TECHNICAL NOTE

Mark L. Miller,[1] Ph.D; Wayne D. Lord,[1] Ph.D.; M. Lee Goff,[2] Ph.D.; Brian Donnelly,[3] Ph.D.; Edward T. McDonough,[4] M.D.; and Jason C. Alexis,[5] B.S.

Isolation of Amitriptyline and Nortriptyline from Fly Puparia (Phoridae) and Beetle Exuviae (Dermestidae) Associated with Mummified Human Remains

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ABSTRACT: The use of anthropophagic fly larvae (maggots) as alternative toxicological specimens is well documented in the entomological and forensic science literature. Detection of various toxins and controlled substances in insects found on decomposing bodies has contributed to the assessment of cause/manner of death. With the development of hair extraction technologies, attention has recently focused on the analysis of chitinized insect remnants which are frequently encountered with mummified/skeletalized remains. In such cases, the standard toxicological specimens are often absent. Herein, we report the first detection of drugs from chitinized insect tissues.

KEYWORDS: toxicology, insects, entomology, drug extraction, amitriptyline

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[1] Research Chemist and Supervisory Special Agent, respectively, Forensic Science Research and Training Center, Laboratory Division, FBI Academy, Quantico, VA.

[2] Professor of Entomology, Department of Entomology, University of Hawaii at Manoa, Honolulu, HI.

[3] Supervisory Special Agent, Chemistry and Toxicology Unit, Laboratory Division, FBI, Washington, DC.

[4] Deputy Chief Medical Examiner, Office of the Chief Medical Examiner, State of Connecticut, Farmington, CT.

[5] Research Assistant, Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, MS.

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In October 1991, the mummified remains of a middle-aged white female were discovered at her residence in New England by a real estate foreclosure agent. A subsequent investigation revealed that the decedent had died of multiple drug intoxication in mid-January 1989. Toxicological analysis of desiccated brain and stomach contents revealed lethal concentrations of amitriptyline and nortriptyline. Empty fly puparia, cast beetle skins (exuviae) and beetle fecal material (frass) were analyzed for amitriptyline and nortriptyline using modified hair extraction techniques. Both amitriptyline and nortriptyline were isolated from the insects sampled. Drug concentrations were greater in fly puparia than in beetle specimens reflecting insect food source partitioning. Drug ratios were consistent with acute amitriptyline overdose.

Subsequently, controlled studies were conducted in the laboratory wherein rabbits were injected with .5, 1.0 and 2.0 median lethal dosages of amitriptyline. Fly larvae were then reared on harvested rabbit organs and empty fly puparia collected and analyzed. Resultant drug concentrations (parent drug: metabolite) demonstrated patterns of acute amitriptyline intoxication and supported case specimen findings.

Over the past decade, there has been a marked increase in drug-related deaths reported in the United States and in other countries. Occasionally, the deceased's remains go undiscovered for a period of several days or more [*1*]. In such cases, decomposition is often advanced and standard toxicological specimens are frequently lacking.

As a result, interest has focused on the potential use of carrion-feeding insects in situations where more traditional specimens, such as blood, urine, or organ tissue is not available. The use of anthropophagic fly larvae (maggots) as alternative toxicological specimens is well documented in the entomological and forensic science literature. Various toxins and controlled substances have been isolated from these insects and such analyses have contributed to the assessment of cause/manner of death [*2,3*]. With the development of hair extraction techniques, attention has recently focused on the analysis of chitinized insect remnants (fly puparia, beetle exuviae) which are frequently encountered with mummified/skeletalized remains.

Basic to understanding the applications of entomological evidence to forensic toxicology is an overview of the life cycles of carrion-frequenting insects. The insects most frequently involved in human toxicological analyses are the true flies or *Diptera.* The predominant species encountered are in the families *Calliphoridae* (blow flies), *Sarcophagidae* (flesh flies), and *Muscidae* (house flies).

The *Calliphoridae, Sarcophagidae,* and *Muscidae* are highly motile, strong flying insects. These flies are typically the first insects to arrive at the remains. *Calliphoridae* and *Sarcophagidae* may arrive within minutes following death, particularly in tropical environments. In more temperate climates, adult fly arrival may require several hours or days depending on the habitat, climatic conditions and method of remains disposal. Studies of carrion communities have demonstrated that muscoid flies generally delay colonization until the late fresh or early bloat stages of decomposition [*4*].

