ZIBELINE INTERNATIONAL™ PUBLISHING

Acta Scientifica Malaysia (ASM)

DOI: http://doi.org/10.26480/asm.02.2024.78.83

ISSN: 2521-5051 (Print)
ISSN: 2521-506X (Online)
CODEN: ASMCCQ

RESEARCH ARTICLE

IS MALATHION DETECTABLE IN BLOW FLY LARVAE? EXPLORING FORENSIC ENTOMOTOXICOLOGY METHODS

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ARTICLE DETAILS

Article History:

Received 8 December 2024
Revised 4 January 2025
Accepted 6 January 2025
Available online 6 February 2025

ABSTRACT

The study focuses on the use of entomotoxicology to detect and quantify malathion, an organophosphorus pesticide, in *Chrysomya megacephala* larvae feeding on malathion-exposed decomposing cow lung. Malathion, often used in agriculture and medicine, can affect insect life cycles, potentially leading to inaccuracies in estimating the minimum post-mortem interval (mPMI) in forensic cases. The study optimized the extraction method of malathion from maggots and used Gas Chromatography-Mass Spectroscopy (GC-MS) for detection. Despite successful chromatographic separation of malathion, the pesticide could not be detected in the larvae, suggesting the need for further research using more realistic models. The findings highlight challenges in forensic entomotoxicology and underscore the need for further research with adequate sample sizes and real-world crime scene models.

KEYWORDS

Forensic entomology, entomotoxicology, *Chrysomya megacephala*, malathion, GC-MS, post-mortem interval

1. INTRODUCTION

Forensic entomology, a subfield of forensic science, plays a critical role in criminal investigations by utilizing insect evidence to estimate the post-mortem interval (PMI) (Iswadi Ismail et al., 2007). By analyzing the insects that colonize decomposing remains, forensic experts can establish timelines and, in some cases, determine the location or cause of death (Rashid et al., 2012). Among the key insect species involved in forensic investigations, blowflies (family Calliphoridae) are usually the first to colonize a decomposing body. Their rapid response to decaying tissues makes them invaluable for PMI estimation, particularly in advanced stages of decomposition where other methods, such as body fluid analysis, may no longer be viable (Bakr et al., 2018; El-Ashram et al., 2022).

Chrysomya megacephala (Fabricius), also known as the Oriental latrine fly, is one of the most forensically important blowfly species in Southeast Asia, including Malaysia. It has been widely used in forensic entomology to estimate PMI because of its abundance and rapid colonization of cadavers (Joshi and Kumare, 2020). The species' life cycle, from egg to adult, is influenced by environmental factors such as temperature and humidity, which are crucial for determining the development rate of its larvae and, consequently, the time since death (Kavitha et al., 2012; Badenhorst and Villet, 2018).

However, estimating PMI is not always straightforward. The accuracy of PMI estimates can be compromised by the presence of toxins or chemicals in the decomposing body, which can affect the development rate of necrophagous insects (Jales et al., 2021; Bhardwaj et al., 2020). This is where the field of forensic entomotoxicology becomes relevant. Forensic entomotoxicology focuses on detecting toxins, such as drugs, pesticides, and chemicals, in insects feeding on decomposing remains (Zehra and Mishra, 2020). By identifying these substances, forensic scientists can gain additional insights into the cause of death, particularly in cases involving poisoning or overdose, and refine PMI estimates when insect development has been altered by the presence of such chemicals (Magni et al., 2018).

One of the common pesticides that may be encountered in forensic entomotoxicology cases is malathion. Malathion is an organophosphorus insecticide widely used in agricultural, public health, and domestic settings. It is frequently applied for controlling pests such as mosquitoes, flies, and parasites (Reed and Rubin, 2014). Despite its low toxicity to humans when used appropriately, malathion can be lethal in cases of acute exposure, whether through accidental poisoning or suicide attempts (Thompson et al., 1998; Pannell et al., 2001; Castillo-Alanis, et al., 2022). In fact, pesticide poisoning is one of the leading causes of suicide in countries such as Malaysia, where access to agricultural chemicals is relatively easy (Kamaruzaman et al., 2020). When malathion is ingested or absorbed into a body, it can be metabolized into malaoxon, a more toxic compound that inhibits acetylcholinesterase, leading to fatal nervous system dysfunction (Reed and Rubin 2014; Castillo-Alanis et al., 2022).

