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Explosives detection in soil using a field-portable continuous flow immunosensor

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Abstract

A field method for quantitative analysis of explosives in contaminated soil samples is described. The method is based on a displacement immunoassay performed in a commercial instrument, the FAST 2000, engineered by Research International Inc. The method can be used on-site to measure 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) within 5 min. For this study, replicate analyses were performed on soil extracts prepared from each field sample as well as appropriate controls, blanks, and laboratory standards. Statistical analyses were done to assess accuracy, bias, and predictability of the method. The results demonstrated that the immunosensor could be used effectively to screen environmental samples for the presence or absence of explosives. In most samples, the method also provided quantitative values that were in good agreement with standard laboratory analyses using HPLC. A limited number of sample matrices interfered with the immunoassay and produced results that varied significantly from the laboratory data. In each case, the compounds causing the problem have been identified and efforts are being made to minimize these matrix interferences in future field evaluations.

Keywords: Explosives; Soil; Immunosensor; Field methods

1. Introduction

Field screening methods to detect environmental contaminants continue to proliferate as microelectronics improve and analytical instruments become miniaturized. One application of on-site testing can be seen in the identification, characterization, and remediation of sites contaminated with 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). These energetic compounds pose a serious site characterization, cleanup, and monitoring problem for the Department of Defense (DoD), Environmental Protection Agency

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(EPA), and other government institutions. The compounds are most commonly encountered in contaminated soils in close proximity to facilities used to manufacture, assemble, test, store, and demilitarize munitions. Once deposited, they do not rapidly decompose and will leach through soils into the groundwater, creating a serious environmental hazard (Spalding et al. [1]).

The current method of choice for analysis of nitroaromatics and nitroamines is the US EPA Method 8330 utilizing HPLC (Crockett et al. [2], SW-846 [3], Jenkins et al. [4]). Though laboratory analyses provide a more complete, exact profile of contaminants present in a given sample than most field methods, remediation goals require a large number of samples to be run at infrequent intervals. It is difficult, costly and time-consuming to send samples off-site for testing. With rigorous sampling plans and careful attention to QA/QC, accuracy, and precision, on-site methods provide real-time information to remediation managers that can improve decision-making, allow better risk assessments, and significantly decrease analytical costs.

There are a number of analytical laboratory methods available for detection of TNT and RDX including thin-layer chromatography (Douse [5], Haas et al. [6]), capillary electrophoresis (Northrop et al. [7], Oehrle [8,9]), liquid chromatography (Nair and Huber [10], Hirata and Okamoto [11], Bauer et al. [12], Kleibohmer et al. [13]), ion exchange resin based tube sensor (Heller et al. [14]), and PVC membrane-based sensor (Zhang and Seitz [15]), GC/MS, HPLC (Kolla [16], Yinon and Zitrin [17], Jenkins et al. [18], Caton and Griest [19]). These laboratory methods are often cost prohibitive, time consuming, and labor intensive for use in a field screening application. In the early 1990s, Jenkins and Walsh [20] pioneered a field method for the detection of explosives using a colorimetric technique employing the Janovsky reaction. Since then, several qualitative and quantitative methods have been validated and commercialized for on site testing, including the D-Tech field test kit (Strategic Diagnositics [21,23]), Ensys immunoassay kit, (Ensys Environmental Products [22]) and other immunochemical based methods (Shriver-Lake et al. [24,25], Narang et al. [26,27]). In general, field measurements are considered to be useful for screening purposes, providing reliable data and timely information on the extent of contamination or remediation progress. Assays utilizing immunological reactions to detect a variety of analytes have been well studied (Keuchel et al. [28–30], Whelan et al. [31], Narang et al. [26,27]). This report demonstrates the effectiveness of the FAST 2000, a commercially available field version of the continuous flow immunosensor (CFI), for detection of TNT and RDX in soil samples obtained from environmental cleanup sites.

2. Materials and methods

2.1. Antibodies and standards

The 11B3 monoclonal antibody specific for TNT (Whelan et al. [31]) and monoclonal anti-RDX (IgG 50518 from Strategic Diagnostics Inc., Newark, DE) were used. Antibodies were immobilized on Porous Immunodyne ABC membranes (Pall Corp., Port Washington, NY), 5.0 μm pore size according to manufacturer's instructions. System buffer used in the analyses was 10 mM sodium phosphate, 0.01% Tween 20, and 2.5% ethanol, pH 7.4.

