



## Original article

# Detection of benzodiazepines in decomposing rabbit tissues and certain necrophagic dipteran species of forensic importance

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## ABSTRACT

The determination of benzodiazepines (carbamazepine and clobazam) in rabbit carcass tissues and larvae of three Calliphoridae flies is described. After feeding the rabbits with lethal and toxic doses, samples of larvae and carcass tissues were studied. Residual drugs were determined using Ultra-high-performance liquid chromatography – quadrupole time-of-flight mass spectrometry (UHPLC/QTOF-MS). Benzodiazepines and its main active metabolites have been detected in the rabbit tissues at different retention times depending on the dosage used (lethal or toxic). A total of 1150 insects were collected and 800 larvae of the flies *Chrysomya albiceps*, *Lucilia sericata* and *L. silvarum* were used in the analysis. The presence of benzodiazepines in the rabbit tissues has been shown to typically affect the larval development cycle of the three necrophagous flies. *Chrysomya albiceps* larvae feed on drugs developed faster, while the development of *L. sericata* and *L. silvarum* larvae slowed. These results indicate that drugs have an impact on the life cycles of insects, which suggests that the presence of these substances is a factor that needs to be taken into account when estimating the post-mortem interval (PMI).

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## 1. Introduction

A key element in the investigation of unexplained deaths is the determination of the cause of death. This particular aspect may be difficult to determine when the body is recovered after it has been severely decomposed (Waghmare et al., 2015). If a person has died from drug abuse, committed suicide or has been ignored, cases of delayed recovery are likely to arise (Archer et al., 2006). In such cases, standard toxicological samples, including tissues, body fluids and internal organs, may have degraded or no longer be available, meaning that insects are the only reliable alternative specimens for forensic purposes (Gosselin et al., 2011; Badenhorst and Villet, 2018). Specifically, arthropods are attracted to dead bodies on

account of their smell, and indeed are typically the first visitors to a dead body. Blowflies, for example, will have oviposited on carion within a few hours of death (Rivers and Dahlem, 2014). Catts and Goff (1992) reported that Calliphoridae fly larvae found in the bodies and in the scenes of death could be used as entomological proof in forensic inquiries, i.e. estimating the PMI and determining toxins, and whether the remains had been moved. After phenobarbital was detected in fly larvae found on skeletonized skin (Beyer et al., 1980), entomotoxicology has been a well-established process, with various studies being performed by different groups to detect drugs at different stages of flies development (Gagliano-Candela and Aventaggiato, 2001).

The pharmacokinetics of drugs in insects depend on the species, the stage of development, the mode of action of the drugs, their absorption, redistribution and metabolism and their stability (Rashid et al., 2008; Charabidze et al., 2017). While actively feeding on the body tissue, toxic substances in the tissue are passed to the metabolic system of the larvae. In fact, these substances have been shown to be capable of being transferred through the food chain to other arthropods preying on the larvae (Tabor et al., 2004). Insect larvae also serve as an effective toxicological specimen, since they

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exist in many cases on the cadaver, and the pupal case remains unchanged and untouched for an extended period of time (Bourel et al., 2001). In this emerging field, the main current avenues of exploration are work on the use of necrophage arthropods as toxicological specimens, and work on the impact of contaminants on the larval life cycle (Boulkenafet, 2016). Furthermore, since the presence of drugs in the carcass can affect insect growth and morphology, and this can introduce a source of error into the estimation of the post-mortem interval (Tracqui et al., 2004; Singh et al., 2016).

Many methods have been used for toxicological testing, including immunoassays (Carvalho et al., 2001), gas chromatography (GC) (Kintz et al., 1994), and liquid chromatography (LC) (Goff et al., 1997). In addition, combining LC with tandem mass spectrometers (MS-MS) can provide increased sensitivity and selectivity, often with much reduced sample preparation and analysis times (Wood et al., 2003). Increased sensitivity may prove to be very useful for the analysis of arthropod tissue. It may, for instance, make it possible to test drugs in single larvae rather than pools. This has important implications for the precision of the estimation of the post-mortem interval (Goff et al., 1997).

Benzodiazepines are among the most used psychoactive drugs in the world, being frequently prescribed for symptomatic treatment of anxiety and sleep disorders. Unfortunately, the misuse of such compounds has often been documented and is associated with increased mortality (Kripke et al., 1998; Belleville, 2010). Benzodiazepines are also commonly used to commit suicide (Drummer and Odell, 2001). As a result, benzodiazepines are commonly seen in post-mortem samples. The effect of the lethal dose of diazepam on *Chrysomya albiceps* and *Ch. putoria* larvae has been studied (Carvalho et al., 2001).

The goal of the experiment reported in this paper was therefore to establish a link between the presence of drugs and their metabolites (benzodiazepine) in larvae and in the decomposing tissues of rabbit carcasses and to establish the effect of these substances on the development of certain forensic insects.

