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REVIEW ARTICLE

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Analysis of poisons and drugs in entomological specimens for forensic applications: a review

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ABSTRACT

Certain poisons and drugs can influence the growth and developmental patterns of necrophagous species, possibly affecting the accuracy of the postmortem interval (PMI) estimation. Since an accurate PMI estimate may prove an alibi (a critical aspect in criminal investigations), failure to consider factors that may affect the growth of necrophagous insects can subsequently result in the miscarriage of justice. In addition, the use of entomological specimens for diagnosing causes and circumstances surrounding death has been suggested for highly decomposed bodies, whereby conventional toxicological specimens are unavailable and/or unsuitable for analysis. In these two aspects, having the ability to determine poisons and drugs in entomological specimens appears imperative. Hence, this paper reviews pertinent articles on the analysis of poisons and drugs in entomological specimens published between 1977 and 2022, emphasizing on advantages/disadvantages of the reported instrumental analytical methods and their applications. It is organized according to the different classes of poisons and drugs, their extraction and analytical methods, as well as the results reported. Where available, information on the validation parameters of the published methods is duly discussed. This review aims to provide suitable references and guidance for future investigators wanting to carry out experiments concerning entomotoxicology for forensic applications.

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1. Introduction

1.1. Forensic entomology and entomotoxicology

Forensic entomology is a scientific discipline where arthropod science interacts with the judicial system in answering pertinent questions relating to criminal and civil laws (Mahat et al., 2019). As such, information on the developmental patterns and behavior of insects, particularly those of necrophagous species, has been interpreted by forensic entomologists for a legal context associated with human and wildlife investigations (Mahat et al., 2016). In addition to the medicolegal aspect that involves homicide, suicide, and suspicious deaths, forensic entomology also deals with civil cases of urban and stored product investigations (Rivers & Dahlem, 2014).

In medicolegal investigations, entomological evidence has been chiefly used to (a) estimate postmortem interval (PMI) and (b) suggest the involvement of drugs and/or poisons as the possible cause or circumstances surrounding deaths (Mahat et al., 2012) as well as (c) physical abuse (Gennard, 2012). Since the entomological assessment of forensic evidence can provide highly accurate PMI for cadavers recovered after 72 h, such evidence is extremely valuable for proving that the suspect was at the crime scene when the murder/suicide occurred (Denis et al., 2018). Moreover, having the ability to identify drugs and/or poisons that caused death would throw some light on the (1) modus operandi of the suspect and (2) the intention for committing the crime, as well as (3) the extent of drug usage before death. Considering that murder is "*the act by which the death is caused is done with the intention of causing death*" (Section 300(a) Penal Code (Act 574), 2015), proving the element of intention is fundamental for conviction. Moreover, researchers have also suggested the potential use of entomological evidence for (d) diagnosing the possible postmortem

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relocation of bodies (Mahat & Jayaprakash, 2013) and (e) identifying human identity (Di Luise et al., 2008). As an applied science, analytical chemistry and bioscience fields appear integral for supporting the current development in forensic entomology research and practice.

Being the relatively underexplored subdiscipline of forensic entomology, entomotoxicology is concerned with two major aspects *viz.* (1) detection of drugs and/or poisons in the necrophagous insects and (2) the possible influences of such compounds on their developmental patterns (Rivers & Dahlem, 2014). The soaring incidence of drug and poison-related deaths, especially those that occurred in remote areas, has led to the discovery of highly decomposing bodies (Mahat et al., 2012), whereby organs may not be identifiable and suitable for toxicological sampling. Meanwhile, necrophagous larvae would accumulate drugs and/or poisons while feeding on the decomposing bodies although its recoverability is dependent on the conditions that the bodies are exposed to, as well as the chemistry of the drugs/poisons themselves. Despite a great deal of studies attempted to associate the concentrations of drugs and poisons in entomological specimens with that of dead bodies and visceral tissues, the quantities of drugs recovered may not be strictly related to the levels present in the body (Gennard, 2012). It appears that providing credible mathematical algorithms for such inference is mostly impossible (Mahat et al., 2012), attributable to the lack of understanding about the "pharmacokinetics" of drugs and poisons in insects. As for the developmental patterns of necrophagous insects, alterations by drugs and poisons shall result in an erroneous estimation of PMI, which can substantially lead to the miscarriage of justice (Denis et al., 2018; Rivers & Dahlem, 2014). Detailed explanations of the contextual importance of these two aspects relating to forensic investigations are provided in the following sub-section.

1.2. Contextual importance for determining drugs and poisons in entomological specimens

Suicide is viewed as a concerning public health issue and is one of the leading causes of death worldwide. It has been estimated that over 700,000 people commit suicide every year (World Health Organization, WHO, 2021). Alarmingly, it is estimated that around 14–20% of all suicide deaths (110,000–168,000 fatalities) are due to pesticide self-poisoning (Gunnell et al., 2017). Considering that homicides and suicides are frequently committed "in rural areas of low-middle income countries where conventional farming is an important economic activity (Gunnell et al., 2017), it is common to discover bodies in highly decomposed conditions. This is particularly prevailing with poisons like pesticides, attributable to their availability in the market with affordable pricing (Magni et al., 2018). As such, obtaining toxicological data can be difficult due to the lack of suitable analyzable tissues (e.g. liver, lung and muscle tissues). Nevertheless, various necrophagous insects are commonly observed feeding on the putrefied, mummified, and skeletonized bodies (Goff & Lord, 2010). Since it is possible that drugs/poisons consumed by the deceased can be detected in necrophagous insects, the application of entomological specimens for toxicological diagnosis has been widely reported (e.g. de Aguiar França et al.,, 2015; Magni et al., 2018), although the opinions remain equivocal when it comes into its real evidential values for toxicological inference.

At one juncture, Tracqui et al. (2004), while analyzing drugs of abuse (e.g. morphine and codeine) and therapeutic drugs (e.g. fluoxetine and lorazepam) from larvae recovered during the autopsy, argued that entomological specimens might not be useful. They concluded that because the drugs determined in larvae can also be determined in fresh dead bodies, and therefore, there was no merit to analyze the larvae. Firstly, interpreting the toxicological findings towards the relatively fresh dead bodies would defeat the basic premise of forensic entomology, whereby its real potential is when the bodies are found in the advanced stages of decomposition (after 72 h) (Gennard, 2012; Mahat et al., 2012, 2019). Secondly, the determination of drugs of abuse and therapeutic drugs in entomological specimens would not throw light on the actual causes of death but only on the circumstances surrounding deaths (e.g. addiction and therapeutic uses). This is because drugs of abuse and/or therapeutic drugs are used for medicinal/abusive purposes and are not known to cause death readily. The momentous contextual difference in detecting the readily causing death poisons (e.g. malathion) in entomological specimens than those of abuse/therapeutic drugs is that even qualitative detection would be enough to suggest the cause of death since these poisons are never abused. Therefore, its detection in necrophagous larvae feeding on the body would signify the deceased's exposure or consumption of such poisons (Mahat et al., 2012).

At another juncture, many authors have reported the influences of drugs and poisons on oviposition and developmental patterns of necrophagous insects (e.g. Gennard, 2012; Mahat et al., 2019; Rivers & Dahlem, 2014). In general, not only the influences of drugs and/or poisons are concentration-dependent, the different necrophagous insects also react

differently to certain drugs and/or poisons. For example, Mahat et al. (2009) reported about the delayed oviposition and prolonged pupation period for *Chrysomya megacephala* (Fabricius) on carcasses treated with malathion, and the influences appeared dose-dependent. In contrast, the development of *Boettcherisca peregrina* Robineau–Desvoidy was evidently accelerated (shorter larval stages) when reared on tissues that contained cocaine than those of controls (Gennard, 2012). As for heroin, faster growth for *B. peregrina* larvae was found; however, the same did not prevail for *Sarcophaga tibialis* Macquart (Gennard, 2012). Prolonged development for *Lucilia sericata* (Meigen) reared on morphine substrates was also observed (Bourel et al., 1999). Here, it is pertinent to quote that "any factors that could influence initial oviposition or the duration of development of necrophagous insects may subsequently affect the accuracy of the PMI estimate" (Mahat et al., 2016). Pertinently, the influx of new drugs and poisons in the market requires rigorous efforts to examine their possible influences on necrophagous insects. This would allow forensic entomologists to properly consider the potential influences of drugs and/or poisons while providing accurate PMI. Considering the importance of accurate PMI estimation in forensic investigations and because drugs and/or poisons can affect its accuracy, having robust and simultaneous analytical methods specifically developed for entomological specimens is therefore imperative, an aspect that will be focused in sections 2 and 3.

2. Analytical methods for determining poisons and drugs in entomological specimens

Owing to its sensitivity and reliability, utilization of entomological evidence by forensic toxicologists in several cases involving putrefied human tissues and remains (Kintz et al., 1994; Kintz et al.,1990a) for the determination of drugs and/or poison (Gosselin et al., 2011) has been reported. In view of such an application, Gosselin et al. (2011) commented on the importance of the appropriate selection of extraction and instrumental techniques, considering the physical and chemical properties of the drugs and poisons of interest. The authors further accentuated that the chosen analytical methods must be highly sensitive and selective to ensure the optimum detection of the drugs and/or poisons for confirming their involvement as the cause of death.

Notwithstanding, it is rather unfortunate to observe the scarcity of specific and validated methods for the simultaneous determination of drugs and/or poisons in entomological specimens (larvae and pupae) in the body of literature. While the majority of reported analytical methods are not specifically developed for such specimens (e.g. Campobasso et al., 2004; Kintz et al., 1990a) and non-simultaneous (e.g. Kintz et al., 1990b; Sadler et al., 1995), complete validation data remained lacking in many entomotoxicological studies (e.g. Gunn et al., 2006; Levine et al., 2000; Zou et al., 2013). Most methods used were developed for the biological samples like adipose tissue, kidney, liver, blood, brain, urine, and fish tissues. The matrix effects of the different specimens may result in compromised analytical efficiency and in due course, the reliability of the results can be questionable, affecting the accuracy of PMI estimation as well as entomotoxicological interpretations. Because entomological specimens (larvae and pupae) are different in body compositions and morphology than such of common toxicological specimens as blood and liver, addressing such a gap in the forensic body of knowledge appears obligatory for the criminal justice system. Considering the pertinence of entomological evidence in court and since the onus of proof in criminal cases is beyond any reasonable doubt (Evidence Act 1950 (Act 56), 2018), inappropriate usage of analytical methods can be a subject of dispute that may result in the inadmissibility of forensic evidence and subsequently, miscarriage of justice. Moreover, while a review of the literature largely reveals the application of chromatographic methods for determining drugs and poisons in entomological specimens, the use of capillary electrophoresis (CE) for the same matrix has never been mentioned so far. The fact that new drugs and poisons are rapidly introduced in the market, utilization of analytical techniques like chromatographic and CE for forensic entomotoxicology deserves consideration. Figure 1 represents the identified loopholes in the analysis of entomotoxicological specimens in the body of literature.

Besides, the analytical method developed in the analysis of the drugs and poisons should be appropriately validated or revalidated in order to prove its suitability for the intended purpose, as prescribed by the International Conference on Harmonization of Technical Requirement for Registration of Pharmaceuticals for Human Use (ICH) (2015). This objective can only be attained when limitations of the methods are identified, namely through method validation. Hence, in the following sub-sections, a review of the literature will revolve around relevant analytical methods used in the identification/determination of drugs and poisons in necrophagous insects as a reference for future investigators wanting to carry out entomotoxicological studies, with a specific focus on appropriate protocols and standardization of the methods.

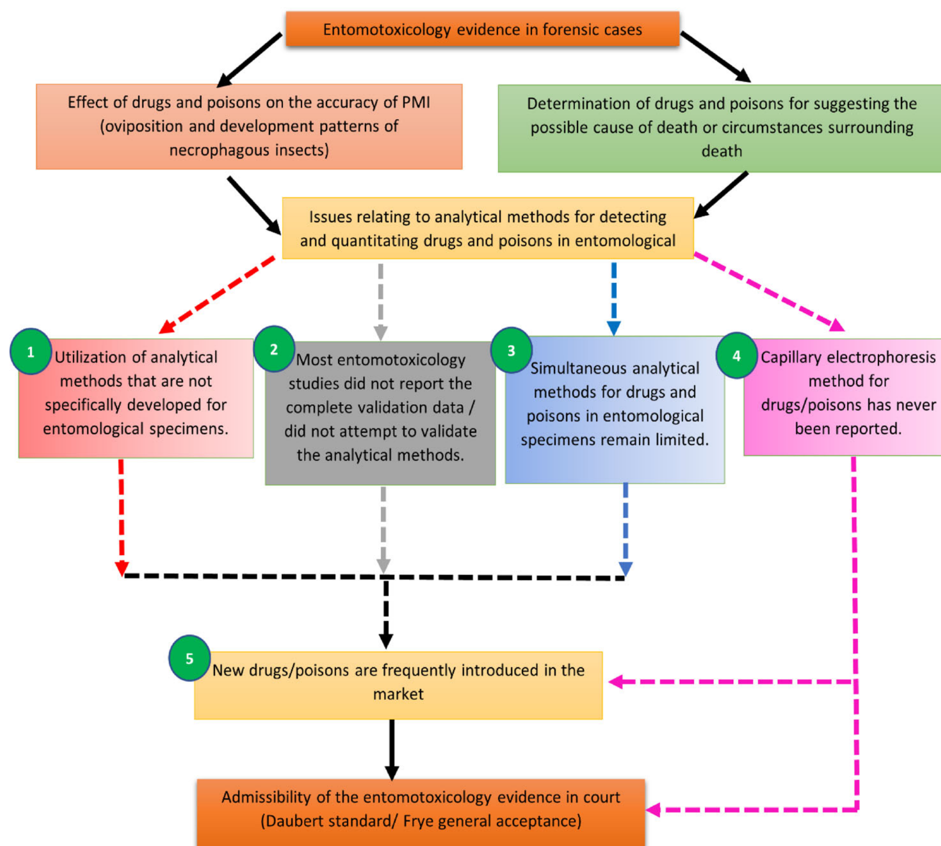

Figure 1. Five loopholes (numbers in green circles) in the analysis of entomotoxicological specimens identified in the body of literature. The dotted lines represent the interrelatedness of the loopholes in the forensic framework.

