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**Development and Validation of a Method for the Detection of  $\alpha$ - and  $\beta$ -Endosulfan (Organochlorine Insecticide) in *Calliphora vomitoria* (Diptera: Calliphoridae)**

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## 1 **1. Introduction**

2

3 Forensic entomology deals with the use of insects and other arthropods in criminal investigations  
4 involving humans and animals (alive or dead), food, products, and in human related environments  
5 (Byrd and Castner 2010a). A forensic entomology examination may be useful to estimate the  
6 minimum Post Mortem Interval (minPMI), the circumstances surrounding a death, and to assess  
7 cases of cruelty or neglect against humans or animals (Byrd and Castner 2010a). A peculiar  
8 feature of forensic entomology is its multidisciplinary status which involves many branches of the  
9 natural sciences such as molecular biology, chemistry, microbiology, physics, and limnology  
10 (Amendt et al. 2010, Byrd and Castner 2010a). "Entomotoxicology", relates to the application of  
11 chemistry and toxicology to insects feeding on remains. These insects may be used to detect  
12 drugs or other toxic substances when extended decomposition processes limit or invalidate the  
13 typical toxicological analyses usually performed on fluids, muscles and internal organs of creature  
14 cadaver. In such circumstances, insects are likely to provide more sensitive and unbiased results,  
15 making entomotoxicology generally accepted as an essential tool of investigation when suspicious  
16 deaths involve drugs or poisons (Beyer et al. 1980, Goff and Lord 1994). Besides, the toxicological  
17 analyses on insects feeding on a dead body may provide considerable information about the  
18 minPMI estimation. In fact, the presence of drugs may severely affect the life cycle and survival of  
19 insects, and invalidate the typical life tables for their growth (Introna et al. 2001, Magni et al. 2014,  
20 Magni et al. 2016b).

21 Since 1980 many drugs have been detected in insect tissues in both forensic context (case work)  
22 and research (Gosselin et al. 2011), but only three research and one case work publication have  
23 focused on the presence and the effects of pesticides on the survival, morphology and  
24 developmental time of necrophagous insects (Gunatilake and Goff 1989, Wolff et al. 2004, Rashid  
25 et al. 2008, Liu et al. 2009). Pesticides are biocides used to control/kill rats, snails, and insects, but  
26 they can also be used for criminal intent. The reason for using these substances is because they  
27 are cheap and simple to obtain. Pesticides are documented in one third of suicide cases by self-  
28 poisoning globally and are commonly used to make poisoned baits (Gunnell et al. 2007). As well,

29 the spread of poisoned baits has become a threat for wild and domestic animals, and many  
30 countries have introduced laws inflicting fines and/or imprisonment to anyone who causes the  
31 death of an animal by cruelty or without necessity (e.g. Italian Law of 20<sup>th</sup> July 2004 n. 189, art.1  
32 and Title IX Bis, art. 544).

33 *Endosulfan* is an organochlorine insecticide and acaricide belonging to the cyclodiene group. It is  
34 produced in variable mixtures of its two isomers ( $\alpha$ - and  $\beta$ - endosulfan) (Budavari 1996).  
35 Endosulfan alters the electrophysiological and enzymatic properties associated with the nerve cell  
36 membrane. Acute poisoning causes hyperactivity, trembling, convulsions, loss of coordination,  
37 jactitation, breathing disorders, nausea, vomit, diarrhea and, in many cases, unconsciousness  
38 (AA.VV. 2015b). Endosulfan is extremely toxic and its use has been forbidden by the Stockholm  
39 Convention since 2012, with 5 more years of limited use (Chan et al. 2007). Humans and animals  
40 are generally exposed to endosulfan by eating food contaminated with it or from poisoned baits. In  
41 Italy, organochlorine pesticides are the third most used chemicals in poisoned baits, with 1223  
42 recorded cases between 2005 and 2009. Endosulfan was present in the 7% of the cases in 2010,  
43 in 8% in 2011, in 12% in 2012 and in 38% in the first four months of 2013 (AA.VV. 2015a). In 2015,  
44 following the analysis of 558 bait samples and tissues from animal necroscopy, 35 samples were  
45 determined to contain endosulfan (as a single pesticide or in association with other biocides such  
46 as methamidophos, dimethoate, diazinon). In 34 cases endosulfan was present as a mixture of its  
47 two isomers while in one case only  $\alpha$ -endosulfan was present (AA.VV. 2015a). Poisoned baits  
48 were identified as meatballs, meat-rolls, sausages, pieces of lard and thick pieces of bread loaf,  
49 which were occasionally mixed with pieces of glass (AA.VV. 2012, 2015a).

50 In the present research, larvae of *Calliphora vomitoria* L. (Diptera: Calliphoridae) were reared on  
51 liver substrates spiked with endosulfan. The spiking concentrations of the pesticide were chosen  
52 from the levels reported in human and animal fatalities involving endosulfan poisoning (Lehr 1996,  
53 Sancewicz-Pach et al. 1997, McGregor 1998, Parbhu et al. 2009). The aim of the present research  
54 was the development and validation of an analytical method dedicated to the detection of  
55 endosulfan on the life history stages of *C. vomitoria*. Furthermore, the effects of this pesticide on *C.*  
56 *vomitoria* morphology (length), survival and developmental time was determined. To the best of the

57 authors' knowledge, this research is the first of its kind in which the QuEChERS method has been  
58 used in entomotoxicology.