The detection of malathion in decomposing bodies, especially in cases where traditional toxicology methods are limited due to advanced decomposition, is critical for understanding the circumstances surrounding a person's death. However, it remains a challenging task, particularly when only insect evidence is available. Studies have shown that the presence of toxic substances in a body can influence the life cycle of necrophagous insects, delaying or accelerating their development. This can lead to errors in PMI estimation, underscoring the importance of detecting and quantifying toxic substances in insect larvae or pupae.

The current study investigates the detection of malathion in *Chrysomya megacephala* larvae using Gas Chromatography-Mass Spectroscopy (GC-MS). The use of GC-MS in forensic toxicology is well-established, given its sensitivity and accuracy in detecting small quantities of chemicals in complex biological matrices (Jangra et al., 2024; Soltaninejad et al., 2024; Djamaa et al., 2020). However, the application of GC-MS to insect samples in forensic entomotoxicology presents unique challenges, such as optimizing the extraction methods and determining the ideal operating conditions for detecting pesticides like malathion. Previous studies have reported varying success in detecting malathion residues in larvae, with

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Cite The Article: Farah Diana Ariffin and Roziah Ali (2024). Is Malathion Detectable in Blow Fly Larvae? Exploring Forensic Entomotoxicology Methods. *Acta Scientifica Malaysia, 8(2): 78-83.*

factors such as sample size, extraction methods, and the concentration of the pesticide influencing the outcomes (Liu et al., 2009; Yan-Wei et al., 2010; Mahat et al., 2012).

This study aims to address some of these challenges by optimizing GC-MS conditions for detecting malathion in blowfly larvae feeding on cow lung spiked with different concentrations of the insecticide. Specifically, this study tests the hypothesis that malathion can be detected in *Chrysomya megacephala* larvae exposed to malathion-treated substrates and explores the effect of malathion on larval development. By determining whether malathion and its metabolite, malaoxon, can be reliably detected in blowfly larvae, this study contributes to the growing body of forensic entomotoxicology research and provides critical insights for improving the accuracy of PMI estimates in cases involving pesticide poisoning.

2. Materials and Method

2.1 Fly experiments

The *Chrysomya megacephala* flies used in this study were obtained from a laboratory colony that has been maintained in the fly rearing colony of the Arthropod Containment Laboratory (ACL), Medical Entomology Unit, Institute for Medical Research (IMR), National Institutes of Health (NIH) Setia Alam, Selangor. The colony was held in the laboratory at 26.5 ± 2.5°C and a photoperiod (h) of 12:12 (L:D) for several generation prior to use. The colony was kept in 12.5 L clear rectangular storage containers. Panel was cut from the lid (250 mm x 185 mm) of the container and covered with mesh organza for ventilation. A circular panel 130 mm diameter was removed from the front to attach an organza sleeve to enable access to the interior of the cage. Water and sugar cubes were provided to adult flies *ad libtium*. Fresh cow lung was exposed to the colony for oviposition. The newly emerged larvae were removed and deposited on the fresh cow lung spiked with different concentrations of malathion.

Fresh cow lung was prepared for three treatment group at various malathion concentration and one control for larvae feeding. The concentrations were calculated considering the estimated fatal dose of 60 g / 60 kg for human (Liu et al., 2009). The fresh cow lung, weighing 100 g, was treated with malathion at concentrations equivalent to 0.5, 1.0, and 1.5 times the estimated lethal dose, corresponding to 0.3 g, 0.6 g, and 0.9 g, respectively. These prepared concentrations were designated as T1, T2, and T3 for experimental replicates. The three different concentrations of malathion were mixed with the cow lung by using chopper to uniformly dispersed and was left out at least 4 hours before introduced to the larvae.

Samples of larvae for each treatment were collected on approximately the 4th day post-exposure, coinciding with the observation of the oldest maggot masses, represented by the largest larvae on each substrate. A total of five larvae samples were used for morphological analysis. The length of individual larvae was measured in millimetres using a LEICA stereomicroscope. These larvae were preserved in 70% ethanol for mounting. The rest of the collected samples were used for toxicology analysis to detect malathion residue. The samples were cleaned individually with tap water to remove external contamination and stored at -20°C prior to use.

