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Analytical Development for the Assessment of Pesticide Contaminations in Blood and Plasma of Wild Birds: The Case of Grey Partridges (*Perdix Perdix*)

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1 Analytical development for the assessment of pesticide contaminations in blood
2 and plasma of wild birds: the case of grey partridges (*Perdix perdix*).

3

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15 **Abstract**

16 In this study, blood and plasma of grey partridges (*Perdix perdix*) were analyzed to assess
17 their potential contamination by plant protection products (PPP) and especially pesticide
18 compounds.

19 The group of pesticides selected is composed of a huge variety of compounds. Therefore, in
20 this study, two methods were optimized and validated to analyze 104 compounds including
21 herbicides, insecticides, fungicides and photoprotectors or synergists.

22 Various extraction methods found in the literature were compared and adapted for the
23 extraction of pesticides from blood and plasma. After extraction, samples were concentrated
24 then injected for quantification simultaneously in LC-MS/MS and ATD-GC/MS/MS with an
25 automatic thermal desorption step (ATD).

26 Both LC-MS/MS and ATD-GC/MSMS analyses were performed using the MRM mode with
27 2 mass transitions for each compound.

28 For both analytical methods, calibrations in triplicate were done by spiking within clean
29 matrices using deuterated internal standard as ISTD. Evaluation of reproducibility, repeatability
30 and determination of extraction yields were also performed with satisfactory results for most
31 compounds then LOD and LOQ were determined with good sensibility results. LODs varied
32 from 0.005 to 0.035 pg mg⁻¹ and LOQs from 0.017 to 0.116 pg mg⁻¹ for both matrices and
33 methods.

34 The two distinct analytical methods were then successfully applied to 70 blood samples and
35 35 plasma samples.

36

37 **Keywords**

38

39 Biomonitoring, pesticides, birds, blood, plasma.

40 **1. Introduction**

41 The constant research of efficiency and rentability in our agriculture and food production
42 has made the use of chemical pesticides more and more trendy because of their high crop yields
43 and economic benefits [1]. Moreover, the European Commission has agreed there are many
44 environmental impacts from using pesticides and recognized the urge of reduce pesticide use
45 [2,3] whereas their number increases exponentially [4].

46 In this environmental context, many studies have showed the importance of biomonitoring
47 as an evaluation tool to assess environmental contamination by pesticides in agrosystems and
48 to determinate the exposition of animals living inside this ecosystem [5–8]. The doses absorbed
49 by animals are often very low resulting in an exposure that does not necessarily lead to an
50 increase in mortality in the short term but could have sublethal effects on individuals in the long
51 term [9]. Therefore, quantifying the exposure of wild animal is a necessary challenge especially
52 since risk assessment studies require data on exposure of organisms [10,11]. Very often, the
53 pesticide exposure of animals is determined after death by analyzing specific organs [12].
54 However, analyzing the amount of pesticides in cadavers is certainly not a good picture of the
55 real exposure of an entire population because it can be assumed that death may be due to
56 pesticide overload, dose that is certainly not representative of all live animals. In addition, it is
57 no longer acceptable to sacrifice animals to determine their exposure to pesticides and there is
58 an urgent need for efficient analytical means to measure their pesticide exposure in matrices
59 that can be easily collected and that do not cause the death of individuals [12]. Blood and plasma
60 of wild birds seem to be good candidates because it is easily to collect once the bird is trapped
61 and can represent a recent pesticide contamination allowing to follow in real time the
62 contamination of environments [12,13].

63 The grey partridge is an iconic European farmland bird, currently declining and exposed to
64 phytosanitary products throughout their lives, as they live in crops and feed with seeds and

65 insects from cultivated fields [14]. Thus, the grey partridge fulfils all the requirements to be a
66 valuable biosentinel of farmlands despite little attention has been paid to its field ecotoxicology
67 [14]. So far, phytosanitary products have been found in wild grey partridges' eggs with 15
68 different compounds detected [14] or in the liver of hunted birds (n.b. only neonicotinoid
69 clothianidin was sought [15]). Investigating the potential presence of such compounds in bird
70 blood and plasma required the development of specific analytical procedures.

