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Effect of Diazepam on the Growth of *Chrysomya albiceps* (Wiedemann 1819) (Diptera: Calliphoridae) in Rabbit Carcass

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ABSTRACT

The larvae of necrophagous fly species are used as forensic tools for the determination of the minimum postmortem interval (PMI). However, any ingested drugs in corpses may affect larval development, thus leading to incorrect estimates of the PMI. This study aimed to investigate the effects of diazepam on the development rate of *C. albcipes* and to evaluate the reliability of this fly species as samples for toxicological analysis. For this purpose, larvae of *C. albcipes* were reared on rabbit carcasses treated with a lethal dose of diazepam. The development rate of the fly was measured, and samples from carcass tissues and fly stages were analyzed for diazepam. Results indicated that diazepam was detected in all tested carcasses tissues and different stages of *C. albcipes;* larvae, pupae, and adults confirming the reliability of these specimens for qualitative toxicology analysis. It was found that the presence of diazepam accelerated larval growth by 12 h, whereas the pupal stage was not affected. Furthermore, a 12 h interval bias on the total duration of development, from first larval instar to imago, has to be considered if diazepam was present in the corpse.

Keywords: Forensic entomology, *Chrysomya albiceps*, Drugs, Entomotoxicology.

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INTRODUCTION

The most common application of entomological evidence during the medico-legal investigation is the determination of the minimum postmortem interval (PMI). The insects most commonly used for this purpose are flies (Diptera), especially family Calliphoridae for being the first to reach and colonize a corpse [1-4]. The blowfly *Chrysomya albiceps* (Wiedemann, 1819) is known to be the first wave of insects that arrive at human remains for oviposition [5]. After hatching, larvae feed on dead tissue [3, 4]. By determining the age of the fly, it is possible to estimate their time of colonization, and hence, the minimum PMI [6]. Although the developmental time of many forensically important fly species is known, some environmental factors influence the growth rates of these insects [7]. These factors include temperature, humidity, body location, larval density, and the drugs presence or toxic substances in a corpse [8]. Previous studies have shown that drugs can alter the development rate of various fly species. They can accelerate [9] or delay the growth rate [10]. Failure to consider the presence of drugs in a corpse may lead to errors in minimum PMI estimates [8, 11]. Also, some deaths that occur by poisoning, remain undiscovered until the body is skeletonized. In that scenario, analysis of body tissues and fluids is almost unfeasible. Recently forensic entomologist has introduced insect evidence as a silent witness interpreting information concerning death. Fly larvae associated with a corpse consumed any toxic substances from the corpse into their tissues. These insects can then be analyzed to detect those toxic substances. This is known as forensic Entomotoxicology,

which is the branch of forensic entomology that investigates the effect of drugs and toxic substances on the development of necrophagous insects and studies the use of insects to detect these substances in animals tissue [12]. The purpose of this research was to study the effect of diazepam in rabbit carcasses on the development of *Chrysomya albcipes* as well as to detect the concentration of diazepam in rabbit tissues and deferent stages of *C. albcipes*; larvae, pupae, and imagos.

MATERIALS AND METHODS

Experimental design

This study was carried out in autumn during the period from 23/11/2017 to 7/12/2017 in Jeddah city which is located on the western coast of the Kingdom of Saudi Arabia (longitude 39.7 & east latitude 29.21 north), in the middle of the eastern shore of the Red Sea. The pharmacy of king Abdulaziz university hospital affiliated with the ministry of health in Jeddah in the Kingdom of Saudi Arabia, supplied the Diazepam. We used sex domestic rabbits weighing 3.75-3.96 kg, which was divided into 2 groups, 3 rabbits on each. In the first group rabbits were treated with an oral suspension of the lethal dose of diazepam; 9 mg/kg body weight, according to [13], whereas in other groups rabbits were treated with distilled water (as control). The rabbits were mechanically sacrificed by cervical dislocation. In each of the treated and control groups, rabbit carcasses were dissected to obtain three replicates weight 5 kg 3 gram of the tissues; heart, lung, liver, kidney, skeletal muscle. Then, each rabbit carcass was transferred outdoor and placed inside a special metal cage floored with soil. The cage had a surface of 2 cm^2 mesh to allow insects access and keep out scavengers. Then, caged carcasses were distributed into 2 lines a part 10 m from each other. Daily weather data of relative humidity and temperature in the study area were obtained from a center of the General Authority for Meteorology and Environment Protection in Jeddah.

