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Development and validation of a GC–MS method for nicotine detection in *Calliphora vomitoria* (L.) (Diptera: Calliphoridae)

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Highlights

1. Gas chromatography–mass spectrometry (GC–MS) can detect both nicotine and cotinine in immatures *Calliphora vomitoria*.
2. Nicotine does not modify the developmental time of *C. vomitoria.*
3. *C. vomitoria* survival during the pupation period is influenced by nicotine.
4. Nicotine also affects *C. vomitoria* larval length.

Abstract

Entomotoxicology is the application of toxicological methods and analytical procedures on

necrophagous insects feeding on decomposing tissues to detect drugs and other chemical components, and their mechanisms affecting insect development and morphology and modifying the methodology for estimation of minimum time since death. Nicotine is a readily available potent poison. Because of its criminal use, a gas chromatography–mass spectrometry (GC–MS) method for the detection of nicotine in *Calliphora vomitoria* L. (Diptera: Calliphoridae) was developed and validated. Furthermore, the effect of nicotine on the development, growth rate, and survival of this blowfly was studied. Larvae were reared on liver substrates homogeneously spiked with measured amounts of nicotine (2, 4, and 6 ng/mg) based on concentrations that are lethal to humans. The results demonstrated that (a) the GC–MS method can detect both nicotine and its metabolite cotinine in immature *C. vomitoria*; (b) the presence of nicotine in the aforementioned three concentrations in food substrates did not modify the developmental time of *C. vomitoria*; (c) during the pupation period, larvae exposed to nicotine died depending on the concentration of nicotine in the substrate; and (d) the resultant lengths of larvae and pupae exposed to 4 and 6 ng/mg concentrations of nicotine were significantly shorter than those of the control.

Keywords: Entomotoxicology; nicotine; GC–MS; *Calliphora vomitoria*

1. Introduction

Entomotoxicology is a scientific term involving the combination of entomology and toxicology. One aspect of entomotoxicology examines the adverse effects of chemicals on living organisms (insects) feeding on the remains of humans and other animals.[1] Toxicological substances (simply referred to as "drugs" in this study) present in remains can also enter necrophagous insects. Many of these drugs affect insects, altering their rate of development and survival.[2] In a forensic context, the identification of drugs in necrophagous insects may help determine the cause of death.[3,4] This is because the common toxicological analyses conducted on decomposed tissues (high decay stage of decomposition or skeletonized remains) were generally less sensitive and yielded almost erroneous results.[2,5–9] As only a modest number of substances and insect species/life instars have been studied so far, reports on analysis of drugs from insects are limited. Moreover, many early studies used analytical procedures that are currently obsolete with little or no validation.[10] While the detection of drugs, metals, pesticides, and alcohol has been reported in entomotoxicological studies, there is no research concerning the detection, analytical quantification, and the effect of nicotine on any necrophagous entomofauna.[10]

Nicotine, 3-(1-methyl-2-pyrrolidinyl)pyridine, is a volatile and water-soluble alkaloid present in the leaves and stems of the plants of *Nicotiana* species (Solanales: Solanaceae), which includes *Nicotiana tabacum* L., the tobacco plant.[11] In such plants, nicotine acts as a botanical insecticide.[12] The tobacco plant, or "holy herb," was first observed by Columbus in the New World, where it was known to exhibit therapeutic properties that can treat a wide range of disorders. The plant was scientifically classified in 1560 in honor of Jean Nicot de Villemain, the French ambassador in Portugal, who introduced tobacco into France and successfully promoted its medicinal use.[13] Although efficacy of tobacco was criticized and people were warned about the negative consequences of tobacco abuse in the 17th century, it has been still suggested for the treatment of several disorders.[13] The outcome of a study of 128 patients treated with tobacco between 1785 and 1860 showed fatal or poisonous *exitus* in only 10% of them.[13,14] In 1851, tobacco became the first vegetable poison ever successfully identified in human tissues: its intake was identified as a contributing factor of death in the investigation of the Bocarmé murder case.[15] Physicians were much aware of using tobacco for medicinal purposes after 1928, when the alkaloid nicotine was isolated from the plant.[13] The therapeutic use of tobacco declined in the 20th and 21st centuries. At present, nicotine is found in tobacco products, such as cigarettes, cigars, pipe, and chewing tobacco, and refill solutions for electronic cigarettes (e-cigarettes). Furthermore, nicotine is present in various formulations of nicotine

replacement therapy (NRT), such as nicotine transdermal patches, nasal sprays, inhalators, gums, sublingual tablets, and lozenges.[12] In some countries, nicotine is used in toothpastes for extra whitening.[16] Moreover, nicotine is used as a synergist in insecticides.[17]

Nicotine acts on brain nicotinic cholinergic receptors to facilitate neurotransmitter release (dopamine and others) and derive pleasure, stimulation, and mood modulation.[18] Many authors have found a positive relationship between tobacco consumption/addiction and suicide.[19] Nicotine is associated with acute toxicity; it is considered one of the most deadly poisons and, at the same time, it can easily come into contact with normal daily life (e.g., buying smoking products).[20] Symptoms of intoxication include parasympathetic as well as sympathetic stimulation, resulting in miosis, diaphoresis, tachypnea with increased secretions, nausea and vomiting, headache, incontinence, tachycardia, paralysis, cardiovascular collapse, and simultaneous respiratory failure.[21] Rapid administration of large doses of nicotine may cause death within a few minutes.[21]

The median lethal doses (LD_{50}) of nicotine are 50 and 3 mg/kg for rats and mice, respectively, whereas a dose of 0.5–1.0 mg/kg can be lethal for humans.[17,22] The fatal dose of nicotine is therefore estimated to be 30–60 mg for adults and 10 mg for children.[23]

The nicotine content of smoking products varies in different countries, over time and between brands. A cigarette typically contains 10–20 mg of nicotine, but only approximately 1–1.5 mg is absorbed during smoking.[24] Many brands of pipe tobacco and cigars contain at least four to six and 10–20 times higher amounts of nicotine, respectively.[25,26] Recently, e-cigarettes have become popular, whose refills contain nicotine concentration of approximately 22 mg/mL.[27]

Nicotine can be readily absorbed by the epithelium of the lung, the nose, skin, and mucosae, regardless of the mode of administration.[28] Therefore, potential poisoning can result from ingestion, injection, inhalation, and absorption of nicotine by skin and rectum.[29] Nonfatal nicotine poisoning sometimes results from accidental intoxication, caused by unorthodox treatments against worms, eczema, and constipation,[30–32] or suicide attempts using insecticides,[21] transdermal nicotine patches,[33] and e-cigarette refills.[25] Most tobacco products contain a considerable amount of nicotine, of which only a small percentage is absorbed by the body during normal activities (e.g., smoking).[24,34] However, standard procedures for the extraction of pure nicotine from smoking tobacco are available on the Internet.[35,36] In addition, the content of e-cigarette refills is potentially lethal for adults and children, if taken other than directed.[27] Furthermore, their pleasant flavors (e.g., cotton candy and bubble gum) could attract children to ingest such solutions.[27] The literature reports a number of accidental/sudden, suicidal, and homicidal cases involving nicotine (alone or mixed with other drugs).[29,36–42]