Analytical standards of 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) were purchased from Radian International LLC (Austin, TX). The calibrant standards were prepared using 20 μl of TNT or RDX (1.0 mg/ml) that was aliquoted into a test tube and dried down under an argon stream. A 2.0 ml aliquot of system flow buffer was added to rehydrate the standard and serial dilutions were made to obtain a range of concentrations from 10 to 1000 ng/ml.

2.2. Experimental setup

The key elements of the continuous flow immunosensor are: (1) antibodies specific for TNT or RDX, (2) analogs of the TNT or RDX labeled with the Cy5 fluorophore (the "labeled antigen"), and (3) a fluorescence detector. The antibodies are first immobilized on a porous membrane and saturated with the labeled antigen. To perform an assay, the porous membrane support is placed in a stream of system buffer and a sample is injected. The explosive of interest competes with the fluorophore-labeled antigen for the binding site of the antibody. When the analyte of interest is present in the sample, there are a proportional number of labeled molecules measured by the fluorometer downstream. Quantitation of the sample is made by comparison of the peak areas of internal standards of explosives run before and after the sample.

The FAST 2000 (Research International, Woodinville, WA) was used to analyze the soil samples for this report. The FAST 2000 is a commercially-available instrument based on the Naval Research Laboratory's CFI technology. It is designed to be a stand-alone instrument comprised of a laptop computer, main assay module, and a fluidics bulkhead. All assay parameters are controlled using a PCMCIA-based PC software program arranged by function. In the FAST 2000, the porous membrane support with the immobilized antibody (RDX or TNT) and labeled antigen is placed into a disposable plastic assay coupon and inserted into the instrument. Samples are injected through a rubber septum in the coupon into a 100 μl injection loop. The fluorescence lifetime of the membrane is dependent upon the number and concentration of positive assays that were run. Since only a limited quantity of the fluorescent antigen is bound to the antibodies on the membrane, it will eventually become depleted of the label. In general, a single coupon can be used for up to 30 injections. Analyses require 2–3 min per injection. Concentrations are determined by comparison to the calibration standards using a simple arithmetic ratio.

2.3. Laboratory tests

To assess system performance under controlled conditions, a series of tests was first run in the FAST 2000 with system flow buffer and samples containing TNT and RDX at known concentrations. To validate CFI results, splits of the sample were analyzed in the laboratory using the EPA-approved high performance liquid chromatography (HPLC) method for explosive analysis in soil and ground water, SW-846 Method 8330 [32]. First, the minimum detection limit (MDL), accuracy and bias of the instrument were examined as well as the false positive/false negative response of the FAST 2000. The MDL is calculated from the low matrix spike standard deviation(s) from the seven replicates as follows:

$$\mathrm{MDL} = 3.143\sigma$$

Accuracy is an indication of how closely the average value of the CFI matches with the HPLC confirmatory method (SW846-Method 8330). Precision is an indication of how close the replicate injections in the CFI are to each other. For the accuracy and precision experiments, RDX and TNT (at concentrations which were 5 and 50 times the detection limit) prepared in system flow buffer were injected into the CFI. Method bias (accuracy) was calculated by the equation:

$$\text{Bias} = \left(\frac{\overline{x}}{x}\right) \times 100$$

where $\overline{x}$ is the mean value for seven or more replicate determinations and X the spiked concentration. To determine the precision of the biosensor, the standard deviation, σ, and the mean are employed as follows:

$$\text{Precision} = \left(\frac{\sigma}{\overline{x}}\right) \times 100$$

The false positive rate was investigated using 20 replicate injections of explosive standards at one-half the detection limit prepared in system buffer. False negative response was examined similarly using standards at two-times the detection limit of the instrument. Triplicate injections of system buffer (blank) were used to establish a reference for signal comparison. A positive signal response was defined as a response greater than three-times the signal intensity of the blank.