2.2 Sample Extraction

The first extraction method was adapted from a study with modifications (Nakamura et al., 1994). Two grams of larvae samples (approximately 30 to 40 larvae) were homogenized with 10 ml of acetone for 3 minutes. The resulting extract was filtered through filter paper, and the residue was re-homogenized with another 10 ml of acetone and filtered again. The combined extracts were concentrated using a hot plate. Subsequently, 10 ml of 10% NaCl solution and 10 ml of ethyl acetate were added to the concentrated extract, and the mixture was shaken vigorously for 5 minutes. The organic layer was collected, and the aqueous layer was re-extracted with an additional 10 ml of ethyl acetate. The combined organic layers were dehydrated with 2 grams of anhydrous Na_2SO_4 and concentrated using a hot plate. The concentrated solution underwent a clean-up process using a column containing activated silica gel suspended in n-hexane. Na_2SO_4 was added continuously as n-hexane drained. Finally, 2 ml of the sample solution was transferred to the silica gel column with 5 ml of an acetone/n-hexane mixture (3:7 v/v). A 2 µl aliquot of the extracted solution was injected into the GC-MS for analysis.

The second extraction method followed a modified version of a study that involved two grams which were approximately 30 to 40 larvae grinded together with two grams of anhydrous Na_2SO_4 (Liu et al., 2009). The mixture was combined with 6 ml of acetone and stirred on a hot plate at 25°C for 30 minutes before being filtered. To the filtrate, 6 ml of dichloromethane and 12 ml of 20% sodium sulphate solution were added, and the mixture was stirred for 2 minutes. The lower layer was collected, and the residue was re-extracted with an additional 6 ml of dichloromethane. The combined extracts were concentrated in a water bath at 60°C. A total of 2 µl aliquot of the final solution was injected into the GC-MS for analysis.

2.3 GC-MS Analysis

The GC-MS analysis was performed using gas chromatograph (Agilent Technologies, Model 7809A GC) coupled with a mass spectrometer (Agilent Technologies, Model 5975C MSD). A total of 2 µl sample was injected into an HP-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness). The MS system operated in full scan mode to qualitatively determine the target analyte, based on the mass spectra and retention time of malathion. The operating conditions, including injector temperature, oven program, and carrier gas flow rate, were varied according to the modifications listed in Table 1 to optimize the conditions for detecting malathion residue in the samples.

Table 1: Operating conditions of GC-MS analysis for the detection of malathion residue

Injector temperature	GC conditions	Carrier gas flow rate	References / Operating conditions
240°C	60° C for 2 min Rise 8° C/min to 235° C	Helium 2 ml/min	Nakamura et al. (1994)
250°C	50° C for 3 min 20° C/min to 130° C 30-190° C/min for 10 min	Helium 1 ml/min	Liu et al. (2009)
250°C	70 to 230° C (1 min hold) at rate 25° C/min	Helium	Mahat, Jayaprakash and Zafarina (2012)
240°C	200° C for 2 min Rise 3° C/min	Nitrogen 4 ml/min	Lofty et al. (2013)
260°C	120° C for 1 min Ramp 20° C/min to 270° C held for 2 min	Nitrogen 3 ml/min	Thabit and Silos (2013)
250°C	150° C for 2 min Rise to 200° C at 5° C/min held for 5 min	Helium2 ml/min	Rezaee at al. (2019)

