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(Article begins on next page)

**Development and validation of an HPLC-MS/MS method for  
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(Diptera: Calliphoridae)**

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1 **Abstract**

2

3 Entomotoxicology studies the detection of drugs or other toxic substances from insects developing  
4 on the decomposing tissues. Entomotoxicology also investigates the effects of these substances  
5 on insect development, survival and morphology to provide an estimation of the minimum time  
6 since death. Ketamine is a medication mainly used for starting and maintaining anesthesia.  
7 Ketamine is also used as a recreational drug and as a sedating drug to facilitate sexual assault,  
8 resulting in several deaths. Furthermore, ketamine has been also implicated in suspicious deaths  
9 of animals. The present study describes for the first time the development and validation of an  
10 analytical method suited to detect ketamine in *Calliphora vomitoria* L. (Diptera: Calliphoridae),  
11 using liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). This study also  
12 considers the effects of ketamine on the survival, developmental rate and morphology of *C.*  
13 *vomitoria* immatures. Larvae were reared on substrates homogeneously spiked with ketamine  
14 concentrations consistent with those found in humans after recreational use (300 ng/mg) or  
15 allegedly indicated as capable of causing death in either humans or animals (600 ng/mg). The  
16 results demonstrated that (a) HPLC-MS/MS method is applicable to ketamine detection in *C.*  
17 *vomitoria* immatures, not adults; (b) the presence of ketamine at either concentration in the food  
18 substrate significantly delays the developmental time to pupal and adult instar; (d) the survival of  
19 *C. vomitoria* is negatively affected by the presence of ketamine in the substrate; (e) the length and  
20 width of larvae and pupae exposed to either ketamine concentration were significantly larger than  
21 the control samples.

22

23 **Keywords:** Entomotoxicology, ketamine, HPLC-MS/MS, blowflies

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## 29 1. Introduction

30

31 In the process of an investigation regarding a suspicious death, toxicological analysis sometimes  
32 plays a pivotal role in identifying the presence of chemical substances that may have caused death  
33 directly (e.g. overdose) or indirectly (e.g. altering the state of awareness) (1). Historically, blood  
34 and urine represent the most commonly used biological matrices for the identification of the  
35 alleged intoxicating substances (simply referred to as “drugs” in this study) in both the living and  
36 the dead. However, over the last few years forensic toxicologists have focused their attention on  
37 the use of non-conventional biological matrices, with the aim of making the sampling less invasive  
38 and more readily available, (2). The criteria used for the selection of non-conventional matrices  
39 must be correlated with the aim of the investigation, the ease of sampling, the cost of the analyses,  
40 the reliability and reproducibility of the results, and the overall analytical complexity (2). Among the  
41 non-conventional biological matrices, the majority of studies have focused on keratine (hairs and  
42 nails), sweat, saliva, amniotic fluid and meconium (2).

43 The insects found on a highly decayed or skeletonized corpse can also be included as a non-  
44 conventional matrix, useful in the identification of drugs, metals, pesticides and poisons. The  
45 discipline of *entomototoxicology* involves the combination of entomology and toxicology by  
46 considering both the presence of the toxic substances in the insects that colonized the remains  
47 and their effects on the insects’ survival and development rate (3). In a forensic context, especially  
48 when the toxicological analyses have to be conducted on highly decomposed tissues, it has been  
49 demonstrated that the use of necrophagous insects provides higher sensitivity and better results  
50 compared to decomposed tissues (4, 5). Furthermore, by studying the effects of the drugs on the  
51 insects it is possible to apply appropriate correction factors to pre-existing tables of growth  
52 concerning the insects’ morphology or survival rate, and obtain a more focused estimation of the  
53 minimum time since death (minPMI) (6). Overall, entomotoxicological studies may provide  
54 information regarding both the cause and the time of death.

55 Ketamine, 2-(2-chlorophenyl)-2-(methylamino)-cyclohexanone, is an arylcycloalkylamine  
56 structurally related to phencyclidine (PCP) and cyclohexamine. It was synthesized in 1962,

57 patented in 1963, and tested on human prisoners in 1964, with the outcome of it being a more  
58 favourable choice over PCP as a dissociative anaesthetic. After its approval in 1970, it was  
59 administered as an anaesthetic to American soldiers during the Vietnam War (7-9). At present,  
60 ketamine is a medication with unique therapeutic value in veterinary medicine, mainly used for  
61 inducing and maintaining anaesthesia (known under the name of Ketalar, Ketaminol Vet.,  
62 Clorketam, Imalgene, Anesketin, Ketamine Ceva, Vetalar Vet., Narketan, Ketaset), and, to a lesser  
63 extent, it is used in human medicine especially in paediatric surgery (Ketalar, Ketamine  
64 Panpharma, Ketolar, Ketanest-S) (10). Nonmedical use of ketamine began in the 1970s, but it was  
65 not until 1999 that ketamine was introduced into the U.S. Food and Drug Administration register  
66 (11). Known also with the street name Special K, K, ket, kitkat, super k, horse trunk, tac et tic, cat  
67 Valium, and vitamin K, ketamine is illegally used for its hallucinogenic effects, that cause the user  
68 to see, hear, smell, feel, and taste non-existing entities different from reality (12). Ketamine also  
69 shows dissociative effects, causing a feeling of disconnection between the mind and the body in  
70 the user ('out-of-body experience') (10). The literature reports a number of accidental/sudden  
71 death cases in which ketamine was used, alone or in combination with other drugs, e.g. cocaine,  
72 amphetamine, cannabis, or alcohol (10, 13). Ketamine has also been used in a number of drug-  
73 facilitated sexual assaults and was implicated in several deaths globally (14). To note, ketamine  
74 has also been associated with the suspicious deaths of animals (e.g. sedation with a wrong dose  
75 of the drug) and in cases of animal cruelty (15).

