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Abstract: The capability to collect timely information about the substances employed on-site at a crime scene is of fundamental importance during scientific investigations in crimes involving the use of explosives. TNT (2,4,6-trinitrotoluene) is one of the most employed explosives in the 20th century. Despite the growing use of improvised explosives, criminal use and access to TNT is not expected to decrease. Immunoassays are simple and selective analytical tests able to detect molecules and their immunoreactions can occur in portable formats for use on-site. This work demonstrates the application of three immunochemical assays capable of detecting TNT to typical forensic samples from experimental tests: an indirect competitive ELISA with chemiluminescent detection (CL-ELISA), a colorimetric lateral flow immunoassay (LFIA) based on colloidal gold nanoparticles label, and a chemiluminescent-LFIA (CL-LFIA). Under optimised working conditions, the LOD of the colorimetric LFIA and CL-LFIA were 1 μg mL-1 and 0.05 μg mL-1, respectively. The total analysis time for LFIAs was 15 minutes. ELISA proved to be a very effective laboratory approach, showing very good sensitivity (LOD of 0.4 ng mL-1) and good reproducibility (CV value about 7%).Samples tested included various materials involved in controlled explosions of improvised explosive devices (IEDs), as well as hand swabs collected after TNT handling tests. In the first group of tests, targets covered with six different materials (metal, plastic, cardboard, carpet fabric, wood and adhesive tape) were fixed on top of wooden poles (180 cm high). Samples of soil from the explosion area and different materials covering the targets were collected after each explosion and analysed. In the second group of tests, hand swabs were collected with and without hand washing after volunteers simulated the manipulation of small charges of TNT.The small amount of solution required for each assay allows for several analyses. Results of immunoassays confirmed that they were suitable to detect post-blast residues in soil and target materials and post transfer residues on hands, allowing further confirmation by more selective techniques. ELISA and LFIAs results obtained from the same solution were consistently in good agreement, and were confirmed by gas chromatography coupled to mass spectrometry (GC-MS). The reported immunoassays data demonstrates the suitability of LFIAs as on-site rapid and effective assays to detect TNT traces. The CL-ELISA proved useful in obtaining very sensitive detection in forensic investigations and testing, while CL-LFIA had performances in between LFIA and CL-ELISA.

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Title Page (with authors and addresses)

Field detection capability of immunochemical assays during criminal investigations involving the use of TNT.

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Highlights

- We detected TNT from typical forensic samples using immunochemical assays.
- LFIA and CL-LFIA showed to be effective as on-site detection tool for TNT traces.
- The CL-ELISA proved useful in obtaining very sensitive detection in forensic cases.
- The small amount of solution required for each assay allows for several analyses.

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Abstract

The capability to collect timely information about the substances employed on-site at a crime scene is of fundamental importance during scientific investigations in crimes involving the use of explosives. TNT (2,4,6-trinitrotoluene) is one of the most employed explosives in the 20th century. Despite the growing use of improvised explosives, criminal use and access to TNT is not expected to decrease. Immunoassays are simple and selective analytical tests able to detect molecules and their immunoreactions can occur in portable formats for use on-site. This work demonstrates the application of three immunochemical assays capable of detecting TNT to typical forensic samples from experimental tests: an indirect competitive ELISA with chemiluminescent detection (CL-ELISA), a colorimetric lateral flow immunoassay (LFIA) based on colloidal gold nanoparticles label, and a chemiluminescent-LFIA (CL-LFIA). Under optimised working conditions, the LOD of the colorimetric LFIA and CL-LFIA were 1 µg mL^{-1} and 0.05 µg mL^{-1}, respectively. The total analysis time for LFIAs was 15 minutes. ELISA proved to be a very effective laboratory approach, showing very good sensitivity (LOD of 0.4 ng mL^{-1}) and good reproducibility (CV value about 7%).

Samples tested included various materials involved in controlled explosions of improvised explosive devices (IEDs), as well as hand swabs collected after TNT handling tests. In the first group of tests, targets covered with six different materials (metal, plastic, cardboard, carpet fabric, wood and adhesive tape) were fixed on top of wooden poles (180 cm high). Samples of soil from the explosion area and different materials covering the targets were collected after each explosion and analysed. In the second group of tests, hand swabs were collected with and without hand washing after volunteers simulated the manipulation of small charges of TNT.

The small amount of solution required for each assay allows for several analyses. Results of immunoassays confirmed that they were suitable to detect post-blast residues in soil and target materials and post transfer residues on hands, allowing further confirmation by more selective techniques.