59

## 60 **2. Material and Methods**

61

### 62 2.1. Preparation of foodstuff and rearing of blowflies

63

64 Calliphorids (Diptera: Calliphoridae) are the most common blowfly species that locate a corpse or  
65 carcass during early or the fresh stage of decomposition. In particular *C. vomitoria* is widely  
66 distributed through the Holarctic region and is commonly found during the colder seasons (Smith  
67 1986). *C. vomitoria* may be found in association with *C. vicina* (Diptera: Calliphoridae), but it has a  
68 more rural distribution than *C. vicina* (Erzinçlioğlu 1985, Smith 1986, Byrd and Castner 2010b).

69 Colonies of *C. vomitoria* are continuously reared in the research facility for different research  
70 purposes. Adult flies are caught from the wild in different seasons, identified by entomologists  
71 using appropriate dichotomous keys (Smith 1986) and maintained in separate rearing containers  
72 (Bugdorm®). Adults are periodically replenished from wild populations to prevent inbreeding. Flies  
73 used in this research were harvested from a fifth-generation laboratory culture, and maintained  
74 following the procedures described by Magni *et al.* (Magni *et al.* 2008, Magni *et al.* 2014, Magni *et*  
75 *al.* 2016b).

76 Newly emerged flies were provided with water and sugar cubes *ad libitum* for five days. On day six,  
77 flies were supplied with fresh beef liver to allow females to develop their ovaries. The liver meal  
78 was removed after 72 hours. On day twelve, flies were provided with a small plastic tray containing  
79 fresh beef liver on water moistened paper, to allow females to oviposit. Oviposition commenced  
80 after 3 hours and egg batches containing approximately 1000 eggs (45 mg (Magni *et al.* 2016a))  
81 were removed from the liver and deposited using a fine paintbrush onto beef liver aliquots already  
82 spiked and homogenized with different concentrations of endosulfan. *C. vomitoria* eggs were  
83 reared on liver substrates containing three concentrations of  $\alpha$  and  $\beta$  endosulfan (10 ng/mg, 25  
84 ng/mg, 50 ng/mg). Another liver substrate containing no drug was used as a control. Technically,

85 endosulfan is a 7:3 mixture of the  $\alpha$  and  $\beta$  isomers, where  $\alpha$  is thermodynamically more stable than  
86  $\beta$ , ultimately leading to  $\beta \rightarrow \alpha$  irreversible conversion (Schmidt et al. 1997, Schmidt et al. 2001),  
87 but several isomer mixtures are available on the market. The current research, involved a 1:1  
88 mixture of  $\alpha$  and  $\beta$  endosulfan to investigate the effects on blowfly development, and for ease of  
89 reading, will be referred to simply as “endosulfan” throughout the remainder of the manuscript. The  
90 appropriate endosulfan concentrations were selected from pesticide levels reported from body  
91 tissues of human and animal fatalities involved in this type of poisoning (Lehr 1996, Sancewicz-  
92 Pach et al. 1997, McGregor 1998, Parbhu et al. 2009).

93 Liver was used as the blowfly food substrate because it is the typical medium used in forensic  
94 entomology experiments (Anderson 2000, Donovan et al. 2006). Furthermore, previous  
95 entomotoxicological studies regarding the effects of pesticides on blowflies successfully used liver  
96 as a food substrate for experimental colonies (Rashid et al. 2008, Liu et al. 2009). Liver aliquots  
97 were homogenized with endosulfan using a A11 basic Analytical mill (IKA®-Werke GmbH & Co.)  
98 and a T18 digital ULTRA-TURRAX (IKA®-Werke GmbH & Co.) to uniformly disperse the analytical  
99 standard. Each experimental liver was placed on small round plastic tray ( $\varnothing$  14 cm) with high  
100 sides (10 cm) to observe the start of the larvae post-feeding instar. Moistened paper was put on  
101 the base of the tray to prevent desiccation. Each plastic tray was placed on 5 cm of sand within a  
102 larger plastic box (22x40x20 cm) covered with a fine mesh cloth and sealed using an elastic band.  
103 Sand was used as the medium to allow post-feeding larvae to pupate. For the entire experimental  
104 period, *C. vomitoria* were reared at a constant temperature of 23°C, the RH was approximately  
105 20% and the photoperiod (h) was 12:12 (L:D). The temperature was recorded by Tinytag data-  
106 loggers every 15 minutes and the average temperature was calculated every hour.

107

## 108 2.2. Sample collection

109

110 Two sets of samples, one consisting of 30 individuals and another amounting to 1g, were collected  
111 from each treatment at each life history stage. Blowflies were collected when they reached the

112 second (L2), third (L3), post-feeding (PF), pupal (P), and adult (A) instars. Empty puparia (EP)  
113 were also collected and the adults were sacrificed four days after their emergence.