Nicotine and its metabolites (e.g., cotinine, the major metabolite of nicotine) can accumulate in human hair and nails, and these matrices can be used to assess long-term exposure to nicotine from tobacco products.[43] However, such tissues do not provide information about the possible misuse and/or

overdose of nicotine.[12] In a nicotine overdose situation, the toxicological examinations will be focused on detecting nicotine in the liver, as nicotine metabolites would provide only accessory information.[12,35,44] This study describes the development and validation of a suitable analytical method, based on gas chromatography–mass spectrometry (GC–MS), to detect nicotine in larvae, pupae, empty puparia (EP), and adults of *Calliphora vomitoria*. Furthermore, the effects of nicotine on the larvae of the necrophagous blowfly *C. vomitoria* L. (Diptera: Calliphoridae) were examined when reared on substrates spiked with three concentrations of nicotine, sufficient to cause death in humans. This study also reports the detection of cotinine, but does not include a method for validating the same.

2. Material and Methods

2.1. Preparation of foodstuff and rearing of *C. vomitoria*

C. vomitoria is a common fly species widely distributed in the Holarctic region.[45] It is an early colonizer of carcasses during the cold season, and mainly found in rural areas as the only colonizing species or in association with *Calliphora vicina* Robineau-Desvoidy.[45] Colonies of *C. vomitoria* were reared following the procedures described by Magni *et al.*[46] The flies were caught from the wild around Turin, Italy, identified by the entomologists using the key of Smith[45] and periodically replenished to prevent inbreeding. *C. vomitoria* species used in this experiment were harvested from a third-generation laboratory culture. Flies were provided tap water and sugar *ad libitum*. Small plastic trays containing fresh beef liver on water-moistened paper were placed in the cages to obtain eggs. The liver was checked every 2 h, and following oviposition, four egg clusters containing approximately 1000 eggs (1.2 g) were deposited using a fine paintbrush onto beef liver aliquots (500 g × 4) already spiked and homogenized with different concentrations of nicotine (control (C): 0; T1: 2; T2: 4; and T3: 6 ng/mg). The appropriate nicotine spiking concentrations were selected based on the concentrations that would most likely cause death in humans.[22] Liver was used as the fly food substrate because it is the typical medium for forensic entomology experiments[47,48] as well as it has the highest affinity for nicotine.[12]

Experimental livers were homogenized with increasing volumes (250, 500, and 750 µL) of a 1000 -ng/mg nicotine solution. The hHomogenization was performed using an A11 basic Analytical mMill (IKA®-Werke GmbH & Co.). To disperse the analytical standard, aA T18 digital ULTRA-TURRAX (IKA®-Werke GmbH & Co.) was used to disperse the analytical standard. Each experimental liver was placed on a roundcircular plastic tray (Ø 14 cm with moistened paper on the base to prevent desiccation) with a height sides (of 10 cm) to observe the start of the larvae post-feeding instar. Each plastic tray was placed on top of 5

cm of dry sand (5 cm height) within a larger plastic box (22 x× 40 x× 20 cm), which was covered with a fine mesh cloth and sealed using an elastic band. Sand was used sofor the post-feeding larvae couldto leave the food substrate and pupate. Immature and adult flies were reared at the laboratory temperature of 23°C°C laboratory temperature with approximately 20% relative humidityRH and a photoperiod (h) of 12:12 (L:D). In this study, Ttemperature data in this study were recorded using Tinytag® data- loggers with data being recordedfor every 1 hour.

2.2. Sample collection

Two samples, one consisting of 30 individuals and another amounting to 1 g from each treatment, were collected when *C. vomitoria* reached the second (L2), third (L3), post-feeding (PF), pupal (P), and adult (A) instars. EP were also collected.

Each sample of 30 individuals was used for morphological analyses. The instar and the length of each individual were determined following the standards and guidelines for the best preserving method in forensic entomology.[49] The length of each individual was measured the day after preserving, using a stereomicroscope (Optika SZM-2) equipped with a graduated lens.[50]

Each sample weighing 1 g from each of the instars was stored at −20°C until the end of the sampling period and was analyzed to detect nicotine. Larvae of L2 and L3 instars were sacrificed and stored only after careful cleaning of each individual with water and neutral soap to remove any external contamination. Adults were not provided with food or water and were sacrificed 2 days after their emergence. The analytical method was validated using 100 mg of control EP, chosen as the target matrix because of their high chitin content. EP were chosen as they have longer lifetime, and in such circumstances they may represent the only reliable sample for toxicological analyses.[51]

When the larvae reached the PF instar, 100 individuals from each treatment were placed in separate boxes. The time to pupation and the total number of pupated individuals, as well as the time to eclosion and the total number of emerging adults were recorded.

2.3 Toxicological analysis

Chemicals and reagents – Liquid (-)-nicotine (≥99%) and (-)-cotinine (1000 mg/L in methanol) were purchased from Sigma-Aldrich® and (2,4,5,6)-d_4-nicotine (98%) was purchased from CDN Isotopes®. Standards solutions of (-)-nicotine in CH_3OH (1000, 100, 10, and 1 mg/L) and (2,4,5,6)-d_4-nicotine

(used as the internal standard, ISTD) in CH_3OH (1000, 100, 10, and 1 mg/L) were prepared from the liquid pure standards. Dichloromethane (CH_2Cl_2), methanol, sodium hydroxide (1 M in water), and trichloromethane ($CHCl_3$) were also purchased from Sigma-Aldrich®.

Sample preparation for GC analysis – Larvae (L2, L3, PF), P, EP, and A samples were placed separately in Falcon tubes (50 mL), and dichloromethane was added as part of the preliminary wash. The tubes with larvae and pupae were then placed in a vortex for 2 min and the organic solvent was discarded. Meanwhile, the EP were dried at room temperature under nitrogen. Following crystallization with liquid N_2, they were crushed using a glass rod and 100-mg aliquot was placed in a new tube. In order to validate the method, control *C. vomitoria* EP were spiked with different amounts of nicotine at this stage, by adding different volumes (0, 5, 10, 25, 35, 50, and 60 μL) of methanol solution of (-)-nicotine (10 mg/L). Then, 1 M NaOH was added to reach a final volume of 2 mL and 50 μL of (2,4,5,6)-d_4-nicotine (1 mg/L in CH_3OH) solution was added as the ISTD. The tubes were sealed and placed at room temperature to extract/dissolve the matrix. The digested sample, after elimination of the solid residues, was extracted twice with 1 mL of 1:3 (V/V) methanol:trichloromethane solution and dried at 50°C under nitrogen stream. After drying, the analytes were recovered with 100 μL of methanol and 2 μL of the solution was injected into the GC–MS instrument.