ELISA and LFIAs results obtained from the same solution were consistently in good agreement, and were confirmed by gas chromatography coupled to mass spectrometry (GC-MS). The reported immunoassays data demonstrates the suitability of LFIAs as on-site rapid and effective assays to detect TNT traces. The CL-ELISA proved useful in obtaining very sensitive detection in forensic investigations and testing, while CL-LFIA had performances in between LFIA and CL-ELISA.

Keywords: 2,4,6-trinitrotoluene (TNT), explosives, chemiluminescence, ELISA, LFIA, on-site tests, crime scene, swabbing.

Introduction

Forensic analytical procedures generally start with a sampling step. In bombing-scene investigations, it is important to provide valuable information to the investigators by collecting the material that is most likely to produce evidence [1]. 2,4,6-trinitrotoluene (TNT) is a very effective and relatively safe explosive because of its high explosive power, high chemical stability and low sensitivity to impact and friction [2, 3]. Royds et al. described very well the investigations after the Bali bombings, where the explosion of three charges containing TNT killed two hundred and two people [1]. In that occasion, a number of presumptive tests (including 3% KOH in ethanol) and ion mobility spectrometry (IMS) were performed in a mobile laboratory using collected debris. The 1993 bombings in Florence, Rome, and Milan provide another good example of the need for on-site testing of explosive traces. TNT was found in samples from all five bombing scenes via mobile explosives detectors, similar to those used in airports to detect hidden explosives. Some suspects from the bombing opted to collaborate to an inquiry and based on their advice, samples were taken from places where explosive charges were prepared and vehicles used for transport [4]. The detectors provided fast preliminary results of samples and positive results supported the suspects' credibility. Another forensic application warranting fast and sensitive detection capabilities is the analysis of samples from people suspected of recently handling explosives.

Several procedures for laboratory analysis of post-explosion residues and traces on suspects or their belongings have been developed since the first studies published in the 1960s [5, 6]. Examples of sources sampled include soil from an explosion crater extracted by solvent [7] and surfaces sampled by swabbing [8], tape lifting [9] or vacuum lifting [10]. General and comprehensive schemes for the analysis of post-explosion residues where first described in 1970s and include the team approach for processing the bomb-scene, the visual examination of debris, and the sample preparation and analysis [11]. Gas chromatographic-thermal energy analysis (GC-TEA) was introduced in 1975 [12] as a very selective and sensitive method for nitrocompounds [13, 14]. The introduction of tandem mass spectrometry (MS) permitted more selective procedures for forensic identification of explosives [15-21]. Each of the laboratory techniques listed above is effective yet requires time consuming procedures. Positive on-site detection saves time in the laboratory by only confirming already positive samples [22]. Although unsuitable for airport security, field tests for TNT based on the formation of coloured Meisenheimer and Janowsky anions in alkaline acetone or methanol [23, 24] can be used for crime scenes. When considering sensitivity and selectivity, research moved from colour changes to fluorescence emission [25], optical chemosensing [26], and ultimately the use of biologically inspired systems [27, 28]. Antibodies in analytical methods can help achieve good detection capabilities needed for forensic applications, especially on-site. Competitive immunoassays have been used to detect many types of small molecules including explosives such as TNT [27, 29]. Three tests were recently developed to detect TNT: an indirect competitive enzyme-linked immunosorbent assay with chemiluminescent detection (CL–ELISA) [30] and two lateral flow immunoassays (LFIAs) particularly suitable for on-site use, the first based on colloidal gold nanoparticles label (colorimetric detection) [30] and the other based on chemiluminescent label detection [31].

This work describes the application of these three immunoassays for the detection TNT traces in typical forensic samples such as samples from an explosion test site and post-handling hand swabs. The aim was to evaluate and compare the effectiveness of these tests to give timely forensic information and contribute to the early stages of criminal investigation.

Materials and Methods

Explosion tests

Explosion tests were carried out in civil and military firing areas in Italy (a quarry near Castel S.Pietro, Bologna, and an army facility near Casal Borsetti, Ravenna) by one bomb squad of the Italian State Police in collaboration with specialists of the Scientific Police of Bologna. Targets of 100 cm^2 each (see Fig.1) covered with different materials (metal, plastic, cardboard, carpet fabric) were fixed at the top of a wooden pole (180 cm high) with adhesive tape and placed at different positions around the explosion point.

A first set of controlled explosions was carried out using TNT charges of 34 g, 67 g, and 100 g to study the feasibility of experimental arrangements. In each test, nine poles with targets were placed around the charge at 1.5 m, 9 m and 15 m from the explosion point (Fig. 2A). Meteorological parameters were recorded using a WS-2300 European Wired/Wireless Weather Center. Temperature was between +30° C and +34° C and wind speed was less than 10 km/h from N/NW.

