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Malathion extraction from larvae of *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) for determining death due to malathion

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Abstract. The use of *Chrysomya megacephala* larvae for detecting malathion for diagnosing the cause of death was investigated. This could prove useful when the visceral organs have become liquefied during decomposition and therefore cannot be sampled. A field experiment was conducted in which *C. megacephala* were allowed to colonise naturally the corpses of rabbits that had died of malathion poisoning. The concentration of malathion increased gradually during the larval stages of *C. megacephala* reaching the maximum concentration in the third instar larvae. The concentration of malathion declined during prepupal stage and reached its lowest level among tenerals. The average malathion concentrations in *C. megacephala* growing in poisoned rabbit corpses left in a sunlit habitat were significantly higher ($p<0.05$) than those growing on poisoned rabbits left in a shaded habitat. The concentrations of malathion in the different stages of development of *C. megacephala* were moderately correlated ($r = 0.51$-0.69) with the administered doses as well as with those estimated in visceral organs. Thus, it would not be reliable to suggest the formulation of mathematical algorithms for relating the concentration of malathion found in the different stages of development of *C. megacephala* with those found in the visceral organs. However, in the context of forensic investigation, the qualitative detection of malathion in *C. megacephala* may prove useful in diagnosing the cause of death, since malathion is a common cause of accidental and suicidal deaths.

INTRODUCTION

Entomotoxicology, a relatively new branch of forensic entomology deals with the qualitative and/or quantitative determination of drugs and/or poisons in insects recovered in decomposing remains (Gagliano-Candela & Aventaggiato, 2001; Introna *et al.*, 2001). While feeding on a decomposing corpse, invertebrates may accumulate drugs and/or poisons consumed by the person before death (Goff & Lord, 2001; Gennard, 2007). However, Tracqui *et al.* (2004) stated that the drugs identified in larvae recovered from a corpse can also be found in cadaver and therefore that analysis of the larvae is of almost no interest for practical forensic casework. In this context, utilization of necrophagous larvae for detecting drugs of abuse and therapeutic drugs (Greenberg & Kunich, 2002; Tracqui *et al.*, 2004; Gennard, 2007) would throw light only on the circumstances surrounding death rather than suggesting the cause of death.

Malathion is an organophosphorous insecticide that is employed for both agricultural and medical purposes. It is used throughout the world in a variety of formulations and is widely available. Although it has relatively low toxicity in humans it is metabolised to the more toxic malaoxon in our bodies and if taken to excess can prove fatal. Owing to its ease of availability, malathion is often used as a

means of committing suicide particularly among agricultural communities (Thompson *et al.*, 1998; Pannell *et al.*, 2001). Malathion may also cause death through accidents (e.g. through not following safety precautions when handling the concentrated insecticide) and when it is used to deliberately poison someone. It is therefore important to be able to detect the presence of malathion if there is a suspicion that it may have been involved as the cause of death. This study describes a field study on the accumulation of malathion residues within the bodies of *Chrysomya megacephala* feeding on rabbits that had died of malathion poisoning and how these compared to the concentrations of malathion recovered from the rabbits' bodies.