3. Results and Discussion

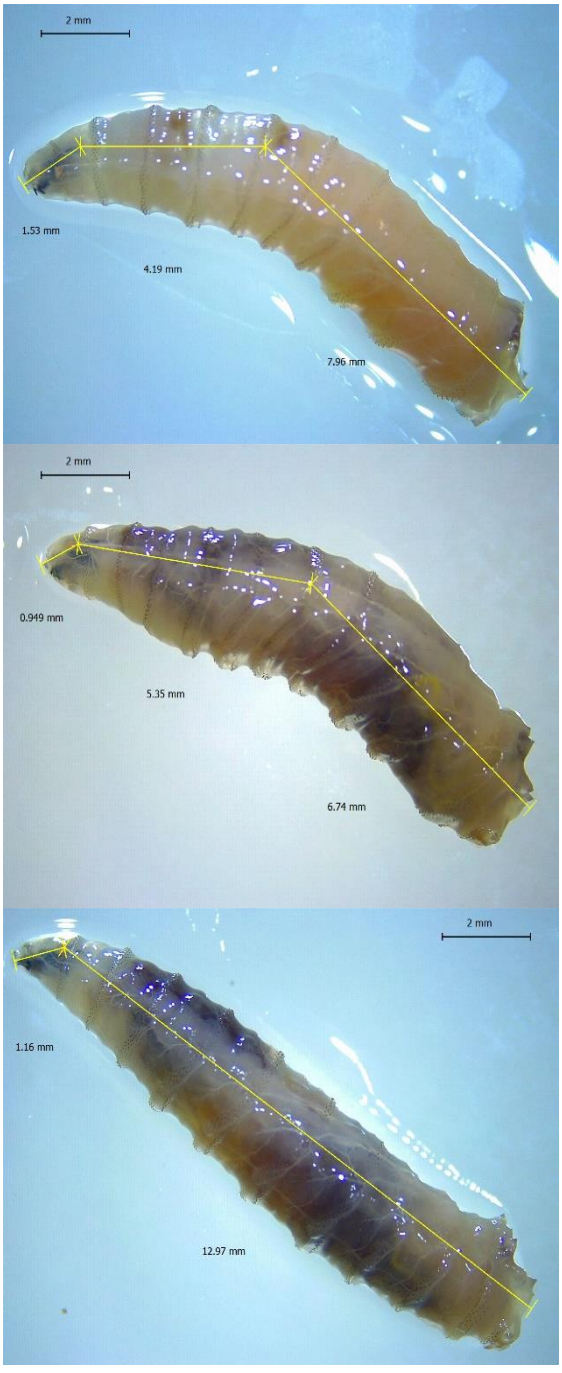
In this study, adult *Chrysomya megacephala* flies exposed to the highest concentration of malathion-treated cow lung which was 50% higher than estimated human fatal dose (T3) did not survive. However, the surviving adults from the 50% lower than estimated human lethal dose, T1 and equivalent to the estimated human lethal dose (T2) concentrations successfully oviposited, and the resulting eggs developed into larvae. Approximately 10 – 15 larvae were retrieved from each treatment and control group for morphological and toxicological analysis.

Larval development was monitored over 144 hours, with body length measurements taken at regular intervals. The average body length was significantly greater in the treatment groups compared to the control group, with an average of 13.89 mm in the treatment group versus 10.64 mm in the control group ($p = 0.005$). A detailed comparison of larval length across different malathion concentrations is presented in Table 2. The results of this study are consistent with previous findings by a group researcher, who observed that malathion-exposed larvae exhibited delayed development in the initial stages of exposure, with control groups showing faster growth (Rashid et al., 2008). In our study, larvae in the control group also grew significantly faster during the first 30 hours.

Table 2: Comparison of means of larvae length (mm) between three different malathion concentration exposed larvae and control		
Group	**Body Length (mm)**	**p-value**
T1	13.68	0.005*
T2	13.04	
T3	14.13	
Control	10.64	

**Significant differences between the treatment and control groups were confirmed using an Independent T-Test ($p < 0.05$).*

After this period, however, malathion-exposed larvae experienced a boost in development, potentially due to the metabolism of malathion within their tissues. These findings align with observations by who reported that malathion slowed the initial development of fly larvae, followed by a compensatory growth boost later (Yan-Wei et al., 2010). The extended larval stage in malathion-exposed groups, coupled with increased body length, suggests that malathion ingestion may stimulate growth once metabolized, likely due to the bioaccumulation of organophosphorus compounds in the larvae. This study contributes to the growing body of forensic entomotoxicology by demonstrating the developmental effects of malathion on *Chrysomya megacephala*. However, future studies should consider optimizing extraction techniques to detect malathion residues more reliably in larvae.



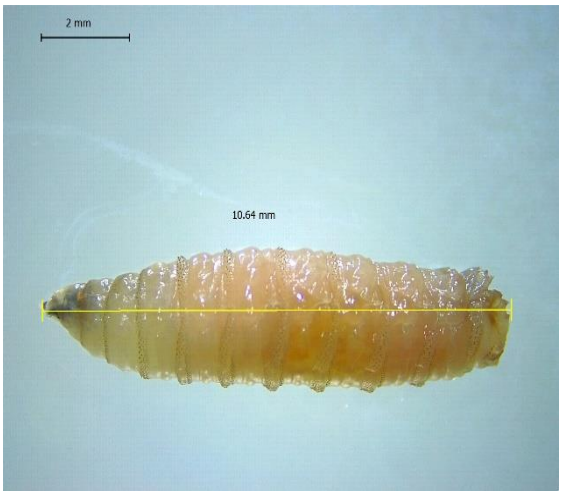


Figure 1: Comparison of body length (in mm) of *Chrysomya megacephala* in a) Treatment group 1, T1; b) Treatment group 2, T2; c) Treatment group 3, T3; and d) Control group