71 Many analytical procedures have already been developed so far for the quantification of
72 contaminants such as perfluoroalkyl substances (PFAs), novel flame retardants (NFRs),
73 neonicotinoids, chlorinated paraffins, parabens, bisphenols but also organochlorine pesticides
74 (OCs), polychlorinated biphenyls (PCBs), neonicotinoids and polycyclic aromatic
75 hydrocarbons (PAHs) in birds of prey, eggs, honey or bees [16–20]. It is already known that,
76 in bird blood and plasma, some compounds are present in high concentrations [15,21–24].
77 However, few studies have reported combined extraction and analysis methods to quantify such
78 a large variety of pesticides and to the best of our knowledge none were reported in grey
79 partridges so far. Basically, Liquid Chromatography coupled to tandem Mass Spectrometry
80 (LC/MSMS) is a reliable method for the quantification of many pesticides at low detection
81 levels [25,26], but does not allow a fine quantification of more volatile and semi-volatile
82 compounds. Therefore, we chose to add a second analysis step using Automated Thermal
83 Desorption Gas Chromatography coupled to tandem Mass Spectrometry (ATD-GC/MSMS) to
84 quantify a wider spectrum of molecules.

85 This study aimed at developing a sensitive analytical methodology to quantify 104 different
86 pesticide compounds among the most used in both blood and plasma of birds using grey
87 partridges (*Perdix perdix*) as model species by optimizing extraction and quantification
88 methods. Pesticides from blood and plasma were extracted by two different liquid-liquid
89 extractions with a mixture of ethyl-acetate and dichloromethane and a mixture of methanol and

90 acetonitrile for blood and plasma respectively. Analyses were then run by internal standard
91 quantification using both LC/MSMS and ATD-GC/MSMS for detection and quantification.
92 The developed methods were finally applied to 70 blood samples and 35 plasma samples for
93 the quantification of the exposure of grey partridges to pesticides.

94

95 **2. Material and methods**

96

97 *2.1. Chemicals and solutions*

98 Formic acid, acetonitrile and water for LC/MS were purchased from Sigma-Aldrich (LPCR,
99 France). The ultra-pure water was obtained through a Milli-Q system (18 MΩ cm) from Merck,
100 Germany. Other solvents of HPLC grade quality (ethanol, ethyl ether, methanol, n-hexane)
101 were purchased from VWR Prolabo (Paris, France).

102 Pesticide high purity standard (>98%) powders were supplied by Sigma Aldrich (l'Isle
103 d'Abeau, France) and/or Dr Ehrenstorfer GmbH (Cluzeau Info Labo, St. Foy la Grande, France)
104 and Riedel de Haën (Sigma Aldrich, St. Quentin Fallavier, France).

105 Internal standards, trifluralin-d₁₄, nitrophenol-d₄, 2-4-D-d₃, pendimethalin-d₅ diuron-d₆,
106 acetamiprid-d₁₁ and carbendazim-d₄, were obtained from Sigma-Aldrich (L'Isle d'Abeau,
107 France) and Cambridge isotope laboratories (Cluzeau Info Labo, St. Foy la Grande, France).

108 Stock solutions of each standard and internal standard were prepared in acetonitrile (ACN)
109 (HPLC grade supplied by Sigma Aldrich, St. Quentin Fallavier, France) at a concentration of 1
110 g L⁻¹ and stored at -20°C.

111 The agent of derivatisation, *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide
112 (MtBSTFA) was purchased from Fluka (Sigma Aldrich, St. Quentin Fallavier, France).