Once a suitable source is located, adult flies will either begin to lay eggs (oviposit) immediately or feed on the various protein-rich fluids seeping from the remains and then begin to oviposit. On corpses that have not suffered traumatic injury, the initial sites of oviposition will generally be the natural body openings (eyes, ears, nose, mouth, and if exposed, anus and the genitalia). Wounds or blood may provide preferential sites for oviposition although this attraction will vary depending on the species of fly involved and the degree of injury.

Blow fly eggs are small (2 to 3 mm) long, elongate, and white to yellow in color. They are typically laid in large clusters and, during warmer parts of the year, may completely fill natural body openings and wound sites. During cooler periods, when adult fly populations

are smaller, the eggs may be few in number and more difficult to locate, being hidden in cryptic sites, such as the inside of the nose or behind the eyelids. Typically the egg stage for blow flies lasts from 1 to 3 days.

When the eggs hatch, they produce larvae, commonly referred to as maggots. These are small, somewhat peg-shaped organisms. The anterior end has a pair of mouthhooks which are used in both feeding and locomotion. The posterior end bears a pair of flattened spiracles through which the maggot breaths. These features, along with size and shape, provide important characters for identification. Maggots grow rapidly, passing through three stages or instars before reaching full size. Eggs laid on a body will typically hatch synchronously, resulting in a mass of maggots which move about the remains feeding as a group. This group feeding behavior results in the dissemination of bacteria and a production of digestive enzymes which enable the maggots to consume most of the soft tissue parts of the remains in a highly efficient manner. Maximum maggot size is reached in a period varying from several days to weeks, depending on the species involved, numbers of maggots present, and environmental conditions.

After reaching maximum size, maggots undergo a dramatic change in behavior. Feeding ceases and the maggots begin to migrate away from the remains. This migration is typically to a drier environment where the maggots burrow into the substrate and begin the process of pupariation. During the pupariation, the outer skin of the maggot becomes hardened and forms a protective encasement. The resulting reddish to dark brown puparium somewhat resembles a small football. Within this protective case, the maggot undergoes a cellular reorganization (metamorphosis) and eventually emerges as an adult fly. The period of time that is required for this transformation varies depending on the species of fly and environmental conditions, particularly temperature. The puparial case itself may be found in the soil surrounding the remains for hundreds of years [*5*]. Blow fly puparial cases can supply valuable forensic information long after the body has decomposed [*6*].

The life cycle of the *Sarcophagidae* or flesh flies is quite similar to that of the blow flies, with one important difference. Flesh flies associated with decomposing remains do not lay eggs (oviposit) but rather deposit first instar larvae (larviposition) on remains. This process requires that the developing fly larvae be retained in the body of the female longer than an egg. Thus the numbers of offspring produced per female are less for *Sarcophagidae* than for the *Calliphoridae* and *Muscidae*. In total numbers of maggots present on a body, generally the *Sarcophagidae* are far outnumbered by other species or flies. There are exceptions to this in specialized circumstances, as may occur in remains found indoors or exposed to severe environmental conditions [*7,8*].

Following the invasion of the corpse by blow flies, other types of flies including house flies, flesh flies, skipper flies, fruit flies and coffin flies also colonize the remaining tissues. Carrion, rove, clown, sap, checkered, scarab and dermestid beetles also become members of the host corpse community feeding and rearing their young on the drier remaining tissues and on the large maggot mass. The corpse eventually comes to support a complex and diverse community of insects often numbering hundreds of species and thousands of individuals.

Case History

In October 1991, the mummified remains of a middle-aged white female were discovered at her residence in New England by a real estate foreclosure agent. Numerous prescription vials, mostly empty, were found near the decedent. These included ampicillin, doxycycline, Ceclor, erythromycin, Lomotil, Elavil, pentazocine, and Tylenol® 3. Blue ovoid tablets labelled "Rugby 0230" were also found. The body was intensely mummified with evidence of considerable insect activity and some loss of tissues (Fig. 1).