## 2. Materials and methods

This study was carried out at the campus of the University of August 20th, Skikda, Algeria, in urban conditions, with temperature between 8 and 25 °C. Five male rabbits, *Oryctolagus cuniculus* Linnaeus, were used (1.3–2.5 kg in weight). Treatments are performed with two psychotropic substances from the benzodiazepine family: Carbamazepine (Carbatol® 200 mg) and Clobazam (Urbanyl® 10 mg). For each substance, both lethal and toxic doses were used (PDL, 2016; TCL, 2018). The five rabbits were divided as follows (Table 1), a control rabbit (R1) was orally given 2 ml of distilled water. The other four rabbits were used to determine the drug's effect. Two were given Carbamazepine (CBZ) (R2: lethal dose and R3: toxic dose) and the other two were given Clobazam (CLB) (R4: lethal dose and R5: toxic dose). For lethal doses, 3271.57 mg/kg of CBZ or 414.08 mg/kg of CLB and for toxic doses, 1710.5 mg/kg of CBZ or 185.94 mg/kg of CLB were dissolved in 2 ml distilled water and given orally using a metal probe. The rabbits were then placed 20 m apart in fine-grained metal cages

(60 × 50 × 50 cm) to prevent predators from attacking, while the cages were designed to allow insects to enter. Immediately after dosing, rabbits treated with lethal doses were dead, and other rabbits killed by slaughter. For each rabbit, two plastic trays (10 cm in diameter) filled with water (20 ml) solution, salts and soap were used as insect traps. The insects trapped in the trays were then collected using a spoonful. Samples were collected for 46 days, three times a day for the first 15 days, twice a day for 15 days, and once a day for the last 16 days. The environmental temperature was noted at each sampling using a Lascar EL-USB-2 data logger. Identification of necrophagous fly larvae was carried out in the laboratory using various identification keys (Wyss and Cherix, 2006; Szpila et al., 2008, 2013). In order to study the life cycles of the flies, some of the collected larvae (LIII) were deposited in plastic boxes with a lid punctured with small holes to allow the air to enter. Other groups of larvae were quickly placed in sterile bottles and then directly in the refrigerator at –20 °C for toxicological analysis.

Toxicological analysis was conducted at the toxicology laboratory at the National Institute of Forensic Science and Criminology (INCC) in Algiers. The analysis was performed on 800 fly larvae and the neck area of the five rabbit. To get the rabbit tissues, a small incision was made in the neck as soon as the rabbit was killed, and a 20 g sample of epidermal and muscle tissue was taken. It was collected and frozen at –4 °C in a dry container. For toxicological study, an additional sample of rabbit tissues was collected every 48 h. Ultra-High-Performance Liquid Chromatography (UHPLC) and Quadrupole Time-of-Flight Mass Spectrometry (QTOF-MS) were used to quantify the amount of benzodiazepine, while samples were prepared (Thompson et al., 1998).

During the analysis of compounds in biological fluids and tissues, it is important to break the xenobiotic/protein bonds, purify the samples and concentrate the toxins in a reduced volume of solvent and thus reduce the detection limits. The larvae and tissues belonging to the five corpses were detached, then crushed, ensuring good sample extraction and representativeness; the crusher used is a mortar crusher. For each sample, the equipment was washed, rinsed once with distilled water and washed twice with methanol. Homogenates were then inserted in plastic centrifuge tubes (50 ml) to which 15 ml of methanol was added; a mixture was then collected (sample-solvent). Three successive stirrings were carried out to dissolve the solutes in methanol and facilitate the extraction: the tubes were first mixed with Vortex for 1 min, and then placed in an ultrasonic bath for 10 min, after ultrasonication, the samples were placed in a mechanical shaker for 5 min. Then the mixtures were centrifuged at 3500 rpm 1 min. Syringes (5 ml) were used to collect the supernatants and for syringe filtration philtre membranes of PTFE (Poly Tetra Fluoro Ethylene, 0.45 µm) were used. The extracts were then moved to Vial bottles (2 ml) to be ready for examination. The UHPLC was used has a dual pumping system that allows online extraction.

The sum of effective temperature (Teff) was calculated by:

$$T_{eff} = \text{time(days)} \times (\text{temperature} - \text{base temperature})$$

## 3. Results

A total of 1150 individuals, including three species, belonging to the family Calliphoridae, order Diptera, were collected over a 46-day period (Table 2). Flies were identified as *Chrysomya albiceps* Wiedemann (82 individuals), *Lucilia sericata* Meigen (446 individuals) and *L. silvarum* Meigen (649 individuals). Each rabbit passed through five stages of decomposition: fresh (day 1–2); bloated (day 3–5); active decomposition (day 6–8); advanced decomposition (day 9–13); and dry remains (day 13+). As shown in Table 2, *L. silvarum* was presented on three rabbits and *L. sericata* was