Estimation of development duration and accumulated degree hour ADH for unadult stages of C. albcipes

Carcasses in the study site were monitored until the flies of *C. albiceps* arrived and laid eggs, then carcasses were examined every 12 h looking for unadult stages of *C. albiceps*. The duration of each pre-incubation period, incubation period, first larval instar, second larval instar, third larval instar, pupal stage, duration from first larval instar to adult eclosion were recorded. Basal temperature (lower development threshold temperature) (Dz) was obtained from previous studies. It was 9.72 ^{0}C for the incubation period [14], and it was also used for the pre-incubation period since it was not available in previous studies. For the first larval instar Dz value was 11.14 ^{0}C [15], and 13.00 ^{0}C for the second larval instar [15]. Due to the lake of Dz for the third larval instar, we used Dz of the larval period, which was 15.04 ^{0}C by Queiroz (1996) [16], 13.92 ^{0}C by [15], the average was 14.48 ^{0}C. For the pupal stage, Dz values were 17.39 ^{0}C [16], 11.65 ^{0}C [14], and 13.65 ^{0}C [15], the average was 14.317 ^{0}C. Dz value for the period from first larval instar to adult eclosion was 15.38 ^{0}C [16]. Because the experiment was conducted under fluctuating temperature; Accumulated degree hours (ADH) for each developmental stage was calculated using the formula; DH= [(maxi. temp. + min. temp.) /2] – Dz, ADH= DH x D Where; Dz= basal temperature (^{0}C), D = duration of development (hour) [17].

1. *Analysis of toxicity in insects and tissues of the rabbit carcasses*

From rabbit carcasses in each of the control and treated groups, three replicates weigh 3 grams of insect samples (third larval instar, pupae, and adult flies of *C. albiceps*) and carcass tissues (liver, kidney, heart, lung, skeletal muscle) were obtained. They were washed twice with distilled water and physiological solution (0.9%) NaCl, then dried by filter paper. Then were placed in plastic bottles and stored in a deep freezer at -20 ^{0}C until analysis.

2. *LC-MS-MS method*

A thermo LCQ fleet coupled with MS/MS Ion Trap was used. The LC was supplied with Gemini C18 (5 µm particle size, 150 mm x 2.0 mm) and protected by a guard column with packing material (4 mm x 2 mm), and the column was maintained at 25^{0}C. One gradient composition and one column were used to optimize the LC part. Mass Spectral was in positive ion mode for all drugs and the probe

voltage was 4.5 kV. Full scan ms/ms have been used to screen the drug.

Drug Tuning and Optimization of drug separation
Tuning was created for the drug and its internal standard separately to optimize the sheath and auxiliary gas flow rate and collision energy. In the tuning process, 10 mg/L working solutions for each drug were used. Separation of compounds occurs in the LC part. The type of the mobile phase, flow rate, the optimizing was done by preparing the drugs (1 mg/L) include the IS.

Sample preparation
The amount of 15 ml of Deionized water was added to 1g of sample in a stomacher bag. The mixture was homogenized by the stomacher at high mode for 2 min. The homogenized mixture (2 ml) was transferred to a centrifuge test tube, then 1 ml of phosphate buffer and 50 µL of I.S were added. The test tube was vortexed and centrifuged for 5 min at 4500 rpm.

SPE extraction
Labeled clean screen columns were placed in the SPE vacuum manifold. For activation, 3 ml of methanol was pipetted to each column, followed by 3 ml of deionized water and 1 ml of phosphate buffer pH 6.0 at flowrate 2 ml/min. Then samples were loaded to the columns at flowrate 1 ml/min. After that deionized water was added to the column followed by 2 ml of 0.1 M acetic acid. Then columns were dried under a high vacuum for 5 minutes. In the first step of elution, labeled collecting tubes were placed into the manifold box then 2 ml of 50 % ethyl acetate and 50 % hexane was added to the column under low or no vacuum pressure. In the second step of elution, the columns were dried for 2 minutes under vacuum, followed by adding 2mL DCM/IPA/NH4 OH (78/20/2) at flowrate 1 ml/min. The collected elution was dried under nitrogen or a Genevac vacuum instrument and reconstituted by adding 200 µL of initial mobile phase and vortexed for 30 seconds. The reconstitution was transferred to an HPLC vial and 20 µL of each sample was injected into the LC-MS/MS. The calibration curve of Diazepam was read to calculate its concentration in the samples.