Experiments representing more realistic criminal event conditions include: a charge of one kg of TNT, surrounded by six target poles at 3 m and 6 m from the explosion site (Fig. 2B); and a charge of two kg surrounded by nine target poles at 3 m, 6 m, and 9 m from the explosion site (Fig. 2C). The recorded parameters were: 991.5 hPa pressure, temperature between -1°C and +4° C, 90 % humidity and 10-20 km/h W/NW wind speed and direction.

For charges up to one kg TNT, one blasting cap N.8 was used; for the test with a charge of 2 kg TNT, two blasting caps N.8 were used.

Handling tests

Handling tests were conducted with three volunteers. Blank samples were taken from their hands before each test. A small block (about 10 g) of military TNT was handled by each person for 3–5 min, simulating its positioning on a working surface. Four swabbing experiments were carried out: sampling immediately after handling, sampling immediately after handling and washing hands only with water, sampling immediately after handling and washing hands with water and soap, sampling two hours after handling.

Sample collection and treatment

Soil samples were collected in the explosion area (the explosion point and the area delimited by the

targets) using a metal spatula and stored in plastic screw-cap vials at room temperature (RT). TNT residues were extracted by adding 500 μL of methanol to 0.4 g of soil and shaking for 3 minutes. Methanol was separated by centrifugation [30].

Two different swabbing systems were tested, one for surface samples and another for handling tests. Surface samples from targets and hands were collected using cotton swabs containing cotton buds from ARTSANA (Grandate, Como, Italy) wetted with methanol RS for HPLC from Carlo Erba (Milano, Italy). Samples were collected from each material on each target. Each swab was then dipped in 500 μL of methanol in a glass vial with screw cap. Explosive residues were extracted from the swabs by shaking the vials for 3 minutes.

For handling tests, round cotton pads from Fort James Italia (Genova, Italy) were cut in four and wetted with isopropyl alcohol (propan-2-ol) RS for HPLC from Carlo Erba (Milano, Italy). These swabs were already successfully tested for several explosives, including TNT [32], TATP [33] and organic gunshot residue [34]. Swabs were dampened with 0.4 mL isopropyl alcohol (propan-2-ol) and shaken to remove any excess solvent. The swab was firmly rubbed numerous times over the entire surface of both hands of each subject: palm, fingers, thumb, back and wrist. Three consecutive swabs were used to sample each subject. After sampling, each swab was immediately enclosed into a small glass screw-cap jar (NEMI 15 mL) (Eurovetrocap Milano, Italy) and sealed with Parafilm® M (Pechiney Plastic Packaging Company, Menasha, WI, USA). Samples were stored at -20° C until analysed. After adding 0.1 mL of propan-2-ol, swabs were emerged in an ultrasonic bath (FALC Instruments, Bergamo, Italy) for 15 minutes. Swabs were removed and squeezed to capture the maximum amount of solution in the glass vial. The solution was filtered on 0.2 μm OlimPeak nylon filters (Teknokroma, Sant Cugat del Vallés, Barcelona, Spain) and evaporated under a nitrogen flux. A solution of musk xylene in acetonitrile (Analytical Standard Fluka) purchased from Sigma Aldrich (Milano, Italy) was used as internal standard after diluting with propan-2-ol. Both solutions (in acetonitrile and dilution in propan-2-ol) were stored at 2° C. An internal standard of 100 μL of propan-2-ol containing musk xylene 5 mg/L was added before GC-MS analysis. The extraction procedure was repeated three times for each swab to obtain three fractions.

Immunoassays

Chemiluminescent ELISA, (CL-ELISA). CL-ELISA was performed according to Girotti, et al., using 96-well microplates as an indirect competitive format. Briefly: the microplate was coated with 1 μg mL^{-1} TNB–OVA conjugate in 0.05 mol L−1 carbonate–bicarbonate buffer (prior to use), pH 9.6 (100 μL/well) and incubated overnight at RT. After washing, 50 μL/well of 1:40,000 dilution of antiserum in phosphate buffer (PBSG; 10 mmol L^{-1}, pH 7.4; 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 10 mmol L^{-1} Na_2HPO_4, 2 mmol L^{-1} KH_2PO_4, 0.5% fish gelatine) and 50 μL/well standard solutions or sample extracts were added and incubated for one hour at RT. Each extract solution was split into three wells; each standard solution was split into six wells. The standard solution of TNT was purchased from AccuStandard (New Haven, USA). Methanol was RS for HPLC from Carlo Erba (Milano, Italy). All other chemicals and organic solvents were reagent grade.