**Table 1**  
Different doses of drugs given to each rabbit.

Rabbit	Weight (g)	Drugs used	DL (mg/kg)	DT (mg/kg)
R1	2470	–	–	–
R2	1844	Carbamazepine	3271.57	–
R3	1555	Carbamazepine	–	1710.5
R4	1294	Clobazam	414.08	–
R5	1352	Clobazam	–	185.94

**Table 2**  
Species and number of necrophagous flies associated with rabbit carcasses.

Origin	<i>Lucilia silvarum</i>	<i>Lucilia sericata</i>	<i>Chrysomya albiceps</i>
R1	263	–	–
R2	–	197	–
R3	167	–	82
R4	–	249	–
R5	219	–	–

presented on two. While *Ch. albiceps* was recorded on only one rabbit. Also, the rabbit (R3) was the only carcass attracted two flies: *L. silvarum* and *Ch. albiceps*.

### 3.1. Detection of benzodiazepine in the rabbit tissues

Chromatograms obtained from rabbit tissues analysis (R2) treated with carbamazepine lethal dose show the drug at a retention time of 7.25 min (Fig. 1) and its major active metabolite (Carbamazepine epoxy) at  $t_r = 5.55$  min (Fig. 2). In the rabbit (R3), the major peak characteristic of carbamazepine was recorded at 7.23 min; also, the presence of the active metabolite of the Carbamazepine lethal dose: epoxide carbamazepine (CBZe) was recorded at a retention time equal to 5.55 min (Fig. 3). Furthermore, the tissue assay from the rabbit (R4), treated with a Clobazam lethal dose, showed a majority peak representing temazepam (a benzodiazepine near the CLB) at  $t_r = 9.40$ , another characteristic peak of clobazam at  $t_r = 9.14$  (Fig. 4), and a third peak at  $t_r = 8.09$  identifies the presence of Clobazam desmethyl metabolite, an active Clobazam metabolite.

### 3.2. Detection of benzodiazepine in larvae

The chromatograms obtained from the larvae assay of the rabbit (R1, control) show that the typical peaks of the two drugs used in this study are missing (Fig. 5). The presence of a lethal dose of Carbamazepine in the rabbit (R2) caused increased mortality of larvae in the first stage, which did not allow us to collect samples neces-

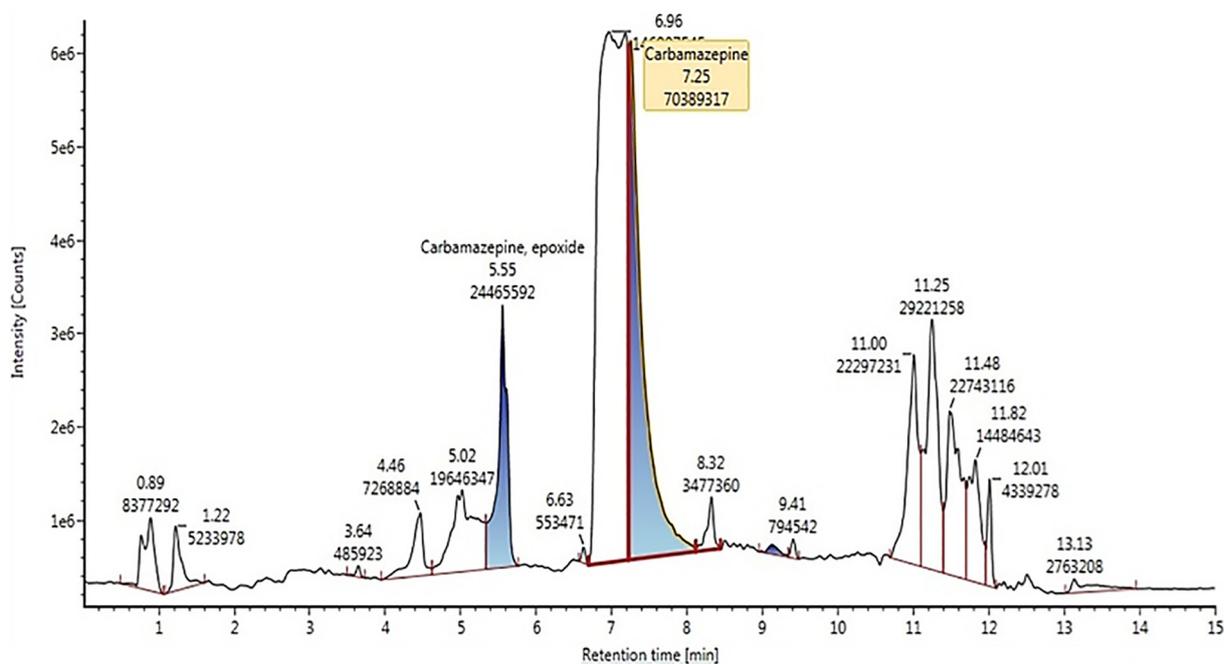
sary for the toxicological analysis. The assay of the larvae of R3 had a majority peak at  $t_r = 7.23$  min, characteristic of the CBZ; and the active metabolite CBZe was presented at 5.56 min (Fig. 6). The larval assay of the rabbit (R4), treated with Clobazam DL showed a peak at  $t_r = 9.22$  min, due to the presence of Clobazam in the cadaver larvae, as well as another peak at 8.09 min, characteristic of oxazepam (Fig. 7), and a total absence of temazepam observed in the same body tissue. Carbamazepine Epoxide and carbamazepine are identified at  $t_r = 5.63$  and  $t_r = 7.23$ . Since these were absent from the corpse tissue, the larvae must have migrated from CBZ-treated carcasses. Chromatograms obtained from the assay of the R5 larvae treated with a toxic dose of Clobazam showed a peak at 9.41 min characteristic of Clobazam and another peak at 8.09, indicating the presence of Oxazepam (active metabolite of Clobazam) in the larvae (Fig. 8).

