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INSECTS AS SILENT WITNESSES: EXPLORING THE ROLE OF ENTOMOTOXICOLOGY IN FORENSIC INVESTIGATION

Souparnika.C[1], Gopika V.P[2], Donkina Nagesh[3]

1.III Year UG Student, Department of Forensic Science, GIET Degree College, Rajahmundry.

2.III Year UG Student, Department of Forensic Science, GIET Degree College, Rajahmundry.

3. Assistant Professor, Department of Forensic Science, GIET Degree College, Rajahmundry.

ABSTRACT

The interdisciplinary field of forensic entomotoxicology, which combines toxicology and forensic entomology, has become essential to improving the precision and breadth of death investigations. This review examines the diverse applications of entomotoxicology in forensic science and synthesizes recent research in the field. The basic ideas of forensic entomotoxicology are examined, along with the significance of this field in resolving mysteries surrounding postmortem durations and causes of death. A detailed examination of the methods employed in forensic entomotoxicology lays the groundwork for understanding how scientists collect, analyze, and assess toxicological data from various insect specimens. This section emphasizes the multidisciplinary nature of the approaches utilized while addressing the challenges and advancements in analytical methods. The range of toxins discovered in insects after their decomposition is illustrated by this review, which compiles research findings from multiple investigations. Exogenous and endogenous sources of poisons are discussed, along with how they impact forensic investigation. This thorough review concludes by highlighting the revolutionary role that forensic entomotoxicology plays in transforming death investigations. Forensic scientists, entomologists, and toxicologists can all benefit greatly from this review's synthesis of the body of knowledge and suggestions for further research.

KEYWORDS: Entomology, Toxicology, Entomotoxicology, Xenobiotics, Toxicological Analysis, Postmortem Interval

1. INTRODUCTION

A combination of the Greek terms "entomon," which means insect, "toxikos," which means poison, and "logos," which means subject matter, results in the phrase entomotoxicology. Etymologically speaking, then, it is the study of xenobiotics as they relate to insects. Pounder initially used the term in 1991 (Hodecek, 2020).

The study of applying toxicological analysis to carrion-eating insects to ascertain the presence of drugs and toxins in their intoxicated tissues is known as entomotoxicology. Entomotoxicology looks on how these compounds affect arthropod growth as well, to help with forensic PMI estimations. The first report of identifying a drug in a corpse by analysing maggots feeding on the body was made in 1980 by Beyer and associates (Introna et.al., 2001).

Entomotoxicology can be divided into two main categories:

1.1 Entomological analysis of substance: caused variations in the rate at which insects developed, which in turn affected how the PMI was calculated.

1.2 Toxicological analysis of drugs within insects: Because of storage excretion and bio accumulation in insect metabolism, which produce concentrations of compounds higher than in the surrounding tissues, toxins may be more easily recognised.

One of the most important parts of a criminal investigation into a death is figuring out the cause of death. Identifying this aspect once the body has completely decomposed could be difficult. Instances of postponed recovery are likely to occur in cases of drug overdose deaths in remote locations or suicide deaths. It may also occur when the deceased are covered up on purpose. Insects are the only reliable substitute specimen that can be utilised for forensic investigations when conventional toxicological samples, such as tissue, bodily fluids, and internal organs, have either degraded or are not available. Insects are vital to forensic research because they feed their larvae on dead bodies. During their active feeding on cadaveric tissue, the larvae's metabolic system absorbs xenobiotics, which include medicines and other hazardous compounds found in the tissue. Moreover, these xenobiotics are transferred up the food chain to other arthropods that consume the larvae. Insects (larvae) also constitute an adequate toxicological sample since the cadaver has a lot of insects on it and the puparial case is not damaged for a long time. The study of whether insects can be used as alternative toxicological samples is known as entomotoxicology. (Chophi et.al., 2019).

Furthermore, in cases where bug evidence is left at a scene after human remains have been deliberately removed, toxicological and molecular investigations of these insects may help identify a victim or even reveal the cause of

death by connecting a larva with its last meal, for example. Certain fly species have the potential to produce myiasis on both living and dead corpses. In these situations, an examination of the larvae can reveal the duration of the animal or human neglect. In court, it will be difficult or impossible to present bug evidence accurately and persuasively without a proper professional gathering of the evidence (Chophi et.al., 2019).

