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High-Performance Liquid Chromatographic Analysis with Fluorescence Detection of Ethyl Centralite and 2,4-Dinitrotoluene in Gunshot Residues After Derivatization with 9-Fluorenylmethylchloroformate

REFERENCE: Meng, H. H. and Caddy, B., **"High-Performance Liquid Chromatographic Analysis with Fluorescence Detection of Ethyl Centralite and 2,4-Dinitrotoluene in Gunshot Residues After Derivatization with 9-Fluorenylmethylchloroformate,"** *Journal of Forensic Sciences,* JFSCA, Vol. 41, No. 2, March 1996, pp. 213–220.

ABSTRACT: A reversed-phase high-performance liquid chromatographic method using 9-fluorenylmethylchloroformate (FMOC) as fluorogenic labeling reagent for the detection of ethyl centralite (EC) and 2,4-dinitrotoluene (2,4-DNT) in gunshot residues is reported. Residues were sampled with cotton wool swabs which were then extracted and the extracts cleaned by TLC. The sample spots on the TLC plate were scraped off and extracted to recover the analytes. The extract corresponding to EC was hydrolyzed while 2,4-DNT was reduced. The hydrolysis and reduction products (N-ethylaniline and 2,4-diaminotoluene, respectively) were derivatized with FMOC in alkaline buffer solution at 52°C for 20 min. The derivatives were analyzed by a reversed-phase HPLC with fluorescence detection. The detection limits for EC and 2,4-DNT were 200 pg and 1 ng per standard sample, respectively. Three out of eleven kinds of gunpowders analyzed were found to contain EC, while another three were found to contain 2,4-DNT. According to the results of gunpowder analysis, two different kinds of ammunition, which were presumed to contain EC in one and 2,4-DNT in the other, were chosen for test firings. Ethyl centralite was detected in cotton swabs sampled from spent cartridge cases of both of these two kinds of ammunition, but 2,4-DNT was not detected in any of these spent cases. Nine out of twelve samples swabbed from shooting hands at various times after firing two rounds of either kind of ammunition were found to contain EC, while none of these swabs were found to contain 2,4-DNT. The quantities of EC recovered from these hand swabs were shown to be in the range of 0.6 to 4.0 ng.

KEYWORDS: forensic science, gunshot residues, pre-column derivatization, fluorescence detection, high-performance liquid chromatography, N,N'-diphenyl-N,N'-diethylurea (ethyl centralite), 2,4-dinitrotoluene

In previous reports the use of dansyl chloride (DNS-Cl) as a labeling agent for the fluorescence detection of the stabilizer ethyl centralite (EC) in gunshot residues was described (1,2) Ethyl centralite was hydrolyzed to yield N-ethylaniline (NEA), which was then derivatized with dansyl chloride to give a fluorescent product. The derivatives were separated by two-dimensional TLC or reversed-phase HPLC the latter using fluorescence detection. Although these methods were successful in detecting EC in some test firing samples, only three out of eleven kinds of analyzed gunpowders were found to contain EC. This clearly restricts the applicability of this method. Furthermore, an improvement in sensitivity was required if a higher detection success rate was to be achieved.

In addition to ethyl centralite, 2,4-dinitrotoluene (2,4-DNT) (3), 2-nitrodiphenylamine (2-NDPA) and 4-nitrodiphenylamine (4-NDPA) (4) were recommended as characteristic components of smokeless powders. 2,4-Dinitrotoluene is used as a flash suppressor in some smokeless powders, while 2-nitro- and 4-nitrodiphenylamine are nitrated products produced from the stabilizer diphenylamine during storage. These aromatic nitro compounds are capable of being reduced to their corresponding aromatic amines which can then be derivatized with labeling agents to give fluorescent products. Many reducing agents have been reported to be useful for the reduction of aromatic nitro compounds to amines (5). In preliminary work, iron (II) ammonium sulphate, sodium dithionite, and stannous and titanous chlorides have all been assessed. Reduction of 2,4-DNT with sodium dithionite at 105°C gave yields lower than 10% in a reaction time varying from 5 to 90 min. Because of the presence of a white colored suspension in the organic layer, the extraction of reduction product from the stannous chloride reaction mixture was very difficult. Iron (II) ammonium sulphate and titanous chloride were therefore chosen for further investigation.

9-Fluorenylmethyl chloroformate (FMOC) has been reported to react rapidly with both primary and secondary amines forming stable and highly fluorescent derivatives. The detection limit of FMO-polyamine derivatives was reported to be less than 40 fmol at a signal to noise ratio of 4 (6), which was much lower than the reported detection limit of their corresponding DNS derivatives of 5–10 pmol at signal to noise ratio of 3 (7). The present report describes the use of reversed-phase HPLC with fluorescence detection for the analysis of EC and 2,4-DNT present in gunpowders and on cotton swabs taken from spent cartridge cases and also from the shooting hands of persons discharging weapons. The hydrolysis product of EC and reduction product of 2,4-DNT have been derivatized with FMOC in alkaline buffer solution prior to HPLC separation. The feasibility of fluorescence detection of 2-nitro- and 4-nitrodiphenylamine in gunshot residues have also been investigated.

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Experimental

Apparatus and Materials

Ethyl centralite was obtained from the Royal Ordnance, UK, while N-ethylaniline was purchased from BDH, UK. 2,4-Dinitrotoluene, 2-nitrodiphenylamine, 4-nitrodiphenylamine, 2,4-diaminotoluene (2,4-DAT), 2-aminodiphenylamine (2-ADPA), and 4-aminodiphenylamine (4-ADPA) were all bought from Aldrich, UK. Stock solutions of the above compounds were prepared in acetone at a concentration of 1 mg/mL. These solutions were used to prepare standards.