### 3.3. Effect of benzodiazepine on the duration of the fly life cycle

The Teff consumed during the development cycle could not be calculated due for *L. silvarum* due to the lack of data on the lower development threshold of this species on the three rabbits (R1, R3 and R5). On the rabbit (R3), the fly took 6 days to reach the third instar larval stage, and it took 13 and 36 days to reach the pupal and adult stage, respectively (Table 3). Data in table 3, indicated that, no difference in the duration of life cycle among the rabbits: R1 (control), R3 (toxic dose of CBZ) and R5 (toxic dose of CLB).

The fly *L. sericata* recorded on the two rabbits with lethal doses of benzodiazepine (R2 and R4). On R2 the fly consumed 34, 100 and 196 °C Teff over 4, 13 and 26 days, respectively to reach the larval, pupa and adult stage. In the other hand, the same fly on R4, consumed 48.5, 119 and 334.5 °C Teff over 6, 16 and 43 days, respectively to reach the same stages (Table 4).

It is noted that the species *Ch. albiceps*, identified on R3, consumed Teff equal to 41.5 °C and 48.6 °C over a period of 6 and 7 days, respectively, to reach the third instar larval stage and pupal stage. The complete cycle was completed after consumption of 117.5 °C over a period of 20 days (Table 5).



**Fig. 1.** Chromatogram illustrating the result of the determination of the toxicology of epidermal and muscular tissues of the rabbit (R2).

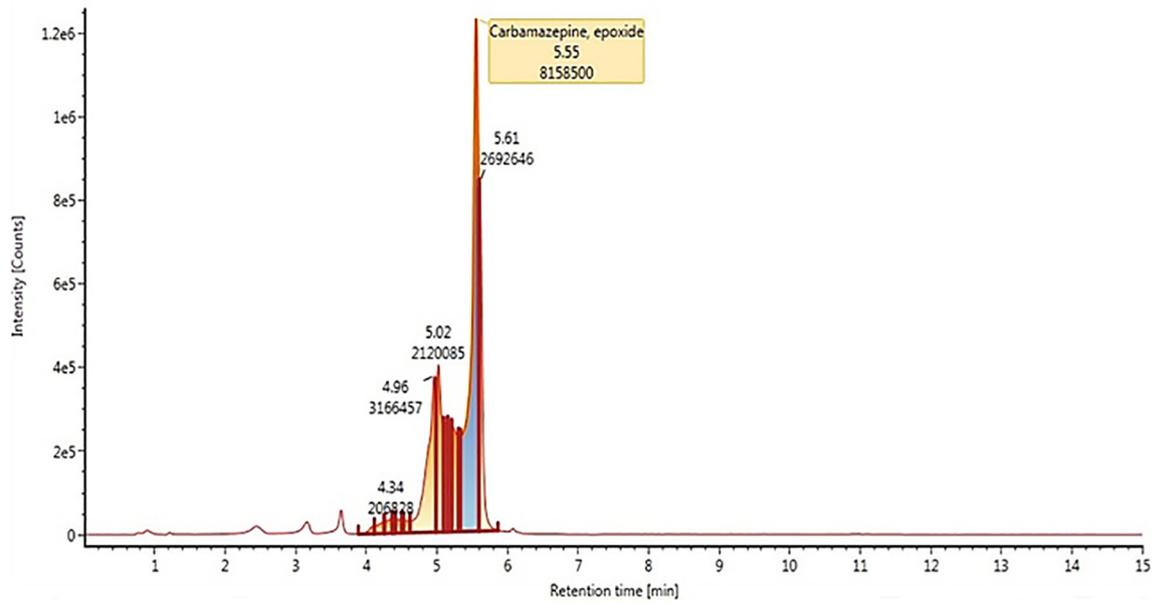


Fig. 2. Chromatogram illustrating the peak of CBZe.