76 Within the entomotoxicological literature (16), only two studies have addressed the effects of  
77 ketamine on blowflies (17, 18). Lü *et al.* (17) investigated the effects of ketamine on *Chrysomya*  
78 *megacephala*'s (Fabricius) (Diptera: Calliphoridae) larval lengths, weights, and the developmental  
79 duration of larval instar, but no analytical method was developed to identify the presence of  
80 ketamine in the insects. Zou *et al.* (18) detected the presence of ketamine in *Lucilia sericata*  
81 (Meigen) (Diptera: Calliphoridae) by gas chromatography-mass spectrometry (GC-MS), and they  
82 also observed the effects of ketamine on the development and morphology of this fly. However, the  
83 analytical method proposed by Zou *et al.* (18) did not take into account all the standard parameters  
84 suggested by the international scientific standards for validation.

85 The present study describes the development and validation of an analytical method based on  
86 liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), suitable to detect ketamine in  
87 immatures of *Calliphora vomitoria* L. (Diptera: Calliphoridae). Furthermore, the effects of the  
88 presence of ketamine were examined on the developmental time, the morphology (length and  
89 width) and survival of *C. vomitoria* immatures, reared on a substrate spiked with the drug.

90

## 91 **2. Material and Methods**

92

### 93 **2.1. Preparation of foodstuff and rearing of *C. vomitoria***

94 Calliphorids (Diptera: Calliphoridae) are blowflies widely distributed in the different continents.  
95 Many species known to be early coloniser of dead bodies, and therefore they are used in forensic  
96 entomology for the estimation of the minPMI (20). *C. vomitoria* is distributed throughout the  
97 Holarctic region and it is mainly present in rural areas during the cold season (21-23). This fly was  
98 chosen for this study, as it is one of the most common species found in cases of forensic relevance  
99 (24).

100 Colonies of *C. vomitoria* were reared following the procedures described by Magni *et al.* (6, 25),  
101 starting from wild flies caught in several rural areas of the north west of Italy. Wild flies were  
102 identified by a taxonomist and regularly added to colonies to prevent inbreeding (21). As in  
103 previous research, *C. vomitoria* used in this experiment were harvested from a third generation  
104 laboratory culture. Adults were provided with distilled water and sugar *ad libitum* for their  
105 sustenance (from eclosion to the end of the experiment), while beef liver was provided as a  
106 medium for the development of oocytes (introduced on day 5 after eclosion and left 48 hours in the  
107 cage) and to obtain eggs (introduced on day 12 after eclosion) (25). The liver was checked every 2  
108 hours and following oviposition, 3 egg clusters containing approximately 1000 eggs (1.2 g) were  
109 deposited with a fine paintbrush onto beef liver aliquots (500 g x 3) already spiked with ketamine at  
110 variable concentration levels and homogenised (control 0 ng/mg, 300 ng/mg, 600 ng/mg – simply  
111 referred as C, T1, T2 respectively). The amounts of ketamine chosen to spike the substrate were  
112 based on the concentrations found in humans after recreational use (300 ng/mg) or that which has

113 been indicated as capable of causing death in either adult humans ( $\approx 80$  Kg) or animals of the  
114 same size (600 ng/mg) (13, 19). Liver was used as the fly food substrate because (a) it is the  
115 typical medium for forensic entomology experiments (26, 27); (b) it was used in previous research  
116 on ketamine and blowflies (18); (c) it is one of the tissues in which ketamine distributes first and its  
117 metabolic evolution starts (28). Experimental livers were homogenized with increasing volumes (0,  
118 12.5, 25, 37.5, 50, 75, and 100  $\mu$ L) of methanol solution of ketamine (1 mg/L) to reach the final  
119 concentration. Homogenization was performed using a A11 basic Analytical mill (IKA®-Werke  
120 GmbH & Co.). Following laboratory standards, a T18 digital ULTRA-TURRAX (IKA®-Werke GmbH  
121 & Co.) was used, to obtain a uniform distribution of the analytical standard. Each experimental liver  
122 was placed on a round plastic tray ( $\varnothing$  14 cm with moistened paper on the base to prevent  
123 desiccation) with high sides (10 cm) to observe the start of the larvae post-feeding instar. Each  
124 plastic tray was placed on top of 5 cm of dry sand within a larger plastic box (22x40x20 cm) which  
125 was covered with a fine mesh cloth and sealed using an elastic band. Sand was used to facilitate  
126 pupation. Immature and adult flies were reared at  $23.3 \pm 1.2^\circ\text{C}$  laboratory temperature with  
127 approximately 20% RH and a photoperiod (h) of 12:12 (L:D). Temperature data in this study were  
128 recorded using Tinytag® data-loggers with data being recorded every hour.

