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REVIEW ARTICLE

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The detection of toxic substances in entomological specimens

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Abstract Entomotoxicology (the study of drugs in insects), a new field of forensic investigations, has still to be precisely defined especially with respect to the toxic substances that can be assessed in entomological specimens. The aim of the present work was to review the relevant entomological publications in order to analyse and describe the various toxic substances that have been detected in biological specimens. Experimental studies have been reviewed separately from case reports. Toxic substances have been classified according to forensic toxicology methodology and on the basis of the chemical and analytical features. This classification will help investigators to identify the compounds that can be found in such biological samples and may stimulate new analytical research investigations. Given the heterogeneity of specimens, the use of non-specific tests (such as immunoenzyme assays) is not recommended and specific and sensitive techniques are suggested. Methods such as GC-MS and HPLC-MS allow the exact identification of the toxic substances and their metabolites.

Keywords Entomotoxicology · Drugs · Larvae · Pupae · Puparial cases

Introduction

The term forensic entomology, although not strictly defined, is generally applied to the study of insects and other arthropods associated with certain suspected criminal events. The objective of forensic entomology is to answer the three questions, what is the post-mortem interval, the cause of death and the site of death.

Entomotoxicology is a specialised area of entomology and forensic science that deals with the qualitative and/or quantitative determination of toxic substances (e.g. drugs, environmental pollutants, pesticides) in insects/arthropods feeding on human remains.

Insects are a useful source of samples for toxicological analysis when the tissues or body fluids normally used are not available. Qualitative determination of toxic substances and drugs can add information on both the cause of death and the geographical area where the cadaver had been laying [1]. Since toxic substances can modify the biological cycle of arthropods, the quantitative determination provides an additional parameter for evaluating the post-mortem interval [2, 3, 4].

The history of entomotoxicology is relatively short. The first reports were by Sohal and Lamb in the late 1970s, who demonstrated the accumulation of different metals, including copper, iron, zinc and calcium, in the tissues of adult house flies [5, 6]. In 1982, Nuorteva and Nuorteva described the recovery of mercury from various species of calliphorid maggots fed on fish tissues containing known concentrations of mercury [7]. Detection of the pesticide malathion was reported by Gunatilake and Goff [8]. In 1980, the first article about a drug, phenobarbital, was published by Beyer et al. [9]. In entomological cases, other authors have also analysed drugs and narcotics, in particular triazolam, oxazepam, phenobarbital, alimemazine, clomipramine [10], bromazepam, levomepromazine and clomipramine [11], morphine and phenobarbital [12], cocaine [13, 14], amitriptyline, propoxyphene and acetaminophen [15], opiates [16, 17, 18, 19], amitriptyline and nortriptyline [20, 21, 22], amitriptyline, temazepam, trazodone and trimipramine [23], salicylates, paracetamol, aminohippurate, amphetamines and barbiturates [24], and amphetamine derivatives [25].

Examination of entomotoxicology publications helps to evaluate the possibilities offered by toxicological chemistry in the field of entomotoxicology, and to define the future applications of this branch of entomology.

Entomotoxicological organic materials

The insects most frequently involved in human toxicological analyses are true flies or *Diptera* (the main species

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encountered belong to the families *Calliphoridae* or blow flies, *Sarcophagidae* or flesh flies, *Muscidae* or houseflies), and beetles (*Dermestidae*).

The organic materials of entomotoxicological interest which can be analysed are larvae, pupae, adult insects, puparial cases, exuviae (cast beetle skins), beetle faecal material (frass), fly predators and scavengers.

The methods for chemical toxicological analysis take into account the substances to be analysed, inorganic (metals) or organic (drugs and pesticides), and the type of biological material to be examined for these substances, as well as major and minor affinities to organic solvents, which depend on lipid, protein and cartilaginous components.

Arthropods are homogenised and then processed in the same manner as human tissues or fluids of toxicological interest.

