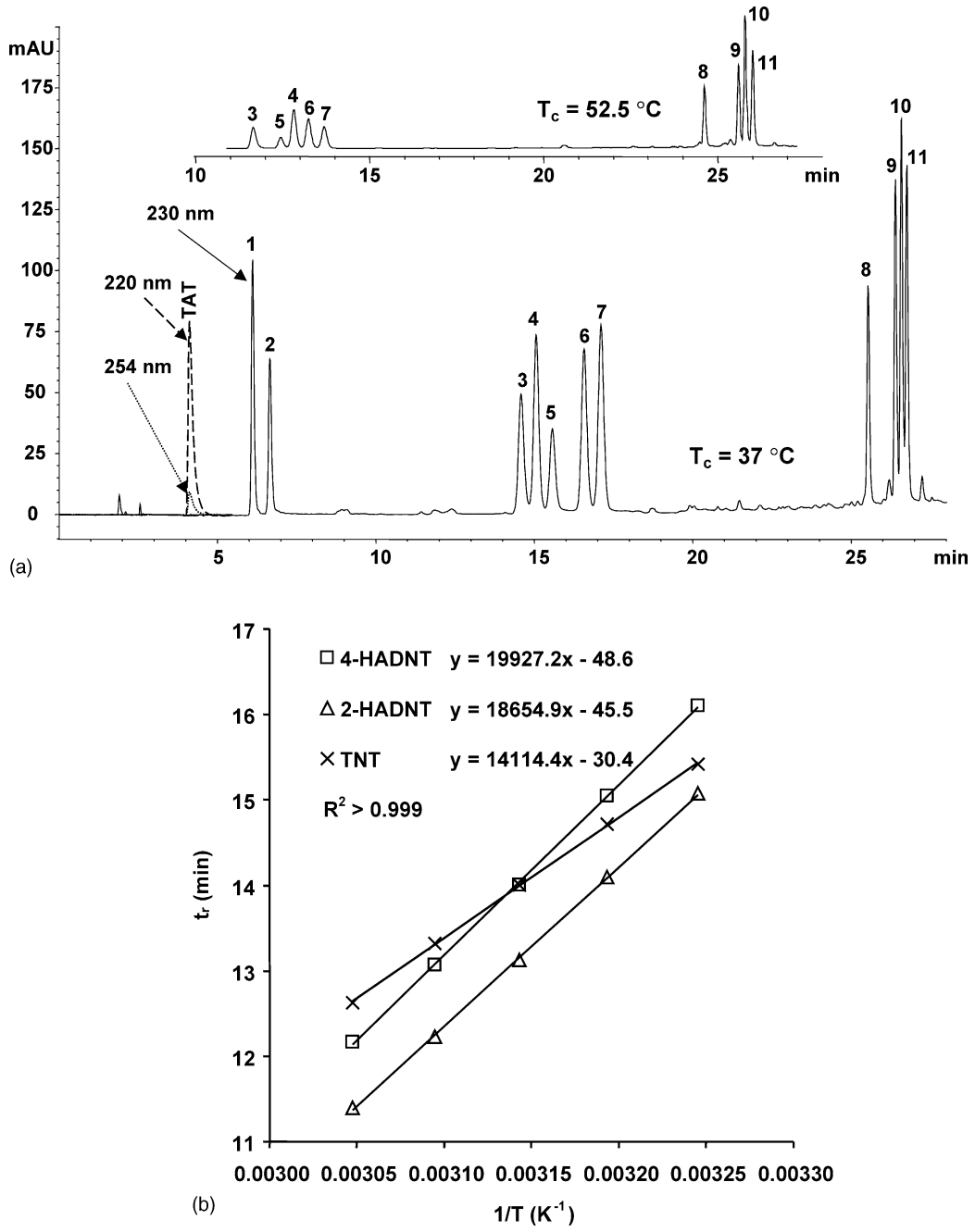

Fig. 3. (a) Optimal separation of TNT metabolites in the absence of an ion-pair reagent at 37 °C (1: 2,6-DANT; 2: 2,4-DANT; 3: 2-HADNT; 4: TNT; 5: 4-HADNT; 6: 2-ADNT; 7: 4-ADNT; 8: 2,2′-Azoxy; 9: 4,4′-Azoxy; 10: 2,2′-Azo; 11: 4,4′-Azo). Note the improved separation of the azoxy and azo compounds with increased temperature and baseline separation of all compounds at 52.5 °C (see inset). 37 °C was chosen as the best column temperature due to the potential for larger instrument-to-instrument variation at higher temperatures. (b) The retention time (t_r) as a function of the absolute temperature for selected compounds.

ADNTs decreased by approximately 4 min which resulted in a drastically improved overall separation of the compounds.