A 9-fluorenylmethylchloroformate solution was prepared by weighing 12.9 mg of 9-fluorenylmethylchloroformate (Sigma, UK) into a 50 mL volumetric flask and diluting to volume with acetonitrile.

Maraour's reagent were prepared as described previously (1). Tetramethyl ammonium hydroxide solution was prepared by adding 8 mL of ethanol and 8 mL of acetone to 2 mL of 25% tetramethyl ammonium hydroxide solution (BDH, UK). A fresh solution was prepared daily.

Reagent grade ammonia solution, boric acid, hydrochloric acid, citric acid, iron(II) ammonium sulphate, monopotassium phosphate, phosphoric acid (88–93%), potassium hydroxide, proline, sodium hydroxide, sodium dithionite, and titanous chloride (>10%, in 20–30% HCl) were all purchased from BDH, UK. HPLC grade acetonitrile (ACN) was obtained from Rathburn Chemicals, Walkerburn, Scotland.

Clark and Lubs buffer solutions were prepared at pH values of 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0 as reported (8).

Eleven kinds of gunpowders, see Table 1, were supplied by the Firearms Section, Strathclyde Police Forensic Science Laboratory. Each kind of gunpowder was obtained from a round of unfired ammunition.

Cotton swabs of two different types of spent cases were obtained immediately after test firings. After a given time interval over time periods 0 to 180 min, the shooting hands of test firers were swabbed. Control samples were obtained by swabbing the samplers' gloves just prior to sampling to eliminate any possible interferants that might be present as a component of the gloves. All test firing and swabbing works were conducted by Northern Ireland Forensic Science Laboratory (NIFSL), UK, according to a procedure previously described (9) except that cotton wool and ethylacetate rather than acrilan fiber and isopropanol were used as swabbing agent. Each test firing was carried out by using either a Colt Python revolver to discharge two rounds of Remington Peters .38 special cartridges or a Colt model 1911 .45 pistol to discharge two rounds of Winchester Repeating Arms Company .45 AC cartridges. The swabbing kits used always contained a blank swab, which was used to take a control from the hand of the firer before discharging the weapon.

TABLE 1—*Details and weight percentage of EC and 2,4-DNT detected in analyzed gunpowders. Wt: weight; WW: Winchester Western; REM-UMC: Remington union munition company; WRAC: Winchester repeating arms company; RM: Remington magnum; RP: Remington peters; WCC: Winchester cartridge company; IMI: Israeli military industry. '—': no analyte detected.*

Manufacturer	Type of Ammunition	Wt of Powder per Round/ mg	EC % (m/m)	2,4-DNT % (m/m)
WW	38 S&W	109	—	—
WW	9 mm LUGER	299	—	—
REM-UMC	9 mm BL	238	—	—
REM-UMC	38 SPL	180	—	—
WRAC	45 AC	404	—	1.99
RM	FC 44	1325	—	0.21
REM-UMC	455 II	159	—	3.10
RP	38 SPL	221	0.48	—
ICI	K 38022	75	—	—
WCC	38 85	232	0.65	—
IMI	357 Magnum Red	10.7	0.49	—
	Black	395	0.02	—

All TLC plates used were plastic backed silica gel 60 purchased from Merck.

The chromatographic system consisted of a Constametric, Model III G pump (Laboratory Data Control, Florida) with a Rhedoyne 7125 sample injector and a 20 μL loop. The analytical column was a home-made ODS column, 125 × 4.9 mm (i.d.), packed with 5 μm particles (Exsil 80 ODS, Alltech Associates, UK).

A Perkin-Elmer MPF-2A Fluorescence Spectrophotometer fitted with a flow cell was employed as fluorescence detector. Excitation and emission wavelengths were 266 and 302 nm, while slit settings for excitation and emission beams were 24 and 40 nm, respectively. All chromatograms were recorded with a Servoscribe 1s, RE 541.20, potentiometric recorder. The full scale and chart speed were set at 50 mV and 10 mm/min, respectively.

Samples were separated at a flow rate of 0.9 mL/min using an isocratic eluent composed of acetonitrile:citrate buffer (pH was adjusted with ammonia solution to 6.6) = 78:22 (v/v) unless otherwise described. The column temperature was ambient.

Method

A cotton wool swab was extracted with ethylacetate using a syringe procedure (1).

The extract was cleaned by TLC as follows: The extract was evaporated to approximate 20 μL in a gentle stream of nitrogen and then completely applied along the baseline of a TLC plate (6.5 cm × 1.5 cm). The plate was developed with a solvent mixture of toluene: ethyl acetate (15:1) for a distance of 5 cm. An 200 ng aliquot of EC and 2,4-DNT standard spotted onto two other plates were run simultaneously. The standard plates were sprayed with either Maraour's reagent or tetramethyl ammonium hydroxide solution, after development, to determine the Rf value of EC (0.16 ± 0.04, n = 5) or 2,4-DNT (0.62 ± 0.04, n = 5), respectively. The areas of the test plate corresponding in Rf values to 0.08–0.24 and 0.54–0.70 were scraped off and separately extracted with two 200 μL portions of ethyl acetate.

The extract corresponding to EC was hydrolyzed with concentrated phosphoric acid at 178°C for 20 min. The reaction mixture was made alkaline and extracted with ethylacetate as described previously (1). The extracts corresponding to 2,4-DNT was transferred into a 1.0 mL reaction vial, evaporated to dryness, and reduced according to the following procedure.

To the evaporated residue, 60 μL of concentrated HCl and approximately 15 mg of $Fe(NH_4)_2(SO_4)_2$ were added. The vial was then tightly covered with a screw cap and heated in a glycerol bath at 155°C for a reaction time of 25 min. The vial was cooled to ambient temperature and the reaction mixture made alkaline by addition of 300 μL of 3 M KOH. The alkaline solution was extracted four times (80 μL + 80 μL + 80 μL + 80 μL) with ethyl acetate.