GC–MS – Analytical determinations for the detection of (-)-nicotine and its metabolites (e.g., cotinine) were performed using an Agilent 6890N Network GC System coupled with an Agilent 5973 Inert Mass Spectrometer operating in the electron impact ionization mode. Samples (1 μL) were injected by programmed temperature vaporization (PTV) into an HP-5MS, 30 × 0.25 mm i.d. and 0.25-μm f.t. capillary column. The initial injector temperature was 60°C for 0.10 min, which was increased by 10°C/min for 2.60 min up to 300°C for a total run time of 15 min. The initial oven temperature was maintained at 60°C for 0.4 min, and increased by 20°C/min for 2.60 min up to 300°C for a total run time of 15 min. The carrier gas was ultrapure He (1.0 mL/min; SIAD, Bergamo, Italy). During preliminary GC–MS analyses, full mass spectra were acquired. The background subtracted mass spectrum for (-)-nicotine and (-)-cotinine (using EI in full scan mode) is shown in Figure 1. In order to complete the quantitative analysis, the mass analyzer was operated in the selected ion-monitoring (SIM) mode. The m/z values of the ions selected to identify nicotine were 162, 133, 84, and 42; and that for cotinine were 176, 119, and 98.

Method validation – Nicotine detection method was validated according to ISO/IEC 17025 requirements and ICH guidelines.[52,53] The validation protocol included quantitative determination of

nicotine in larvae, P, and EP: specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), extraction recovery (ER%), repeatability, and carry over were determined.

Specificity – A total of 10 samples of the control EP were used to ascertain specificity of the method, of which five were spiked with 1 mg/L of ISTD. For all ion chromatograms, the specificity test was successful if the S/N ratio was <3 at the expected retention time of the target analytes.

Linearity – The linearity of the calibration model was checked by analyzing control EP samples (100 mg) spiked with nicotine solution at concentrations of 0.5, 1, 2.5, 3.5, 5, and 6 ng/mg, and cotinine solution at concentrations of 1, 2, 3, 4, 5, and 6 ng/mg; (2,4,5,6)-d_4-nicotine with a final concentration of 1 ng/mg was used as the ISTD. The linear calibration parameters were calculated by least-squares regression, and the correlation coefficient (R^2) was used for a rough estimation of linearity. Quantitative results from area counts were corrected using the ISTD signal.

LOD and LOQ – These parameters were calculated for both nicotine and cotinine as the analyte concentrations, whose response provided a signal-to-noise (S/N) ratio of 3 and 10, respectively, as determined from the least abundant qualifier ion. The S/N ratios at the lowest calibration level (LCL) were used to extrapolate the theoretical LOD and LOQ, which had been subsequently verified by blank EP samples spiked at concentrations close to LOD and LOQ.

ER% – ER% was evaluated at two concentrations of nicotine in control EP: 2 and 6 ng/mg. For each of these concentrations, five samples were spiked before the digestion step of the matrix and five after extraction. ER% was calculated by the average ratio of the analyte concentration determined after its extraction (first set) to the one determined on the spiked extract (second set).

Repeatability (intra-assay precision) – Repeatability was calculated as the percent coefficient of variance (CV%) after spiking 10 samples of control EP with two concentrations of nicotine: 2 and 6 ng/mg. Repeatability was considered acceptable when CV% < 20%.

Carry Over – Carry-over effect was evaluated by injecting an alternate sequence of five negative EP samples and five blank EP samples spiked with nicotine at a concentration of 6 ng/mg. S/N ratio < 3 indicates the absence of carry-over effect.

2.4 Statistical analysis

Concentrations of nicotine and cotinine in larvae and pupae as well as their respective lengths in different treatments were analyzed by one-way analysis of variance (ANOVA) and Tukey test. Pupation and eclosion rate were analyzed by one-way ANOVA and Pearson's chi-squared test. Statistical significance was set at $p < 0.05$. Calculations were performed using IBM SPSS Statistics 22 software package.

3. Results

Entomotoxicological analyses by GC–MS confirmed the possibility to detect both nicotine and cotinine in different life instars of *C. vomitoria* reared on food substrates containing different concentrations of nicotine. However, this study is focused on the development of a GC–MS method for the determination of nicotine only, because the parent drug represents the target analyte in the cases of lethal poisoning. The results concerning cotinine are provided as supplementary information.

3.1 Method validation

The following parameters were obtained: coefficient of linearity (R^2), LOD, LOQ, ER%, and repeatability (CV%) (Table 1). Specificity was satisfactory and no carry-over effects were observed.

3.2 Nicotine and cotinine concentration

A summary of concentrations of nicotine and cotinine found in different treatments and instars of C. *vomitoria* is reported in Table 2.

GC–MS analyses confirmed that the nicotine artificially added to food substrates was present in the different instars of *C. vomitoria* as well as in the EP. Nicotine treatments revealed the absence of nicotine (lower than LOD) in L2 and A samples and all control samples. The highest concentration of nicotine was found in the EP of *C. vomitoria* from the T3 treatment, whereas lower concentrations were determined overall from the T1 and T2 treatments. The amount of nicotine found in all treatments and instars was found to be significantly different from that in the controls. Statistical differences were also found between T1, T2, and T3 treatments (Table 2).

Cotinine was observed only in P and EP of C. *vomitoria*, with the highest concentration recorded in EP from T3 treatment. As with nicotine, these cotinine concentrations proved significantly different from the control and between treatments (Table 2).

3.3 Growth rates and survival

The presence of nicotine in food substrates had no significant effect on the development time of flies (Table 3): the time from oviposition to eclosion was similar for control larvae and for larvae feeding on liver containing various concentrations of nicotine (Table 3). On the contrary, nicotine present in food substrates did not affect *C. vomitoria* survival during the early instars of development (until the P instar). Later, the presence and increasing concentration of nicotine (and cotinine) significantly affected the fly survival during metamorphosis (Table 3). Table 3 shows that during the PF instar, only a small number of larvae died before pupation (2/100, 2/100, 3/100, and 5/100 in C, T1, T2, and T3, respectively), while after metamorphosis, lesser flies eclosed in the treatment groups (83/98, 77/97, and 62/95 in T1, T2, and T3, respectively) than in the control (90/98) (Table 3). The survival of the adults reared in T3 was significantly lower than all the other treatments and the control. The survival of the adults reared in T1 was not significantly different from that of the control and T2. The survival of the adults reared in T2 was significantly different from that of the control and T3 treatments.

3.4 Larval and pupal length

Significant differences were observed in the average length of larvae and pupae between control and treatment groups (Table 4). Significant differences occurred in the length of L3, PF, and P instar for all treatments with respect to the control, but not in the length of L2 for all the treatment groups, which were not significantly different from control (Table 4; Fig. 2). No significant differences were observed between the treatment groups.

4. Discussion

Relatively large amounts of nicotine are currently found in smoking products, NRT products, dentifrices, and a few insecticides.[12,16,17] Cases of death by nicotine poisoning, either accidental or intentional, are occasionally reported. The necrophagous insects feeding on highly decomposed remains are likely to be the only reliable resource for conducting toxicological analyses for a fatal event involving missing people.