114 The samples of 30 individuals were used for morphological analyses (length). The specimens were  
115 collected using metallic forceps and preserved following the standards and guidelines for forensic  
116 entomology, that recommends to sacrifice the specimens by immersion for 30 seconds in hot water  
117 (>80°C) and preserving them in 70-95% ethanol (Amendt et al. 2007). The length of each  
118 individual was measured using a stereomicroscope (Optika SZM-2) with a graduated lens.  
119 Measurements were performed no longer than two weeks following the preservation.

120 The 1g samples were used for toxicological analyses to detect endosulfan. All samples were  
121 stored at -20°C after careful cleaning of each individual with water and neutral soap to remove any  
122 external contamination.

123 The method validation was performed using 100 mg of control EP. The high chitin content and the  
124 long lifetime in the environment make EP the best target matrix for entomotoxicological studies  
125 (Magni et al. 2014, Magni et al. 2016b).

126 To consider the effects of the pesticides on the blowflies life cycle and survival, 100 PF individuals  
127 from each treatment were placed in separate boxes. The time to pupation and the total number of  
128 pupated individuals, as well as the time to eclosion and the total number of emerging adults were  
129 recorded. The viability of the first generation (F1) of the flies in the different treatments was not  
130 considered in this research.

131

### 132 2.3 Toxicological analysis

133

134 *Chemicals and reagents* – Solid  $\alpha$  endosulfan ( $\geq 98\%$ ), solid  $\beta$  endosulfan, triphenylphosphate  
135 ( $\geq 99\%$ ), magnesium sulphate (MgSO<sub>4</sub>), sodium chloride (NaCl), disodium citrate, trisodium citrate,  
136 dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>), methanol and acetonitrile (ACN) were purchased from Sigma Aldrich®.  
137 Standard solution of  $\alpha$  and  $\beta$  endosulfan in CH<sub>2</sub>Cl<sub>2</sub> (10 mg/L), and triphenylphosphate (used as the  
138 internal standard, ISTD) in CH<sub>2</sub>Cl<sub>2</sub> (100 mg/L) were prepared from the solid pure standards.

139

140 *Sample preparation for GC-MS analysis and QuEChERS extraction* – *C. vomitoria* larvae (L2, L3,  
141 PF), P, EP and A samples were placed separately in 50 mL falcon tubes. Dichloromethane was  
142 added as part of the preliminary wash. The tubes with larvae and P were then placed in a vortex  
143 for two minutes and the organic solvent was discarded. Meanwhile, EP and A were dried at room  
144 temperature under nitrogen. Following crystallisation using liquid N<sub>2</sub>, they were crushed with a  
145 glass rod and a 100-mg aliquot was placed in a new tube.

146 In order to validate the method, 100 mg of control *C. vomitoria* EP were spiked with different  
147 amounts of  $\alpha$  and  $\beta$  endosulfan, by adding increasing volume concentrations of dichloromethane  
148 solution of  $\alpha$  and  $\beta$  endosulfan (0, 7.5, 10, 100, 250, 500  $\mu$ L of each isomer).

149 The QuEChERS extraction involved, 4.00 g of magnesium sulphate, 1.00 g of sodium chloride,  
150 0.50 g of disodium citrate, 0.50 g of trisodium citrate and 10 mL of acetonitrile which were added to  
151 the samples. Furthermore, 25  $\mu$ L of triphenylphosphate solution in a concentration of 100 ng/mg  
152 was also added as the ISTD. The tubes were sealed and placed in a vortex for two minutes,  
153 centrifuged for five minutes at 3000 rpm and then placed in the freezer overnight. 5 mL of solution  
154 were moved into clean-up tubes and, after two minutes of vortex and five minutes of centrifugation  
155 at 3000 rpm, 1 mL of solution was taken and dried at room temperature under nitrogen. The  
156 residue was dissolved with 100  $\mu$ L of dichloromethane and injected (1  $\mu$ L) into the GC-MS  
157 instrument.

158

159 *GC-MS analysis* – Analytical determinations for the detection of  $\alpha$ - and  $\beta$ -endosulfan were  
160 performed using an Agilent 6890N Network GC System coupled with an Agilent 5973 Inert Mass  
161 Spectrometer operating in the electron impact ionization mode. Splitless sample injection was  
162 effected at a temperature of 250°C and the injection volume was 1  $\mu$ L. The capillary column used  
163 was a HP-5MS, 30 m x 0.25 mm i.d. x 0.25  $\mu$ m f.t.. The oven temperature was programmed as  
164 follows: initial column temperature was 120°C for 1.5 min, then increased by 25°C/min to 290°C in  
165 4.3 min and lastly it was kept steady to 290° for 6.8 min, for a total run time of 12.6 min. The carrier  
166 gas was ultrapure He (1.0 mL/min; SIAD, Bergamo, Italy). During preliminary GC-MS analyses, the  
167 full mass spectra were acquired. The background subtracted mass spectrum for  $\alpha$ - and  $\beta$ -

168 endosulfan (using EI in full scan mode) is given in Fig. 1. For the quantitative analysis the mass  
169 analyser was operated in the selected ion monitoring (SIM) mode. The ions selected to identify  $\alpha$   
170 and  $\beta$  endosulfan were:  $m/z$  195, 197, 207, 241, 243, and 339. Triphenylphosphate characteristic  
171 ions  $m/z$  51, 169, and 326 were selected.