Externally, the body was found to be covered with a combination of mummified integument, adipocere, and filamentous insect frass. Internally, a variety of mummified organs

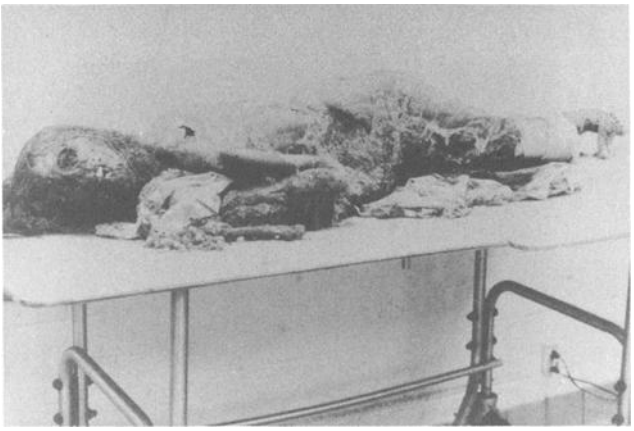

FIG. 1—*Mummified remains of a middle-aged, white female discovered during October 1991, at her New England residence.*

were present, many of which displayed extreme insect destruction. Within the stomach, approximately 50 cc, of granular material admixed with small fly larvae was retrieved. Within the skull partially preserved brain tissue was found. No evidence of antemortem trauma or significant disease was noted during autopsy.

A subsequent investigation, including a detailed victimology, revealed that the decedent had died of multiple drug intoxication in January 1989. Toxicological analysis of desiccated brain and stomach contents revealed lethal concentrations of amitriptyline (25.38 mg/kg-brain; 4.25 mg/38 gms-stomach contents) and nortriptyline (8.14 mg/kg-brain; 0.28 mg/38 gms-stomach contents). Diphenhydramine (1.67/mg/kg-brain; 0.09 mg/38 gms-stomach contents) and cocaine (trace only) were also detected in lesser amounts. Empty fly puparia, beetle exuviae and beetle fecal material (frass) were collected at the scene and at autopsy for entomo-toxicological analyses. Additionally, controlled studies were undertaken to correlate the presence of drugs in tissues with the levels detected in fly puparia and other chitinous insect remnants.

Toxicological Methods

The dry empty fly puparia, cast beetle skins and beetle fecal material (frass) were collected from the scene and at the autopsy and forwarded to the FBI Laboratory for extraction and analysis (of amitriptyline and nortriptyline.) Two extraction techniques were employed. One technique utilized a strong acid extraction and the other a strong base extraction.

Extraction of Basic Drugs from Puparial Cases with Acid and SPE

Approximately 100 to 200 mg of puparial cases were weighed out and placed in a test tube. The test tube had been previously rinsed with nanopure water and methanol before weighing out samples. Three or four milliliters (enough to cover casings) of 1 N hydrochloric acid were added to the samples. Internal standards were added to each tube: 970 ng of d_6-amitriptyline and 1760 ng of d_3-nortriptyline. The puparial casings were crushed to expel any trapped air and extracted overnight at 65°C.

Once extraction was complete, the acid solutions were removed from the puparial casings and adjusted to pH 4 using concentrated ammonium hydroxide. Nanopure water was added to adjust the total volume to 5 mL.

Tox-Clean RC SPE tubes (Alltech-Applied Science Labs) were used for sample clean up and concentration according to the procedure in Alltech Bulletin No. 175 for urine drug screening. To each solution 150 microliters of 1 N acetic acid was added. The Tox-Clean tube was conditioned with 2 mL of methanol, 2 mL of deionized water followed by 2 × 0.5 mL aliquots of 1 N acetic acid. Then the extract solutions were passed through the conditioned tubes at a rate of approximately 1 mL/minute. The Tox-Clean tubes were washed with 2 mL of deionized water and the columns were dried by passing air through them at full vacuum for 15 minutes. The tubes were washed with 2 mL of hexane and then the acid/neutral fraction was eluted by passing 3 mL of methylene chloride through them. The basic drug fractions were collected in clean test tubes by passing 2 mL of basic methanol (2% NH_4OH) through the Tox-Clean tubes. To each test tube 3 mL of deionized water was added along with 200 microliters of methylene chloride. Samples were vortexed 15 seconds and then centrifuged 5 minutes. The methylene chloride layer was removed for GC/MS analysis.