The isocratic retention as a function of temperature can often be described by the van't Hoff relationship ($\log k' = a + b/T$; a and b are constants for a given compound and T is the absolute temperature) [28]. The following empirical relationship, $t_r = a' + b'(1/T)$ where a' and b' are constants for a given solute as T is varied and all other conditions are kept constant, was derived by Zhu et al. [29] from the van't Hoff relationship based on assumptions such as $k'_0 \gg 1$ (value of k' at start of separation) and that S does not vary with temperature; S is a solute parameter (please refer to Zhu et al. [29] for further details). A linear relationship ($R^2 > 0.99$) was obtained when plotting the t_r as a function of $1/T$ for the herein investigated compounds. The following order of temperature sensitivity was observed based on the calculated

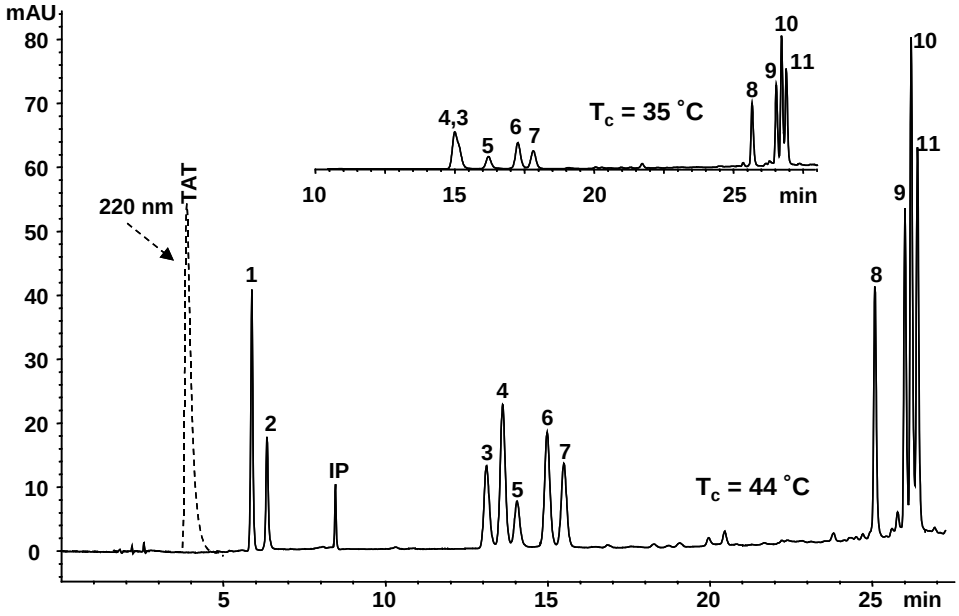

Fig. 4. Optimal separation of TNT metabolites in the presence of an ion-pair reagent at 44 °C; the peak labeled "IP" is an inherent peak caused by the addition of the ion-pair reagent (1: 2,6-DANT; 2: 2,4-DANT; 3: 2-HADNT; 4: TNT; 5: 4-HADNT; 6: 2-ADNT; 7: 4-ADNT; 8: 2,2′-Azoxy; 9: 4,4′-Azoxy; 10: 2,2′-Azo; 11: 4,4′-Azo). Note the co-elution of TNT and 2-HADNT at 35 °C (see inset).

slopes (b'): 4-ADNT ($b' = 21\,738$) > 2-ADNT ($b' = 21\,231$) > HADNTs ≫ TNT ≫ 2,4-DANT ($b' = 3581$) > 2,6-DANT ($b' = 2464$) (Fig. 3b). The linear relationship for the studied explosives can potentially be used to predict the retention time as a function of temperature and thereby help improving the separation of closely eluting compounds, which is in agreement with detailed studies by Zhu et al. [29,30].

In a recent review by Dolan [25], it was reported that a change of temperature especially influenced the selectivity of ionizable and polar compounds due to a possible concurrent change of the pK_a, which is consistent with the results observed in this study. The impact of temperature on the DANTs was attenuated compared to the ADNTs possibly due to a significantly higher pK_a value or other physical–chemical interactions, which were not further investigated as part of this study [25,29–32]. However, when comparing the relative shift in retention time of the polar TAT with TNT and 4-ADNT as a result of an increase in column temperature from 37 to 52.5 °C similar relative shifts were observed (i.e. 13, 16, and 19%, respectively).

The increased temperature sensitivity of 2- and 4-HADNT, as compared to TNT, was used to optimize the peak separation (Fig. 3b). Most compounds were separated with baseline resolution ($R_s \geq 1.5$) at a temperature of 37 °C except for 2,2′-azo and 4,4′-azo which had R_s values of 1.3 and 1.2, respectively (Fig. 3a). The resolution of the azo isomers was improved to $R_s \geq 1.4$ by increasing the column temperature to 52.5 °C (inset in Fig. 3a and Table 1). However, 37 °C was chosen as the best-suited chromatography temperature due to potentially increased relative standard deviation, and potentially larger instrument-to-instrument variation at elevated column temperatures (Table 1) [26].

