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Optimization of two methods for the analysis of hydrogen peroxide: High performance liquid chromatography with fluorescence detection and high performance liquid chromatography with electrochemical detection in direct current mode☆,☆☆

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ABSTRACT

Two complementary methods were optimized for the separation and detection of trace levels of hydrogen peroxide. The first method utilized reversed-phase high-performance liquid chromatography with fluorescence detection (HPLC–FD). With this approach, hydrogen peroxide was detected based upon its participation in the hemin-catalyzed oxidation of *p*-hydroxyphenylacetic acid to yield the fluorescent dimer. The second method utilized high performance liquid chromatography with electrochemical detection (HPLC–ED). With this approach, hydrogen peroxide was detected based upon its oxidation at a gold working electrode at an applied potential of 400 mV vs. hydrogen reference electrode (Pd/H_2). Both methods were linear across the range of 15–300 μM, and the electrochemical method was linear across a wider range of 7.4–15,000 μM. The limit of detection for hydrogen peroxide was 6 μM by HPLC/FD, and 0.6 μM by HPLC/ED. A series of organic peroxides and inorganic ions were evaluated for their potential to interfere with the detection of hydrogen peroxide. Studies investigating the recovery of hydrogen peroxide with three different extraction protocols were also performed. Post-blast debris from the detonation of a mixture of concentrated hydrogen peroxide with nitromethane was analyzed on both systems. Hydrogen peroxide residues were successfully detected on this post-blast debris.
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1. Introduction

In recent years there has been increasing concern over the use of hydrogen peroxide in improvised explosive devices. When properly mixed with appropriate fuels, this material can become a powerful explosive. Because hydrogen peroxide is widely used as a strong, environmentally friendly oxidant compared to bleach, it is easy to obtain in a variety of forms. Most municipal and industrial applications utilize 35–50% concentrated solutions of hydrogen peroxide. Household applications involving 3% solutions of hydrogen peroxide include cleaning wounds and bleaching clothing, while the bleaching of hair typically utilizes 15% solutions of hydrogen peroxide. Industrial applications of hydrogen peroxide include pulp and paper bleaching, organic and inorganic chemical processing, the treatment of metal, catalysis of polymerization reactions, and industrial waste treatment [1].

Though stable if uncontaminated, hydrogen peroxide solutions will decompose slowly into oxygen gas and water with the evolution of heat. There is considerable evidence that this process occurs as a chain reaction involving free radicals [2]. Decomposition of hydrogen peroxide into perhydroxy and hydroxyl radicals is accelerated in the presence of near UV light or an iron catalyst. In dilute solutions, the water present can absorb the heat which is evolved through the decomposition of hydrogen peroxide. In more concen-

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trated solutions, the heat evolved from decomposition raises the temperature of the solution, which in turn increases the rate of decomposition. High alkalinity can also increase the decomposition rate of hydrogen peroxide. The catalytic decomposition of hydrogen peroxide at a concentration of 70% or greater proceeds rapidly, and with sufficient heat released that the products are oxygen and steam. The thrust from this reaction can be used to propel torpedoes and small missiles [2].

Hydrogen peroxide based explosives may be prepared as a blend of separate fuel and oxidizer compounds, such as mixtures of concentrated hydrogen peroxide with carbon-based fuels. Alternatively, hydrogen peroxide may be used as a starting component in the production of explosives which contain the fuel and oxidizer within the same molecule. Triacetone triperoxide (TATP) and hexamethylene triperoxide diamine (HMTD) are examples of the latter type.

Research has been published on the development of HPLC-based methods for the analysis of trace levels of hydrogen peroxide and selected organic peroxides which are present in the atmosphere [3–6]. The combination of an acidic mobile phase with a C-18 analytical column permits the elution of hydrogen peroxide within 6 min [3–6]. Trace levels of hydrogen peroxide can be detected using the reaction of hydrogen peroxide with *p*-hydroxyphenylacetic acid in the presence of an iron containing catalyst [3–7]. The product of this reaction is the dimer 6,6′-dihydroxy-3,3′-biphenyldiacetic acid, the anionic form of which is readily detectable using a standard HPLC fluorescence detector. In addition to the analysis of atmospheric peroxides, this technique has also been applied to the analysis of organic peroxide explosives such as TATP and HMTD [7–8]. In this procedure, the peroxide explosives are photolytically degraded post-column to form hydrogen peroxide, which then reacts with *p*-hydroxyphenylacetic acid in the presence of the iron containing catalyst.

While work has been performed on the use of electrochemical detection to analyze hydrogen peroxide in biological samples [9], many biological-based assays require the use of specially modified electrodes [10], which are not commercially available and therefore may be impractical for use in forensic laboratories. There has been a deficiency of peer-reviewed work published on the use of this technology for the analysis of hydrogen peroxide in improvised explosives. However, Schulte-Ladbeck et al. published a study in 2003 detailing a method for the detection of TATP and HMTD by reversed phase HPLC with post-column UV irradiation and electrochemical detection [11]. This method employed a C-18 analytical column in conjunction with a mobile phase which consisted of 65% acetonitrile and 35% aqueous 4 mM sodium phosphate buffer at pH 8.

The goal of this research was to develop and optimize two distinct methods, based on different principles of separation and detection, which could separate and detect trace amounts of hydrogen peroxide. This research could provide a basis for the analysis of hydrogen peroxide residues in post-blast situations. Experiments were also conducted regarding the effects of sampling materials on the collection of hydrogen peroxide. As part of the evaluation, post-blast samples from the detonation of a mixture of concentrated hydrogen peroxide with an organic fuel were collected and analyzed using both methods.

2. Experimental

2.1. Chemicals

HPLC grade water was used as received from Burdick & Jackson (Honeywell Burdick & Jackson, Morristown, NJ, USA). Deionized water (18.3 MΩ) was obtained using a Nanopure Infinity™ water purification system (Barnstead/Thermolyne, Pittsburgh, PA, USA). Reagent grade hemin powder (Sigma–Aldrich, St. Louis, MO, USA), 4-hydroxyphenylacetic acid (Acros Organics, Geel, Belgium), sodium acetate (Sigma–Aldrich), 10 N sodium hydroxide, sulfuric acid, ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific, Pittsburgh, PA, USA), ammonium hydroxide, ammonium chloride (Sigma–Aldrich), methanol (Honeywell Burdick & Jackson), acetonitrile (Acros Organics), 50% hydrogen peroxide (Sigma–Aldrich) and 30% hydrogen peroxide in water (Riedel-de-Haen, Seelze, Germany) were purchased from chemical supply houses. Reagent grade benzoyl peroxide (Fluka, Pittsburgh, PA, USA), 70% *tert*-butyl hydroperoxide in water, 98% dicumyl peroxide, 97% urea hydrogen peroxide (Sigma–Aldrich), 2-butanone peroxide (Fluka), and 80% cumene hydroperoxide (Sigma–Aldrich) were purchased from chemical supply houses.

