Hsien-hui Meng,[1] *M.A. and Brian Caddy,*[1] *Ph.D.*

Fluorescence Detection of Ethyl Centralite in Gunshot Residues

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ABSTRACT: A fluorescence method has been developed to detect ethyl centralite in gunshot residues. Residues were recovered from shooters' hands with cotton wool swabs. The swabs were extracted by a syringe elution procedure and the extracts cleaned by TLC. The cleaned samples were hydrolyzed in phosphoric acid at 178 °C for 20 min to yield N-ethylaniline. Any N-ethylaniline was dansylated directly on a TLC plate, which was then developed two-dimensionally. The fluorescent dansyl-N-ethylaniline spot was scraped off and extracted for fluorometric determination. The detection limits for ethyl centralite standards and spiked hand samples are 1 ng and 5 ng respectively. Three out of six test firings of a .38 revolver gave extracts that were found to contain ethyl centralite. One of these was swabbed 180 min after firing three rounds of ammunition.

KEYWORDS: forensic science, gunshot residues, fluorescence detection, stabilizer, ethyl centralite, clean-up, hydrolysis

The detection of organic gunshot residues (GSRs) has been of great interest to forensic scientists. In connection with its work on a mass spectrometric approach to the analysis of GSRs, the FBI Laboratory has compiled a list of 23 organic compounds that may occur in smokeless gunpowders [*1*]. In addition to these compounds, dinitrotoluene and nitroguanidine are also used as flash suppressors in smokeless powders (Table 1). Among this list are two commonly used stabilizers, N,N'-diphenyl-N,N'-diethylurea (ethyl centralite, EC) and diphenylamine (DPA). These have attracted most attention for the characterization of organic GSRs. Unfortunately, because it is widely used in rubber products and in the foodstuffs industry as a post-harvest treatment for apples, DPA has been regarded as an evidentially irrelevant component [*2,3*]. In contrast, EC was recommended by Mach et al. [*4*] as the most characteristic material found in smokeless powders. However, the amount of EC in residues was reported as being low and well below the detection limits of techniques available at that time [*3*]. Other more sensitive methods will detect EC [*5*]. However, the development of detection techniques showing high sensitivity and selectivity is important for the identification of GSRs by means of EC.

Fluorescence detection is much more sensitive and selective than many techniques used for organic GSRs analysis, but, these organic compounds present as residues are, in general, not fluorescent. It is of no surprise therefore to find that the application of fluorescence

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[1] Ph.D. Student and Professor/Director, respectively, Forensic Science Unit, University of Strathclyde, Glasgow, Scotland.

TABLE 1—*Organic compounds that may be found in smokeless gunpowder.*

Cresol	RDX (Cyclonite)
Resorcinol	Diethyl phthalate
Carbazole	Nitroglycerin
Diphenylamine	Trinitrotoluene
Dimethyl phthalate	Dimethylsebacate
N-Nitrosodiphenylamine	N,N-Dimethylcarbanilide (Methyl centralite)
Dinitrocresol	2,4-Dinitrodiphenylamine
Carbanilide	N,N-Diethylcarbanilide (Ethyl centralite)
Nitrodiphenylamine	Dibutyl phthalate
Triacetin	PETN (Pentaerythritol Tetranitrate)
Nitrocellulose	N,N-Dibutylcarbanilide (Butylcentralite)
Nitrotoluene	Dinitrotoluene
Nitroguanidine	

for the detection of GSRs has not been pursued. As a compound, ethyl centralite is similar to the urea herbicides and fluorescence detection of these has been reported by several workers [*6–10*]. The urea herbicides were hydrolyzed in various ways to their corresponding anilines, which were then labeled with dansyl chloride to yield fluorescent derivatives. Ethyl centralite is, however, resistant to hydrolysis under the conditions used by these workers, which included boiling with aqueous solutions of base and catalytic-hydrolysis on silica gel. For the most resistant amides and substituted amides, Cheronis and Entrikin [*11*] recommended heating with phosphoric acid as a means of hydrolysis. This work describes a procedure that makes use of the hydrolysis of ethyl centralite to N-ethylaniline and the coupling of this hydrolyzed product to the fluorophore dansyl chloride.

Experimental

Apparatus and Materials

Ethyl centralite (Royal Ordance, UK) was dissolved in ethyl acetate at a concentration of 1 mg/mL. This solution was used to prepare standards and spiked samples.