172

173 *Method validation* – The method validation was performed according to ISO/IEC 17025  
174 requirements and ICH guidelines (AA.VV. 2005a, b). The validation protocol included the  
175 quantitative determination of  $\alpha$ - and  $\beta$ -endosulfan in larvae, P, EP and A. Specificity, linearity, limit  
176 of detection (LOD), limit of quantification (LOQ), extraction recovery, repeatability and carry over  
177 were determined.

178

179 *Specificity* – Ten samples of the control EP were used to ascertain the method's specificity. Five of  
180 them were spiked with 25  $\mu$ L of ISTD. The specificity test was successful if the S/N ratio was lower  
181 than 3 at the retention time of the target analytes, for all the specific ion chromatograms.

182

183 *Linearity* – The linear calibration model was checked by analysing control EP samples (100 mg)  
184 spiked with  $\alpha$  and  $\beta$  endosulfan solutions at concentration of 0, 0.75, 1, 10, 25 and 50 ng/mg. The  
185 linear calibration parameters were calculated by least-squares regression, and the squared  
186 correlation coefficient ( $R^2$ ) was used to estimate linearity. Quantitative results from area counts  
187 were corrected using the ISTD signal.

188

189 *Limit of detection and limit of quantification (LOD and LOQ)* – LOD was estimated as the analyte  
190 concentrations whose response provided a signal-to-noise (S/N) ratio of 3, as determined from the  
191 least abundant qualifier ion. LOQ was estimated as the analyte concentrations whose response  
192 provided a signal-to-noise (S/N) ratio of 10 The S/N ratios at the lowest concentration (LCL) were  
193 used to extrapolate the theoretical LOD and LOQ.

194

195 *Extraction recovery (ER%)* – ER% was evaluated at two endosulfan concentrations in control EP:  
196 10 ng/mg and 25 ng/mg. For each concentration, ten samples were spiked before QuEChERS  
197 extraction and ten after the extraction. ER% was calculated by the average ratio between the  
198 analyte concentration determined after its extraction (first set) and the one determined on the  
199 spiked extract (second set).

200

201 *Repeatability (intra-assay precision)* – Repeatability was calculated as the percent coefficient of  
202 variance (CV%), after spiking ten samples of control EP with two endosulfan concentrations: 10  
203 ng/mg and 25 ng/mg. Repeatability is considered acceptable when the CV% is lower than 25% at  
204 low analyte concentrations and lower than 15% at high concentration.

205

206 *Carry Over* – Carry-over effect was evaluated by injecting an alternate sequence of five negative  
207 EP samples and five blank EP samples spiked with  $\alpha$ - and  $\beta$ -endosulfan at 25 ng/mg  
208 concentration. To ensure the absence of any carry-over effect, the signal-to-noise ratio (S/N) for  
209 each transition from negative samples was lower than 3.

210

## 211 2.4 Statistical analysis

212

213 Concentrations of endosulfan in insects and their remains as well as their respective lengths in  
214 different treatments were analyzed by one-way analysis of variance (ANOVA) and Tukey test.  
215 Pupation and eclosion rate were analyzed by one-way ANOVA and Pearson's chi-squared test.  
216 Statistical significance was set at  $p < 0.05$ . Calculations were performed using IBM SPSS Statistics  
217 22 software package.

218

## 219 3. Results

220

### 221 3.1 Method validation

222

223 The following parameters were obtained for  $\alpha$  and  $\beta$  endosulfan: coefficient of linearity ( $R^2 > 0.99$ ),  
224 detection limit (LOD), quantification limit (LOQ), extraction recovery %, and repeatability (CV%). A  
225 summary of validation parameters are reported in Table 1. Specificity was satisfactory and no carry  
226 over effects were observed.

227

### 228 3.2 Endosulfan concentrations

229

230 GC-MS analyses showed that endosulfan concentration was absent (lower than the LOD) in all the  
231 samples collected from T1 and A samples for all treatments. Endosulfan was present in the  
232 different developmental instars of *C. vomitoria* in T2 and T3, however only in T3 the amount of  
233 endosulfan detected was higher than the LOQ (Table 2). Toxicological analyses on T3 samples  
234 were limited to the early larval stages, since no larvae of T3 reached P, EP and A instars. Actually,  
235 a peak of  $\alpha$ - and  $\beta$ -endosulfan concentration was found in the L2 instar from T3 samples, to  
236 decrease in the following stages. In contrast, the T2 treatment yielded the more stable  
237 concentrations of endosulfan, equal or below the LOQ (range: 0.41-0.54 ng/mg ( $\alpha$ ) and 0.12-0.45  
238 ng/mg ( $\beta$ )). In general, the amount of endosulfan in all treatments and instars was found to be  
239 significantly different from the control. A summary of the  $\alpha$  and  $\beta$  endosulfan concentration found in  
240 the different treatments and instars of *C. vomitoria* is reported in Table 2.