The hydrolysis and reduction extracts were pooled and extracted two times (180 μL + 180 μL) with distilled water to remove any

KOH coextracted in the ethyl acetate solution. The ethyl acetate solution was transferred into a reaction vial and evaporated to nearly dryness in a gentle stream of nitrogen. The residue was redissolved in 20 μL of acetone. To the acetone solution, 30 μL of FMOC solution and 60 μL of borate buffer (pH 8.0) were added unless otherwise specified. The reaction vial was sealed and heated in an oven at 52°C for 20 min, and then cooled to room temperature. To the reaction mixture, a 10 μL aliquot of proline (15 M) was added to scavenge the reagent excess. After a reaction time of 10 min, the mixture was diluted to 600 μL with HPLC eluent and an aliquot of 20 μL was injected into the HPLC.

Fluorescence Characteristics of FMO Derivatives

Six 300 ng aliquots each of NEA, 2,4-DAT, 2-ADPA, or 4-ADPA were separately spotted onto a TLC plate. A 6 μL aliquot of a FMOC solution was over spotted onto each sample spot. The plate was covered with two sheets of clean glass plate and kept in the dark at room temperature for a reaction time of 10 minutes and then developed with a solvent of toluene: ethyl acetate (7:1) over a distance of 5 cm. One reagent blank and an analyte-only spot were simultaneously treated. After the plate was dried, chromatograms of one derivatized spot, analyte-only spot, and reagent spot were cut off and visualized with concentrated sulphuric acid to determine the Rf value and identity of each separated spot. The area with equivalent Rf value to each identified derivatization product on each unvisualized chromatogram was marked with a pencil. The thin layer material (silica gel) of the marked area was scraped off and extracted with two 200 μL portions of ethyl acetate. The extract was evaporated to nearly dryness in a stream of nitrogen. The evaporated residues of the five derivatized samples of each amine were separately redissolved in 1100 μL each of the five different solvents cyclohexane, ethyl acetate, acetone, methanol, or acetonitrile. The solution was then fluorometrically recorded to identify the excitation and emission wavelength maxima. The extracts were also subjected to HPLC analysis to determine the retention time of each derivative and to identify the analyte peak for the following investigation.

Detection Limits of FMO Derivatives

A 300 ng aliquot of each amine was derivatized with FMOC in alkaline solution as described above. The final reaction mixture was sequentially diluted with acetonitrile to obtain quantities of amine per injection into the HPLC in the range from 5 pg to 10 ng. The diluted solutions were subjected to HPLC separation. Minimum detection limit was obtained by recording the smallest amount of amine that still produced a peak at a signal to noise ratio of 3:1 while the sensitivity of the detector and the full scale of the recorder were set to 5 and 50 mV, respectively. Any peak height recorded at other sensitivity settings was corrected to sensitivity at 5 according to the operational manual of the Perkin-Elmer MPF-2A Fluorescence Spectrophotometer.

Optimization of the Derivatization Reaction

Three series of various aliquots of a mixture of NEA (200 ng), 2,4-DAT (200 ng), 2-ADPA (600 ng), and 4-ADPA (600 ng) were derivatized with FMOC in alkaline solution as described. In one of these three series the pH value of buffer solution was varied from 7.5 to 10.0, while in another series the reaction time was varied from 3 to 60 min. In a third series the amount of buffer solution added was varied from 5 μL to 120 μL. The reaction mixtures were diluted to 600 μL with HPLC eluent and subjected to HPLC separation where the sensitivity of the detector was set at 2 for FMO-NEA and FMO-2,4-DAT or at 5 for FMO-2-ADPA and FMO-4-ADPA.

Optimization of the Reduction of 2,4-Dinitrotoluene

Reduction with Iron (II) Ammonium Sulphate—Two series of 300 ng aliquots of 2,4-DNT were separately reduced with $Fe(NH_4)_2(SO_4)_2$ as described, except that the reaction temperature was either 145 or 155°C and the reaction time varied from 5 to 60 min. The ethyl acetate extract of reduction product was extracted two times (180 μL + 180 μL) with distilled water to remove KOH coextracted with ethyl acetate solution. The ethyl acetate solution was derivatized with FMOC in alkaline solution as described above and a 20 μL aliquot of diluted reaction mixture was subjected to HPLC separation. A blank vial was treated simultaneously with every sample series. The yield of the reduction reaction was calculated.

Reduction with Titanous Chloride—Two series of 300 ng aliquots of 2,4-DNT were separately transferred into 1.0 mL reaction vials and reduced with 60 μL of $TiCl_3$ (>10%, in 20–30% HCl) at either 138 or 145°C for reaction times varying from 5 to 45 min. The vial was cooled to ambient temperature and the reaction mixture made alkaline by addition of 400 μL of 2 M KOH and extracted four times (80 μL + 80 μL + 80 μL + 80 μL) with ethyl acetate. Each extract was extracted with distilled water to remove coextracted KOH and derivatized with FMOC in alkaline solution as described above. A 20 μL aliquot of diluted derivatization mixture was subjected to HPLC separation. A blank vial was treated simultaneously with every sample series. The yield of the reduction reaction was calculated.

Recovery of 2,4-Dinitrotoluene in Clean-Up Procedures

A 300 ng aliquot of 2,4-DNT was cleaned-up using either the TLC procedure described here or by means of an XAD-7 column previously described (1). The cleaned samples and various amounts (30 ng, 50 ng, 150 ng, 300 ng) of 2,4-DNT standards used for calibration were analyzed as described. The recovery of 2,4-DNT was calculated from the peak height of each sample and a calibration curve produced from the standards.