The microplate was washed and 100 μL/well goat antimouse IgG-HRP diluted 1:2000 in PBSG (according to the manufacturer's guidelines) was added before incubating for 90 minutes at RT. Ultimately, after washing, 100 μL of luminescent mixture (45 μL luminol 1 mmol L^{-1}; 10 μL p-iodophenol 0.5 mmol L^{-1} both from Sigma, Hamburg, Germany), 9845 μL borate buffer (0.2 mol L^{-1}, pH 8.5; 50 mmol L^{-1} $Na_2B_4O_7.10H2O$; 200 mmol L^{-1} H_3BO_3) and 100 μL of 1 mmol L^{-1} hydrogen peroxide (Merck, Germany) was added to each well and the absorbance at 425 nm was immediately recorded by a Victor 1420 luminometer (Wallac–Perkin–Elmer, Waltham, MA, USA). Four readings per well were recorded. The chemiluminescent data, expressed as relative luminescent units (RLU) were normalized according to the expression:

$$\%B/B_0 = 100 (A\text{-}A_{excess}) / (A_0 - A_{excess})$$

and mathematically fit to a four-parameter logistic equation using SigmaPlot Version 8.0. LOD was calculated in correspondence of the RLU value obtained according to the expression:

$RLU_{LOD} = (A_0 - A_{excess}) \times 0.9 + A_{excess}$

The CL-ELISA showed very good sensitivity (LOD of 0.4 ng mL^{-1}) and good reproducibility (CV value about 7%) [30].

Colorimetric lateral flow immunoassay, (LFIA). LFIA was prepared according to Girotti et al. [30]. The lateral-flow assay was performed by dipping the strips in a well containing 50 μL running buffer (PBS 1X–1% BSA), 10 μL rabbit anti mouse-colloidal gold, and 3 μL mouse anti-TNT antibody. The upper part of the strip was in contact with a piece of filter paper, which forced complete migration of the liquid throughout the membrane. After 5 minutes, two visible red lines confirmed the assay had been conducted correctly on a sample not containing TNT (Fig. 3). The sample extracts were added into the well containing the reagents described above. The presence of the analyte was revealed by the colour intensity decrease of the test line, directly proportional to the amount of analyte in the sample, until its complete disappearance. The colorimetric LFIA showed to be very selective in cross reactivity studies with a LOD of 1 μg mL^{-1} [30].

Chemiluminescent lateral flow immunoassay, (CL-LFIA). CL-LFIA was performed according to [31], using nitrocellulose LFIA strips. The assay was performed by dipping the LFIA strip in a microtiter plate well containing 50 μL of running phosphate buffer (PBS; 10 mmol L^{-1}, pH 7.4; 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 10 mmol L^{-1} Na_2HPO_4, 2 mmol L^{-1} KH_2PO_4, with 3%, w/v BSA, 1:1000, v/v anti-TNT mouse antibody, and 1:20,000, v/v anti-mouse HRP-labelled rabbit antibody) and 1 μL of sample (or TNT standard solution for generating calibration curves in the range between 0.02 and 5 μg mL^{-1}). When flow through the membrane ended (about 10 min), 50 μL of HRP luminol-based CL substrate was dispensed in the well and flowed for 3 minutes. The strip was then transferred in the portable imaging device and the CL image was acquired using an integration time of 5 seconds. During the acquisition, the CCD sensor temperature was fixed at −10 °C. The imaging system was based on a CCD camera (model MZ-2PRO, MagZero, Pordenone, Italy) equipped with a thermoelectrically cooled monochrome CCD image sensor, coupled with a Computar 2/3 in. 8 mm, f1.4 objective (CBC Corp., Commack, NY, USA), and connected to a light-tight dark box to image LFIA strips without interference from ambient light [31]. The camera was

controlled by a laptop computer powered by a 12 V battery to allow portability of the imaging system. Images were recorded in the Flexible Image Transport System format and analysed using the WinLight 32 Version 2.91 (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). The reference instrument was a research-grade luminograph (NightOWL LB 981, Berthold Technologies GmbH & Co. KG) equipped with a back-illuminated thermoelectrically cooled CCD camera.

For quantitative analysis, the CL signal was measured in the LFIA membrane areas corresponding to the analytical and control lines, as well as in two couples of adjacent areas that were used to evaluate the CL background signal of each line. The calibration curve was obtained by plotting the fraction of bound anti-TNT antibody displaced versus TNT concentration, then performing a non-linear regression with a four-parameter logistic equation using GraphPad Prism Version 5.03 (GraphPad Software, San Diego, CA). The limit of detection of the CL-LFIA was 0.05 μg mL^{-1}, requiring 15 minutes for analysis [31].