Maraour's reagent was prepared as follows: 5 g of potassium dichromate was dissolved in 100 mL of distilled water. To this solution was added sequentially 100 mL of glacial acetic acid and 50 mL of concentrated sulphuric acid.

Dansyl chloride solution was prepared by weighing 0.25 g of dansyl chloride (Aldrich, UK) into a 50 mL volumetric flask and diluting to volume with acetone.

Phosphoric acid (88% to 93%) was purchased from BDH, UK.

Non-absorbent cotton wool was successively Soxhlet extracted with acetone and ethyl acetate for 5 h and 8 h, respectively and was then allowed to dry in a fume hood at room temperature.

A 10 g wet mass of Amberlite XAD-7 beads (BDH, UK, 0.3 to 0.78 mm) was cleaned by washing successively with 100 mL of distilled water, methanol, ethyl acetate, ether, and pentane. The beads were stored under ether until required for use.

A Pasteur pipette was plugged at its constricted end with glass wool and an appropriate amount of XAD-7 beads was poured into the pipette to form a 2 cm column. The column was successively washed with 2 mL of ethyl acetate and 3 mL of pentane just prior to use.

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

Fluorescence spectra were recorded on a Perkin-Elmer MPF-2A Fluorescence Spectrophotometer.

A Smith & Wesson .38 Special revolver and Israeli Military Industry .357 Magnum semi-jacketed hollow-point bullets were employed for test firings.

Method

Swabbing—A small strip of Soxhlet extracted cotton wool was wrapped around the tips of a pair of tweezers to form a pea sized ball. Swabbing was performed by repeat scrubbing the web and back area of a hand with the cotton ball that was moistened with ethyl acetate prior to use. Swabbing was repeated three times using different cotton wool swabs to maximize recovery.

Swab Extraction—The cotton swabs were inserted into a 5 mL glass syringe and the plunger was pressed down hard to expel any solvent. The cotton swabs were then washed through with 200 μL of ethyl acetate and then pressed out. The procedure was repeated 5 times and the extracts pooled in an Eppendorf tube that was then centrifuged at 1000 g for 5 min to remove skin debris and cotton fibres. The supernatant was transferred into a second Eppendorf tube.

Clean-Up—The extract was evaporated to a small volume (approximate 20 μL) in a gentle stream of nitrogen. The concentrated solution was completely applied along the baseline of a TLC plate (6.5 cm × 1.5 cm) and the plate developed with a solvent mixture of toluene:ethyl acetate (15:1) for a distance of 5 cm. A similar TLC plate to which a 200 ng EC standard was applied and was run simultaneously and sprayed with Maraour's reagent, after development, to determine the Rf value of EC (0.16 ± 0.04, $n = 5$). An area of the test plate corresponding in Rf value to 0.08–0.24 was scraped off and extracted with three 200 μL portions of ethyl acetate. The extracts were pooled into a 1.0 mL reaction vial.

Hydrolysis of Ethyl Centralite—The sample solution in this reaction vial was evaporated to dryness in a gentle stream of nitrogen and 40 μL of phosphoric acid added. The vial was then loosely covered by a screw cap and heated in a glycerol bath at 178° C for 20 min. The vial was cooled to ambient temperature and the reaction mixture made alkaline by addition of 160 μL of 15M KOH. The alkaline solution was extracted three times (80 μL + 80 μL + 60 μL) with ethyl acetate. The extracts were pooled.

Dansylation and TLC Development—The hydrolyzed product was evaporated in a stream of nitrogen to a volume of about 10 μL and the total volume spotted onto a TLC plate (6.7 cm × 6.7 cm). A 4 μL aliquot of a DNS-Cl solution was over spotted onto the sample spot. The plate was covered with two sheets of clean glass plate and kept in the dark for a reaction time of 45 min and then developed with a solvent of toluene:ethyl acetate (15:1) in one direction and with dichloromethane at rightangles for a second, over distances of 5 cm. The plate was observed under 365 nm UV light and the fluorescent DNS-NEA spot or the area with equivalent Rf values in blank or unknown samples was marked with a pencil. The thin layer marked area was scraped off and extracted with two 250 μL portions of ethyl acetate and the extracts pooled.