2.2. Instrumentation

2.2.1. High performance liquid chromatography with electrochemical detection

The HPLC/ED system consisted of a Waters Alliance 2695 Separations Module connected to a Waters 2465 Electrochemical Detector. The Alliance system included the following components: pump, autosampler, injector, solvent mixing system, and membrane degasser. Separation was carried out using CarboPac™ PA10 guard and analytical (4.6 mm × 75 mm) columns (Dionex Corporation) and a mobile phase consisting of 150 mM sodium acetate (NaOAc) at pH 10.5 and a flow rate of 1.00 mL/min. The injection volume varied between 20 μL and 100 μL. The guard and analytical columns were housed in the oven of the electrochemical detector. The columns and flow cell were maintained at 45 °C. A 3-mm gold working electrode was used in conjunction with a palladium-hydrogen reference electrode and a palladium auxiliary electrode. A 50 μm spacer was used in the flow cell. Detection occurred in DC mode at an applied potential of +400 mV.

2.2.2. High performance liquid chromatography with fluorescence detection

The HPLC/FD system consisted of a Waters Alliance 2695 Separations Module, two Waters 515 HPLC Pumps, a post-column reaction cell, and a Waters 474 Fluorescence Detector. The Alliance system included the following components: pump, autosampler, injector, solvent mixing system, and membrane degasser. Separation was carried out using an Acclaim 120 C-18 (4.6 mm × 250 mm) column (Dionex Corporation). The mobile phase consisted of 1.00 mM H_2SO_4 and 0.10 mM EDTA, which was delivered by the Waters Alliance 2695 Separations Module at a flow rate of 0.60 mL/min. Injection size varied between 10 μL and 100 μL. The reagent solution contained 80 μM *p*-hydroxyphenylacetic acid and 8 μM hemin dissolved in an ammonia buffer; this solution was delivered by the Waters 515 pump at a flow rate of 0.20 mL/min. The post-column reactor consisted of a length of Teflon® tubing 0.5 mm ID × 2.0 m long (Microsolv Technology, Eatontown, NJ, USA). A solution of 0.10 M NaOH was delivered by the second Waters 515 HPLC pump at 0.20 mL/min. The Waters 474 Fluorescence Detector with grating technology included a standard flow cell of 100 μL; λ_{ex} was 320 nm and λ_{em} was 405 nm.

2.3. Preparation of mobile phase for HPLC/ED

The composition of the mobile phase was adapted from a Dionex application note [12]. This mobile phase was prepared by dissolving 12.305 g NaOAc in 1.0 L of HPLC-grade water. The resulting solution contained 150 mM NaOAc. The pH of this solution was initially 7.8, before the pH was adjusted to 10.5 through drop-wise addition of 0.50 M NaOH. The mobile phase was vacuum filtered through a

0.45 μm nylon filter prior to use. The mobile phase was stored in glass bottles at room temperature.

2.4. Preparation of mobile phase and reagents for HPLC/FD

The composition of the mobile phase was adapted from Kok et al. [5]. The mobile phase was prepared by diluting 55.0 μL of 96.1% H_2SO_4 and 0.047 g EDTA in 1.0 L of HPLC grade water. The resulting solution contained 1.00 mM H_2SO_4 and 0.10 mM EDTA. The mobile phase was filtered through a 0.45 μm nylon filter prior to use. The base solution was generated by diluting 5.00 mL of 10.0 N NaOH in 495 mL of DI water to yield 0.50 L of 100 mM NaOH. Both the mobile phase and base solution were stored in glass bottles at room temperature.

The composition of the post-column reagent solutions was adapted from Qi et al. [6]. Stock solutions of hemin were prepared by dissolving 13.0 mg hemin in 10.0 mL of 100 mM NaOH. This solution was stored in a glass screw-top vial at 4 °C. Stock solutions of hemin were made approximately once per month. Stock solutions of *p*-hydroxyphenylacetic acid were prepared by dissolving 30.4 mg *p*-hydroxyphenylacetic acid in 10.0 mL DI water. This second solution was stored in a glass screw top vial at room temperature. Stock solutions of *p*-hydroxyphenylacetic acid were made approximately once per month. The ammonia buffer was prepared by dissolving 2.68 g NH_4Cl and 25.0 mL 29% NH_4OH in 475 mL of DI water to yield 0.50 L of solution. This third solution was stored at room temperature in a glass bottle. Base solution was made as needed, typically about once per month. The reagent solution was prepared by diluting 0.20 mL stock solution of hemin and 0.15 mL stock solution of *p*-hydroxyphenylacetic acid to 50.0 mL with the ammonia buffer. The pH of this solution was 9.5. The reagent solution was filtered through a 0.2 μm cellulose acetate filter prior to use. This solution was made fresh daily.

2.5. Preparation of hydrogen peroxide standards

Standard solutions of hydrogen peroxide were generated weekly by diluting 50% reagent grade hydrogen peroxide in DI water to yield a 0.15 M hydrogen peroxide standard. The 0.15 M hydrogen peroxide standard was diluted with DI water to yield a 1.50×10^{-3} M hydrogen peroxide standard. These standards were stored in the refrigerator at 4 °C. The 1.50×10^{-3} M hydrogen peroxide standard was diluted with DI water to yield the daily working standard of 15.0×10^{-6} M hydrogen peroxide.

2.6. Preparation of cotton sampling media

The cotton sampling media employed in this study were standard round white cotton balls of average mass 0.325 g, manufactured by Johnson & Johnson (New Brunswick, NJ, USA). Each cotton swab was subjected to the same cleaning procedure prior to use. Five swabs at a time were placed inside a disposable 25.0 mL plastic luer-lock syringe (Sigma–Aldrich). The syringes were used to force 50.0 mL DI water through the swabs, followed by 50.0 mL isopropanol, and then a second aliquot of 50.0 mL DI water. The swabs were allowed to air dry at bench top conditions before they were packaged inside of a double layer of heat-sealed nylon bags. Analysis of water extracts from the cleaned swabs by HPLC/FD revealed no background signal, while analysis of water extracts from the cleaned swabs by HPLC/ED in DC mode yielded a small background peak at 1.50 min which did not interfere with the detection of hydrogen peroxide. The typical height of this peak was 5 nA.

2.7. Application and recovery of hydrogen peroxide

For the study regarding the effects of sampling on peroxide recovery, aliquots of 4.1 mg H_2O_2 were applied to the surface of paint chips and were dried with a heat gun. The use of the heat gun was necessary to "fix" the peroxide sample onto the surface of the substrate. Simple evaporation rarely left behind sufficient residue to permit detection. Hydrogen peroxide residues were recovered either by performing a direct water rinse with 1.0 mL DI water, swabbing with a cotton ball moistened with 0.5 mL DI water followed by a water extraction in 2.5 mL DI water, or dry swabbing followed by a water extraction of the cotton in 3.0 mL of DI water. Ten replicates were performed of each. The extracts were analyzed by HPLC/FD and HPLC/ED in DC mode. For this experiment, only cotton which had been cleaned according to the procedure outlined previously was used.