2.4. Statistical methods

The CFI values were compared to the HPLC method using linear regression, relative percent difference and other statistical methods. A brief description of each follows.

2.4.1. Relative percent difference (RPD)

The RPD values between Method 8330 concentrations and the field screening results were calculated with the following equation:

$$\text{RPD} = \left[\frac{D_1 - D_2}{(D_1 - D_2)/2}\right] \times 100$$

where D_1 is the field screening concentration and D_2 the Method 8330 concentrations. The smaller the RPD value, the closer are the concentrations of the two methods and the more accurate the field screening method. A positive RPD indicates that the field screening method gave higher concentrations than Method 8330 results. The reverse is true for a negative RPD. A value of ±50% RPD is considered acceptable (Thorne and Myers [33], Crockett et al. [34]). RPD values outside this range have little use as a means of evaluating the agreement between two methods. The maximum value of the equation is never greater than ±200 indicating complete disparity between the two methods.

2.4.2. Linear regression

Linear regression plots were also constructed to evaluate the accuracy of the field screening method. The FAST 2000 results were plotted versus the HPLC results for each sample

and a best-fit line was calculated. Under ideal conditions, perfect agreement of the two analytical measures would produce a line with a slope of 1.0, a *y*-intercept of 0, and a coefficient of determination, $r^2 = 1.0$. A slope >1.0 indicates that the field screening methods generally give higher concentrations than Method 8330, and the reverse is true for slopes <1.0. The r^2 indicates the amount of scatter in the data, with 1.0 indicating no scatter.

2.4.3. Other statistical values

Other statistics used to evaluate the field data were the paired student's *t*-test and *F*-test. *t*-Test results on the slope from linear regression analyses were measured at 95% confidence levels. The paired *t*-test indicates whether or not the immunosensor method predicted the same analyte concentrations as the HPLC method (i.e. accuracy). If the immunosensor is generating accurate numbers, the result of the paired *t*-test will be that of no significant difference between the methods. The *F*-test assesses the variance of the data generated by the methods. In most cases, an accurate method will predict analyte concentrations that span the same range as those from the HPLC and there will be no significant difference between the variances. The *t*-tests on the slope from regression analyses determine whether or not these values differ significantly from 0. The ideal case would be a slope of one. From these properties, the following set of criteria was employed to assess the predictive capability of the immunosensor method for a given analyte: (1) the paired *t*-test result from the raw data must not be significant, (2) the *F*-test result from the raw data must not be significant, and (3) the slope of the regression analysis must be positive and significantly different from 0. A method must satisfy all three criteria to be deemed predictive.

2.5. Field sample tests

Authentic munitions contaminated soils were obtained from 5 sites: (1) Hawthorne AAP (Hawthorne, NV), a former Navy-operated load, assemble, and pack facility; (2) the US Army Ammunition Depot (Umatilla, Oregon) where explosives were removed and recovered from munitions; (3) Nebraska Ordnance Plant (Mead, NE); (4) Raritan Arsenal, (Edison, NJ); and (5) Fort Ord (Monterey, CA). T. Jenkins of CRREL provided 10 characterized soil samples (TJ00x) and H. Craig of US EPA Region 10 provided soil samples (Gxx-xx-A) from Umatilla Army Depot, Hermiston OR. The soils were extracted using a modified version of a field method described by Jenkins and Walsh [20]. Approximately, 5.0 g of soil were placed into an amber screw-top vial and extracted by shaking using 20 ml of reagent-grade acetone (Sigma–Aldrich) for 3 min. The sediment in the extracts was allowed to settle out of suspension. The supernatant was filtered through a disposable 0.45-micron syringe tip filter and stored in a certified clean vial under refrigeration until analysis. To perform analysis of the sample, 1.5 ml of the acetone extract was placed into a 12 mm × 75 mm test tube, dried down using an argon stream, and rehydrated using the system buffer.

For statistical comparisons, seven replicates of each soil extract were analyzed in the CFI using three internal standards per sample. The FAST 2000 software was used to calculate the peak area that corresponded to the beginning and end of the peak, as defined by the operator. A summary of the overall soil assay protocol is shown in Fig. 1. The data were averaged and standard deviations were calculated for each sample.