Increased temperature decreased the peak width (W) of all peaks and increased the number of theoretical plates (N) for the majority of peaks. In a few cases, a decrease of N was observed. In the absence of an ion-pair reagent for instance, a temperature increase from 35 to 52.5 °C resulted in an approximately 33% increase in N for 2,6-DANT, but a 13% decrease in N for TNT. N should in theory increase as temperature increases, however, experimental studies have shown that this is not always the case in gradient elution [29].

An ion-pair reagent has often been used for the chromatography of TNT metabolites in order to prolong the retention time of the polar TAT [13]. The addition of an ion-pair reagent to the aqueous mobile phase in the present study resulted in the co-elution of 2-HADNT and TNT at the commonly used column temperature of 35 °C (inset in Fig. 4). However, the co-elution was completely avoided by increasing the temperature to 44 °C (Fig. 4).

A relatively low shift in retention time was observed in the presence of an ion-pair reagent compared to previously published results with Supelcosil C-18 and ABZ+ columns (25 cm by 4.6 mm; 5 μm particles) [13,24]. The relatively low shift in t_r compared to these results could be due to a lower ion-pair reagent affinity to the (15 cm long) C-8 column packing material, differences in the mobile phase, or differences in the gradient elution. However, the exact reasons remain unknown and were beyond the scope of this investigation. The retention time of TNT decreased by 2.6% in the presence of the ion-pair reagent possibly due to a change of polarity in either the mobile phase or the stationary phase. The temperature sensitivity in the presence of an ion-pair reagent decreased by 8–17% for all compounds

Table 1
Retention time factor (k') and peak resolution (R_s) calculated for the TNT metabolites (37 °C; Fig. 3a), EPA Method 8330 compounds (50.5 °C; Fig. 7a), and TNT metabolites combined with the EPA Method 8330 compounds (50.5 °C; Fig. 8a)

Compound ($n = 6$)	TNT metabolites[a]		EPA compounds[a]		Metabolites and EPA compounds[a]	
	k'[b]	R_s[b]	k'[b]	R_s[b]	k'[b]	R_s[b]
TAT[c]	1.05	>1.5			0.83	>1.5
2,4-DHANT[d]	1.95	n.a.				
2,6-DANT	2.05	>1.5			1.91	>1.5
HMX			1.89	>1.5	1.91	0.0
2,4-DANT	2.32	>1.5			2.11	>1.5
RDX			2.74	>1.5	2.74	>1.5
1,3,5-TNB			3.46	>1.5	3.47	>1.5
1,3-DNB			4.17	>1.5	4.19	>1.5
NB			4.94	>1.5	4.97	>1.5
2-HADNT	6.27	>1.5			5.08	0.82
Tetryl			5.27	>1.5	5.28	>1.5
4-HADNT	6.77	>1.5			5.49	1.4
TNT	6.51	>1.5	5.61	>1.5	5.63	0.94
2-ADNT	7.27	>1.5	5.89	>1.5	5.91	>1.5
4-ADNT	7.53	>1.5	6.13	>1.5	6.14	>1.5
2,4-DNT			6.36	>1.5	6.38	>1.5
2,6-DNT			6.60	>1.5	6.62	>1.5
2-NT			7.52	>1.5	7.55	>1.5
4-NT			7.73	1.3	7.75	1.3
3-NT			8.06	>1.5	8.08	>1.5
2,2′-Azoxy	11.7	>1.5			11.4	>1.5
4,4′-Azoxy	12.2	>1.5			11.8	>1.5
2,2′-Azo	12.3	1.3			11.9	1.4
4,4′-Azo	12.4	1.2			12.0	>1.5

[a] Ranges of relative standard deviation (R.S.D.) of the retention time (t_r): $T = 37$ °C (0.01–0.06%); $T = 50.5$ °C (0.01–0.17%).
[b] Relative standard deviation (R.S.D.) of k' and $R_s \ll 1\%$ for all compounds.
[c] TAT was measured at 220 nm and run separately ($n = 7$).
[d] 2,4-DHANT was run separately because the chemical was obtained just before submission of this manuscript ($n = 3$).

based on comparisons of the calculated slopes (b') (4-ADNT ($b' = 19\,985$) > 2-ADNT ($b' = 19\,483$) > 4-HADNT ($b' = 18\,103$) > 2-HADNT ($b' = 16\,269$) > TNT ($b' = 12\,014$) > 2,4-DANT ($b' = 3031$) > 2,6-DANT ($b' = 2054$)) with the calculated slopes in the absence of an ion-pair reagent (see above and Fig. 3b).