Entomotoxicology literature reports only a limited number of studies focusing on the search and detection of alkaloids (e.g., amphetamine, cocaine, codeine, mitragynine, methadone, morphine, pholcodine, propoxyphene, and other opiates) in insects (mostly blowflies, but also beetles and their

residuals).[10,54] Unfortunately, majority of them were case studies or simply reports on the extraction of alkaloids from insects (sometimes not identified beyond the Family level). Furthermore, the analytical method of validation and the effects of such drugs on the morphology, development, and survival of the insects are generally omitted.

To the best of the authors' knowledge, this study is the first of its kind to determine the comprehensive effects and residual presence of the alkaloid nicotine in *C. vomitoria* flies reared on liver homogenized with nicotine. The validated GC–MS analytical procedure detected the presence of both nicotine and cotinine in *C. vomitoria* larvae, pupae, and EP. Furthermore, nicotine artificially added to their food substrates produces a significant decrease in the survival of these flies during the period of metamorphosis, from pupa to adult.

Nicotine and cotinine concentration – As stated, no information is available pertaining to the effects of nicotine on blowflies. However, comparisons and analogies can be made with morphine, a more complex alkaloid than nicotine. The toxicological effects on calliphorids reared on morphine showed that (1) larvae grown on meat contaminated with a higher dose of drug contained more drug than those grown on meat with a lower dose[55–58]; (2) larvae fed with a high drug dose contained less drug than that of the preceding instar[55]; (3) the highest percentage (70%) of the drug adsorbed by the feeding instars was incorporated into the cuticle and excreted with the exuviae (puparium), while only a minor percentage (30%) was retained in the tissues of the adult fly and excreted with the meconium (waste products discarded with the first excretion upon emergence of adult age of the fly[59])[51,55] and (4) immunohistochemical studies of *C. vomitoria* larvae reared on food substrates containing morphine showed an intense immunoreaction at the boundary between exocuticle and endocuticle.[60]

The current results on nicotine are similar: (1) A higher spiking dose in the liver resulted in more nicotine detected by GC–MS in L, P, and EP of C. *vomitoria* with the only exception of PF in T2 treatment, where the amount of nicotine detected was lower (and statistically different) than that in the T1 treatment (Table 2); (2) larvae fed on any nicotine dose and their subsequent pupae contained less nicotine than the preceding instar, with the exception of PF in T1 treatment, where the amount of nicotine was higher than L3; (3) the maximum amount of nicotine in any of the treatments was found in the EP, while the amount of nicotine in the adult was found to be lower than the LOD of the method. During pupation, the endocuticle of calliphorids becomes sclerotized, and during the transformation of the hard dark shell of the puparium, it retains the majority of nicotine. This is also evident when larvae are fed with a substrate containing morphine.[51,55] The fact that EP are generally evident around the remains for long time after death underlines the toxicological interest of such samples and the reason why EP were specifically used in this study to validate the analytical method.

The GC–MS method was also capable of detecting a nicotine metabolite cotinine. Similarly, metabolites were identified entomotoxicologically for another alkaloid methadone.[61] In humans, approximately 70–80% of nicotine is converted to cotinine.[12] It is important to note that cotinine has a longer half-life than nicotine in the host body and it is therefore considered a good indicator of smoking and nicotine poisoning.[62] However, in cases of nicotine overdose and consequent death, the metabolic transformations are stopped and the toxicological examinations are concerned mainly on the presence of nicotine.[12]

As stated by Gosselin *et al.,*[61] origin of metabolites cannot be clearly elucidated, as they may result from larvae metabolism or be produced by substrate enzymes. In the case of metabolism of nicotine to cotinine, both hypotheses are conceivable. In both humans and bovines, nicotine is metabolized to cotinine primarily by the liver enzyme P450 2A6 (CYP2A6),[12,63,64] which may have a residual postmortem activity.[65] In this study, the postmortem activity of the beef liver enzymes could be accentuated by liver homogenization, releasing the enzymes. Furthermore, the homogenized liver had never been exposed to denaturing agents (e.g., extreme temperatures, acids, and solvents) that could inactivate its enzymes. In insects, P450 is a well-known enzyme family that performs many important tasks such as synthesis and degradation of ecdysteroids and juvenile hormones and metabolism of xenobiotics.[66] In particular, the P450 enzymes appear fundamental for insects that feed on tobacco plants [*Manduca sexta* (L.)], while in *Musca domestica* L., they are responsible for insecticide resistance.[66]

As Kharbouche *et al.*[67] stated, a better understanding of drug metabolism in blowflies facilitates the interpretation of toxicological results. In this study, an appreciable concentration of cotinine was present only in P and EP samples, which is attributed to the different chemical structure of cotinine or different kinetics/excretion rate of the metabolite with respect to nicotine. Alternatively, the presence of cotinine may be due to its longer half-life or the increase of its concentration during the postmortem period, caused by the residual activity of the liver enzymes.

Effects of nicotine and cotinine on growth rate and survival of flies – Growth rate of *C. vomitoria* is unaffected by the presence of nicotine in the food substrate. Similar results were obtained on *Calliphora stygia* (Fabricius) reared on substrates containing morphine.[55,68] Accordingly, several authors note that insects may be capable of excreting drugs efficiently, which allows them to maintain their concentration at levels lower than their food, and grow and survive despite the presence of drugs.[55,58,67,69] Malpighian tubules are considered the place where the physiological mechanism of excretion takes place. Observations on *Drosophila melanogaster* Meigen suggest that the rate of secretion of a drug by Malpighian tubules increases when the insect feeds on a substrate containing that drug.[55,60] Furthermore, the rate of excretion of a drug is related to its chemical structure and

pharmacological properties.[67]

Survival data show an interesting result, that is, the survival of C. *vomitoria* during metamorphosis decreases with the increasing dose of nicotine in the food substrate. This effect may be attributed to the ingestion of nicotine during the feeding period, which is not surprising, as nicotine is a natural insecticide.[12] However, GC–MS results clearly show that the concentration of cotinine was higher than nicotine in P samples. Such a high concentration of cotinine, rather than nicotine, may be considered the real cause of death of a large percentage of P during metamorphosis.

Effects of nicotine and cotinine on larval and pupal lengths – Bourel *et al.,*[70] Kharbouche *et al.,*[67] and Rashid *et al.*[54] analyzed the length of immature calliphorids reared on food substrates containing morphine, codeine, and ketum extract. Results of their studies showed significant differences in the length of blowflies reared on drug-positive substrates compared with the control. In agreement with these results, this study shows that larvae (L3 and PF) and P of C. *vomitoria* reared on a substrate containing nicotine are significantly shorter in length than the control. In particular, the results of both the experiments on codeine and nicotine show an "all or nothing effect" on the length of the immatures: the presence of the drugs in any of the treatments has similar effects compared with the control, and this effect is not subjected to the amount of nicotine in the food substrate, but only to its presence.[67] As a consequence, as well as stated for other alkaloids, when nicotine is present in the food substrate, caution must be taken in the estimation of the age of immatures based on their length.