129

## 130 **2.2. Sample collection**

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

134 Each sample of 30 individuals was used for morphological analyses. Specimens were sacrificed by  
135 immersion in hot water ( $>80^\circ\text{C}$ ) for 30 seconds and preserved in 70% ethanol (29). Following  
136 preservation, larvae and pupae were measured with a digital calliper (Terminator®) under a  
137 stereomicroscope (Optika SZM-2). As described by Day and Wallman (30) the length of each larva  
138 was measured between the most distal parts of the head and the eighth abdominal segment, while  
139 the width of each larva was measured between the ventral and dorsal surfaces at the junction of

140 the fifth and sixth abdominal segments. Regarding the pupa, the length was measured between  
141 the most distal parts, while the width was measured in the largest part of the pupal case.

142 Each sample weighing 1 g from each of the instars was stored at -20°C until the sampling period  
143 finished and then they were analysed to detect ketamine. Larvae of L2 and L3 instars were  
144 sacrificed and stored only after careful cleaning of each individual with water and neutral soap to  
145 remove any external contamination. Adults were not provided with any food or water source and  
146 were sacrificed 2 days after their emergence. The analytical method was validated using 50 mg of  
147 control EP, chosen as the target matrix because of their high chitin content. Empty puparia were  
148 also chosen because they can be found at the scene for a much longer period after emergence,  
149 and in such circumstances they may represent the only reliable sample for toxicological analyses  
150 (31).

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

154

### 155 **2.3 Toxicological analysis**

156

157 **Chemicals and reagents** – Liquid ketamine ( $\geq 99\%$ ) and d4-ketamine 100  $\mu\text{g}/\text{mL}$  in methanol (as  
158 free base) ampule of 1 mL, certified reference material Cerilliant® were purchased from Sigma  
159 Aldrich® (Milano, Italy). Standard solutions of ketamine in  $\text{CH}_3\text{OH}$  (0.5 mg/L, 1 mg/L, 10 mg/L, 100  
160 mg/L, 1000 mg/L) and d4-ketamine (used as the internal standard, ISTD) in  $\text{CH}_3\text{OH}$  (10 mg/L and  
161 1 mg/L) were prepared from the pure liquid standards. Dichloromethane ( $\text{CH}_2\text{Cl}_2$ ), methanol,  
162 trifluoroacetic acid were also purchased from Sigma Aldrich® (Milano, Italy).

163

164 **Sample preparation HPLC-MS/MS analysis** – Larvae (L2, L3, PF), P, EP and A samples were  
165 placed separately in falcon tubes (50 mL) and dichloromethane was added as part of the  
166 preliminary wash. The tubes with larvae and pupae were then placed in a vortex for two minutes  
167 and the organic solvent was discarded. Meanwhile, the EP were dried at room temperature under



168 nitrogen. Following crystallisation using liquid N<sub>2</sub>, they were crushed with a glass rod and a 50-mg  
169 aliquot was placed in a new tube. To validate the method, control *C. vomitoria* EP were spiked with  
170 different amounts of ketamine at this stage, by adding different volumes (0, 12.5, 25, 37.5, 50,  
171 75, and 100 µL) of methanol solution of ketamine (1 mg/L). In addition, 2 ml of CH<sub>3</sub>OH was added  
172 and 10 mL of d4-ketamine (10 mg/L in CH<sub>3</sub>OH) solution was added as the ISTD. The tubes were  
173 sealed and placed in heating-blocks at 60°C to extract/dissolve the matrix, for 4 hours. After  
174 elimination of the solid residues, at the digest sample was added trifluoroacetic acid (30µL) then  
175 the sample was dried at 70°C under nitrogen stream. After drying, the analytes were recovered  
176 with 200 µL of methanol. 10 µL of the solution was injected into the HPLC-MS/MS instrument.

177

178 **HPLC-MS/MS analysis** – Analytical determinations for the detection of ketamine was performed  
179 with LC Varian 920 coupled with Varian 320 MS operating in the electrospray ionization mode.  
180 Samples (10 µL) were injected into a Luna C18, 150mm x 2mm x 3 µm, with C18 precolumn filter  
181 (Security Guard, Phenomenex Inc., Torrance, CA-US). Elution mixture was composed by 87%  
182 formic acid 0.1% and 13% acetonitrile 0.1%. Temperature of drying gas was 200°C and  
183 nebulization temperature was 55°C, electron multiplier potential was 1500V. In order to complete  
184 the quantitative analysis, the mass analyzer was operated in Multiple Reaction Monitoring (MRM)  
185 and transition followed to identify ketamine were reported in Table 1.