The use of an ion-pair reagent for improved compound selectivity often has an unfavorable impact on various factors such as slow column equilibration and method ruggedness, however it is still one of the favored ways to increase the selectivity factor (α) of ionizable compounds in RP-HPLC [29]. The use of an ion-pair reagent in this study resulted in the appearance of an inherent peak (IP). In this case, the inherent peak did not interfere with other peaks of interest (e.g. IP observed at 8.45 min in Fig. 4), however the presence of such an IP could complicate the detection of TNT related compounds in certain situations. Since the overall chromatography was not improved by use of an ion-pair reagent in the aqueous mobile phase, it is suggested to perform the chromatography in its absence, which will result in a significant cost reduction.

3.1.2. Method performance

An acceptable level of reproducibility must be established before any separation method can be applied to the analysis of intricate environmental samples. Therefore, the chromatographic performance and reproducibility of the developed gradient method for the analysis of TNT and its metabolites was investigated at 37 and 52.5 °C (data not shown), which had demonstrated optimal separation in the absence of an ion-pair reagent, and at 44 °C (data not shown) in the presence of an ion-pair reagent. A mixture of TNT and its metabolites (solubilized in acetonitrile) was repeatedly injected and monitored for variability in retention time (t_r), retention time factor (k'), and peak resolution (R_s) (Table 1). TAT was run separately, due to its poor solubility and stability in acetonitrile.

The retention time factors (k') for the less polar azoxy and azo compounds were significantly higher than for the other metabolites (Table 1). However, in order to obtain sufficient separation of the azoxy and azo compounds, a slow increase of the methanol concentration was necessary. If the chromatography of the azo or both the azo and azoxy compounds is not desired, the total run time can be significantly decreased by use of a steeper final gradient. If the chromatography of the azo compounds is not desired, the methanol concentration can be increased to 100% over 8.5 min instead of 12.5 min, if neither azo nor azoxy compounds are of interest, the methanol concentration can be increased over 1 min.

Very good reproducibility and performance was obtained under all three conditions, however a slightly increased variation in the retention times was observed at 52.5 °C (range of R.S.D.: 0.02–0.27%; $n = 3$). The retention time factors were less than 12.4 and the resolution was better than 1.2 for all compounds with baseline resolution ($R_s \geq 1.5$) for most of the investigated compounds (Table 1).

Since sorption of an ion-pair reagent to the column packing material can change the characteristics of a column, the reproducibility of the presented results was tested by reconfirming the quality of separation with a new Supelcosil LC-8 column. The new column had never been exposed to ion-pair reagent. The retention times of the 14 EPA 8330 compounds at 50.5 °C changed by less than 2.2% between the columns and the resolution was of the same quality as with the LC-8 column that had been exposed to ion-pair reagent. The slight change in t_r may be due to column aging rather than sorption of the ion-pair reagent.

In order to test the developed method in a real case scenario, samples were taken from a TNT (bio)transformation experiment ([33], and unpublished data) and analyzed using the developed gradient method at 37 °C. No significant retention time shift was observed over a period of 86 days after analysis of more than 500 samples from various TNT degradation studies using the same Supelcosil LC-8 chromatography column. The two chromatograms in Fig. 5 demonstrate the degradation of TNT by strain ES6 in the presence of hydrous ferric oxide and the electron shuttling compound 9,10-anthraquinone-2,6-disulfonate