5. Conclusions

Smoking habits and products containing nicotine are common in society, and hence intoxication and toxicity caused by nicotine could be missed by pathologists, particularly when remains are highly decomposed, skeletonized, or without additional clues left by the deceased.[36] However, nicotine-containing products are easily available and highly toxic to living beings; therefore, the possibility of nicotine overdose, accidental or intentional, should not be ignored.[35] Murder in Three Acts by Agatha Christie and Behold, Here's Poison by Georgette Heyer[71] are some of the examples found in the literature concerning murders by nicotine poisoning.

This study validates a GC–MS method to detect the presence of human lethal doses of nicotine in blowflies. It also shows that *C. vomitoria* immatures accumulate both nicotine and its metabolite cotinine and that the length and survival of *C. vomitoria* feeding on nicotine-containing liver can be significantly affected by the presence of the drug. Interestingly, although the effect on survival is dose dependent, that on length is not. Furthermore, C. *vomitoria* growth rate is not affected by the presence

of nicotine in the food substrate.

This research underlines the need of further studies concerning nicotine and its effects on blowflies in topics such as: (a) how does the chronic use of smoking products by people who have committed suicide affect blowfly development and could this affect the estimate of the minimum time since death[19]; (b) the effects of higher nicotine doses on blowflies: LD_{50} of nicotine is higher in other animals than humans; (c) how nicotine mixed with other drugs affects blowflies[39–41]; (d) the presence of nicotine in blowfly meconium; and (e) nicotine metabolites and their effects on blowflies.

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References

[1] Byrd JH, Peace MR. Entomotoxicology: Drugs, Toxins, and Insects. In: Kobilinsky L, editor. Forensic Chemistry Handbook 2011. p. 483-99.

[2] Carvahlo LML. Toxicology and forensic entomology In: Amendt J, Campobasso CP, Goff ML, Grassberger M, editors. Current Concepts in Forensic Entomology: Springer Science; 2010. p. 163–78.

[3] Magni P, Massimelli M, Messina R, Mazzucco P, Di Luise E. Entomologia Forense. Gli insetti nelle indagini giudiziarie e medico-legali: Ed. Minerva Medica; 2008.

[4] Haskell NH, Williams RE. Entomology & Death: A Procedural Guide. 2 ed: Forensic Entomology Partners, Clemenson, SC, USA; 2009.

[5] Kintz P, Godelar B, Tracqui A, Mangin P, Lugnier AA, Chaumont AJ. Fly larvae: a new toxicological method of investigation in forensic medicine. J Forensic Sci 1990;35:204–7.

[6] Kintz P, Tracqui A, Mangin P. Toxicology and fly larvae on a putrefied cadaver. J Forensic Sci Soc 1990;30:243–6.

[7] Nolte KB, Pinder RD, Lord WD. Insect larvae used to detect cocaine poisoning in a decomposed body. J Forensic Sci 1992;37:1179–85.

[8] Amendt J, Richards CS, Campobasso CP, Zehner R, Hall MJ. Forensic entomology: applications and limitations. Forensic Sci Med Pathol 2011;7:379-92.

[9] Introna F, Campobasso C, Goff M. Entomotoxicology. Forensic Sci Int 2001;120:42-7.

[10] Gosselin M, Wille SM, Fernandez Mdel M, Di Fazio V, Samyn N, De Boeck G, Bourel B. Entomotoxicology, experimental set-up and interpretation for forensic toxicologists. Forensic Sci Int 2011;208:1-9.

[11] Schep LJ, Slaughter RJ, Beasley DM. Nicotinic plant poisoning. Clin Toxicol 2009;47:771-81.

[12] Benowitz NL, Hukkanen J, Jacob P. Nicotine chemistry, metabolism, kinetics and biomarkers. Handbook of Experimental Pharmacology 2009:29-60.

[13] Charlton A. Medicinal uses of tobacco in history. J R Soc Med. 2004;97:292-6.

[14] Stewart GG. A history of the medicinal use of tobacco 1492-1860. Med Hist. 1967;11:228-68.

[15] Wennig R. Back to the roots of modern analytical toxicology: Jean Servais Stas and the Bocarme murder case. Drug testing and analysis. 2009;1:153-5.

[16] Agrawal SS, Ray RS. Nicotine contents in some commonly used toothpastes and toothpowders: a present scenario. J Toxicol. 2012;2012:1-11.

[17] Hayes WJ. Pesticides studied in man. Baltimore: Lippincott Williams & Wilkins; 1982.

[18] Benowitz NL. Neurobiology of nicotine addiction: implications for smoking cessation treatment. Am J Med 2008;121:S3-10.

[19] Hughes JR. Smoking and suicide: a brief overview. Drug Alcohol Depend. 2008;98:169-78.

[20] Faulkner JM. Nicotine poisoning by absorption through the skin. J Am Med Assoc. 1933;100:1664-5.

[21] Rogers AJ, Denk LD, Wax PM. Catastrophic brain injury after nicotine insecticide ingestion. J Emerg Med 2004;26:169-72.

[22] Mayer B. How much nicotine kills a human? Tracing back the generally accepted lethal dose to dubious self-experiments in the nineteenth century. Arch Tox 2014;88:5-7.

[23] Etter JF, Bullen C, Flouris AD, Laugesen M, Eissenberg T. Electronic nicotine delivery systems: a research agenda. Tobacco Control 2011;20:243-8.

[24] Benowitz NL, Jacob Pr. Daily intake of nicotine during cigarette smoking. Clin Pharmacol Ther 1984;35:499-504.

[25] Cervellin G, Luci M, Bellini C, Lippi G. Bad news about an old poison. A case of nicotine poisoning due to both ingestion and injection of the content of an electronic cigarette refill. Emerg Care J 2013;9:18.

[26] Taghavi S, Khashyarmanesh Z, Moalemzadeh-Haghighi H, Nassirli H, Eshraghi P, Jalali N, et al. Nicotine content of domestic cigarettes, imported cigarettes and pipe tobacco in Iran. Addict & Health 2012;41:28-35.

[27] Cameron JM, Howell DN, White JR, Andrenyak DM, Layton ME, Roll JM. Variable and potentially fatal amounts of nicotine in e-cigarette nicotine solutions. Tobacco Control 2014;23:77-8.

[28] Nicotine. US Department of Health and Human Services The Health Consequences of Smoking—50 Years of Progress: A Report of the Surgeon General. Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health; 2014. p. 109-38.

[29] McNally WD. A report of seven cases of nicotine poisoning. J Lab Clin Med. 1922;8:83-5.

[30] Davies P, Levy S, Pahari A, Martinez D. Acute nicotine poisoning associated with a traditional remedy for eczema. Arch Dis Child. 2001;85:500-2.

[31] Oberst BB, Mcintyre RA. Acute nicotine poisoning; case report. Pediatrics. 1953;11:338-40.