Statistical analysis
The duration of each pre-incubation period, incubation period, first larval instar, second larval instar, third larval instar, pupal stage, the period from first larvae to adult eclosion were calculated using the mean value according to Arkin & Colton [18]. Diazepam content in each of rabbit carcass tissues and deferent stages of *C. albcipes* was analyzed statically using the method of factorial experiments which was achieved in randomized complete block design with three replicates for one factor; diazepam content which included eight levels, represented by rabbit carcass tissues and deferent stages of *C. albcipes*. The statistical analysis included the "F test", and its results summarized in "ANOVA table", and then "Dancun's test" was utilized for the comparison of means, according to Snedicor (1958) [19].

RESULTS AND DISCUSSION

During this study, larvae of C. *albiceps* fed and developed on dead rabbit carcasses of diazepam and normal rabbit carcasses. The experiment was conducted under fluctuating temperature, where minimum temperature was varied from 21.8^{0}C to 25.8^{0}C, and maximum temperature was varied from 27.37^{0}C to 31.8^{0}C. The maximum relative humidity was varied from 68 % to 73.5 %.

Effect of diazepam on development rates of immatures of C. albiceps
Data are given in **Table 1** illustrated that the duration of the pre-incubation period for *C. albcipes* on treated rabbits (20 h) was shorter than on control rabbits (30 h), whereas the incubation period on treated rabbits (18 h) was longer than on control rabbits (12 h). Each of the first larval instar and late third larval instar in the treated group (12 h and 24 h, respectively) took half the time of the control group (24 h and 48 h, respectively). The development time for both second larval instar and early third larval instar in the treated group (24 hours) was double compared to the control group. The total period of the larval stage in the treated group (84 h) was shorter as compared with the control group (96 h). For the pupal stage, the development time was similar in both groups (24 h). The development time from the first larval instar to adult eclosion in the treated

group (108 h) was shorter than in the control group (120 h) **(Figure 1)**.

Results in the **Table 1** showed that accumulation degree-hours (ADHs) required for the pre-incubation period in control rabbits (482.4 DH) was more than in treated rabbits (319.6 DH), and for the incubation period, ADHs in control rabbits (220.56 DH) was less than in treated rabbits (306.72 DH). The ADHs required for the first larval instar and late third larval instar in the diazepam group (187.32 DH and 298.08 DH, respectively) were about half that required for the control group (374.64 DH and 534.48 DH, respectively). But, each second larval instar and early third larval instar in the diazepam group required about double ADHs in the control group (337.2, 295.68 DH and 172.8, 147.84 DH, respectively). The ADHs for the pupal stage in the control group were 320.88 DH and in the treated group was 299.28 DH. The first larval instar in the control group required more heat to complete development to the adult stage (1412.4 DH) than those in the treated group (1238.76 DH).

Detection of diazepam in carcass tisseus and C. albcipes stages

Carcass tissues and deferent stages of *C. albcipes* were analyzed to detect diazepam. As shown from the **Table 2**, all analyzed carcass tissues and *C. albcipes* stages were positive for diazepam. The liver had the highest concentration of diazepam significantly (472) then each of pupae (211.5), kidney (182.6), lung (180.3), and heart (162.3). The lowest content of diazepam significantly was in adult flies (38.833), 3rd larval instar (35), and skeletal muscle (32.8).