Recovery of Ethyl Centralite and 2,4-Dinitrotoluene from Spiked Hands and Cotton Swabs

An aliquot of a mixture of 300 ng each of EC and 2,4-DNT was spiked onto precleaned cotton wool ball. The cotton ball was extracted using the syringe elution procedure. The extract was cleaned-up and then analyzed according to the developed procedures.

An aliquot of a mixture of 300 ng each of EC and 2,4-DNT was spiked onto the back of a clean hand. After 20 min, the hand was swabbed three times with precleaned cotton balls that were moistened with ethyl acetate prior to use. The cotton swabs were then extracted, the extracts were cleaned and analyzed as described.

A series of a mixture of 50 ng, 100 ng, 200 ng, or 300 ng each of EC and 2,4-DNT and a blank sample were simultaneously cleaned-up and analyzed for the preparation of calibration data. The recoveries of EC and 2,4-DNT of each spiked sample were calculated.

Analysis of Ethyl Centralite and 2,4-Dinitrotoluene Standards

A number of samples containing a mixture of EC and 2,4-DNT standards in the range 200 pg to 200 ng were cleaned-up and then analyzed according to the developed procedures. For samples containing less than 5 ng of EC or 2,4-DNT, only 5 μL of FMOC and 15 μL of buffer were used in the derivatization procedure and the final reaction mixture was diluted to 200 μL with eluent prior to HPLC separation. A blank reaction vial was simultaneously treated as a control.

Analysis of Gunpowders

A 10 mg aliquot of gunpowder from each ammunition type listed in Table 1 was extracted with 3 mL of dichloromethane in a sealed vial for at least 48 h. An exception to this was that only 1.3 mg of the red flakes of ammunition IMI was extracted with 500 μL of dichloromethane. A 10 μL aliquot of each extract was cleaned-up and analyzed according to the developed procedures except that the derivatization mixture was diluted to 800 μL with eluent prior to HPLC separation. For the gunpowder which contained EC or 2,4-DNT, the weight percentage of EC or 2,4-DNT was calculated by using a calibration curve, account being taken of the recovery of the clean-up procedure and the yield of hydrolysis and reduction procedures.

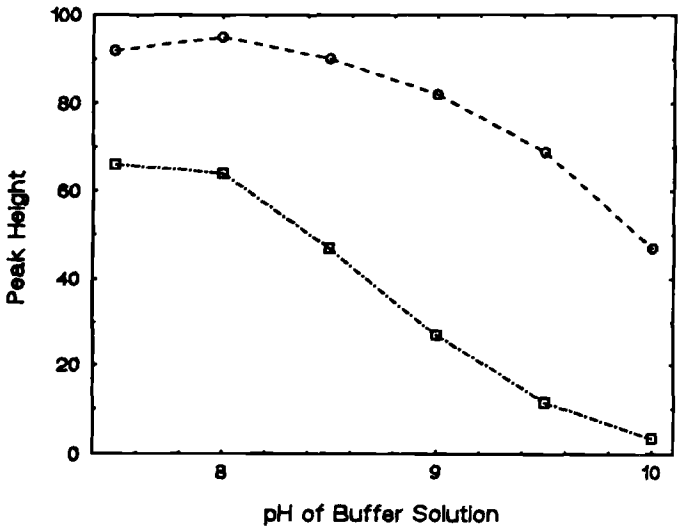

FIG. 1—*The effect of pH on the derivatization of NEA* (upper) *and 2,4-DAT* (lower) *with FMOC; reaction temperature: 52°C, reaction time: 20 min.*

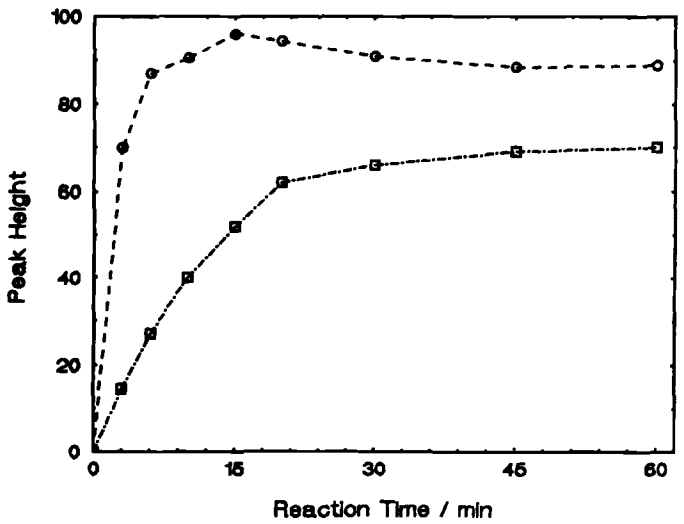

FIG. 2—*The effect of reaction time on the derivatization of NEA* (upper) *and 2,4-DAT* (lower) *with FMOC; reaction temperature: 52°C, pH of buffer solution: 8.0.*

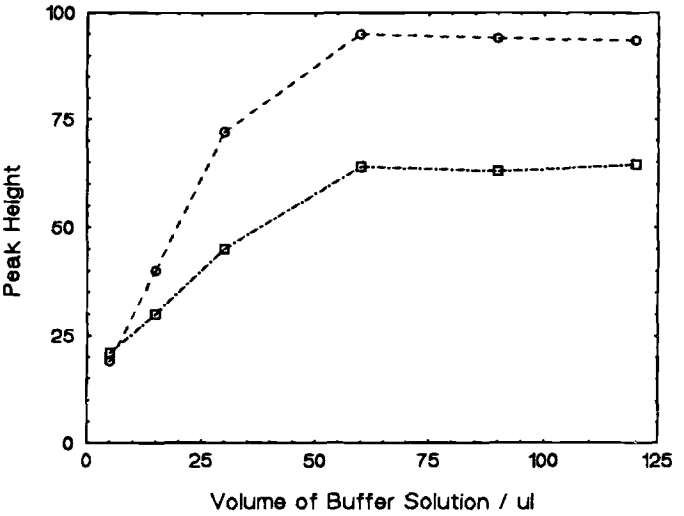

FIG. 3—*The effect of the amount of buffer solution on the derivatization of NEA* (upper) *and 2,4-DAT* (lower) *with FMOC; pH of buffer: 8.0, reaction temperature: 52°C, reaction time: 20 min.*