113 Perkin Elmer® ATD-empty tubes (89 mm x 5 mm i.d.) were purchased from PerkinElmer
114 Corp. (Norwalk, CT, USA).

115

116 2.2. *Blood and plasma materials*

117

118 To validate those methods, 70 seven-month-old captive-born grey partridges were monitored
119 in a commercial game farm in south-western France. All birds were fitted with an alphanumeric
120 metal ring and lived in two large pens (100 m × 10 m × 4 m) equipped with feeders, drinkers
121 and metal sheet for shelter. They were fed a mixture of corn, wheat, faba bean and pea *ad*
122 *libitum* since they were born. Blood samples from all 70 grey partridges were collected in March
123 2021 after five months of exposure to the two different diets; 200 µL of blood were taken when
124 it was possible from the brachial vein using a sterile needle (Ø 0.06 mm) and heparinized micro-
125 capillary tubes. One hundred microliters of these blood samples were directly stored at -20°C.
126 When there was 100 µL of blood left, it was centrifuged immediately after collection (9000
127 rpm. at 20°C for 10 min) to recover the plasma which was then stored in Eppendorf tubes also
128 at -20°C until analysis. For the 70 partridges, a total of 70 blood samples and 35 plasma samples
129 were collected. All experiments complied with French laws on animal experimentation and all
130 experimental protocols were approved by the Deux-Sèvres Committee of Animal
131 Experimentation (APAFIS#9465-201703101551625).

132

133 2.3. *Plasma extraction*

134 Plasma samples were defrosted and weighted within the Eppendorf. Before extraction, 10
135 µL of a solution of carbendazim-d₄ at 1 g L⁻¹ were added to each sample and homogenised by
136 vortex. For the extraction procedure, the protocol of Hao *et al.* [27] was adapted and consisted
137 in a precipitation step of the lipids and then a liquid-liquid extraction. The figure 1 resumes the
138 adapted extraction procedure.

139 Twenty microliters of methanol and 2,5 μL of a formic acid solution (pH 2.8) were added to
140 plasma samples to precipitate the proteins. After homogenization by vortex, the samples were
141 put under a fume hood for gentle evaporation. After ca. 4 hours of evaporation, 500 μL of a
142 mixture of methanol and acetonitrile (20:80, v/v) was added. Samples were then centrifugated
143 20 minutes at a speed of 4000 rpm for elimination of the proteins' residues.

144 Finally, 500 μL of the supernatant were collected and stored at -20°C until further analyses
145 by ATD-GC/MSMS and LC/MSMS. The empty and dried Eppendorf tubes were weighted
146 again to determinate the exact weight of the samples.

147

148 2.4. Blood extraction

149 Blood samples were defrosted and weighted within the Eppendorf. Before extraction, 10 μL
150 of a solution of carbendazim- d_4 at 1 g L^{-1} were added to each sample and homogenised by
151 vortex. For the extraction procedure, the protocol of Goutner *et al.* [28] was adapted and
152 consisted in a liquid-liquid extraction and then a purification step. The Figure 1 resumes the
153 adapted extraction procedure.

154 Two millilitres of a mixture of dichloromethane and ethyl acetate (1:1) were added to each
155 sample and homogenised 1 minute by vortex. Extracts were then transferred to a centrifugation
156 tube of 10 mL and sonicated for 10 minutes. This step of extraction was repeated 3 times
157 successively. The supernatant was collected each time and put under a fume hood for gentle
158 evaporation until a volume of 500 μL .

159 The final extract of 500 μL was collected and stored at -20°C until analyses by ATD-
160 GC/MSMS and LC/MSMS. The empty and dried Eppendorf tubes were weighted again to
161 determinate the exact weight of the samples.

162 2.5. *Analysis by LC/MSMS*

163 A Thermo Scientific TSQ Quantum Access Triple Quadrupole Mass Spectrometer operating
164 in heated positive electrospray ionization mode (HESI+) coupled with a Thermo Accela 1250
165 pump and a Thermo Combi Pal autosampler were used. The sampler is equipped with a 20 μ L
166 injection loop and the samples were kept at a temperature of 5°C. The analysis was performed
167 on a Nucleodur C₁₈ Pyramid column (150 mm \times 3 mm, 3 μ m) at room temperature. Samples
168 were analyzed using a mobile phase water / ACN both containing 0.1% formic acid, at a flow
169 rate of 0.3 mL min⁻¹. The composition of the mobile phase started at 70:30 (v/v) then changed
170 to 50:50 in 5 min, then 20:80 in 11 min and 5:95 in 18 min (hold for 6 min) and finally returned
171 at 70:30 in 25 min (hold 8 minutes) for a total run of 33 minutes.

