

1 **Determination of ochratoxin A in tissues of wild boar (*Sus scrofa L.*) by enzymatic**
2 **digestion (ED) coupled to high-performance liquid chromatography with a**
3 **fluorescence detector (HPLC-FLD)**

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15

16 **Abstract**

17 Ochratoxin A (OTA) is a secondary toxic metabolite synthesized by *Aspergillus* or
18 *Penicillium* species, which can contaminate various crops. The International Agency for
19 Research on Cancer (IARC) classified OTA as a group 2B possible human carcinogen.
20 The aim of the present study was to assess OTA concentrations in tissues of wild boar
21 (*Sus scrofa L.*) from Tuscany (Italy). Over a period of 2 years, samples of muscle, liver
22 and kidney from 48 wild boars were collected and concentrations of OTA were
23 determined by enzymatic digestion (ED) coupled to high-performance liquid
24 chromatography with a fluorescence detector (HPLC-FLD). The highest concentrations
25 of OTA were found in the kidneys of the 48 wild boars analyzed. No difference in
26 concentrations were found based on years of collection and sex while a significantly
27 higher OTA concentration was found in the kidney of the young wild boars respect to
28 the adult one. Monitoring the quality of meat destined for transformation is a priority in
29 order to decrease the possibility of toxin carry-over to humans. The present study
30 showed that contamination of wild boar meat products by OTA represents a potential
31 emerging source of OTA.

32

33 **Keywords:** Ochratoxin A, wild boar, muscle, kidney, liver, HPLC

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35

36 **Introduction**

37 Ochratoxin A (OTA) is a secondary toxic metabolite of various *Penicillium* and
38 *Aspergillus* fungi, which is widely distributed in various food commodities, including
39 cereals, coffee, beer, wine and spices (Malir et al., 2016). OTA has carcinogenic,
40 nephrotoxic, teratogenic, immunotoxic and possibly neurotoxic properties (EFSA,
41 2006). International Agency for Research on Cancer (IARC) classified OTA as a
42 possible human carcinogen (Group 2B) (IARC, 1993). Long-term exposure to OTA in
43 humans has been implicated in Balkan endemic nephropathy (BEN) and is associated
44 with urinary tract tumors because of the high OTA levels detected in food samples and
45 in biological fluids from affected patients (Fuchs and Peraica, 2005; Pfohl-Leszkowicz
46 et al., 2002). As cereals are widely used in animal feed, animals are potentially exposed
47 to OTA through the consumption of contaminated feed, which can lead to the
48 accumulation of this mycotoxin in meat and meat products (EFSA, 2004). Ranging
49 OTA levels of 0.1-1 µg/kg have been detected in foodstuffs of animal origin (pork and
50 chicken meat, dry-cured ham) (European Union, 2002; EFSA, 2004; Ostry et al., 2015).
51 The European Union has established regulatory levels of OTA in cereals and in a wide
52 variety of other food, but **has not established** maximum OTA levels in meat-based
53 products (EC, 2006a). However, some countries have set maximum levels of OTA in
54 meat or animal products, such as Denmark (pig kidney 10 µg/kg, pig blood 25 µg/ml)
55 and Romania (pig kidney, liver, and meat 5 µg/kg) (EFSA, 2004). Also, Italy through
56 the Italian Ministry of Health Circular No 10, dated 9 June 1999 (Italian Ministry of
57 Health, 1999), establishes, as a guideline, a maximum level (**ML**) for pork meat and
58 meat products of 1 µg/kg OTA.
59 Wild ungulate populations are increasing in Italy, especially in Tuscany where wild
60 ungulate meat derives mainly from wild boar. It is estimated that these animals are

61 around 300,000 and the consumption rises to significant levels until 4 kg per capita/year
62 (Ramanzin et al., 2010). The significant increase in the wild boar population has
63 resulted in an increased prevalence of wild boar meat and ready-made products in the
64 food industry. Furthermore, an increasing interest in meat from animals kept in
65 conditions as close as possible to natural one has been developed in recent years,
66 because of special sensory properties desired by consumers (Vergara et al., 2003;
67 Soriano et al., 2006). For these reasons, there is an increasing awareness of the
68 importance of implementing good practices to ensure safety of meat obtained from
69 game.