Extraction of Basic Drugs Using a Strong Base

Approximately 80 mg of puparial cases were weighed out in a test tube that was previously rinsed with deionized water and methanol. Two milliliters of 0.1 N NH_4OH was added to cover the puparial cases. To this was added 100 microliters of internal standards: 970 nanograms of d_4-amitriptyline and 880 nanograms of d_3-nortriptyline. Test tubes were capped, placed on a lab shaker and heated at 80°C for one hour. After the solution was heated, 10 mL of n-butyl chloride was added to each test tube and samples were rotated on a lab quake shaker for 30 minutes. Samples were then centrifuged at half speed (approximately 2500 rpm). Supernatant was collected and back extracted with 5.0 mL of 1.0 N HCl on a lab quake shaker for 20 minutes. Tubes were then centrifuged for 10 minutes at 2500 rpm. Subnatant was collected and the pH was raised by adding 1 mL of concentrated ammonium hydroxide. 3.0 mL of chloroform was added to the tubes with the subnatant, capped and then vortexed. The tubes were then centrifuged for 10 more minutes. Subnatant was collected and filtered through a pipette that was prepared with a plug of glass wool and 0.5 inches of sodium sulfate. The chloroform layer was dried down under nitrogen gas. The concentrate was reconstituted in methanol (10 microliters) for GC/MS analysis. Strong base extraction was utilized on collected samples of cast beetle skins (exuviae) and beetle fecal material (frass).

Additionally, laboratory rabbits were injected with zero, 0.5, 1.0, and 2.0 median lethal dosages of amitriptyline. After the drug was administered, the rabbits were sacrificed and the livers harvested for feeding to flesh fly (*Parasarcophaga ruficornis*) larvae. The larvae were reared to adulthood, and the empty fly puparia were collected and analyzed for amitriptyline and nortriptyline using the forementioned techniques.

Both fly and beetle specimens collected from the decedent's remains were forwarded to the Department of Entomology, United States National Museum, Smithsonian Institute, Washington, D.C., for confirmation of their species identifications (Fig. 2.). Fly puparia were subsequently confirmed as *Megaselia scalaris* (Loew) (Diptera: Phoridae) and the beetles as *Dermestes maculatus* (DeGreer) (Coleoptera: Dermestidae). Both are common inhabitants of mummified remains.

Results and Discussion

The use of insects and their remnants as forensic indicators of time since death, relocation of remains, and antemortem injury has been well established. Only recently, however, have insect puparia and exuviae been recognized as potential alternative toxicological resources [*9–13*]. Development of new methodologies for extracting and quantifying toxins and

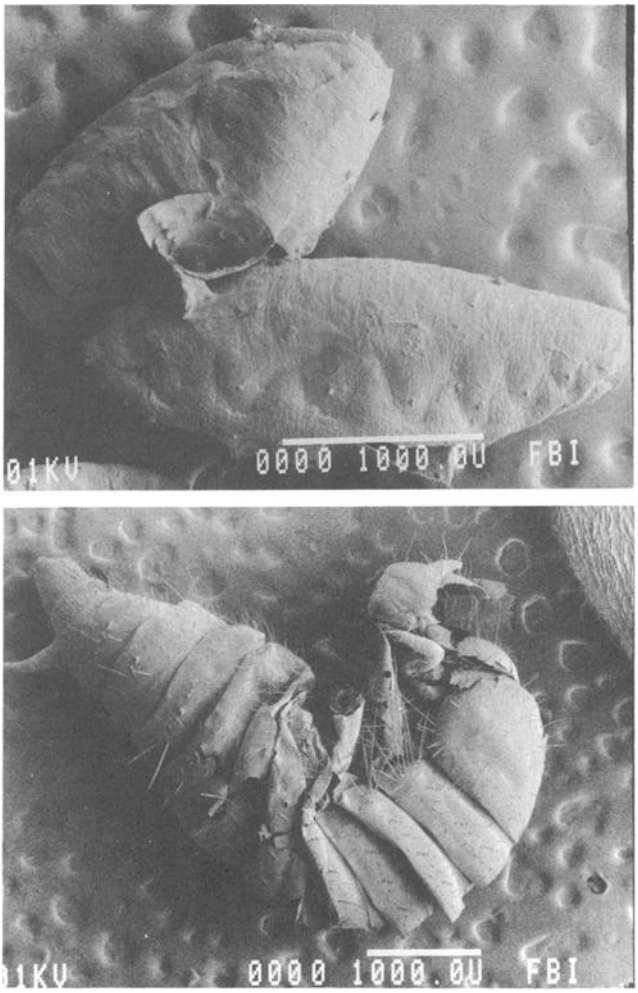

FIG. 2—*Scanning electron micrographs of Megaselia scalaris (Diptera: Phoridae) puparium (A) and Dermestes maculatus (Coleoptera: Dermestidae) exuvia (B). Magnification 1000x.*

controlled substances from such remnants continues to prove important particularly in cases of advanced decomposition where traditional toxicological specimens are lacking.