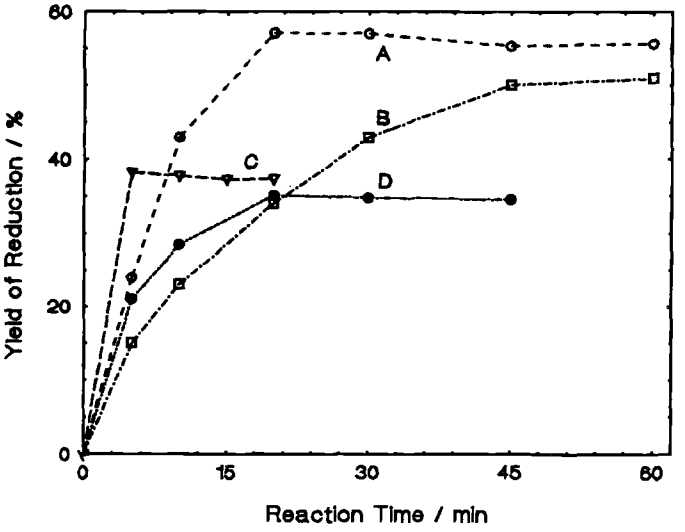

FIG. 4—*Influence of time and temperature in the reduction of 2,4-dinitrotoluene with* $Fe(NH_4)_2(SO_4)_2$ *(A: 155°C, B: 145°C) and* $TiCl_3$ *(C: 145°C, D: 138°C).*

Analysis of Gunshot Residues from Spent Cartridge Cases and Shooting Hands

Cotton swabs from spent cartridge cases, shooting hands, and the samplers' gloves were extracted. The extract was cleaned-up, and then hydrolyzed or reduced according to the developed procedures. One fifth of the swab extracts from the spent cartridge cases and the whole swab extracts from the other samples were subjected to analysis. The hydrolysis and reduction products from spent cartridge cases were derivatized with FMOC as previously

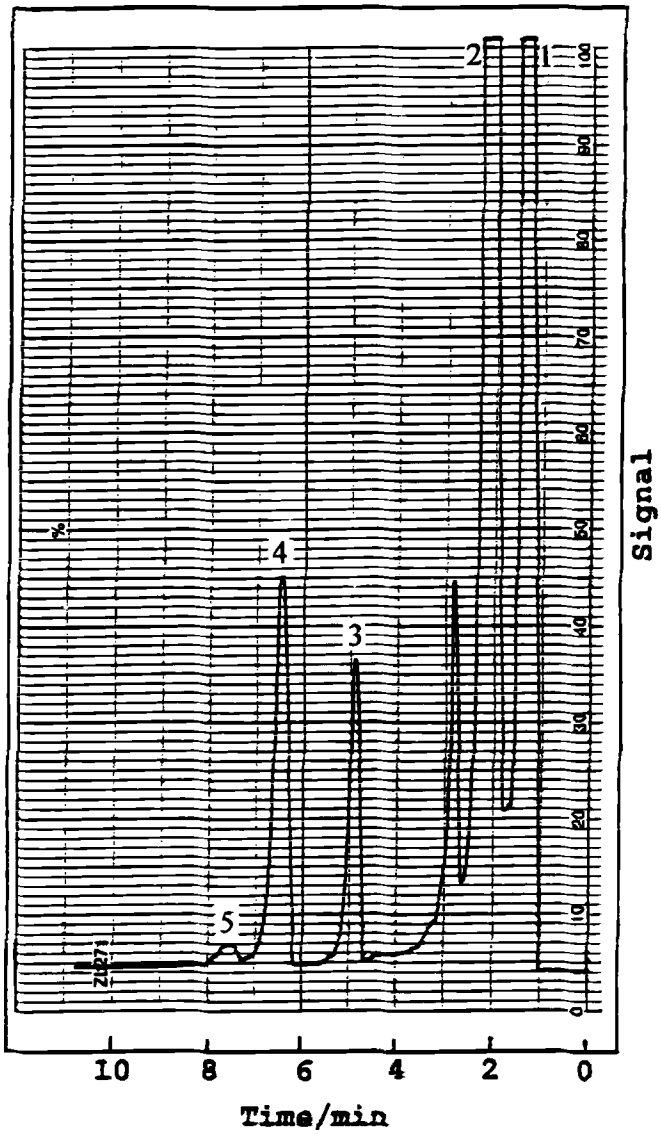

FIG. 5—*HPLC chromatogram of FMO derivatives from EC and 2,4-DNT standards. Peak 1: FMO-proline; peak 2: FMOH; peak 3: FMO-NEA; peak 4: FMO-2,4-DAT; peak 5: FMOC.*