Metals in larvae

Mercury has been found in adult flies emerging from larvae reared on tissues containing methyl mercury, by neutron activation analysis (NAA) and flameless atomic absorption spectroscopy (FAAS) analysis. Moreover, a clear bioaccumulation process was observed, which ultimately resulted in an average mercury content 4.3 times higher than that in the food given to the larvae. Although some larvae had difficulty in beginning the process of pupation, the overall development was not altered [7]. Sohal and Lamb detected other metals, copper, iron, zinc and calcium, in the tissues of adult flies, in a similar manner [5, 6].

Pesticides in larvae

Analysis of fly larvae found on the decomposing human remains of a male suicide showed the presence of an organophosphate, malathion. While the level of malathion present in larvae was substantially higher than the LD_{50} established for adult flies, data are lacking on the effect of this substance on fly larvae.

Malathion was detected in pooled larvae (2050 μg/g) by gas chromatography with nitrogen-phosphorus detection following solvent extraction. It was also observed that the presence of malathion on remains may alter the rate of development of fly larvae feeding on them and delay oviposition for several days; this was supported by the presence of only two species of fly larvae on the remains. All these factors contribute to shorten the estimated post-mortem interval (PMI) [8].

Drugs in larvae

Following the work of Beyer et al. [9], demonstrating the presence of phenobarbital in larvae found on a skeletonised female body, numerous experiments were carried out by other groups [10, 11, 12, 13, 14, 15, 16, 20, 21, 23, 24] to detect controlled substances and drugs (e.g. opiates, benzodiazepines, antidepressants, cocaine, acetylsalicylic acid and amphetamine derivatives) and to gain useful information on the cause of death, when the toxicologist was unable to examine the usual biological fluids and tissues. Introna et al. [17] suggested a quantitative correlation between the level of opiates present in larvae and the liver, while Goff et al. demonstrated a correlation between 3,4-methylendioxymethamphetamine concentrations in skeletonised tissues and larvae [25]. Moreover, it was demonstrated that heroin accelerates the growth of larvae, while the pupal stage lasts longer. The total times required for development from larval to adult stages were found to be longer in colonies feeding on tissues containing heroin [18].

The articles examined, and related references, are detailed in Table 1.

Metals and pesticides in pupae

Although pupae are suitable for analysing metals and pesticides, there are no specific reports on these substances in the literature.

Drugs in pupae

Experiments have been carried out in pupae for the detection of amitriptyline and nortriptyline [21], and MDMA and its metabolite, MDA [25]. The presence of a parent drug, in the absence of its metabolite, can serve to confirm acute intoxication.

In some reports, pupae were analysed for drug content, but none were detected [15, 24]. The reported absence of some drugs in pupae may either be attributable to the elimination of drugs during the larval stage, or to a concentration below the detection limits of the methods used. In practice, the sharp decrease in drug concentrations in non-feeding larvae and at pupation makes it only worthwhile to sample larvae actively feeding on a corpse [23].

Entomological publications on drugs in pupae are detailed in Table 2.

Drugs in puparial cases

The presence of empty pupae could provide useful forensic information because they can be found in the proximity of human remains even after many years [14, 19, 26].

With the development of hair extraction technologies, attention has recently focused on the extraction of drugs from chitinised insect remains, which are frequently encountered in mummified or skeletonised remains. An analytical technique similar to that for extraction of drugs from hairs is employed. Miller et al. [20] described the first detection of drugs (amitriptyline) from chitinised insect tissues (puparial cases and exuviae), associated with mummi-

Table 1 Entomotoxicology publications on drugs in larvae: experimental or case report, analytical methods adopted, results of drug concentrations in larvae (*MS* mass spectroscopy, *RIA* radioimmunoassay, *EIA* enzyme immunoassay, *HPLC* high-performance liquid chromatography, *FPIA* fluorescence polarisation immunoassay, *NPD* nitrogen-phosphorus detection *MLD* median lethal dose)