241

### 242 3.3 Developmental time and survival

243

244 The presence of endosulfan had significant effects only on the survival of the *C. vomitoria* reared in  
245 the treatment with the highest concentration of endosulfan in the food substrate (T3). No larvae in  
246 T3 reached pupation and, therefore, no adult instar resulted. On the other hand, the presence of  
247 endosulfan in the food substrate had no significant effects on *C. vomitoria* survival and  
248 development time for both T1 and T2, and were found to be similar to the C A summary of the  
249 effects of endosulfan on *C. vomitoria*'s developmental time and survival is reported in Table 3.

250

251 3.4 Larval and pupal length

252

253 The presence of endosulfan had a significant effect only on the length of the *C. vomitoria* reared on  
254 T3. Larvae were significantly shorter than C, T1 and T2 with no larvae feeding on T3 pupated. No  
255 significant differences were observed in the average length of larvae and pupae between control  
256 and the other treatment groups. A summary of the effects of endosulfan on *C. vomitoria*'s larval  
257 and pupal lengths is reported in Table 4.

258

#### 259 4. Discussion

260

261 Pesticides are a class of biocide used to decimate, suppress or alter the life cycle of any pest, e.g.  
262 fungi, weed, insects, and mammalian vermin. They are extremely important for pest control in both  
263 agriculture and urban environment. These substances or mixtures of them are generally cheap and  
264 available worldwide. However, pesticides although effective in the control of target species, they  
265 have also been reported poisoning humans and animals, either accidentally or purposefully. Case  
266 reports in both newspapers and the scientific literature are not uncommon regarding poisoned food  
267 and baits in homicides, and suicides (Shemesh et al. 1989, Lehr 1996, Sancewicz-Pach et al. 1997,  
268 Parbhu et al. 2009, Giorgi and Mengozzi 2011). A forensic investigation regarding a suspicious  
269 death by poisoning requires a toxicological analysis of the remains in order to identify the type and  
270 amount of poison(s) that may have caused or contributed to the death. When only highly  
271 decomposed remains of the victim are discovered, necrophagous insects are likely to represent  
272 the most reliable resource for conducting entomotoxicological analyses. In regards to the literature  
273 only a limited number of studies have focused on the detection of pesticides in insects, typically  
274 involving organophosphate compounds. Two research studies and one case report involving  
275 malathion (Gunatilake and Goff 1989, Rashid et al. 2008, Liu et al. 2009) and one research  
276 publication involving parathion (Wolff et al. 2004), but no research has been conducted in the past  
277 on organochlorine insecticides such as endosulfan, in blowflies. The current research has

278 demonstrated that the presence of high concentrations of this pesticide in the food substrates  
279 significantly affected the survival, the developmental time and the morphology (length) of *C.*  
280 *vomitioria* immatures.

281

282 **Endosulfan concentration** – The entomotoxicological analyses conducted by GC-MS confirmed  
283 that when the food substrate was spiked with only 10 ng/mg endosulfan (T1), the pesticide could  
284 not be detected in any life stage of *C. vomitoria*. Endosulfan was present in a quantity below the  
285 LOQ in immature instars and EP of *C. vomitoria* reared on a concentration of 25 ng/mg endosulfan  
286 (T2). Instead, both  $\alpha$ - and  $\beta$ -endosulfan was positively detected in the early immature instars of *C.*  
287 *vomitioria* if the insects were reared on food substrates containing 50 ng/mg (T3). However, in T3  
288 no larvae survived to the pupal stage.

289 The spiking concentration ratio of  $\alpha$ - and  $\beta$ -endosulfan was initially 1:1, but a higher concentration  
290 of  $\alpha$ -isomer was expected because of the tendency of endosulfan  $\beta$ -isomer to convert to  $\alpha$ -isomer  
291 (Schmidt et al. 1997). This trend was not observed in the experimental data. It is also worth noting  
292 that in this research no metabolites of any of these pesticide isomers was detected.

293 Although no information is available in the literature on the effects of organochlorine pesticides on  
294 blowflies, comparisons and analogies can be made with the studies and case reports conducted  
295 on the organophosphate pesticides malathion and parathion (Gunatilake and Goff 1989, Wolff et al.  
296 2004, Rashid et al. 2008, Liu et al. 2009). The analytical methods used in these research papers  
297 were able to detect the insecticides spiked in the food substrates. Parathion was detected by high  
298 performance liquid chromatography (HPLC) in numerous arthropod species on decomposing  
299 rabbits sacrificed following poisoning, in particular *Phaenicia sericata* (Meigen) (Diptera:  
300 Calliphoridae) (L3), *Chrysomya albiceps* (Wiedemann) (Diptera: Calliphoridae) (L3) and in P and  
301 EP of undetermined species of muscids (Diptera: Muscidae) (Wolff et al. 2004). Malathion, on the  
302 other hand, was detected in *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) and  
303 *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) larvae found on a corpse using GC  
304 analyses (Gunatilake and Goff 1989), *Ch. megacephala* larvae, P and A using solid phase

305 extraction (SPE) followed by GC-ECD detection (Rashid et al. 2008) and in *Ch. megacephala*  
306 larvae and prepupae using GC-MS analyses (Liu et al. 2009).