70 The available knowledge on xenobiotics contamination of meats from wild ungulates
71 reported data regarding heavy metals, organochlorine pesticide and polychlorinated
72 biphenyl contamination (Bilandžić et al., 2009; Chiari et al., 2015; Mulero et al., 2016;
73 Naccari et al., 2004), whereas few data are available on mycotoxins contamination in
74 these species (Bozzo et al., 2012; Grajewski et al., 2012). In general compared to farm
75 animals, these species showed higher levels of contamination by xenobiotics such as
76 heavy metals and organochlorine compounds, which can constitute a greater
77 toxicological threat (Larsen et al., 2010; Rudy, 2010).

78 The aim of the present study was to determine OTA concentrations in muscle, kidney
79 and liver of wild boars hunted in Tuscany between 2014 and 2015 by using an
80 enzymatic digestion (ED) method coupled to high-performance liquid chromatography
81 with a fluorescence detector (HPLC-FLD).

82

83 **Materials and methods**

84 *Samples*

85 The research was conducted with the participation of the Migliarino, San Rossore,
86 Massaciuccoli (MSRM) Natural Park staff. The MSRM is a regional park stretching
87 along the Tyrrhenian coast on an area of about 240 sq km, located in Pisa and Lucca
88 provinces. Inside the park there are several species of wild animals specially ungulates
89 as wild boar. This specie is the most numerous and represents environmental interest.

90 The management of the animals includes a series of reliefs and catches during the
91 seasons. Periodically biologists estimated the population across the transect method to
92 verify the real number of the individuals. The management of these ungulates is
93 essential to sustain the population avoiding that it can reduce the natural resources
94 available for the future.

95 For the present study, sampling of animal was carried out during periods of planned
96 felling performed by the MSRM Park staff; the killings were carried out according to a
97 planning in the different areas where the animals appeared to be in surplus. The culled
98 wild boars were recovered, transported to a slaughterhouse and eviscerated in
99 accordance with the EU regulations (EC 2004a; EC 2004b). For each animal was
100 compiled a data sheet where it was reported the day of killing, abatement area, sex and
101 age-class choice of three periods (0-4 months; 4-12 months, > 12 months) depending on
102 the pigmentation of the coat of the animal killed (light brown coat; reddish coat; dark
103 brown cloak). During the cutting operations the carcass was weighed and portions of
104 liver, kidney, ~~fat~~ and muscle were taken from each one. **Muscle samples were portions**
105 **of the inner muscle of the back thigh of the animal.** The samples were stored at a
106 temperature of -18 ° C pending the analytical determinations.

107 **Forty eight wild boars (male n = 25, female n= 23) culled from 2014 to 2015 have been**
108 **slaughtered and the carcass weight were determined (from a min. of 13.8 kg and a max.**

109 of 72.0 kg).

110 We had selected these provinces due to the large number of wild boars being shot there,
111 in order to decrease damage to crops caused by wild boars.

112 *Reagents*

113 OTA (from *Aspergillus ochraceus*) (M 403.8) reference standard was purchased from
114 Sigma (Milan, Italy). The OTA standard was dissolved in a toluene-acetic acid mixture
115 (99:1 %, v/v) to give a stock solution of 200 µg/ml, which was stored at -20 °C until
116 use. Working solutions (0.01, 0.02, 0.1, 0.2, 0.5 and 1 µg/ml) were prepared by diluting
117 the stock solution with the mobile phase consisting of a methanol-sodium phosphate
118 buffer (pH 7.5) 50:50 % v/v. HPLC-grade water, methanol, ethylacetate and acetonitrile
119 were purchased from VWR (Milan, Italy). The pancreatin enzyme (from porcine
120 pancreas) was purchased from Sigma (code P1750, Milan, Italy), and was stored at – 20
121 °C until use.