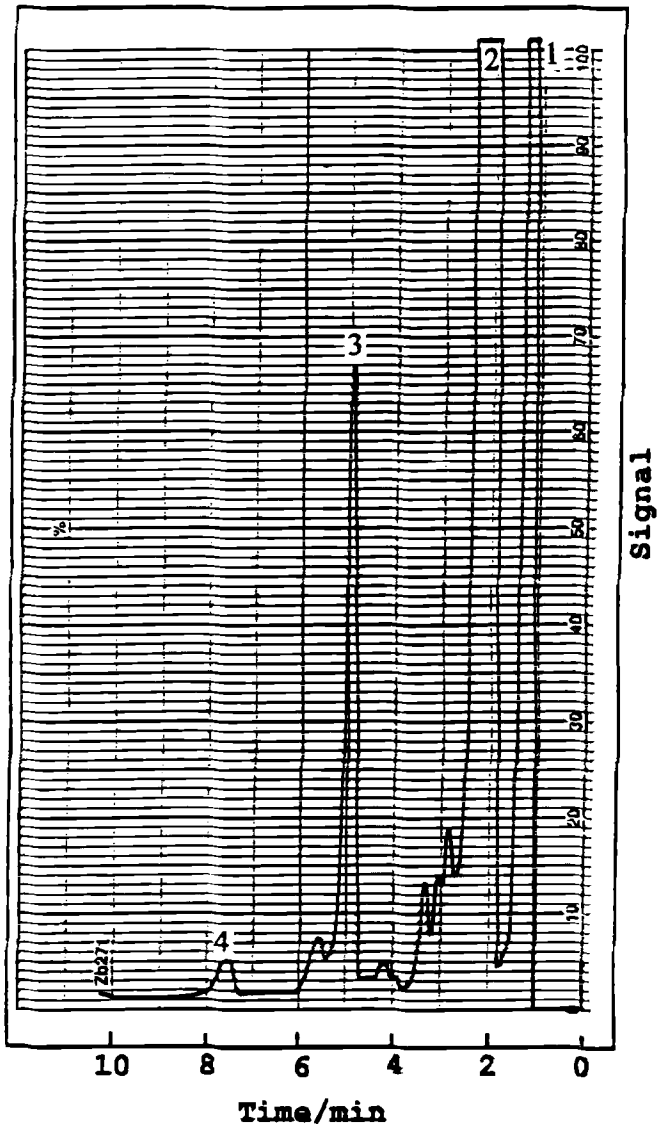

FIG. 6—*HPLC chromatogram of FMO-NEA detected in gunshot residue sample swabbed from a spent cartridge case (WRAC 45 AC). Peak 1: FMO-proline; peak 2: FMOH; peak 3: FMO-NEA; peak 4: FMOC.*

described and diluted with HPLC eluent to 600 μL. Hydrolysis and reduction products from shooting hands or samplers' gloves were derivatized with 10 μL of FMOC and 20 μL of pH 8.0 buffer and the final reaction mixture was diluted with eluent to 400 μL. The diluted solutions were subjected to HPLC separation. The quantities of EC or 2,4-DNT recovered from cotton swabs were calculated.

Results and Discussion

Although no visible or fluorescent spot was observed on the developed TLC plate under visible, 254 nm UV, and 365 nm UV light, a number of dark blue spots were revealed after visualization with concentrated sulphuric acid. The Rf values of these spots were recorded as: 0.33 ± 0.04, 0.46 ± 0.03, 0.54 ± 0.04, 0.60 ± 0.03, 0.67 ± 0.03, and 0.86 ± 0.03 for FMOH (hydrolysis product of FMOC), FMO-2,4-DAT, FMO-4-ADPA, FMO-NEA, FMO-2-ADPA, and FMOC, respectively.

The excitation and emission maxima of FMO derivatives in acetonitrile were 266 nm and 302 nm. Both excitation and emission maxima of FMO derivatives were in the UV light range, which explains why no fluorescent spot was observed on the TLC plate radiated at 254 and 365 nm. In contrast to DNS derivatives (1), the excitation and emission maxima of FMO derivatives with the exception of acetone remained unchanged when dissolved in solvents of different polarity. There was neither excitation nor emission spectra observed for FMO derivatives dissolved in acetone. Since the cut-off wavelength of acetone is 330 nm, the excitation beam and emission beam (if there was any) were completely absorbed by acetone.

After HPLC separation, the ratio of peak heights of FMO derivatives relative to the fluorescent intensity produced by 5 ng of each underivatized amine was: FMO-NEA: FMO-2,4-DAT: FMO-4-ADPA: FMO-2-ADPA = 200 : 120 : 2 : 1. FMO-NEA gave the highest peak which was two hundred times of that of FMO-2-ADPA. The detection limit per injection of each FMO derivative was 5 pg, 10 pg, 1 ng, and 2 ng for NEA, 2,4-DAT, 4-ADPA, and 2-ADPA, respectively. The detection limit of 5 pg for NEA is