Notably, larvae exposed to the higher malathion concentrations (T2 and T3) exhibited a distinct colorization in their segmented bodies (Figure 1). The difference in colour between malathion exposed larvae and control larvae can be explained by several physiological and biochemical effects induced by the insecticide. Malathion induces oxidative stress by generating reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide anion radicals which can lead to deterioration of proteins, lipids and DNA within the larvae (Abdelfattah and El-Bassiony, 2022). Additionally, malathion can inhibit acetylcholinesterase (AChE), an enzyme essential for breaking down acetylcholine in the nervous system (Badr, 2020). This inhibition disrupts normal nerve function, leading to further physiological stress and potential discoloration. The activation of oxidative stress and upregulation of enzyme indicate that malathion can have a toxic effect on fly by affecting development and metabolic processes.

Malathion standard dissolved in acetone was analyzed under various operating conditions, as outlined in Table 1, to determine the optimal parameters for GC-MS detection of malathion. Six different GC-MS operational settings were tested to assess their impact on the chromatographic separation of standard malathion. The parameters examined included variations in i) injector temperature, ii) oven programming, and iii) carrier gas flow rate, while all other GC-MS conditions were kept constant. The mass spectrum of malathion was characterized by the presence of three key ion peaks at m/z 125, m/z 173, and m/z 93, in agreement with the findings of a study (Mahat et al., 2012). To optimize the GC-MS conditions, the retention time, peak shape, and quality of the detected peaks were evaluated. The retention time of malathion ranged from 4.71 to 21.18 minutes, with all peak qualities exceeding 90.

Additionally, three of the tested conditions detected malaoxon, a primary metabolite of malathion, with retention times of 6.95 minutes, 7.29 minutes, and 10.87 minutes (Table 3). As illustrated in Figure 2, the best peak quality and separation without interference were achieved using the conditions outlined by followed closely by those from (Thabit and Silos, 2013; Nakamura et al., 1994). Although the peak obtained from a group researcher had a lower area compared to the others, it was well-separated from adjacent peaks, and the detected compound was confirmed to be malaoxon (Mahat et al., 2012). The total ion chromatogram of malaoxon is presented in Figure 3. The full scan mass spectra of malaoxon, shown in Figure 4, indicate the formation of a parent ion at m/z 127, with a molecular weight of 314, confirming the presence of the metabolite. Additionally, malaoxon was detected prior to malathion under the operating conditions from (Thabit and Silos, 2013; Rezaee et al., 2019).

Table 3: Retention time and area of the standard malathion diluted with acetone

Method	Retention time (min)	Area	Quality of peak
Nakamura et al. (1994)	21.187	33.02	91
Liu et al. (2009)	15.444	27.08	94
Mahat et al. (2012)	7.294*	1.75	91
Thabit and Silos (2013)	6.946* and 7.328	1.26* and 55.02	91
Lofty et al. (2013)	4.712 and 4.831	88.39 and 11.61	91
Rezaee et al. (2019)	10.871* and 12.141	4.42* and 94.39	45 and 91

*Note: Malaoxon, the oxygen analog of malathion, was detected at the indicated retention times.

The obtained total ion chromatogram of standard malathion with retention time, area and quality of peak as per Table 3 were taking into consideration for modifying optimized GC-MS conditions. The operating conditions from were referred as both obtained the best peak (Thabit and Silos, 2013; Nakamura et al., 1994). The injector was set at 230°C, oven was programmed at 90°C and held for 1 minute immediately ramp up at 15°C/min to 220°C and held for 5 minutes with the flow rate of Helium was set at 1 ml/min (Table 4). Malathion standard dissolved in acetone was detected as parent compound at 10.83 minutes by using these modified optimal conditions (Figure 5). These optimal operating conditions were selected for further analyses of malathion detection in the current study.