186

187 **Method validation** – Ketamine detection method was validated according to the guidelines of  
188 Raposo (32), the ISO/IEC 17025 requirements and ICH guidelines (33, 34). The validation protocol  
189 included quantitative determination of ketamine in larvae, P, EP and adults: specificity, linearity,  
190 back calculation, limit of detection (LOD), limit of quantitation (LOQ), extraction recovery (ER%),  
191 repeatability, matrix effect and carry over were determined.

192

193 **Specificity** – Ten samples of the control EP were used to ascertain the method's specificity. Five  
194 of them were spiked with 1 mg/L of ISTD. The specificity test was successful if the S/N ratio was  
195 lower than 3 at the expected retention time of the target analytes, for all ion chromatograms.

196

197 **Linearity** – The linearity of the calibration model was checked by analyzing control EP samples  
198 (50 mg x 5 repetitions for each calibration point) spiked with ketamine solution at concentrations of  
199 0, 0.5, 0.75, 1, 1.5 and 2 ng/mg. d4-ketamine with a final concentration of 10 ng/mg was used as  
200 the ISTD. The linear calibration parameters were calculated by least-squares regression, and the  
201 correlation coefficient ( $R^2$ ) was used for a rough estimation of the linearity. For determination of  
202 linearity were considered Mandel and Olivieri's principles (35, 36). Another parameter used to  
203 evaluate linearity was back calculation, which, from calibration curve point, calculates backwards  
204 the concentration of ketamine in sample starting from the instrumental signal. Back calculation is  
205 useful to evaluate calibration curve goodness. Quantitative results from area counts were  
206 corrected using the ISTD signal.

207

208 **Limit of detection and limit of quantitation (LOD and LOQ)** – LOD and LOQ were calculated  
209 according to Hubaux and Vos (37). This method is based on calibration curve so the result is more  
210 relevant and sturdy to the method that has been developed than standard calculation of LOD and  
211 LOQ.

212

213 **Extraction recovery (ER%)** – ER% was evaluated at two concentrations of ketamine in control EP:  
214 0.75 and 2 ng/mg. For each of these concentrations, five samples were spiked before the digestion  
215 step of the matrix and five after the extraction. ER% was calculated by the average ratio of the  
216 analyte concentration determined after its extraction (first set) to the one determined on the spiked  
217 extract (second set).

218

219 **Repeatability (intra-assay precision)** – Repeatability was calculated as the percent coefficient of  
220 variance (CV%) after spiking ten samples of control EP with two concentrations of ketamine: 0.75  
221 and 2 ng/mg. Repeatability was considered acceptable when CV% <20%.

222

223 **Carry Over** – Carry-over effect was evaluated by injecting an alternate sequence of ten blank EP  
224 samples spiked with ketamine at concentration of 0.5 ng/mg and ten blank EP samples spiked with  
225 ketamine at a concentration of 2 ng/mg. To ensure the absence of any carry-over effect, for each  
226 transition, the signal-to-noise ratio (S/N) from negative samples had to be lower than 3.

227

228 **Matrix-effect** – Matrix effect was evaluated following the Matuszewski's criteria (38) analysing five  
229 EP samples (chitinic matrix) spiked with ketamine at concentration of 0.25 ng/mg and five samples  
230 at 2 ng/mg both five sample of cheratin matrix at the same concentration.

231

## 232 **2.4 Statistical analysis**

233

234 Ketamine concentration in larvae, pupae and adults as well as their respective lengths and widths  
235 in different treatments were analysed by one-way ANOVA and Tukey test. Pupation and eclosion  
236 rate were analysed by a one-way ANOVA and Pearson's Chi-squared test. The level of  
237 significance was set at  $P < 0.05$ . Calculations were performed using IBM SPSS Statistics 22  
238 statistical software package.

239

## 240 **3. Results**

241 Entomotoxicological analyses by HPLC-MS/MS confirmed the possibility that ketamine can be  
242 detected in different instars of *C. vomitoria* reared on food substrates containing ketamine in  
243 concentrations of 300 ng/mg and 600 ng/mg.

### 244 **3.1 Method validation**

245

246 The following parameters were obtained: coefficient of linearity ( $R^2$ ), limit of detection (LOD), limit  
247 of quantitation (LOQ), extraction recovery (%), and repeatability (CV%) (Table 2). Specificity was  
248 satisfactory, while no matrix effects and carry over effects were observed.

249

### 250 3.2 Ketamine concentration

251

252 A summary of the ketamine concentration found in the different treatments and instars of *C.*  
253 *vomitorea* detected by HPLC-MS/MS is reported in Table 3.