2.8. Collection and analysis of post-blast samples

Experiments were conducted to test the optimized HPLC/ED and HPLC/FD methods, and to determine whether sufficient hydrogen peroxide survived detonation to be detected on post-blast debris. In an effort to provide guidance on peroxide recovery, the effectiveness of different types of substrates at retaining hydrogen peroxide residues was evaluated. *Mixtures of concentrated hydrogen peroxide with fuel are extremely dangerous and prone to spontaneous detonation, and should only be handled by trained professionals wearing the proper safety gear*. Personnel from the FBI Laboratory Explosives Unit prepared and detonated a small amount of a mixture of 70% concentrated hydrogen peroxide and nitromethane under controlled conditions.

A bottle of the hydrogen peroxide/nitromethane mixture was contained inside a metal ammunition can which had been spray-painted bright pink to aid in the recovery of the fragments post-blast. Three steel witness plates (6 in. × 6 in.) were set in a triangular pattern around the ammunition can at distances of 2.5 ft, 5 ft, and 10 ft. The face of each plate was oriented toward the ammunition can and rested 16 in. from the ground to the top of the plate. The ammunition can was covered by a white plastic mesh cover to prevent overheating of the mixture prior to detonation. The explosive mixture was initiated with an electric blasting cap. Following detonation of the explosive, the witness plates were collected and packaged on-site inside of a double layer of heat-sealed nylon bags. Fragments from the ammunition can and the white cover were collected and packaged inside of a double layer of heat-sealed nylon bags. Soil samples from the blast crater were collected and stored inside of glass screw-top vials. The samples were collected within 1 h of detonation and placed into storage the same day. All of these samples were stored in darkness at −4 °C until they were analyzed. The witness plates were analyzed the day after collection, one of the ammunition can fragments was analyzed 1 month after collection, and the rest of the post-blast debris was not analyzed until approximately 5 months after collection.

Hydrogen peroxide residues from the witness plates were extracted either by washing the front and back faces separately with 2.0 mL 18.3 MΩ DI water each, or by washing both faces with the same aliquot of 3.0 mL 18.3 MΩ DI water to avoid sample dilution. Residues from the ammunition can fragments were extracted either by directly rinsing the fragment with 2.0 mL 18.3 MΩ DI water, or by first using a dry cotton swab to collect the residues, and then extracting the cotton in 5.0 mL 18.3 MΩ DI water. Residues were extracted from swatches cut from the plastic covers by rinsing the swatches in 2.0 mL 18.3 MΩ DI water. Soil samples were extracted by sonicating 2.0 g of pulverized soil in 2.0 mL of 18.3 MΩ DI water for 20 min, followed by filtration using 0.2 μm

nylon syringe filters with 3 mL luer-lock plastic syringes (Sigma–Aldrich)

3. Results and discussion

The goal of this study was to test and optimize two methods developed for the analysis of trace levels of hydrogen peroxide. The HPLC/ED and HPLC/FD methods optimized for the analysis of hydrogen peroxide are based upon different principles of separation and detection. Either of the two methods could provide a greater degree of certainty as a confirmation method after an initial screening test. This is particularly important in the event that these techniques are applied to forensic samples.

3.1. Optimization of HPLC/ED method

In acidic solution, the standard reduction potential for hydrogen peroxide is E_0 = +1.80 V (see reaction (1)) [2], while the standard reduction potential for water is E_0 = −0.83 V (see reaction (2)) (all values are given relative to a standard hydrogen reference electrode).

$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O \quad (1)$$

$$2H_2O + 2e^- \rightarrow H_2(g) + 2OH^- \quad (2)$$

Due in part to problems with the reduction of dissolved oxygen in the mobile phase at high working potentials, detection of hydrogen peroxide is much more effective when performed in oxidative mode [11].

Additional reactions for hydrogen peroxide are listed below [2]:

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e^- \quad (3)$$

$$H_2O_2 + OH^- \rightarrow HOO^- + H_2O \quad (4a)$$

$$OH^- + OOH^- \rightarrow O_2 + H_2O + 2e^- \quad (4b)$$

$$H_2O_2 + 2OH^- \rightarrow O_2 + 2H_2O + 2e^- \quad (5)$$

At pH 0, when hydrogen peroxide is in the form H_2O_2, the oxidation of hydrogen peroxide will occur at E_0 = −0.66 V (see reaction (3)). Because hydrogen peroxide is a weak acid with a pK_a of 11.65, in strongly basic solution, hydrogen peroxide will form the perhydroxyl ion HOO^- (see reaction (4a)). At pH 14, when hydrogen peroxide is in the form HOO^-, the oxidation of hydrogen peroxide will occur at E_0 = +0.08 V (see reaction (4b)) [2]. At this pH, oxidation of hydrogen peroxide to oxygen is accomplished with the concomitant reduction of hydroxide ions to water. This reaction can be combined with reaction (4a) to yield reaction (5), which represents the nature of the oxidation reaction of hydrogen peroxide at the surface of the working electrode.

It should be noted that under certain conditions, the two-electron transfer that occurs during the oxidation of hydrogen peroxide can be mediated by adsorption onto a catalytic site. As a result of this process, oxidation of hydrogen peroxide can occur at lower potentials. For example, Johnston et al. generated electrocatalytic gold/palladium composite surfaces which resulted in the oxidation of hydrogen peroxide at +0.4 V vs. SCE (a difference of 300 mV in comparison to a platinum working electrode) [12]. Although it is possible that electrocatalysis may occur at the gold working electrode in the present experiment, it seems unlikely. The authors of the Johnston paper attributed the electrocatalytic activity of the composite electrode to the presence of the palladium oxides, not the gold oxides. In our experiment the response of the gold working electrode was stable over time and replacing it with a platinum working electrode did not change the electrochemical response.

3.1.1. Optimization of HPLC separation

Due to specific concerns regarding mixtures of concentrated hydrogen peroxide with sugars, the starting point for development of the HPLC/ED method was the standard method for carbohydrate analysis by the International Commission for Uniform Methods of Sugar Analysis (ICUMSA), as described by Dionex Technical Note #20 [13] and Application Update #152 [14]. Mobile phase compositions of 5 mM, 50 mM, 150 mM, and 300 mM sodium acetate (NaOAC) were tested with a CarboPac™ PA 10 column. Hydrogen peroxide eluted at approximately 1.90 min for all four mobile phase compositions. The average peak area for a 15 μM hydrogen peroxide sample was 5.40×10^3 at 5 mM NaOAc, 1.00×10^4 at 50 mM NaOAc, 1.03×10^4 at 150 mM NaOAc, and 9.10×10^3 at 300 mM NaOAc. Peak response was notably lower for the 5 mM NaOAc mobile phase compared to the other three compositions, presumably because the 5 mM NaOAc mobile phase was too dilute to conduct current through the flow cell [15]. The combination of the 150 mM NaOAc mobile phase at pH 10.5 with the PA10 column resulted in the greatest peak area, lowest relative standard deviation of the peak area, and best peak symmetry for the hydrogen peroxide standard, with baseline resolution of the peroxide peak from the unretained peak. Therefore, this mobile phase was selected to undergo further testing and optimization.