Trends in the application of analytical methods in entomotoxicological research are depicted in Figure 2. A review of literature pertaining to forensic entomotoxicology (*via* Scopus and Web of Science databases between 1977 and 2022; keywords: forensic entomotoxicology, forensic science, analytical) has identified 70 relevant articles published in the field. The more frequently reported analytical methods for analyzing entomological specimens included high-performance liquid chromatography (HPLC) (24%), followed by gas chromatography-mass spectrometry (GC-MS)/gas chromatography-tandem mass spectrometry (GC-MS/MS) (24%), liquid chromatography-mass spectrometry (LC-MS)/liquid chromatography-tandem mass spectrometry (LC-MS/MS) (19%) and spectroscopy (*viz.* ATR-FTIR, UV-vis, AAS, ICP-OES and ICP-MS) (16%) as well as immunoassay (*viz.* RIA and FPIA) (5%) techniques. Table 1 represents the summary of analytical instrumentations used for analyzing poisons and drugs in entomotoxicological specimens, focusing on the advantages and disadvantages of each instrument.

The fact that HPLC is a versatile instrument, simple to operate with minimum sample preparation while providing high resolution and recovery the vast interest in applying this instrument in entomotoxicology is highly expected. Furthermore, HPLC is able to analyze any soluble compounds (regardless of volatility), as opposed to GC-MS and GC-MS/MS which can only analyze volatile and thermally stable substances, expanding the operation capacity of the former, particularly useful for forensic laboratories. Since HPLC utilizes a pressure pump, its maintenance can be performed relatively quickly when compared with GC-MS and GC-MS/MS which operate with an oven to build heat over time. Needless to say, the price for purchasing and maintaining GC-MS and GC-MS/MS can be substantially higher than that of HPLC although both the GC-MS and GC-MS/MS may have better sensitivity (lower LOD) and better identification ability (presence of NIST library) than HPLC (Lynch, 2017). Nonetheless, the use of HPLC has been associated with large consumption of solvents (Flanagan et al., 2020; Douglas et al., 2022; Wolstenholme et al., 2021). While better LOD and LOQ as well as identification ability can also be associated with LC-MS and LC-MS/MS than that of HPLC, they require higher capital and running costs as well as skilled personnel to operate them. In this context, it is pertinent to indicate here that ideally the state-of-the-art instruments shall be used for forensic analysis to ensure the robustness of the findings (Gosselin et al., 2011). However, these instruments are costly, and therefore, they are not always available in all laboratories, especially in developing and least-developed countries. Hence, developing validated analytical methods that can be performed

Table 1. Summary of analytical instrumentations used for analyzing poisons and drugs in entomotoxicological specimens.

Instrumentations (Analytes)	Advantages	Disadvantages
1. HPLC (Organic poisons & drugs)	• Simple to operate with minimum sample preparation. • High resolution and recovery. • Able to analyze nonvolatile compounds at low temperatures. • Faster separations with high specificity.	• Large consumption of solvents. • Non-conclusive identification.
2. GC-MS & GC-MS/MS (Organic poisons & drugs)	• High level of sensitivity (lower LOD). • Conclusive identification: library (e.g. NIST). • Faster separations with high specificity. • Simultaneous analysis of multiple analytes.	• Limited to volatile and thermally stable compounds, • May require derivatization, • High purchase, maintenance, and operational costs.
3. LC-MS & LC-MS/MS (Organic poisons & drugs)	• High level of sensitivity (lower LOD) and accuracy. • Able to analyze nonvolatile compounds at low temperatures. • Conclusive identification: library (e.g. NIST) • Faster separations with high specificity. • Simultaneous analysis of multiple analytes.	• Large consumption of solvents • High purchase, maintenance, and operational costs. • Limited selection of solvent system.
4. UPLC/MS (Organic poisons & drugs)	• High level of sensitivity (lower LOD). • Able to analyze nonvolatile compounds at low temperatures. • Conclusive identification: library (e.g. NIST). • Faster separations with high specificity. • Simultaneous analysis of multiple analytes. • Low solvent consumption.	• High purchase, maintenance, and operational costs. • Requires frequent maintenance due to high pressure used.
5. Voltammetry (Organic & inorganic poison & drugs)	• Low purchase, maintenance, and operational costs. • Simple to operate with high sensitivity for organic and inorganic species. • Availability of solvents and electrolytes in market. • Rapid analysis times (seconds). • Simultaneous analysis of multiple analytes. • Able to determine kinetic and mechanistic parameters.	• Low reproducibility and less sensitivity (LOD and LOQ). • Substance must be oxidizable or reducible in the range were the solvent and electrode are electrochemically inert. • Non-conclusive identification. • Sample must be in liquid form.
6. UV-Spectroscopy (Organic poisons & drugs)	• Low purchase, maintenance, and operational costs. • Simple to operate. • Non-destructive. • Short analysis time.	• Unable to analyze compounds that do not interact with light in the UV and visible areas of the spectrum. • Less specific. • Long preparation time.
7. NIRS (Organic poisons & drugs)	• Low purchase, maintenance, and operational costs. • Simple to operate with minimum preparation. • Non-destructive. • Short analysis time.	• Low sensitivity and accuracy. • Requires the development of a multivariate calibration model.
8. ATR-FTIR (Organic poisons & drugs)	• Non-destructive. • Able to analyses solids (powder), polymer, and semi solid and liquid sample. • Simple to operate with minimum sample preparation. • Fast and easy sample analysis.	• Lacks of sensitivity. • The accessory is costly.
9. RIA (Organic poisons & drugs)	• A sensitive assay (picogram quantities). • High specificity. • Able to analyze multiple type of samples. • Indirect analysis method.	• The use of radioisotopes and scintillation fluids. • The high cost of waste disposal. • Sophisticated and time-consuming. • High purchase, maintenance, and operational costs.
10. FPIA (Organic poisons & drugs)	• Fast and simple to operate with high sensitivity (LOD). • No separation step is required. • Suitable to screen a large number of samples.	• Background interference (e.g. Serum) (requires blank measurement). • High purchase, maintenance, and operational cost. • Can suffer from autofluorescence.
11. AAS/FAAS (Inorganic poisons)	• Simple to operate with high sensitivity (LOD). • Low operational cost.	• Destructive. • Difficult to analyze solids. • Requires large volume of sample. • Mono-elemental detection.
12. NAA (Inorganic poisons)	• Non-destructive. • Simultaneous analysis of multiple analytes.	• Requires access to a nuclear reactor for neutrons. • The high cost of waste disposal.
13. ICP-MS (Inorganic poisons)	• High level of sensitivity (lower LOD). • High throughput and simultaneous analysis of multiple analytes. • Capable of distinguishing isotopes. • Conclusive identification: library (e.g. NIST).	• High purchase, maintenance and operational cost. • Low tolerance for total dissolve solid. • Destructive.
14. ICP-OES (Inorganic poisons)	• High throughput and simultaneous analysis of multiple analytes. • Limited spectral interferences (vs. ICP-MS). • High tolerance for total dissolve solid (vs ICP-MS). • Relatively low purchase, maintenance, and operational cost compared to ICP-MS.	• Liquid samples only. • Destructive.

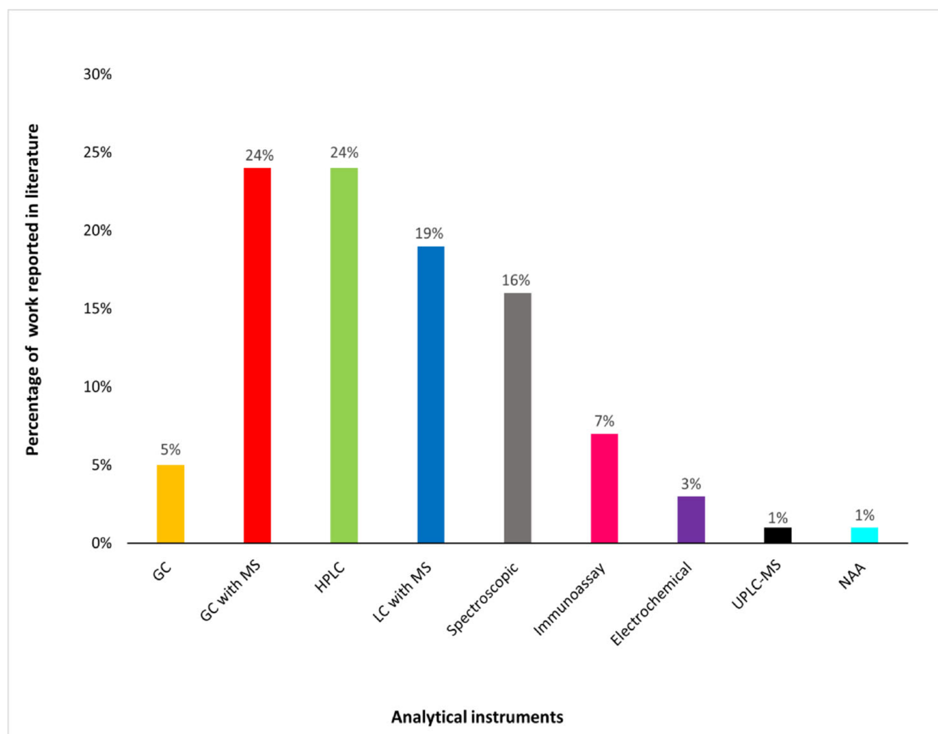

Figure 2. Trends in the application of analytical methods in entomotoxicological research reported in the literature between 1977 and 2022 (70 articles).

using moderately priced instruments like HPLC (but robust and accurate) for analyzing forensic specimens like larvae and pupae still proves relevant and necessary.

Owing to advantageous like low purchase, maintenance, and operational costs, simple to operate, non-destructive and short analysis time, utilization of UV-Spectroscopy has been reported in many scientific fields including forensic entomotoxicology. However, this technique also suffers from several disadvantages that include inability to analyze compounds that do not interact with light in the UV visible spectrum, less specific and requiring long preparation time. Non-destructive techniques such as Near-infrared spectroscopy (NIRS) and attenuated total reflection-Fourier transform infrared (ATR-FTIR) have also been used in forensic entomotoxicology. NIRS method is simple to operate with minimum sample preparation (Wolstenholme et al., 2021); however, it is associated with low sensitivity and accuracy as well as it requires the development of a multivariate calibration model (which can be time consuming). As for ATR-FTIR, it is able to analyze a wide range of samples (powder, polymer, semi solid and liquid sample) with minimum sample preparation and short analysis time. Several disadvantages have been associated with the use of ATR-FTIR which include the lack of sensitivity and the higher cost of accessory (Flanagan et al., 2020; Douglas et al., 2022; Wolstenholme et al., 2021).

In view of its advantages namely high sensitivity and specificity as well as able to analyze multiple type of samples, radioimmunoassay (RIA) has also been utilized in forensic entomotoxicological study. The method also has several drawbacks *viz.* sophisticated and time-consuming, utilization of radioisotopes and scintillation fluids that requires high cost of waste disposal resulting in high purchase, maintenance, and operational costs. As for the fluorescence polarization immunoassay (FPIA), the method is fast and simple to operate with high sensitivity (LOD). FPIA is also able to screen a large number of samples with no separation step required. Nonetheless the use of FPIA may suffer with background interference (requires blank measurement), require higher capital, maintenance and operational costs as well as issues of auto-fluorescence (Flanagan et al., 2020).

For determination of inorganic poisons, spectroscopy techniques *viz.* atomic absorption spectroscopy/Flame atomic absorption spectroscopy (AAS/FAAS), inductively coupled plasma-mass spectrometry (ICP-MS) and inductively coupled plasma-optical emission spectroscopy (ICP-OES) have been utilized in forensic entomotoxicology. As for AAS/FAAS, the methods are simple to operate with high sensitivity (LOD) and low operational cost. However, the method can only detect single element, destructive, and difficult to analyze solids samples as well as it requires large volume of sample. ICP-MS is a method that has high level of sensitivity (lower LOD), high throughput and able to analyze multiple analytes simultaneously. Using ICP-MS, isotopes can be distinguished and the analytes can be conclusively identified (presence of NIST library). However, the method is destructive with low tolerance for total dissolve solid

and it requires high purchase, maintenance and operational cost. In contrast to ICP-MS, ICP-OES has limited spectral interferences and it has high tolerance for total dissolve solid. Furthermore, the method is also able to simultaneously analyze multiple analytes with high throughput. Compared to ICP-MS, the purchase, maintenance and operational costs for ICP-OES are relatively lower (Flanagan et al., 2020; Douglas et al., 2022; Wolstenholme et al., 2021).