Quantitative analysis by GC-MS

All samples were analysed via a GC-MS developed for this purpose as the reference method to obtain forensic confirmation and quantitative results to be compared to the results obtained by immunoassays.

A gas chromatograph (Agilent Technologies 7890) equipped with a mass spectrometer (Agilent Technologies 5975C MSD) used in electron impact ionisation mode and an autosampler (Agilent Technologies 5973 B) was employed. The software was Agilent Chemstation. The injector was a multi mode injector and operated in split mode (5:1). A fused-silica capillary column (Agilent 19091S-433HP-5MS) with chemically bonded phase 5% phenyl-95% dimethylpolysiloxane 10 m x 250 μm i.d., 0.25 μm film thickness was used. Conditions were as follows: injection volume, 1 μL; injection port temperature, 175°C; carrier gas, helium 99,9995% purity; flow 1 mL min^{-1}; column temperature, 50°C for 2 min; then programmed 25°C/min to 200°C for 3 minutes, after post-run at 250°C for 5 minutes; transfer line temperature, 200°C. The damping gas was helium 99,9995% purity; flow 0,3 mL min-1; the source was set at 200°C; the scan range of the mass spectrometer was between m z^{-1} 35 and 350.

The GC-MS method reached a LOD of 0,4 μg mL^{-1} and a LOQ of 0,8 μg mL^{-1}. Linearity of the response was tested in the range 1-10 μg mL^{-1} obtaining the following calibration curve: y=0.5606x - 0.0922, r^2 = 0.9851 and in the range 10-100 μg mL^{-1} obtaining: y=0.8583x - 0.4536, r^2 = 0.9984. The intra-day coefficient of variation (CV) was 1 % and the inter-days CV~~RSD~~ was 8 %, both at 1 μg mL^{-1}. The accuracy was 10% at 1 μg mL^{-1}**.**

Results and discussion

Explosion tests

Preliminary tests performed using the smaller charges of TNT (34 and 67 g) showed that traces can be revealed by CL-ELISA and GC-MS only in the soil samples from craters. Larger charges were then employed. Samples from the targets and soil after the explosion of the 100 g TNT charge were analysed by

colorimetric LFIA, CL-ELISA and GC-MS. Positive results from this test were found on some materials located 1 m from the detonation site and in the wind direction moving the explosion cloud on target 3. A soil sample taken close to target 3 also resulted positive to TNT residues. All the remaining targets and soil samples were negative. LFIA gave clearly positive results for the swabs used on the adhesive tape and the carpet fabric. The samples from plexiglass, wood, and soil were classified as positive (Fig. 3). These results were confirmed by CL-ELISA and GC-MS. Both assays gave positive results for all the same samples from target 3 and for soil. The quantitative values from CL-ELISA and GC-MS were in good agreement (Table 1).

These findings suggested planning new tests with larger charges to be performed in a suitable military area. The detonation of a 1 kg TNT charge was executed; post-explosion samples were all analysed by LFIA, CL-ELISA and GC-MS. Results are reported in Table 2.

In this case, all targets from the inner circle (3 m far from the charge) and the soil sampled close to the pole of target 1 gave positive results. The quantitative data shown in Table 2 confirm good agreement between the CL-ELISA and GC-MS determination.

A further explosion test was performed by employing a 2 kg charge and a higher number of targets. Sample extracts were tested by all four methods. Data obtained by CL-LFIA, CL-ELISA and GC-MS analyses are reported in Table 3. All samples positive to the other assays were confirmed by the colorimetric LFIA. Again, only the targets placed in the inner circle (3 m from the exploding TNT charge) were contaminated by the explosion residues. The soil samples taken close to the poles of the targets 7 and 8 contained TNT residues. All remaining soil samples resulted negative to all analytical methods here employed.

It is interesting to observe the good agreement between the CL-LFIA and GC data. The two values obtained were very similar with the exception of one, underlining the capability of this rapid and simple test to assess the presence and amount of the analyte with acceptable accuracy.

Handling tests

During handling tests, the TNT sample was manipulated for about 3-5 min by the three volunteers (A, B and C). The four different experiments were Handling Test 1 (HT1): samplings were carried out immediately after handling; Handling Test 2 (HT2): sampling immediately after handling and washing the hands with water but without soap; Handling Test 3 (HT3): sampling immediately after handling and washing the hands with water and soap; Handling Test 4 (HT4): sampling was performed two hours after handling, without washing. The swab extracts were analysed separately by CL-ELISA, LFIA and GC-MS as done before in case of explosion test sample. The results obtained from handling tests are reported in Table 4.