307

308 **Effects of endosulfan on fly development time and survival** – The experimental data  
309 demonstrated that the development time and the survival of *C. vomitoria* were unaffected by the  
310 presence of endosulfan in the food substrate if present at a concentration of 10 ng/mg (T1) and 25  
311 ng/mg (T2) (Table 3). These results were unexpected, since endosulfan is in fact an insecticide  
312 that is potentially active on *C. vomitoria*. However, when reared on a substrate containing 50  
313 ng/mg endosulfan (T3), *C. vomitoria* failed to moult into the pupal instar, and the larvae were  
314 visibly different to the other treatments e.g. the larvae moved around very slowly in the food  
315 source.

316 Considering the low and/or undetectable concentration of endosulfan in the larvae fed on  
317 substrates spiked with the lower concentration of the pesticide (Table 2), it is possible to speculate  
318 that, *C. vomitoria* may efficiently excrete the pesticide and develop and survive despite the its  
319 presence. Accordingly, the mechanism of excretion in blowflies has been observed for several  
320 toxicological substances, including nicotine, morphine, and codeine (Kharbouche et al. 2008, Parry  
321 et al. 2011, Magni et al. 2016b).

322 From the comparison with other insecticides, *Ch. megacephala* reared on a food substrate spiked  
323 with malathion showed a lower survival and a slower development time with respect to the control,  
324 but none of the experimental concentrations affected the survival rate of the fly (Rashid et al. 2008,  
325 Liu et al. 2009). No studies on insect survival and developmental time have been conducted on  
326 parathion (Wolff et al. 2004).

327

328 **Effects of endosulfan larval and pupal length** – The length of the larvae and pupae of *C.*  
329 *vomitoria* feeding on a substrate spiked with low concentrations (10-25 ng/mg, T1 and T2) of  
330 endosulfan was not significantly different to the control (Table 3). Larvae reared on the highest  
331 concentration of endosufan (50 ng/mg, T3) were visibly affected by the presence of the pesticide:  
332 they were smaller and slower, and they never pupated (Table 3). Under such circumstances, the

333 presence of *C. vomitoria* pupae on the remains may be delayed which underlines why a forensic  
334 entomologist must always consider the context of where and how a deceased person was  
335 discovered, before any inference can be made. The toxicological analyses is important as it may  
336 play a role in the final calculation of the minPMI. If the remains are highly decomposed, such  
337 analyses should preferentially be conducted on the necrophagous insects. While previous studies  
338 on the effects of parathion on blowflies larva and pupa morphology provide only partial information  
339 (Wolff et al. 2004), the results regarding malathion on the same fly species are quite contrasting  
340 (Rashid et al. 2008, Liu et al. 2009). This suggests that the effects of this insecticide group on  
341 blowfly development are inconclusive and should be investigated in more detail.

342

## 343 **5. Conclusions**

344

345 GC-MS analyses combined with QuEChERS represents a useful technique for detecting  $\alpha$ - and  $\beta$ -  
346 endosulfan in all immature stages of *C. vomitoria*, reared on medium and high concentrations of  
347 this pesticide. High concentrations of endosulfan incorporated into the rearing substrate affect the  
348 morphology, the survival and the development time of this blowfly species, while low  
349 concentrations of endosulfan did not result in any detectable change.

350 This research provides a valuable addition to the present knowledge in entomotoxicology, because  
351 pesticides are lethal poisons that are easily available, and cheap to purchase; consequently, they  
352 may be involved in cases of accidental death, animal poaching, suicides and homicides, with  
353 higher frequency than other highly toxic substances.

354

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455 Table 1

456

457 Validation parameters calculated for  $\alpha$ - and  $\beta$ -endosulfan.

458

Parameter	Value	
	$\alpha$ -endosulfan	$\beta$ -endosulfan
correlation coefficient, $R^2$	> 0.99	> 0.99
Limit of detection, LOD	0.22 ng/mg	0.21 ng/mg
Limit of quantification, LOQ	0.73 ng/mg	0.71 ng/mg
Extraction recovery low concentration (%)	94.2%	78.5%
Extraction recovery high concentration (%)	93.2%	85.5%
CV% low concentration	5.7%	8.7%
CV% high concentration	12.6%	17.2%

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463 Table 2

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465  $\alpha$ - and  $\beta$ -endosulfan quantification (ng/mg  $\pm$  S.E.) in *C. vomitoria* (L2=second instar, L3=third instar,

466 PF=post-feeding instar, P=pupa instar, EP=empty puparium, A=adult instar) through GC-MS analysis.

467 Quantification was calculated using 3 replicates. The groups indicated in brackets (i.e. C, T1, T2, T3) are the

468 ones whose results proved significantly different ( $P < 0.05$ ) from the group indicated in the corresponding

469 column.