3.1.2. Optimization of electrochemical detection

Schulte-Ladbeck et al. previously found success in detecting TATP by first degrading the compound to hydrogen peroxide, then detecting the hydrogen peroxide at a glassy carbon working electrode using an applied potential of 900 mV versus a palladium-hydrogen reference electrode [11]. To determine the optimal detection potential for hydrogen peroxide in a 150 mM NaOAc mobile phase with a gold working electrode, a hydrodynamic voltammogram was constructed across the range of E = 0 mV to E = +800 mV. This graph is presented in Fig. 1. Although this experiment was repeated twice, the graph in Fig. 1 is a single representative plot. The optimal detection potential is the point where the graph just begins to plateau, in this case, at E = +400 mV. At potentials lower than +400 mV, the signal strength of hydrogen peroxide is weaker, the peak height is less stable, and the limit of detection will be poorer. At potentials higher than +400 mV, there is a greater chance that additional, interfering species will be oxidized that could potentially mask the signal from hydrogen peroxide. A negative response was noted when the potential was 0 mV, indicating the onset of a reductive process. A chromatogram of a hydrogen peroxide standard analyzed using this optimized method is presented in Fig. 2.

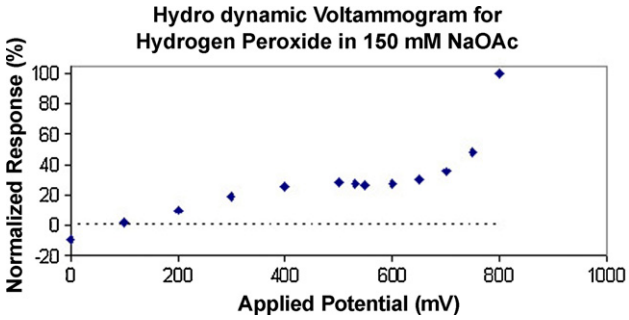

Fig. 1. Hydrodynamic voltammogram of hydrogen peroxide in 150 mM NaOAc mobile phase. The optimal detection potential for hydrogen peroxide is E = +400 mV vs. Pd/H_2. The response at 0 mV vs. Pd/H_2 was a negative peak.

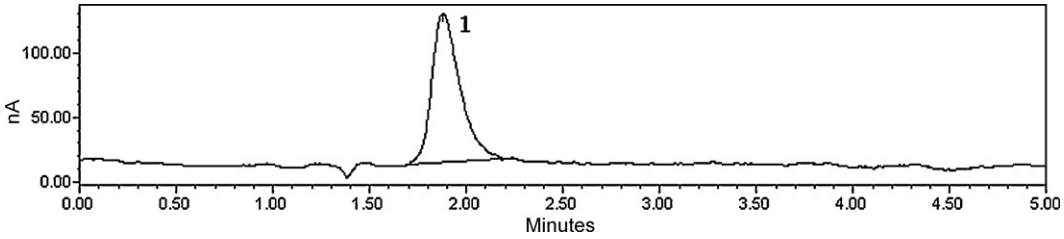

Fig. 2. Analysis of a 15 μM hydrogen peroxide standard by HPLC/ED in DC mode. Conditions: Dionex CarboPac PA10 guard and analytical columns; isocratic elution with 150 mM NaOAc at pH 10.5; flow rate 1.0 mL/min; 20 μL injection, $T = 45\,^{\circ}C$, DC mode with $E = +400$ mV vs. Pd/H_2. Peak 1, hydrogen peroxide, R_t 1.90 min. Under these conditions, the hydrogen peroxide peak displays minimal tailing, and baseline resolution from the unretained peak (1.40 min) is achieved.

3.2. Optimization of HPLC/FD method

3.2.1. Optimization of HPLC separation

Previous research demonstrated that the combination of an acidic mobile phase with a C-18 analytical column was sufficient to resolve hydrogen peroxide from a series of organic peroxides [3,5,6]. The composition of the mobile phase was adapted from Kok et al. and consisted of 1.0 mM H_2SO_4 with 0.1 mM EDTA [5]. The combination of this mobile phase with a C-18 column resulted in the elution of hydrogen peroxide within 7 min. Two additional mobile phase compositions were tested to determine if the addition of more acid to the mobile phase would affect retention time or peak area: 2.0 mM H_2SO_4 with 0.1 mM EDTA and 1.5 mM H_2SO_4 with 0.1 mM EDTA. Use of the second two mobile phase compositions did not affect the retention time or level of detector response for hydrogen peroxide. Therefore, they were not explored further.

The effect of changing the flow rate of the 1.0 mM H_2SO_4/0.1 mM EDTA mobile phase on the detection of the fluorescent dimer formed during the post-column reaction was measured. It was determined 0.60 mL/min was the optimal flow rate for this configuration, as it allowed for both maximal detection of the p-hydroxyphenylacetic acid (POPHA) dimer and a relatively quick separation of hydrogen peroxide. The effect of organic solvent on the separation and detection of hydrogen peroxide was also investigated. The percentage of acetonitrile in the mobile phase was varied between 0% and 15%. It was clear that increasing the percentage of acetonitrile in the mobile phase resulted in suppression of the hydrogen peroxide signal. It is theorized that acetonitrile was interacting negatively with either the hemin catalyst or the *p*-hydroxyphenylacetic acid, leading to a reduction in formation of the dimer. Therefore, no organic solvent was added to the mobile phase in order to keep the signal strength of hydrogen peroxide as high as possible to achieve a low limit of detection.

3.2.2. Optimization of post-column derivatization reaction

The composition of the post-column derivatization reagent was adapted from the work of Qi et al. [6]. By independently varying the concentrations of *p*-hydroxyphenylacetic acid and hemin in the reagent solution, Qi et al. determined that the optimal conditions to maximize fluorescence intensity were 8 μM hemin and 80 μM *p*-hydroxyphenylacetic acid in ammonia buffer at pH 10 [6]. This reagent solution was utilized in the HPLC/FD method for hydrogen peroxide analysis. A post-column reactor was designed for these experiments using Teflon® tubing 2.0 meters in length by 0.50 mm internal diameter. Optimal detection of hydrogen peroxide was achieved when the residence time of the reagents (8 μM hemin, 80 μM *p*-hydroxyphenylacetic acid, and hydrogen peroxide) in the tubing was at least 20 s to allow for maximum formation of the POPHA dimer. This occurred when the reagent solution was delivered at a flow rate of 0.20 mL/min. A solution of 100 mM NaOH was utilized to raise the pH of the post-column derivatization reaction via a mixing tee to pH 10.0 prior to entering the fluorescence detector. The optimal flow rate of the sodium hydroxide was 0.20 mL/min. The base was added 20 s after the POPHA in order to optimize the reaction time.

Experiments were conducted to optimize the temperature of the post-column reactor in order to maximize the signal strength of the fluorescent dimer. The greatest peak area for the fluorescent dimer was obtained at 25 °C with a 20 s residence time and flow rate of 0.20 mL/min. This was the lowest temperature setting for the reactor housing. As the temperature increased, the signal response decreased. Higher temperatures can cause decomposition of the hemin catalyst, which can lead to a reduction in sensitivity for hydrogen peroxide.