A key difference between the extraction of insect puparia/exuviae and standard pathological samples lies in the need to break down a rigorous chitin/protein matrix so that drugs and toxins can be released. Chitin, a complex polysaccharide composed mostly of n-acetylglucosamine and glucosamine, constitutes 25 to 50% of the dry weight of insect exoskeletons. The remainder consists largely of protein. Chitin sugars form chains which are embedded in the surrounding protein matrix, forming extremely resilient microfibers (micelles) [*14*]. Chitin remnants are shed by forensically important insects during molting (exuviae) and metamorphosis (puparia). Both exuviae and puparia are known to survive in the environment long after softer tissues have decomposed.

The use of either strong acids or bases to break down the chitin/protein matrix may be central to drug/toxin release. After the acid or base treatment and pH adjustment, some of the more routine drug screen extraction methodologies can be employed for analyte isolation.

GC/MS with chemical ionization was utilized to detect the presence of amitriptyline and its metabolite nortriptyline in extracts studied here.

Quantitative extraction data for both our case and controlled study samples is presented in Tables 1, 2, and 3. It should be emphasized that the use of either the strong base or strong acid extraction protocol resulted in the successful isolation of amitriptyline and its metabolite, nortriptyline, from chitinous fly puparia. Base extraction successfully isolated amitriptyline from beetle exuviae and fecal material. We suggest, therefore, that this tech-

TABLE 1—*Case findings for concentrations of Amitriptyline and Nortriptyline detected in decedent and insect tissue specimens by GC/MS.*

Sample	Amitriptyline ng/mg	Nortriptyline ng/mg	Ratio Ami/nor
Dermestid skins	3.4	ND	
Dermestid frass	3.6	ND	
Phoridae puparia	5.4	2.5	2.2
Brain tissue	25.4	8.1	3.1
Stomach contents	112	7.4	15

ND = None detected.

TABLE 2—*Controlled study findings for concentrations of Amitriptyline and Nortriptyline detected in empty flesh fly* (Sarcophagidae) *Puparia following exposure to livers from rabbits previously injected with .5, 1.0, and 2.0 median lethal doses of Amitriptyline. Acid extraction protocol followed by GC/MS.*

Median Lethal Dose	Amitriptyline ng/mg	Nortriptyline ng/mg	Ratio Ami/nor
0	1.0	ND	
0.5	9.9	5.7	1.7
1.0	169	51.4	3.3
2.0	7.0	2.5	2.8

ND = None detected.

TABLE 3—*Controlled study findings for concentrations of Amitriptyline and Nortriptyline detected in empty flesh fly* (Sarcophagidae) *puparia following exposure to livers of rabbits previously injected with .5, 1.0, and 2.0 median lethal doses of amitriptyline. Base extraction protocol followed by GC/MS.*

Median Lethal Dose	Amitriptyline ng/mg	Nortriptyline ng/mg	Ratio Ami/nor
0	ND	ND	
0.5	19.7	7.0	2.8
1.0	154	31.2	4.9
2.0	7.9	Trace	

ND = None detected.

nique may be applicable to other forensically important toxins and pharmaceuticals, and to other insect species.

Amitriptyline concentrations were greater in puparia than exuviae or frass. This most likely reflects the food source preferences characteristic of the carrion flies and beetles examined. Flesh flies (*Sarcophagidae*) and scuttle flies (*Phoridae*) have a propensity for soft tissues where acute drug concentrations are likely to be higher. While skin beetles (*Dermestidae*) feed primarily on dried integument.

The ratio of amitriptyline to nortriptyline in all puparia examined reflected a pattern consistent with acute drug exposure (parent drug concentration > metabolite concentration) [*15,16*]. Nortriptyline concentrations were detected at a level of approximately one fifth that of the parent compound. These findings were similar to the ratios of amitriptyline/nortriptyline found in decedent brain tissue and dry stomach content samples. All toxicological results were suggestive of acute amitriptyline overdose. Case investigative information, developed independently, supported these conclusions.

In our laboratory studies, the highest level of amitriptyline was found in the puparia of flies reared on tissues from the 1.0 median lethal dosage rabbit. This may reflect the fact that the rabbit given the 2.0 median lethal dosage died rapidly from an overdose. The sudden onset of death may not have allowed enough time for the drug to circulate as fully to the liver as did the other dosages. This idea is also supported by the fact that only a trace of nortriptyline was found in the 2.0 median lethal dosage fly puparia.