Forensic medicine is highly interested in this area due to the growth in drug-related mortality, primarily from heroin and cocaine, as well as deaths from poisoning and/or hazardous chemical intake that occur accidentally or suicidally. Standard toxicological techniques such as gas chromatography (GC), thin-layer chromatography (TLC), high pressure liquid chromatography (HPLC–MS), radio-immune analysis (RIA), and gas–mass analysis (GC–MS) can be used to easily analyse insects after the most representative specimens have been homogenised. The person's consumption of drugs and toxins during their life is integrated into the metabolism of Diptera larvae, who feed on human tissues that have been intoxicated. These chemicals are not only transferred from the human organism to Diptera at this stage in the food chain, but they are also transferred when beetles eat blowfly larvae. (Introna et.al., 2001).

2. A Brief History

The field of entomotoxicology is very new. The first paper addressing the use of phenobarbital in entomotoxicology was released in 1980. A 22-year-old female was found in the first skeletonized form, 14 days after she was last seen alive. There were no fluids or tissues since the decomposition was so advanced. Phenobarbital was discovered when toxicological screening was performed on fly larvae samples (Introna et.al., 2001). Since then, numerous medications and harmful substances have been found in various insect and larval tissues. These include Amitriptyline, Propoxyphene, Acetaminophen (Wilson et.al., 1993), Steroids (Musvasva et.al., 2001), Trazodone, Trimipramine, and Temazepam (Salder et.al., 1995), Benzodiazepines, Barbiturates, and Meprobamate (Levine et.al., 2000), Methylphenidate (Bushby et.al., 2012), Methamphetamine (Mullany et.al., 2014), Clomipramine, Bromazepam, Levomepromazine, Cocaine, and Nortriptyline (Goff et.al., 1989), Opiates and Opioids (Kintz et.al., 1994), Nordiazepam (Wood et.al., 2003), Phencyclidine (Goff et.al., 1994), Codeine (Kharbouche et.al., 2008), Insecticides and Pesticides (Shi et.al., 2010), Mercury50 (Nuorteva & Nuorteva, 1982), etc.(fig 1)

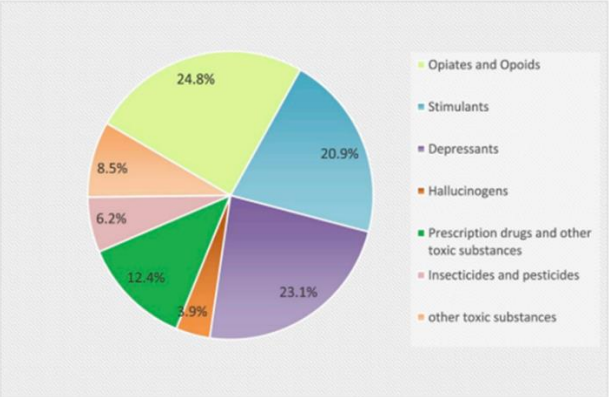

Fig. 1. Various drugs and toxic substances reported in forensic entomotoxicology.

(Chophi et.al., 2019)

3. Using Insects as a Toxicological Sample

Five stages can be distinguished in the decomposition process, which begins as soon as a person dies:

- Fresh
- Bloated
- Decay
- post-decay
- skeletal

The smell that a dead body releases as it decomposes attracts a wide range of insects and terrestrial arthropods (Rivers & Dahlam, 2014). Insects are typically the first animals to arrive near a dead body. Blowflies can deposit their eggs on carrion some hours after a death. Metamorphosis is the process by which an insect changes from one life stage to the next. First, the corpse is covered in a vast number of eggs. After hatching from the eggs, the maggot consumes the body and proceeds through three larval instars. At the third instar, mature maggots leave the food source to pupate in the proper place, usually the soil. Occasionally, empty puparia cases left behind by the adults emerging from the pupa can be seen on the clothing of the deceased, under the ground, or beneath the carpets in the room (Byrd & Castner, 2009). Puparia case serves as a last resort for toxicological samples when bodies are recovered at the skeletonized state. Drugs and other dangerous substances may accumulate inside the larvae's cuticle throughout growth and development, and during pupariation, these compounds may transform into sclerotized puparium (Bourel et.al., 2001). Entomotoxicologists can examine the following organic components of interest: fly scavengers and predators (Candela & Aventaggiato, 2001); larvae, pupae, and adult insects; puparial cases; exuviae (cast beetle skins); and beetle excrement (frass). In toxicological examinations, beetles (Coleoptera) and true flies (Diptera) are the two most utilised insects. The bug species that have been examined the most within the family are Lucilia sericata and Calliphora vicina.