Fluorometric Determination—To reduce the working volume of sample solution required for fluorometric determination using a standard 1 cm × 1 cm × 5 cm cell, a 9 mm thick plastic stand was placed beneath the sample cuvette to allow the excitation beam to pass through the solution just above the bottom of the cuvette, and two pieces of glass plate (10 mm × 10 mm × 2 mm) were used to line opposite sides of the cuvette parallel to the excitation beam. The emission spectrum of a 470 μL sample solution was recorded over the range of 420 to 620 nm using an excitation wavelength of 336 nm.

Fluorescence Characteristics of Dansyl-N-Ethylaniline

A standard of 300 ng of EC was hydrolyzed, dansylated, and developed according to the procedure described. The DNS-NEA solution was determined directly or evaporated to dryness by a stream of nitrogen and redissolved in 500 μL of cyclohexane, acetone, or methanol. The DNS-NEA solutions in these different solvents were fluorometrically recorded to identify their excitation and emission wavelength maxima.

Optimization of the Hydrolysis Condition for the Conversion of Ethyl Centralite into N-Ethylaniline

A series of four 30 μL aliquots of a EC standard solution (1 mg/mL) were transferred to conical flasks and allowed to evaporate to dryness in a fume hood. To these were added 15 mL of phosphoric acid and the flasks loosely stoppered and heated in a glycerol bath at 140°C, 159°C, 168°C, or 178°C, respectively. Approximately 0.5 mL of each reaction mixture was transferred into a glass vial after a reaction time in the range 3 to 120 min and immediately cooled by immersing the vial in cold water. A 40 μL aliquot of each of the mixtures was then transferred to a reaction vial, made alkaline and extracted as described. The extracts were dansylated, chromatographically developed and their emission spectra recorded as described.

Recovery of Clean-Up Procedures

A 300 ng aliquot of EC (2 μg/mL) was cleaned-up using either the TLC procedure described or by means of an XAD-7 column [*12*] as follows: The EC solution was evaporated to dryness in a stream of nitrogen, and the residue was moistened with 10 μL of diethylether and dissolved in 500 μL pentane. The pentane solution was passed through the XAD-7 column and discarded. An additional 500 μL volume of pentane was used to ensure the quantitative transfer of any EC to the column. The column was then washed with 2 mL of pentane, which was again discarded. Ethyl centralite was eluted with two 200 μL portions of ethyl acetate. Slight pressure was required to completely elute the ethyl acetate. The ethyl acetate eluates were pooled and transferred to a reaction vial. The cleaned samples and various amounts (30 ng, 50 ng, 150 ng, 300 ng) of EC standards used for calibration were further treated as described. The recovery of EC was calculated from the fluorescence intensity of each sample and a calibration curve produced from the standards.

Choice of Extracting Solvent

Two 300 ng aliquots of EC were spiked onto separated precleaned cotton wool balls. Each cotton ball was extracted using the syringe elution procedure employing either diethylether or ethyl acetate as eluent. The extracts were then hydrolyzed, dansylated, and analyzed as described.

Two 300 ng aliquots of EC were cleaned directly by the TLC procedure using either diethylether or ethyl acetate to extract the scraped-off thin layer material. Each extract was then hydrolyzed, dansylated, and analyzed as previously described.

Two separate 300 ng aliquots of EC were spiked at different times on the back of a clean hand. In each instance the hand was then swabbed using either diethylether or ethyl acetate and processed as described using the corresponding solvent to elute the cotton ball and the TLC plate extract. Each extract was then hydrolyzed, dansylated, and analyzed as previously described.

For each stage of the experiment a set of EC standard samples (30 ng, 50 ng, 150 ng, 300 ng) and a blank sample were simultaneously submitted to hydrolysis, dansylation, and

analysis for the preparation of calibration data. The recoveries of each sample were calculated.

Optimization of the Derivatization Reaction

Three series of various aliquots of 100 ng N-ethylaniline were dansylated on TLC plates according to the procedure described above except that the reaction temperature was at room temperature, 53°C, or 70°C, respectively. The TLC plates were chromatographically developed after a reaction time in the range 5 to 60 min and the emission spectra of DNS-NEA spots were recorded as previously described.

Enhancement of Fluorescence

A 300 ng aliquot of EC was hydrolyzed and dansylated as described. The DNS-NEA solution was then diluted to a volume of 2.5 mL with ethyl acetate. A series of volumes in the range 100 to 2000 μL were taken and their spectra recorded using a standard cell with or without modification to reduce the sample volume required by means of a plastic stand or glass plate inserts described.