In summary, our results further demonstrate how insects and other arthropods can prove to be valuable tools in investigations of homicide, suicide, or other unattended deaths. In addition to the recognized applications (estimation of postmortem intervals), insects may serve as reliable alternate specimens for toxicological analyses in the absence of tissues and fluids normally taken for such purposes. While our data are limited, medical examiners, toxicologists, and other forensic practitioners should be cognizant of the potential use of these organisms as nontraditional toxicologic specimens.

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References

[*1*] Goff, M. L., Brown, W. A., Amori, A. I., and La Pointe, D. A., "Preliminary Observations of the Effects of Amitriptyline in Decomposing Tissues on the Development of *Parasarcophaga ruficornis* (Diptera: Sarcophagidae) and Implications of this Effect to Estimation of Postmortem Interval," *Journal of Forensic Sciences*, Vol. 38, No. 2, March 1993, pp. 316–322.

[*2*] Goff, M. L. and Lord, W. D., "Entomotoxicology: A New Area of Forensic Investigation," *American Journal of Forensic Medicine and Pathology*, 1993, in Press.

[*3*] Nuorteva, P. and Nuorteva, S. L., "The Fate of Mercury in Sarcosaprophagous Flies and Insects Eating Them," *Ambio*, Vol. 11, 1982, pp. 34–37.

[*4*] Catts, E. P. and Haskell, N. H., Eds., *Entomology & Death: A Procedural Guide*, Joyce's Print Shop, Inc., Clemson, SC, 1990.

[*5*] Gilbert, B. M. and Bass, W. M., "Seasonal Dating of Burials from the Presence of Fly Pupae," *American Antiquity*, Vol. 32, 1967, pp. 534–535.

[*6*] Nuorteva, P., "Empty Puparia of Phormia Terraenovae R.D. (Diptera, Calliphoridae) as Forensic Indicators," *Annals of Entomology*, Fenn. 1987, Vol. 53, pp. 53–56.

[*7*] Goff, M. L., "Comparison of Insect Species Associated with Decomposing Remains Recovered Inside Dwellings and Outdoors on the Island of Oahu, Hawaii," *Journal of Forensic Sciences*, Vol. 36, 1991, pp. 748–753.

[8] Lord, W. D. and Burger, J. F., "Arthropods Associated with Herring Gull (*Larus argentatus*) and Great Black-backed Gull (*Larus marinus*) Carrion on the Islands in the Gulf of Maine," *Environmental Entomology,* Vol. 13, 1984, pp. 1261–1268.

[9] Wilson, Z., Hubbard, S., and Pounder, D. J., "Drug Analysis in Fly Larvae," *American Journal of Forensic Medicine and Pathology,* Vol. 14, 1993, pp. 118–120.

[10] Beyer, J. C., Enos, W. F., and Stajic, M., "Drug Identification Through Analyses of Maggots," *Journal of Forensic Sciences,* Vol. 25, No. 2, April 1980, pp. 411–412.

[11] Introna, F., Lo Dico, C., Caplan, Y. H., and Smialek, J. E., "Opiate Analysis in Cadaveric Blowfly Larvae As an Indicator of Narcotic Intoxication," *Journal of Forensic Sciences,* Vol. 35, No. 1, Jan. 1990, pp. 118–122.

[12] Kintz, P. B., Godelar, B., Tracqui, A., Mangin, P., Lugnier, A. A., and Chaumont, A. J., "Fly Larvae: A New Toxicological Method of Investigation in Forensic Medicine," *Journal of Forensic Sciences,* Vol. 35, No. 1, pp. 204–207.

[13] Nolte, K. B., Pinder, R. D., and Lord, W. D., "Insect Larvae Used to Detect Cocaine Poisoning in a Decomposed Body," *Journal of Forensic Sciences,* Vol. 37, No. 4, 1992, pp. 1179–1185.

[14] Chapman, R. F., "The Insects: Structure and Function," *Elsevier Publishing Company,* New York, NY, 1971, pp. 425–448.

[15] Bailey, D. N. and Shaw, R. F., "Tricyclic Antidepressants: Interpretation of Blood and Tissue Levels in Fatal Overdose," *Journal of Analytical Toxicology,* Vol. 3, 1979, pp. 43–46.

[16] Spiehler, V., Spiehler, E., and Osselton, M. D., "Application of Expert Systems Analysis to Interpretation of Fatal Cases Involving Amitriptyline," *Journal of Analytical Toxicology,* Vol. 12, 1988, pp. 216–224.

Address requests for reprints or additional information to
Wayne D. Lord, Ph.D.
FSRTC
FBI Academy
Quantico, VA 22135