Table 1. Development duration and thermal requirements (ADH) for different stages of *C. albiceps* feeding on control and treated rabbit carcasses with diazepam at fluctuating temperatures

The stages	Base Temp. (Dz)	Control rabbit carcasses					Treated rabbit carcasses				
		Temp (^{0}C)		R.H. (max %)	Duration (Hour)	ADH (^{0}C)	Temp. (^{0}C)		R.H. (max %)	Duration (Hour)	ADH (^{0}C)
		Min	Max				Min	Max			
Pre-incubation period	9.72	21.9	29.7	68	30	482.4	21.8	29.6	70	20	319.6
Incubation period	9.72	25.8	30.4	69.7	12	220.56	23.5	30.02	69.7	18	306.72
1st larval instar	11.14	23.5	30	69.8	24	374.64	23.5	30	69.8	12	187.32
2nd larval instar	13.00	24.6	30.2	69.5	12	172.8	24	30.1	69.5	24	337.2
Early 3rd larval instar	14.48	23.6	30	72	12	147.84	23.6	30	73.5	24	295.68
Late 3rd larval instar	14.48	23.5	27.73	70	48	534.48	23.5	30.3	71.3	24	298.08
Larval stage	14.48	23.8	29.5	70.3	96	1168.32	23.65	30.1	71.0	84	1041.18
Pupal stage	14.23	23.4	31.8	71	24	320.88	23.4	30	70	24	299.28
1st larval instar to adult eclosion	15.38	24.2	30.1	70	120	1412.4	23.6	30.1	70.8	108	1238.76

n= 30 insects for each development stage

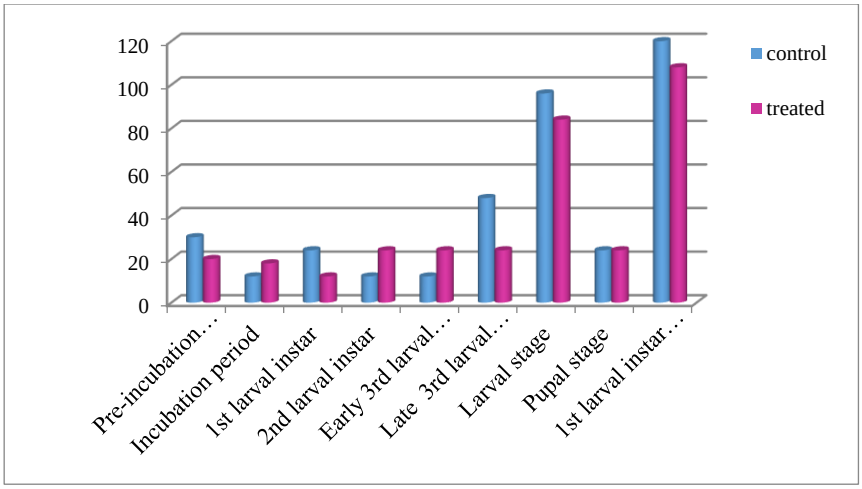

Figure 1. Development duration for different stages of *C. albiceps* feeding on control and treated rabbit carcasses with diazepam at fluctuating temperatures.

Table 2. Diazepam quantitation (ug/mg) in samples of rabbit tissues and deferent stages of *C. albiceps*

Type of tissue	Mean of diazepam content
Heart	162.3 B
Lung	180.3 B
Liver	472 A
Kidney	182.6 B
Skeletal muscle	32.8 C
3rd larval instar	35 C
pupae	211.5 B
Adult flies	38.833 C

Different letters indicate that there are significant differences between the averages

In this research, *C. albcipes* females deposited their eggs on control carcasses after 30 h from death and in treated groups after 20 h. This observation agreement with Ekrakene & Odo (2017) who proved that when rabbits were treated with cypermethrin and tramadol, eggs of *C. albcipes* in the control group were found at 24 h post-death and in the treated group at 6.25 h post-death [20]. Whereas, the recent study illustrated that duration of the incubation period in the control and treated group were 12 h and 18 h, respectively. Fouda *et al*. (2017) found that the incubation period of *C. albiceps* was not defer significantly on both dog carcasses treated with ephedrine sulphate and normal carcasses [21].

From this study, it was clear that in the larval stage diazepam accelerated growth in early and late phases, but delayed growth in middle phases, the total result was the larval stage in the control group (96 h) was shortened to (84 h) in the treated group. This observation was supported by previous studies, where Carvalho *et al*. (2001) found that *C. albiceps* larvae reared on rabbit tissues containing diazepam developed more rapidly than larvae from control colonies [11]. Fathy *et al.* (2008) recorded acceleration in the larval duration of *C. albcipes* after feeding on intoxicated rabbit carcasses with codeine phosphate, which was 72 h in the treated group and 96 h in the control group [22]. As well as Carvalho *et al.* (2012) observed that larvae of *C. albiceps* which fed on the cocaine-containing livers developed faster than those fed on livers from control animals [23].