MATERIALS AND METHODS

Animal model

This study utilized the same animals described in our previous published research (Mahat *et al.*, 2009) (Ethical Approval Reference Number: PPSG/07(A)/044). During each month, eight rabbits were used in two sets, one set for sunlit habitat and the other for the shaded habitat throughout one year from July 2006 to June 2007. The sunlit habitat was in open grassland, while the shaded habitat was a sandy area below are reinforced cement concrete roof, which was fully protected from rain and open on all sides. During that period, the monthly average ambient temperature recorded by the meteorological department ranged between 26.2°C and 28.3°C (annual mean of 27.3°C), while the monthly average ambient temperature recorded at the decomposition site ranged between 24.4°C and 28.7°C (annual mean of 27.0°C) and the monthly total rainfall ranged between 7.0 and 510.4 mm. Among the four rabbits that formed one set, one was used as control (PC) and the remaining three designated as T1, T2 and T3 were used for administering malathion in three different doses in increasing order (Table 1). These doses were calculated on the basis of the estimated fatal acute oral toxicity of malathion in humans of 0.86 g/kg (Carlton *et al.*, 1998). This is slightly lower than the estimated acute oral toxicity of malathion in rabbits which is 1.2 g/kg (Weeks *et al.*, 1977). It should be noted that published estimates of the LD50 of malathion in rodents vary considerably as a consequence of impurities. In the ascending order, the above doses were 50% lower than the estimated human fatal oral dose (0.43 g/kg) (T1), equivalent to the estimated human fatal oral dose (0.86 g/kg) (T2) and 50% higher than the estimated human fatal oral dose (1.29 g/kg) (T3) (Table 1). The rabbits were administered malathion orally via gastric intubation. The rabbits were not anaesthetized before or after receiving the malathion in case the anaesthetic interfered with the absorption of the insecticide. All the rabbits that received malathion died within one hour of treatment. The control rabbits were killed in a carbon dioxide chamber (Table 1). After death a midline abdominal incision was made in the abdomen of each rabbit to enable sampling of liver (approximately 5 g), gastric content (5 mL) and femoral venous blood (5 mL), after which the incision was stitched as per standard stitching procedure (Bright, 2000). The carcasses placed in separate self-lock plastic bags were transported and deposited

Table 1. Details of the rabbits that formed one set

Serial Number	Experimental use	Method of sacrifice	Surgical incision	Alphabetical designation used for sampling
1	Control	Carbon dioxide chamber	Present	PC
2	Treated 1	Commercial malathion (0.43 g/kg)[a]	Present	T1
3	Treated 2	Commercial malathion (0.86 g/kg)[a]	Present	T2
4	Treated 3	Commercial malathion (1.29 g/kg)[a]	Present	T3

[a]Gastric intubation procedure

in the respective decomposition sites before 9.00 am.The carcasses (8 each month) were placed on the ground in contact with soil or grass and were covered with slotted plastic baskets (height: 25.0 cm; length: 60.0 cm; width: 40.0 cm; slot width: 2.5 cm; slot length: 3.0 cm; mould width between slots: 0.5 cm) with two to three bricks on top to safeguard from vertebrate scavengers, yet permitting free ingress and egress of insects. An inter-carcass distance of at least 50 m was maintained to minimize interruption of flies from the adjacent colonies. Representative specimens of the different stages of *C. megacephala* (3.0 g for every developmental stage) were collected randomly from each carcass until the completion of the first lifecycle i.e. approximately 200-250, 100-150 and 75-100 larvae were collected during the first instar, second instar and third instar, respectively.

Statistical analyses were conducted using the SPSS 13.0 software. Independent-Samples T test was used for comparing the concentrations of malathion in the different stages of *C. megacephala* between sunlit and the shaded habitats. Pearson correlation coefficient (r) was used to correlate the concentrations of malathion found in every stage of development in *C. megacephala* sampled from the three groups of malathion-treated carcasses versus those found in the visceral organs of the carcasses as well as versus the dosages administered.

Extraction of malathion from visceral organs and *C. megacephala* samples

Malathion was extracted using the acidic extraction method standardized by the National Poison Centre, Malaysia (2006). 10 µL gastric contents was placed in a screw-capped glass test tube followed by the addition of 10 ng of chlorpyrifos as the internal standard and the mixture was vortex-mixed, briefly. Hydrochloric acid, HCl (0.5 M, 50 µL) was added to the mixture, followed by the addition of n-hexane (250 µL) and the tube containing the mixture was vortex-mixed for one minute at 1800 rpm. The hexane layer was transferred into a clean glass test tube followed by the addition of anhydrous sodium sulphate (~0.1 g) and the mixture was vortex-mixed, briefly. The extract was left at room temperature (about 25ºC) for one minute to settle and the clear extract was transferred into a vial insert and analysed by GC-MS. Analysis was conducted in triplicates and the mean concentration was recorded. Following the same extraction procedure, malathion was extracted from femoral venous blood (50 µL), ground liver tissue (50mg) and the different stages of ground *C. megacephala* samples (1.0 g).