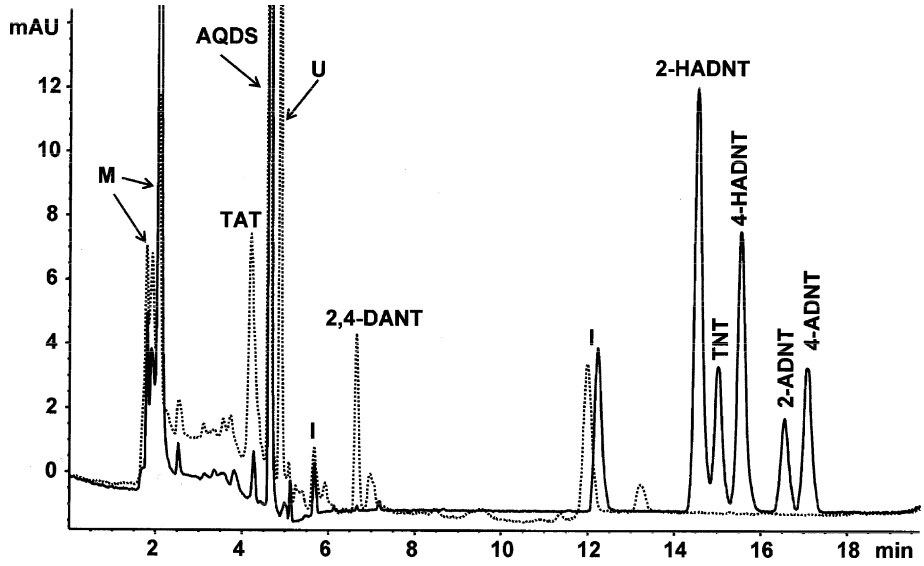

Fig. 5. Randomly selected chromatograms from the analysis of aqueous samples of a TNT (bio)degradation experiment [33]. Test tubes contained synthetic ground water [38], carbonate buffer (pH 7), hydrous ferric oxide, 9,10-anthraquinone-2,6-disulfonate (AQDS), sucrose, and strain ES6, tentatively identified as a *Cellulomonas* sp. [33]. The test tubes were injected with 52 μM of TNT after 14 days of inoculation and TNT transformation and metabolite patterns were observed over time. The solid line and the dotted line show TNT and its metabolites after 3 days and 86 days of incubation, respectively. The chromatograms are shown at 230 nm and were obtained using the developed gradient elution method at 37 °C. M: microbially related metabolites, U: unidentified peaks, I: sample inherent peaks.

(AQDS). TNT was completely transformed via the HADNTs and ADNTs metabolites to 2,4-DANT and TAT after 86 days.

3.2. Analysis of EPA Method 8330 explosives

Fourteen explosives, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), 1,3,5-trinitrobenzene (TNB), 1,3-dinitrobenzene (DNB), nitrobenzene (NB), TNT, 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), 2,4,6-trinitrophenylmethylnitramin (Tetryl), 2-ADNT, 4-ADNT, 2-nitrotoluene (2-NT), 3-nitrotoluene (3-NT), and 4-nitrotoluene (4-NT) are included in the EPA Method 8330 [34].

The main difficulty with the isocratic EPA Method 8330 is the reported co-elution of the DNTs and ADNTs when employing the recommended primary C-18 (250 mm × 4.6 mm, 5 μm) RP-HPLC column [3,27,34]. Consequently, a second confirming run is needed for the separation of the DNTs and ADNTs, which is commonly performed by utilizing a CN (250 mm × 4.6 mm, 5 μm) RP-HPLC column [27,34]. The co-elution problem of DNTs and ADNTs was solved in a recent study by use of a two-phase approach in a single RP-HPLC run. Lang and Burns [3] used a CN guard column in series with a C-18 column, while keeping the EPA Method 8330 specifications. Lang and Burns [3] demonstrated that, although, the two-phase approach resulted in a total run time of approximately 32 min compared to approximately 24 min for the EPA Method 8330, it only required one HPLC run since the co-elution of the DNTs and ADNTs was avoided [3,34].

The goal of this part of the study was to develop an alternative method, that would decrease the total run time, reduce the consumption of solvents (mobile phases) compared to the discussed methods, and still baseline separate all 14 EPA Method 8330 compounds.

3.2.1. Influence of temperature and ion-pair reagent

Baseline resolution was achieved for all compounds (except for 4-NT; $R_s = 1.3$) by optimizing the column temperature of the described gradient elution method. Fig. 6 illustrates how the increased temperature sensitivity of the ADNTs can be used to separate them from the DNTs. 4-ADNT and 2,4-DNT co-eluted at 42 °C but were completely separated at 50 °C. The higher temperature sensitivity of the ADNTs might be due to the ionizable character of these compounds [25]. An optimum temperature of 50.5 °C was found to rapidly (18 min), reliably (R.S.D. < 0.2%), and fully separate all the EPA Method 8330 compounds (Fig. 7a and Table 1).

The flow rate in this study was 1 ml/min as compared to 1.5 ml/min used in the EPA Method 8330 and the method proposed by Lang and Burns [3,34]. Additionally, the total run time in this study was reduced by approximately 7 min (≈22%) compared to Lang and Burns [3], based on a 7 min column conditioning time following the elution of 3-NT. The combined decrease in total run time and flow rate resulted in an approximately 48% reduction in solvent consumption as compared to Lang and Burns and a 30% reduction compared to the EPA Method 8330 [3,34].