172 Detection and quantitation of all compounds were performed using multiple reactions
173 monitoring (MRM). The ion source was operated in positive ion mode with a spray voltage of
174 4,500 V and a vaporizer and capillary temperature of 300°C each. Nitrogen was used for sheath
175 and auxiliary gas pressure (20 and 10 arbitrary units) while argon was used for collision
176 pressure (1.5 arbitrary unit). Two precursors product ion transitions for each analyte and each
177 internal standard were used for quantitation. The selected transitions for MSMS analysis, the
178 collision energy and the retention times are presented in Table A.1 in a subsequent appendix
179 (Appendix A). Data were acquired and processed using Excalibur software.

180

181 2.6. *Analysis by ATD-GC/MSMS*

182 First, prior to GC–MS/MS analysis, 100 μ L of the obtained extracts were diluted into 20 mL
183 using briny water (1.5% NaCl) allowing their reconcentration by SPME using a CTC
184 CombiPAL autosampler according to Levy *et al.* [29]. Pesticides were extracted at 60 °C for
185 40 min using a polyacrylate (PA) 85 μ m fiber SUPELCO (Sigma Aldrich, St. Quentin Fallavier,
186 France).

187 Finally, instead of SPME, a thermal desorption step was added by spiking a Tenax®-TA
188 passive tubes by deposition upside down of 100 µL of extract. For this, conditioned Tenax®-
189 TA clean passive sampling tubes, placed in empty stainless-steel tubes (89 mm × 5 mm i.d.),
190 were used as support for analysis. Ten microliters of a mix of internal standards (trifluralin-d₁₄,
191 nitrophenol-d₄, 2-4-D-d₃ and pendimethalin-d₅) and 10 µL of a derivatization agent, M_tBSTFA,
192 were deposited. The derivatization agent was selected because of its efficiency and ease of use
193 [30–32]. Moreover, the mass spectra of derived compounds using this agent, possess a
194 characteristic fragment of $m/z = M-57$ (M corresponding to the molecular ion of the derived
195 molecule) which can be, in the most cases, the most intense fragment, allowing its use in MSMS
196 using an electronic impact source. Figure 2 presents the sialylation reaction of a primary
197 alcohol.

198 For derived molecules, formation of the derived product, which has a mass [M+114] relative
199 to the mass of the molecular ion of the compound alone was observed. This by-product was
200 fragmented in the mass spectrometer source and then gave several characteristic masses
201 including the mass [M-57] representing the loss of the C(CH₃)₃ group of M_tBSTFA and the
202 mass [M-131] representing the loss of the OSi(CH₃)₂C(CH₃)₃ group.

203 The liquid was then allowed to spread during 5 min before returning the tube and inserting
204 it in the automated thermal desorber (ATD) autosampler. Thermal desorption was carried out
205 by using an automatic thermal desorption system (ATD 350, PerkinElmer Corp.; Norwalk, CT,
206 USA) connected to a Trace 1300 GC coupled to an ITQ 900 mass spectrometer (Thermo
207 Scientific). ATD 350 was coupled to a GC/MSMS system *via* a valve and a transfer line
208 maintained at 280°C and 300°C respectively. The thermal desorption consisted of a two steps
209 desorption. First, a 5 min tube purge with helium (He) at a flow rate of 45 mL min⁻¹; then, a
210 desorption step occurs when the sample tube is heated at 300°C during 30 min under a He flow
211 stream (45 mL min⁻¹). All compounds are refocused in a cold trap maintained at 20°C by Peltier

212 effect. At the end, the trap is heated to 300°C by induction (temperature rate 20°C s⁻¹) for a
213 flash desorption step.