Analysis of Ethyl Centralite Standards

A number of samples containing 50 ng, 25 ng, 10 ng, 5 ng, 2 ng, 1 ng, and 500 pg ethyl centralite were analyzed according to the developed procedure using a blank reaction vial as a control.

Analysis of Spiked Hand Samples

Various amounts of EC (100 ng, 50 ng, 25 ng, 10 ng, 5 ng, 2 ng, 1 ng) were spiked onto the back of the precleaned hands of a number of subjects. These hands and their corresponding uncontaminated hand used as a blank were swabbed and the samples analyzed according to the developed procedure.

Analysis of Gunpowders and the Hands of Subjects Exposed to GSRs from Some Test Firings

Three blank samples were obtained by swabbing the shooters' left hands just prior to test firing. The shooters used both hands to fire the revolver. The firing regimen is listed in Table 2. After a given time interval in the range 30 to 180 min, both hands were swabbed and the swabs stored in tightly sealed glass vials at −23°C until analyzed.

The hand swabs were analyzed according to the developed procedure alongside two EC standard samples containing 1 ng and 5 ng.

To verify the presence of EC in the ammunition used, gunpowder from a round of ammunition of the same type was obtained. The gunpowder consisted of two kinds of flakes, one red and the other black. The quantity of each flake type was 10.7 mg and 394.7 mg respectively. One flake of each type was analyzed as follows: The flake was placed in an eppendorf tube and 200 μL of ethyl acetate added. The mixture was vigorously vortexed for 5 min and then centrifuged at 10,000 g for 5 min. An aliquot of 20 μL of the supernatant was then processed in the normal way.

Results and Discussion

Heating with phosphoric acid was found to be efficient for the hydrolysis of EC and the hydrolyzed product was verified to be N-ethylaniline from its IR spectrum.

The rate of hydrolysis is highly dependent on temperature as illustrated in Fig. 2. However, a high temperature results not only in quick hydrolysis of EC but also in drastic

TABLE 2—*Details and results of fluorescence detection of ethyl centralite in test firing hand swabs, hand blanks, and gunpowder flakes.*

Sample	Rounds Fired	Time Lapse	Hand Sampled	Result
GSR01-R			R (Right)	−
GSR01-L	1	30 min	L (left)	−
GSR02-R			R	+
GSR02-L	2	30 min	L	−
GSR03	3	30 min	R + L	−
GSR04-R			R	−
GSR04-L	6	30 min	L	+
GSR05	3	60 min	R + L	−
GSR06	3	180 min	R + L	+
Blank01	0	—	L	−
Blank02	0	—	L	−
Blank03	0	—	L	−
Powder Flake (Red)	—	—	—	+
Powder Flake (Black)	—	—	—	−

decomposition of any N-ethylaniline formed. A 15 to 20 minutes heating time at a temperature of 178°C represents a good compromise for minimizing the time of hydrolysis with maximizing the yield of N-ethylaniline.

The excitation and emission maxima of DNS-NEA in ethyl acetate are 336 nm and 495 nm respectively. The excitation maximum remains unchanged in different solvents, while the emission maximum, in general, shifts to longer wavelength and the quantum yield decreases with increasing dielectric constant of the solvent as shown in Fig. 1.