[32] Garcia-Estrada H, Fischman C. An unusual case of nicotine poisoning. Clin Toxicol. 1977;10:391-3.

[33] Montalto N, Brackett CC, Sobol T. Use of transdermal nicotine systems in a possible suicide attempt. J Am Board Fam Pract. 1994;7:417-20.

[34] Armitage AK, Dollery CT, Houseman TH, Kohner EM, Lewis PJ, Turner DM. Absorption of nicotine by man during cigar smoking. Br J Pharmacol 1977;59:493P.

[35] Schneider S, Diederich N, Appenzeller B, Schartz A, Lorang C, Wennig R. Internet suicide guidelines: report of a life-threatening poisoning using tobacco extract. J Emerg Med 2010;38:610-3.

[36] Corkery JM, Button J, Vento AE, Schifano F. Two UK suicides using nicotine extracted from tobacco employing instructions available on the Internet. Forensic Sci Int 2010;199:e9-13.

[37] Dillon N. California nuclear power plant engineer convicted of killing wife with nicotine. NY Daily News2014.

[38] McNally WD. A report of five cases of poisoning by nicotine. J Lab Clin Med. 1919;5:213-7.

[39] Chaturvedi AK, Rao NGS, McCoy FE. A multi-chemical death involving caffeine, nicotine and malathion. Forensic Sci Int 1983;23:265-75.

[40] Moriya F, Hashimoto Y. A fatal poisoning caused by methomyl and nicotine. Forensic Sci Int 2005;149:167-70.

[41] Solarino B, Riesselmann B, Buschmann CT, Tsokos M. Multidrug poisoning involving nicotine and tramadol. Forensic Sci Int 2010;194:e17-9.

[42] Lardi C, Vogt S, Pollak S, Thierauf A. Complex suicide with homemade nicotine patches. Forensic Sci Int 2014;236:e14-8.

[43] Crooks PA, Dwoskin LP. Contribution of CNS nicotine metabolites to the neuropharmacological effects of nicotine and tobacco smoking. Biochem Pharmacol. 1997;54:743-53.

[44] Kemp P, Sneed GS, George CE, Distefano RF. Postmortem distribution of nicotine and cotinine from a case involving the simultaneous administration of multiple nicotine transdermal system. J Anal Toxicol. 1997;21:310-3.

[45] Smith KGV. A Manual of Forensic Entomology. London: Trustees of the British Museum, Natural History and Cornell University Press; 1986.

[46] Magni PA, Pacini T, Pazzi M, Vincenti M, Dadour IR. Development of a GC-MS method for methamphetamine detection in Calliphora vomitoria L. (Diptera: Calliphoridae). Forensic Sci Int 2014;241:96-101.

[47] Donovan S, Hall M, Turner B, Moncrieff C. Larval growth rates of the blowfly, *Calliphora vicina*, over a range of temperatures. Med Vet Entomol. 2006;20:106-14.

[48] Anderson G. Minimum and maximum development rates of some forensically important Calliphoridae (Diptera). J Forensic Sci. 2000;45:824-32.

[49] Amendt J, Campobasso CP, Gaudry E, Reiter C, LeBlanc HN, Hall M. Best practice in forensic entomology. Standards and guideline. Int J Legal Med. 2007;121:104-9.

[50] Richards CS, Rowlinson CC, Hall MJR. Effects of storage temperature on the change in size of *Calliphora vicina* larvae during preservation in 80% ethanol. Int J. Leg Med 2013;127:231-41.

[51] Bourel B, Tournel G, Hedouin V, Deveaux M, Goff M, Gosset D. Morphine extraction in necrophagous insects remains for determining ante-mortem opiate intoxification. Forensic Sci Int 2001;120:127-31.

[52] AA.VV. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Validation of analytical procedures: text and methodology Q2 (R1). 2005.

[53] AA.VV. ISO/IEC 17025:2005. General requirements for the competence of testing and calibration laboratories. 2 ed: International Organization for Standardization; 2005.

[54] Rashid AR, Siti AS, Siti FR, Reena AR, Sharifah HSS, Nurul FZ, et al. Forensic implications of blowfly *Chrysomya rufifacies* (Calliphoridae: Diptera) development rates affected by ketum extract. IJIRSE. 2013;7:70-4.

[55] Parry S, Linton SM, Francis PS, O'Donnell MJ, Toop T. Accumulation and excretion of morphine by *Calliphora stygia*, an Australian blow fly species of forensic importance. J Insect Physiol 2011;57:62-73.

[56] Hedouin V, Bourel B, Martin-Bouyer L, Becart A, Tournel G, Deveaux M, Gosset D. Determination of drug levels in larvae of *Lucilia sericata* (Diptera: Calliphoridae) reared on rabbit carcasses containing morphine. J Forensic Sci. 1999;44:351-3.

[57] Gunn J, Shelley C, Lewis S, Toop T, Archer M. The determination of morphine in the larvae of *Calliphora stygia* using flow injection analysis and HPLC with chemiluminescence with detection. J Anal Toxicol. 2006;30:519-23.

[58] Hedouin V, Bourel B, Becart A, Tournel G, Deveaux M, Goff ML, Gosset D. Determination of drug levels in larvae of *Protophormia terraenovae* and *Calliphora vicina* (Diptera: Calliphoridae) reared on rabbit carcasses containing morphine. J Forensic Sci. 2001;46:12-4.

[59] Stocker H, Gallant P. Getting started: an overview on raising and handling Drosophila. Methods Mol Biol 2008;420:27-44.

[60] Bourel B, Fleurisse L, Hedouin V, Cailliez J-C, Creusy C, Gosset D, Goff ML. Immunohistochemical contribution to the study of morphine metabolism in Calliphoridae larvae and implications in forensic entomotoxicology. J Forensic Sci. 2001;46:596-9.

[61] Gosselin M, Ramirez-Fernandez MDM, Wille SMR, Samyn N, Boeck GD, Bourel B. Quantification of methadone and its metabolite 2-ethylidene-1, 5-di-methyl-3,3-diphenylpyrrolidine in third instar larvae of *Lucilia sericata* (Diptera: Calliphoridae) using Liquid Chromatography–Tandem Mass Spectrometry. J Anal Toxicol 2010;34.

[62] Moriya F, Hashimoto Y. Nicotine and cotinine levels in blood and urine from forensic autopsy cases. Leg Med (Tokyo). 2004;6:164-9.

[63] Bao Z, He XY, Ding X, Prabhu S, Hong JY. Metabolism of nicotine and cotinine by human cytochrome P450 2A13. Drug Metab Dispos 2005;33:258-61.

582 [64] Hirano Y, Mizutani T. Study of inhibition of CYP2A6 by some drugs derived from quinoline. J
583 Pharm Pharmacol 2003;55:1667-72.

584 [65] Kozik MB. Postmortem activity of respiratory enzymes in human brain. Acta Histochem.
585 1981;68:79-90.

586 [66] Feyereisen R. Insect P450 enzymes. Annu Rev Entomol. 1999:507-33.