214 Compounds from ATD desorption were separated on a Macherey-Nagel OPTIMA XLB
215 capillary column (30 m × 0.25 mm i.d.; 0.25 µm film thickness) with He as carrier gas with an
216 electronically regulated constant flow of 1.2 mL min⁻¹. The GC oven temperature was initially
217 set at 50°C for 3 minutes, then increased to 160°C at 36.6°C.min⁻¹ and then to 206°C at
218 5.8°C.min⁻¹ (held for 2 minutes) and then programmed to 230°C at 1.5°C.min⁻¹ and then to
219 300°C at 6°C.min⁻¹ where it was held for 6 minutes for a total analysis time of approximately
220 50 minutes.

221 Spectra of compounds were obtained in electron impact (EI) ionization mode at 70 eV
222 electron energy. Transfer line temperature was set up at 300°C and source temperature at
223 210°C. The most abundant ion of a full scan analysis of each compound was selected as
224 precursor ion for the second ionization step. Collision induced dissociation (CID) was
225 performed in resonant mode. The highest abundant product ions were then selected as
226 characteristic ions for each compound. The selected ions for MSMS analysis, the CID excitation
227 voltage, and the retention times are presented in Table B.2 in a subsequent appendix (Appendix
228 B). Data were acquired and processed using Excalibur software.

229

230 2.7. Calibration and uncertainties

231 For all sample types, calibration was performed with curves obtained by spiking, within
232 clean matrices, increasing amount of all 104 pesticide compounds. The concentration of internal
233 standard solutions was of 1 mg L⁻¹ for 10 µL injected in GC and 0.1 mg L⁻¹ for 10 µL injected
234 in LC. In LC/MSMS, diuron-d₆, acetochlore-d₁₁ and pendimethalin-d₅ were used for calibration
235 and carbendazim-d₄ was used to evaluate the extraction performance. Extraction yields were
236 determined for each sample. In ATD-GC/MSMS, nitrphenol-d₄ was used to evaluate the

237 derivatization performance and trifluar-in-d₁₄, 2,3-D-d₄ and pendimethalin-d₅ were used for
238 calibration.-For calibration, multi-compound solutions ranging between 0.2 pg and 0.2 ng for
239 20 µL injected in LC and 0.01 to 50 ng for 100 µL injected in GC were prepared. In order to
240 evaluate the potential matrix effect, samples with and without matrix were spiked for each
241 concentration range and responses were compared. The analytical procedures as described
242 before were performed three times on each calibration sample. The blank samples were
243 obtained from birds guaranteed that no pesticides contamination has occurred.

244 Limits of quantification (LOQs) and detection (LODs) were determined for all compounds
245 as the concentrations giving signal to noise (S/N) ratios of 3 and 10 respectively. Calibrations
246 were done in triplicate using deuterated compounds as internal standards. Standard deviation
247 of the slope was determined and considered acceptable for all compounds (RSD < 15%).
248 Uncertainties were determined for both analytical methods (LC and GC) and each sample types
249 (plasma and blood) by repeating and analyzing three time all calibration levels of all compounds
250 from spiked samples on different days for reproducibility and on the same day for repeatability.

251

252 **3. Results and Discussion**

253

254 *3.1. Choice of the column*

255 A Nucleodur C₁₈ pyramid (150 mm × 3 mm diameter, 3 µm particle size) column was used
256 for separation of non-volatile compounds in LC/MSMS. This column has already shown good
257 results and efficiency in air and biological samples [33,34].

258 For volatile and semi-volatile compounds analyzed in GC, the method proposed by Sonnette
259 *et al.* [30] was adapted to consider only pesticides. The length of the column was reduced to 30
260 m permitting to decrease the time of analysis and increase the stability of some pesticides
261 sensible to long period exposure at high temperature. By decreasing the length of the column,

262 the sensitivity obtained for example for deltamethrin and tebuconazole was increased by a
263 factor of 2 in comparison to a column with 60 m length [35]. Some other molecules, like
264 cymoxanil and metrafenone, which cannot be detected with a 60 m column, were identified
265 with a good sensitivity on the 30 m column.