122 *Chromathographic method*

123 The chromatographic system consisted of a Jasco 880 pump and a Jasco 821
124 fluorescence detector (Jasco, Tokyo, Japan). JascoBorwin software was used for data
125 processing. The excitation wavelength (λ_{ex}) and emission wavelength (λ_{em}) were set at
126 380 and 420 nm, respectively. The reversed-phase column was a HAISIL HL, C₁₈, 5
127 µm, 150 mm x 4.6 mm (Higgins Analytical, USA). The column was kept at room
128 temperature. The HPLC was operated with a mobile phase system consisting of a
129 methanol-phosphate buffer solution pH 7.5 (0.03 M Na₂HPO₄, 0.007M NaH₂PO₄) 50/50
130 % v/v at flow rate of 1 ml/min.

131 *Enzymatic digestion method*

132 Samples were extracted using the method of Luci et al. (2016). Five grams of muscle,
133 liver, or kidney sample aliquot were homogenized with 5 ml of a phosphate buffer
134 (sodium phosphate monobasic dihydrate 0.2 M and sodium phosphate dibasic 0.2 M

135 20:80 % v/v pH 7.5) using an Ultra Turrax T25 homogenizer for a few minutes. A 2.5 g
136 aliquot of the homogenate was transferred into a tube and incubated for 1 hour at 37°C
137 with 5 ml of 1% pancreatin solution of in a phosphate buffer (sodium phosphate
138 monobasic dihydrate 0.2 M and sodium phosphate dibasic 0.2 M 20:80 % v/v pH 7.55).
139 The incubation was performed in a rotatory shaker, after which step samples were
140 acidified with 85% H₃PO₄ until pH 2-3. These samples were then extracted with the
141 same volume of ethylacetate, vortexed for 1 min, and centrifuged for 10 min at 3000
142 rpm. The organic phase was evaporated to dryness under nitrogen stream, reconstituted
143 in 1000 µl mobile phase, and a 100 µl aliquot was injected into HPLC.

144 *Method validation*

145 The HPLC-FLD method was validated according to (EC, 2002, EC 2006b) by
146 evaluating: specificity, recovery, trueness, decision limit (CC α), detection capability
147 (CC β) of the method, linearity, LOD and LOQ, repeatability and reproducibility. A ML
148 of 1 µg/kg (1 ppb) OTA in pork meat and derived products was established by the
149 Italian Ministry of Health in 1999 (Italian Ministry of Health, 1999). The validation
150 procedure was performed taking into account the ML of 1 µg/kg OTA.

151 The linearity was evaluated by spiking muscle, liver and kidney samples with OTA at
152 0.05, 0.1, 0.5, 1, 2.5 and 5 µg/kg and analysing them using the extraction and HPLC-
153 FLD method. The experiment was repeated three times. The repeatability was tested by
154 analysing muscle, liver and kidney samples spiked with OTA at the levels of 0.1 µg/kg,
155 1 µg/kg, and 5 µg/kg. All samples were measured in triplicate on the same day. For the
156 within-laboratory reproducibility test, each of the fortification levels was tested in
157 triplicate over a period of five days. The results of these experiments were also used for
158 the determination of the recovery. No certified reference material was available for the
159 trueness assessment of OTA analysis in wild boar tissues samples. Repeatability and
160 reproducibility data corrected with the mean recovery were used for trueness

161 determination; trueness (%) was calculated as the mean (recovery corrected)
162 concentration of added known amount x 100 /added amount. The LOD and LOQ were
163 determined by the signal-to-noise approach, defined at levels resulting in signal-to-noise
164 ratios of 3 and 10, respectively. The analytical response and the chromatographic noise
165 were measured from the chromatogram of a blank sample extract (1 ml) to which an
166 OTA solution was added. The decision limit was estimated by spiking 10 muscle, liver
167 and kidney samples at the current limit taken as the reference value (1 µg/kg). The
168 concentration at this limit plus 1.64 times the corresponding standard deviation equals
169 the decision limit ($\alpha = 5\%$). Decision capability was estimated by spiking 10 muscle,
170 liver and kidney samples at the corresponding CC α level. The value of the decision
171 limit plus 1.64 times the corresponding standard deviation equals the decision capability
172 ($\beta = 5\%$).