As expected, the amounts of TNT residue determined immediately after handling were much larger than in samples from the explosion tests. In spite of the great differences among the residue amounts in HT1 positive samples, all of them resulted positive to LFIA. In a typical forensic context, one swab is used for sampling both hands, or maximum one swab per hand. According to these data, a single swab is expected to result clearly positive to LFIA if sampling occurs early after handling TNT.

The TNT residues collected by the swabs in HT2 were very low compared to the results of HT1. At least one of the samples was recognized as positive by all the three method of analysis, confirming their fitness for purpose in forensic application. The results obtained from analysis of the HT3 swabs were practically the same results obtained from the HT2 test, concluding that the use of soap during hand washing does not have significant influence on the removal of TNT residues. Only two subjects were swabbed in HT4. The first swab (taken two hours after handling the TNT residues) from Subject A contained about 20 $\mu g\ mL^{-1}$, while the first swab from Subject B was close to the results obtained after washing tests. The responses of the three assays were still in agreement.

The reported results show that the tested immunoassays are useful forensic tools to obtain fast detection of TNT presence at a bombing scene or on the hands of suspects during criminal investigations.They also provide accurate quantitative data.

Conclusions

The preliminary results obtained during this research support the importance of portable tests for a most effective selection of the material to collect for further analytical work in places where explosive charges were used or prepared, or in vehicles used to transport an IED. The LFIA is particularly promising because it is the fastest, easy-to-use, portable and the cheapest technique, which also produced results continuously in agreement with reference methods such as GC-MS. Further research is needed; however, the results obtained by CL-LFIA and CL-ELISA (more sensitive) showed to be sensitive, selective and accurate enough to be used in forensic procedures. These procedures can be very useful to select the samples needing

forensic confirmation by more expensive and time consuming procedures. The small amount of solution required for each assay allows for several analyses. Because only samples testing positive to immunoassays are analysed for confirmation, this approach saves time and money as well as produces faster results, consequentially resulting in optimisation of resources. These traits are always desirable in criminal investigations. The authors believe the reported results show the fitness for purpose of LFIA, CL-LFIA and CL-ELISA in forensic analytical approaches used to seek TNT traces. Results confirmed that immunoassays were suitable to detect post-blast residues in soil and target materials and post transfer residues on hands. Results from ELISA were consistently in good agreement with results by LFIAs and repeatedly confirmed by GC-MS. The reported immunoassays data demonstrate the suitability of LFIAs as on-site rapid and effective assays to detect TNT traces and the utility of CL-ELISA to obtain very sensitive detection in forensic investigations and testing, with CL-LFIA having performances in between LFIA and CL-ELISA.