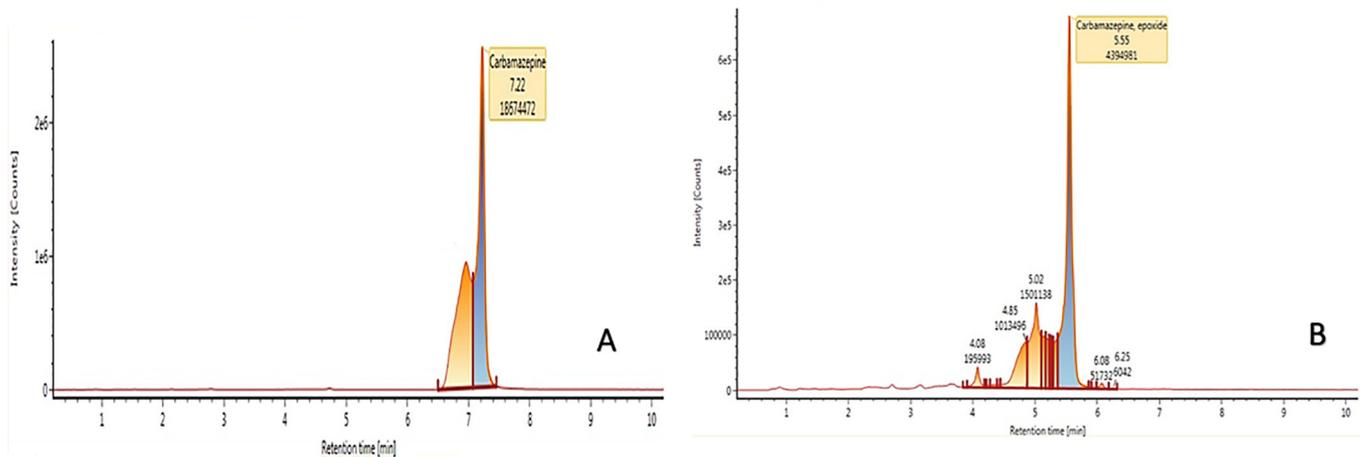


Fig. 3. Chromatogram illustrating peak of CBZ (A) and CBZe (B) from the tissues in the rabbit (R3).

#### 4. Discussion

Entomotoxicology not only assesses the effect of drugs on insects, but also uses insects as an alternative matrix (Goff and Lord, 1994; Introna et al., 2001). Toxicological analyses on highly decomposed bodies are easier with insects rather than with traditional matrices (blood, urine), as there are fewer disturbances due to decomposition (Nolte et al., 1992; Kharbouche et al., 2008). During the study, three species of necrophagous Diptera were identified from the carcasses of the five rabbits deposited at the study site, namely *Ch. albiceps*, *L. sericata* and *L. silvarum*. These species belong to the largely dominant Calliphoridae family (Grassberger and Frank, 2004), which are the most important species for forensics, whose larvae can complete their cycle on both animal or human carcasses. Zumpt (1965) and Marchenko (1985) indicated that Calliphorid flies were recognized as a species of great forensic importance. Four of the rabbits attracted only one fly species and only the rabbit (R3) attracted two species. This could be explained by the predatory behaviour of the species towards other necrophagous

larvae or by the size of the carrion which limits the food availability. Faria et al. (1999) showed that the predation rate of *Ch. albiceps* larvae can be as high as 80%. Also, Denno and Cothran (1975) revealed that carrion size limits the food availability, which would in turn affect the life history of each species. There may be competition or predation among species when resources are limited (Faria et al., 1999).

It is observed that the duration of the life cycle of the decomposed rabbits does not differ between the control bodies and the treated bodies with toxic doses, whereas there was a difference in the life-cycle duration between rabbit carcasses with lethal doses. This can be explained by the effect of the concentration of drugs affecting the development of insects present in the treated carcasses. This is consistent with Abd El-Bar and Sawaby (2011), who argued that certain chemical compounds may affect the activity and development of insects, in particular diptera present on the corpses. Drugs may influence the development of larvae on the corpse (Introna et al., 2001; George et al., 2009). Again, Carvalho et al. (2001) reported that diazepam (and/or its metabolites,