Gas Chromatography-Mass Spectrometry (GC-MS) condition

Chemical analysis was conducted using a GC-MS (a HP6890 GC coupled with a HP5973 MS detector). The GC column (HP-5MS) used was a fused-silica capillary column (30.0 m X 0.25 mm X 0.25µm, Agilent Technologies, Australia). Helium was used as the carrier gas and the analysis was performed at constant pressure of 14.5 psi. The GC inlet was a split/splitless type and 1.0 µL of sample was injected with the injector temperature maintained constantly at 250°C. The oven temperature was programmed to increase from 70 to 230ºC (1 min hold) at a rate of 25ºC/min and the total separation time was 7.4 minutes. The post-run temperature was set at 300ºC for 5 minutes and the temperature of the interface was maintained constantly at 280ºC. The MS detector was set in the Selective Ion Monitoring mode (SIM). For confirming the identity of malathion, three ions used by Kralj *et al.* (2007) (m/z 173, 125 and 285) were chosen. Additionally, ions (m/z) 314, 197 and 258 were used for confirming the identity of chlorpyrifos (Oliva *et al.*, 1999). Quantitation of malathion was based on the integration of peak area ratios of ion m/z 173 against ion m/z 314, respectively, while the remaining ions served as qualifying ions for the corresponding analytes.

Calibration curves

Calibration curves were used to quantify the concentrations of malathion in visceral organs as well as in *C. megacephala* samples. A calibration curve of malathion in gastric content was prepared by spiking blank samples of gastric content obtained from

malathion-free rabbits (10 µL) with the known amounts of malathion (50, 75, 100, 150, 200, 225 and 250 ng), equivalent to 5.0 to 25.0 µg/mL of malathion via serial dilution. Then, chlorpyrifos as the internal standard (10 ng) was added and the mixture was extracted as per standard method described earlier. Following the same protocol, known amounts of malathion (50, 75, 100, 150, 200, 225 and 250 ng), equivalent to 1.0 to 5.0 µg/mL and 1.0 to 5.0 µg/g of malathion were added to the blank samples of femoral venous blood (50 µL) and ground liver tissue (50 mg), respectively. A calibration curve for malathion in *C. megacephala* was prepared by spiking blank samples of stages of *C. megacephala* obtained from malathion-free carcasses (1.0 g) with the above amounts of malathion (3.0, 10.0, 50.0, 75.0, 100.0, 125.0 and 150.0 ng). All calibration curves were constructed using the obtained peak area ratio of malathion/chlorpyrifos versus the concentrations of malathion added via linear regression analysis. The obtained calibration curves were accepted only when (1) the regression was ≥ 0.995 and (2) the coefficient variation (CV) was < 20% (National Poison Centre, Malaysia, 2006; Man *et al*., 2006)

Method standardization

The extraction method used for extracting malathion from the visceral organs was standardized by the National Poison Centre, Malaysia, indicating analytical recoveries of 85.2%, 87.3% and 86.5% for gastric content, femoral venous blood and liver tissues, respectively. On the other hand, for extracting malathion from *C. megacephala*, the method was standardized once again following the protocol prescribed by the National Poison Centre, Malaysia (2006) and Man *et al*. (2006) that included (1) linearity and sensitivity, (2) precision and accuracy and (3) recovery.

RESULTS

Concentration of malathion in visceral organs

Malathion was detected in all the visceral organs that were collected from all the malathion-treated rabbits (T1, T2 and T3) but not in any of the controls (PC) (Table 2). Malathion concentrations in visceral organs were the highest in the T3 carcasses, those orally administered with the highest concentration of malathion (1.29 g/kg) followed by T2 (0.86 g/kg) and T1 (0.43 g/kg) carcasses. Among the visceral organs in all the malathion-treated carcasses (T1, T2 and T3), the highest mean of malathion concentration was found in gastric content followed by femoral blood and liver tissue (Table 2).