3.2.3. Optimization of fluorescence detection

A series of excitation and emission wavelengths were investigated to determine the combination of wavelengths which would maximize detection of the POPHA dimer. Samples of hydrogen peroxide at 15 μM were analyzed in duplicate by varying first the emission wavelength and second the excitation wavelength. It was determined that detection of the POPHA dimer was maximized when an excitation wavelength of 320 nm was paired with an emission wavelength of 405 nm. This set of wavelengths is similar to what has been employed in previous studies [5,7,16]. The analysis of a 15 μM hydrogen peroxide standard using the optimized HPLC/FD method is presented in Fig. 3. The small peak at approximately 0.65 min is a system peak which was present in every sample, including water and mobile phase blank samples. It should be noted that the individual derivatization reagents did not yield a fluorescence signal.

3.3. Validation

Validation included providing a demonstration of linearity, documentation of the limit of detection, demonstration of specificity and selectivity, demonstration of accuracy and precision, and demonstration of method robustness. A calibration curve was generated on the HPLC/FD system for hydrogen peroxide across the range of 15–300 μM. Peroxide concentrations greater than 300 μM resulted in detector saturation. A calibration curve was generated on the HPLC/ED system (in DC mode) for hydrogen peroxide across the range of 7.4–15,000 μM. At sample concentrations greater than 15,000 μM, the calibration became non-linear and carryover was a problem. The limit of detection (LOD) was calculated by determining the hydrogen peroxide concentration equal to three times the standard deviation of ten replicates of a low level peroxide sample. For the HPLC/ED system, a 0.7 μM hydrogen peroxide sample was used; for the HPLC/FD system, a 6.0 μM hydrogen peroxide sample was used (with an injection size of 20 μL). The LOD for hydrogen peroxide on the HPLC/ED system in DC mode was 0.6 μM, while the LOD for hydrogen peroxide on the HPLC/FD system was 6 μM.

Studies were conducted to determine the within-day repeatability of the retention time and peak area of standard samples of hydrogen peroxide on both the HPLC/ED and HPLC/FD systems.

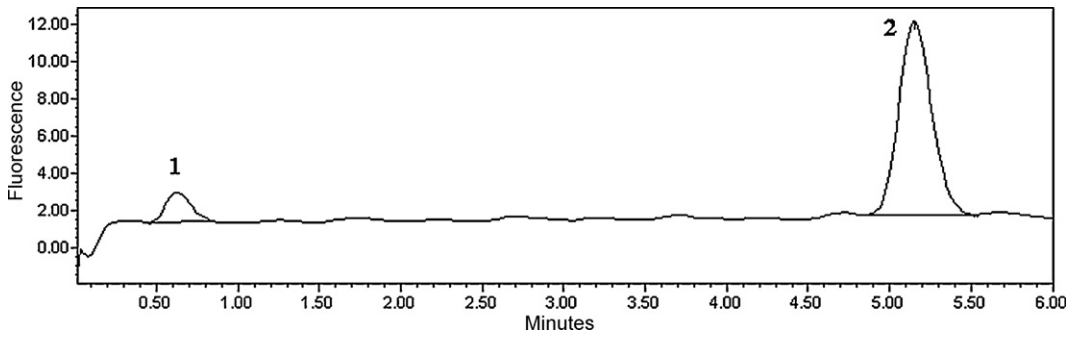

Fig. 3. Analysis of a 15 μM hydrogen peroxide standard by HPLC/FD. Conditions: Dionex C-18 analytical column, isocratic elution with 1 mM H_2SO_4/0.1 mM EDTA at flow rate 0.6 mL/min, 20 μL injection, reagent 8 μM hemin/80 μM POPHA in ammonia buffer pH 9.5 at flow rate 0.2 mL/min, post-column reactor 2 m, T = 25 °C, base 100 mM NaOH at flow rate 0.20 mL/min, λ_{ex} = 320 nm λ_{em} = 405 nm. Peak 1, unretained peak, R_t 0.65 min; peak 2, hydrogen peroxide R_t 5.15 min.

This test involved the analysis of ten sequential injections of both a 15 μM hydrogen peroxide standard, and a 150 μM hydrogen peroxide standard. This data is presented in Table 1. To explore the between-day repeatability of each HPLC method, peak area and retention time data for standard solutions of hydrogen peroxide at 15 μM and 150 μM were compared over 10 days of data analysis. This data is presented in Table 1. The standard deviation for the retention time for both standards on both methods was less than or equal to 0.25%, which is typical for HPLC. For both methods, the between-day variability of the peak area of hydrogen peroxide was greater than the within-day variability.

A brief series of experiments were conducted to evaluate the effects of several different types of compounds that might reasonably be expected to interfere with the detection of hydrogen peroxide in explosives analysis. In this study, three groups of compounds were tested: the inorganic anions nitrate, nitrite, chloride, chlorate, and perchlorate; the organic acids ascorbic acid, citric acid, formic acid, lactic acid, and oxalic acid; and the sugars glucose, fructose, and sucrose. Each analyte was tested individually at a concentration of 100 μM on both the HPLC/FD system, and the HPLC/ED system in DC mode. Of the potential interferences which were tested, none yielded a detectable signal on the fluorescence system, and only ascorbic acid gave a signal on the electrochemical system. This signal took the form of a broad peak which eluted at approximately 2.12 min. This peak was absent when ascorbic acid was injected at a concentration of 60 μM. Samples which consisted of 100 μM hydrogen peroxide with 100 μM of each of the potential interferences were also analyzed on both systems. The presence of the additional analytes with the hydrogen peroxide standard resulted in minimal suppression of or addition to the hydrogen peroxide signal. Only the combination of ascorbic acid with hydrogen peroxide resulted in an electrochemical signal which was greater than one standard deviation from the response of the peroxide standard, as measured by the intra-day repeatability studies. None of the samples resulted in an electrochemical signal which was greater than one standard deviation from the response of the peroxide standard.

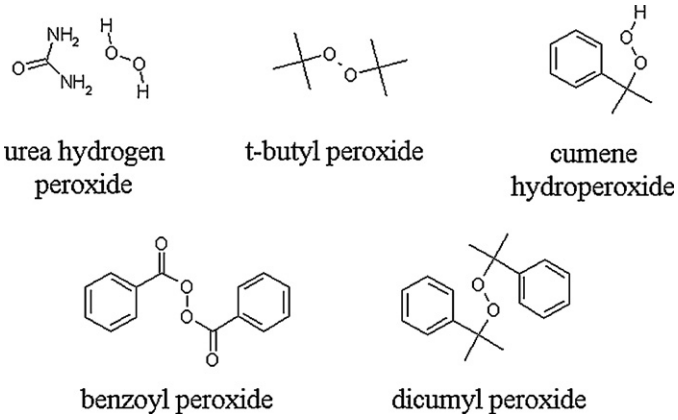

Fig. 4. Structures of organic peroxides. Each of these compounds is commercially available. It was unknown whether they would interfere with the separation and detection of hydrogen peroxide by HPLC/FD or HPLC/ED in DC mode..