On the other hand, nuclear activation analysis (NAA), electrochemical techniques (voltammetry) and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) are the least preferred analytical methods for analyzing drugs and poisons in entomological specimens. The possible reasons for the NAA being among the least preferred methods include the fact that NAA requires access to a nuclear reactor as the source of neutrons which can be associated with the intensive handling of radioactive samples in specially designated laboratories and the stringent compliance towards specific safety regulations as well as higher cost of waste disposal (Kucera, 2009). As for the voltammetry, the method is associated with the consumption of the analyte, and as such it would detrimentally affect measurements at low concentrations as well as due to slow movements of the analyte form the sample bulk to the electrode especially in cases involving catalyzed oxidation (Muratova et al., 2015). Such condition would translate in low reproducibility and less sensitivity (LOD and LOQ) of the method although the costs for setting up the instrument, as well as analysis are markedly cheaper when compared with GC-MS, LC-MS, and HPLC. However, voltammetry also has its own advantages *viz.* simple to operate for simultaneous analysis of organic and inorganic species, easy availability of solvents and electrolytes, rapid as well able to provide kinetic and mechanistic parameters (Scholz, 2015). In addition to the fact that the UPLC-MS retains the practicality of HPLC with significant improvement in the interrelated attributes of speed and resolution, its utilization in forensic entomotoxicology remains limited, probably because it is a relatively new category of analytical separation science (Sherathia et al., 2012) and therefore, requires time to gain popularity. UPLC-MS has been associated with faster separations with high specificity while requiring lower solvent consumption when compared with that of HPLC although the former can be associated with higher cost of maintenance and operation. Interestingly, the use of CE for analyzing drugs and poisons in entomological specimens has not been reported. Specific discussion on the possible use of CE is provided as part of the Section 3 of this article.

2.1. Determination of single-class poisons in entomological specimens

2.1.1. Pesticides

In a jointly published document, WHO and Food and Agriculture Organization (WHO & FAO, 2016), defines pesticides as "substances or a mixture of substances of chemical or biological ingredients generally intended for repelling, destroying, or controlling any pest or regulating plant growth". In the context of entomotoxicology, having the ability to determine the concentration of pesticides in entomological specimens proves relevant since many studies have reported the negative impact of pesticides on insects' growth (e.g. Abd Al Galil et al., 2021; Denis et al., 2018; Mahat et al., 2009, 2014). It is evident that the previous analytical studies on this aspect are limited to only a few pesticides although studies on the influence of pesticides on the growth of necrophagous insects are extensively reported in the literature. As such, a review of the literature only reveals the analysis of organophosphorus (OP) (malathion, parathion, and dichlorvos) (Dowling et al., 2022; Guntilake & Goff, 1989; Liu et al., 2009; Mahat et al., 2012; Rashid et al., 2009; Wolff et al., 2004; Yan-Wei et al., 2010), organochlorine insecticides (OC) (α and β endosulfan) (Magni et al., 2018) as and herbicide (paraquat) (Lawai et al., 2015; Wan Mahmood et al., 2015) as well as aluminum phosphide (AlP) (El-Ashram et al., 2022) in the entomotoxicological context. However, because studies have revealed the possible influence of chlorpyrifos and dimethoate on necrophagous insect development patterns (Abd Al Galil et al., 2021; Denis et al., 2018), and since diazinon is readily available in the market as a pesticide in agriculture, developing specific methods for analyzing these poisons in entomological samples merits forensic considerations. Peculiarly, a review of the literature reveals limited attempts to develop simultaneous determination of poisons such as pesticides in entomological specimens. As a matter of fact, most of the reported studies focused on single analyte analysis which may limit suitable interpretation to be drawn in entomotoxicology assessment as a result of lesser analytical efficiency. Summaries of the sample preparation and analytical procedures for pesticides in necrophagous insects reported by previous researchers are discussed below.

For the extraction method, solid-liquid extraction (SLE) has been identified as the most common method for extracting pesticides in entomological specimens, followed by solid-phase extraction (SPE). The favor towards SLE than that of SPE in forensic entomotoxicology studies may be due to the fact that the former has a simple operation that requires simple apparatus, resulting in a lower cost of

extraction than that of the latter. However, because SLE (a) requires the use of large volumes of solvents (high purity), (b) small enrichment of analytes, (c) is associated with low selectivity, and (d) formation of emulsions that may complicate the handling of sample of large volumes, its popularity as the method of choice may be reduced in near future. Since SPE (a) enables the storage of enriched analytes on its solid sorbent that would allow easy transportation, (b) requires smaller volumes of toxic solvents (c) with reduced formation of emulsion problems, its utilization has been acquiring popularity. Interestingly, in a relatively recent work published by Magni et al. (2018), quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction method was used for extracting endosulfan (α and β). QuEChERS has been recently utilized for extracting a myriad of analytes that included pesticides, therapeutic drugs, and toxins from the different matrices. This may be due to the fact that the method is rapid, simple, and cost-effective, as well as having the ruggedness and high throughput extraction procedure; favorable characteristics in analytical chemistry (Wolstenholme et al., 2021). Despite being routinely used in regulatory laboratories throughout the world for pesticides residues analysis in food, its application for extracting pesticides from entomological specimens appears nascent. Taking into account its advantages, utilization of QuEChERS for forensic application should be encouraged. As for the analytical instrumentations used for analyzing pesticides in entomological specimens, it is observed that gas chromatography (GC) (Guntilake & Goff, 1989; Liu et al., 2009; Magni et al., 2018; Mahat et al., 2012; Rashid et al., 2009; Yan-Wei et al., 2010) and HPLC (El-Ashram et al., 2022; Wan Mahmood et al., 2015; Wolff et al., 2004) as the methods of choice with the former being the more prevalent.

It is evident that malathion has been the most common OP insecticide investigated by researchers in forensic entomotoxicology. This may be due to its apparent role as a poison that can readily cause death (which can be seen in many suicide cases), as well as its possible influence as an insecticide that can directly affect the oviposition and developmental patterns of necrophagous insects, and hence the accuracy of PMI estimation (Liu et al., 2009; Mahat et al., 2012, 2019). Gunatilake and Goff (1989) were the first to describe a case study in which malathion was determined in the pooled larvae of *C. megacephala* and *Chrysomya rufifacies* (Macquart) (2,050 μg/g) collected from a dead body in Hawaii, emphasizing the possible use of necrophagous larvae as an alternative toxicological specimen for the determination of malathion. In addition to the direct use of the SLE method which was not specifically developed for entomological specimens, Gunatilake and Goff (1989) also did not attempt to optimize and validate the extraction method used. Moreover, the condition of the gas chromatography-nitrogen phosphorus detector (GC-NPD) used for the analysis was also not reported.

Rashid et al. (2009) used gas chromatography with an electron capture detector (GC-ECD) to quantitate the concentration of malathion in larvae (0.157 ± 0.092 μg/mg), pupae (0.202 ± 0.184 μg/mg) and adults (0.045 ± 0.021 μg/mg) of *C. megacephala*. A sample of *C. megacephala* (0.5 g) was extracted using SPE (HLB cartridge) with methanol: diethyl ether (10:90) as the elution solvent. However, the extraction method used in this study was neither developed nor optimized for entomological specimens, and the condition of GC-ECD used was also not reported. Furthermore, the authors did not provide the complete validation data; they only reported the R^2 value of 0.9497 for the calibration curve which appears unacceptable from an analytical point of view that would require the minimum value of R^2 of 0.995 (Food Drugs Administration, FDA, 2018) for the calibration curve to be accepted. Nonetheless, the authors used a small amount (0.5 g) of entomological specimen for analysis that can be favorable in the context of green chemistry since the extraction would only require small amounts of solvents while introducing lesser contaminants to the GC-ECD analysis.

Liu et al. (2009) reported the amounts of malathion in rabbit tissues (57.80–556.38 ng) and necrophagous larvae (3.53–35.96 ng), following the two dichloromethanes (30 mL) extraction steps and analyzed using GC-MS. The extraction method involved two evaporation steps that can potentially result in the loss of analytes. Moreover, considering that the extraction method involved a large amount of sample (10 g), the use of high volumes of solvents may be required, affecting the overall greenness assessment of the method as well as potentially introducing larger numbers of contaminants for the instrumental analysis. Despite using the same extraction method for both types of specimens (rabbit tissues and larvae), the optimization and complete validation data for larval specimens remain unreported. Although the authors reported the generally acceptable calibration curves ($R^2 > 0.999$), LOD (0.1 μg/mL), LOQ (0.3 μg/mL), recoveries (75% and 85%), and RSD (4.7–12.8%), evaluation of the matrix effects for necrophagous insect specimens was not reported. Interestingly, the calibration curves were prepared in acetone without using any sample matrix for quantifying malathion in animal tissues and necrophagous insect specimens. In a later study, Yan-Wei et al. (2010) attempted to correlate the

concentrations of malathion in necrophagous larvae (0.44–3.64 μg/g) with that of the initial quantity administered to rabbits (1/2 to 4 times of lethal dose) and found no reliable correlation. They utilized a similar extraction method as reported by Liu et al. (2009) and therefore, this study too can be affected by issues of extraction efficiency and matrix effects discussed earlier. Similarly, the authors did not report the complete validation data for the GC-MS analysis.

Using GC-MS, Mahat et al. (2012) reported the concentrations of malathion in entomological specimens that ranged between 6.10 and 137.20 ng/g. The authors concurred with the indication made by Yan-Wei et al. (2010) that no reliable correlation can be drawn between the concentration of malathion in entomological specimens with that of the initial quantity administered and the concentration in the tissues of the animal model used. Using the single tube SLE method (n-hexane, 250 μL) developed by the National Poison Centre of Malaysia (2006) for visceral tissues, the method was once again optimized and revalidated to extract malathion from the necrophagous insect samples (1 g each). The fact that the authors used a small amount of entomological sample, the extraction method appears in adherence with the principle of green chemistry. However, despite indicating that they performed the complete method validation (linearity and sensitivity, precision and accuracy, and recovery), such data are not available in the article.

Wolff et al. (2004) reported an analytical method aiming to determine parathion (another OP) in rabbit liver tissues and the 10 different species of arthropods using HPLC with a diode array detector (DAD); however, the analyte was not quantified for the arthropod samples. As for liver samples, the concentrations ranged between 1.38 and 2.97 mg/kg. In addition to the direct use of the SLE method, which was neither specifically developed for parathion (but malathion) nor entomological specimens, Wolff, et al. (2004) did not perform the optimization and validation processes for the analysis of parathion in their article. Furthermore, the authors prepared the calibration curves (R^2 = 99.532%) in a mixture of ACN: water (70:30) without the use of any sample matrix, and hence, the matrix effects pertaining to the use of necrophagous insects as specimens remain unknown.

Magni et al. (2018) quantitated the concentrations of α- endosulfan (0.44–15.50 ng/mg) and β- endosulfan (0.12–20.20 ng/mg) in *Calliphora vomitoria* L. collected from liver tissues using GC-MS. The QuEChERS extraction method developed for fruits and vegetables was revalidated by the authors to extract α- and β-endosulfan from the necrophagous insect samples (1 g), following the requirement of ISO/IEC 17025 and ICH guidelines. The authors reported highly sensitive LODs ($\alpha = 0.22$ ng/mg; $\beta = 0.21$ ng/mg) and LOQs ($\alpha = 0.73$ ng/mg; $\beta = 0.71$ ng/mg) as well as acceptable recoveries ($\alpha \approx 94\%$; $\beta = 78.5–85.5\%$), repeatability and specificity (no carry-over effects) parameters. In addition, the authors used a small amount of entomological sample for analysis, which is advantageous in the context of green chemistry, presumably implicating fewer contaminants into the GC-MS analysis. The fact that this was the first record of the successful use of the QuEChERS method in entomotoxicology (involving extraction/partitioning and dispersive-solid phase extraction steps), future endeavors capitalizing on this technique should be promoted.

Commonly used on crops and aquatic as a plant growth regulator and defoliant, paraquat dichloride is an herbicide known for its high toxicity towards humans and can readily cause death (Lawai et al., 2015). Besides OP (malathion, parathion, and dichlorvos) and OC pesticides (endosulfan and aldicarb), there were two studies conducted to detect the presence of paraquat in necrophagous specimens (Lawai et al., 2015; Wan Mahmood et al., 2015). Lawai et al. (2015) analyzed reduced paraquat using GC-MS in the necrophagous insect samples collected from the decomposing paraquat-exposed rabbit carcasses. To improve the recovery and reproducibility of the result for the GC-MS analysis, the reduction of quaternary ammonium presence in paraquat using sodium borohydride prior to the extraction process was conducted (Lawai et al., 2015). The reduced paraquat was extracted using the SLE method with diethyl ether (5 mL) as the extraction solvent. Moreover, the authors utilized a small amount of entomological sample (0.5 g), and the extraction process seemed to follow the green chemistry principle. However, despite proposing a specific method for analyzing paraquat dichloride in entomological specimens, validation data (*viz* analytical figures of merit, precision and accuracy as well as recovery) were not provided.

Wan Mahmood et al. (2015) reported the concentrations of paraquat dichloride in *C. rufifacies* that ranged between 0.16 and 0.57 mg/L. The sample of *C. rufifacies* was extracted using SPE with C18 cartridges, preconditioned using methanol (10 mL) and phosphate buffer (5 mL, pH 8.0) prior to HPLC-DAD analysis. However, the authors used the extraction method reported by Rashid et al. (2009), which was specifically developed for analyzing malathion, not paraquat. Although the authors indicated minor modification of the method, such modification was not explicitly described. While providing partial validation data that included linearity (1.0–50 mg/L, R^2 = 0.9982), LOD (0.03 mg/L), LOQ (0.1 mg/L), precision

(RSD = 3.75%) and recovery (62%), the LOQ (0.1 mg/L) reported by the authors was peculiarly outside of the analytical linear range (1.0–50 mg/L). Moreover, the precision data were derived from only 1 level of concentration calibrator (with no accuracy data provided), and the recovery percentage (62%) was evidently lower than the acceptable range of 80–120% (FDA, 2018).