587 [67] Kharbouche H, Augsburger M, Cherix D, Sporkert F, Giroud C, Wyss C, et al. Codeine
588 accumulation and elimination in larvae, pupae, and imago of the blowfly *Lucilia sericata* and effects on
589 its development. Int J Leg Med 2008;122:205-2011.

590 [68] George K, Archer M, Green L, Conlan X, Toop T. Effect of morphine on the growth rate of
591 *Calliphora stygia* (Fabricius) (Diptera: Calliphoridae) and possible implications for forensic
592 entomology. Forensic Sci Int. 2009.

593 [69] Campobasso C, Gherardi M, Caligara M, Sironi L, Introna R. Drug analysis in blowfly larvae and
594 in human tissues: a comparative study. Int J Legal Med. 2004;118:210-4.

595 [70] Bourel B, Hédouin V, Martin-Bouyer L, Bécart A, Tournel G, Deveaux M, et al. Effects of
596 morphine in decomposing bodies on the development of *Lucilia sericata* (Diptera: Calliphoridae). J
597 Forensic Sci 1999;44:354–8.

598 [71] Bardell EB. Literary reflections of pharmacy-XII: the toxicity of nicotine. Pharm Hist.
599 1989;31:126-8.

600

600 Table 1

601

602 Parameters calculated for nicotine and cotinine. NC = not calculated.

603

Parameter	Value	
	Nicotine	Cotinine
Coefficient of linearity, R^2	0.9954	0.9930
Limit of detection, LOD (ng/mg)	0.13	0.38
Limit of quantification, LOQ (ng/mg)	0.43	1.2
Extraction recovery at 2-ng/mg concentration (%)	71.11	NC
Extraction recovery at 6-ng/mg concentration (%)	69.23	NC
CV % at 2-ng/mg concentration	14.65	NC
CV % at 6-ng/mg concentration	15.80	NC

604

605

Table 2

Nicotine and cotinine quantification (ng/mg ± S.E.) in *C. vomitoria* (L2 = second instar, L3 = third instar, PF = post-feeding instar, P = pupa instar, EP = empty puparium, A = adult instar) through GC–MS analysis. Quantification was calculated in triplicates. Nicotine LOD = 0.13 ng/mg; Cotinine LOD = 0.38 ng/mg. The groups indicated in brackets (i.e., C, T1, T2, and T3) are the ones whose results proved significantly different ($p < 0.05$) from the group indicated in the corresponding column.

Treatment		Control (C)		T1		T2		T3	
Amount of nicotine spiked with liver		0 ng/mg		2 ng/mg		4 ng/mg		6 ng/mg	
Quantification (ng/mg ± S.E.)		Nicotine	Cotinine	Nicotine	Cotinine	Nicotine	Cotinine	Nicotine	Cotinine
Life instar	L2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	L3	<LOD	<LOD	0.77 ± 0.12 (C, T3)	<LOD	1.53 ± 0.23 (C, T3)	<LOD	3.08 ± 0.46 (C, T1,T2)	<LOD
	PF	<LOD	<LOD	0.98 ± 0.15 (C, T2)	<LOD	0.53 ± 0.08 (C, T1, T3)	<LOD	1.06 ± 0.16 (C, T2)	<LOD
	P	<LOD	<LOD	0.39 ± 0.06 (C, T3)	<LOD (T2, T3)	0.50 ± 0.08 (C, T3)	1.11 ± 0.17 (C, T1, T3)	0.89 ± 0.13 (C, T1,T2)	1.84 ± 0.25 (C, T1, T2)
	EP	<LOD	<LOD	0.82 ± 0.07 (C, T3)	0.90 ± 0.01 (C, T3)	1.82 ± 0.27 (C, T3)	1.41 ± 0.12 (C, T3)	3.29 ± 0.46 (C, T1, T2)	2.78 ± 0.29 (CT, T1, T2)
	A	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

Table 3

Time (hour mean ± S.E.) from oviposition to pupation and to eclosion of *C. vomitoria* larvae, which were exposed to either liver containing different amounts of nicotine or the control liver. The number of larvae died before pupation, the number of nonemerged adults, and the number of survivals are also reported. The groups indicated in brackets (i.e., C, T1, T2, and T3) are the ones whose results proved significantly different ($p < 0.05$) from the group indicated in the corresponding column.

Treatment	Control (C)	T1	T2	T3
Amount of nicotine spiked with liver (ng/mg)	0	2	4	6
Larvae third instar N=	100	100	100	100
Time (h) from oviposition to pupation	163.82 ± 1.01	164.52 ± 1.21	164.62 ± 1.30	163.98 ± 1.31
Larvae died before pupation	2	2	3	5
Pupae	98	98	97	95
Pupae %	98	98	97	95
Pupae N=	98	98	97	95
Time (h) from oviposition to eclosion	468.92 ± 1.25	470.92 ± 1.05	470.04 ± 1.24	469.08 ± 1.51
Nonemerged adults	8	15	20	33
Survival	90 (T2, T3)	83 (T3)	77 (C, T3)	62 (C, T1, T2)
Survival %	92	84.70	79.38	65.26

627
628 Table 4

629

630 Mean lengths (mm ± S.E.) of *C. vomitoria* larvae and pupae related to time of exposure (h) and instar
631 of life (L2 = second instar, L3 = third instar, PF = post-feeding instar, P = pupa instar). The notation C
632 indicates significant difference from the control group ($p < 0.05$). For each time of exposure and each
633 treatment, $N = 30$.

634

C. vomitoria mean length (mm ± S.E.)					
Treatment		Control (C)	T1	T2	T3
Amount of nicotine spiked with liver (ng/mg)		0	2	4	6
Hours of exposure (Instar)	60 (L2)	5.30 ± 0.18	5.32 ± 0.18	5.32 ± 0.18	5.32 ± 0.18
	120 (L3)	18.68 ± 0.33	12.45 ± 0.45 (C)	11.24 ± 0.54 (C)	12.72 ± 0.53 (C)
	168 (PF)	17.62 ± 0.29	14.57 ± 0.52 (C)	12.97 ± 0.54 (C)	13.27 ± 0.63 (C)
	216 (P)	10.07 ± 0.12	8.42 ± 0.31 (C)	8.14 ± 0.33 (C)	7.93 ± 0.35 (C)