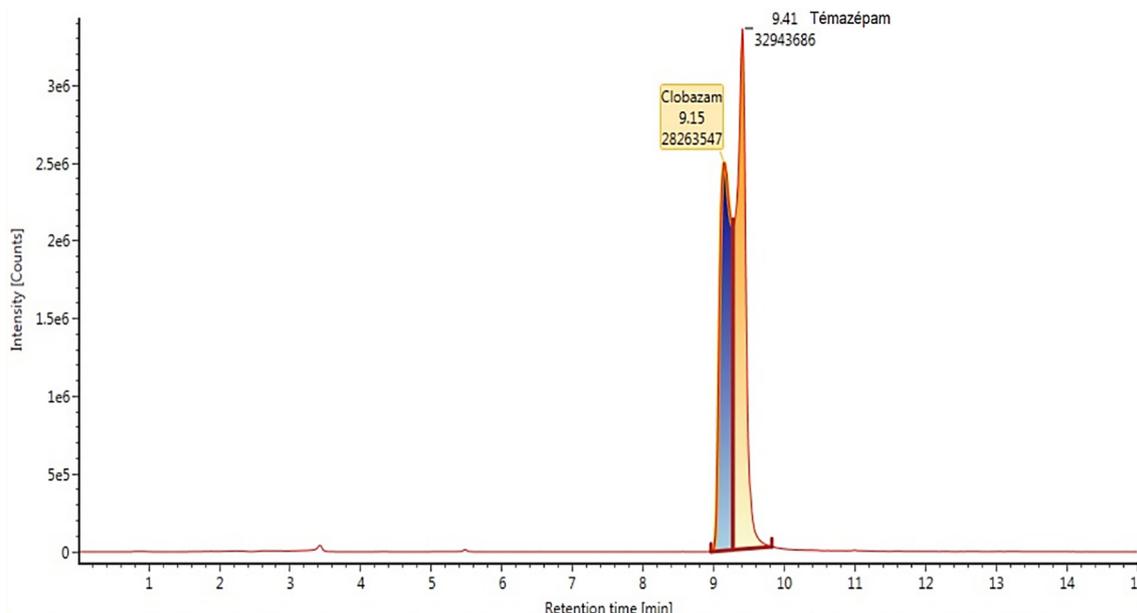


Fig. 4. Chromatogram illustrating CBZ and Temazepam peak from the tissue in the rabbit (R4).

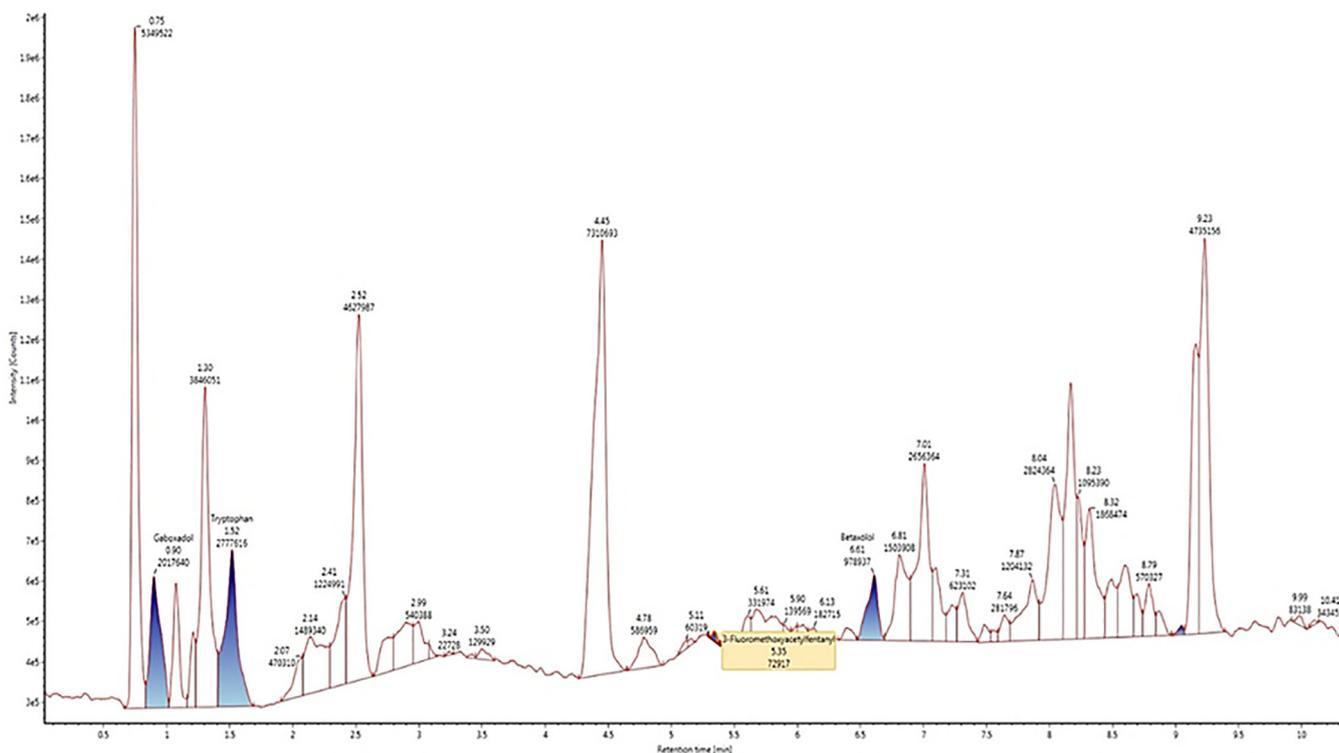


Fig. 5. Chromatogram illustrating the results of the assay of the larvae of the rabbit (R1, control).