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471

Treatment		Control (C)		T1		T2		T3	
Amount of endosulfan $\alpha$ and $\beta$ spiked with liver		0 ng/mg		10 ng/mg		25 ng/mg		50 ng/mg	
Quantification (ng/mg $\pm$ S.E.)		$\alpha$	$\beta$	$\alpha$	$\beta$	$\alpha$	$\beta$	$\alpha$	$\beta$
Life instar – sampling day	L2 – day 4	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	15.50 $\pm$ 0.02 (C, T1, T2)	20.30 $\pm$ 0.06 (C, T1, T2)
	L3 – day 5	< LOD	< LOD	< LOD	< LOD	0.41 $\pm$ 0.02 <sup>b</sup> (C, T1, T3)	0.12 $\pm$ 0.06 <sup>a,b</sup> (C, T1, T3)	6.21 $\pm$ 0.03 (C, T1, T2)	0.98 $\pm$ 0.01 (C, T1, T2)
	PF (T2) – day 7 L3 (T3) – day 9	< LOD	< LOD	< LOD	< LOD	0.53 $\pm$ 0.08 <sup>b</sup> (C, T1)	0.29 $\pm$ 0.03 <sup>b</sup> (C, T1, T3)	0.44 $\pm$ 0.02 <sup>b</sup> (C, T1)	1.26 $\pm$ 0.05 (C, T1, T2)
	P – day 11	< LOD	< LOD	< LOD	< LOD	0.54 $\pm$ 0.02 <sup>b</sup> (C, T1)	0.45 $\pm$ 0.07 <sup>b</sup> (C, T1)	N/A	N/A
	EP – day 20	< LOD	< LOD	< LOD	< LOD	0.44 $\pm$ 0.04 <sup>b</sup> (C, T1)	0.21 $\pm$ 0.08 <sup>a,b</sup> (C, T1)	N/A	N/A
	A – day 23	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	N/A	N/A
$\alpha$ -endosulfan LOD=0.22 ng/mg and LOQ=0.73 ng/mg $\beta$ -endosulfan LOD <sub><math>\beta</math></sub> =0.21 ng/mg and LOQ <sub><math>\beta</math></sub> =0.71 ng/mg <sup>a</sup> results are below or equal to the LOQ <sup>b</sup> results are below the LOQ N/A = not applicable, since larvae belonging to T3 never reached the stage of pupa.									

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476 Table 3

477

478 Time (day mean  $\pm$  S.E.) from oviposition to pupation and from oviposition to eclosion of *C. vomitoria* larvae,479 which were exposed to either liver containing different amount of  $\alpha$ - and  $\beta$ -endosulfan, or to the control liver.

480 The table shows also the number of larvae dead prior to pupation, the number of not emerged adults, and

481 the number of survivals. The groups indicated in brackets (i.e. C, T1, T2, T3) are the ones whose results

482 proved significantly different ( $P < 0.05$ ) from the group indicated in the corresponding column. N/A = not

483 applicable, since larvae belonging to T3 never reached the stage of pupa.

484

Treatment	Control (C)	T1	T2	T3
Spiking $\alpha$ - and $\beta$ -endosulfan concentration in liver	0 ng/mg	10 ng/mg	25 ng/mg	50 ng/mg
Larvae third instar N=	100	100	100	100
Time (h) from oviposition to pupation	10.25 $\pm$ 0.08	10.33 $\pm$ 0.13	10.42 $\pm$ 0.05	Never reached the pupation
Larvae dead prior to pupation	3	4	6	100
Pupae	97	96	94	0
Pupae %	97%	96%	94%	0%
Pupae N=	97	96	94	0
Time (h) from oviposition to eclosion	19.54 $\pm$ 0.05	19.62 $\pm$ 0.04	19.58 $\pm$ 0.05	N/A
Not emerged adults	0	4	3	100
Survival	97 (T3)	93 (T3)	91 (T3)	0 (C, T1, T2)
Survival % during the metamorphosis	97%	96%	96%	0%
Survival % from oviposition to eclosion	97%	93%	91%	0%

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500 Table 4

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502 *C. vomitoria* larvae and pupae mean lengths (mm  $\pm$  S.E.) related to time of exposure (days) and instar of life

503 (L2=second instar, L3=third instar, PF=post-feeding instar, P=pupa instar). The groups indicated in brackets

504 (i.e. C, T1, T2, T3) are the ones whose results proved significantly different ( $P < 0.05$ ) from the group505 indicated in the corresponding column. For each time of exposure and each treatment  $N=30$ . N/A = not