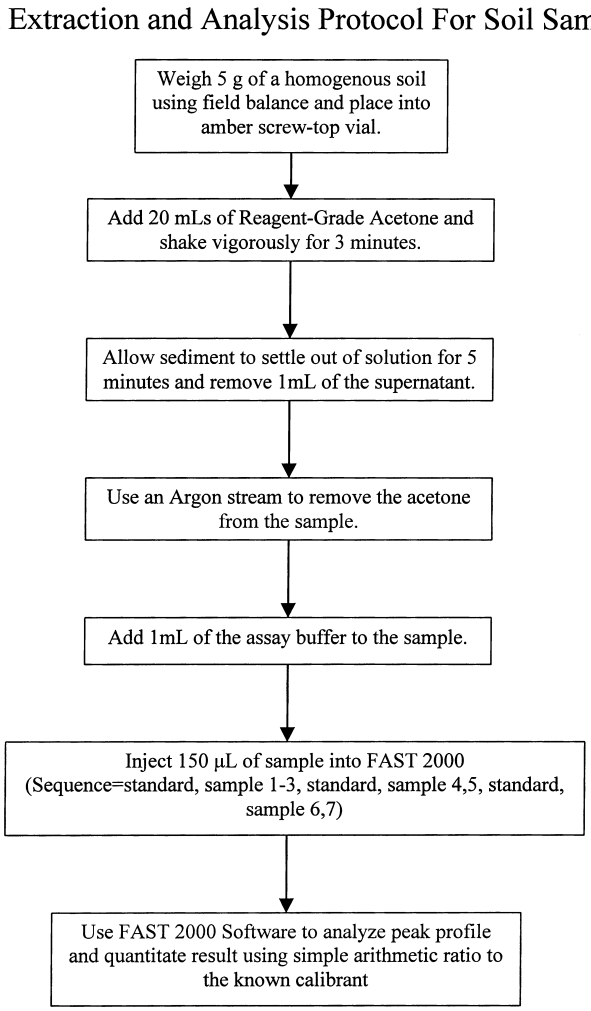

Fig. 1. Schematic of the field extraction protocol and continuous flow immunosensor analysis procedure.

3. Results and discussion

3.1. Laboratory standards

Experiments were performed using standards of the explosives prepared in the system buffer. Table 1 lists the results of the accuracy and precision tests that demonstrate a high degree of accuracy between RDX and TNT, with values that range from 93 to 99%. The precision of the sensor was also calculated, with percentages that range from 6 to 15%. Using the criteria outlined in the methods, the instrument is capable of a detection limit of

Table 1
Accuracy and precision of the CFI[a]

Sample concentration	Bias	Precision
50 μg/l TNT (nine replicates)	99	7
500 μg/l TNT (seven replicates)	93	14
50 μg/l RDX (seven replicates)	98	15
500 μg/l RDX (seven replicates)	99	6

[a] Samples prepared in system buffer, MDL = 1 μg/l).