266

267 3.2. *Extraction optimization*

268 Different extraction methods were evaluated and two were finally selected, adapted and
269 optimized according to the compounds of interest and the matrices. First, solvent extraction
270 efficiency was evaluated by comparing several solvents or mixture of solvents. According to
271 Goutner *et al.* [28], a mixture of hexane and ethyl ether (1:1, v/v) was used to extract PCBs
272 from blood of vultures as previously used for OCs determination in blood [36,37]. Due to poor
273 sensibility for some compounds, efficiency of other extraction solvents was evaluated. For
274 plasma extraction, several solvents were also tested for extraction and reconstitution. The
275 Figure 3 presents an example of the extraction recovery obtained for carbendazim,
276 epoxiconazole, pymetrozine and spinosade-A with different extraction solvents or mixtures for
277 blood and plasma procedures.

278 Finally, in regard of the extraction yields obtained, a mixture of DCM and ethyl acetate (1:1,
279 v/v) was selected for blood extraction with the best average recovery for all compounds of 93.11
280 %. The combination of methanol with formic acid was conserved for plasma extraction as
281 specified by Hao *et al.* [27] with the best average recovery of 94.33 % but the reconstitution of
282 the sample after evaporation was adapted with a mixture of methanol and ACN (20:80, v/v) to
283 avoid water in the final extract and giving the best results for detection of the compounds of
284 interests in this study with the best average recovery of 78.85 % for all compounds of interest
285 (Figure 4).

286 For blood extractions, the purification step was considered as unnecessary according to the
287 very small amount of sample used for this method (35.80 ± 13.15 mg) compared to the previous
288 studies [28,36,37] and it was decided that the extraction step was sufficient with good extraction
289 yields for most of the compounds.

290 The rest of the extraction procedures consisting on extraction cycles, centrifugation and
291 evaporation were also tested and conserved as described by Goutner *et al.* [28] and Hao *et al.*
292 [27] given the good results obtained for most of the compounds of interest of this study. All
293 tests were done within blank matrices in triplicate by the same operator within the same day.

294

295 3.3. Comparison of SPME and ATD

296 Prior to GC/MSMS analysis, two different pre-concentration and extraction step were
297 compared. Solid phase micro extraction (SPME) using three different fibers
298 (Polydimethylsiloxane 100 μm , polyacrylate 85 μm and Polydimethylsiloxane/Divinylbenzene
299 65 μm) was first considered. Based on detection and intensity of signal of each compound, the
300 polyacrylate (PA) fiber was selected. These results were consistent with previous studies
301 [33,38–40]. With this pre-concentration method, extraction had to be adapted because of the
302 non-miscibility of hexane used for blood extraction with water needed for SPME extractions.
303 Indeed, poor sensibility was observed with this extraction solvent due to the non-
304 homogenization of the extract with the SPME saltwater solution. Also, many compounds were
305 non-detectable using SPME. In fact, only 54 compounds out of the 71 of interest were
306 considered well-detected with good signal intensities.

307 According to these results, thermo desorption (ATD) was then considered. Indeed, this
308 method has already been used for analysis of pesticides in dust and air samples with high
309 efficiency [30]. This method shown good efficiency and recoveries results in both spiked blood

310 and plasma samples with lower LODs and LOQs as SPME-GC/MSMS. Method performance
311 characteristics are presented in Table 1.