173 *Statistical analysis*

174 Statistical analysis was performed with GraphPad Prism (v. 6) software (La Jolla, CA,
175 USA). All data were tested for normality by means of the Kolmogorov-Smirnov test.
176 The data are reported as median and range. A value of $p < 0.05$ was considered
177 significant. Linear regression analysis and Spearman correlation coefficient analysis
178 were used to assess the correlation between OTA concentrations in muscle, liver,
179 kidney and wild boar body weight. Mann-Whitney test was used to compare OTA
180 tissues concentrations between female and male wild boars, between old and young
181 wild boars and between wild boars from the two different years of the study.

182

183 **Results and discussion**

184 Environmental contamination and food safety are a major public health concern
185 worldwide and several studies showed that mycotoxins occurred very frequently in food
186 (Alshannaq and Yu, 2017; Capcarova et al., 2016). ~~The increase in the wild boar
187 population in Europe is correlated with an increase in the consumption of wild boar
188 meat, which may involve a risk of xenobiotics carry-over to consumers.~~

189 The analytical method used in the present study has been showed to be easy and time-
190 saving, accurate and can be applied at levels of OTA contamination considerably lower
191 than the **ML**, which is indicated by Italian Ministry of Health Circular **No 10** dated 9
192 June 1999 (Italian Ministry of Health, 1999). The extraction phase is a critical point of
193 the analytical procedure, because is highly influenced by the matrix. The most common
194 extraction procedures for the determination of OTA in animal tissues are performed by
195 using chloroform, followed by a clean-up with immunoaffinity columns or liquid-liquid
196 partitioning (Valenta, 1998; Curtui et al., 2001; Monaci et al., 2004). These techniques
197 need a large amount of organic solvents, which are environmentally harmful.

198 The **enzymatic digestion** technique used in this study allowed to reduce the use of
199 organic solvents in comparison to other methods and to avoid the use of immunoaffinity
200 columns which are expensive. **Furthermore, enzymatic digestion method is simple, easy
201 to apply and shows very satisfactory performance criteria.** Figure 1 showed
202 chromatograms of muscle, liver and kidney samples naturally contaminated by OTA of
203 one wild boar. Results of the validation study are reported in Table 1. **The method has
204 been validated according to EU criteria for the confirmatory methods for contaminants
205 and has shown to be suitable for accurate quantitative determination of OTA in different
206 tissues of wild boars.**

207 **The median and the range of OTA concentrations found in the muscle, liver and kidney
208 of wild boars sampled in the two years analyzed are shown in Table 2. The highest**

209 concentrations of OTA were found in the kidneys of the 48 wild boars analyzed. The
210 levels found in the liver were 4 (year 2014) and 2 (year 2015) fold lower than in the
211 kidneys. The lowest concentrations were found in muscle samples.

212 There weren't significant differences between OTA concentrations in muscle, liver and
213 kidney samples between the two years analyzed. No significantly differences were
214 found in OTA concentrations between female and male wild boars in the entire studied
215 period and also in single years (Table 2).

216 The present results are in agreement with previous study conducted in Italy and Poland
217 in wild boars (Bozzo et al., 2012; Grajewski et al., 2012). Swine are particularly
218 sensitive to OTA, kidneys showed the highest accumulation of the toxin, followed by
219 liver and muscle tissue. ~~finally the lowest accumulation is represented in adipose tissue.~~

220 The present results showed the same type of accumulation in wild boar tissues. ~~The year
221 of sampling of wild boar did not influence the OTA concentrations in muscle, liver and
222 kidneys samples as the sex of animals. The age of animals influenced the OTA
223 concentrations particularly in the kidney samples.~~

224 Spearman analysis revealed statistically significant correlation between muscle, liver
225 and kidney OTA concentrations (Table 3). Spearman analysis for the relationship
226 between body weight and OTA in kidneys showed a statistically significant negative
227 correlation. As body weight increased the OTA content decrease and animals with a
228 body weight > 50 kg can be considered to have only trace amounts of OTA in their
229 kidneys (Figure 2). Spearman tests revealed no significant correlation between body
230 weight and OTA concentrations in both muscle and liver (Figure 2). The OTA
231 concentrations in the kidneys were significantly higher in young than in adult wild
232 boars, while no significantly differences were found in OTA concentrations in muscle
233 and liver between young and adult wild boars (Table 4).