In a recent study, El-Ashram et al. (2022) evaluated the concentration of aluminum phosphide (AlP) (is a low-cost insecticide, rodenticide, and fumigant) in a pooled sample of the 3rd instar larvae of *Chrysomya albiceps*, in view of mimicking human poisoning from suicide attempts and incidental environmental exposure. While the authors reported that the concentration was 11.24 µg/g (standard deviation was not provided), the article also did not describe the extraction method used for extracting AlP from the larvae. The authors cited a review article written by Gagliano-Candela and Aventaggiato (2001) although that review article did not discussed specifically on the extraction of AlP. In addition, El-Ashram et al. (2022) also did not report any analytical validation data to support the validity and reliability of the chemical analysis. Notwithstanding, they reported that the presence of AlP would delay the growth of *C. albiceps* as well as causing deformation to the larvae exhibited by "small-sized respiratory spiracles and deformed small posterior end with hypogenesis of the posterior respiratory spiracles".

2.1.2. Toxic metals, other elements and toxins

Pesticides apart, toxic metals (mercury, cadmium, and lead) and other trace elements (antimony, thallium, copper, zinc, iron, and barium) as well as toxins (chemical warfare simulants (CWAs) and hydrolysis products) have been detected in entomological specimens for various purposes (Gennard, 2012; Dowling et al., 2022). Older studies on toxic metals in necrophagous insects utilized atomic absorption spectroscopy (AAS) and NAA (Nuorteva & Nuorteva, 1982). On the other hand, the relatively new ones employed more sophisticated instrumentations like inductively coupled plasma-mass spectrometry (ICP-MS) (LaGoo et al., 2010; Malejko et al., 2020; Roeterdink et al., 2004) and ICP-optical emission spectroscopy (ICP-OES) (Motta et al., 2015; Rashid et al., 2012a) as well as square wave anodic stripping voltammetry (SWASV) (Bessa et al., 2021). As for chemical warfare simulant and hydrolysis products in necrophagous insects, the use of LC-MS/MS was reported (Dowling et al., 2022).

In contrast to pesticides whereby their determinations are solely made for the purposes of toxicological inference and alteration of PMI, the determination of toxic metals, trace elements and toxins in entomological specimens revolves around four common purposes. The purposes include (a) determination of the geographic origin of an unidentified victim (Nuorteva, 1977) and (b) detection of gunshot residues (GSR) (Bessa et al., 2021; LaGoo et al., 2010; Motta et al., 2015; Rashid et al., 2012a; Roeterdink et al., 2004) and other toxicants (Malejko et al., 2020) in blowfly for diagnosing the cause of death. In addition, such an analysis on entomological specimens is also used as (c) sensors for detecting CWAs and hydrolysis products (Dowling et al., 2022). Nonetheless, (d) to provide better biological understanding, evaluations on the changes in the physiology of flies (intracellular deposition of minerals as well as storage-excretion) following minerals exposures have also been reported (Sohal & Lamb,1977, 1979). Summaries of the sample preparation and analytical procedures for toxic metals, trace elements, and toxins in necrophagous insects reported by previous researchers (in view of the purposes for analyzing such compounds in entomological specimens) are discussed in the following paragraphs.

Perhaps Nuorteva (1977) was the first researcher that analyzed mercury (Hg) in entomological specimens for the purpose of determining the geographic origin of an unknown body discovered in a rural area of Inkoo, Finland. Citing the low Hg concentrations (0.12–0.15 ppm) observed in the specimens, it was inferred that the victim may have come from a relatively free Hg area, which was later confirmed by the police that the victim was a student for the city of Turku (free of Hg pollution area). Hence, it can be seen that the information gathered from the Hg analysis of entomological specimens had directed the police in the right direction for resolving the case. Notwithstanding, the fact that the elements of analysis and validation data were not reported, the extent of the accuracy, precision, and trustworthiness of the chemical analysis remains uncertain. Therefore, it is suggested that future research in this regard should focus on the analytical aspect of the analysis, so that the results to be reported would not be disputed in the court of law.

The analysis of trace metals (Pb, Ba, and Sb) that are commonly observed in GSR in entomological specimens has been acquiring popularity as a potential means for establishing the fact that a victim may have been shot dead (Bessa et al., 2021; LaGoo et al., 2010; Motta et al., 2015; Rashid et al., 2012a; Roeterdink et al., 2004). Roeterdink et al. (2004) reported about the determination of Pb, Ba, and Sb in the necrophagous insect samples. Each sample was digested using nitric acid (HNO_3) and perchloric acid ($HClO_4$) (4:1), followed by the analysis with the ICP-MS. From their Figures 1, 2, and 3, it can be seen that the concentrations of Pb, Sb, and Ba in the

samples analyzed ranged from 0–1000 ppb, 0–1200 ppb, and 0–140 ppb, respectively. However, the fact that the validation data are not provided by the authors, the robustness of the analytical methods used is unknown.

Using ICP-MS, LaGoo et al. (2010) reported the concentrations of Pb, Sb, and Ba in entomological specimens that ranged between 10.3–80.0 μg/g, 0.15–0.75 μg/g, and 0.42 μg/g–6.26 μg/g, respectively. The microwave digestion method (with HNO_3 and hydrogen peroxide (H_2O_2)) was used prior to analyzing Pb, Ba, and Sb in the necrophagous insect samples. The authors reported on the partial method validation data that included linearity (0–500 ng/mL, R^2 values were not reported), LODs (Sb = 0.106, Ba = 0.074, and Pb = 0.017 ng/mL), and LOQs (Sb = 0.10 to 0.25 ng/mL, Ba = 0.10 to 1.0 ng/mL and Pb= 0.10 to 1.0 ng/mL). Should the authors report on the complete method validation data, the integrity of the reported result may be enhanced.

In another study, Rashid et al. (2012a) used inductively coupled plasma-optical emission spectrometry (ICP-OES) to quantitate the concentrations of Pb (1.1825 mg/kg) and Ba (0.557 mg/kg) in a larval sample of *C. megacephala*, without any replication. The sample was subjected to the acid digestion method using HNO_3 (5 mL) on a hotplate until completely digested. Then, a volume (2 mL) of the mixture of HNO_3 and H_2O_2 (4:1) was added to the residue, heated until dryness, and subsequently solubilized with ultrapure deionized water. Calibration curves for Pb (R^2 = 0.997382) and Ba (R^2 = 0.997566) apart, other validation data (*viz* precision and accuracy as well as recovery) were not provided. Peculiarly, the amount of entomological specimen used for one particular analysis was reported as "10 larvae" without specifying the actual weight utilized, which can limit the reproducibility of the data that they tabulated.

Motta et al. (2015) reported the concentrations of Pb, Ba, and Sb in entomological specimens that ranged between 382.26–522.66 μg/L, 140.50 μg/L, and 39.18–56.14 μg/L respectively. The sample (0.05 g) was prepared using the microwave digestion method with the addition of concentrated HNO_3 (6 mL) and H_2O_2 30% (w/w), subjected to ICP-OES analysis. The use of the small amount of entomological sample may appear favorable for a greener extraction process as well as introducing a lesser number of contaminants during instrumental analysis. However, the authors did not provide the complete validation data, only the LODs (Pb:1.49, Ba: 0.15, Sb: 4.79 μg/L) and LOQs (Pb: 4.97, Ba: 0.50, Sb: 15.97 μg/L), were provided.

Using electrochemical as an alternative method for metal analysis, Bessa et al. (2021) determined Pb from the GSR in larvae samples *via* SWASV measurements. The sample (0.3 g) was subjected to the acid digestion method using HNO_3 (1.5 mL) at 150 °C on a hot plate for 2 h with the addition of H_2O_2 (0.5 mL), and adjusted to pH 7 prior to analysis. It was observed that the larvae exposed to 50 μg Pb g^{-1} had 11.5 μg/g of Pb, while the same was not detected for larvae exposed to 1.0 and 10 μg Pb g^{-1}. Since only a small amount of sample (0.3 g) was used for the analysis, the method appears in compliance with the principle of green chemistry, reducing the amount of contaminants during analysis. However, the authors didn't report the complete method validation data. Only data on linearity (50–400 μg/L), LOD (6.5 μg/L), calibration curve (R^2 = 0.995), and selectivity were reported.

Malejko et al. (2020) quantitated the concentrations of Cd and Tl in entomological samples that ranged between 2.04–21.10 ng/g and 0.07–5.90 ng/g, respectively. Microwave-assisted acid digestion using concentrated HNO_3 (2 mL) and H_2O_2 (1 mL) was used to prepare the samples (liver and necrophagous samples) before analysis using 1CP-MS. The analytical method was validated that included the instrumental detection limit, IDL (Cd= 0.049 ng/g and Tl = 0.015 ng/g), instrumental quantification limit, IQL (Cd= 0.16 ng/g and Tl = 0.051 ng/g), RSD of the slopes method detection limit (Cd= 0.8–4.1%, Tl = 3.2–6.7%), MDL (Cd =1.6–3.4 ng/g; Tl = 0.034–0.15 ng/g), method quantification limit, MQL (Cd = 5.5–11.4 ng/g; Tl = 0.11–0.51 ng/g) and RSD (Cd = 1.4–34.1%, Tl = 2.5–31.4%) as well as acceptable recoveries (from certified reference materials, CRM) of Cd (91.3%) and Tl (94.3%). Hence, it can be construed that the analytical method utilized by the authors was robust and sensitive for Cd and Tl analysis in entomological samples.

While it can be seen that the recent studies reported in the literature focused on contemporary issues related to the use of insect evidence, older literatures (Nuorteva & Nuorteva, 1982; Sohal & Lamb, 1977, 1979) emphasized in providing a suitable physiological understanding on the intracellular deposition of minerals as well as storage-excretion in flies and beetles. Using AAS, Sohal and Lamb (1977) measured the concentrations of Cu and Fe in insect specimens. From their reported Figures 1 and 2, the concentration (dry weight) were about 0–60 μg/g and 0–80 μg/g, respectively. Entomological specimens were dried at 120 °C (12 h), ashed at 650 °C (24 h), and the resultant ash was dissolved in 2 mL of concentrated hydrochloric acid (HCl) and diluted to 50 mL with distilled water prior to chemical analysis. However, the analytical condition of the AAS and method validation data were not provided. In another study, Sohal and Lamb (1979), reported the

concentrations of Zn, Ca, and Cu in the same specimens that ranged between 2–182 μg/g, 40–402 μg/g, and 1–77 μg/g respectively. Before the chemical analysis with AAS and atomic emission spectrophotometer (AES), the tissues were prepared by wet ashing in 4 mL HNO_3 (24 h) in a steam bath. The mixture was then evaporated to dryness and redissolved in 4 mL of 1 M HCl. Interestingly, these two studies (Sohal & Lamb, 1977, 1979) reported the use of "25 flies of both sexes" and "15 flies" of unspecified sexes, respectively. As such the actual weights used in both studies remained unknown and can possibly confuse the interpretation to be made by other researchers. Moreover, the conditions of the instruments as well as the validation data are not provided in these two studies.

Nuorteva and Nuorteva (1982) reported the bioaccumulation and excretion of Hg in the entomological specimens (blowflies and beetles). The concentrations detected in pooled samples ranged between 0.6 and 62.4 ppm. Samples (eggs, larvae, and pupae) were analyzed for Hg based on a fresh-weight basis and later converted to dry weight prior to NAA and FAAS analyses. However, a description on the sample preparation and analytical processes as well as the conditions of NAA and FASS were not provided. Furthermore, validation data (*viz.* analytical figures of merit, precision and accuracy as well as recovery) were also not reported.

As for the effect of heavy metals on the development of necrophagous insects, a review of literature revealed limited studies in this aspect (e.g. Al-Misned, 2003; Altunsoy & Başaran, 2011; Bessa et al., 2021; Diener et al., 2015; Ilahi et al., 2020; Kökdener et al., 2022; Kökdener & Yılmaz, 2021; Malejko et al., 2020; Nuorteva & Nuorteva, 1982; Rashid et al., 2012a; Servia et al., 2006; Singh & Kaur, 2017; Xie et al., 2014; Zhan et al., 2017). It has been reported that the accumulation of heavy metals in the bodies of necrophagous insects may alter the ion balance at cellular level, the acidity, the permeability as well as the polarity of the cell membrane, causing metabolic disorders, retarded growth and life-history changes at the organism level (Xie et al., 2014). Moreover, studies also revealed the adverse impacts on population dynamics, survival rate as well as reproduction and biodiversity that are attributable to heavy metal accumulation in necrophagous insects (Ilahi et al., 2020). Specifically, the increased amounts of Cd, Zn and Cu in necrophagous insects is associated with higher rates of mortality as well as reduction in infecundity and development (Al-Misned, 2003; Kökdener et al., 2022; Singh & Kaur, 2017; Xie et al., 2014). While Cu has been reported as an essential trace mineral for insects, responsible for a variety of metabolic processes (e.g. consumption of oxygen, efficiency of energy and enzymatic functions), its excessive accumulation can lead to negative implication on insects' reproductive process as well as development, behavior and survival rate (Servia et al., 2006; Kökdener et al., 2022). With regards to the presence of Pb and Ba as the component of GSR, necrophagous insects that were exposed to this particular substance demonstrated slower developmental rate and total duration of life cycle, requiring approximately 12 h longer than that of controls (Rashid et al., 2012a). In this context, Bessa et al. (2021) attributed such an observation to the changes larval metabolism related to the absorption and excretion of food by necrophagous insects such as *Lucilia cuprina* and *L. sericata*. Interestingly, while Hg can be extremely toxic to human, Nuorteva and Nuorteva (1982) reported that no clear health effects were observed in sarcosaprophagous flies exposed to high Hg concentrations (up to 62.4 ppm fresh weight). The fact that any substance that can affect the development of necrophagous insects can subsequently affect the accuracy of PMI estimate and since suitable literature regarding heavy metals influence remains limited, suitable empirical endeavor to clarify such an aspect merit forensic consideration. On the positive notes, the fact that insects are sensitive to environmental changes, particularly heavy metals contamination, their utilization as the possible bioindicator for detecting the environmental pollution has been suggested by the previous researcher (Diener et al., 2015).