4. Sample Collection and Preservation

Sample collection and preservation are crucial for providing a more accurate and reliable assessment of the postmortem interval (PMI) and for serving as a specimen for further toxicological examination. Given that drug redistribution can result in variations in drug concentration in larvae, samples can be taken from the corpse as well as from various body parts (Gosselin et.al., 2011). To determine the most appropriate medium, researchers have examined a variety of killing and preservation techniques as well as how they affect the duration of the larvae. In a single experiment, specimens of larvae were either killed in hot water at 80°C and 100°C for one, thirty, sixty, and ninety minutes, and then they were submerged in a 10% formalin, 80% ethanol, and 95% ethanol preservative (Adams & Hall, 2003). It was discovered that the larvae's length differed greatly based on how they died. The type of preservative used also had varying effects on the length of the larvae and the larvae of different insect species. It was discovered that larvae died at 80ºC for 30 seconds of immersion bring the least amount of change in length from their starting point, making this mode of death appropriate. It was stated that the ideal preservation medium was 80% ethanol. Nevertheless, research by Midgley and Millet (Midgley & Villet, 2009) shown that beetle larvae cannot be killed using the previously described technique. The length of the beetle larvae varied significantly across the three methods employed to kill them: immersion in 70% ethanol for one minute, freezing at -20°C for one hour, and immersion in hot water at 90°C for one second. Therefore, the authors recommended measuring beetle larvae while they are still alive, if possible. The easiest way to measure the length of huge larvae collected is to calculate the mean length after the larvae are killed with ethanol. The entire corpse, including the area up to ten metres surrounding it, can be used to collect insects. Dead insects and eggs can be preserved in 70–95% ethanol. Larvae can be killed in hot water at 80°C for thirty seconds and then stored in 70–95% ethanol. Pupae can be stored in a temperature range of 2–6°C with a punched lid. Adult flies can be killed in a vial frozen at -20°C. The dead specimen can be stored in 70–95% ethanol (Amendt et.al., 2007). For toxicological analysis, specimens are kept at -4 °C. The procedure is the same as for toxicologically interesting human tissues or fluids.

5. Extracting Samples

When it comes to human tissue, there are some advantages to the extraction of xenobiotic compounds from insect samples. As is occasionally the case with human tissue, emulsion does not obstruct the analysis, and sampling is easy and quick (Wood et.al., 2003). In one experiment, measurements from larvae were successful, but due to matrix interferences, measurements from human tissue could not be made. Samples of larvae are retrieved and processed similarly to samples of human tissue. Different drugs and poisons are extracted using different extraction techniques, such as liquid-liquid and solid phase extraction, depending on the chemical properties of the substances that need to be located. Solid phase extraction is believed to provide the best organic toxicant purification from aqueous extracts of entomological specimens (Candela & Aventaggiato, 2001) (fig 2).