Toxic drugs detected and reference	Analytical findings
Barbiturates: phenobarbital [9]	Case report Analytical method: GC/MS Results: 100 μg/g
Opiates: morphine [17]	Experimental report: larvae were reared on 40 different samples of liver collected from bodies in which initial blood testing produced a positive opiate result Analytical methods: RIA, GC/MS Results: 8–1208 ng/g
Bromazepam, levomepromazine [11]	Case report Analytical methods: EIA, HPLC Results: bromazepam 0.81 μg/g, levomepromazine 45 ng/g
Morphine, phenobarbital [12]	Case report Analytical methods: FPIA, GC Results: morphine 0.18 μg/g, phenobarbital 0.50 μg/g
Benzodiazepines, phenobarbital, clomipramine, alimemazine [10]	Case report Analytical methods: FPIA, HPLC Results: triazolam 204 ng/g, oxazepam 153 ng/g, phenobarbital 761 ng/g, clomipramine 28 ng/g, alimemazine 22 ng/g
Opiates: heroin [18]	Experimental report Analytical method: RIA Results: positive for morphine and codeine. Flesh fly larvae feeding on tissues containing morphine developed more rapidly than those feeding on tissues from control. The differences observed in the rates of development were sufficient to alter post-mortem interval estimates based on larval development by up 29 h
Cocaine and metabolite [14]	Case report Analytical methods: RIA, GC/NPD and GC/MS Results: cocaine 0.49 μg/g, metabolite 0.03 μg/g
Amitriptyline, nortriptyline [22]	Experimental report: *Sarcophagidae* larvae were reared on tissues from rabbits administered different dosages of amitriptyline, to study the effects of this drug on the development of these insects. The rabbits were given 300, 600 (median lethal dose), and 1000 mg of amitriptyline Analytical method: HPLC Results: positive results (larval stage was longer)
Propoxyphene, acetaminophen, amitriptyline, nortriptyline [15]	Case and experimental report Analytical methods: acetaminophen, amitriptyline, nortriptyline, HPLC; propoxyphene, GC/NPD Results: propoxyphene 0.06 μg/g, acetaminophen 0 μg/g, amitriptyline 0.28 μg/g, nortriptyline 0.18 μg/g
Opiates: morphine, codeine [16]	Case report Analytical method: GC/MS Results: morphine 90 ng/g, codeine 12 ng/g
Amitriptyline, temazepam, trazodone, trimipramine [23]	Case and experimental report Analytical methods: amitriptyline, GC/MS; other drugs, HPLC Results: amitriptyline 0.10 μg/g, temazepam 0.52 μg/g, trazodone 0.13 μg/g, trimipramine 0.28 μg/g
Amitriptyline, nortriptyline [21]	Experimental report: *Calliphora vicina* larvae, reared on artificial foodstuffs spiked with 100 ng/g (human therapeutic dose), 300 ng/g (toxic), 500 ng/g (lethal) concentrations of amitriptyline, nortriptyline were harvested at various stages of development and analysed for drug contents Analytical methods: HPLC and GC/MS Results: there was a large degree of biological variation in larval drug concentration, therefore any quantitative extrapolation from drug concentrations measured in larvae and in the food source is unreliable

Table 1 (continued)

Toxic drugs detected and reference	Analytical findings
Acetylsalicylic acid, paracetamol, aminohippurate, amphetamine, biturates [24]	Experimental report: *Calliphora vicina* larvae were reared on artificial foodstuffs, spiked with a range of concentrations of different barbiturates and analgesics. Larvae were harbar vested at different days of development and analysed for drug contents Analytical method: HPLC Results: human therapeutic dose showed only phenobarbitone; human LD (lethal dose) showed acetylsalicylic, amphetamine, amylobarbitone, barbitone, paracetamol; LD of other barbiturates was lethal to larvae
MDMA (ecstasy) and MDA (metabolite) [25]	Experimental report: rabbits were given 22.5 mg (1.0 MLD) of MDMA Analytical method: HPLC/MS Results: MDMA 1.5 μg/g

Table 2 Entomotoxicology publications on drug poisons in pupae: experimental or case report, analytical methods adopted, results of drug concentrations in pupae (*MDMA* 3,4-methylenedioxymethamphetamine, *MDA* 3,4-methylenedioxyamphetamine, *MLD* median lethal dose)