2.2. Determination of single-class drugs in entomological specimens

In comparison to poisons, studies that detected and determined drugs in entomological specimens are more extensively reported in the body of literature. According to the sequence of popularity, the drugs included opioids, benzodiazepines, antidepressant, barbiturates, illicit/recreational drugs as well as miscellaneous drugs. Among the opioids, morphine, heroin, and codeine are prevalently determined in entomological specimens followed by methadone and its metabolites, tramadol as well as mitragynine (ketum) (e.g. Elshehaby et al., 2019; Gosselin et al., 2011; Kintz et al., 1990a; Kintz et al., 1994; Rashid et al., 2012b). As for the benzodiazepines, determinations of flunitrazepam, diazepam, nordiazepam, oxazepam, bromazepam, temazepam, triazolam and prozepam in the same matrix have also been reported (e.g. Bugelli et al., 2017; Oliveira et al., 2014; Wood et al., 2003). While amitriptyline, nortriptyline, trimipramine, and trazodone being the common antidepressant agents detected (e.g. Miller et al., 1994; Sadler et al., 1995; Wilson et al., 1993),

phenobarbital and secobarbital are barbiturates reportedly observed in entomological specimens (Kintz et al., 1990a; Kintz et al., 1990b; Levine et al., 2000). Moreover, the common cannabinoid detected are Δ^9-tetrahydrocannabinol (THC) and its metabolite 11-carboxyl-delta-9-tetrahydrocannabinol (THCA) (Karampela et al., 2015). In addition to the illicit/recreational drugs (methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA) and their metabolites *viz.* 3,4-methylenedioxyamphetamine (MDA), methylphenidate, ρ-hydroxy methamphetamine (ρ-OHMA)), other drugs like ketamine, nicotine and paracetamol (acetaminophen) have also been detected in entomological specimens (Bushby et al., 2012; Goff et al., 1997; Magni et al., 2014).

In this context, the detection and determination of drugs in entomological specimens pertained to (a) the interpretation of the influence of such drugs on the developmental patterns of the necrophagous insects as well as (b) suggesting the causes and circumstances surrounding deaths. A review of the literature reveals liquid-liquid extraction (LLE) and SPE as the prevalently used extraction methods for drugs in entomological specimens, attributable to the analytical benefits and advantages discussed earlier in Section 2.1.1. As for the instrumental analysis, the usages of HPLC (e.g. Khan et al., 2015) GC-NPD (e.g. Levine et al., 2000), GC-MS (e.g. Ishak et al., 2019), LC-MS (e.g. Karampela et al., 2015), LC-MS/MS (e.g. Dowling et al., 2022), NIRS (Oliveira et al., 2014) and ATR-FTIR spectroscopy as well immunoassay (Baia et al., 2016) have been reported. Taking into account the necessity to develop simultaneous determination analytical procedures for forensic entomotoxicological assessment, recently published articles that explored such an aspect have been identified, the detailed discussion is provided in Section 2.3.

2.2.1. Opioid

The utilization of entomological specimens in the opiates analysis was first reported by Kintz et al. (1990a) and Kintz et al. (1994). The first case involved the putrefied corpse of a 31-years old white man identified from an autopsy known to be a chronic heroin abuser who had been dead since 4 July, and the body was found on 13 July 1989 at his home. Internal organs (heart, liver, whole blood, kidney, and brain) and calliphorid larvae collected from the putrefied corpse were subjected to toxicological analysis (Kintz et al., 1990a). FPIA was used for initial screening, and further analysis was conducted using GC-NPD for both internal organs and larvae. From the analysis of FPIA and GC-NPD, morphine was detected in two larvae samples at concentrations of 182 µg/kg and 165 µg/kg, respectively.

Kintz et al. (1994) described another case of a male body who had been dead for 10 days and was known to be a chronic heroin abuser. The body was completely decomposed and covered with calliphorid larvae. The analysis was performed on the biological samples (blood and bile) and the larvae collected on the corpse. The homogenized larvae sample was subjected to enzymatic hydrolysis with β-glucuronidase and solvent extraction using a mixture of v/v, 50:17:23 of chloroform-isopropanol n-heptane. The bis-(trimethylsilyl, trifluoroacetamide) (BSTFA) containing 1% trimethylchlorosilane (TMCS) was used to derivatize the drug extract followed by the detection *via* GC-MS with ion trap detector and selected ion monitoring (SIM) mode (Kintz et al., 1994). While the morphine concentrations of 168 µg/L, 357 µg/kg, and 90 µg/kg were found in blood, bile, and larvae, approximately 37 µg/L, 88 µg/kg, and 12 µg/kg were detected for codeine, correspondingly. Even though the concentration of morphine and codeine in larvae was lower than those detected in blood and bile, it showed that the analysis of drugs in larvae as alternative specimens for decomposed cadaver was feasible. Hence, analyzing this drug in necrophagous insects can help in indicating the circumstances surrounding death such as therapeutic use and addiction. However, both articles (Kintz et al., 1990a; Kintz et al., 1994) did not provide suitable validation data, in fact, they utilized the extraction method for human tissues (Kintz et al., 1989) which was not specifically developed for entomological specimens. As a matter of fact, the method by Kintz et al. (1989) also did not provide any validation data. Hence, the efficiency of the extraction methods as well as the robustness, reproducibility, and repeatability of the analytical methods used by these authors (Kintz et al., 1990a; Kintz et al., 1994) remain uncertain. Besides, unreliable analytical data might not only be contested in court but could also lead to unjustified legal consequences.

In 2001, a group of researchers analyzed the concentration of morphine in beetles (*Dermestes frischi* Kugelmann and *Thanatophilus sinuatus* Fallen) (Bourel et al., 2001) and flies (*Protophormia terraenovae* and *Calliphora vicina*) (Bourel et al., 2001) using the same radioimmunoassay method (RIA). While both authors reported the LOD of 1 ng/mL, other validation parameters (including LOQ) were not available in their articles. Determination of morphine in *Calliphora stygia* (Fabricius) through an analysis conducted by HPLC with acidic potassium permanganate chemiluminescence was first reported by Gunn et al. (2006). The drug was extracted from the larvae of *C. stygia* collected from minced beef homogenized with different morphine concentrations (0,

500, 1000, 2500, 5000, and 10,000 ng/g). Preliminary Flow injection analysis (FIA) was conducted and revealed that the maggot matrix produced a significant chemiluminescence signal with the presence of potassium permanganate which can interfere with the analysis. Thus, several sample clean-up experiments were conducted before being subjected to LLE to avoid false positive detection (Gunn et al., 2006). Because the LOD established in the study was 2500 ng/g, the morphine concentration in larvae reared on the substrate below than the LOD cannot be detected and improvement to the sample clean-up should be performed. The results showed that the analytical method proposed by the authors was sensitive to analyze morphine in the larvae, as low as 2500 ng/g (Gunn et al., 2006). While no significant correlation was identified, the concentration of morphine incorporated in the substrate was directly related to morphine concentrations in larvae. Peculiarly, while the authors reported about the LOD (2500 ng/g), the LOQ was not provided. The fact that the authors reported the lowest concentration of morphine as 765 ng/g, which was much lower than the LOD itself, the validity of the data they reported appeared questionable.

In another study, Parry et al. (2011) investigated the presence of morphine isolated from the Malpighian tubules of *C. stygia*. The analysis of pooled secreted droplets of morphine was performed using HPLC with acidic potassium permanganate chemiluminescence detection. This work attempted to explain the pharmacological aspects (excretion and accumulation) of morphine in necrophagous flies, which are generally lacking in many forensic entomotoxicology studies. The analysis was done semi-quantitatively, rendering the actual amount of morphine as uncertain. Therefore, suitable quantitative analysis using the same experimental model may provide better insights into the matter. It was postulated that morphine was metabolized into a yet to be characterized metabolite, following its transportation across the Malpighian tubules' cells. As such, further studies for specifying the identity of the metabolite are indeed required.

Salimi et al. (2018) identified and quantified morphine in *C. albiceps* and *Creophilus maxilosus* reared on rabbit carcasses administered with different concentrations of morphine. Upon completion of acid digestion (ammonium sulfate in 1 L of 20% HCl), the drug was extracted using LLE. Screening of morphine was performed by thin-layer chromatography (TLC), and confirmatory analysis was done using HPLC. The concentrations of morphine in entomological samples ranged between 25.64 and 210 ng/g with satisfactory selectivity; however, the possible matrix effect that can influence the validity of the data was not reported in the article.

Kharbouche et al. (2008) reported the determination of codeine, morphine, and norcodeine in the different stages of blowfly *L. sericata* (larvae, prepupae, pupae, and imago) using LC-MS operating with SIM mode. The larvae reared on minced pig liver substrates treated with different concentrations of codeine. The drugs were extracted by LLE using a mixture of 50: 17: 33, v/v of chloroform, isopropanol, and n-heptane, respectively. In this study, codeine and its metabolites (morphine and norcodeine) were detected in all larvae samples and the concentrations corresponded to the initial concentrations of codeine spiked in the substrates. This method had a linear range between 1 and 500 ng/500 mg for codeine, morphine, and norcodeine with $R^2 > 0.99$, within and between-day precision (11.9 and 18.7%), LODs (codeine = 0.5, morphine = 1.0 and norcodeine = 1.5 ng/500 mg), and LOQ (fixed at 5 ng/500 mg). The percentage recoveries established at three concentrations (10, 150, and 400 ng/500 mg) were about 54 to 85% for codeine, 19 to 21% for morphine, and 39 to 65% for norcodeine. The extraction recoveries obtained in this study were relatively low (<80%), probably attributable to the unsuitable condition of the extraction method used to isolate the analytes. Hence, further improvement on the suitable condition of the analytical method for larvae samples needs to be investigated since the method did not show satisfactory results for the determination of codeine and its metabolites in *L. sericata*. In addition, performing the matrix effect study also proves useful.

Ishak et al. (2019) attempted to detect heroin metabolites (morphine and hydromorphone) in each developmental stage of *L. cuprina* (Weidemann) reared on minced buffalo meat substrates homogenized with five different concentrations of heroin. Successful detection of morphine and hydromorphone in the 2nd and 3rd instar larvae fed on 5000 and 10,000 ng/μL of heroin-containing substrates using acid-alkali extraction and GC-MS was reported. However, the method used was not specifically developed for analyzing heroin and its metabolites in entomological specimens, neither that optimization and re-validation of the method were attempted by the authors. As indicated by the authors, a specific emphasize on understanding the pharmacokinetics of the drug in the different species of necrophagous larvae proves pertinent for forensic entomotoxicological inference. This understanding is particularly important for explaining the influence of heroin on the developmental pattern of *B. peregrina* reported by previous researchers (Goff et al., 1991),

in view of its practical value in estimating accurate PMI.

Gosselin et al. (2010) developed and validated an analytical method using LC-MS/MS for the determination of methadone and its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) in the third instar larvae and adult of *L. sericata*. The quantitation of both methadone and EDDP was conducted by the integration of the area under the specific multiple reaction monitoring (MRM) chromatogram. While reporting varying concentrations of methadone and EDDP, the analytical method utilized by the authors suffered from unsatisfactory recovery percentages. The recoveries for the 3rd instar larvae (methadone: 77.1–79.2%; EDDP: 67.7–71.4%) and pupae (methadone: 77.0–98.2%; EDDP: 8.0–74.6%) were observably lower than that of adults (methadone: 90.1–96.8%; EDDP: 84.2–95.3%). Such relatively low recovery percentages may possibly translate into inaccurate concentrations of the analytes reported by the authors.

One year later, Gosselin et al. (2011) reported a new method using UPLC-MS/MS for analyzing methadone and EDDP in *L. sericata* empty puparia. The heart beef substrates were spiked with five different methadone concentrations and used to rear larvae of *L. sericata*. The extraction of the drugs was done by utilizing the LLE method that demonstrated high and reproducible recoveries of more than 86% for all analytes with no carry-over observed. The analytical method proposed by the authors demonstrated successful quantification of methadone in the larvae fed with heart beef spiked with high methadone concentrations. As proposed by the authors, EDDP is more hydrophilic than methadone, leading to rapid elimination by the larvae. Beneficially, the analytical method proposed was fast, where all the analytes eluted within 6.5 min compared to the previous study using LC-MS/MS (total run-time: 15 min). The LOQ established in this study for methadone and EDDP was 4.13–4.13 pg/mg and 3.81–3.82 pg/mg, respectively, which showed the sensitivity of the analytical method. Additionally, the analytical method developed by the authors was validated according to the USFDA guideline and publications regarding the validation of bioanalytical methods. The use of the small amount of entomological sample in this study supported the principle of green chemistry as well as introducing a lesser number of contaminants during instrumental analysis.