nordiazepam and oxazepam) accelerated larval growth in *Ch. albiceps* and *Ch. putoria*. Meanwhile, *L. sericata* found on R4, which had been treated with a lethal dose of clobazam, took 43 days to reach the adult stage, consuming a Teff of 334.5 °C. This contrasts with data that states that *L. sericata* must consume a heat constant of 207 °C to complete its cycle (Marchenko, 2001). On the other hand, *L. sericata* identified on R2 treated with an CBZ DL accumulated 196 °C Teff. The finding that the existence of CLB in R4 tissues delayed the overall development of *L. sericata* was consistent with

the results of the El-Samad et al. (2011) study, which found that tramadol delayed the development of *L. sericata*. Goff et al. (1989) noted that control and sublethal batches (DL25) developed at approximately the same rate in a study on the effects of cocaine on the development of sarcophagides. On the other hand, batches fed to doses of tissue (DL50 and DL100) increased faster. This is consistent with studies on benzodiazepines (Carvalho et al., 2001), anticholinergic (Oliveira et al., 2009), opioid (Gosselin et al., 2011) and cocaine (Carvalho et al., 2012).

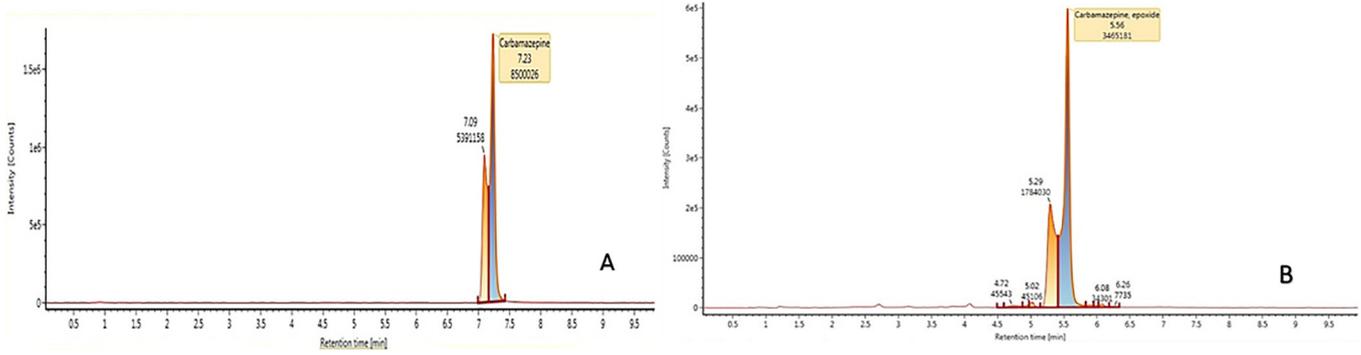


Fig. 6. Chromatogram illustrating peak of CBZ (A) and CBZe (B) of the larvae assay on the rabbit (R3).

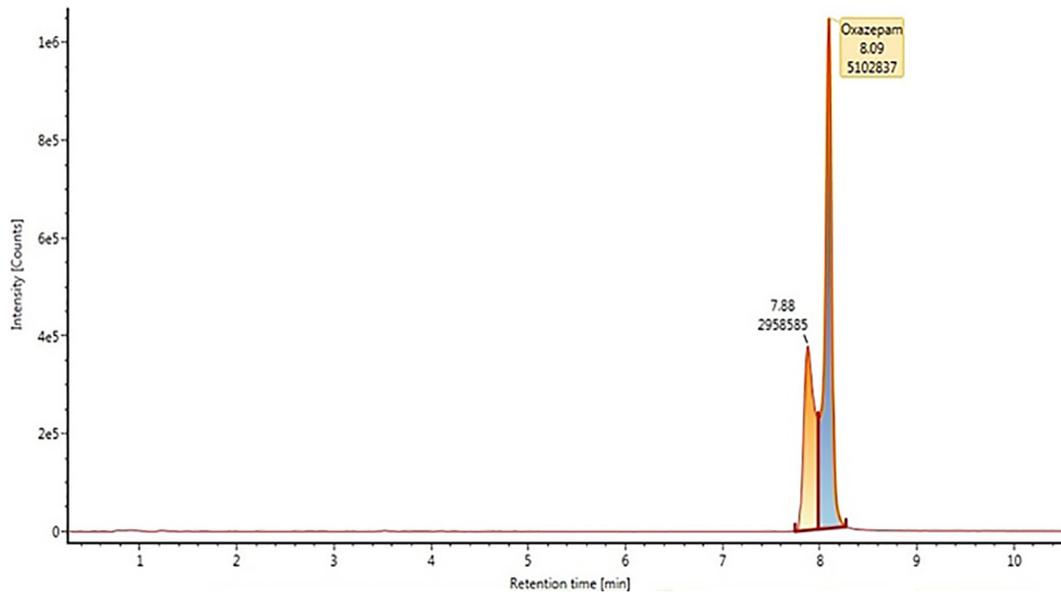


Fig. 7. Chromatogram illustrating the peak of oxazepam of the larval assay on the rabbit (R4).