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Figure

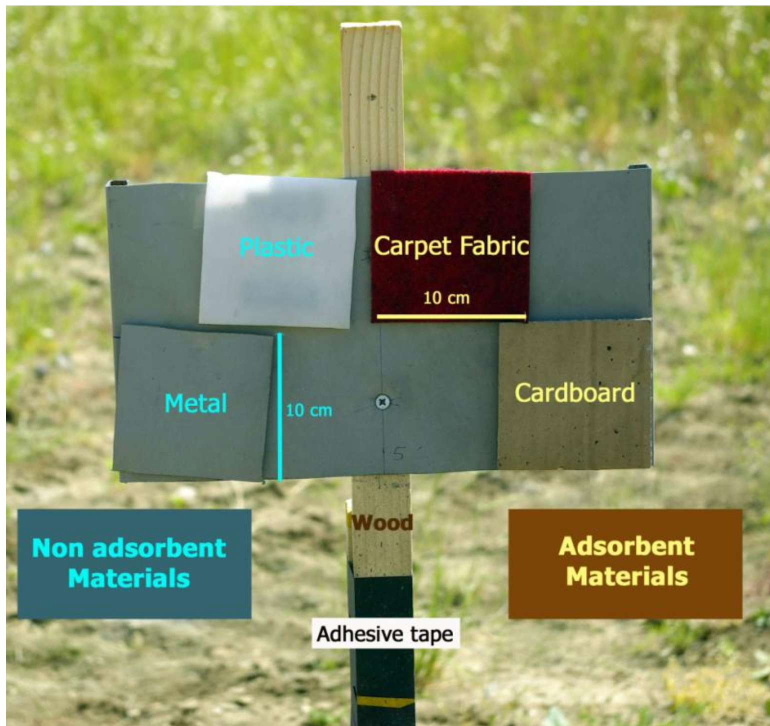


Figure 1

Figure

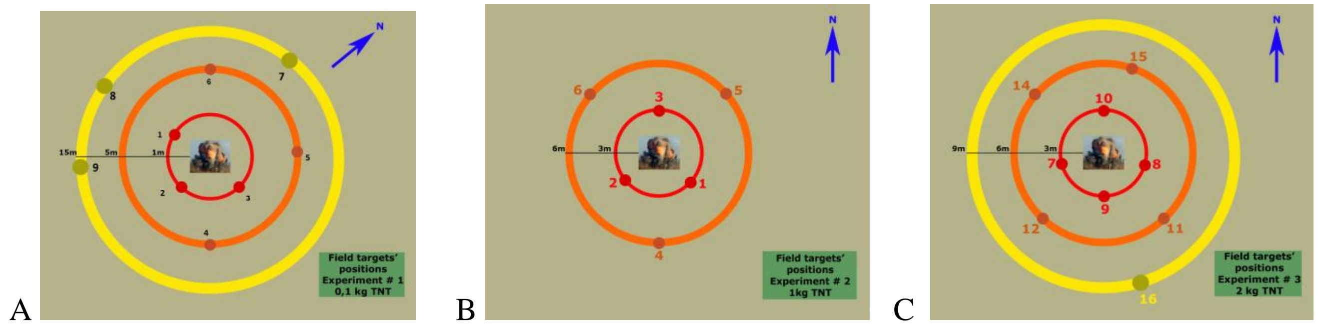
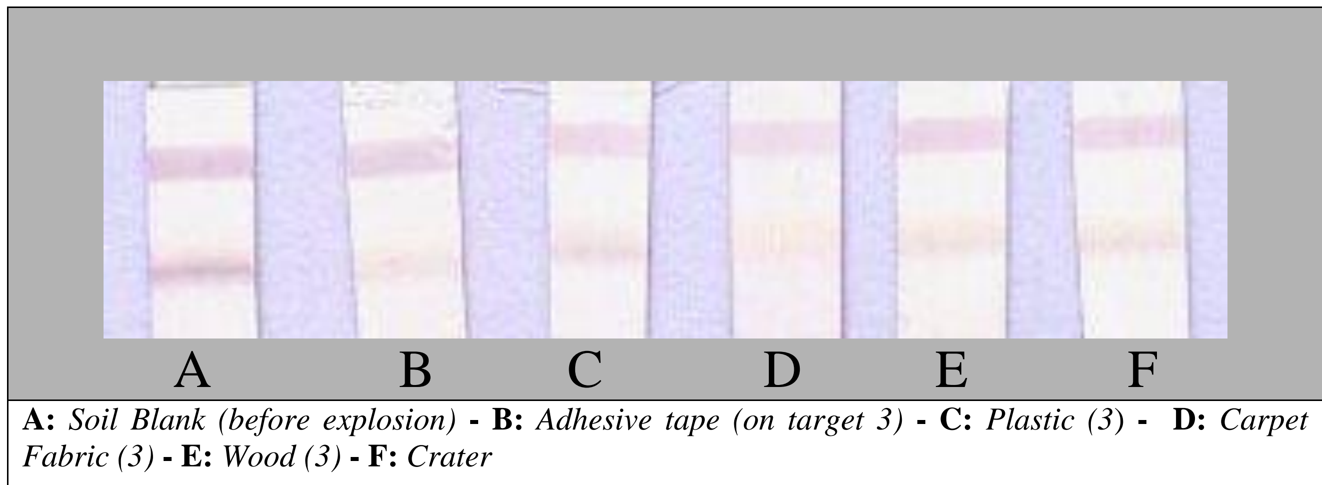


Figure 2



A: *Soil Blank (before explosion)* **- B:** *Adhesive tape (on target 3)* **- C:** *Plastic (3)* **- D:** *Carpet Fabric (3)* **- E:** *Wood (3)* **- F:** *Crater*

Figure 3

Figure captions

Figure 1: Aspect and distribution of the materials compositing the targets employed during the explosion experiments

Figure 2: Distribution and distances of the targets in the 0,1 kg (A), 1 kg (B), and 2 kg (C) TNT explosion experiments (images not scaled).

Figure 3: LFIA strips as they resulted after the analysis of positive samples from the 100 g TNT charge explosion test.

Table

Table 1. Data from the positive target samples after the explosion of 100 g charge of TNT. The remaining samples were negative.