312 For plasma samples and considering all 104 compounds of the study, LODs ranged from
313 0.001 ng mg⁻¹ to 0.078 ng mg⁻¹ in LC/MSMS and from 0.005 ng mg⁻¹ to 0.625 ng mg⁻¹ in
314 ATD-GC/MSMS and LOQs from 0.003 ng mg⁻¹ to 0.263 ng mg⁻¹ in LC/MSMS and from 0.005
315 ng mg⁻¹ to 0.625 ng mg⁻¹ in ATD-GC/MSMS. Accordingly, LODs ranged from 0.002 ng mg⁻¹
316 to 0.111 ng mg⁻¹ in LC and from 0.004 ng mg⁻¹ to 0.188 ng mg⁻¹ in GC; LOQs from 0.007 ng
317 mg⁻¹ to 0.370 ng mg⁻¹ and LOQs from 0.001 ng mg⁻¹ to 0.625 ng mg⁻¹ for blood samples, in
318 LC/MSMS and ATD-GC/MSMS respectively.

319

320 3.4. *Choice of the internal standards*

321 In LC/MSMS, four deuterated standards were selected with different chemical
322 characteristics and different retention times covering all run. Nicosulfuron-d₆ (t_R = 7.1 min),
323 diuron-d₆ (t_R = 10.21 min), acetochlore-d₁₁ (t_R = 12.46 min), and pendimethalin-d₅ (t_R = 17.22
324 min).

325 Carbendazim-d₄ (t_R = 2.22 min) was used as a recovery standard for extraction and was
326 added to each sample at a concentration of 1 mg L⁻¹ prior to extraction. Table 2 presents the
327 extraction recovery observed in each sample according to the carbendazim-d₄ concentration
328 determined.

329 Trifluaraine-d₁₄ (t_R = 11.51 min) and pendimethalin-d₅ (t_R = 19.29 min) were used for
330 quantification of most of the molecules in ATD-GC/MSMS, whereas 2,4-D-d₃ (t_R = 16.88 min)
331 was used for the quantification of derived molecules. In these standards, 4-nitrophenol-d₄ (t_R =
332 13.19 min) was added as an efficiency standard for the derivatization reaction.

333 3.5. *Calibrations*

334 The calibration in LC/MSMS were done in triplicate by spiking within a blank matrix and
335 without matrix to quantify 33 non-volatile compounds. For plasma and blood samples, a good
336 linearity was observed for the responses with correlation coefficients ≥ 0.98 , using the linear
337 regression model. The matrix effect was evaluated by comparing the standard deviation of the
338 slope for both calibrations with and without the matrix and no variation of more than 15% was
339 observed.

340 About the ATD-GC/MSMS method, the same manipulation was conducted to quantify the
341 71 volatiles or semi-volatiles compounds. Here again, calibrations with a good correlation
342 coefficient were obtained. However, for some fewer volatile compounds, calibration lines of
343 the quadratic type were observed. This can be explained by the desorption kinetics of the
344 compounds (in ATD as in SPME) because for most volatile compounds a curve that is close to
345 a linear regression was obtained. Therefore, for these compounds, a quadratic regression model
346 was used to minimize this deviation. The correlation coefficients were still ≥ 0.98 .

347

348 3.6. *Methods performance characteristics*

349 To evaluate the performance characteristics of those methods, number of interest compounds
350 analyzed, LODs and extraction recoveries obtained were compared with previous extraction
351 methods optimized in this work. To our knowledge, no previous studies investigated the
352 quantification of all those compounds of interest in grey partridges. Even if several previous
353 studies proposed methods for determining pesticide contaminations in other birds, only a few
354 of them included both plasma and blood analyses for such a large number and variety of
355 compounds. However, Goutner *et al.* analyzed 7 PCBs and 16 OCs in blood samples of vultures
356 obtained similar recoveries (between 72–94%) and similar LODs ranged from 0.01 to 0.05 ng
357 g⁻¹ [28]. The main difference was the amount of blood extracted, which was about 50 mg in this

358 study but 2 g in the previous study, giving better sensitivity to this method and allowed to
359 eliminate the purification step. In the study of Hao *et al.*, 8 neonicotinoid compounds and 1
360 metabolite were monitored in songbirds' plasma samples with higher LODs ranged from 2.3 to
361 177.7 pg mL⁻¹ for a similar amount of plasma of 50 µL and with similar recoveries (between
362 58–101%) [27]. This present study led to develop more sensitive analytical methods for more
363 compounds with similar or lower LODs. In terms of repeatability and extraction recovery, this
364 work is in the same range than most of the other studies.