Optimal chromatographic separation in the presence of an ion-pair reagent was obtained at 41.5 °C (Fig. 7b). The

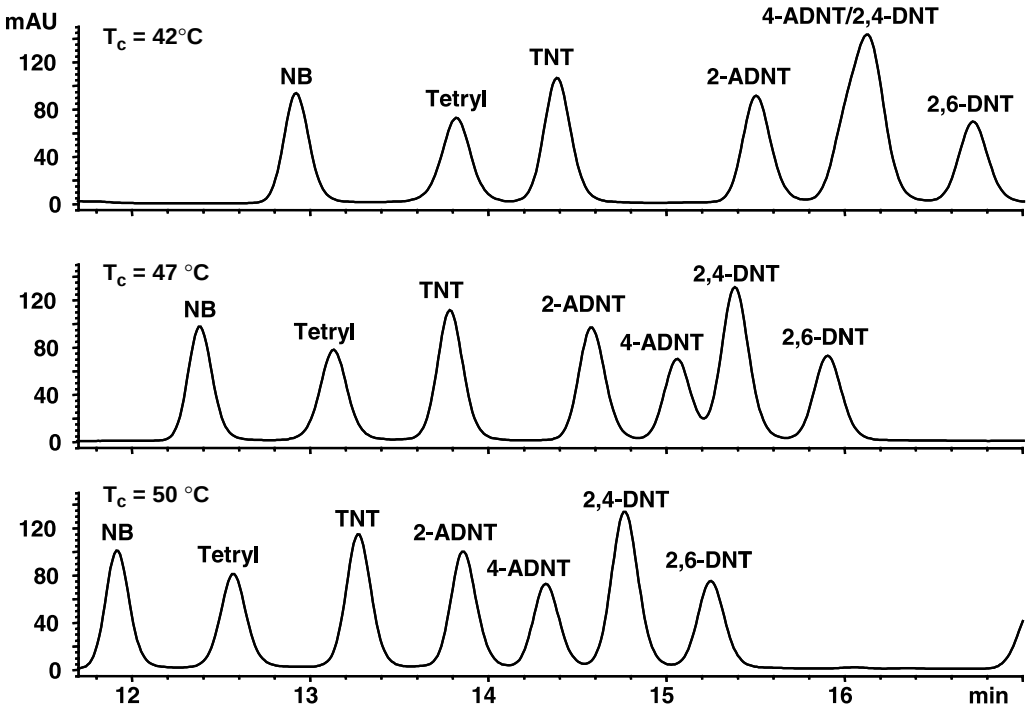

Fig. 6. Influence of temperature (42, 47, and 50 °C) on the separation of selected EPA Method 8330 chemicals in the absence of an ion-pair reagent. Note the co-elution of 4-ADNT and 2,4-DNT at 42 °C and the improved separation with increased temperature.