SAMPLE	**CL ELISA TNT(ng mL^{-1})±CV(%)**	**GC/MS TNT(ng mL^{-1}) ± CV(%)**	**LFIA(*)**
Adhesive tape	141 ± 11	157 ± 14	++
Plexiglass	57 ± 8	39 ± 12	+
Carpet fabric	107 ± - 5	122 ± - 15	++
Wood	40 ± 18	48 ± 11	+
Cardboard	38 ± 9	46 ± 9	
Soil near the target	98 ± 8	123 ± 14	+
Soil (Blank)	Below LOD	Below LOD	

(*) + : positive sample; ++ clearly positive.

Table

Table 4. Handling test results in μg mL^{-1}. The CV values for these data were consistently below 10%.

Samples	**CL-ELISA**				**LFIA** (§)				**GC-MS**			
	HT1	HT2	HT3	HT4	HT1	HT2	HT3	HT4	HT1	HT2	HT3	HT4
A, 1st swab	643.0	0	2.0	18.0	++	-	+	+	580.0	0.9	0.9	22.0
A, 2nd swab	212.0	4.0	0	3.0	++	+	-	+	187.0	1.1	0	1.1
A, 3rd swab	47.0	0	0	1.0	+	-	-	+	11.0	0	0	0.8 1
B, 1st swab	52.0	3.0	3.0	1.0	+	+	+	+	67.0	0.9	0.9	0.9
B, 2nd swab	12.0	0	0	1.0	+	-	-	+	21.0	0.9	0	0.8
B, 3rd swab	0	0	1.0	0	-	-	-	-	0	0	0	0
C, 1st swab	128.0	2.0	2.0	n.s*	++	+	+	n.s*	147.0	0.9	0.9	n.s*
C, 2nd swab	30.0	1.0	0	n.s*	+	-	-	n.s*	23.0	0	0	n.s*
C, 3rd swab	5.0	0	0	n.s*	+	-	-	n.s*	2.7	0	0	n.s*

(§) + : positive sample; ++ clearly positive.

* n.s : not sampled.

Table 2

Table 2: Results from the positive samples (targets 1, 2 and 3 and e soil collected close to the pole of target1) analyzed after the explosion of a 1 kg charge of TNT. All the remaining samples resulted negative when analyzed by all methods.

SAMPLE	**CL-ELISA** **TNT (ng mL^{-1}) ± CV (%)**	**GC-MS** **TNT (ng mL^{-1}) ± CV (%)**	**LFIA (*)**
CARDBOARD 1	62 ± 8	55 ± 9	+
PLEXIGASS 1	27 ± 10	31 ± 11	+
CARPET FABRIC 1	46 ± 9	40 ± 9	+
WOOD 1	30 ± 10	25 ± 8	+
SOIL TARGET 1	23 ± 10	32 ± 6	+
CARDBOARD 2	33 ± 10	41 ± 12	+
PLEXIGASS 2	120 ± 8	136 ± 11	++
WOOD 3	32 ± 10	27 ± 8	+
CARPET FABRIC 3	24 ± 9	31 ± 6	+

(*) + : positive sample; ++ clearly positive.

Table 3

Table 3. Results from samples collected from targets 7 and 8 and the soil taken close to the pole of the targets 7 and 8 after the explosion of a 2 kg charge of TNT. All the remaining samples resulted negative.

SAMPLE	CL-ELISA TNT (ng mL^{-1}) ± CV (%)	CL-LFIA TNT(ng mL-1) ± CV (%)	GC-MS TNT (ng mL^{-1}) ± CV (%)
CARDBOARD 7	34 ± 7	39 ± 8	44 ± 8
PLEXIGASS 7	206 ± 11	199 ± 13	200 ± 14
METAL 7	200 ± 14	180 ± 18	221 ± 10
CARPET FABRIC 7	94 ± 8	111 ± 18	122 ± 8
WOOD 7	34 ± 10	28 ± 11	26 ± 8
SOIL (Target7)	10 ± 9	4 ± 7	18 ± 12
CARDBOARD 8	23 ± 10	19 ± 12	30 ± 14
SOIL (Target 8)	12 ± 11	19 ± 14	21 ± 10

October 13th, 2014

Dear Prof. Saukko (Editor-in-Chief),

Please find enclosed our revised manuscript entitled: **Field detection capability of immunochemical assays during criminal investigations involving the use of TNT.**

Thank you for the positive comments.

We have modified the manuscript following the comment: "The expectation is that samples would be subsequently analysed in a lab. Therefore, sample availability after immunoassay needs to be considered or at least mentioned".

A complete review of the manuscript was carried out by a specialist whose first language is English..

Sincerely,