Because the fluorescence and electrochemical systems were optimized for the separation and detection of hydrogen peroxide, it was unknown whether organic peroxides might interfere with the detection of hydrogen peroxide. In this study, a set of five commercially available peroxides were analyzed on both HPLC systems. This group included urea hydrogen peroxide, dicumyl peroxide, di-tert-butyl peroxide, benzoyl peroxide, and cumene hydroperoxide. Urea hydrogen peroxide is also called carbamide peroxide, and it is used as a tooth whitening agent. Dicumyl peroxide and di-tert-butyl peroxide are utilized as cross-linking agents in polymer chemistry. Benzoyl peroxide is the active ingredient in many acne medications. Cumene hydroperoxide is used in the synthesis of phenols. The structures of these compounds are presented in Fig. 4. This group of commercially available organic peroxides was included in the study to test the specificity of the method.

Each of the organic peroxides was initially tested without the analytical columns in place to determine whether or not they

Table 1
Repeatability data for the retention time and peak area of 15 μM hydrogen peroxide and 150 μM hydrogen peroxide. For both methods, the between-day variability of the peak area and retention time of hydrogen peroxide was greater than the within-day variability. See text for discussion.

Method		Sample	Ave R_t (min)	RSD R_t	Ave Peak Area	RSD Area
HPLC–FD	Within-day	15 μM	5.15 min	0.11%	2.19×10^5	6.1%
		150 μM	5.17 min	0.01%	1.22×10^6	1.0%
	Between day	15 μM	5.16 min	0.15%	2.07×10^5	18%
		150 μM	5.17 min	0.01%	1.21×10^6	4.6%
HPLC–ED	Within-day	15 μM	1.91 min	0.18%	5.00×10^3	12%
		150 μM	1.88 min	0.01%	3.95×10^4	1.3%
	Between day	15 μM	1.89 min	0.25%	6.00×10^3	17%
		150 μM	1.88 min	0.03%	3.95×10^4	1.8%

would yield a signal under the standardized detection conditions. At a concentration of 100 μM in mobile phase, the organic peroxides each yielded a detectable signal on the fluorescence system, with the exception of dicumyl peroxide. Even at concentrations up to 1 mM, this compound remained undetectable. At a concentration of 100 μM in mobile phase, the organic peroxides each yielded a detectable signal on the electrochemical system, with the exception of di-tert-butyl peroxide. Increasing the concentration of di-tert-butyl peroxide failed to yield a detectable signal. The signal strengths of dicumyl peroxide, benzoyl peroxide, and cumene hydroperoxide were almost two orders of magnitude lower than the signal yielded by samples of hydrogen peroxide and urea hydrogen peroxide at the same concentration.

The organic peroxides were re-tested on both systems with the analytical columns in place. Each of the peroxides was initially prepared in ethanol; they were then diluted to 100 μM in the appropriate mobile phase. For both the fluorescence and electrochemical methods, only urea hydrogen peroxide gave a detectable peak. This peak was most likely the result of the breakdown of urea peroxide into hydrogen peroxide, as the retention time observed was similar to hydrogen peroxide. In contrast, dicumyl peroxide, benzoyl peroxide, di-tert-butyl peroxide, and cumene hydroperoxide did not elute from either column within 60 min of injection. It is likely that these compounds were excessively retained on the column due to a lack of an organic solvent in the mobile phase. As discussed earlier, addition of organic solvent to the mobile phase resulted in suppression of the hydrogen peroxide signal. The organic peroxides were flushed from the columns with a 50% acetonitrile mobile phase before analysis of additional hydrogen peroxide samples continued.

3.4. Effect of extraction procedure on recovery of hydrogen peroxide

Experiments were performed to determine the effect of the sampling technique on the recovery of hydrogen peroxide from the surface of a given substrate. Three different procedures were examined: dry swabbing, wet swabbing, and a water rinse. As mentioned earlier, simply drying a standard on a substrate produced negative results. It is proposed that the heat of an explosives blast fixes some of the peroxide to the surface. This effect is simulated in the laboratory by using a heat gun to flash-dry the peroxide aliquots onto the surface of the paint chips. Approximately 4.1 mg of H_2O_2 was deposited onto the surface. The extracts from these chips were analyzed by HPLC/FD and HPLC/ED in DC mode. The results of this experiment are presented in Table 2.

The data in Table 2 illustrates that on average approximately 0.1% of the deposited sample was actually recovered by each of the three extraction protocols. While direct rinsing appeared to produce the best recovery, statistical analysis with a *t*-test indicated little difference between the various methods due to the high variation in recovery, 89–150% RSD. This large variance may also be due to variation in the decomposition and evaporation of the peroxide when heated. It should be noted that there was no difference in the level of background signal on the HPLC/FD or HPLC/ED instruments amongst the different extraction protocols, nor were there any additional peaks present in the chromatograms besides hydrogen peroxide.

Table 2
Effect of mode of extraction on recovery of hydrogen peroxide residue. Aliquots of 4.1 mg H_2O_2 were applied to the surface of paint chips and dried with the heat gun. Peroxide residues were recovered from the paint chips by a direct water rinse procedure, wet swabbing, or dry swabbing. Standard HPLC/FD and HPLC/ED in DC mode analysis conditions were applied. Ten replicates were performed of each extraction procedure.

Extraction procedure	Average mass of recovered H_2O_2	Standard deviation of recovered H_2O_2	RSD of recovered H_2O_2
Dry swab	2.36 μg	±2.7 μg	110%
Wet swab	1.82 μg	±1.6 μg	89%
Direct rinse	7.89 μg	±12 μg	150%

Table 3
Results of analysis of materials collected from a small scale detonation of hydrogen peroxide/nitromethane inside of an ammunition can. Hydrogen peroxide residues were detected on the ammunition can fragments and the plastic remnants by HPLC/FD and HPLC/ED in DC mode. Peroxide was not detected in the soil samples by either method. The witness plates were not analyzed by HPLC/ED in DC mode.

Material	H_2O_2 by HPLC/ED in DC mode		H_2O_2 by HPLC/FD	
Ammunition can fragments (6)	No (1)	Yes (5)	No (1)	Yes (5)
Plastic remnants (3)	No (0)	Yes (3)	No (0)	Yes (3)
Soil (3)	No (3)	Yes (0)	No (3)	Yes (0)
Witness plates (3)	N/A	N/A	No (1)	Yes (2)

3.5. Analysis of post-blast samples

A field test was performed in September of 2008. The test involved the detonation of a small-scale mixture (less than one pound) of hydrogen peroxide/nitromethane in a plastic bottle which was contained inside of a metal ammunition can. Post-blast materials from these tests were collected, including three plastic remnants, six pieces of metal debris, three witness plates, and three soil samples from the area directly under the explosion. The results of the analysis of the ammunition can fragments, plastic debris, soil samples, and witness plates are presented in Table 3. Extracts from the witness plates were only analyzed by HPLC–FD due to the limited amount of sample. The standard practice at the FBI Laboratory Explosives Unit is to qualitatively identify post-blast explosives residues. Therefore the level of hydrogen peroxide present on this set of post-blast debris was not determined. The electrochemical and fluorescence analyses were performed as qualitative analyses only. Samples were reported as positive if the signal strength of hydrogen peroxide was greater than the limit of detection of 0.6 μM for the HPLC/ED system, and 6 μM for the HPLC/FD system.

