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

Paola A. Magni [a,*], Marco Pazzi [b], Marco Vincenti [b], Eugenio Alladio [b], Marco Brandimarte [b], Ian R. Dadour [c]

[a] Department of Molecular and Biomedical Sciences, School of Veterinary and Life Sciences, Murdoch University, Perth, Australia
[b] Dipartimento di Chimica, Università di Torino, Via Pietro Giuria 7, Torino 10125, Italy
[c] Program in Forensic Anthropology, Department of Anatomy & Neurobiology, Boston University School of Medicine, 72 East Concord St (L 1004) , Boston, MA 02118, USA

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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.

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 the efficacy of tobacco

* Corresponding author. Tel.: +61 8 9360 7563.
E-mail addresses: p.magni@murdoch.edu.au (P.A. Magni), marco.pazzi@unito.it (M. Pazzi), marco.vincenti@unito.it (M. Vincenti), eugenio.alladio@unito.it (E. Alladio), brandimarte.marco@yahoo.it (M. Brandimarte), idadour@bu.edu (I.R. Dadour).p.magni@murdoch.edu.au

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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 this drug.

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 ng/mg; treatment 1, T1: 2 ng/mg; T2: 4 ng/mg; 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 homogenization was performed using an A11 basic Analytical Mill (IKA®-Werke GmbH & Co.). A T18 digital ULTRA-TURRAX (IKA®-Werke GmbH & Co.) was used to disperse the analytical standard. Each experimental liver was placed on a circular plastic tray (Ø 14 cm with moistened paper on the base to prevent desiccation) with a height of 10 cm to observe the start of the larvae post-feeding instar. Each plastic tray was placed on dry sand (5 cm height) within a larger plastic box ($22 \times 40 \times 20$ cm^3), which was covered with a fine mesh cloth and sealed using an elastic band. Sand was used for the post-feeding larvae to leave the food substrate and pupate. Immature and adult flies were reared at the laboratory temperature of 23 °C with approximately 20% relative humidity and a photoperiod (h) of 12:12 (L:D). In this study, temperature data were recorded every hour using using Tinytag data loggers.