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Development of chemiluminescent methods for explosives detection

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Abstract. The present work was finalised to develop chemiluminescent assays more sensitive and easy to use than the analytical methods currently in use to detect traces of TNT (2,4,6-trinitrotoluene), TATP (Triacetone triperoxide) and HMTD (Hexamethylene triperoxide diamine). For TNT detection we developed an indirect competitive ELISA with chemiluminescent end-point (CL-ELISA) and a Lateral Flow ImmunoAssay (LFIA) for on-site analysis, based both on monoclonal antibody commercially available and conjugates specifically synthesized. For the peroxide-based explosives TATP and HMTD we developed an indirect assay which measures, by luminol emission, the H_2O_2 released by these compounds. Both methods showed good sensitivity and reproducibility, with detection limit, LOD, and IC50 values in the ng mL^{-1} range, CV values lower than 10%. This indirect assay was finally optimized for using in a portable luminometer.

Keywords: explosives, TNT, TATP, HMTD, chemiluminescence, CL-ELISA, LFIA, portable luminometer.

1. Introduction

The terroristic attacks performed in the last ten years have focused the attention on the need for more effective strategies and tools for the protection of citizens. The rapid and sensitive detection of the various types of explosives must be included among these strategies. The 2,4,6-trinitrotoluene (TNT) is one of the most employed explosive in the 20th century [1] and, at the same time, it is dangerous as environmental pollutant because its well known toxicity [2, 3]. For these reasons its detection is required concerning terrorism acts or to have an early sign of environmental pollution.

Many methods have been reported for TNT detection: the EPA standard method, based on high performance liquid chromatography (HPLC) with UV detection, the gas chromatography [2] with various detectors, the spectrometric and spectroscopic assays [4-6, 7-8], the immunoassays [9-11].

TATP (Triacetone triperoxide) and HMTD (Hexamethylene triperoxide diamine) are compounds extremely instable because they contain peroxide groups [12]. Due to their simple synthesis, which requires the simple mixing of compounds easily available at any supermarket, they have been frequently used in terrorist attacks [13].

Peroxide explosives are usually analyzed by chromatographic techniques, according to the EPA method N 8330 [14]**.** However, these methods cannot be used on-site to analyze immediately the scene of an explosion. Other methods identify these explosives by means of spectrophotometric and fluorescent detections, but with low sensitivity and a significant possibility of false positives results [12, 13].

In a recent review Smith [15] provides an exhaustive list of publications describing the use of biosensors and biologically-inspired system for explosives detection underling the necessity of fast,

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highly specific, reliable and low cost tests in this field.

For this reason we decided to apply the rapid and sensitive luminescent detection to the determination both at laboratory and on site of TNT and peroxide-based explosives.

2. Experimental

Sample extraction: for TNT analysis three different extraction methods were tested: 10 minutes of shaking followed by 1 hour of sonication (Starsonic 60 LIARRE®) in ice bath (SS), 3 minutes of shaking followed by 3 minutes immersion in hot water (almost 70°C) (SW), 3 minutes of shaking (S). Samples were represented by free TNT soils spiked with 0.5, 1 and 5 µg mL^{-1} of standard.

In case of the peroxide based explosives the fortified soil samples (100 µL of TATP 58.8 µg mL^{1}, in acetonitrile (ACN), added to 500 mg of soil) were extracted with 300 µL of ACN simply by shaking for two min. The spiked samples were extracted at different times after addition: 5, 30 and 60 min to evaluate the evaporation rate of TATP from the soil.

CL-ELISA: a checkerboard titration was effectuated in order to define the optimum conditions for the indirect competitive assay. The competitive reaction between each one of the 13 especially synthesized conjugates and a commercially available monoclonal anti-TNT antibody (Strategic Diagnostic INC.), the tolerance to organic solvents (methanol and acetone at 1, 3, 5 and 10% of final dilution), the time required for the competitive and washing steps were evaluated. The antibody cross-reactivity with two nitro aromatic compounds related to the analyte, the 4-amino-2,6-dinitrotoluene (4-A-DNT) and the 2-amino-4,6-dinitrotoluene (2-A-DNT) was also evaluated.