Table 2. Concentrations of malathion in the visceral organs of malathion-treated carcasses

Malathion-treated groups	Concentration of malathion in visceral organs		
	Gastric content (µg/mL)	Femoral blood (µg/mL)	Liver tissue (µg/g)
T1 [dose: 0.43 g/kg] Total number of rabbits used: 24 [12 sunlit; 12 shaded]	7.61±0.38[a] 7.09–8.10[b]	1.64±0.06[a] 1.52–1.70[b]	1.46±0.05[a] 1.40–1.51[b]
T2 [dose: 0.86 g/kg] Total number of rabbits used: 24 [12 sunlit; 12 shaded]	12.13±0.49[a] 11.73–12.59[b]	2.15±0.12[a] 2.04–2.28[b]	1.91±0.07[a] 1.82–1.98[b]
T3 [dose: 1.29 g/kg] Total number of rabbits used: 24 [12 sunlit; 12 shaded]	19.55±0.88[a] 18.66–20.43[b]	3.34±0.14[a] 3.19–3.48[b]	2.95±0.17[a] 2.82–3.13[b]

[a]Concentrations of malathion as mean±standard deviation
[b]Ranges of malathion concentration

Concentration of malathion in the stages of *C. megacephala*

Malathion was detected in all the stages of development of *C. megacephala* and the concentrations were the highest in the specimens sampled from the T3 carcasses followed by those from the T2 and T1 carcasses (Table 3). The concentration of malathion increased gradually during larval stages reaching the maximum concentration in the third instar larvae. However, the concentration started to decline during prepupal stage and reached its lowest level among tenerals. In addition, the means of malathion concentrations in the feeding larvae in the malathion-treated carcasses (T1, T2 and T3) in sunlit habitat were found to be significantly higher ($p<0.05$) than those in the corresponding stage in the shaded habitat.

Correlation coefficient between administered malathion, its concentrations in visceral organs and those in stages of *C. megacephala*

The correlation coefficient between the concentrations of malathion in the stages of *C. megacephala* versus the administered dosages of malathion (0.43, 0.86 and 1.29 g/kg) as well as the concentrations of malathion in visceral organs of the corresponding carcasses are presented in Table 4. The correlation coefficient between concentrations of malathion in the different stages of *C. megacephala* sampled from T1, T2 and T3 carcasses in both sunlit and the shaded habitats versus the administered dosages (0.43, 0.86 and 1.29 g/kg) ranged between 0.51-0.62 (Table 4). It was observed that the correlation coefficient between concentrations of malathion in the different stages of *C. megacephala* in both sunlit and the shaded habitats versus those in the visceral organs of T1, T2 and T3 carcasses ranged between 0.52 to 0.69 (Table 4). Based on the 'strength of correlation coefficient' prescribed by Munro (2005), the concentrations of malathion in *C. megacephala* versus the administered dosages as well as versus the concentrations in the visceral organs were moderately correlated. The highest correlation coefficient was observed between the concentrations of malathion in the third instar larvae of *C. megacephala* versus those in gastric content (sunlit: r =0.69; shaded: r = 0.67). The lowest correlation coefficient was found between malathion concentrations among tenerals of *C. megacephala* versus those in liver tissues (sunlit: r = 0.53; shaded: r = 0.51).

DISCUSSION

The highest concentration of malathion was found in gastric content, while the lowest concentration was observed in liver tissues. These findings were in concurrence with those findings previously reported in acute malathion fatal poisoning cases (Farago, 1967; Morgade & Barquet, 1982; Jadhav *et al.*, 1992). The concentrations of malathion in liver tissues were lower than those in femoral blood, which was in agreement with the findings reported by Farago (1967) and Lewin *et al.* (1973). In a suicide victim the ratio of malathion found in gastric content as compared to that in blood or other organs varies over a wide range from 1.2:1 to 80000:1 (Thompson *et al.*, 1998). In this research, the consistency in the detection of malathion in all the visceral organs can be attributed to the controlled administration of specified dosages. In instances of suicide where the amount consumed remains arbitrary, failure to detect malathion in organs such as liver may occur when the amount absorbed from the stomach was just sufficient to cause death.