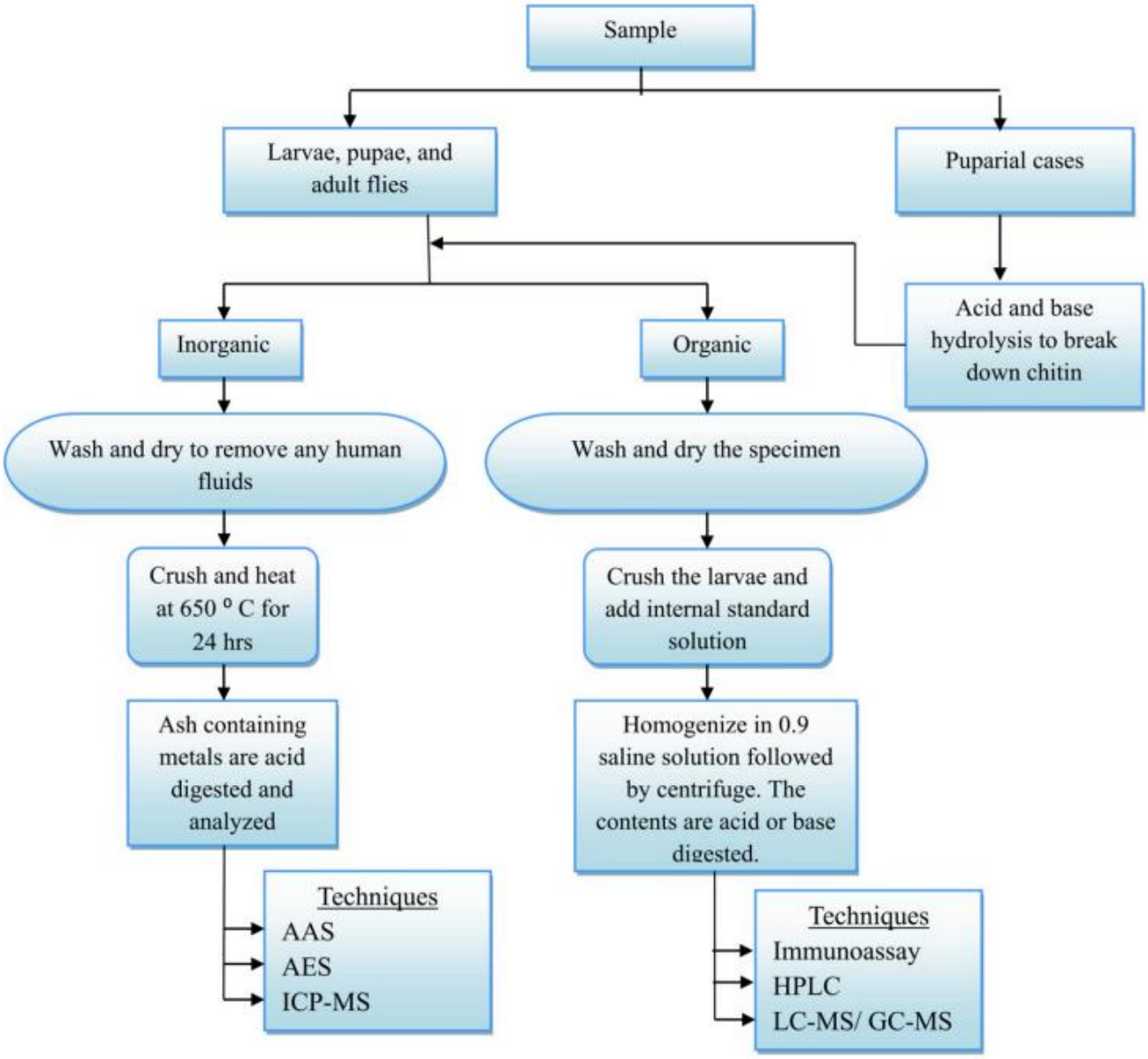

Fig. 2. Layout of the analysis of insect specimen for toxicological analysis.

(Chophi et.al., 2019)

6. Toxicological Analysis

To investigate the possibility of identifying, quantifying, and correlating pharmaceuticals and other hazardous chemicals between different substrates and larvae (insects), numerous researchers have employed a variety of animal models (rabbits, rats) and meat substrates spiked with these substances. The processes of drug recovery from larvae feeding on the dead corpse and drug breakdown are faithfully simulated by these models. The extraction method used, and its efficacy determine whether a drug may be successfully detected and recovered from an insect specimen (Chophi et.al., 2019) (fig 2).