The microplate treatments and the data processing were performed as previously reported [16].

Lateral flow: gold nanoparticles of 40 nm were obtained according to Frens [17]. The better dilution of the secondary antibody, a rabbit anti-mouse IgG (Sigma, Germany), labelled by the gold particles, was determined. A nitrocellulose membrane (Millipore, USA), 2.5 cm high, was cut in strips of 10 cm length. The test and control lines were coated, by using an Easy Printer LPM02 printer device (Advanced Sensor Systems P.LTD., India), with 100 µL of the trinitrobenzene hapten conjugated with ovoalbumin (TNB-OVA) 250 µg mL^{-1} and of Goat anti-rabbit immunoglobulin 200 µg mL mL^{-1}, respectively, both dissolved in Phosphate Buffered Saline (PBS) 1X. 50 µL of running buffer (PBS 1X containing 1% of BSA) will flow through the membrane, transporting the primary antibody, the gold labelled secondary antibody, the blank or the sample extract. In the first case two red lines will appear in correspondence of the test and control lines. In the second case the red colour of the test line will be less intense, inversely to the analyte concentration.

The coated membranes were stored over night at 37°C and then cut in pieces of 0.5 cm width at the moment of use.

Indirect luminol assay: 100 µL of 0.1 M HCl were added to 100 µL of TATP-HMTD solutions in ACN and after 5 minutes of shaking an equal volume of 0.1 M NaOH was added blocking the production of hydrogen peroxide radicals. In the light-emitting oxidation of Luminol these radicals are employed as substrates by the horseradish peroxidase (HRP), and then quantified by measuring the intensity of the emitted light. Two luminometers have been used: the 96 wells microplate reader Luminoskan Ascent (Labsystem, Italy) and the portable, single sample device System Sure II (Hygiena, USA)

After the determination of the optimum parameters for TATP and HMTD detection, the analysis was applied to fortified sample extracts, divided into three aliquots of 100 µL to perform the analysis in triplicate.

3. Results and Discussions

CL-ELISA: the highest sensitivity was reached by using 1 µg mL^{-1} of TNB-OVA conjugate, 0.33 µg mL^{-1} of mouse anti-TNT antibody, time periods of 90 min for the washing and the competitive steps, a final dilution to 10%.by using methanol as solvent. The average IC50 and LOD values of the calibration curves resulted equal to 2.97 ng mL^{-1} and 0.41 ng mL^{-1}, respectively (Fig. 1). The reproducibility of these values, expressed as the coefficient of variation (CV) was 7%, showing a good sensitivity and reproducibility.

Concerning cross reactivity it resulted of the 16% for 2-A-DNT, whereas for 4-A-DNT no cross reactivity was determined.

The results obtained from the analysis of spiked soils are shown in Table 1. It is important to note that the three extraction methods gave almost the same results, the differences were not significant.

This finding was very important for the development of the lateral flow assay, which requires effective but simple methods of extraction, easy to be performed on-site.

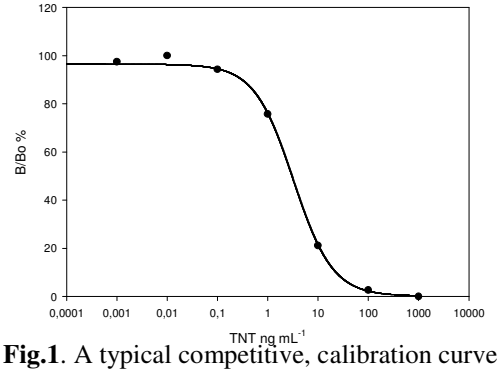

Fig.1. A typical competitive, calibration curve for 2,4,6-trinitro toluene.

Table 1. Mean recovery of TNT spiked soils extracted with different methods: SS; SW; S.

spiked TNT (ng mL^{-1})	Extraction Method	Mean Recovery (ng mL^{-1})	CV (%)	Mean Recovery (%)
500	SS	216	12	43.2
	SW	186	8.2	37.2
	S	260	9.2	52.0
1000	SS	912	7.7	91.2
	SW	884	9.6	88.4
	S	882	9.9	88.2
5000	SS	3672	7.4	73.4
	SW	5320	7.6	106.4
	S	4560	10	91.2

Lateral flow assay (LFIA). In order to assess the detection limit of the system we add to the strip 1 µL of standard TNT solutions in a concentration range between 100 and 0.01 µg mL^{-1}. The minimum amount leading to a visible decrease of the test line was 1 µg mL^{-1} (Fig. 2).