It was observed that the concentrations of malathion in the stages of *C. megacephala* were lower than the concentrations of malathion in all the visceral organs of rabbits. The concentrations of malathion in the larvae of *C. megacephala* were also lower than that reported from a pooled sample of second and third instar larvae of *C. megacephala* and *C. rufifacies* (i.e. 2050 µg/g) recovered from a highly decomposed dead body wherein the quantity consumed was unknown (Gunatilake & Goff, 1989).

The concentration of malathion increased gradually up to the third instar

Table 3. Concentrations of malathion in the stages of *C. megacephala* in all the malathion-treated carcasses in sunlit and the shaded habitats

Stages of development	Concentration of malathion in *C. megacephala* (ng/g)						Comparison of concentrations of malathion in *C. megacephala* between sunlit and the shaded habitats		
	Sunlit habitat			Shaded habitat					
	T1 carcasses (12 replicates)	T2 carcasses (12 replicates)	T3 carcasses (12 replicates)	T1 carcasses (12 replicates)	T2 carcasses (12 replicates)	T3 carcasses (12 replicates)	T1 (sunlit) versus T1 (shaded)	T2 (sunlit) versus T2 (shaded)	T3 (sunlit) versus T3 (shaded)
1st instar									
Mean±SD	25.90±1.38	35.74±2.15	55.88±3.02	18.94±1.14	26.97±1.89	41.42±2.49	p<0.05	p<0.05	p<0.05
Range	21.97-28.22	32.55-38.93	52.08-57.67	16.58-21.30	24.57-29.38	39.31-43.53			
2nd instar									
Mean±SD	47.63±2.44	71.25±3.47	108.33±6.38	36.70±2.02	52.26±2.62	80.25±4.82	p<0.05	p<0.05	p<0.05
Range	42.57-54.68	63.07-75.43	100.91-111.74	32.13-41.27	47.60-56.93	76.16-84.34			
3rd instar									
Mean±SD	65.75±3.14	85.35±4.47	137.20±8.24	47.36±2.14	67.44±3.38	103.55±5.70	p<0.05	p<0.05	p<0.0001
Range	54.93-70.56	81.38-97.33	130.21-144.19	41.46-53.25	61.42-73.45	98.27-108.82			
Prepupae									
Mean±SD	45.92±2.20	66.55±3.13	94.04±4.33	33.15±1.66	47.21±2.36	72.48±3.65	p<0.05	p<0.05	p<0.05
Range	38.45-49.39	56.97-68.13	91.15-100.93	29.02-37.28	42.99-51.42	68.79-76.17			
Pupae									
Mean±SD	21.84±1.21	31.95±1.70	44.13±2.61	16.00±0.99	22.63±1.30	33.35±1.98	p<0.05	p<0.05	p<0.05
Range	20.87-26.81	30.92-36.98	43.48-54.79	15.75-20.24	21.34-27.91	32.34-41.35			
Tenerals									
Mean±SD	8.71±0.57	12.80±0.67	18.58±1.05	6.10±0.36	10.12±0.51	15.53±0.78	p<0.05	p<0.05	p<0.05
Range	7.24-10.58	11.21-14.60	17.53-21.63	5.22-7.99	9.21-11.02	14.74-16.32			

A total of 24 rabbits (12 in each habitat) were used for T1, T2 and T3 groups, respectively. Specimens of the different stages of *C. megacephala* (3.0 g for each stage) infesting every malathion-treated carcass were collected and 0.05 g of the sample was used for analysis using GC-MS. Independent-Samples T test was used for comparing the concentrations of malathion in *C. megacephala* between sunlit and the shaded habitats

Table 4. Correlation coefficient (r) between administered malathion, its concentrations in visceral organs and those in *C. megacephala*