254 HPLC-MS/MS analyses confirmed that the ketamine artificially added to the food substrate was  
255 present in the different immature instars of *C. vomitoria* as well as in the EP. The ketamine  
256 concentration was not found to be present in *C. vomitoria* adults analysed by HPLC-MS/MS.

257 The ketamine concentration was absent (lower than the LOD) in all the control samples, in the L2  
258 of both the treatments and in all the A samples analysed by HPLC-MS/MS.

259 The peak of ketamine concentration was found in the L3 of both treatments and analytical methods.

260 Overall, ketamine shows an increase in concentration until the larvae reach L3, then a decrease in  
261 the following larval instars and an increase in the P and EP. The amount of ketamine found in all  
262 treatments and instars was found to be significantly different from the controls. Statistical relevant  
263 differences were also found between T1 and T2 treatments (Table 3).

264

### 265 3.3 Growth rates and survival

266

267 The presence of ketamine in the food substrate had significant effects on fly development time,  
268 especially in the time from oviposition to eclosion (Table 3). The time from oviposition to pupation  
269 was similar for control larvae and for T1 larvae, but it was significantly different between control  
270 larvae and T2 larvae, that needed approximately one day more to complete pupation. The time  
271 from oviposition to eclosion was significantly different between control and larvae feeding on liver  
272 containing the two concentrations of ketamine (1-2 days more to complete metamorphosis). The  
273 difference between the treatments was not significant for either the time from oviposition to  
274 pupation and oviposition to eclosion (Table 4).

275 Ketamine present in the food substrate significantly affected *C. vomitoria* survival during the early  
276 instars of development (until the P instar), but it was only during metamorphosis that the effects of  
277 the presence of ketamine were extreme. Table 4 shows that during the PF instar only a maximum

278 of 15% of larvae died prior to pupation (2% in C; 10% in T1; 15% in T2), while during  
279 metamorphosis survival was 85% in C, 37% in T1 and 9% in T2. The survival of pupae was  
280 significantly different only between the control and both the treatments, while the survival of the  
281 adults was significantly different between all treatments.

282

### 283 **3.4 Larval and pupal length**

284

285 Significant differences were observed in the average length of larvae and pupae between the  
286 control and treatment groups (Table 5). However, significant differences occurred only in the length  
287 of advanced L3 for T2 treatment with respect to the control and T1, and in the length of P for T2  
288 treatment with respect to the control. The length of L2, early L3 and PF of all the treatment groups  
289 were not significantly different from control (Table 5).

290

### 291 **3.4 Larval and pupal width**

292

293 Significant differences were observed in the average width of larvae and pupae between control  
294 and treatment groups (Table 6). The width of control larvae and pupae was significantly smaller  
295 than T2 individuals during the whole cycle of life. Larvae of T1 were found to have a larger width  
296 with respect to the control only in the advanced L3 stage, while during the PF instars were  
297 significantly smaller in width with respect to the T3 individuals.

298

## 299 **4. Discussion**

300 The use of ketamine in a medical and veterinary setting has been shown to be efficient and safe.  
301 However, in the recent past the abuse of ketamine has caused severe harm to individuals (39). A  
302 2006 US report shows that approximately 2.3 million teens and adults have used ketamine in their  
303 lifetime (40). Ketamine it is extremely popular amongst drug users at parties all over the world and  
304 in the last 10 years the number of ketamine-related deaths have significantly increased (41). There  
305 have been major concerns in regards to driving under the influence of ketamine and the use of this

306 drug to facilitate sexual assault (39).

307 The entomotoxicology literature reports only two studies which focused on the presence of  
308 ketamine in the food substrate and its effects on blowfly development (17, 18). One study (17)  
309 considers colonies of *Ch. megacephala*, a blowfly occurring in Australasia, South Africa, Southern  
310 United States and South America (20), reared on food substrates spiked with different  
311 concentrations of ketamine. The aim of this research was to determine the effects of ketamine on  
312 blowfly development when reared at different temperatures (17). The other study (18) considers  
313 colonies of the cosmopolitan necrophagous blowfly *L. sericata*, reared on the tissue of rabbits  
314 killed following different doses of ketamine. The aims of this study were the detection of ketamine  
315 in larvae by GC-MS and the observation of the effects of ketamine on the larval morphology and  
316 development of *L. sericata* (18). The current research is the first comprehensive study regarding  
317 the effects of ketamine on *C. vomitoria* flies reared on liver homogenised with two concentrations  
318 of ketamine. The validated HPLC-MS/MS analytical procedure detected the presence of ketamine  
319 in *C. vomitoria* larvae, pupae and empty puparia. Furthermore, ketamine artificially added to the fly  
320 food substrate produces a significant increase in larval and pupal size (length and width), a  
321 significant increase in the time required to complete development and a significant decrease in the  
322 survival of this fly species especially during the period of metamorphosis.