234 ~~Adult wild boar showed lower OTA concentrations in kidneys than the young.~~ These
235 results were also confirmed by correlation analysis between OTA concentrations in
236 kidney samples and body weight of wild boar. As previously reported by Grajewski et
237 al. (2012) we also found that animals weighing < 50 kg accumulated much OTA in their
238 kidneys. Adult wild boars migrate in search of food resulting in the ingestion of more
239 diversified (i.e. varied) feed and in a reduction of the time they spend in each feeding
240 area. However, hungry young pigs consume large quantities of feed at a time that makes
241 them subjects to a more rapid and intense mycotoxin exposure. These results are
242 different from studies of heavy metal contamination in wild boar tissues that showed a
243 positive linear relationship between OTA content and weight of animal (Larsen et al.,
244 2010, Rudy, 2010). This result could be explained by the different metabolism of OTA
245 respect to inorganic compounds such as lead.

246 In 16 of the tissue samples examined in this study (10 kidney, 6 liver), the
247 concentrations of OTA were higher than the ML (1 µg/kg) established by the Italian
248 Ministry of Health Circular No 10 dated 9 June 1999 (Italian Ministry of Health, 1999).
249 None of the muscle samples exceeded the limit. The correlation between the OTA
250 content in muscle and in kidneys was positive and high, indicating a positive
251 proportional increase of OTA between the two tissues. The higher level of OTA in the
252 kidney was probably connected to the high affinity of OTA for this tissue and to the low
253 rate of excretion. Its bioavailability in monogastric species is high and it accumulates in
254 the blood and edible organs, especially in the kidneys (Ringot et al., 2006).

255 OTA can be carried over to wild-boar meat products and can also be present in the
256 muscle tissue. ~~Maximum levels of some mycotoxins have been legally set for several
257 food products but until now such regulations do not apply to meat products.
258 Nevertheless, a revision of the OTA limit in meat products is expected, according to the
259 risk assessment updating (EC, 2005).~~

260 Furthermore, traditionally in several Italian regions, wild boar meats are used to
261 produce niche products, especially coppa and salami. These derived products
262 throughout the ripening time could be contaminated with higher OTA concentration
263 because of water loss of in the drying stage. For example, Markov et al (2013) reported
264 OTA contamination in wild boar fermented sausage, range of concentrations 2.70-3.07
265 µg/kg, suggesting the enrichment of OTA levels in ripened meat products. For these
266 reasons dried wild boar meat may contribute to overall OTA intake by carry-over
267 effects into processed meats. Monitoring the quality of meat destined for transformation
268 is a priority in order to decrease the possibility of toxin carry-over to humans.
269 Several recent studies indicated OTA occurrence in pig tissues from sub-chronically
270 treated pigs (Persi et al., 2014; Pleadin et al., 2016). OTA was also found in pig tissues
271 naturally contaminated (Matrella et al., 2006). OTA tissues levels reported in the
272 present study are close to the levels of naturally contaminated pigs tissues and lower
273 than sub-chronically treated pigs. Despite of the fact that OTA levels established in
274 game meat within this study results appear not to be hazardous for human health if
275 entering the human body via game meat consumption route, and despite of the fact that
276 game meat is generally not consumed in large amount, MLs for various types of meat
277 and meat derived products should be defined.

278

279 **Conclusion**

280 The present study confirms that contamination of meat products by OTA represents a
281 potential emerging source of OTA. Because wild ungulates meat is being consumed in
282 increasing quantities and no EU legislation on minimum risk levels in their meat exists,
283 consumers may be at risk from the effects of potentially highly toxic contaminants such
284 as OTA. Therefore, there is a clear need to establish a specific regulatory framework for
285 contaminants in wild ungulates meat.