Stages of development	Correlation coefficients (r) in sunlit habitat between the stages (rows) and the administered dosages of malathion as well as the concentration of malathion in organs (columns)				Correlation coefficients (r) in shaded habitat between the stages (rows) and the administered dosages of malathion as well as the concentration of malathion in organs (columns)			
	administered dosages of malathion	gastric content	femoral blood	liver tissue	administered dosages of malathion	gastric content	femoral blood	liver tissue
1st instar	0.53	0.57	0.55	0.55	0.51	0.55	0.53	0.53
2nd instar	0.54	0.60	0.57	0.56	0.52	0.58	0.55	0.54
3rd instar	0.62	0.69	0.66	0.64	0.60	0.67	0.64	0.62
Prepupae	0.57	0.65	0.61	0.59	0.55	0.63	0.59	0.57
Pupae	0.55	0.62	0.56	0.54	0.53	0.60	0.54	0.52
Teneral	0.53	0.59	0.54	0.53	0.52	0.59	0.53	0.51

larval stage after which it decreased. This phenomenon can be explained by the fact that larval stages with increased malathion concentrations corresponded with a period of rapid feeding and thus excluded the postfeeding prepupal stage (Hedouin *et al.*, 1999). Campobasso *et al.* (2004) indicated that maggots' crop that are located at the anterior end of the digestive system expanded greatly during the feeding stage and deflated very rapidly during the postfeeding stage. During the feeding stage, the rate of absorption exceeds the rate of elimination (Hedouin *et al.*, 1999), while during the post-feeding stage the rate of elimination exceeds the rate of absorption (Campobasso *et al.*, 2004). These observations in the feeding behaviour explain the drastic decrease in the concentrations of malathion during the period that succeed the vigorously feeding stages. Additionally, the concentrations of malathion in *C. megacephala* infesting the malathion-treated carcasses in sunlit habitat (ambient temperature range: 24.58-28.71°C) were significantly higher ($p<0.05$) than those in the shaded habitat (ambient temperature range: 24.41-28.16°C). This finding may be attributable to relatively higher ambient temperature (by about 0.2-0.8°C) recorded in sunlit habitat, which may have resulted in higher larval growth and increased feeding behaviour of the larvae and consequently higher concentration of malathion accumulated in the larvae.

Statistically, for an association to be useful in making decisions, the 'strength of the correlation coefficient' must be very high, approximately 0.95 (Munro, 2005). In this research, it was observed that the concentrations of malathion in the different stages of development of *C. megacephala* were moderately correlated (r = 0.51-0.69) with the administered doses as well as with those estimated in visceral organs. Thus, it is impossible to suggest the formulation of credible mathematical algorithms for relating the concentration found in larvae with those found in the visceral organs.

Therefore, we conclude that the qualitative detection of malathion in all the larvae and pupae that fed on the malathion-treated carcasses supports its use for inferring the presence of malathion in the source. However, reliable quantitative relationship could not be established. Malathion chosen for this research is not a drug of abuse; instead it is a common poison used for committing suicide and hence its qualitative detection *per se* can enable inferring cause of death. The need for such inference is augmented in view of the fact that suicides are increasingly committed in remote areas with least ease for access leading to more frequent findings of highly decomposed bodies.

Although the concentration of malathion in *C. megacephala* from sunlit and the shaded habitats was analysed separately; the same was not done for the gastric contents, liver, and blood samples. Here the limitation is that

rabbit carcasses those were left in sunlit habitat may have lost water faster due to dehydration than that of in the shaded habitat and therefore this might have concentrated the amount of malathion in the carcasses. That reason can also be attributed to higher concentrations of malathion found in *C. megacephala* sampled from those carcasses in sunlit habitat when compared to that of in the shaded habitat. Although this could be a limitation to this current research, we reiterate that the overall findings of this research still hold good in indicating that it is impossible to determine the dose of poison a person might have consumed from the levels recovered from maggots feeding on the body.

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