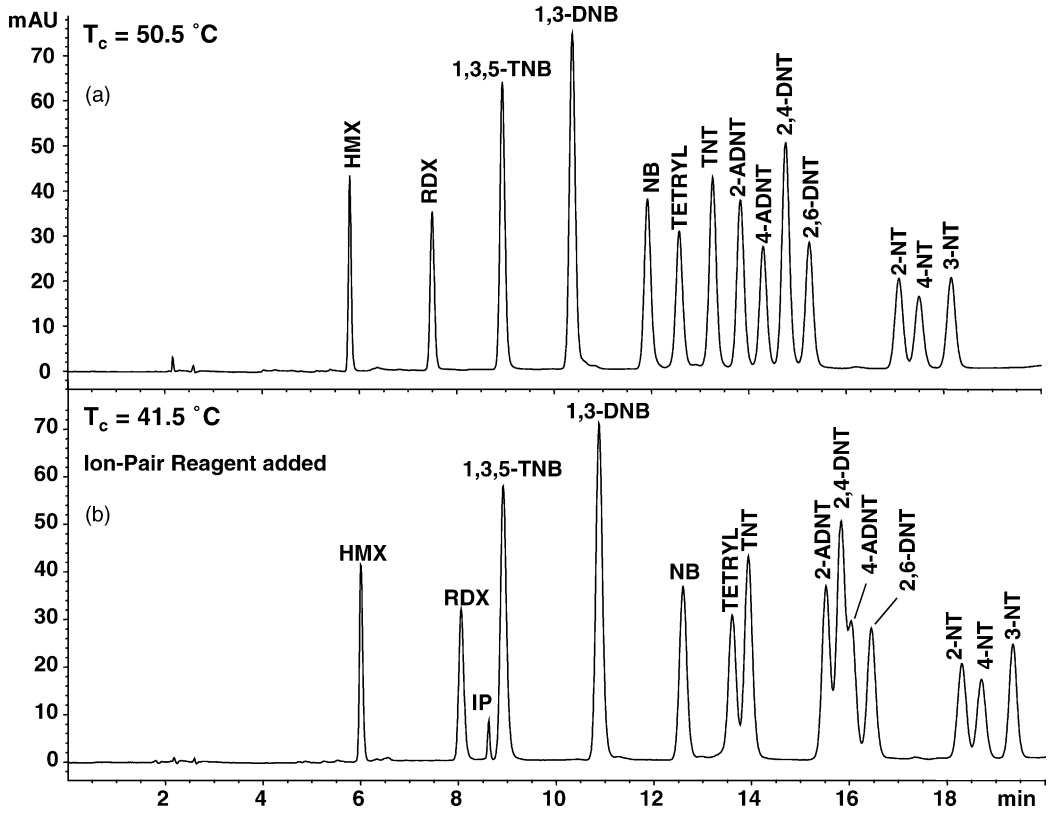

Fig. 7. (a) Baseline separation of EPA Method 8330 compounds at the optimized temperature of 50.5 °C (no ion-pair reagent added). (b) Separation of the fourteen EPA Method 8330 chemicals in the presence of an ion-pair reagent at the optimized temperature of 41.5 °C. Note how the ion-pair reagent prevents baseline separation of several compounds.

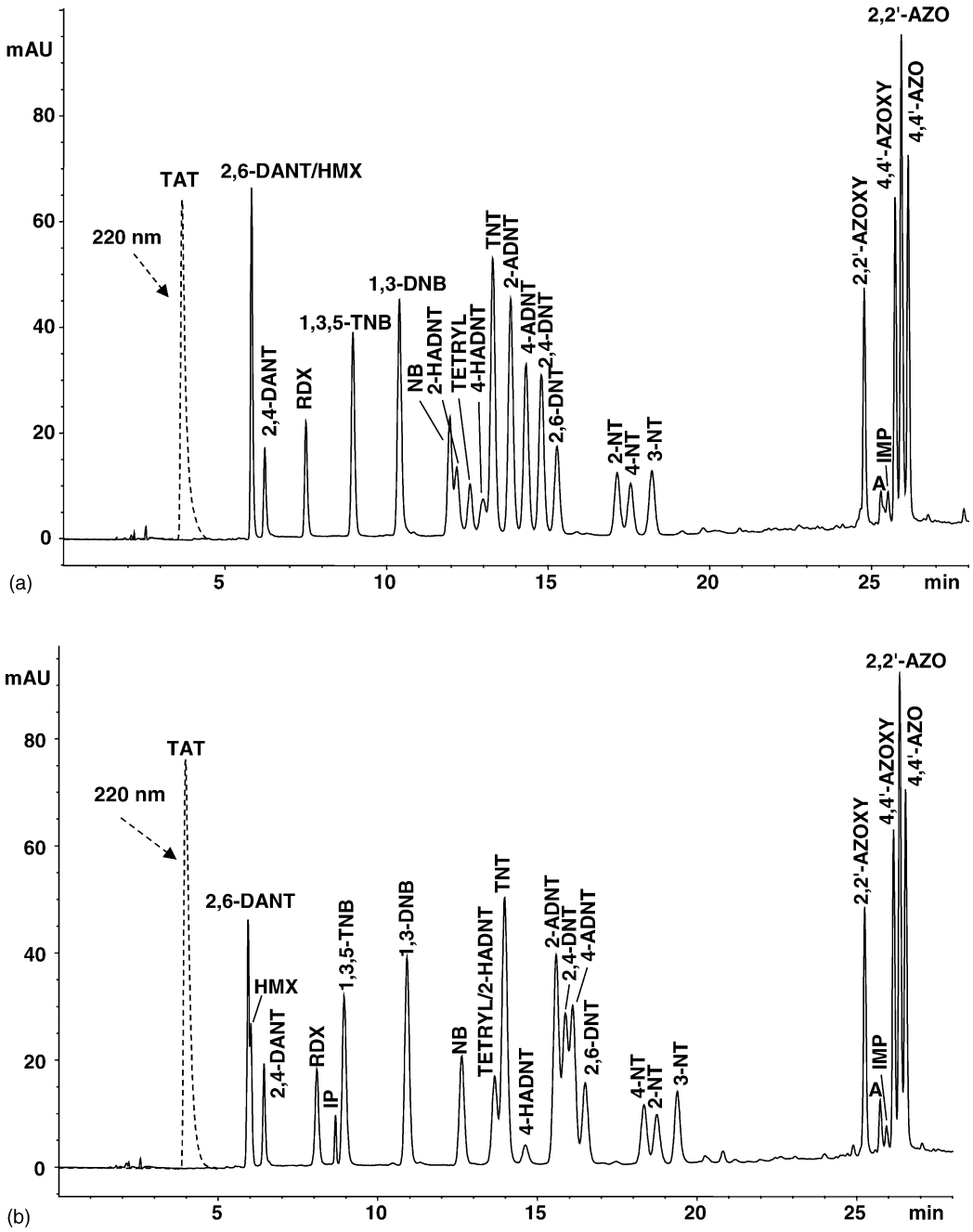

Fig. 8. (a) Separation of the 23 TNT metabolites and EPA Method 8330 compounds at 50.5 °C in the absence of an ion-pair reagent. (b) Separation at 41.5 °C in the presence of an ion-pair reagent. Note the slightly improved separation of 2,6-DANT and HMX in the presence of an ion-pair reagent but the overall decreased chromatographic resolution. "A" is a possible transformation product (probably 2,4′,6,6′-tetranitro-2′,4-azoxytoluene, see text for discussion) and "IMP" is an inherent impurity contained in the 4-HADNT standard.