Table 4: Modified operating conditions for malathion residue in *Chrysomya megacephala* larvae sample

Parameters	GC-MS conditions
Column	HP-5 MS capillary column (30m x 0.25 mm, 0.25 µm)
Inlet/Injector temperature, °C	230°C
Detector temperature, °C	280°C
Oven programme	90°C for 1 min Rise to 220°C at 15°C/min for 5 min
Carrier gas	Helium
Flow rate, ml/min	1 ml/min
Injection volume, µl	2 µl
Injection mode	Splitless
Detector	MS Detector
Total time, minutes	10.83

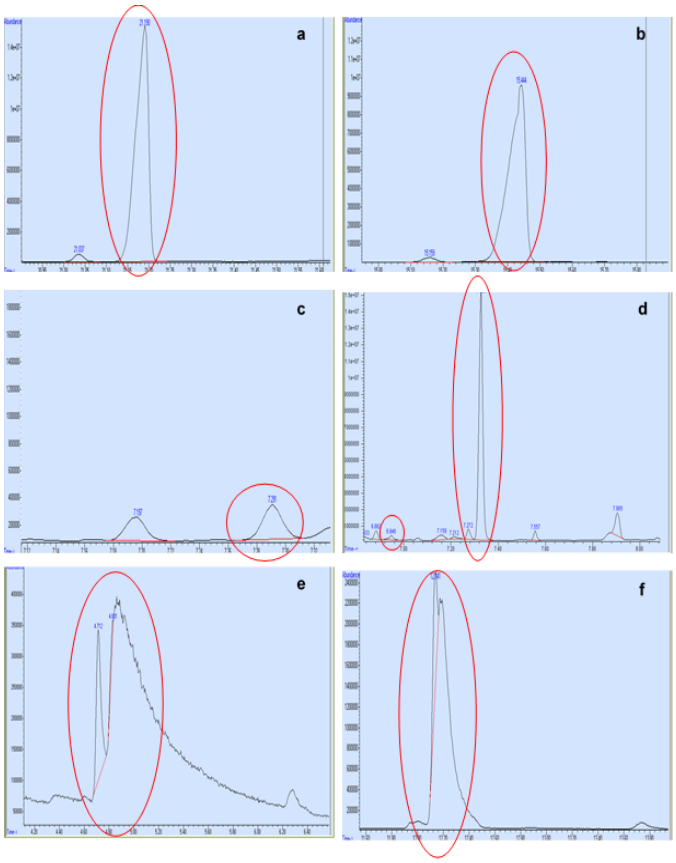


Figure 2: Total ion chromatogram of malathion for standard sample in acetone (50 mg/ml) of six different operating conditions ((a) Nakamura et al., 1994; (b) Liu et al., 2009; (c) Mahat et al., 2012; (d) Thabit and Silos, 2013; (e) Lofty et al., 2013; (f) Rezaee et al., 2019)

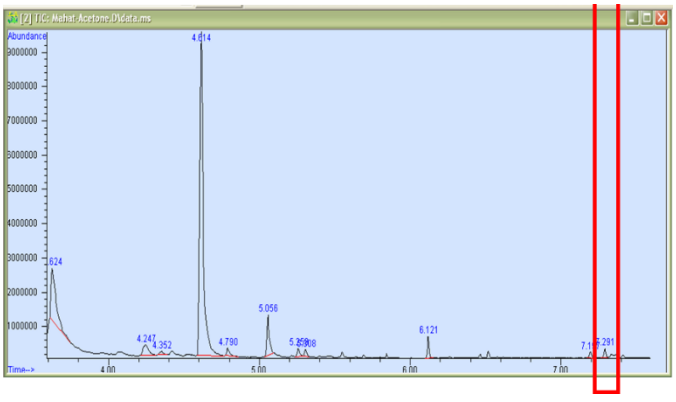


Figure 3: The total ion chromatogram of malaoxon in malathion standard sample with acetone

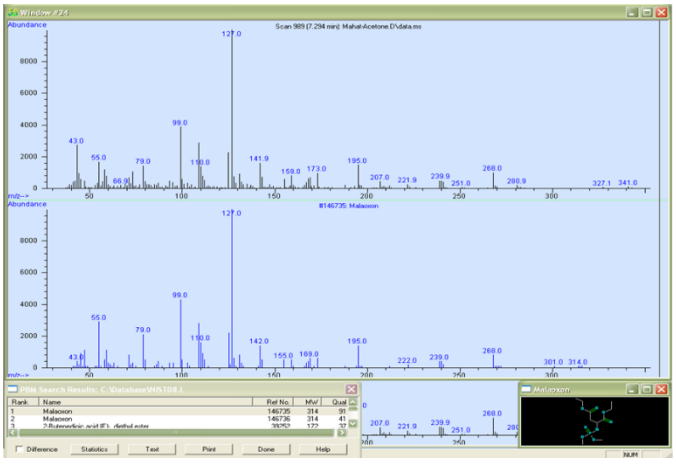


Figure 4: Full scan mass spectra of malaoxon for malathion standard

Optimal conditions of GC-MS can provide high sensitivity for detecting low concentrations of analytes, high resolution for separating compounds within similar chemical properties, accurate mass measurements for precise determination of molecular weights and structures, and rapid analysis for efficient processing of samples.