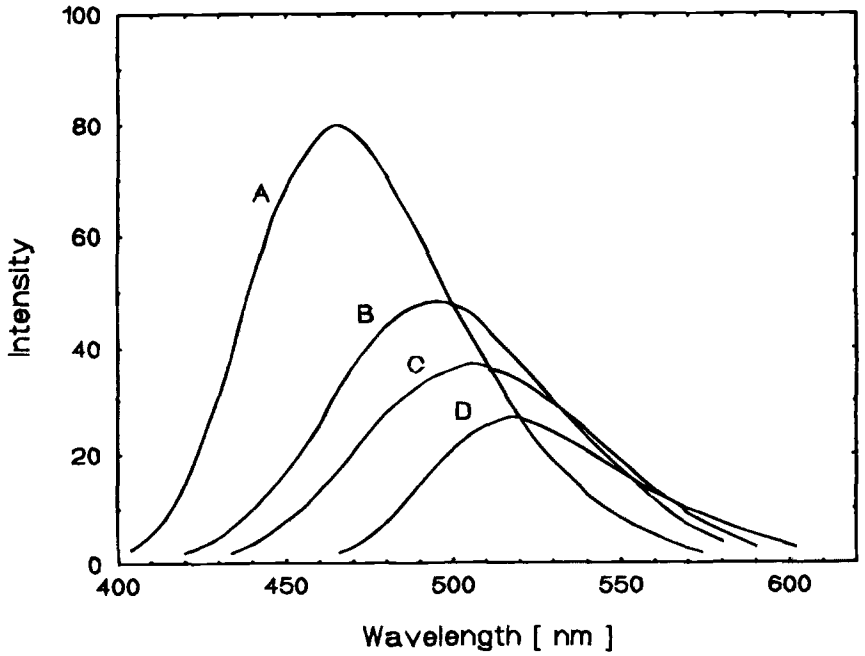

FIG. 1—*Emission spectra of DNS-NEA in different solvents, solvent A: cyclohexane, B: ethyl acetate, C: acetone, D: methanol,* λ_{EX}: 336 nm.

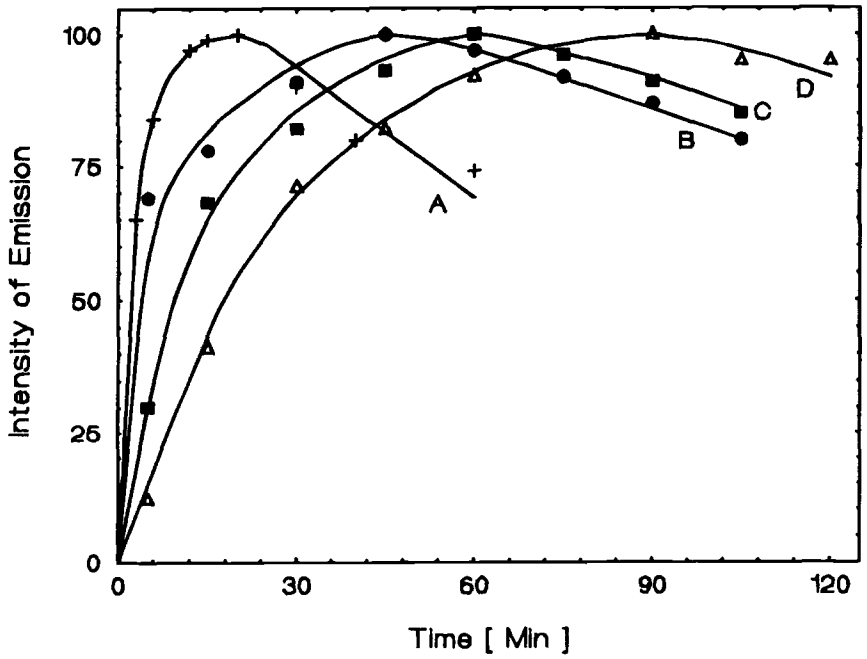

FIG. 2—*Influence of time and temperature in the hydrolysis of ethyl centralite, A: 178°C, B: 168°C, C: 159°C, D: 140°C,* λ_{EX}*: 336 nm,* λ_{EM}*: 495 nm.*

The recovery of EC using different clean-up procedures and different solvents at each stage of the analysis is shown in Table 3. Recovery using the TLC clean-up procedure is over 90% compared with only about 25% when an XAD-7 column was used. Thus the TLC method was chosen as the preferred clean-up procedure for the remainder of this study. The results in Table 3 also show that ethyl acetate is a better recovery solvent than diethylether for obtaining EC from spiked hands, cotton wool, and silica gel. Recoveries of EC from cotton wool and silica gel were over 90% when ethyl acetate was used, but these values were reduced to around 60% and 80% respectively when diethylether was used. Because of its high recovery, the syringe elution method using ethyl acetate as eluent was considered the most suitable for extracting EC from cotton wool swabs. The total recovery of EC from spiked hands after processing procedures is between 40% to 55% with ethyl acetate but is only between 25% to 35% when diethylether was used. Although it takes less time to evaporate diethylether than ethyl acetate in the procedures for sample

TABLE 3—*Recovery of EC using different clean-up methods and solvents.*

		Solvent						
		Ethyl Acetate Recovery (%)			Diethylether Recovery (%)			
Step	Method							
Extraction of Cotton Swabs	Syringe Elution	90.3	92.2	97.0	58.3	60.9	62.7	67.3
Clean-up	TLC Method	92.6	95.5		78.2	84.3		
	XAD-7 Column	22.9	25.6		—			
Whole Procedures	—	41.3	52.9	55.0	26.8	35.5		

concentration, the better recovery of low quantities of EC predicate that ethyl acetate should be the solvent of choice.