365

366 **4. Application to blood and plasma samples**

367

368 The developed methods were successfully applied to the analysis of 35 plasma samples and
369 70 blood samples collected in birds. For LC/MSMS analyses, all the measurements were
370 confirmed by the qualification transition, with a deviation of the (product ion 1/ product ion 2)
371 ratio within an accepted tolerance of 20%. In ATD-GC/MSMS analyses, a percentage of
372 fragmentation of minimum 90% was necessary to confirm detection. The Table 3 resumes
373 principal results. The concentrations ranged between 1.552 to 255.7 pg mg⁻¹ for LC and GC
374 analyses of plasma with a positivity ratio of 7.10 to 12.31 % respectively. In contrast, for blood
375 samples, the concentrations were lightly superior and ranged from 7.012 to 369.5 pg mg⁻¹ with
376 a lower positivity ratio of 4.46 to 7.34%.

377 Indeed, lower concentrations were monitored in plasma samples with concentrations
378 sometimes close to the LOQs in both LC and GC. This can be explained by the smaller amount
379 of material used for plasma extraction ($m = 35.8 \pm 13.2$ mg) than for blood extraction ($m = 44.2$
380 ± 6.8 mg).

381 However, although the concentrations found were consistent with previous studies
382 [19,27,28,41], they were still lower. This may probably be due to the origin of the samples. In

383 fact, blood and plasma samples were collected from young captive-born grey partridges. They
384 have likely not been subjected to many sources of contamination so far.

385

386 **5. Conclusions and perspectives**

387

388 In this project, two distinct analytical methods which showed very good analytical
389 characteristics were developed and validated. Both analytical methods achieved a good linearity
390 for the calibration responses in plasma and blood. Methods allowed sensitive detection and
391 quantification in complex biological matrices such as plasma and blood in both LC and GC.
392 For plasma samples and considering all 104 compounds of the study, the average LOD was
393 0.005 ng mg^{-1} in LC/MSMS and 0.035 ng mg^{-1} in ATD-GC/MSMS and the average LOQ was
394 0.017 ng mg^{-1} and 0.116 ng mg^{-1} in LC/MSMS and ATD-GC/MSMS respectively.
395 Accordingly, the average LOD for blood samples was 0.011 ng mg^{-1} in LC and 0.028 ng mg^{-1}
396 in GC whereas the average LOQ was 0.038 ng mg^{-1} and 0.094 ng mg^{-1} in LC/MSMS and ATD-
397 GC/MSMS respectively.

398 Those methods were successfully applied to real samples with an average contamination
399 level of $122.35 \text{ ng.mg}^{-1}$ for a positivity ratio of 9.12% in plasma and $216.08 \text{ ng mg}^{-1}$ in blood
400 samples for a positivity ratio of 5.35%. Results were consistent to previous studies even though
401 these analyses were restricted to assess contaminations to specific compounds. In this sense,
402 this study aimed to propose a simple and efficient methodology for the analysis of
403 contamination with a wild spectrum of pesticides from different chemical groups with two
404 complementary analytical techniques for eco-toxicological studies.

405 The choice of several complementary matrices such as blood and plasma allowed a good
406 assessment of environmental contaminations for a selected population of birds. This study also
407 showed blood and plasma from grey partridges were appropriated matrices for biomonitoring

408 and more accurate evaluations of environmental pollution. However, blood seems to be a better
409 matrix than plasma, allowing higher levels of detection with easier sampling to obtain a larger
410 amount of sample. The scope of this technique is substantial and has the potential for application
411 to testing of a wider range of birds and with other vertebrates in natural populations thus
412 providing a useful tool for monitoring programs in environmental studies. In addition, as this
413 monitoring technique is not destructive, it can be applied to the same animal over a period of
414 time to follow the dynamics of their contamination. Therefore, this technique is promising and
415 opens new dimensions for future studies and applications for ecological risk assessment.

416

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