Toxic drugs	Analytical findings
Amitriptyline, temazepam, trazodone, trimipramine [23]	Case and experimental report Analytical methods: amitriptyline, GC/MS; temazepam, trazodone and trimipramine, HPLC Results: amitriptyline < 0.01 μg/g, temazepam 0.01 μg/g, trazodone < 0.01 μg/g, trimipramine < 0.04 μg/g. There was a precipitous fall in pupal drug concentrations associated with the pupation process
Amitriptyline, nortriptyline [21]	Experimental report Analytical methods: HPLC, GC/MS Results on 15th day of pupation: amitriptyline 20.4 ng/g, nortriptyline 1.2 ng/g
MDMA (ecstasy) and MDA (metabolite) [25]	Experimental report: the rabbits were given 22.5 mg (1.0 MLD) of MDMA Analytical method: HPLC/MS Results: MDMA, 6.6 μg/g; MDA, 1.8 μg/g. Correlation between MDMA concentration in tissues and pupae

Table 3 Entomotoxicology publications on drug poisons in puparial cases: experimental or case report, analytical methods adopted, results of drug concentrations in puparial cases (*FPIA* fluorescence polarisation immunoassay)

Toxic drugs	Analytical findings
Cocaine [14]	Case report Analytical method: GC Positive results
Amitriptyline, nortriptyline [20]	Case report Analytical method: GC/MS Results: amitriptyline 5.4 ng/mg, nortriptyline 2.5 ng/mg
Opiate [19]	Experimental report Analytical method: FPIA Positive results

fied human remains. The drug concentration was found to be higher in the puparia than in the beetle exuviae analysed.

Other drugs (e.g. cocaine, opiates) have been found in puparial cases by other authors [14, 19]. The articles are reported in detail in Table 3.

Metals in adult flies

Besides pupae and larvae, adult flies found at the site can also be analysed. The accumulation of various metals, including mercury, copper, zinc and calcium in the tissues of adult flies has been demonstrated [5, 6, 7]. In particular, there is a clear correlation between the mercury concentration in adult flies and the metal concentration in the tissues which the larvae have fed on. Adult flies, like larvae, show a high mercury tolerance and effective excretory functions for eliminating mercury, especially inorganic mercury [7]. On the other hand, it has been demonstrated that adult houseflies can tolerate high levels of metals (e.g. copper, iron) in their diet without any deleterious effects on the life span [5]. Neither pesticides nor other drugs have been found in adult flies.

Drugs in exuviae (cast beetle skins)

Metals and pesticides have not been investigated in beetle exuviae. Only few drugs have been examined using a method similar to that applied for hair drug extraction. Miller et al. [20] reported the first detection of drugs (amitriptyline) in exuviae in a case of mummified human remains. The drug concentrations found were greater in the fly pu-

paria than in the beetle specimens which had fed on them.

Drugs in beetle faecal material (frass)

In beetle faecal materials, metals and pesticides have not been investigated. Only few drugs, such as cocaine [13] and amitriptyline [20] have been found using a modified hair extraction technique.

Metals, pesticides and drugs in fly predators and scavengers

Nuorteva and Nuorteva [7] noted that beetles, predators of blowfly larvae, bioaccumulate mercury present in high concentrations in such larvae. Moreover, this metal was not found to be toxic for beetles and no change in life-span was noted for beetles fed on mercury-containing maggots.

Pesticides and drugs have not been investigated in fly predators and scavengers.

Entomotoxicology analytical procedures

Sampling of entomological specimens

After sampling of entomological specimens (e.g. larvae, pupae, adult flies) at the scene and at autopsy, they were washed with copious deionized water [10, 11, 12] or in cold [21, 23, 24] or luke-warm tap water [15] and dried on absorbent materials, then killed by freezing and finally stored at 4 °C or at –20 °C for entomotoxicological analyses.

Entomological chitinised tissues (puparial cases and exuviae) are washed with methanol or detergent, vortexed for 10 s, centrifuged for 5 min and dried under nitrogen.