635
636
637

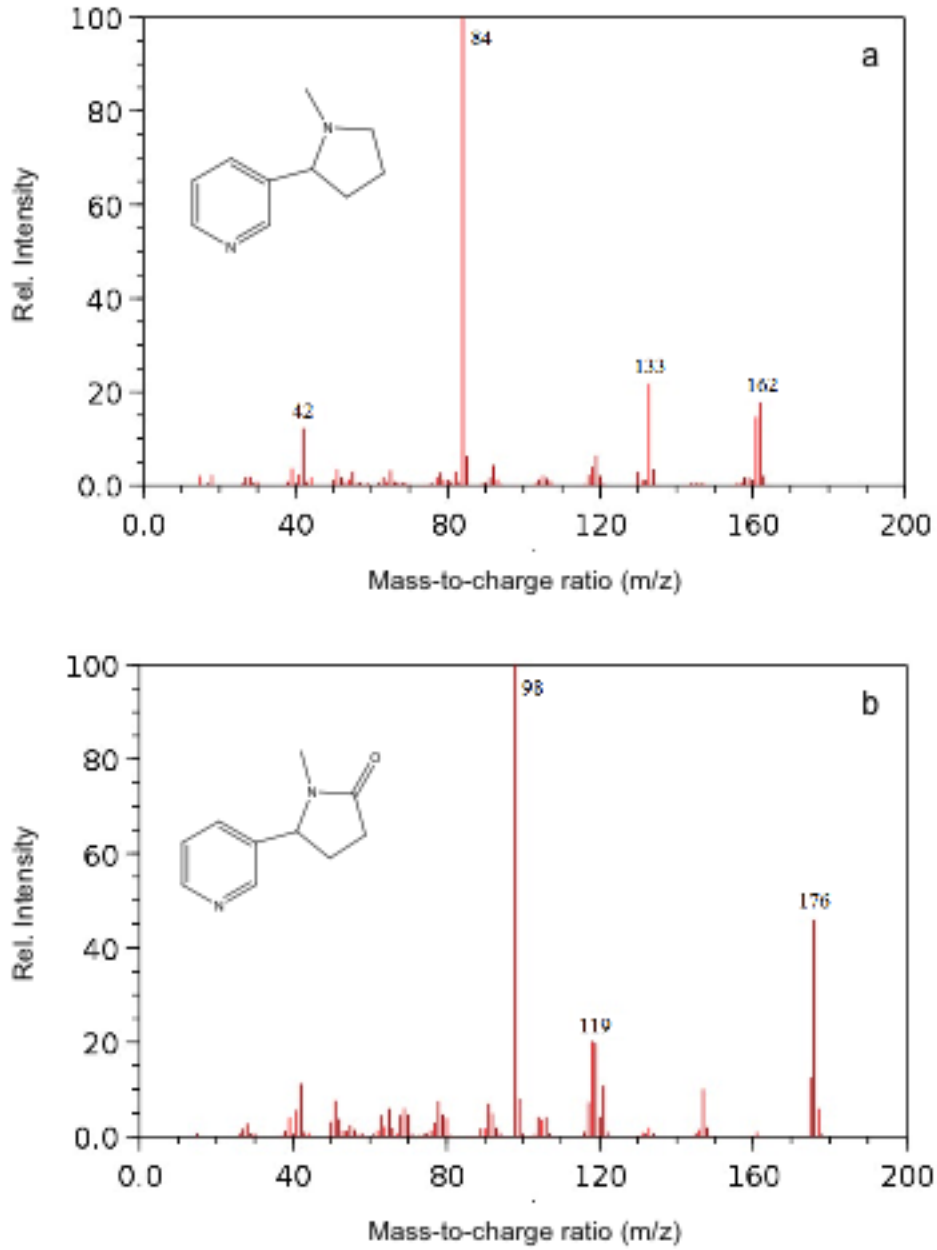

Fig. 1. Background subtracted mass spectrum of nicotine (a) and cotinine (b) obtained with electronic impact (EI) ionization. The mass-to-charge ratios (m/z) for nicotine and cotinine are 162 and 176, respectively.

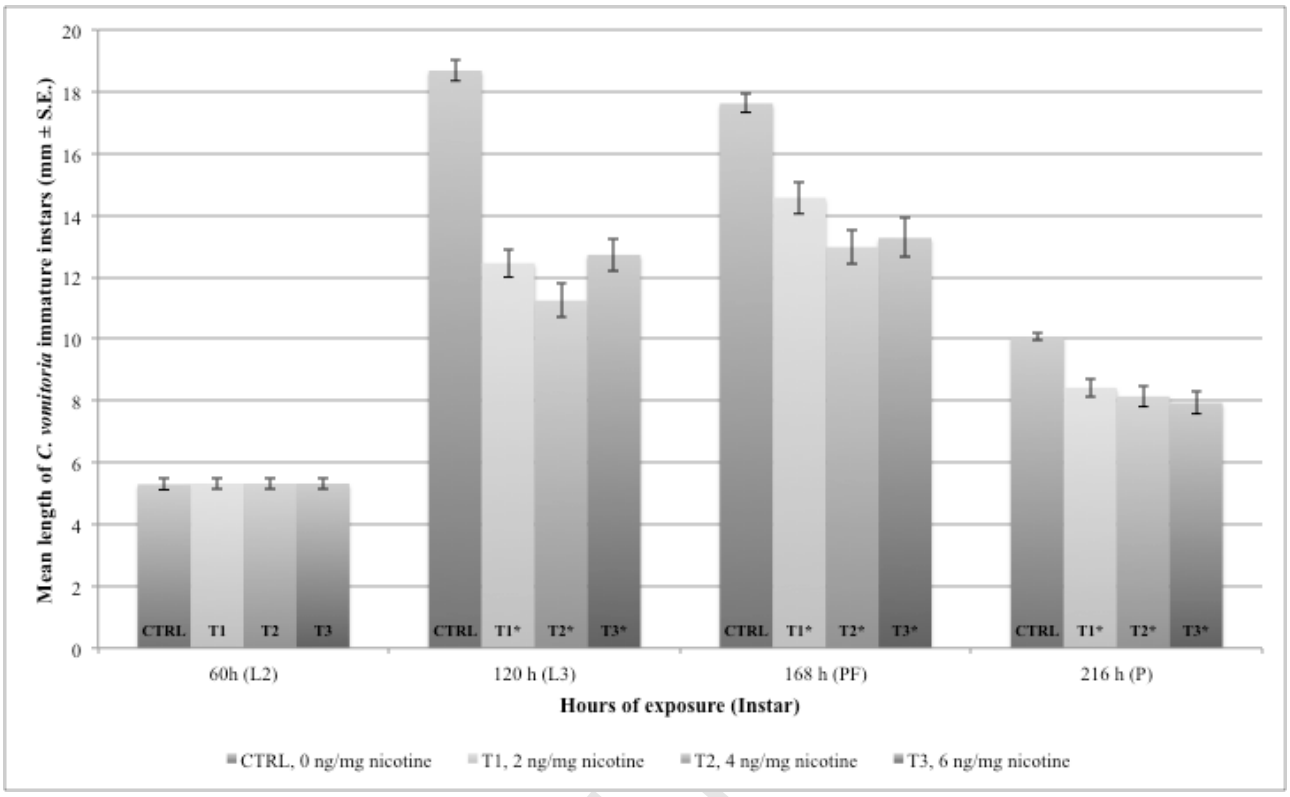

Fig. 2. Mean length (mm ± S.E.) of *C. vomitoria* immature instars according to treatment type, time of exposure, and developmental instar. (*) indicates significant difference compared with the control group ($p < 0.05$).