6.1 Qualitative Analysis

In cases where appropriate samples such as tissue, blood, or urine are unavailable for toxicological analysis, insects may serve as a useful indicator of possible drug exposure in poisoning-related deaths. For instance, cocaine in the skeletal muscles and larvae of a deceased individual at the skeletonized stage was identified using GC-MS. Similarly, amphetamine and alcohol were discovered in the maggots on a deceased body that was estimated to be one month old. The analyses were conducted using methods including immunoassay, HPLC, GC, LC-MS, LC-MS/MS, and GC-MS. The immunoassay technique is a useful tool for screening a variety of drug classes for a certain class of drug, even though the results are not definitive (Moody, 2006). It offers quick sample analysis and an answer to the question of whether further confirmatory tests need to be performed. Forensic entomotoxicology has been the primary use of the immunoassay method for the detection of morphine substances from a range of substrates and materials. Using this technique, it was possible to demonstrate the distribution of morpholine throughout the insect's body, including the larvae's integument, cuticle, and haemolymph. An HPLC method revealed that flies could efficiently metabolise 76% of morphine, excreting only 24% of the drug in its original form. This approach has also proven useful for both quantitative and qualitative research on a range of drugs and pesticides. Insects that consumed rabbit carcasses that had been slaughtered with parathion were examined by Wolff, and parathion was found in a variety of bug species using the HPLC technique. In certain species, the low concentration of parathion makes quantitative estimation impossible. The "gold standard" method for verifying the presence of chemicals in samples is the combination of mass spectrometry and chromatography techniques (Wallace, 2017). Increased selectivity, sensitivity, accuracy, and reproducibility are achieved by this method, which calls for tiny amounts of samples. This technique has been used in the forensic investigation of drug addiction cases to identify different substances on different matrices.

6.2 Quantitative Analysis

When compared to the substrate that they are feeding on, the drug concentration in larvae and insects decreases significantly. It is typically discovered that the larvae consuming the substrate that has been treated with a higher dose or concentration have higher concentrations in their tissues. When morphine was present in two different diets at concentrations of 17.5 $nmolg^{-1}$ and 7 nmolg-1, for example, the larvae fed on the former diet had a higher morphine concentration. This might not always be the case, though. When three distinct medication doses were administered to larvae, the drug concentration in the larvae fluctuated over time. The drug concentration in the larvae decreases during the post-feeding stage because there is no active xenobiotic uptake from the meal. The larva successfully eliminates medications and other dangerous compounds from its body as it matures, leaving fewer of them in its adult form (Liu et.al., 2009). However, it has been discovered that the concentrations of several drug classes, such as antidepressants, are higher during the post-feeding stage. Bioaccumulation might be the cause of this occurrence. Since a small number of pharmaceuticals is known to build up in the puparium's cuticle, medications can be recognized from puparial instances that are empty. However, the technique must be sensitive enough to detect these low drug concentrations in cases involving insects, larvae, and pupae. In this

research, a drug molecule from larvae grown on minced beef meat containing 0.01 mg/kg of morphine was not detectable by the immunoassay approach. To measure the drug, only larvae fed on minced meat containing 0.1 mg/kg and 1 mg/kg of morphine were employed. Similarly, the HPLC method was unable to quantify a material from larvae fed on minced beef containing 500 ng/g and 1000 ng/g of morphine. For the purpose of measuring the medicines, only larvae feeding on beef minced with morphine above 2500 ng/g were permitted. However, it was noted that larvae fed on 2500 ng, 5000 ng, and 10,000 ng of minced morphine meat, respectively, had drug levels as low as 765 ng, 2720 ng, and 3010 ng. The LC-MS and GC-MS procedures are the most effective ways to measure pharmaceuticals at lower levels of production. Pien demonstrated that, beginning with a single larva and puparia, the drug Nordiazepam and its metabolite Oxazepam could be identified and measured down to the Pg level. This method has been utilised by several researchers to measure drugs from different human and animal body sections. Different drug concentration levels in the liver, heart, lung, blood, brain, urine, and skin have been reported (Bachmann et.al., 2018) (Gunn et.al., 2006) (Bushby et.al., 2012). Since the liver is where xenobiotic processing takes place, drug concentrations in the liver are often higher than in other organs. Thus, it is important to carefully analyze the quantitative results.