Preparation of entomological samples for extraction of inorganic substances

Inorganic substances (e.g. mercury, iron, copper, zinc, calcium) ingested by maggots were detected in emerging adult flies. Samples containing 15–25 flies (15–25 mg/fly) of both sexes are dried and ashed in porcelain crucibles at 650 °C for 24 h. The resultant ash is dissolved in strong acids (e.g. HCl, HNO_3) [5, 6]. Nowadays, metals are prepared for analysis by an acid digestion procedure with 70% HNO_3 in Teflon vessels using a microwave sample preparation system [27].

Preparation of entomological protein samples for extraction of organic toxic substances

Before entomotoxicological analyses, cleaned tissues (e.g. larvae, pupae, adult flies) are washed again with distilled water and dried with filter paper. This multiple washing treatment is necessary to prevent contamination by human fluid exudate or transudate [16] and 1–10 g [9, 10, 11, 12, 13, 16, 17, 21, 23, 24] of cleaned larvae are weighed and finely chopped with scissors. In the works where pupae have been analysed, a lower amount, 2.5–5 g, was used [21, 23]. Organic substances have never been detected in adult flies. Normally, the entomological samples, spiked with an appropriate internal standard solution, are homogenized in a 0.9% saline solution. The resulting homogenate is centrifuged.

Preparation of entomological chitinised samples for extraction of organic toxic substances

An appropriate internal standard solution is added to 100–200 mg (a similar amount to that used for hair) of chopped puparial cases in test tubes. The use of strong acids or bases has been recommended to break down the chitin/protein matrix and allow the release of toxic substances [20]. Our laboratory utilises acid hydrolysis and HCl (0.1 N) is added to cover the tissues. Test tubes are capped, and extraction is carried out overnight at 65 °C. Once the extraction is complete, the acid solutions are removed from the chitinised tissues and adjusted to a suitable pH. For cast beetle skins (exuviae) and beetle faecal material (80 mg), basic hydrolysis is reported to give the best results [20].

Poison and drug extraction

The preparation and extraction of toxic substances from entomological samples presents some advantages with respect to human normal tissues: sampling is fast and easy and no emulsion was noted during the extraction step, whereas this is sometimes observed with human tissue. Moreover, chromatograms obtained during analysis presented less endogenous peaks and this is particularly important in the case of putrefied material. However, the techniques for extraction of poisons and drugs vary according to the chemical features of the substances to be detected (metal or organic poisons) and the kind of material to be used.

For protein entomological material (larvae, pupae, adult flies), the same extraction techniques as for skeletal muscles or fluids of toxicological interest have been adopted, while chitinised insect remnants, which are frequently encountered with mummified or skeletonised remains (e.g. puparial cases and exuviae) are best analysed by a modified hair extraction technique. A main difference between the extraction of insect puparia/exuviae and standard samples is to break down the resistant chitin matrix so that drugs and toxins can be released. For this reason, acid or base hydrolysis and pH adjustment are important so that some of the more routine drug screening extraction methodologies can be employed for analyte isolation.

Toxicological analysis

For the detection of inorganic substances in entomological tissues, analytical procedures have included FAAS, atomic emission spectroscopy or AES (calcium), or optical ICP (inductively coupled plasma) or ICP/MS, to measure low concentrations.

For the detection of organic substances in entomological tissues, analytical procedures have included as screening tests, radio immunoassay (RIA) and fluorescence polarisation immunoassay (FPIA), and as confirmation tests, chromatography techniques (e.g. TLC, GC, GC/MS, HPLC).

The aqueous extracts are analysed as fluids, so they are ready for the subsequent purification analyses such as liquid-liquid extraction (LLE) or solid-phase extraction (SPE).

Normally, entomological samples are analysed in the same manner as human tissues. The tricyclic antidepressants amitriptyline [15, 20, 21, 22, 23, 28, 29], trimipramine, and trazodone [23] were extracted after adding sodium hydroxide, with a LLE procedure using a heptane-isoamyl alcohol mixture, acid back-extracted, and re-extracted with toluene-isoamyl alcohol. The analysis was carried out with the HPLC technique.