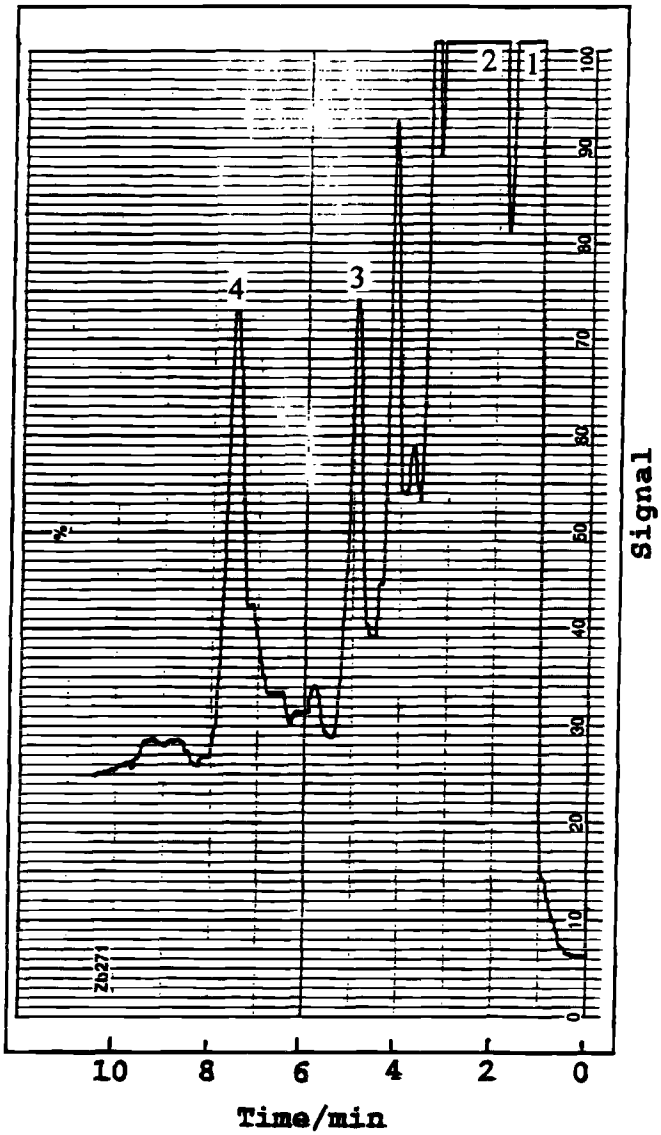

FIG. 7—*HPLC chromatogram of FMO-NEA detected in gunshot residue sample swabbed from a shooting hand 60 min after discharging two rounds of RP 38 SPL ammunition. Peak 1: FMO-proline; peak 2: FMOH; peak 3: FMO-NEA; peak 4: FMOC.*

much lower than previously reported of 60 pg using DNS-Cl as labelling agent (1). The calibration curve of peak height of FMO derivative versus picograms of corresponding amine injected showed good linearity over the range of 5 pg and 5 ng of NEA ($r > 0.999$) and over the range of 10 pg and 5 ng for 2,4-DAT ($r > 0.999$). The amounts of nitrated products of diphenylamine including 2-NDPA and 4-NDPA in gunshot residues were reported to be very low (10). Considering the high detection limits of 2-ADPA and 4-ADPA, the loss of 2-NDPA and 4-NDPA during swab extraction and clean-up procedures, the yields of the reduction reaction, and sample dilution, the detection limits for 2-NDPA and 4-NDPA per sample would be expected to be higher than if these two compounds existed in gunshot residue swabs. The feasibility of using the proposed method to detect 2-NDPA and 4-NDPA in gunshot residues is therefore low.

The reaction of FMOC with amines was found to be pH dependent. Figure 1 shows the effect of pH value on the derivatization process for NEA and 2,4-DAT, pH 8.0 and 7.5 giving the best result for NEA and 2,4-DAT respectively. At higher pH values the hydrolysis of FMOC is favored. A derivatization pH of 8.0 was chosen for all subsequent experimental work. Figure 2 shows the effect of reaction time on the formation of FMO derivatives of NEA and 2,4-DAT at 52°C. Derivatization of NEA is essentially completed within 15 min and the peak height slowly decreased after 20 min. Derivatization of 2,4-DAT is nearly completed at 20 min and the peak height slowly increased to a maximum at 45 min. An optimal reaction time of 20 min was therefore chosen. The effect of the amount of buffer solution on derivatization of NEA and 2,4-DAT is shown in Fig. 3, a volume ratio of buffer solution to reagent of 2:1 reaching the plateau conditions. The optimization results for derivatization of 2-ADPA and 4-ADPA are similar to those of NEA and 2,4-DAT. The optimal conditions were pH 7.5, reaction time 40 min, and buffer/reagent ratio 2:1 for 2-ADPA, and pH 8.0, reaction time 20 min, buffer/reagent ratio 2:1 for 4-ADPA. However, the peak heights of FMO-2-ADPA and FMO-4-ADPA are too small to enable these to be illustrated on the same scale as FMO-NEA and FMO-2,4-DAT.

The rate of reduction is highly dependent on temperature as illustrated in Fig. 4. However, since the reaction vial was tightly covered to prevent the evaporation of reaction mixture, reaction temperature should be lower than the boiling point to avoid a drastic increase of pressure. Reduction with $Fe(NH_4)_2(SO_4)_2$ at 155°C for a reaction time of 20 min gave the best yield (57.1% ± 4.1% $n = 9$) for all reaction conditions investigated. Reduction with $TiCl_3$ at 145°C is essentially completed within 5 min, however, the yield (38.3% ± 1.9%, $n = 3$) is lower than that of the former conditions. The reduction product of 2,4-DNT was verified to be 2,4-DAT from its IR spectrum. An experiment to determine the yield of the reduction of 2-NDPA and 4-NDPA using the proposed reduction procedure was conducted. There was no 2-ADPA (complete reduction product of 2-NDPA) detected, and the yield of 4-ADPA was found to be lower than 20%.

The recovery of 2,4-DNT from the TLC clean-up procedure was 59.2% ± 2.3%, $n = 3$, while that from the XAD-7 column procedure was only 22.3% ± 2.9%, $n = 3$. This is consistent with Douse's results (11) where the recovery of 2,4-DNT using the XAD-7 method was 27%. Since the TLC method gave better recovery for both EC (1) and 2,4-DNT, it was chosen as the preferred clean-up procedure for this study. The recovery of EC and 2,4-DNT from the spiked cotton wool through the syringe extraction procedure were 86.5% ± 4.0% and 78.3% ± 1.7% ($n = 3$), respectively. The recovery of EC and 2,4-DNT from the spiked hand through swabbing combined with syringe extraction procedures were 66.7% ± 2.5% and 53.4% ± 3.4% ($n = 3$), respectively. These results suggest that the proposed sample pretreatment procedures are more efficient in the recovery of EC than that of 2,4-DNT.