The lateral flow immunoassay for testing the cross reactivity showed for 2-4-DNT a little less intense colour of the test line with respect to the standard one, whereas for 4-A-DNT there was no colour intensity changes, indicating that the first compound was partially recognized while the second one was not at all recognized by the anti-TNT antibody.

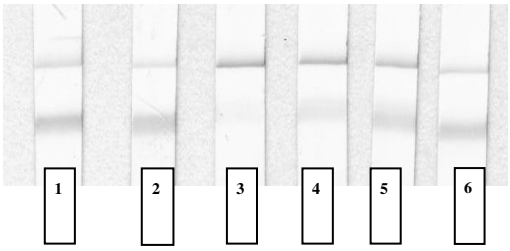

Fig.2. LFIA of TNT standard at different concentrations. 1) no TNT; 2) no TNT, 1 µL of Methanol; 3) 1 µL of 100 µg mL^{-1} TNT; 4) 1 µL of 10 µg mL^{-1} TNT; 5) 1 µL of 1µg mL^{-1} TNT; 6) 1 µL of 0.1 µg mL^{-1} TNT.

Peroxide based explosives. The interval of linearity for the calibration curves was 0.45-4 µM for TATP (figure 3), and 0.25–8 µM for HMTD (figure 4), respectively, when the portable System SURE II was employed. The same intervals were: TATP 22 µM – 14 µM, HMTD 0.24 µM – 16 µM for measurements on the microplate reader.

The LOD value for TATP was 0.5 µM and for HMTD 0.2 µM by using the system SURE II and 0.3 µM and 0.1 µM, respectively, by using the microplate reader. The analysis of the spiked soil extracts revealed that the recovery was very good, close to the 90% when the extraction was performed after short time from spiking, but the added TATP evaporated very quickly from the soil (Table 2).

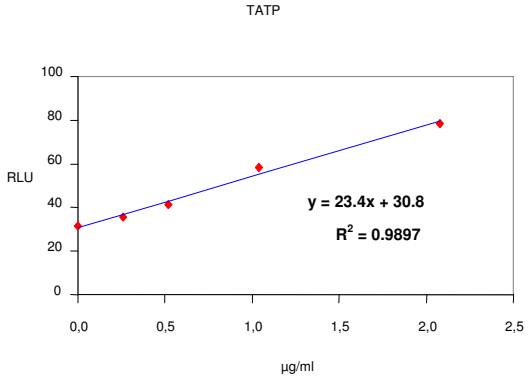

Fig. 3. Calibration curve for TATP

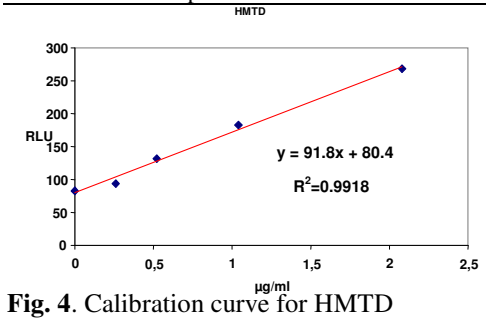

Fig. 4. Calibration curve for HMTD

Table 2. Mean recovery of TATP from soil by extraction at different times after spiking.

TIME (min)	Measured TATP (µg/ml)	CV (%)	Recovery (%)
5	0.57	6.2	88
30	0.05	10.6	8
60	0	0	0

4. Conclusions

The results obtained during this preliminary work were very satisfying and encouraging. The chemiluminescent assays here developed confirmed the high sensitivity of these revealing systems and they will be the basis on which the luminescent determination will be tested on other classes of explosive compounds. In particular, the on field methods showed high sensitivity and reproducibility, as well as to be perfectly suitable for real samples analyses.

5. Acknowledgments

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