The benzodiazepines triazolam and oxazepam [10], bromazepam [11], temazepam [23, 28, 29], were also extracted for HPLC determination with a LLE procedure by heptane or diethyl ether and then further purified by partition between acetonitrile and heptane.

The opiates, morphine and codeine [12, 16, 17, 19] were extracted from entomological samples by a chloroform-isopropanol-n-heptane mixture in the presence of phosphate buffer at a basic pH, re-extracted into an acidic solution and back-extracted into chloroform. After evaporation of the solvent and derivatisation

with BSTFA (bis-(trimethylsilyltrifluoroacetamide) containing 1% TMCS (trimethyl-chlorosilane), the drugs were identified by GC/MS.

The SPE procedure used normally for human tissues and biological samples, has also been applied to entomological samples. This is carried out with commercial columns (e.g. Bond Elute Certify, Tox-Clean RC, Chem Elut, Clean Screen DAU), filled with various materials (usually C18, silica gel derivatised with n-octadecylsilane), and using suitable organic solvents [30, 31].

Tox-Clean RC SPE cartridges were used for amitriptyline and nortriptyline clean up and concentration from aqueous extracts of chitinised tissues for GC/MS analysis [20].

Purification of cocaine from aqueous extracts obtained from decomposed soft tissues, including maggots, and beetle faeces [13, 32], was performed on Clean Screen DAU columns for GC/MS analysis.

Some drugs, acetaminophen [15], acetylsalicylic acid and paracetamol [24], were extracted by a non-preconditioned Chem Elut column for HPLC analysis.

Our experience in entomological material analysis

In our laboratory we have had the opportunity of detecting some toxic substances (e.g. opiates, cocaine, benzodiazepines and other tranquillisers) in fly larvae and puparial cases collected on the decomposing bodies of suicide cases and narcotic deaths.

In our experience, the best organic toxicant purification from aqueous extracts of entomological specimens is obtained with solid phase extraction (SPE), and the best analytical results are obtained by GC/MS with single ion monitoring (SIM).

To analyse metals, an acid digestion procedure in Teflon vessels is necessary using a microwave sample preparation system [27] and the optical ICP (inductively coupled plasma) technique, which is able to measure low concentrations.

Discussion

Entomotoxicology is a special area of both entomology and forensic toxicology, with the common objective of establishing the cause of death, site of cadaver permanence and post-mortem interval. The specific analytic methodology pertaining to forensic toxicology is applied.

The specimens commonly used are of protein and chitinised tissue origin with a composition roughly similar to that of human tissues or hair samples. Entomotoxicological investigations therefore replace forensic toxicology analysis whenever cadaver material of protein origin is not available due to advanced decomposition processes, and the search for specific substances can consequently be performed only on *Diptera* recovered in the immediate proximity of the human remains. It has been found that drug concentrations are higher in puparia than in exuviae or frass. This most likely reflects the food source preferences characteristic of the fly and beetle remnants examined. *Sarcophagidae* and *Phoridae* flies mostly feed on soft tissues where acute drug concentrations are likely to be higher, while *Dermestidae* beetles feed essentially on dried integuments [20].

Entomological sampling from human tissues is simple. Fly larvae feed on human tissues, even putrefied, and are able to metabolise them due to an enriched enzyme system. Analyses of these larvae are therefore devoid of putrescent substances that could interfere with the chemical assays. The amounts of entomological sample needed have decreased according to the kind of analytical technique used. The mass spectrometry technique combined with liquid chromatography and gas-chromatography, which is highly sensitive and specific, has contributed to this reduction.

Entomological specimens include larvae, the most useful, together with pupae and puparial cases, which are recovered at longer intervals after death has occurred. Adult flies are suitable only for the determination of inorganic compounds (such as metals). The finding of a specific toxic substance allows extrapolation of the toxic substances that were present in the human tissue of origin. However, the quantitative relationship between the concentration of toxicants in entomological specimens and in human tissues is affected by several variables such as the main human organ the larvae fed on, the development of cadaver *Diptera* and the type of toxic substances.

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