A typical chromatogram of FMO derivatives from EC and 2,4-DNT is shown in Fig. 5. In addition to FMO-NEA and FMO-2,4-DAT, a derivatized by-product which has not been identified, the hydrolysis product of FMOC (FMOH), FMO-proline, and FMOC were also present in the chromatogram. All peaks were well separated. The retention times of FMO-NEA and FMO-2,4-DAT were 5.0 min ± 0.1 min ($n = 7$) and 6.5 min ± 0.1 min ($n = 7$), respectively. The detection limits for EC and 2,4-DNT were 200 pg and 1 ng per sample, respectively. However, the reproducibility of peak heights for samples containing less than 15 ng of 2,4-DNT or 5 ng of EC were poor, the standard deviation of peak heights was usually higher than 20%. This probably reflects the

low reproducibility of the yields of hydrolysis, reduction, and derivatization when the amounts of EC and 2,4-DNT were low.

For test firing samples, no EC or 2,4-DNT was detected either on the control samples from the samplers' gloves or on the blanks taken from the firer's hands before discharging the weapon. The results of the analysis of gunpowders are shown in Table 1. For the eleven gunpowders, three were found to contain EC, and another three were found to contain 2,4-DNT. The results of the presence of EC in gunpowders are consistent with the authors' previous findings (2). The amount of 2,4-DNT contained in the powder of ammunition was generally higher than that of EC. Remington Peters .38 special cartridges and Winchester Repeating Arms Company .45 AC cartridges, which contained EC or 2,4-DNT, respectively, were chosen for test firings.

The results of the analysis of swabs from spent cartridge cases and shooting hands are shown in Table 2. Based on the results of gunpowder analysis, two different kinds of ammunition which contained either EC or 2,4-DNT were purposely chosen for the test firings. Both kinds of spent cartridge cases were found to contain EC but none of them were found to contain 2,4-DNT. Since the gunpowder samples and the test firing samples were obtained from different sources, the ammunition used, although from the same manufacturer, might be from different production lots. Ammunition manufactures are known to use different sources of powders in response to supply vagaries.[3] This suggest that using the results of gunpowder analysis to differentiate gunshot residues or vice versa may be misleading. For gunshot residue swabs of shooting hands, four out of six samples from the discharge of RP 38SLP ammunition and five out of six samples from the discharge of WRAC 45AC ammunition were found to contain EC. There was no 2,4-DNT detected on any shooting hand samples. The success rate is better than that of previous work where three out of test six firing samples were found to contain EC (1). The amount of EC detected in swabs obtained from spent cases of RP 38SPL and WRAC 45AC were 183.2 ng and 770.2 ng, respectively. The amounts of EC detected in shooting hand samples were in the range of 0.6 to 4.0 ng. Because after test firings the activities of the discharges were not restricted, gunshot residues deposited on the shooting hands were continuously lost and as a consequence there was no EC detected in some samples. One of these was swabbed only 30 min after test firing. However, for those samples which were shown to contain EC, the amount of EC detected decreased with increase in time to sampling except for sample G4505 (Table 2), which was swabbed 3 h after test firing but contained more EC than that of sample G4503 which was swabbed 1 h after test firing.

TABLE 2—*Details and amount of EC detected in gunshot residue samples. WRAC: Winchester Repeating Arms Company; RP: Remington Peters. '—': No analyte detected; RP 38SPL was discharged by a .38 revolver; WRAC 45AC was discharged by a .45 pistol.*

Sample	Sampling Area	Ammunition Used	Time Lapse (min)	EC Detected
G38SC	Spent Case	RP 38SPL	0	183.2 ng
G3801	Shooting Hand	RP 38SPL	0	3.89 ng
G3802	Shooting Hand	RP 38SPL	30	—
G3803	Shooting Hand	RP 38SPL	60	2.14 ng
G3804	Shooting Hand	RP 38SPL	120	0.61 ng
G3805	Shooting Hand	RP 38SPL	180	—
G45SC	Spent Case	WRAC 45AC	0	770.2 ng
G4501	Shooting Hand	WRAC 45AC	0	4.09 ng
G4502	Shooting Hand	WRAC 45AC	30	4.00 ng
G4503	Shooting Hand	WRAC 45AC	60	1.84 ng
G4504	Shooting Hand	WRAC 45AC	120	—
G4505	Shooting Hand	WRAC 45AC	180	2.25 ng

[3]P. R. DeForest, personal communication.

Chromatograms of one spent case sample and one shooting hand sample are shown in Figs. 6 and 7, respectively. When compared with the chromatogram of standards, there are clearly more by-product peaks present in chromatograms of gunshot residue samples especially those swabbed from the shooting hands. The identities of these by-products were not investigated. To prevent possible interference from derivatization by-products, chromatographic methods that possess higher separation efficiency and compatibility with fluorescence detection, such as supercritical fluid chromatography and micellar electrokinetic capillary electrophoresis, might be required for application to real gunshot residue samples.

Only six out of eleven kinds of gunpowders were found to contain EC or 2,4-DNT. Moreover the proposed procedure was not sensitive enough to detect 2-NDPA and 4-NDPA in gunshot residues, even though these compounds have been reported to be more prevalent than EC and 2,4-DNT in gunpowders (4). The potential for the fluorescence detection of gunshot residues requires a more thorough investigation of fluorescent labeling reagents and reduction procedure, which might enable fluorescence detection of 2-NDPA nd 4-NDPA to be achieved.

Acknowledgments

The authors thank Dr. W. J. Rodger and the Firearms Section, Strathclyde Police Laboratory for providing gunpowders, and Mr A. Hall, Director and Mr P. Boyce of the Northern Ireland Forensic Science Laboratory for their assistance with test firing and sampling.

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