The recent study indicated that diazepam did not affect the growth of the pupal stage. Similarly, Goff *et al*. (1989, 1997) reported that there were no differences noted in the duration of the pupal stage for colonies of *B. peregrina* and *P. ruficornis* fed on tissues containing cocaine and methylenedioxymethamphetamine, respectively [24, 25]. This result was not consistent with the work of Carvalho *et al*. (2001) who demonstrated that feeding of *C. albcipes* on rabbit carcasses treated with diazepam caused inhibition of growth in the pupal stage compared to the control group [11]. As well, Ekrakene & Odo (2017) recorded that each cypermethrin and tramadol in rabbit carrions prolonged the pupal stage comparably to control carrions [20].

The difference in diazepam effect in different stages of the fly was consistent with the work of Kharbouche *et al*. (2008) who found that larvae of *Lucilia sericata* which fed on liver samples treated with codeine developed more rapidly than control larvae, but codeine did not affect on the pupal development [26]. Khedre (2003) found that when larvae of *Wohlfahrtia nuba* reared on liver tissues obtained from rabbits administered a lethal dosage of diazepam, results revealed that the presence of diazepam in liver tissues shortened the duration of the larval stage, while the pupal stage was not affected [27]. Fouda *et al*. (2017) used a dog carcass treated with ephedrine sulphate to study its effect on the development of *C. albiceps* [21]. He found that larval duration was not affected significantly, but pupal development in the treated group was longer significantly than in the control one.

This study proved that there was an acceleration in the life cycle duration of *C. albcipes* by 12 h when diazepam was used compared to control. Previous researches involving insects and drugs indicated that they act as a feeding stimulant in some fly species. Carvalho *et al*. (2001) recorded acceleration in the total time of adult emergence of *C. albiceps* which fed on tissues containing diazepam compared to control [11]. O'Brien & Turner (2004) demonstrated that feeding of *Calliphora vicina* larvae on pig liver containing paracetamol resulting a 12-hour difference in the duration of the life cycle [28]. As well, Fathy *et al*. (2008) noticed that after feeding of *C. albcipes* larvae on intoxicated rabbit carcasses with codeine phosphate, there was the acceleration in the life cycle duration led to bias in the estimation of postmortem interval up to 24h when estimation

based on larval development and 48h when estimation based on pupal development [22]. Abd El-Gawad *et al*. (2018) found that the duration period from first instar larvae to adults of *C. albcipes* which resulted in treated rabbit carcasses with warfarin was faster than in control ones [29]. By contrast, some previous studies recorded the opposite of this result. Rashid *et al*. (2008) proved that larvae of *Chrysomya megacephala* in the control group developed more rapidly (7 days) than larvae in the treated group with malathion (10 days) [30]. Rezende *et al*. (2014) found that when *C. albiceps* larvae grew on an artificial diet containing phenobarbital, methylphenidate hydrochloride, and association of these two drugs, developmental time of the larvae from the first instar to adult stage for all treatments was longer than the control group [31]. Ekrakene & Odo (2017) proved that cypermethrin and tramadol in rabbit carcasses prolonged the total developmental period from egg to adult *C. albiceps* compared to the control [20]. Fouda *et al*. (2017) found that the total development of *C. albiceps* which fed on ephedrine sulphate dog carcass was highly significantly longer (17 ± 0.25 days) as compared to (16 ± 0.25 days) for control [21].

In this study, heat requirements at fluctuating temperature (minimum was 23.4 ^{0}C - 24.6 ^{0}C, and the maximum was 27.73 ^{0}C -31.8 ^{0}C) for each larval stage, pupal stage, and from first larvae to adult eclosion were; 1168.32 DH, 320.88 DH and 1412.4 DH, respectively. Al-Shareef and Al-Qurashi (2016) found that heat requirements at constant temperature (29.5 ^{0}C) for larval stage, pupal stage, and adult eclosion were 75.01, 62.73, and 129.138 DD, respectively [32]. Shiravi *et al*. (2011) in Tehran city recorded data on the same stages of *C. albiceps* at 28 ^{0}C which were 30-45, 62-93, 130-195 DD, respectively [33].