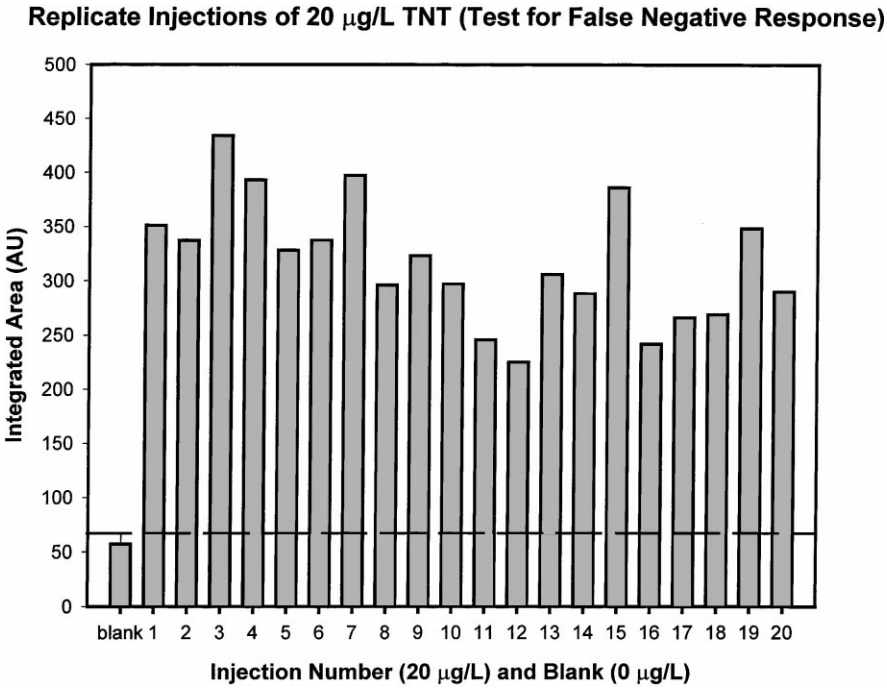

Fig. 2. Test for false negative response using twenty injections of TNT (20 μg/l) prepared in system buffer. Signals are compared to triplicate injections of system buffer (blank). Positive signals are considered to be those greater than three times the intensity of the blank injections.

10 μg/l in system flow buffer. Fig. 2 is included to illustrate the reproducibility of response to multiple injections of the explosive standards in system buffer obtained during the test for false negative response.

3.2. Soil field samples

A summary of the results for the FAST 2000 and SW-846 Method 8330, arranged by sample origin, is presented in Tables 2 and 3. Because the assays employ different antibodies

Table 2
Extract concentration (μg/l), relative percent difference (RPD)

Site/sample	TNT + TNB	TNT + TNB	TNT	TNB	RPD
	CFI	Lab	Lab	Lab	
Hawthorne AAP, (Hawthorne, NV)					
2	370	551	551	BDL	−39
5	342200	1205	1205	BDL	199
7	183200	368058	251548	116510	−67
Nebraska ordnance plant (Mead, NE)					
6	963000	347088	82118	264970	94
8	7300	BDL	BDL	BDL	−25
9	14200	18212	BDL	18212	
10	87100	65482	434	65048	28
Raritan Arsental (Edison, NJ)					
3	1027000	915965	915965	BDL	11
4	482000	54216	49054	475162	−8
Fort Ord (Monterey, CA)					
1	20	BDL	BDL	BDL	
Umatilla Army Depot (Umatilla, OR)					
11	5530	2660	2660	BDL	70
12	27200	32575	12797	19778	−18
13	219400	231011	231011	BDL	−5
14	27900	20636	3698	16938	30
15	50600	46939	23482	23457	8

for recognition and detection of energetic material, the results for TNT and RDX will be discussed separately. A combined TNB and TNT concentration for the samples analyzed is included for discussion due to the high cross-reactivity of the 11B3 antibody to TNB [31]. The samples listed in Table 2 fall into several categories: no detectable levels of TNT or TNB (BDL), high levels (>250 μg/l) of TNT, high levels of TNB (>250 μg/l), and high levels of both TNT and TNB. Nine of the fifteen samples have RPD values that fall within the acceptable range of ±50%. The following is an explanation for samples that fall outside the acceptable RPD range. Sample 1 resulted in a false positive that can be attributed to a sample response generated near the limit of resolution of the instrument and by the internal calibrant used for quantitation. Subsequent analysis of sample 5 revealed high levels of 2,4,6-trinitrophenol (picric acid), which has been shown to affect the antibody recognition capacity (Zeck et al. [35]). This interference is significant enough to interrupt the steady state of fluorescence released under the dynamic flow conditions of the assay. The result for such a sample will vary depending on the antibody selected for the analysis. There is some discrepancy regarding the HPLC analysis of TNT in sample 8, since two independent laboratories reported values that were in conflict. However, the bias reported in the RPD of samples 6 and 7 likely represents both the effect of cross reactivity of the antibody for TNB and mass action. This detection scheme provides the antibody and analyte a limited amount of time for interaction and resulting fluorescence response. The proportion of TNT

Table 3
Extract concentration (μg/l), relative percent difference (RPD)