323

324 **Ketamine concentration** – As stated, at present only two studies pertain to the effects of  
325 ketamine on blowflies. However, comparisons and analogies regarding the concentration of the  
326 drug in the flies can be made only with the research of Zou (18), since the other published  
327 research (17, 42) lacks any toxicological analyses of the flies reared on the food substrate spiked  
328 with ketamine.

329 In the research of Zou *et al.* (18) ketamine was identified by GC-MS in *L. sericata* immatures  
330 (larvae only) when reared on rabbits killed after receiving an intravenous injection of ketamine at  
331 different concentrations (1/4LD50, 1/2LD50, LD50, 2LD50). Rabbit liver and muscle containing  
332 different amounts of ketamine were used as food to rear the fly colonies. Results show that  
333 ketamine concentrations were more consistent (higher in concentration and present in several

334 immature instars) in treatments that had liver as food rather than muscle tissue. This is the most  
335 parsimonious explanation because (a) following GC-MS analyses of both the organs, ketamine  
336 was found to have a higher concentration in liver than in muscles and (b) liver is the organ in which  
337 ketamine metabolism occurs (28). To note, the analytical method used in the research of Zou *et al.*  
338 (18) was validated only for linearity and only 10 larvae at the different instars were used for the  
339 toxicological analysis. As well this sample consisted of 10 larvae aging from 12 to 120 hours (from  
340 L2 instar to P instar) which is not consistent in terms of analytical weight and drug content. In order  
341 to obtain reliable results, the same amount of sample should be used at the same life stage  
342 throughout the experiment.

343 In the present research, ketamine was identified by HPLC-MS/MS and the analytical method was  
344 validated following a set of international standards (33, 34). During the study 1 g of insect material  
345 at each instar was used as a sample for the toxicological analyses. Ketamine was detected by  
346 HPLC-MS/MS in all immature instars and pupal remains of *C. vomitoria*, Negative results in *C.*  
347 *vomitoria* adults were surprising, because it is known that upon emergence as an adult, the flies  
348 rapidly eliminate the drug introduced with the diet during the immature life stages (42, 43). Lastly,  
349 accordingly, to Zou *et al.* (18) ketamine was present in higher concentrations in larvae of the  
350 treatments with higher concentration of ketamine and no metabolites of ketamine were detected.

351

352 **Effects of ketamine on fly growth rate and survival** – *C. vomitoria* growth rate is affected by the  
353 presence of ketamine in the food substrate. In the treatment with recreational-use concentration  
354 (T1) only the time of metamorphosis was affected by the presence of the drug, while in the higher  
355 dose treatment both the period needed to reach the pupation and metamorphosis were affected.  
356 These results are in agreement with the findings regarding the effects of different ketamine  
357 concentrations on *Ch. megacephala* (17). However, they are in contrast with findings regarding *L.*  
358 *sericata* reared on different ketamine concentrations that showed a delay in the early development,  
359 but an overall reduction of the time needed to reach the pupal stage (18). Furthermore,  
360 *Parasarcophaga ruficornis* (Fabricius) (Diptera: Sarcophagidae) reared on different concentrations  
361 of PCP, another dissociative drug similar to ketamine, showed no significant difference in the larval

362 growth when comparing control vs treatment groups (42).

363 When considering survival data the only available information regarding ketamine and blowflies  
364 demonstrates that by increasing the ketamine dosage in the food substrate the survival of *C.*  
365 *vomitioria* will decrease, especially during the period of metamorphosis. A similar trend was  
366 observed in *P. ruficornis* reared on different concentrations of PCP (42).

367 All previous research (17, 18, 42) underlines how similar drugs can play a role in the physiology of  
368 different fly species, but before such assertions, the limitations of these studies regarding the lack  
369 of repetition needs to be addressed

370

371 **Effects of ketamine on larval and pupal length and width** – Lü *et al.* (17) analysed the length of  
372 *Ch. megacephala* reared on food substrates containing ketamine in doses associated with  
373 1/2LD50, LD50, 2LD50 for an adult male of approximately 70 kg. It is important to note that larval  
374 samples were sacrificed with a 50:50 v/v blend of ethanol and xylene and preserved in 75%  
375 alcohol (17). This preservative method makes the estimation of real length difficult to compare due  
376 to larval shrinkage. It is not the method recommended as a standard of best practice in forensic  
377 entomology (44). Regardless of the preservation method used by Lü *et al.* (17), this research  
378 showed that the relative average length of *Ch. megacephala* larvae in all the treatment colonies  
379 was significant less than the control for larvae between 16 to 64 hours (= until the L3). However,  
380 since the overall duration of the PF instar of the treatments was longer compared with the control,  
381 the PF *Ch. megacephala* larvae in all the treatment colonies were significantly larger in length  
382 compared to the control. These results, however, are not absolute measures and cannot be  
383 compared with this study (47).