Experiments designed to assess the minimum working volume required for successfully recording a fluorometric spectrum made use of a plastic stand to the quartz cell, which allowed the lowest end of the excitation beam to pass through the solution just above the bottom of the cuvette. The length of the beam passing through the cuvette remained at 10 mm, so the level of liquid was restricted to 10 mm above the bottom of the cuvette. The useable dimensions of the cuvette were therefore 10 mm × 10 mm × 10 mm. It would be expected that the intensity of fluorescence for different volumes of the same concentration would remain constant. This was the case when the volume was reduced from 2000 to 1000 μL. However, a further reduction in volume size produced an enhancement in fluorescence which reached its maximum when the sample volume was 800 μL. Fluorescence intensity rapidly faded as the volume further decreased as illustrated in Fig. 3.

One possible explanation of this phenomenon is the optical properties of the meniscus of the sample solution, Fig. 4. When the excitation light rays traveling through the solution, strike the meniscus, part is refracted and comes back into the solution after travelling a distance in the air, while the remainder are totally reflected back into the solution. This results in a longer optical path than the original optical path of the cuvette. By this means both refracted and reflected rays contribute to the enhancement of fluorescence. When the volume is further decreased, the upper part of the beam passed through the air above the solution rather than the solution itself and the optical path of meniscus-reflected rays is shortened as the depth of solution decreases. This results in a rapid decrease of fluorescence. The maximum of fluorescence enhancement of DNS-NEA obtained by using smaller volumes of ethyl acetate solution was about 25%.

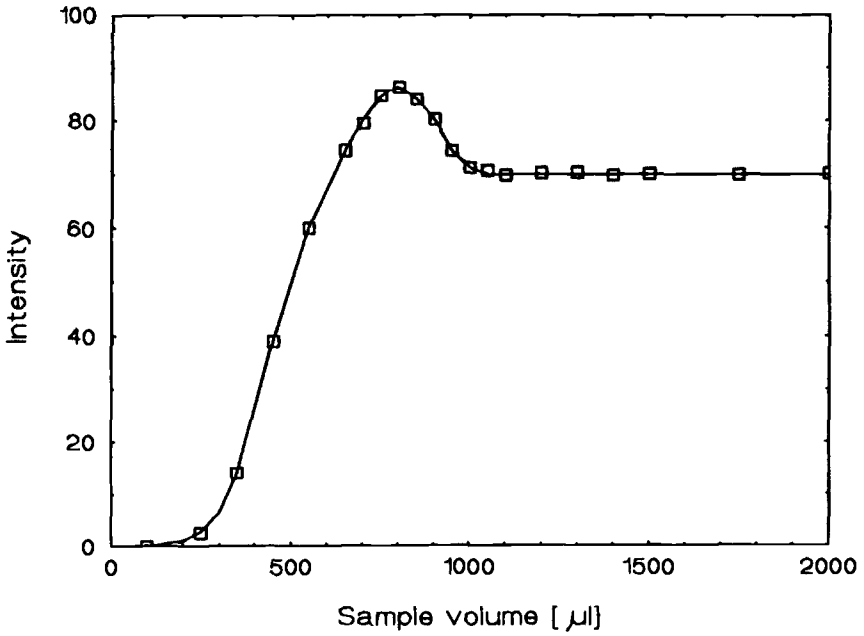

FIG. 3—*The effect of sample volume variation in the intensity of fluorescence,* λ_{EX}*: 336 nm,* λ_{EM}*: 495 nm.*