Concerning tramadol, a study (El-Samad et al., 2011) using HPLC-with UV-Visible spectrophotometric detector was conducted to determine the concentration of the drug in the larvae of *L. sericata* as well as its effect on the insect development. The spiked rabbit carcasses with different concentrations of the drug (R0 (control), 550 (R1), 1100 (R2), and 2200 (R3) mg/kg) were used to rear the larvae of *L. sericata*. The larvae were subjected to LLE with ethyl acetate as the extraction solvent. Detection of tramadol showed its presence in the feeding third instar larvae of *L. sericata* in lower concentrations than those in the tissues used as the food source. However, the authors found that the tramadol concentrations in the larvae were significantly correlated ($p < 0.01$) with the initial dosages and concentrations found in the tissue used as a food source. This finding was consistent with two other similar studies by Introna et al. (1990) and Tantawi et al. (1996) who found strong correlations between the concentration of drugs administered and that determined in the tissue samples (El-Samad et al., 2011). However, the fact that the analytical method used was neither developed nor optimized for entomological specimens and the complete validation data were not provided, the robustness, accuracy and precision of the method used by the authors are unknown. In another study, Elshehaby et al. (2019) while investigating the effect of tramadol on the microscopic morphology on *C. albiceps* third instar larvae, in order to estimate PMI, reported that the concentration of the analyte as 29.62 µg/g. However, the authors did not report the extraction method used as well as the validation data, rendering uncertainty in the appropriateness of the chemical analysis.

Mitragynine is the primary active alkaloid found in *Mitragyna speciosa* Korth (ketum) that possess a potent opioid agonistic activity, and is widely available in the black market. In the context of entomotoxicology, researchers attempted to detect the presence of mitragynine in entomological specimens for inferring its negative influence on the survival rate and growth patterns of *C. megacephala* (Rashid et al., 2012b) and *C. rufifacies* (Rashid et al., 2013). Both groups of authors (Rashid et al., 2012b; Rashid et al., 2013) utilized the same extraction method (SPE), prior to performing the HPLC-UV analysis. However, the fact that they reported the R^2 values $<$ 0.995 for their calibration curves, the robustness of the chemical analysis appears unsupported.

2.2.2. Benzodiazepines

Wood et al. (2003) developed a simultaneous and sensitive analytical method for determination of ten different benzodiazepines in larvae and puparia of *C. vicina* using acetonitrile (ACN) precipitation analyzed *via* reversed-phase LC-MS/MS. The benzodiazepines were alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nordiazepam, oxazepam, prazepam, temazepam. In developing the method, the authors used a set of larvae and puparia of *C. vicina* as calibrators that were spiked with different

concentrations of a mixture of benzodiazepines standards. The validation data for all the ten analytes in both the larvae and puparia of *C. vicina* were within the acceptable ranges for the analytical method to be considered as accurate, sensitive, and robust. The linearity, LOD, LOQ, recovery, as well as precision and accuracy (both as CV %) for larvae matrix were $R^2 > 0.999$, 1.88–5.13 pg/mg, 7.63–20.63 pg/mg, >90%, 0.4–11.2%, and 1.4–11.7%, respectively. As for the pupae the same parameters were $R^2 > 0.995$, 6.28–19.03 pg/mg, 25.23–73.93 pg/mg, > 90%, 2.0–14.8%, and 2.8–19.8%, correspondingly. Then, the validated method was applied on *C. vicina* (larvae and puparia) reared on beef-heart treated with 1 µg/g nordiazepam. The authors then reported the concentrations of nordiazepam (larvae: 311–467 pg/mg; puparia: <50.0–98.9 pg/mg) and oxazepam (larvae: 571–>750 pg/mg; puparia: 74.7–311.0 pg/mg) in true samples of larvae and puparia of *C. vicina*.

For studying the influence of the different concentrations of nordiazepam and oxazepam on the growth pattern of *C. vicina,* Pien et al. (2004) utilized the simultaneous analytical method developed by Wood et al. (2003). The result showed that nordiazepam and oxazepam were present in the larvae and puparia of *C. vicina*. The analytical methods proposed by these authors (Pien et al., 2004; Wood et al., 2003) had demonstrated higher sensitivity using LC-MS/MS because of their ability to detect small amounts of the drugs (ρg/mg) in single larvae and puparia. Thus, the method can be considered sensitive and robust for determining nordiazepam and oxazepam in entomological specimens (Pien et al., 2004).

In the other study, Carvalho et al. (2001) qualitatively determined the presence of diazepam in larvae of *C. albiceps* and *Chrysomya putoria* (Weidman) using GC-MS with multiple ion monitoring. The effects of this drug on the development of these two species were also investigated by given twice the lethal dosage of diazepam to the rabbits and the tissue of the rabbit that was used as a food source to rear the larvae of both species. For the extraction, the homogenized larvae sample was subjected to the LLE method initially developed for human blood, prior to analysis with GC-MS. While only reporting a single value of $R^2 = 0.98$ for the calibration curve, other important validation data that can prove the validity and robustness of the analytical method used by the authors were not provided. In this context optimization of the extraction method as well as the GC-MS conditions shall be attempted, considering the differences that might occur in the matrix effects for entomological specimens than that of blood.

In view of providing non-destructive/less destructive analytical methods that were statistically supported, Oliveira et al. (2014), Baia et al. (2016) and Lima et al. (2018) reported the integration of spectroscopic techniques with the chemometrics analysis for identification and classification of flunitrazepam at varying concentrations in the different stages of fly development. In addition to the spectroscopic technique, Lima et al. (2018) also investigated the feasibility of integrating the voltametric technique with chemometrics for the same purpose. While these studies (Baia et al., 2016; Lima et al., 2018; Oliveira et al., 2014) advocated the potential use of such a combinatory approach of spectroscopic/voltametric and pattern recognition techniques in forensic entomotoxicology, its real application may prove as farfetched considering the issues relating to the repeatability and reproducibility of the analysis. In particular, the performances of differential pulse voltammetry (DPV) and fluorescence excitation-emission matrix (EEM) spectroscopy are extremely sensitive to environmental variations namely pH, temperature and solvent properties as well as the presence of fluorescence quenchers (for EEM). Therefore, a great deal of further studies is required for empirically supporting the notion made by these groups of researchers (Baia et al., 2016; Lima et al., 2018; Oliveira et al., 2014) before the approach can be admissible in court, especially in criminal trials. In this context, legal factors such as the onus of proof as well as admissibility standard for forensic evidence (e.g. Daubert standard and Frye's general acceptance) must be duly considered to avoid the possibility of miscarriage of justice.

Bugelli et al. (2017) reported a case study that presumably a suspected maternal filicide is by fire. The authors reported the concentrations of diazepam and its metabolites (nordiazepam and oxazepam) in both the visceral tissues of the victims and larvae of *L. sericata*. The concentration of diazepam and metabolites were determined using GC-MS/MS. Expectedly, the concentrations of diazepam and its metabolites were markedly lower in the larvae when compared with that of visceral tissues, and concluded that the analysis of entomological specimens would reveal not only the cause of death but also circumstances surrounding death such as sedation. However, the toxicological inference made using victim's blood and other visceral tissues (if available) remains the more reliable forensic assessment than that of the necrophagous insects in death investigations.

In another study, Al-Shareef et al., (2021) reported that the larval growth of *C. albiceps* alone was accelerated by 12 h in the presence of diazepam in decomposing rabbit tissues, which can potentially

affect the estimation of PMI. The authors utilized SPE for extracting the analyte followed by LC-MS/MS. While only reporting the conditions of LC-MS/MS, the authors did not provide suitable analytical validation data to substantiate the validity and reliability of the analysis. As for clonazepam, Afifi et al., (2022) investigated the effect of this benzodiazepines on the development of *Sarcophaga argyrostoma* (Robineau-Desvoidy). They reported that larvae of *S. argyrostoma* that feed with minced meat mixed with high concentration (50 & 100 mg/ml) of clonazepam demonstrated faster larval growth rate which may affect the accuracy the PMI estimation for forensic investigations. While utilizing the extraction method (Rojas et al., 2017) that was specifically developed for extracting clonazepam in human oral fluid, extraction of the same analyte from the larvae of *S. argyrostoma* was attempted. In this instance, Afifi et al. (2022) did not provide the data on the matrix effect of the larvae as well as the complete validation data for proving the reliability and validity of the analysis. Although the authors reported the R^2 values that ranged between 0.81 and 0.91, such values were far lower than the acceptable R^2 value of $\geq$0.995 prescribed by FDA, 2018.

2.2.3. Antidepressant

A review of literature on the detection/determination of single-class antidepressants in entomological specimens revealed the works by four different groups of researchers, focusing on amitriptyline and nortriptyline (Goff et al., 1993; Miller et al., 1994) as well as fluoxetine (Zanetti et al., 2021; Zanetti et al., 2016). While investigating the effect of the different concentrations (300, 600, and 1000 mg in 10 mL normal saline) of amitriptyline (and nortriptyline, the metabolite) on the development of *Parasarcophaga ruficornis* (Fabricius), Goff et al. (1993) indicated that the drugs would sufficiently alter a PMI estimate by up to 77 h. The authors further reported the concentrations of amitriptyline and nortriptyline in the larvae collected at 48 h from liver substrates that ranged between 2.0–27 mg/kg and 0.6–3.7 mg/kg, respectively. While the authors explicitly indicated that the visceral tissues collected from the rabbits were submitted to Chemical Toxicology Institute, Foster City, California, it remains unclear who performed the HPLC analysis for amitriptyline and nortriptyline in the larvae specimens. In a later study, Miller et al. (1994) modified the unspecified extraction method for amitriptyline and nortriptyline, originally developed for hair specimens, for analyzing the compounds in Phoridae puparia, dermestid skins, and frass. However, neither that the authors (Miller et al., 1994) revealed the original extraction method for hair nor that they specified the modification made to the existing ones for entomological specimens' analysis. They reported the concentrations of amitriptyline in the puparia (5.4 ng/mg), dermestid skin (3.4 ng/mg), and frass (3.6 ng/mg). As for nortriptyline, 2.5 ng/mg was determined in the puparia; the metabolite was not detected in both the dermestid skin and frass. Pertinently, the suitable validation data were not provided by both groups of researchers (Goff et al., 1993; Miller et al., 1994).

Zanetti et al. (2016) determined the concentrations of fluoxetine in *Dermestes maculatus* (Degeer) (larvae, pupae, and adults) using a UV spectrophotometer at two different wavelengths (270 and 277 nm). At 270 nm, the concentrations of fluoxetine were 16.1–23.0 µg/µL, 18.4–24.2 µg/µL, 14.9–29.15 µg/µl in larvae, adult, and exuvia, respectively. The same were 31.1–42.2 µg/µL, 22.1–43.7 µg/µL, 27.2–41.85 µg/µL at 277 nm, correspondingly. Although the authors indicated that they performed the calibration curve, the R^2 and RSD for the calibration curve, as well as other important validation data (e.g. recovery, precision, and accuracy) that can prove the validity and robustness of the analytical method, were not provided. Subsequently, Zanetti et al. (2021) while attempting to study the effect of fluoxetine on the development of *D. maculatus* using pork muscle (non-living animal model) and pig (living animal model), validated the GC-MS method to analyze the concentrations of such a drug in the beetles. The authors reported that fluoxetine in pork muscle (2000 mg/kg) and orally administered to pigs (833 mg/kg) "had no detectable effect on the development of *D. maculatus*", and concluded that the PMI estimates remain accurate even in the presence of fluoxetine. A close examination of the analytical figures of merit (Table S4 of the supporting information) revealed the calibration range, LOD and LOQ of 1000–8000 ng/mL, 61.02 ng/g and 1000 ng/mL, respectively. Other validation data were not provided. Peculiarly, not only the LOQ (1000 ng/mL) reported by the authors was markedly more than 100% higher than that of LOD (61.02 ng/g), the concentrations of fluoxetine in larvae (11.60–12.16 ng/mL) reported in Table 1 were largely inconsistent with that of the calibration range (1000–8000 ng/mL). Typically, the LOD and LOQ should be at 3:1 and 10:1 of signal-to-noise ratio (ICH, 2015). As such, the validity of the analytical method used by the authors would require suitable clarification.

2.2.4. Barbiturates

A review of literature on the analysis of barbiturates (as a single-class drug) in entomotoxicological specimens reveals two articles that were written by Beyer et al., (1980) and Levine et al. (2000). Beyer et al. (1980) while analyzing the maggots recovered from a decomposed dead body of a 22- year-old white

female reported the 100 µg/g of phenobarbital. The maggots were subjected to the protein precipitation using 10% sodium tungstate (22 mL), 10% sodium hydroxide (4 mL), and 3 N sulfuric acid (20 mL), and extracted twice with chloroform (50 mL each time). The determination of phenobarbital was made using gas chromatography (unknown detector), the identity of which was confirmed using TLC and GC-MS. Similarly, Levine et al. (2000) reported about the successful qualitative identification of secobarbital in maggots collected from a highly decomposed dead body (other conventional toxicology specimens were not suitable for analysis). Upon completion of extraction using the Chem Elut column with dichloromethane, the qualitative identification of secobarbital was made using GC-MS. Because Levine et al. (2000) did not quantitatively determine the concentration of secobarbital in the maggots, suitable interpretation on the cause of death could not be fully supported since the drug is medically used for treating insomnia and sedative. As such, the effect of the drug is dose-dependent. However, the fact that the qualitative identification of secobarbital can still provide circumstances surrounding death could not be ruled out. It can be seen that both groups of authors (Beyer et al., 1980; Levine et al., 2000) emphasized on the relevance of analyzing entomological specimens for barbiturates in the forensic context. Nonetheless, since they used analytical methods that were not specific for larval matrix, neither that they provide the appropriate method validation parameters, the robustness, repeatability, and reproducibility as well as the matrix effect for the methods used can be questioned. As for the analysis of multi-class drugs (including barbiturates) in entomological specimens, a specific discussion is provided in Section 2.3.2.