6.3 Correlational Analysis

There is a lot of disagreement over the connection between drug concentrations in the substrate and insects. Even though correlation studies with maggots may be conducted using both qualitative and quantitative methods, some scientists remain sceptical of this approach. Their thesis is predicated on the notion that a number of factors, such as drug pharmacokinetics, metabolism, drug redistribution, drug accumulation, feeding activity of larvae, and several others, may have an impact on correlation studies but are currently unknown. In the discipline of entomotoxicology, a thorough examination of 29 human cadavers suspected of poisoning deaths was carried out. The researchers were unable to discover any correlation between the drug content in samples of larvae and human tissue (Tracqui et.al., 2004). There was no repeatability found when inter-larval and inter-site variance were examined. The highly unpredictable mobility of the larvae was the explanation for the variation in drug concentration in the larvae, as reasoned. The authors came to the conclusion that any attempt to determine the cause and circumstances of death using this method is very doubtful and dishonest from a scientific standpoint, given the profusion of ambiguous components in the analysis. It is improbable that there is a quantifiable relationship, they added. It was predicted that unless there was significant insight gained, this subject would remain, at best, a lab curiosity and, at worst, a scientific hoax. Several writers also conducted trials in which a quantitative link could not be established, hence providing evidence against correlation research. On the other hand, a few investigations have shown a connection between drug concentrations in substrates and larval specimens. Liu found a connection between the liver tissues and the amount of malathion present in larvae. Burel found that feeding beef steak contaminated with morphine hydrochloride to Thanatophilus sinuatus larvae in their second and third instars resulted in the strongest relationship. An association was found by (Hedouin et.al., 2001) between third-instar Lucilia sericata larvae fed on rabbit corpses treated with morphine. Similarly, a number of

authors have observed a quantitative relationship between the number of drugs in the substrate and the larvae. Since the larvae in most of these trials were fed minced meat rather than allowing the drug to go through its usual metabolic pathway, the results may not correspond to real-world poisoning scenarios. To date, very few studies have looked closely at how drugs are metabolized, redistributed, accumulated, and excreted in insects. Consequently, caution must be used when interpreting the findings of such a study until suitable baseline data are available (Liu et.al., 2009).

7. Trends in Analytical Techniques

The capacity to identify and detect drugs in decomposed or putrefied tissue depends on how well extraction techniques and analytical technologies are applied. In the past, materials could be difficult to analyze due to the damaged matrix when using methods like chromatography and immunoassay. The matrix affected the analysis's findings and increased the possibility of falsely positive outcomes for the method. Additionally, the methods used were less sensitive. Because fewer interferences were seen when employing larvae and insects as proxies, these samples were sought after as an alternative. Prior to the development of sensitive techniques such as LC-MS and GC-MS, which enable the confirmatory identification and quantification of low-level toxic concentrations in a highly degraded matrix, maggots held some forensic value as alternative toxicological samples. However, their usefulness has since diminished. Only once the corpse has reached the skeletonized stage and there are only insects, puparial cases, and insect frass remaining for analysis is this field useful and applicable. For entomotoxicological research, chromatographic methods in conjunction with mass spectrometry have been the most often utilised analytical methodology, and this combination is the preferred strategy (Chophi et.al.,2019).

These days, spectroscopic methods backed by statistical tools like PCA (Principal Component Analysis), SPA (Successful Projection Algorithm), and GA (Genetic Algorithm) are being utilised to study entomological specimens non-destructively. Spectroscopic techniques are employed because they overcome the accessibility barrier caused by pricey instruments such as GC-MS and are sensitive, repeatable, and need few samples with minimal sample preparation (Baia et.al., 2016) (Lagoo et.al., 2010). In this (Zenetti et.al., 2016) UV-spectrophotometry is used to identify the antidepressant medication fluoxetine from entomological specimens. The substance was identified by the authors from exuviae and from D. Maculatus in all developmental stages. Statistical techniques are combined with Near Infrared (NIR) Spectroscopy to analyse flunitrazepam in insect specimens. Statistical techniques were used to gather information regarding potential variations in flurazepam concentration (classification) and biochemical changes for insects (larvae, puparia, and adults). As a result, the application of statistical methods facilitates the objective interpretation of data that is precise, trustworthy, and repeatable. Although the results were encouraging, the authors suggested that more research methods be done and that a larger vibrational spectra database containing a greater variety of fly and insect species be produced (Oliveira et.al., 2014).