In comparison to data provided by Shiravi *et al*. (2011) on the same stages of *Chrysomya albiceps* reared at 28°C in Tehran city, which were 30-45, 62-93, 130-195 DD [33].

Detection of diazepam in carcass tisseus and C. albcipes stages

In the recent study, the toxicological analysis revealed that all tissue samples from treated rabbit carcasses were positive for the drug, as well as deferent stages of *C. albcipes*; 3rd instar larvae, pupae, and adult flies. The concentrations of diazepam in deferent stages of *C. albcipes* were lower than those in rabbit tissues. This result is in line with those of earlier researchers such as Khedre (2003) who proved that concentrations of diazepam in liver tissues were higher than those in the developmental stages, 3rd instar larvae, prepupae and empty puparial of *Wohlfahrtia nuba* [27]. As well, Kharbouche *et al*. (2008) illustrated that codeine concentrations measured in treated pig liver were significantly higher than those in larval samples [26]. Mahat *et al*. (2012) proved that the concentration of malathion in *C. megacephala* larvae which fed on rabbit carcasses treated with the drug was less than those in rabbit tissues [34].

Results in this study showed that in rabbit tissues, the highest concentration of diazepam was recorded in the liver followed by the heart, lung, kidney then skeletal muscles. Whereas, for insects, the drug was found in the pupal stage in a higher concentration than each of the 3rd instar larvae and adult flies. These results were supported by Rashid *et al*. (2008), who proved that after feeding *Chrysomya megacephala* larvae on a rat carcass administrated malathion, the liver showed containing the highest concentration of malathion compared to heart and lung, but for insects, the drug was detected in higher concentration in pupae followed by larvae and adult flies [30]. While, As well as, Carvalho *et al*. (2012) reported that after administering rabbits with a twice lethal dose of diazepam, the highest concentration of the drug was found in the heart and liver, but for insects diazepam in the pupal stage was higher than in larvae and adult flies [23]. The high content of diazepam in liver tissues is due to the metabolic process of drugs and toxins that takes place in the liver [35]. Generally, drugs were present in the heart, lung, kidney, and skeletal muscles of a mammal because of the presence of the metabolic enzyme, mixed-function oxidase [36]. Whereas, obtained results in the present work differ from those obtained by Introna *et al*. (1990) in which the concentrations of morphine in *C. vicina* larvae were quite similar to those in the human tissues used [12]. As a general rule, if the larvae feed on a tissue containing some kind of drugs or substances, two processes may happen: a bioaccumulation or an excretion of

the drug [11]. The present study illustrated that in larval stage drug effect on larval growth, which indicates that diazepam entered into the metabolic processes. Sadler *et al*. (1995) suggested that drug-contaminated tissues consumed by larvae are stored in the crop until they reach the pupal stage [37], therefore inducing an increase in the rate of absorption than elimination in the larval stage [38].

In the current study, the concentration of diazepam in the pupal stage was significantly higher than in the larval stage. Although it did not affect the length of the pupal period, this may be due to the accumulation of the drug in the cuticle of the puparium, without entering into the metabolic processes. Bourel (2001) suggested that the cuticle in pupae acts as a storage organ similar to that of adipocytes and pericardial nephrocytes [39]. Anderson (2000) also showed that in the pupal stage there is an unknown lipid layer below the cuticle, and this layer stores drugs [40]. Miller (1994) illustrated that pupal cases can retain toxins that were present even if the adult insect exits and migrates away from the carcass [41]. Accumulation or elimination of toxins from insect bodies may be due to its molecular weight. Bourel (2001) showed that within the body of the larva the water-soluble molecules are excreted from the haemolymph by malpighian tubules, whereas the less-soluble molecules are located near the pore canals in the cuticular matrix, moreover, nephrocyte cells also accumulate these drugs within their cytoplasm [39].

The results of this study proved the content of diazepam in adult flies was less than pupae, and this is in agreement with Bourel (2001) who showed that a small amount of morphine can be extracted from dead adult flies of *L. sericata* [39].

CONCLUSION

This study proved that the presence of diazepam accelerates larval growth, but did not affect pupal development. Furthermore, a 12 h interval bias has to be considered if diazepam is present in the corpse. The results also indicate that it is possible to use insects as alternative samples for toxicology analysis, due to the availability of diazepam in insect samples in conjunction with carcass tissues.

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