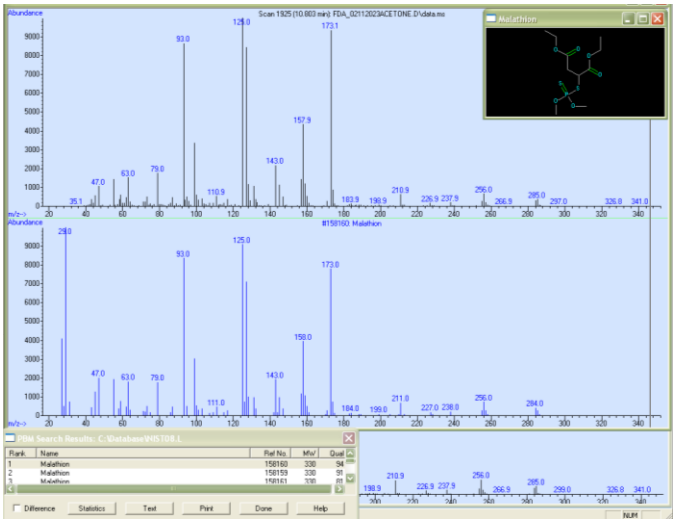


Figure 5: Full scan mass spectra of malathion in malathion standard sample using optimized conditions of GC-MS

In this study, both extraction methods following some study with modifications unable to detect the presence of malathion residue in *Chrysomya megacephala* larvae for all the treatment groups (T1, T2 and T3) (Nakamura et al., 1994; Liu et al., 2009). Malathion residue also cannot be detected from the dead adult samples collected from T3 group. The undetected malathion residue in this study could be due to insufficient samples for the extraction procedure. Both study use 30 to 40 larvae for extraction which was a number that was not favourable for our study as we only manage to collect 10 – 15 larvae for each treatment (Nakamura et al., 1994; Liu et al., 2009). The number of samples can significantly impact the extraction and detection of malathion in studies using larval samples. When the number of larvae is lower than the recommended amount, the total quantity of malathion extracted decreases, which can lead to challenges in accurately detecting and quantifying the compound. This reduced concentration can result in a lower signal-to-noise ratio, making it difficult to distinguish malathion from background noise. Furthermore, smaller sample sizes can increase the variability and reduce the reliability of the results, as the data may be more susceptible to minor experimental variations and matrix effects (Tarelli et al., 2009).

The current study successfully optimized GC-MS conditions for malathion detection using standard malathion solutions in acetone. Retention times and peak quality were assessed under six different sets of operating conditions, with some of research yielding the most favourable results (Thabit and Silos, 2013; Nakamura et al., 1994). Malaoxon, a more toxic metabolite of malathion, was detected under three conditions, further demonstrating the sensitivity of the method. Although the retention time and peak quality varied, the overall detection of both malathion and malaoxon across the different conditions indicates that GC-MS is a viable method for analyzing these compounds. However, the inability to detect malathion in *Chrysomya megacephala* larvae samples in subsequent experiments highlights the need for further refinement in sample extraction methods or increased sample sizes to improve detection sensitivity. These findings emphasize the importance of selecting the appropriate operating conditions for forensic entomotoxicology, as varying parameters can significantly impact the detection of toxic compounds such as malathion and its metabolites. Future studies should consider utilizing more complex sample matrices to mimic real-world forensic scenarios and ensure reliable detection in insect tissues.

4. Conclusion

This study contributes to the understanding of malathion detection in forensic entomology. Although no malathion was detected in the larvae, the optimized GC-MS conditions can be applied in future studies. Further research using larger sample sizes and real-world models is recommended.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgement

The authors would like to thank the Director General of Health Malaysia and the Director, Institute for Medical Research (IMR) for their permissions to publish this article. Special appreciation to SEAMEO Tropmed Network Malaysia, National Institute of Health and all Medical Entomology Unit staff for their supports throughout this study.

Conflicts of Interest

The authors declare no conflict of interest.

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