8. Determination of PMI

Fly estimation of the postmortem interval (PMI) is used in judicial investigations. In order to identify the bug and determine its size and stage, juvenile larvae, pupa, and insects are removed from the body (Byrd & Castner, 2009). The PMI from the insect succession on the cadaver is determined by taking into account a number of variables, such as climate, season, geographic region, exposure to solar radiation, synanthropy, substrate type, latitude, altitude, body location and position, size, cause of death, presence of clothing, intra- and inter-specific competition, and larval migration. It has been noted that when toxic compounds are present, the pace at which larvae grow can either increase or decrease. It was shown that the presence of malathion decreased the growth rate of fly larvae. It was found that the larvae feeding on poisoned tissues were smaller in size, and that the postmortem interval estimate changed by 36 hours for the larvae collected from poisoned liver tissues and 28 hours for those taken from poisoned muscle tissues. Methamphetamine was found to alter the PMI and speed up larval growth when assessed from puparia and larvae, respectively, by 18 and 48 hours (Goff et.al., 1992). The presence of codeine in the tissue also altered the postmortem interval estimation by 48–96 hours when the larval age was determined using the larvae weight (Kharbouche et.al., 2008). It was shown that in tissues containing butyl-scopolamine bromide, fly development lagged by 54 hours. However, if enough doses of butyl-scopolamine bromide are ingested before to death, this drug may considerably distort estimations of the postmortem period due to its effect on the growth rate of larvae (Oliveira et.al., 2009). Therefore, erroneous PMI assessment may arise if the presence of dangerous compounds in tissues is ignored.

9. Forensic Significance

9.1 Calculating the PMI (Postmortem Interval):

Forensic investigations require the determination of the deceased's time of death. Insects such as flies and beetles have a predictable technique of colonising a body. Using entomotoxicology, forensic investigators can determine the postmortem interval by examining the chemicals present in these insects. When the dead were exposed to harmful chemicals, this can be inferred from the chronology of toxin buildup in insects (Chophi et.al., 2019).

9.2 Identification of Poisoning:

Entomotoxicology can help determine the presence of pharmaceuticals or toxins in situations where poisoning is suspected. When insects feed on a dead body, they can gather materials from the surrounding area, which could lead to information on the cause of death. Determining if poisoning happened can be aided by analyzing the tissues of the insect to determine the presence and concentration of poisons.

9.3 Verification of Drug Use:

To verify drug use or overdose, entomotoxicological analysis may be utilized. Drugs and their metabolites can encounter insects; examining the tissues of the insects can identify the existence of these chemicals. In situations involving drug-related deaths or criminal activity, this information may be very helpful.

9.4 Environmental Context:

When examining a crime or death, entomotoxicology takes the surrounding circumstances into account. Insects may be exposed to different compounds in different contexts, and examining these substances can provide information about what was going on at the time of the incident.

10. Limitation

Entomological specimens can yield high-quality toxicological specimens. However, not enough research has been done to develop an assessment that assesses a drug's concentration in tissue using entomological data. Samples of pupae and third-instar larvae do not contain any drug concentrations, and drugs are only found in larvae when the rate of absorption outpaces the rate of excretion. These findings suggest that medication does not bioaccumulate during the larvae's life cycle. This suggests to entomologists that toxins eventually find their way out of larvae's bodies when there isn't a reliable source of toxins. Since entomotoxicology is still in its infancy, there is a great need for more study in this area (Sankhla et.al., 2017).

11. Conclusion

As the previous discussions have demonstrated, insects and larvae are reliable toxicological specimens, especially when the materials required for a standard toxicological test are not accessible. Before concluding that there could be no quantitative association and that maggots only play a qualitative function, much research is needed to understand the pharmacokinetics of drugs in insects. The majority of entomotoxicological investigations have been conducted to identify medications in insect specimens; very few studies have been conducted on cases of pesticide and insecticide poisoning. Because insecticides and pesticide poisonings are so common in our culture, researchers should continue to explore this area. Lastly, as poisons can either raise or decrease postmortem interval estimation, the postmortem interval estimation should be properly completed while accounting for their presence in the tissues.

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