384 In the present research as well as in the research of Zou *et al.* (18) fly immatures are preserved  
385 according to the standards and guidelines for forensic entomology, by sacrificing specimens in hot  
386 water and preserving them in 70% ethanol (44). Similarly, the two studies show that larvae reared  
387 on substrates enriched with ketamine are significantly longer in length when compared to the  
388 respective controls (18).

389 In the present research the width of larvae and pupae was also considered. The length of fly larvae



390 is often used to help provide an entomological estimate of the minPMI, but the curved shape of the  
391 larvae can affect the accuracy of length measurements. The width is not affected by the curved  
392 shape of the larvae, it has been demonstrated to be comparable with body length for larval age  
393 prediction (30) and it has been used in previous entomotoxicology research (48). Despite the width  
394 measurement not often being used to measure larvae size, this data was considered in the present  
395 research as it provided a comparison with the control treatment. Statistical results on *C. vomitoria*  
396 showed that larvae and pupae reared on substrates enriched with ketamine are significantly larger  
397 in width compared to the control. As a consequence, when ketamine is present in the food  
398 substrate both width and length can be taken to estimate the age of immatures with larval width  
399 being a more accurate.

400

## 401 **5. Conclusions**

402 Although ketamine is important in medical and veterinary practice, it is also illegally used by  
403 humans to hallucinate and to facilitate sexual assaults. It has been a drug of choice found amongst  
404 some high profile investigations, e.g. the death of the world famous singer Amy Winehouse (2011).  
405 This research validates an analytical method based on HPLC-MS/MS to detect the presence of  
406 human recreational and lethal doses of ketamine in blowflies.

407 This research shows that *C. vomitoria* immature and adults accumulate ketamine and that the  
408 development and survival of *C. vomitoria* feeding on liver containing ketamine can be significantly  
409 affected by the presence of the drug.

410 This research underlines the need of further entomotoxicology studies, such as: a) effects of  
411 ketamine on different fly species reared at different temperature; b) the effects of “ketamine  
412 cocktails” on blowflies; c) the effects of ketamine on subsequent generations; d) the validation of  
413 alternative analytical methods (e.g. GC-MS) with the aim of allowing laboratories in possession of  
414 other analytical techniques to benefit from this type of research.

415

416

417

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528

529 **Table 1**

530 Triple quadruple monitored transitions and applied collision energy.

531

<b>Substance</b>	<b>Precursor Ion</b>	<b>Fragment Ion</b>	<b>Collision Energy (V)</b>	<b>Use of transition</b>
Ketamine	238	125	20	Quantitation
	238	179	13	Identification
	238	207	10	Identification
	238	220	10	Identification
Ketamine-d4	242	129	7.5	Identification
	242	211	23.5	Identification
	242	224	10	Quantitation

532

533

534 **Table 2**

535 Parameters calculated for ketamine using HPLC-MS/MS.

536

537

Parameter	Value
	HPLC-MS/MS
Coefficient of linearity, $R^2$	0.99677
Limit of detection (ng/mg), LOD	0.015
Limit of quantitation (ng/mg), LOQ	0.031
Extraction recovery (%) at 0.75 ng/mg concentration	99.4
Extraction recovery (%) at 2 ng/mg concentration	100
CV % at 0.75 ng/mg concentration	14
CV % at 2 ng/mg concentration	16

538

539

540



541 **Table 3**

542 Ketamine quantitation (ng/mg  $\pm$  S.E.) in *C. vomitoria* (L2=second instar, L3=third instar, PF=post-  
 543 feeding instar, P=pupa instar, EP=empty puparium, A=adult instar) detected through HPLC-MS/MS  
 544 analysis. Quantitation was calculated using 3 replicates. Ketamine LOD<sub>HPLC-MS/MS</sub>=0.015 ng/mg and  
 545 LOQ<sub>HPLC-MS/MS</sub>=0.031 ng/mg. The groups indicated in brackets (i.e. C, T1, T2) were significantly  
 546 different ( $P<0.05$ ) from the group indicated in the corresponding column.

547

Treatment		Control (C)	T1	T2
Amount of ketamine spiked with liver		0 ng/mg	300 ng/mg	600 ng/mg
Sampling day (Life instar)	Day 3 (L2)	<LOD	<LOD	<LOD
	Day 4 (L3)	<LOD (T1, T2)	14.9 $\pm$ 0.03 (C, T2)	180.0 $\pm$ 0.28 (C, T1)
	Day 5 (L3)	<LOD (T1, T2)	7.40 $\pm$ 0.03 (C, T2)	16.9 $\pm$ 0.24 (C, T1)
	Day 7 (PF)	<LOD (T1, T2)	0.15 $\pm$ 0.02 (C, T2)	0.97 $\pm$ 0.19 (C, T1)
	Day 9 (PF)	<LOD (T1, T2)	0.05 $\pm$ 0.02 (C, T2)	0.35 $\pm$ 0.23 (C, T1)
	Day 11 (P)	<LOD (T1, T2)	0.20 $\pm$ 0.28 (C, T2)	0.81 $\pm$ 0.11 (C, T1)
	EP	<LOD (T1, T2)	1.00 $\pm$ 0.08 (C, T2)	2.06 $\pm$ 0.21 (C, T1)
	A	<LOD	< LOD	< LOD

548

549

550

551 **Table 4**

552 Time (days  $\pm$  S.E.) from oviposition to pupation and to eclosion of *C. vomitoria* larvae, which were  
 553 exposed to either liver containing different amount of ketamine, or to the control liver. The table  
 554 shows also the number of larvae dead prior to pupation, the number of not emerged adults, and  
 555 the number of survivals. The groups indicated in brackets (i.e. C, T1, T2) were significantly  
 556 different ( $P < 0.05$ ) from the group indicated in the corresponding column.