2.2.5. Illicit/recreational drugs

A study by Goff et al., (1992) evaluated the effects of MA on the developmental rates of *P. ruficornis* reared on liver tissues harvested from rabbit carcasses administered with varying concentrations of MA *via* ear vein infusion. The authors reported that the larvae of *P. ruficornis* reared on liver substrates that presumably contained 71.4 and 142.9 mg of MA had developed more rapidly than those of control and substrates that contained the lowest concentration (37.5 mg) of MA. In contrast, the time required for pupariation was longer for *P. ruficornis* reared on MA-containing liver substrates when compared with that of control substrates. The authors concluded that the alteration in the developmental rates by MA may potentially affect the accuracy of PMI estimates. Interestingly, the authors utilized the semi-quantitative determination of MA using RIA, with the cut-off value >50 ng/mL to indicate the positive presence of the analyte in both the entomological and conventional toxicological specimens. As such, the authors themselves reported that the RIA analysis performed may not be accurate, causing substantial doubts in the validity of the interpretation on the growth patterns since they indicated that the influence of MA on *P. ruficornis* as dose-dependent. Considering the semi-quantitative nature of the RIA, the accuracy of the concentrations of MA that they reported in blood and liver samples may require further clarification. The fact that an adequate description on sample preparation as well as method validation was not provided in the article, a suitable discussion on the validity of the chemical analysis performed could not be made.

In another study, Goff et al. (1997) attempted to interpret the influence of the different concentrations of MDMA and its metabolite MDA on *P. ruficornis* reared on liver substrates containing MDMA. The chemical analysis for both entomology and conventional toxicology specimens (blood and liver of rabbits) was performed using LC-MS with atmospheric pressure ionization (API) electrospray interface. Not only that the authors did not report the analytical validation data for the LC-MS analysis, the method also appeared as non-optimized for entomological specimens, which can be clearly seen in the broaden and tailed chromatograms reported as Figure 1 in the article. Moreover, without the appropriate optimization and evaluation of the matrix effect, the extraction of MDMA and MDA from the entomological specimen was performed using the same base LLE method specifically developed for the blood and liver specimens. The authors, however, reported that the concentrations of MDMA and MDA in entomological specimens that ranged between 0.4–22.8 ng/mg and 0.8–10.1 ng/mg, respectively. The authors also indicated that MDMA may possibly reduce the duration of larvae development, an aspect that needed to be considered when estimating PMI.

To investigate the effect of MA and p-hydroxymethamphetamine (ρ-OHMA) on *C. stygia* in Australia, Mullany et al., (2014) utilized spiked minced kangaroo meats as substrates. The authors utilized a somewhat complex SLE method involving several solvents *viz.* dichloromethane (2 × 10 mL), di-tert-butyl dicarbonate, and tetrahydrofuran, followed by the HPLC-UV analysis. They reported the faster larval growth with increased sizes for all the different stages during metamorphosis and longer pupation period, attributable to the presence of MA and ρ-OHMA. While concluding that the estimates of minimum PMI for corpses with MA using the growth data of *C. stygia* could be incorrect, the authors did not

quantitate these two compounds in *C. stygia* although many entomotoxicological studies reported about dose-dependent relationships. As such, a suitable categorical inference on the extent of the influence could not be made. In addition to the authors' own indication that the method of homogenization was relatively crude and may subject to complete lysis of cells affecting the released of stored MA, the use of HPLC-UV also appeared inappropriate due to its low sensitivity.

In another study, Magni et al. (2014) quantified 5 ng/mg and 10 ng/mg of MA in larvae of *C. vomitoria* using their newly developed GC-MS (with electron impact ionization operated using SIM mode) analytical method. The sample of *C. vomitoria* (larvae and pupae) were extracted *via* the SLE method (methanol), followed by derivatization of the residue using trifluoroacetic anhydride and recovered by tert-butyl methyl ether before the GC-MS analysis. The GC-MS method for determining MA in *C. vomitoria* by the authors can be considered as fairly robust, accurate and sensitive, reporting the acceptable validation data *viz.* linearity ($R^2 > 0.99$), LOD (0.10 ng/mg), LOQ (0.33 ng/mg), extraction recovery (ER% = 78.77–88.91%) and repeatability (CV%= $5.8 - 19.05\%$) as well as specificity. The concentrations of MA in *C. vomitoria* ranged between 0.39 and 6.70 ng/mg.

Wang et al. (2020) reported the LLE method for extracting MA in another dipteran species (*Aldrichina graham* (Aldrich)), followed by the GC-MS analysis. Upon completion of LLE method, N-Methyl-N-(trimethylsilyl) trifluoroacetamide: methanol (1: 1, 100 µL) mixture was used to recover the residue. The authors reported the linearity ($R^2 > 0.99$), LOD (0.10 ng/mg), LOQ (0.33 ng/mg), extraction recovery (ER% = 73.9–102.1%), selectivity, carryover and accuracy (CV = $7.7 - 19.2\%$). However, the fact that the concentrations of MA in *A. graham* (as low as 0.06 ng/mg) were lower than the LOQ (0.33 ng/mg), causing considerable doubt in the accuracy and sensitivity of the method.

Karampela et al. (2015) developed a simultaneous and sensitive analytical method for determination of THC and its metabolite, THCA in *L. sericata* analyzed using LC-MS method (acetonitrile-ammonium acetate (2 mM) (30:70, v/v)) with ESI- detector and SIM mode. In developing the method, the authors used a set of larvae of *L. sericata* as calibrators that were spiked with different concentrations of THC and THCA standards.

In this study, several extraction methods (LLE, SPE, back titration) and their relevant parameters were optimized for yielding higher analytical recovery with less matrix effect for both THC and THCA. The parameters optimized included, sampling larvae amount, centrifugation speed, and ultracentrifugation, as well as sample filtration. The LLE method with hexane-ethyl acetate (50:50, v/v under acidic pH) showed to provide optimum recovery for THC and THCA at 98% and 95%, respectively. In addition, method optimization and validation were conducted according to European Union guidelines that included calibration curve ($R^2 > 0.998$), LOD (3.2 pg/mg), LLOQ (10 pg/mg), precision and accuracy (<20%) as well as recovery (THC: 98%; THCA: 95%) with satisfactory selectivity and stability for both analytes. Then, the validated method was applied to *L. sericata* collected from a suicide body of a 30-year-old woman (with a history of psychiatric disorder). The authors then reported that the concentrations of THCA were detected at 43 pg/mg while THC was not detected. The method established from the study showed that the method was robust and sensitive for the determination of THC and its metabolite THCA in the *L. sericata*.

Zou et al. (2013) attempted to investigate the effect of ketamine on the developmental pattern and morphology of *L. sericata* reared on the liver (L1-L4) and muscle (M1-M4) from rabbit carcasses previously injected with ketamine at different concentrations. It was evident that ketamine exerted its effect *via* a dosage-and-time-dependent pattern, significantly reducing the period for larval stages of *L. sericata*. This finding can be attributable to the higher growth of trophocytes (in the fat body) of *L. sericata*. However, despite the fact that ketamine did not appear to significantly affect the larval body length and weight, the progressive feeding intervals plunged considerably with the increment of ketamine dosages. This observation can be particularly important since the dropped in progressive feeding intervals may result in decreased absorption rate (but a higher elimination rate *via* the gut emptying process) of the analyte by the larvae, requiring improved sensitivity of the analytical method. The authors determined the concentrations of in *L. sericata* using the LLE method (with ethyl acetate as extraction solvent) prior to GC-MS analysis and reported the concentration detected in larvae of *L. sericata* that ranged between 0.08 and 2.09 µg/g. The analytical method utilized by the authors only reported on the calibration curve ($R^2 = 0.9977$) of the method. However, the authors did not report on other important validation parameters (selectivity, accuracy (bias, precision), LOD, LOQ, recovery, and specificity) for supporting the robustness, accuracy, and reliability of the method used, suggested by the international scientific standard like FDA (2018).

Magniet al. (2018), reported on the development and validation of an analytical method using HLPC-MS/MS to investigate the presence of ketamine in *C.*

vomitoria (larvae, pupae, empty puparia, and adults) reared on the liver homogenously spiked with the different concentrations of ketamine (300 ng/mg and 600 ng/mg). In addition, the authors investigated the effect of this drug on the developmental time, the morphology, and survival rates of *C. vomitoria* immatures. Ketamine was extracted from the larvae samples using SLE (methanol) prior to HPLC-MS/MS analysis. The method successfully determined the concentrations of ketamine in the different stages of immatures of *C. vomitoria* that ranged between 0.05 and 180.0 ng/mg. The LOD and LOQ established were ng/mg: 0.015 and 0.031, respectively, and the method was fully validated by complying to the requirements of international standards (ISO/IEC 17025:2005; ICH, 2015; Raposo, 2016). In addition to the LOD and LOQ, other validation parameters were: linearity ($R^2 = 0.99677$), extraction recovery (ER% = 99.4–100) and repeatability (CV%<20). Considering the satisfactory attainment of the validation parameters with no matrix or carry-over effects, the method reported by the authors can be consider as robust, sensitive and reliable for the analysis of the ketamine in the entomological specimens.

2.2.6. Miscellaneous drugs

O'brien and Turner (2004) reported that paracetamol slightly increased the growth rate of *C. vicina* larvae during the first 2–4 days of decomposition, which may likely affect the accuracy of PMI estimation. A similar study conducted by Khan et al. (2015) revealed that *C. rufifacies* infesting rabbit carcasses (administered with 5600 mg/kg of paracetamol) had accelerated growth rates when compared with the control. The authors further analyzed the concentrations of paracetamol in the larvae, pupae, and adults using SPE followed by HPLC-DAD (octadecyl carbon chain (C18)-bonded silica HPLC column) with a total analysis time of 5.5 min. The authors reported that concentrations of paracetamol that ranged between 0.13 and 3.05 ppm; paracetamol was not detected in the adults of *C. rufifacies*. Although the authors reported the R^2 of 0.9999 for the calibration curve, LOD, and LOQ of 0.048 µg/mL and 0.144 µg/mL respectively, the percentage recovery (63.7%) appears unsatisfactory. This can be attributed to the poor resolution (presumably less than 1.5) that can be seen in Figure 2(b) of the article. The authors did not report the result on precision and accuracy as well as the matrix effect for the extraction process.

Magni et al. (2016) developed and validated an analytical method using GC-MS for determining nicotine in the different stages of development for *C. vomitoria* collected from liver substrates spiked with 2, 4, and 6 ng/mg of nicotine. The concentrations reported for nicotine ranged between 0.39 and 3.29 ng/mg; nicotine was not detected in the second instar larvae and adults as well as all entomological specimens from controls. The authors further investigated the potential effect of nicotine on the developmental pattern and survival of the said necrophagous insect. Their results revealed that nicotine did not significantly affect the growth rate of *C. vomitoria* although the length of the larvae was significantly shorter than that of controls. Moreover, the survival rate of *C. vomitoria* was significantly decreased with the increment of nicotine concentrations. Despite reporting the acceptable linearity ($R^2 > 0.995$), LOD (0.13 ng/mg), LOQ (0.43 ng/mg), as well as precision and accuracy (CV% < 20), the percentage recovery of 69.23–71.11% reported by the authors were lower than the acceptable value of 80%, requiring further improvement in the analytical method. Interestingly, Magni et al. (2016) calculated the dosages for the substrates used in their study based on the estimated LOD of 60 mg, presumably from Mayer (2014). As a matter of fact, Mayer (2014) himself indicated that "Kobert estimated the lethal dose of nicotine on the basis of highly dubious self-experiments performed in the mid of the nineteenth century while ignoring conflicting data". His excellent reputation as a leading scholar in toxicology has apparently led to uncritical acceptance and citation of the 60-mg dose by contemporary fellows and successive researchers. Therefore, further clarifications on the lethal dose of nicotine along with its suitable inference on the development and survival rates of necrophagous insects prove necessary.

In an attempt to investigate the feasibility of calliphorid larvae as the potential reservoir for alkaline-labile drugs that may be vulnerable to postmortem decomposition of human tissues, Bushby et al. (2012) developed and validated an analytical method for the determination of methylphenidate (MPH) in *L. sericata* using LLE followed by LC-MS/MS analysis. The LLE method utilized small amounts of larvae sample (500 µL larvae homogenate) and solvent (1 mL of hexane: dichloromethane, 9:1, v/v) with the promising percentage recovery (> 80%). It can be seen that the linearity ($R^2 = 0.9973$) within the analytical range of 200–2300 ng/mL was acceptable with the LLOD and LLOQ of 24 ng/mL and 80 ng/mL, respectively. The analytical method also appears accurate (97–99.6%) and precise (RSD%=1.8–2.8) with a percentage recovery that ranged between 94.5 and 108.8%. Therefore, it can be construed that the analytical method reported by Bushby et al. (2012) is sensitive and robust for determining MPH in entomological specimens. Hence, further studies focusing on method development for other alkaline-labile substances (that can be easily deteriorated by

the decomposition process) in entomological specimens should be encouraged.