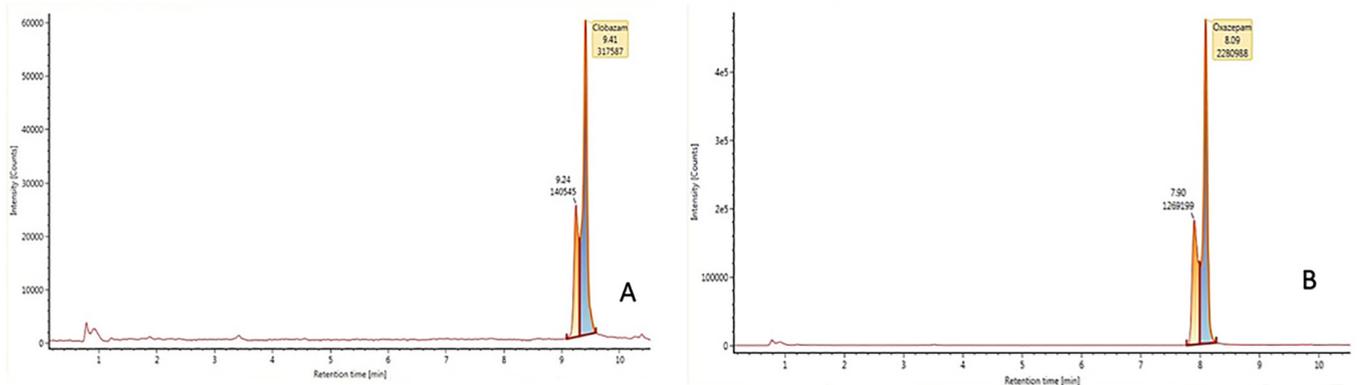


Fig. 8. Chromatogram illustrating the peak of Clobazam (A) and Oxazepam (B) from the larval assay on the rabbit (R5).

When larvae are fed to tissues treated with a drug or a poison, two processes occur in their systems, bioaccumulation or excretion of the drug or its metabolites (Carvalho et al., 2001). In our study, a remarkable bioaccumulation was reported by the detection of the two substances (carbamazepine and clobazam) in the larvae and carcass tissues of the treated rabbits, as well as the metabolites

**Table 3**  
Length of the development cycle of *Lucilia silvarum* identified on the rabbit carcasses (R1, R3, R5).

Life stage	Date of laying and end of stage	Number of days
Eggs - L3	April 15 – 20, 2019	6
Eggs - Pupae	April 15 – 27, 2019	13
Eggs - Adults	April 15 – May 20, 2019	36

**Table 4**Length of the *Lucilia sericata* development cycle identified on the rabbit carcasses (R2 and R4).

Carcass	Life stage	Date of laying and end of stage	Number of days	∑Teff °C
R2	Eggs - L3	April 17 – 20, 2019	4	34
	Eggs - Pupae	April 17 – 29, 2019	13	100
	Eggs - Adults	April 17 – May 12, 2019	26	196
R4	Eggs - L3	April 15 – 20, 2019	6	48.5
	Eggs - Pupae	April 15 – 30, 2019	16	119
	Eggs - Adults	April 15 – May 27, 2019	43	334.5

**Table 5**Length of the development cycle of *Chrysomya albiceps* identified on the rabbit carcass (R3).

Life stage	Date of laying and end of stage	Number of days	∑ Teff °C
Eggs - L3	April 15 – 20, 2019	6	41.5
Eggs - Pupae	April 15 – 21, 2019	7	48.6
Eggs- Adult	April 15 – May 04, 2019	20	117.5

of these substances (carbamazepine epoxy, oxazepam, desmethyl clobazam) and temazepam, a molecule adjacent to clobazam. The presence of metabolites in insect tissues confirms the internal metabolism of drugs by necrophagous diptera (Gosselin et al., 2010). This is the case with the temazepam detected in the rabbit tissue (R4), as this substance was not present in the larvae of the same carcass. The absence of Temazepam in larvae while oxazepam is being recorded. This is because oxazepam is one of the metabolites of temazepam after demethylation, so it is likely that the temazepam accumulated in the carcass tissue was metabolised to oxazepam in the larvae.

## 5. Conclusion

Insects provide details of a person's life before death when an individual has used drugs that can be detected in arthropods that feed on the body. Furthermore, ingestion of drugs from the body tissues can alter the developmental sequence of the insect in predictable ways. Benzodiazepines (Carbamazepine and clobazam) were detected in the rabbit tissues and larvae of three necrophagous diptera *Chrysomya albiceps*, *Lucilia sericata* and *L. silvarum*. The effect of drugs on the development of different insect species has also been reported. This influence should be considered in the alteration of the life cycle since it has a direct impact on the estimation of the PMI.

## Declaration of Competing Interest

The authors declare that they have no known competing interests or personal relationships that could have appeared to influence the work reported in this paper.

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