temperature selectivity of the ADNTs relative to the DNTs was decreased in the presence of an ion-pair reagent, exemplified by the reverse elution order of 4-ADNT and 2,4-DNT (Fig. 7a and b). The affinity of the ion-pair reagent for the ADNTs resulted in increased retention times and consequently a poor separation of 2-ADNT, 2,4-DNT ($R_s = 0.83$), and 4-ADNT ($R_s = 0.63$) (Fig. 7b). Hence, it is not recommendable to use an ion-pair reagent for the separation of compounds included in the EPA Method 8330.

3.3. Combined analysis of TNT metabolites and EPA Method 8330 compounds

Soils and water contaminated with TNT and its metabolites often contain co-contaminants such as the compounds included in EPA Method 8330 [18,27]. Thus, it is important to minimize the potential for co-elution of chemicals to prevent false positive results when analyzing complex environmental samples. It was therefore tested whether the

developed gradient method would be capable of separating all 23 chemicals investigated in this study ranging from the very polar TAT to the less polar nitrotoluene, azoxy, and azo compounds in a single run.

Various temperatures were investigated (data not shown) and it was found that the same temperature used for the optimal separation of EPA Method 8330 compounds (50.5 °C) gave the most satisfying chromatographic separation (Fig. 8a and Table 1). 2,6-DANT and HMX co-eluted and 2-HADNT and TNT had a peak resolution of less than 1. Nevertheless, reliable identification was obtained for the majority of the investigated compounds including the 7 pairs of isomers contained in the analyte mixture (Table 1).

Decreasing the steepness of the proposed initial methanol gradient can prevent the co-elution of 2,6-DANT and HMX. However, this will result in a poor separation of compounds with longer retention times (data not shown). The use of an ion-pair reagent in the aqueous mobile phase at 41.5 °C allowed for a slightly better separation of 2,6-DANT and HMX ($R_s \sim 0.7$), however, it also resulted in an overall decreased chromatographic resolution (Fig. 8b).

The peak labeled IMP was an inherent impurity (IMP) originating from the 4-HADNT standard. The peak labeled A in the chromatogram is a possible transformation product, which accumulated over time. It is believed, based on previous studies [5,35], that the degradation product (A) originated from the spontaneous dimerization of the oxygen sensitive HADNTs potentially producing an additional azoxy-isomer (e.g. 2,4′,6,6′-tetranitro-2′,4-azoxytoluene or 2′,4,6,6′-tetranitro-2,4′-azoxytoluene). This was supported by an abiotic experiment investigating the resulting transformation product after oxygen-purging a standard mixture containing only 2- and 4-HADNT by comparison of the DAD spectra (data not shown).

4. Conclusion

This study demonstrates for the first time the importance of optimizing the temperature for the improved separation of complex mixtures containing explosives-related compounds. The findings herein evoke that column temperature should not always be used as the last parameter to optimize RP-HPLC methods. Additionally, this work is a supplement to research illustrating the importance of column temperature for the improved separation of compounds like chlorophylls, herbicides, peptides, and drugs (e.g. anticancer agents) [25,30,36,37].

The developed gradient method is unique because the same method at different temperatures can be used to completely separate TNT and 12 of its reduced metabolites as well as the compounds targeted in the EPA Method 8330. The TNT metabolites included 2,4,6-triaminotoluene (TAT), the DANTs, ADNTs, HADNTs, 2,4-DHANT, tetranitroazoxytoluenes, and tetranitroazotoluenes.

The proposed chromatographic method for the EPA Method 8330 compounds does not only provide improved separation of the 14 target compounds but also a significant reduction of total run time and solvent consumption when compared to previous studies [3,27,34].

The use of an ion-pair reagent for increased selectivity was also investigated. However, the use of the costly ion-pair reagent could be totally avoided by optimizing the column temperature.

Finally, the gradient method proved to be capable of separating all 23 explosives-related compounds, including the 12 reduced TNT metabolites tested, and the EPA Method 8330 compounds in a single run except for 2,4-DANT and HMX, which co-eluted. Thus, the gradient elution method described here can become a valuable tool for the fast and reliable analysis of complex samples containing mixtures of nitroaromatics, aminoaromatics, and nitramines.

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