Site/sample	RDX		
	CFI	Lab	RPD
Hawthorne AAP, (Hawthorne, NV)			
2	530	352	40
5	598	50456	−195
7	8560	8633	−1
Nebraska Ordnance Plant (Mead, NE)			
6	193400	147985	27
8	92900	138500	−39
9	365	526	−36
10	3470	2203	21
Raritan Arsenal (Edison, NJ)			
3	61	209	−110
4	36	407	−167
Fort Ord (Monterey, CA)			
1	400	BDL	
Umatilla Army Depot (Umatilla, OR)			
11	3550	2203	47
12	36800	14850	85
13	74400	135885	−58
14	14355	10259	33
15	80500	19492	122

to TNB in a particular sample that interacts with the antibodies then determines the high or low bias. The linear regression analysis for TNT + TNB (Fig. 3) indicates that only three of the samples (5, 6, and 7) fall outside the 95% confidence interval. A false positive determination (13%) resulted for 2 of the 15 samples, (8 and 1), and there were no false negatives.

The CFI uses a commercial monoclonal antibody for RDX analysis that is considerably more specific to the RDX molecule and exhibits minimal cross reactivity to other energetic materials when compared to the antibody used for TNT analysis. Eight of the fifteen samples analyzed for RDX using the CFI have RPD values that fall within the acceptable range of ±50%. There are several likely explanations for the remaining samples that lie outside of the acceptable RPD range. Sample 1 contained high levels of HMX (488 mg/l) and demonstrates the potential for minimal cross reactivity to HMX resulting in a false positive determination. As with the TNT assay, the high quantity of picric acid in sample 5 interfered with the antibodies' capacity to recognize the RDX present in the sample and effectively masked the normal signal generated from a calibrant at that concentration (50 mg/l). Samples 3 and 4 from Raritan Arsenal both showed a matrix-related effect in the fluorescence response caused by some factor present in the extraction that appears to be particular to that site. Samples from Umatilla Army Depot generally showed a high bias with positive RPD values ranging from 33 to 122 that may indicate a site-specific component present in the extraction

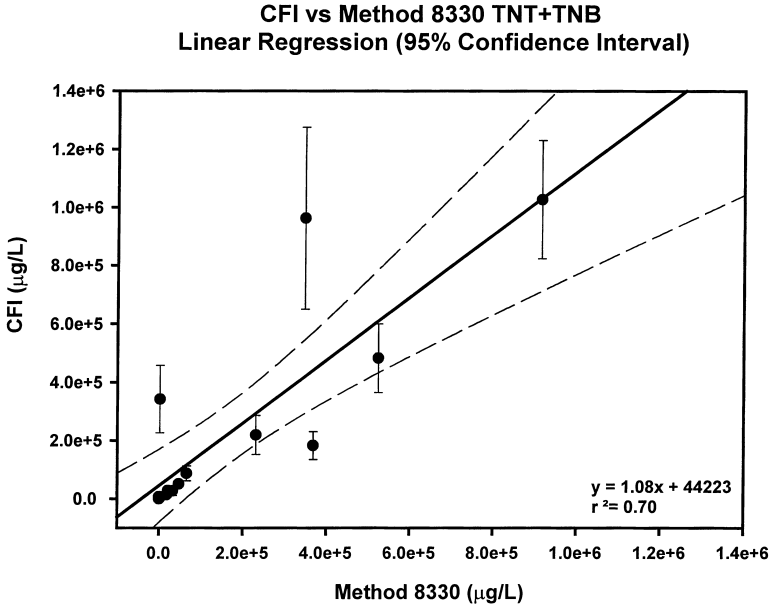

Fig. 3. Linear regression analysis of TNT + TNB concentration (μg/l) from the continuous flow immunosensor (seven replicate injections) vs. Method 8330 (HPLC). 95% Confidence interval identified by dashed lines.

that accentuates the fluorescence response of the assay. The linear regression analysis of the samples for RDX (Fig. 4) shows a coefficient of determination, (r^2) of 0.69 and indicates that 4 (5, 6, 13, and 15) of the 15 samples lie outside the 95% confidence interval. Only 1 of the 15 samples (1) was identified as a false positive (7%).