It was hypothesized that because the improvised explosive mixture was contained within the ammunition can, the ammunition can fragments would be more likely to yield hydrogen peroxide residues than the plastic remnants, soil samples, or witness plates. In fact, hydrogen peroxide residues were detected on all three of the plastic remnants, two out of the three witness plates, and five out of the six ammunition can fragments. Hydrogen peroxide was not recovered from any of the soil samples. Data from a sample extracted from one of the plastic remnants is presented in Figs. 5 and 6. Hydrogen peroxide can clearly be seen in both chromatograms; no additional peaks or interferences are present on either chromatogram. Data from a sample extracted from an ammunition can fragment is presented in Figs. 7 and 8. Peaks corresponding to hydrogen peroxide can be seen on the chromatograms from the electrochemical and fluorescence systems. It should be noted that the intensity of the hydrogen peroxide signal is approaching the limit of detection for the fluorescence method. Because the intensity of the peroxide signal is so low, baseline noise that is the result of small fluctuations in fluid flow from the three pumping systems is apparent in this chromatogram. The absence of interfering peaks should also be noted. Hydrogen peroxide standard samples were run at the beginning and end of each day, while blanks were run in-between the post-blast samples. Samples featured in Figs. 5–8 were not analyzed until approximately 5 months after collection. They were stored in a laboratory freezer until analysis. For all of these post-blast samples, the retention

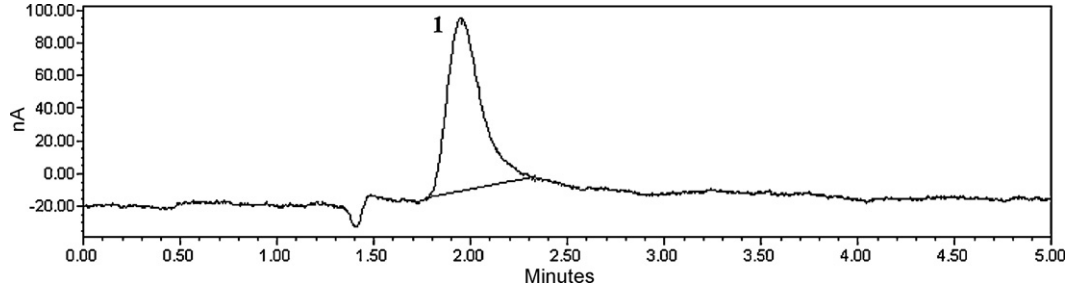

Fig. 5. Analysis of a post-blast plastic remnant by HPLC/ED in DC mode. Conditions: Dionex CarboPac PA10 analytical column, isocratic elution with 150 mM NaOAc at pH 10.5, flow rate 1 mL/min, 100 μL injection, $T = 45\,^{\circ}C$, DC mode with $E = +0.4$ V vs. Pd/H_2. Peak 1, hydrogen peroxide, R_t 1.95 min. Hydrogen peroxide residues were readily recovered from all of the plastic remnants which were analyzed.

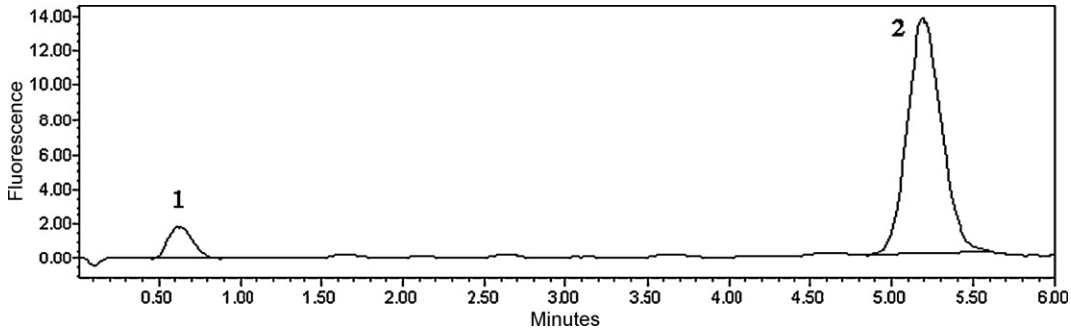

Fig. 6. Analysis of a post-blast plastic remnant by HPLC/FD. Conditions: Dionex C-18 analytical column, isocratic elution with 1×10^{-3} M H_2SO_4/1×10^{-4} M EDTA at flow rate 0.6 mL/min, 100 μL injection, reagent 8 μM hemin/80 μM POPHA in ammonia buffer pH 9.5 at flow rate 0.2 mL/min, post-column reactor 2 m, $T = 25\,^{\circ}C$, base 100 mM NaOH at flow rate 0.2 mL/min, $\lambda_{ex} = 320$ nm, $\lambda_{em} = 405$ nm. Peak 1, unretained peak, R_t 0.65 min; peak 2, hydrogen peroxide, R_t 5.20 min. Hydrogen peroxide residues were readily recovered from all of the plastic remnants which were analyzed.

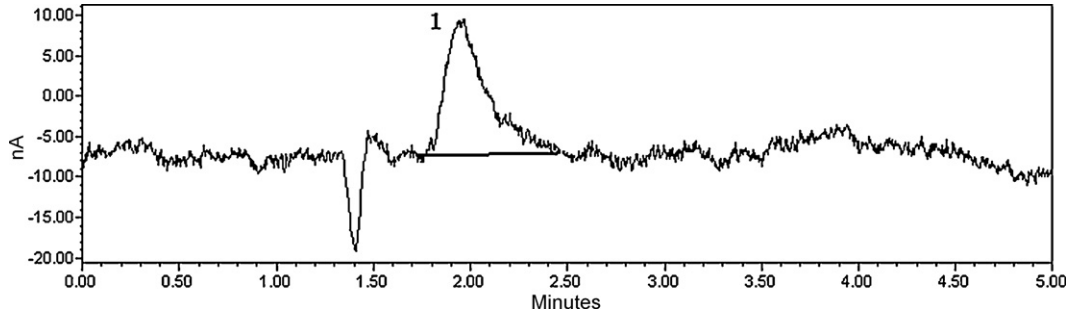

Fig. 7. Analysis of water rinse of exterior of ammunition can post-blast debris by HPLC/ED in DC mode. Conditions: Dionex CarboPac PA10 analytical column, isocratic elution with 150 mM NaOAc at pH 10.5, flow rate 1 mL/min, 100 μL injection, $T = 45\,^{\circ}C$, DC mode with $E = +0.4$ V vs. Pd/H_2. Peak 1, hydrogen peroxide, R_t 1.94 min. Hydrogen peroxide residues were present on five of the six ammunition can fragments which were analyzed.