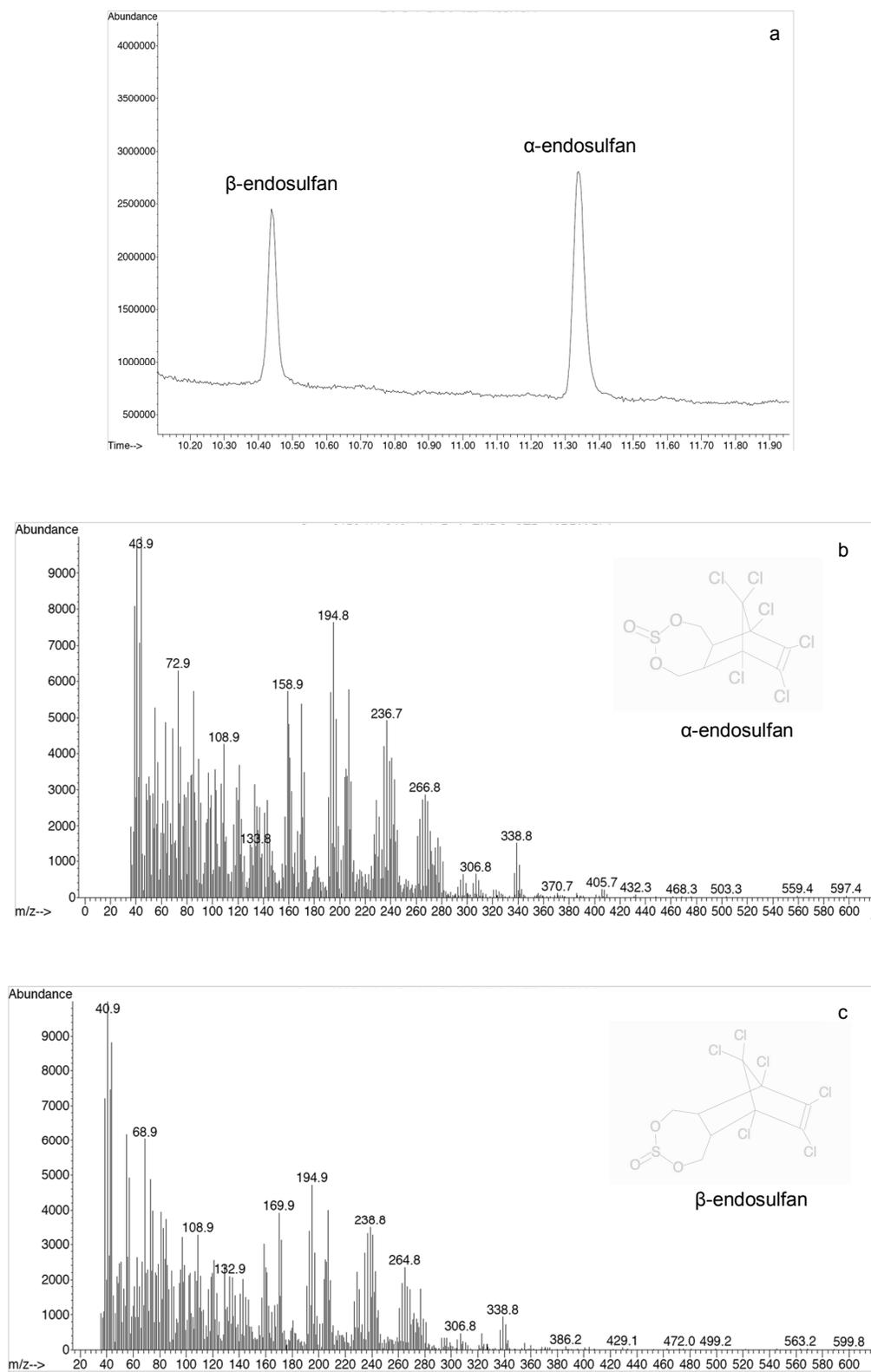
506 applicable, since larvae belonging to T3 never reached the stage of pupa.

507

Treatment		Control (C)	T1	T2	T3
Spiking $\alpha$ - and $\beta$ -endosulfan concentration in liver		0 ng/mg	10 ng/mg	25 ng/mg	50 ng/mg
days of exposure (instar)	L2 – day 4	3.46 $\pm$ 0.11	3.58 $\pm$ 0.12	3.50 $\pm$ 0.18	3.42 $\pm$ 0.12
	L3 – day 5	16.37 $\pm$ 0.40 (T3)	16.63 $\pm$ 0.42 (T3)	16.53 $\pm$ 0.56 (T3)	8.07 $\pm$ 0.40 (C,T1,T2)
	PF (C, T1,T2) – day 9 L3 (T3) – day 9	10.79 $\pm$ 0.36 (T3)	9.90 $\pm$ 0.36 (T3)	10.78 $\pm$ 0.44 (T3)	8.29 $\pm$ 0.56 (C,T1,T2)
	P – day 11	8.71 $\pm$ 0.13	8.66 $\pm$ 0.13	8.92 $\pm$ 0.13	N/A

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 511  
 512 Fig. 1. Chromatogram of an  $\alpha$ - and  $\beta$ -endosulfan standard solution (100ng/mg) (a);

513 full scan mass spectra of  $\alpha$ -endosulfan (b) and  $\beta$ -endosulfan (c).

514