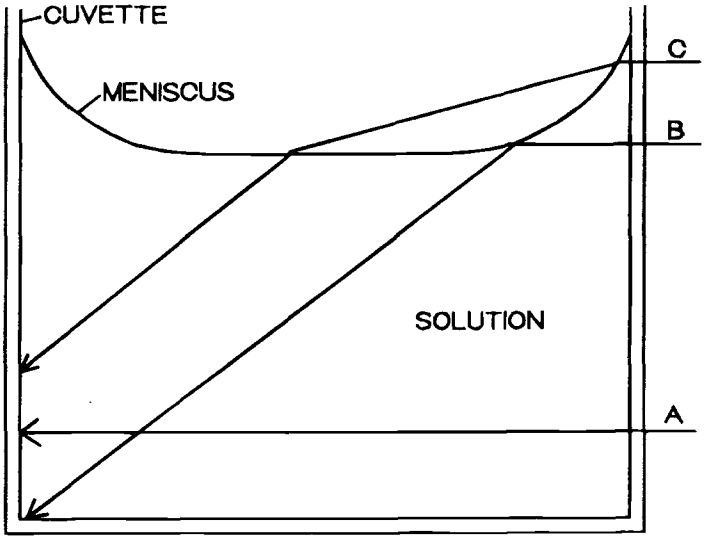

FIG. 4—*Optical paths of normal(A), reflected(B), and refracted(C) excitation rays in the sample solution.*

When the glass plates were introduced into the sample cell, the maximum enhancement happened at a sample volume of 470 μL. Thus 470 μL was considered the working volume for fluorometric determinations used in this work.

The Rf values of DNS-NEA after two-dimensional development are 0.45 ± 0.04 for the first direction and 0.49 ± 0.04 for the second direction. Dansylchloride is easily hydrolysed to DNS-OH on the silica gel layers, and this can be observed as blue fluorescent spots on the start line and blue fluorescent streaks on the chromatogram. When the quantity of EC is equal to or lower than 10 ng, the greenish yellow fluorescent spot of DNS-NEA cannot be distinguished from the fluorescent streak produced on first development. Thus the second development is essential for the separation of DNS-NEA spot from any hydrolyzed reagent. For this work, the detection limit was defined as: the minimum quantity of EC that leads to a emission spectra that is distinguishable from the fluorescence spectra of blank sample over the wavelength range of 420 nm and 620 nm. By using freshly distilled ethyl acetate for the extraction of DNS-NEA from the TLC plate to minimize interference the detection limits for EC standards and EC spiked hand samples were 1 ng and 5 ng respectively.

The reaction time for the derivatization of N-ethylaniline could be reduced from 40 min to 10 min when the reaction temperature was increased from room temperature to 70°C. Heating the TLC plates at 53°C and 70°C during derivatization also resulted in a minor increase in fluorescent yield, Fig. 5. Reaction temperatures greater than 70°C were not attempted because the reaction time of 10 min was considered to be acceptable and the heating of TLC plates resulted in a change of chromatographic activity of the silica gel. The Rf value of DNS-NEA for the first development was decreased as the reaction temperature and heating time increased. The Rf value of the second development was not affected. For one sample, approximately 2 h was required to accomplish the procedure from swab extraction to TLC development where a preliminary result was obtained. The average time required per sample was shorter at approximately 1.5 h for simultaneous processing of samples.

The results of analyses of gunpowder and test firing samples are shown in Table 2. For the gunpowder only the red flakes contained EC. The red flakes are usually considered as markers not different powders and the ratio of approximately 40:1 corresponds well to the