286

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290

291 **Conflict of interest None.**

292

293

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405

406 **Figures legend**

407 **Figure 1. HPLC-FLD chromatograms of muscle naturally contaminated at 0.74 µg/kg**
408 **A), liver naturally contaminated at 1.61 µg/kg B) and kidney naturally contaminated at**
409 **1.90 µg/kg C) samples of one wild boar.**

410 **Figure 2. Correlation between body weight and OTA concentrations in muscle (r = -**
411 **0.074, p = 0.628) liver (r = - 0.189, p = 0.214) and kidney (r = - 0.338, p = 0.023)**
412 **samples.**

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416 **Table 1. Validation parameters of ED method coupled with HPLC-FLD according to (EC, 2002,**
 417 **EC 2006b); (LOD = limit of detection, LOQ = limit of quantification, r² = coefficient of correlation,**
 418 **SD = standard deviation, RSD = relative standard deviation).**

Parameters		Muscle	Liver	Kidney
LOD (µg/kg)		0.001	0.001	0.001
LOQ (µg/kg)		0.002	0.002	0.002
r²		0.997	0.999	0.998
Repeatability				
0.1	Mean concentration ± SD	0.088 ± 0.003	0.095 ± 0.002	0.093 ± 0.004
	RSD (%)	3.01	2.11	4.93
1.0	Mean concentration ± SD	0.92 ± 0.03	0.92 ± 0.02	0.94 ± 0.04
	RSD (%)	3.80	2.75	4.44
5.0	Mean concentration ± SD	4.92 ± 0.05	4.98 ± 0.02	4.96 ± 0.01
	RSD (%)	1.00	0.40	0.12
Reproducibility				
0.1	Mean concentration ± SD	0.090 ± 0.003	0.094 ± 0.003	0.093 ± 0.003
	RSD (%)	3.95	2.82	3.63
1.0	Mean concentration ± SD	0.94 ± 0.03	0.93 ± 0.02	0.94 ± 0.04
	RSD (%)	3.50	2.68	3.92
5.0	Mean concentration ± SD	4.93 ± 0.06	4.97 ± 0.03	4.92 ± 0.10
	RSD (%)	0.93	1.11	0.81
Recovery %				
0.1		86.11 ± 3.54	94.50 ± 2.67	93.50 ± 3.39
1.0		94.00 ± 3.29	93.33 ± 2.50	93.68 ± 3.67
5.0		98.70 ± 0.92	98.63 ± 1.10	98.67 ± 0.80
CC_α		1.031	1.069	1.059
CC_β		1.06	1.100	1.098

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421 **Table 2. Median and range of OTA concentrations ($\mu\text{g}/\text{kg}$) in the two years analyzed; LOD = limit**
 422 **of detection.**

	2014		
	Muscle	Liver	Kidney
Female (n = 12)	0.10 (<LOD-0.77)	0.15 (0.06-1.93)	1.10 (0.24-1.90)
Male (n = 14)	0.08 (<LOD-0.77)	0.15 (0.04-1.76)	0.63 (0.19-3.23)
Total (n = 26)	0.08 (<LOD-0.77)	0.15 (0.04-1.93)	0.68 (0.19-3.23)
	2015		
	Muscle	Liver	Kidney
Female (n = 11)	0.18 (0.03-0.38)	0.23 (0.05-1.31)	0.45 (0.07-1.72)
Male (n = 11)	0.12 (0.06-0.50)	0.20 (0.02-0.91)	0.25 (0.09-1.15)
Total (n = 22)	0.13 (0.03-0.50)	0.23 (0.02-1.31)	0.34 (0.07-1.72)

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Table 3. Spearman correlation analysis between muscle, liver and kidney OTA concentrations.

	p	R²	r
Muscle vs Liver	<0.0001	0.33	0.64
Muscle vs Kidney	<0.0001	0.70	0.62
Kidney vs Liver	<0.0001	0.46	0.57

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429 **Table 4. Median and range of OTA concentrations ($\mu\text{g}/\text{kg}$) in young and adult wild boars; LOD =**
430 **limit of detection; different letters indicate significant difference ($p < 0.05$) between samples of young**
431 **and adult using Mann-Whitney test.**

	Young (n = 9)	Adult (n = 39)
Muscle	0.10 (<LOD-0.77) ^a	0.08 (<LOD-0.77) ^a
Liver	0.15 (0.06-1.93) ^a	0.15 (0.04-1.76) ^a
Kidney	1.10 (0.24-1.90) ^a	0.63 (0.19-3.23) ^b

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