557

Treatment	Control (C)	T1	T2
Amount of ketamine spiked with liver	0 ng/mg	300 ng/mg	600 ng/mg
Larvae third instar N=	100	100	100
Time (days) from oviposition to pupation	9.89 $\pm$ 0.13 (T2)	10.30 $\pm$ 0.10	10.46 $\pm$ 0.09 (C)
Larvae dead prior to pupation	2	10	15
Pupae	98 (T1, T2)	90 (C)	85 (C)
Pupae %	98%	90%	85%
Pupae N=	98	90	85
Time (days) from oviposition to eclosion	18.40 $\pm$ 0.10 (T1, T2)	19.27 $\pm$ 0.18 (C)	20.00 $\pm$ 0.10 (C)
Not emerged adults	17	57	77
Survival	81 (T2, T3)	33 (C, T2)	8 (C, T1)
Survival %	83%	37%	9%

558

559

560

561 **Table 5**

562 *C. vomitoria* larvae and pupae mean lengths (mm  $\pm$  S.E.) related to instar of life (L2=second instar,  
 563 L3=third instar, PF=post-feeding instar, P=pupa instar). The groups indicated in brackets (i.e. C, T1,  
 564 T2) were significantly different ( $P<0.05$ ) from the group indicated in the corresponding column. For  
 565 each time of exposure and each treatment  $N=30$ .

566

<i>C. vomitoria</i> means length (mm $\pm$ S.E.)				
Treatment		Control (C)	T1	T2
Amount of ketamine spiked with liver		0 ng/mg	300 ng/mg	600 ng/mg
Sampling day (Life instar)	Day 3 (L2)	6.56 $\pm$ 0.25	6.79 $\pm$ 0.21	7.19 $\pm$ 0.16
	Day 5 (L3)	16.87 $\pm$ 0.15	16.54 $\pm$ 0.25	17.20 $\pm$ 0.29
	Day 6 (L3)	16.79 $\pm$ 0.26 (T2)	17.25 $\pm$ 0.27 (T2)	18.50 $\pm$ 0.23 (C, T2)
	Day 7 (PF)	11.91 $\pm$ 0.24	12.59 $\pm$ 0.31	12.45 $\pm$ 0.28
	Day 8 (PF)	11.56 $\pm$ 0.21	11.57 $\pm$ 0.13	11.69 $\pm$ 0.28
	Day 11 (P)	9.27 $\pm$ 0.11 (T2)	9.49 $\pm$ 0.09	9.82 $\pm$ 0.12 (C)

567

568

569

570 **Table 6**

571 *C. vomitoria* larvae and pupae mean widths (mm  $\pm$  S.E.) related to instar of life (L2=second instar,  
 572 L3=third instar, PF=post-feeding instar, P=pupa instar). The groups indicated in brackets (i.e. C, T1,  
 573 T2) were significantly different ( $P<0.05$ ) from the group indicated in the corresponding column. For  
 574 each time of exposure and each treatment N=30.

575

<i>C. vomitoria</i> mean width (mm $\pm$ S.E.)				
Treatment		Control (C)	T1	T2
Amount of ketamine spiked with liver		0 ng/mg	300 ng/mg	600 ng/mg
Sampling day (Life instar)	Day 3 (L2)	0.89 $\pm$ 0.04 (T2)	1.03 $\pm$ 0.02 (C)	1.05 $\pm$ 0.03 (C)
	Day 5 (L3)	2.31 $\pm$ 0.50	2.30 $\pm$ 0.06	2.37 $\pm$ 0.07
	Day 6 (L3)	2.33 $\pm$ 0.07 (T1, T2)	2.70 $\pm$ 0.06 (C)	2.86 $\pm$ 0.05 (C)
	Day 7 (PF)	2.51 $\pm$ 0.07 (T2)	2.39 $\pm$ 0.06 (T2)	2.92 $\pm$ 0.04 (C, T1)
	Day 8 (PF)	2.26 $\pm$ 0.04 (T2)	2.39 $\pm$ 0.05 (T2)	2.70 $\pm$ 0.05 (C, T1)
	Day 11 (P)	2.88 $\pm$ 0.05 (T2)	3.08 $\pm$ 0.04	3.10 $\pm$ 0.06 (C)

576

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578