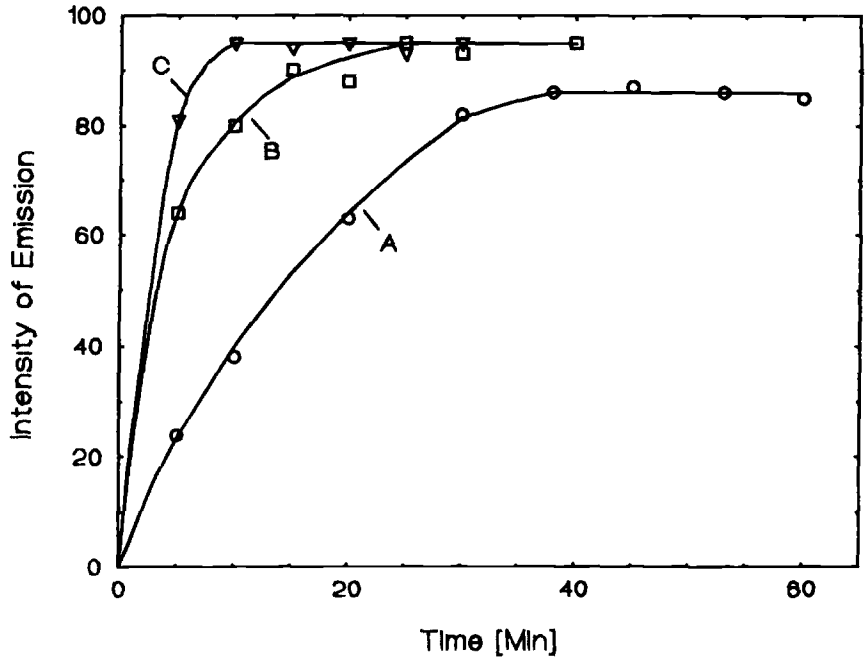

FIG. 5—*Influence of time and temperature in the dansylation of N-ethylaniline on TLC plate, A: room temperature, B: 53°C, C: 70°C.*

reported usage of about 2.5% markers. Normally this markers would be the same composition as the powder but in these tests they differed. In retrospect this was an unfortunate choice of powder for testing. There was no EC detected in any of the hand blank samples. Three out of six test firings were found to contain EC and one of these was swabbed 180 minutes after firing three rounds of ammunition. However, for the samples from the remaining three test firings the results were all negative. Emission spectra of three EC standards (1 ng, 5 ng, 10 ng), one test firing sample (GSR06), and one hand blank sample are shown in Fig. 6. One possible explanation for the variation of the results may be the fact that EC is only contained in the red gunpowder flakes which represent 1/40 of the total gunpowder present. These powders may be unevenly distributed and EC may be randomly rather than evenly deposited on the firing hand during the explosion. It is also possible that the negative results contained quantities of EC below the detection limit of this method. If this fluorescence detection technique can be utilized in an HPLC procedure rather than TLC analysis, then the detection limit could possibly be reduced from nanogram to picogram levels.

N-ethylaniline is a toxic substance and seldom exists in the environment. Even if it is present in the swabs, it will be expelled in the TLC clean-up step. Thus the fluorescence detection of EC through DNS-NEA is highly selective.

Although the sensitivity of this technique is still not high enough to detect EC in all test firing samples examined, the feasibility of the application of fluorescence detection to GSRs analysis is high.

Acknowledgments

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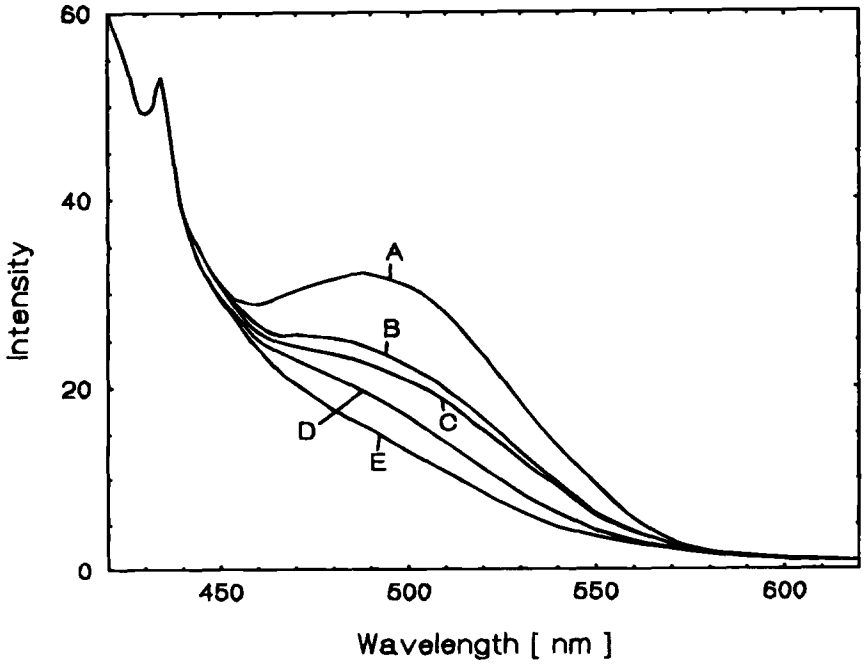

FIG. 6—*Emission spectra of EC standards (A: 10 ng, B: 5 ng, D: 1 ng), test firing sample (C: GSR06), and hand blank sample (E: Blank01),* λ_{EX}*: 336 nm.*

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Address requests for reprints or additional information to
Brian Caddy, Ph.D.
Forensic Science Unit
University of Strathclyde
204 George St.
Glasgow, G1 1XW
Scotland