2.3. Determination of multi-classes poisons and drugs in entomological specimens

2.3.1. Multi-classes of poisons

Dowling et al. (2022) simultaneously detected methyl parathion, malathion and dichlorvos as well as chemical warfare agents (CWAs) *viz.* dimethyl methylphosphonate (DMMP), diethyl phosphoramidate (DEPA), and diisopropyl methylphosphonate (DIMP)) and DIMP hydrolysis products (ethyl methylphosphonate (EMPA), isopropyl methylphosphonate (IMPA), and pinacolyl methylphosphonate (PinMPA) in the gut specimens of three different blow fly species. The semi-quantitative determination of CWAs in blow flies as "a wild, untrained organism to naturally acquire important chemical information from its environment" was intended to minimize any physical harm to the frontline workers. This approach can be potentially prudent for assessing the safety of a CWAs contaminated areas without the need for direct physical human intervention. Each sample was extracted by sonicating in methanol (100 µL) for 30 min, nitrogen evaporation, and reconstitution in water (200 µL) followed by its semi-quantitative analysis using LC-MS/MS. For detecting the hydrolysis products, a benchtop experiment was performed where a solution of DIMP (1 mg/mL) was acidified with formic acid (0.1%) and heated at 80 °C for 8 h. Aliquots were sampled at 0, 0.5, 1, 2, 4, and 8 h and were diluted in water to 1000 ng/mL followed by semi-quantitative analysis using the same instrument.

Although the authors reported complete validation data (*viz.* linearity and sensitivity, precision, accuracy, and recovery), evaluation of the matrix effect for necrophagous specimens was not conducted. They reported the R^2 of values of $0.9546 - 0.9894$, $0.9840 - 0.9985$, and $0.9706 - 0.9936$ for methyl parathion, malathion, and dichlorvos, respectively with recovery percentages of 58%, 55% and 49% for the same analytes. As for CWAs and hydrolysis products, the R^2 values ranged between 0.9464–0.9970 and 0.8982–0.9987, correspondingly. It can be seen that the R^2 values and the recovery percentage reported for a number of analytes did not fulfill the minimum requirement for accepting the calibration curves ($R^2 \geq 0.995$) as well as the recovery (80–120%) (FDA, 2018). Except for DIMP (a CWA simulant), the recovery percentages (determined at a high QC level) reported for other CWA simulants and hydrolysis products were lower than the acceptable range of 80–120% (FDA, 2018). The fact that the calibration curves violated the minimum requirement of acceptance, and the accuracy of LODs and LOQs reported by the authors remain unclear. Since the chemical analysis was performed on an individual fly (unknown weight), the quantitative determination of CWAs concentrations cannot be made. Without disputing the ability of the method to detect the qualitative presence of CWAs and hydrolysis products in the blowfly species, a significant improvement in the quantitative determination of the same must be made before the method can be used for routine forensic analysis.

2.3.2. Multi-classes of drugs

Kintz et al. (1990a), Kintzet al. (1990b), Kintz et al.,(1990c) was the earlier group of researchers that determined multi-classes of drugs in entomological specimens *viz.* antidepressant (clomipramine), benzodiazepines (bromazepam, triazolam, and oxazepam), barbiturate (phenobarbital), phenothiazines (alimemazine and levomepromazine) and opioid (morphine) for forensic entomology purposes. However, instead of analyzing these drugs simultaneously, the authors utilized individual analytical methods described by the earlier researchers. While conveniently indicating that "the preparation and extraction from larvae are the same as those from any other human tissues" (Kintzet al., 1990a), the authors of these three articles did not provide any validation data to support the accuracy and robustness of the individual analytical methods used.

In an attempt to explain the pharmacology of antidepressant (amitriptyline and nortriptyline) and miscellaneous (propoxyphene and acetaminophen) drugs in the empty and full crop of *C. vicina*, Wilson et al. (1993) performed the non-simultaneous analyses of these drugs using HPLC (amitriptyline, nortriptyline, and acetaminophen) and GC-NPD (propoxyphene). They reported the concentrations of amitriptyline, nortriptyline and propoxyphene that ranged between 0.19–0.29 µg/g, 0.16–0.25 µg/g, and 0.05–0.06 µg/g, respectively. As for the acetaminophen, it was non-detectable in the larvae. Later, Sadler et al. (1995) while interpreting the influence of the different concentrations of antidepressant and antipsychotics (amitriptyline, trimipramine and trazodone) and benzodiazepine (temazepam) utilized the individual analytical methods for those drugs as described by earlier researchers. Despite Sadler et al. (1995) indicated that all the drugs analyzed had the same LOD (0.1 µg/g), other relevant validation parameters such as linearity, LOQ, recovery as well as precision and accuracy were not reported. In general, similar observation made for the work reported by Sadler et al. (1995) also prevailed in the works reported by Kintzet al. (1990a), Kintz et al. (1990b), Kintz et al. (1990c) and Wilson et al. (1993) in which analytical validation data were not reported, causing

doubt in the accuracy and robustness of the analyses.

As for the simultaneous detection and determination of drugs, Campobasso et al. (2004) compared the analytical results from maggots with that of human tissues from 18 bodies that died due to drug-related intoxication. The drugs analyzed were amitriptyline, clomipramine, nortriptyline, phenobarbital, morphine, levomepromazine, thioridazine, benzoylecgonine, and cocaine. They were qualitatively and semi-quantitatively analyzed using ONLINE Abuscreen, whereas the quantitative analysis was performed using GC-MS. Similarly, Tracqui et al. (2004) reported the concentrations of several drugs that included morphine, codeine, pholcodine, propoxyphene, amitriptyline, fluoxetine, dothiepin, venlafaxine, amobarbital, alprazolam, bromazepam, lorazepam, nordiazepam, triazolam, pholcodine, alimemazine, chlorpromazine, cyamemazanine, levomepromazine, cocaine, THC, digoxin, meprobamate, nefopam and propoxyphene in larvae collected during autopsies. These authors (Campobasso et al., 2004; Tracqui et al., 2004) concluded that the drugs detected in the maggots were also detected in human tissues although the concentrations were higher in the latter. While reporting on the concentrations from GC-MS and LC-MS analyses, these authors (Campobasso et al., 2004; Tracqui et al., 2004) did not provide adequate information on the analytical methods, neither that they provided the required validation data.

In contrast to the indication made by Tracqui et al. (2004) that "larvae analysis is of almost no interest for practical forensic casework" because drugs detected in larvae can also be detected in cadavers, Groth et al. (2022) reported that some drugs such as the new psychoactive substances EAM-2201 and U-47700 were detected in larvae and hair samples alone. Such a finding from the simultaneous determination of drugs in larvae would advocate on the possibility of gaining important information in atypical/less common toxicological specimens (such as larvae and hair), especially when dealing with bodies that are at extensive postmortem decomposition stage. Similar to the previously discussed articles by Campobasso et al. (2004) and Tracqui et al. (2004), the analytical method for human tissues was analogously used by Groth et al. (2022) for analyzing the larvae (without offering any method validation data) in the two postmortem forensic cases.

2.3.3. Combination of poisons and drugs

de Aguiar França et al. (2015) attempted to quantify combination of poisons (aldicarb, an OC as well as its sulfone and sulfoxide metabolites) and drugs (amitriptyline, carbamazepine bromazepam, clonazepam, diazepam, flunitrazepam, cocaine and benzoylecgonine) in necrophagous insects using LC-MS/MS. A sample of necrophagous insect (1 g) was extracted using the specifically optimized SLE-low temperature programming (SLE-LTP) method in a 15 mL falcon tube with the addition of 0.5 mL and 2 mL of water and ACN, respectively. Since the extraction process involved the use of a small amount of insect sample (1 g), the method can be corroborated as a green process while reducing the amounts of contaminants injected during the LC-MS/MS analysis. The method developed was fully validated according to the recommendation by bioanalytical methods (FDA, 2018). The LOQ for all the analytes ranged between 1 and 40 ng/g, with a wide range of precision (2.83 and 16.9%) . Using the optimized and validated analytical method, the authors analyzed actual larvae samples (28 samples from 27 cases) obtained from the mortuary. Results revealed that at least one of the compound was positively detected in 11samples with 2 of the samples contained more than 1 compound. While neither aldicarb nor its sulfoxide/sulfone metabolites were detected in any of the samples, amitriptyline (16.3 ng/g), cocaine (1.00–392 ng/g), carbamazepine (detected-503 ng/g), benzoylecgonine (0.84–177 ng/g) and diazepam (3.39–6.70 ng/g) and were positively determined in 1, 3, 3, 4 and 4 cases, repectively. In this regard, the authors (de Aguiar França et al., 2015) indicated that since information on the circumstances of the death in most cases is unclear, and because various substances may be involved in poisoning cases, having the simultaneous analytical methods for determining multi-classes of poisons and drugs proves imperative for a forensic laboratory. On the other end, the determination of multi-classes of poisons and drugs in necrophagous insects is also important for forensic entomologists in assessing the possible influence of such poisons and drugs on the accuracy of the developmental data for estimating accurate PMI.

3. Challenges and future insights for the use of entomological evidence and its analytical techniques for forensic inference

Despite its possible usage for suggesting the cause and circumstances surrounding deaths (Mahat et al., 2019), the development of specific and validated analytical methods for drugs and poisons in necrophagous specimens proves nascent. The selection of analytical methods depends heavily on the physicochemical properties of the drugs and poisons, as well as the required selectivity and sensitivity. Besides, the sample preparation/extraction technique is also vital for the successful detection of drugs and

poisons. However, entomological specimens have been either processed like common toxicological specimens (e.g. visceral tissues and fluids) or underwent extraction steps similar to rigorous tissues (e.g. nails) (e.g. Campobasso et al., 2004; Goff & Lord, 2010; Kintz et al., 1990a). Considerable number of studies did not report the extraction method used which can gravely affect the effectiveness of analysis, reliability of the results, and the robustness of conclusions. Since necrophagous insects are different in composition and morphology than those of common toxicological specimens (Mahat et al., 2019), analyzing them using the extraction and analytical methods specifically developed for human and/or animal tissues may result in inaccurate interpretations. Considering the relevance of entomological evidence in criminal courts (Rivers & Dahlem, 2014), and because utilizing inappropriate analytical methods would cause reasonable doubts (Evidence Act 1950 (Act 56), 2018), the admissibility of such evidence can be disputed.

In addition to the incomplete validation data (e.g. Gunn et al., 2006; Levine et al., 2000; Zou et al., 2013), a review of literature further revealed the use of non-simultaneous determination of drugs and poisons in necrophagous insects (e.g. Kintz et al., 1994; Sadler et al., 1995). The lack of complete validation data in several studies may compromise the robustness and reproducibility of the analytical methods used, particularly if such methods are to be adopted for forensic practical casework. Relevantly, a number of drugs and poisons (e.g. malathion, chlorpyrifos, and endosulfan) that can potentially affect the oviposition and developmental patterns of necrophagous insects are, indeed, readily available in the market. Therefore, the fact that (a) these compounds are used for committing homicides and suicides, as well as (b) creating "unlawful and dubious alibi may cause significant confusion in forensic investigation" (Denis et al., 2018) cannot be neglected. Hence, concerted efforts to develop validated simultaneous yet simple and robust extraction and analytical methods, specifically for entomological specimens, would prove forensically and scientifically obligatory. In fact, the inclusion of simultaneous screening and/or determination of drugs and poisons in the overall operational forensic entomology framework must be seriously considered, in order to provide forensic entomologists with the necessary information in making as accurate PMI estimate as possible. Mindfully, specific consideration must be given to the less accessibility to the state-of-the-art instruments by forensic laboratories in developing and under-developing countries due to budgetary, logistic, and trained analysts constraints. Therefore, while developing the cutting-edge technologies (e.g. LC-MS/MS) for improving the sensitivity and specificity of drugs/poisons analysis must be encouraged, attempts to develop the moderately priced instrumental analysis with acceptable sensitivity and specificity (e.g. HPLC-DAD) still prove appropriate.

In addition, utilization of CE (e.g. CZE-DAD) for entomological specimens remains unreported so far. The fact that CZE-DAD has a high separation efficiency, the development of a simultaneous determination for various compounds (inorganic and organic ions) is feasible. The technique allows online pre-concentration steps and the separation of different compounds within the same capillary. Hence, CZE only requires small amounts of solvents and samples with a relatively shorter total analysis time (Flanagan et al., 2020; Douglas et al., 2022; Wolstenholme et al., 2021). Considering that forensic specimens are usually limited and degraded, the advantages of CZE prove appealing for forensic use. Therefore, the specific attempt to develop the CZE-DAD methods for the simultaneous determination of drugs and/or poisons acquires forensic and scientific significance.

4. Conclusion

Considering (a) the importance of forensic entomological evidence in crime investigation and (b) factors affecting its admissibility in court, as well as the fact that (c) drugs and poisons can potentially influence the accuracy of PMI estimates, performing the specifically developed simultaneous and validated analytical determination of drugs and poisons appears forensically imperative. A number of analytical limitations (*viz.* non-specific analytical method for entomological specimens, incomplete validation data and non-simultaneous determination) have been associated with the analysis of drugs and poisons in entomological specimens in substantial number of articles. If unaddressed, these limitations may result in the inadmissibility of forensic entomological evidence in the court of law, necessitating suitable corrective measures to be taken. Additionally, continuous development and validation of analytical methods prove necessary, considering the rapid introduction of new drugs and poisons that can potentially affect the growth pattern of the necrophagous insects.

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