The statistical analyses used to evaluate the predictability of the TNT and RDX assays were the paired *t*-test, *F*-test and the slope of the regression line. The *t*-test and *F*-test (14 d.f.) values for the CFI method for TNT detection were 0.256 and 0.325, respectively. The slope of the regression analysis line was 1.08 with a coefficient of determination $r^2 = 0.70$. The *t*-test and *F*-test (14 d.f.) values for the RDX assay were 0.425 and 0.946, respectively. Soil extract analyses for the combined TNT + TNB and RDX using the FAST 2000 showed no significant difference from Method 8330 using the criteria outlined in this report and can therefore be considered predictive. A study demonstrated that the CFI is capable of providing quantitative information for environmental samples from a variety of field sites. There are numerous advantages to using an immunosensor for monitoring the progress of remediation efforts. The ability to analyze samples on-site provides the project manager with timely information concerning the remediation progress. A diagnostic instrument that is reliable and relatively simple to operate allows personnel with limited training to obtain quantitative data from a wide variety of sample matrices. The technology is not intended to replace the need for certified laboratory analysis of samples and is not without its limitations. This study revealed that a simple field extraction, while sufficient to remove energetic material, might also contain other factors that have a negative effect on the assay. The extraction method

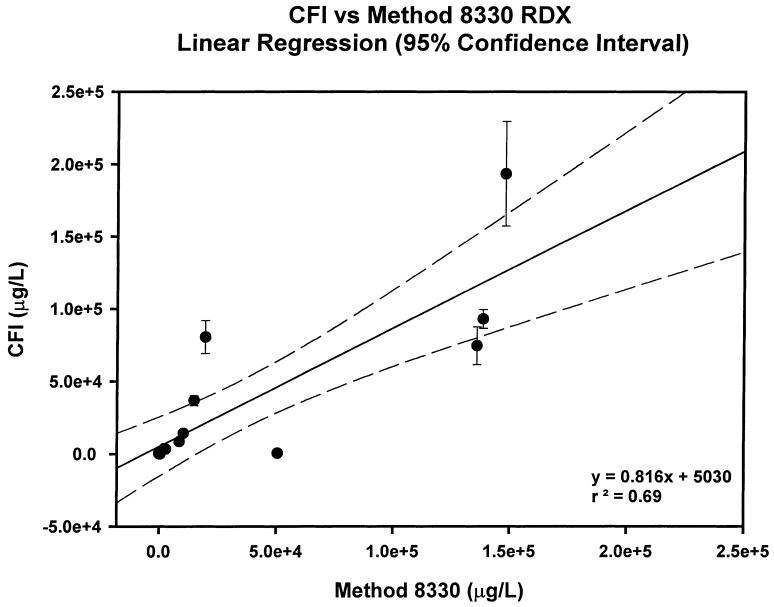

Fig. 4. Linear regression analysis of RDX concentration (μg/l) from the continuous flow immunosensor (seven replicate injections) vs. Method 8330 (HPLC). 95% Confidence interval identified by dashed lines.

employed for this study uses a strongly polar solvent, acetone, that removes a wide variety of other materials contained in the sample that may cause discrepancies during analysis. The nature of these matrix-related effects is not specifically known, but they do appear to be ubiquitous in soils collected from many different sites. In several cases, the HPLC analysis showed high levels of cross-reacting species, further emphasizing the importance of a complete site characterization prior to implementing any routine monitoring program. These effects can be mitigated by further treatment of the acetone extract using solid phase extraction (SPE) protocol, reducing the number of possible interferents contained in the sample.

4. Conclusion

Overall, the NRL biosensor performance suggests that the FAST 2000 instrument is a promising field technology for determinations of explosives in soil. The instrument is simple to use, requires minimal sample preparation, is easily carried to the field and generates minimal waste. Determinations of TNT and RDX levels were accurate and precise down to 10 μg/l in system buffer, with acceptable levels of false positive/false negative values. We found that the highly heterogeneous nature of soils can lead to a high degree of variability in the amount of explosives material found in the extract. Though further study to identify a suitable method of SPE to remove the matrix-related effects is required to improve the

overall performance of the FAST 2000 on environmental samples, this initial investigation demonstrates the feasibility of employing a field portable devise for the detection of explosives in soils.

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