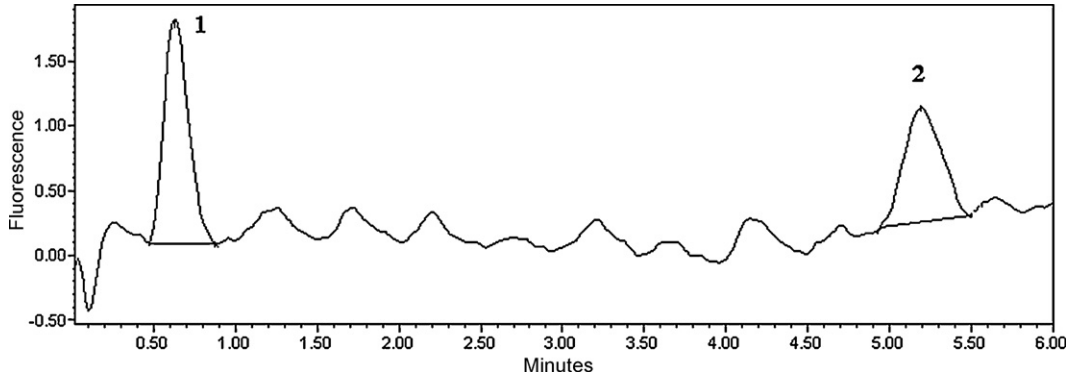

Fig. 8. Analysis of water rinse of exterior of ammunition can post-blast debris by HPLC/FD. Conditions: Dionex C-18 analytical column, isocratic elution with 1.00 mM H_2SO_4/0.010 mM EDTA at flow rate 0.6 mL/min, 100 μL injection, reagent 8 μM hemin/80 μM POPHA in ammonia buffer pH 9.5 at flow rate 0.2 mL/min, post-column reactor 2 m, $T = 25\,^{\circ}C$, base 100 mM NaOH at flow rate 0.2 mL/min, $\lambda_{ex} = 320$ nm, $\lambda_{em} = 405$ nm. Peak 1, unretained peak, R_t 0.65 min; peak 2, hydrogen peroxide, R_t 5.20 min. Hydrogen peroxide residues were present on five of the six ammunition can fragments which were analyzed.

time of hydrogen peroxide on the HPLC/ED system was shifted by 0.03 min, while the retention time of hydrogen peroxide on the HPLC/FD system was shifted by 0.04 min relative to Figs. 2 and 3. Due to the day-to-day drift in retention time, these shifts are not significant. Hydrogen peroxide standards analyzed at the beginning and end of each batch of post-blast samples showed the same shifts in retention time.

To determine whether the HPLC–ED and HPLC–FD methods differed significantly in their precision, an *F*-test was performed [17]. A two-tailed *F*-test was performed to compare the precision of the retention time of hydrogen peroxide for the two methods. The calculated value of $F(1.381)$ did not exceed the critical value of $F(4.026$ at $P = 0.05)$. Therefore, there was no significant difference between the two methods at the 5% probability level. A one-tailed *F*-test was also performed to determine whether the precision of the HPLC–FD instrument response was significantly greater from the HPLC–ED instrument response. The calculated value of F (99.11) exceeded the critical value of F (3.179 at $P = 0.05$), indicating that there is a significant difference between the two methods at the 5% probability level. This result is most likely attributed to the fact that the gold working electrode was susceptible to potential interferences from matrix components and sample impurities, which affected its response to hydrogen peroxide. It is important to realize, however, that a qualitative result is sufficient for a screening test. A more sophisticated confirmatory test can be used to quantify the amount of sample present.

In addition to statistical tests, the two methods can be compared based upon their ease of use and selectivity. The HPLC–ED method is the simpler of the two in terms of required equipment and chemicals, as well as operator skill. The limit of detection for this method was lower by a factor of ten in comparison to the HPLC–FD method. However, the HPLC–FD method is more selective to interfering species and matrix components.

4. Conclusions

Two methods were optimized for the analysis of trace levels of hydrogen peroxide: HPLC/FD and HPLC/ED in DC mode. Each method offered the benefits of a low limit of detection (0.6 μM for the HPLC/ED system and 6 μM for the HPLC/FD system), a linear dynamic range from 15 to 300 μM, selectivity to hydrogen peroxide, and insensitivity to a select group of potential interferences. The repeatability of the retention time of hydrogen peroxide for both methods was excellent. Post-blast debris from the detonation of a mixture of concentrated hydrogen peroxide with nitromethane was analyzed. Hydrogen peroxide residues were successfully detected on three out of the four types of post-blast debris collected. The electrochemical and fluorescence methods appeared to be fairly robust and insensitive to matrix compounds. This was particularly helpful given the notoriously complex nature of post-blast samples. A series of *F*-tests revealed that the two methods do not differ significantly at the 5% probability level in the precision of the retention time for hydrogen peroxide, but a significant difference in the precision of the instruments response to hydrogen peroxide did exist at the 5% probability level.

Acknowledgements

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References

[1] R. Hage, A. Lienke, Angew. Chem. Int. Ed. 45 (2005) 206.
[2] W. Eul, A. Moeller, N. Steiner, Hydrogen peroxide. Kirk-Othmer Encyclopedia of Chemical Technology, John Wiley & Sons, 2001.
[3] S. Francois, I. Sowka, A. Monod, B. Temime-Roussel, J. Laugier, H. Wortham, Atmos. Res. 74 (2005) 525.
[4] E. Hellpointner, S. Gaeb, Nature 337 (1989) 631.
[5] G. Kok, S. McLaren, T. Staffelbach, J. Atmos. Ocean. Technol. 12 (1994) 282.
[6] B. Qi, Y. Zhu, M. Hu, Y. Zhang, X. Tang, Anal. Lett. 34 (2001) 1247.
[7] R. Schulte-Ladbeck, P. Kolla, U. Karst, Analyst 127 (2002) 1152.
[8] R. Schulte-Ladbeck, P. Kolla, U. Karst, Anal. Chem. 75 (2003) 731.
[9] D. Grieshaber, R. MacKenzie, J. Voros, E. Reimhult, Sensors 8 (2008) 1400.
[10] P. Osborne, K. Yamomoto, J. Chromatogr. B 707 (1998) 3.
[11] R. Schulte-Ladbeck, U. Karst, Chromatographia 57 (2003) S61.
[12] D. Johnston, M. Cardosi, D. Vaughan, Electroanalysis 7 (1995) 520.
[13] Dionex, Technical Note #20, Sunnyvale, CA, 2000.
[14] Dionex, Application Update #152, Sunnyvale, CA, 2006.
[15] Installation and Operations Manual for Model LC4B/17 Amperometric Controller and Transducer Package, Bioanalytical Systems Inc., West Lafayette, IN, 1984.
[16] H. Hwang, P. Dasgupta, Anal. Chem. 58 (1986) 1521.
[17] J. Miller, J. Miller, Statistics for Analytical Chemistry, 3rd ed., PTR Prentice Hall, New York, NY, 1993.